Volume 1

Advances in Experimental Surgery

Surgery - Procedures, Complications, and Results

Nova Biomedical

Huifang Chen, M.D., Ph.D.
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Editors

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SURGERY - PROCEDURES, COMPLICATIONS, AND RESULTS

ADVANCES IN EXPERIMENTAL SURGERY

VOLUME 1

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ADVANCES IN EXPERIMENTAL SURGERY

VOLUME 1

HUIFANG CHEN
AND
PAULO N. MARTINS
EDITORS

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FOREWORD

Pierre Daloze, CM, CQ, MD, FRCSC
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Experimental surgery is a key link for developments in clinical surgery, research and teaching. Throughout the history of medicine, many discoveries and techniques developed from experimental surgery. Most modern surgeons are now learning or improving their surgical techniques firstly via experimental surgery. Reviewing the 20th century surgical developing history, most clinical achievements were related to experimental surgery. Some were real landmarks, such as successful vascular sutures, opening the striking advances of vascular surgery and organ transplantation. In recent years, experimental surgery has achieved new advances, like laparoscopic and robotic surgery, tissue engineering and gene therapy, which are now applied in clinic and have saved many patients.

Both editors of this book, Drs. Huifang Chen and Paulo Martins as well as their colleagues have been contributing to experimental surgery at the University of Montreal, Canada and the University of Massachusetts, US. Their achievements in experimental surgical models in small and large animals, including nonhuman primates, have been applied in clinical trials.

It is my pleasure to write this foreword for this impressive book, *Advances in Experimental Surgery*, which Drs. Chen and Martins provide as a reference for surgeons, residents, surgical researchers, physicians, immunologists, veterinarians and nurses in surgery. I am sure these two volumes will provide an abundance of imperative information for these individuals and their patients.

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PREFACE

Huifang Chen and Paulo N. Martins

Experimental surgery is an important link for developments in clinical surgery, research and teaching. Experimental surgery has been a part of the most important surgical discoveries in the past century. Since 1901, nine Nobel Prizes have been awarded to the pioneers who had remarkable achievements in basic or practical surgery. In recent years, experimental surgery has achieved new advances, like laparoscopic and robotic surgery, tissue engineering, and gene therapy which are widely applied in clinic surgery.

The present book covers wide experimental surgery in preclinical research models subdivided into two volumes. Volume I introduces basic surgical notions, techniques, and different surgical models involved in basic experimental surgery, and reviews the biomechanical models, ischemia/reperfusion injury models, repair and regeneration models, and organ and tissue transplantation models, respectively. Volume II introduces several specific experimental models such as laparoscopic and bariatric experimental surgical models. The second volume also introduces graft-versus-host diseases, and other experimental models. A review of the advances and development of recent techniques such as tissue engineering, organ preservation, wound healing and scarring, gene therapy and robotic surgery is included. This book documents the enormous volume of knowledge scientists have acquired in the field of experimental surgery.

The editors have invited experts from the United States, Canada, France, Germany, China, Japan, Korea, UK, Sweden, Netherlands, Hungary and Turkey to contribute 21 chapters in the fields of their expertise. This volume is a compilation of basic experimental surgery and updated advances of new development in this field that will be invaluable to any experimental surgery lab.

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The editors are grateful to Dr. Pierre Daloze for the writing of this foreword and reviewing these volumes. The editors also want to thank all authors for their contributions and the time they spent to make this book successful. They also appreciate Dr. Muhammad Zafarullah and Dr. Lijun Song for the contributing of their valuable time in proofreading the entirety of this book.

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SECTION I. BASIC NOTIONS AND TECHNIQUES IN EXPERIMENTAL SURGERY RESEARCH

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Chapter 1

HISTORY, DEVELOPMENT AND THE IMPORTANCE OF EXPERIMENTAL SURGERY

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ABSTRACT

It is an aphorism to say that surgery composes both science and art, only one of which still true. The science has often competently made medical and surgical progress both in the clinic and in research.

In this chapter, the history of medicine and surgery is reviewed in Babylon, Egypt, India, China, Japan, America, Canada, Russia, and during the eighteenth and nineteenth centuries in Europe. The clinical history of surgery remains the basic element of medicine. All medical history in this chapter will guide students through by covering all routine clinical situations, and will give the students the courage and tenacity they need. It also includes a series of clinical cases where surgical skills can be improved. The introduction to confirm the information is efficiently estimated in the history of treatment of bleeding, pain, and infection. We sincerely hope that from this information, the student and resident doctor will learn faster in their initial steps on the way to becoming a proficient and caring professional.

Keywords: experimental surgery, Babylon, Egypt, India, China, Japan, Europe, America, bleeding, pain, infection

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Abbreviations

B.C.	Before Christ
A.D.	Anno Domini (in the year of our Lord)
C.E.	Christian Era

Introduction

Modern surgery's success is the result of thousands of years of efforts by the pioneers encouraged by the same ideals as surgeons today. Chirourgia, is from the Greek, "cheir" meaning "hand" plus "ergon" meaning "work." A sixteenth century French surgeon named Ambroise Paré defined performing surgery as "to eliminate that which is overmuch, restore that which has been disjointed, separate that which has been integrated, join that which has been divided and repair the deformity of nature." (Bishop1960).

Bleeding, pain and infection were three major barriers for surgeons before the industrial revolution. Successes in these fields have reformed surgery from a delicate "art" into a scientific principle for the treatment of many kinds of diseases (Woodward 1963).

Babylonian Surgery

Babylonian medicine (Leonardo 1943) and surgery supposedly started off as the most primordial in nature. According to the records from unearthed cuneiform, diseases were assumed to be caused by evil essences and cures were obtained with the help of astrologers. Gradually, things changed and Babylonia became famous as a spiritual center around 2000 B.C. At that time, there was the constant existence of priests. Medical activity in this period also changed, accomplished by a medical god of the moon named Sin. Medical plants were picked up by moonlight to be used for medical preparation. Magic symbols played an important role even in the late Babylonian age.

One of the most famous documents of Babylonian medicine is named the *Code of Hammurabi* from about 2000 B.C. which detailed regulations about the legal side of the medical profession, but also described the usefulness of surgery in Babylonia. It was the first time dealing with operating fees in the *Code of Hammurabi*: "If a physician shall cause on anyone a severe operation wound with a bronze operating-knife and cure him, or if he shall open tumor (abscess or cavity) with a bronze operating-knife and save the eye of the patient, he shall have ten shekel of silver; if it is a slave, his owner shall pay two shekels of silver to the physician."

In spite of such details about management, nothing is known of Babylonian surgical techniques. At the beginning of seventeenth century B.C., the whole Assyro-Babylonian kingdom was destroyed by the war and almost all specific cultural practices and development were suffocated.

The Medes and Persians were a little more progressive in their medicine and surgery than were the Babylonians. Their book named *Zend-Avesta*, was written by Zoroaster himself. According to these fearful writings, the world is administered by good or bad spirits, and both

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their religion and medicine were based on a dual deity. At that time, there were three classes of physicians, the knife, the herb, and the word professional, but because of the lagging behind of the three groups, when patients were sick they had to consult Egyptian doctors. At late age, they resorted to inviting the best Greek physicians. The next step in the evaluation of human surgery was in the Nile Valley, in the kingdom of the Pharaohs, where the builders of Pyramids were developing an essential system of medicine and where dogmatic circumcision was being started.

EGYPTIAN SURGERY

Because of literature records, we know much more about Egyptian medicine and surgery. The *Edwin Smith Papyrus* is one of the crucial and brightening documents in surgery which was written in about 3000 B.C., around as old as the Pyramids themselves. The book was standardized and written as a textbook of surgery and not mixed with magic of later centuries, but was incomplete. It started with injuries of the head and sprains of spinal columns, but was missing abdominal organs and lower extremities. Forty-eight ordinary cases were described and each one was carefully examined before the diagnosis was made. The manuscript was exceptional and impressive, and of high scientific class. In the case of an injured head, the pulse was recorded and a digital determination of the wound was attempted in order to decide whether there was a fracture of the skull in the intact surface. The surgeon should have been familiarized with the symptoms from the brain, and discouraged fractured fragment of the bony skull with an elevator. The treatment of wounds was considered to be quite modern, where the wound edges were placed close together and bandages obtained from an embalmer to hold them together in position. Fractures were nicely detailed and there was even excellent technical management, e. g., in the fracture of the clavicle, where the patient should lie flat on the back for a prolonged time, with a sand-bag between the should-blades, a method which is often employed even today to ensure a good therapeutic result. All the details were specifically explained. Bandages and splints of linen soaked in glue were used. A crushed fracture of a vertebra of the neck, caused by an accident with the death of the patient, was specifically expressed and, in a lesion of the spinal cord, paralysis of intestines and bladder was reported (Smith 1972).

In the ancient surgical thesis, which was like "*Secret Book of Physician,*" it was advised how to treat a dislocated mandible. It showed directly how to place the doctor's hands to reduce the dislocation. A manuscript of the Fourth Dynasty (2900−2750 B.C.) showed a drill-hole in the mental gap for the purpose of draining an abscess under a molar tooth for which specialized metal instruments, probably made of bronze was approved. Thus, in this age, surgical instruments already existed which appeared in the surgical treatise (The Oriental Institute of the University of Chicago 1930).

The original edition of *Edwin Smith Surgical Papyrus,* perhaps written around 5000 years ago, was an essential book for the practitioner. Another valuable medical commencement was *Kahun Papyrus*, found in Faijum in 1889 by Sir Flinders Petrie. It mainly concerns gynecological subjects which were written around 2000 to 1800 B.C. The Brugsch Papyrus which was very small, consisted of many recipes, but was not very important for surgery. More important is Papyrus Ebers written about 1550 B.C. and found during exploration by

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Ebers at Luxor in 1872 A.D. This collection contains many medical texts of various ancient periods, especially dealing with medical treatment. Ebers himself considered that Papyrus was one of the lost sacred books.

From the dig, Egyptians found a variety of instruments, such as knives, hooks, cupping glasses, needles, forceps, etc. These instruments, found in a tomb near Thebes, are characteristic of the Bronze Age. The *Ebers Papyrus* contains almost one thousand prescriptions and therapies. But they mostly contain elements which either were ridiculous or fill us with dread and esteem. In more ancient times, it seems that the real pharmaceutical preparations were known. Later, however, appeals and prayers, magic hymns and sentences had to prepare the prescriptions. In this age, the following wounds were treated; gangrene, pus, pustules, abscesses and especially tumors of different kinds (fatty tumors, abscesses of the neck, enlarged glands, mammary growth), hemorrhoids, and foreign bodies. How did they treat the wounds? They used perfect methods of dressing; bandages of linen, soaked with honey and myrrh, and all had to be removed four days after surgery.

The history of Egyptian medicine has other important sources of information such as hieroglyphics carved in stones and monuments. Many surgical instruments were unearthed, and the discovery of metallic sacred improvement, usually made of gold or silver, describing parts of the human body which presented evidence of deformity or damage. Furthermore, the dead bodies were embalmed and protected, so that both physicians and embalmers had the opportunity to learn the basic knowledge of human anatomy, especially the interior anatomy of the body. The first anatomy book is said to have been written by King A, the son of King Menes who was of the first dynasty that ruled in 5241 B.C. (Manetho) or in 5702 B.C. (Boekh).

From the Papyrus Ebers and other sources, we know that some, but not all Egyptian physicians were priests. Herodotus and Diodorus Siculus were our main sources for the positions of physicians in Egypt in the fifth century B.C. According to the document, Herodotus described: “The art of medicine is thus divided among them: Each physician applies himself to one disease only, and not more. All places abound in physicians; some physicians are for the eyes, others for the head, others for the teeth, others for the intestines, and others for the internal disorders.” According to Diodorus Siculus: “in expeditions and journeys from the country, all treated free of charge, for the physicians received their salary from the state. They provided their services according to a written law compiled by many famous physicians in ancient Egypt…and if after following the laws from this sacred book they are not able to save the patient, they are safe from any allegation, but if they act contrary to the written law, they may expect capital punishment.”

For dentistry, the Papyrus only showed a few prescriptions, but when they examined mummies, it showed that the dentist possessed a certain degree of background in conservative treatment and replacement.

Ophthalmic surgery was relatively deficient. The Papyrus mentioned only cleanup for trichiasis, known as conjunctivitis, inflammatory corneal opacity, abscess of cornea, epiphora, ptosis, myosis, ecchymosis of lids, and milium.

Circumcision and sterilization were old practices in Egypt as established by many headstones of most ancient origin and excavations from 4000 to 5000 year B.C. They showed circumcision on a series of male corpses. The procedure was generally used on girls consisting of excision of the prepuce of the clitoris. It is believed to have been performed on both sexes during the fourteenth year of age.

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For centuries, Egypt was the world's principal contributor of sterilization, and castration was either performed by cutting with a knife of flint or by battering the testes to total extinction with a stone. Lithotomy was known from more recent sources and this surgery had been performed constantly for centuries. For many countries, these were a recognized part of medical and surgical training and practice.

Egyptian medicine from various sources and from many centuries of information formed the picture we have today. Priestly influences, tradition and cabalism, scientific cogitation and research were all incorporated later. Only when Persia became predominant did Egyptian culture, arts and sciences become plentiful for later generations, Egypt perpetuated some of the best medical and surgical conceptions (Tomnsend 2005).

CHINESE SURGERY

Chinese medicine and surgery were closely developed and connected with the history of Chinese advancements. There was an Emperor named Shen Nong who lived about 2700 B.C., who was respected as the chief god and primary architect of Chinese medicine. He was also respected as the father of acupuncture and medical herbs (Leonardo 1943).

There was plenty of medical literature recorded in ancient China. The most famous Chinese book was written by the Emperor entitled Huang-Di Nei Jing (2688−2599 B.C.) which represents the most ancient medical texts. Similar to other nations and countries, demonology and magic aided in the development of Chinese medicine. However, the influence of Chinese culture and philosophy gradually changed the exceptional cosmogony combined with a finding of experimentation that was designed into a kind of medicine which is totally different from any other nations except Japan and other Asian countries which were monopolized by Chinese medicine.

Confucius religion regulated the whole theory of the universe and hence also of medicine. The whole life is galvanized by two fundamentals: the positive *Yang*, which is always male and decisive, and the negative *Yin*, being female. These two fundamentals are always in the condition of balance and do not change with time. Yang means sun, force, light and heavenly quality. Yin means humidity, darkness, cold, etc. The domination of Yang or Yin regulates character, sex, sickness or prosperity. Yang, the male predominant, should always be an influence and in suitable relation to Yin. The Yin-Yang theory is still used in Chinese traditional medical practice at present.

Hua Tuo was a famous Chinese surgeon in Eastern Han and Three Kingdoms. He was the first surgeon to perform surgery with the aid of herbal anesthesia, around 1600 years before the practice was adopted by Europeans (Sherer 2004). Hua Tuo performed many laparotomies and the curettement of scapulas of soliders to cure caries of the bone. Bian Que (PienCh'iao) was a "marvel doctor" described by a Chinese historian named Sima Qian in his famous history book entitled Shiji, where he was cited with many skills. Another book entitled: Liezi (Tang Wen Pian) described that Bian Que exchanged two patients' hearts to cure their diseases (Kahan 1988; Graham 1961). This was a legendary story which means the concept of organ transplantation was fantasized around 300 CE ago in China (Schlich 2010).

Acupuncture and moxibustion are typical surgical procedures in the history of Chinese medicine which are currently in clinical practice. Moxibustion is a traditional Chinese

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medicine treatment using *moxa* made from dry mugwort (*Artemisia argyi*). Modern scientific evidence does not prove moxibustion can prevent or treat cancer (list of unproven and disproven cancer treatment 2013). But it still be widely used in traditional medicine in China, Korea, Japan, Vietnam, and Mongolia. According to the ancient theory, the decisive Yang and Yin are contained in twelve hypothetical and invisible channels named Chin, which are situated deep in the musculature. This channel system is not directly connected with blood vessels and nerves but nevertheless influences the whole human body, particularly the blood circulation. The distribution of Yang and Yin, the vital elements, is always changing, but they keep balancing each other all the time.

Chinese surgery has not made big progress over the past 1,000 years, but fortunately it did during last century due to the peaceful invasion of foreign missionaries. Modern medical schools and hospitals were established in most of provinces of China, not only by European and American doctors, but also by Chinese co-workers who had learned the philosophy and scientific knowledge. Since 1949, the Chinese government has authorized a policy to combine western medicine with Chinese medicine. In each county, there is at least one Chinese medical hospital. In each province, there is one Chinese Traditional Medical School to train Chinese traditional doctors. In any western medical school, there are courses to train medical students to combine western medicine with Chinese traditional medicine. To date, Chinese medicine has been transferred to 171 countries and districts in the world, according to the statistical data from WHO (Chan 2014). Chinese medicine has been legally accepted in Australia, Canada, Austria, Singapore, Vietnam, Thailand, and South Africa, etc., a total of 29 countries and districts. There are 18 countries or districts that have accepted Chinese medicine as their health insurance system (Chan 2008).

The Nobel Prize winners in physiology or medicine in 2015 were William C. Campbell, Satoshi Omura, and Youyou Tu. A number of serious infectious diseases are caused by parasites spread by insects. Malaria is caused by a single-cell parasite that causes fever. Traditional Chinese medicine uses sweet wormwood to treat fever. In the 1970s, after studies of traditional herbal medicines, Youyou Tu managed to extract a substance, artemisinin, which inhibits the malaria parasite. Drugs based on artemisinin have led to the survival and improved health of millions of people (The Nobel Prize in Physiology or Medicine 2015).

JAPANESE SURGERY

As in other countries, Japanese medicine had a mythical period with demons and evil continuity in the early stages. Magical incantations, prayers and sacrifices were the sole treatment methods in the ancient period. Bloodletting, covering of wounds with sawdust, and other very primeval therapies were the surgical treatments (Traditional Japanese Medicine 2017).

Very early on (around 1500 B.C.), Japanese culture and medicine were influenced and impacted by Chinese culture and medicine. Korea was the link between China and Japan, transmitting not only Chinese medicine, mainly for Buddhist acquaints but also to indoctrinate (about 560 A.D.). Young Japanese doctors were sent to China to learn medicine sponsored by Japanese government. They learned midwifery, the treatment of wounds, bandaging and acupuncture. Hospitals took care of old and sick patients in the temple of Nara

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and other associations. The Empress Gemmyo equipped the largest hospital for poor, sick people around 758 A.D.

Surgery in the Heian Period (784−1186 A.D.) mainly consisted of incisions and external abscesses with a knife, and cauterization of ulcers and dog bites. Abdominal surgeons liked using the bark of a mulberry tree to suture with threads the intestinal wounds, and also operated on atresia vagina.

The author named Fukuyoshi Omura in the Showa Era (834−847 A.D.) wrote the first dissertation on surgery, based entirely on Chinese surgery. During the Muromachi Period (1334−1568 A.D.), the wound surgeon emerged. Wounded soldiers and weak patients who were unable to do further military work learned medicine and took care of other wounded soldiers when the opportunity arose (Park 2012).

An important improvement was achieved when the Portuguese clergy came to Japan in 1568 A.D. Two physicians named Yariis and Geri-Gori, who belonged to this group, founded the “Surgery of the Southern Barbarian School.” They performed brilliant operations, treated the lepers and built hospitals. In 1585 A.D. the Christian religion was totally abrogated from Japan and all foreign physicians were discharged. Two Japanese students continued the practice of Portuguese medicine, settling in Sakai, and thus spreading foreign medicine by practice and by publication of medical books. Worthy of mention is a method of wound treatment and Gense Yamamoto’s *MangaiSbuyo*, a handbook of surgery. Other books were written by various authors concerning internal medicine, surgery, diseases of eyes, diseases of women and children acupuncture and moxa, mouth and tooth diseases, materiamedica and hygiene. 1765 was an important year for obstetrics because Gen-Etsu Kagawa published his work, and succeeded in taking away midwifery from midwives to the medical art.

In fact, Portuguese physicians had developed good and productive relations with Japan, at least for a certain period of time, which induced other nations to try the same. The Dutch were very successful establishing trade relations by starting a mission to come in contact with Japanese patients. In the seventeenth century, many Dutch medical books were translated into Japanese entitled *GekaSoden* (1706). Afterwards, Paré’s surgery was so demanded and definitive in Japan that it became almost equivalent with European surgery. Some years later, Dutch books were prohibited until 1702, then they were allowed again and their scientific knowledge was even promoted (1745).

A book on German anatomy, written by Johann Adam Kalmus was translated from Dutch to Japanese by several Japanese physicians, and other books followed. In the eighteenth century, there were many European medical books translated into Japanese, i.e., “*A New Work on Surgery*” or “*YoiShinsho”* by Gempaku Sugita and Gentaku Otsuki which mainly translated Lorenz Heister’s work. In 1832 the first institutionalized textbook on European surgery was translated into Japanese by Kincho Sugita. Many Japanese doctors followed and translated many medical textbooks. Medicine and especially surgery made great attainments during that time. In 1857, a medical school was established at Yeddo by Dutch physicians and then many medical facilities were established in the Kyoto Medical School and University of Tokyo. Soon, Muller, Hoffmann, Wernich, Gierke, Schultz, Baelz and others taught medical students in these institutes.

During the Meiji Period (1868 to the present), Japanese Medical Science closely followed Europe, America and international advances, building-up many modern medical centers, medical schools, medical institutes. Medicine and surgery are now distinctly state-of-the-art. Japanese medicine has given up the old methods as well as the transitory stage of

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learning.The Japan of today has contributed international humanity by their medical practice and major medical research discoveries (Traditional Japanese Medicine 2017).

HINDU SURGERY

In India, the earliest Hindu medicine and surgery developed in the Vedic era. The Vedic era acquired the name from four *Vedas*, which create the Sanskrit language and literature and were nominated as the Rigveda, Yagveda, Sâmaveda and Atharvaveda (1500 B.C.). Among these four, Rigveda and Atharvaveda possessed medico-surgical interest. In this epoch, the surgery of ancient Hindus reached an outstanding level. Atreya, Agnovesa, Charaka, Dhanvantari and Susruta who were the students of the latter, were all the first Indian physicians. During that epoch, the most important medical text books were *Charaka-Sambita* and *Susruta-Sambita*. At that time, Susruta concluded a more succinct treatment of his practice and full consideration of surgery. Susruta classified operations into eight different kinds: incision, division, scarification, puncture, probing, extraction of foreign bodies, evacuation of fluids and suturing. A surgeon who intended to perform this kind of surgery should equip himself with the following tools: blunt instruments, cutting instruments, caustics, probe, horn, leeches, bitter gourd, a tent or bougie made of black stone, cotton, pieces of cloth, thread, jute leaves, honey, clarified better, suet, milk, oil, emollient and astringent fluids, liniments, pastes, fan, cold water, hot water, iron pans and steady, calm and able-bodied assistants. Surgical operations should be performed on a propitious lunar day, star, and moment. The surgeon should be careful not to injure any vital part, vessel, nerve, joint, and bone.

Susruta enumerated 101 blunt instruments, the most important being the human hand. The instruments are usually made of iron. But sometimes made of other materials when iron is not available, like: "hooks, scalpels, scissors, saws, lancets, needles, probes, sounds, directors, trocars, forceps, syringes, catheters, bougies, vaginal and rectal specula."All the instruments are accurately described for the shapes and applications. There were many kinds of sutures: "winding like a sling, continued and interrupted sutures." And there were three kinds of needles: "round needles, three-sided needles, and abdomen and scrotum, a needle curved like a bow."

Before operations, the patients were prepared a diet. If it was an abdominal surgery, the patient should fast. Charaka and Sambita used wine as anesthesia to produce an anaesthesia effect to prevent pain. For the same purpose, Indian hemp (*Cannabis indica*) was used by inhaling the fumes. In 927 A.D., two surgeon brothers operated on a brain tumor of the King Dhar.A drug named Samohini was used for anesthesia. After trepanation of the skull, the tumor was removed and the wound was closed by stitches. The king was aroused by using another drug (Sanjivani). Sambita applied the forceps in case of a tough surgery. He described the procedure of craniotomy and caesarean section methodically.

In ancient Hindus, amputation of limbs was performed and the bleeding was controlled by pressure, boiling oil or cautery, but there was no mention of ligatures to control bleeding. They also could perform perineal lithotomy and via suprapubic operation. The author described detailed procedures for the surgery, pre- and post-surgery for the patient in the book of *Susruta-Samhita*.

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Plastic surgery was very crucial in ancient India. At that time, to cut the nose and ears was the retribution reserved for adultery and also often for retaliation. Rhinoplasty was described in detail. The original Hindu method of extracting cataracts is still applied in India. Their surgical work also involved hernioplasty, fractures and luxations. Susruta said: "Surgery is the first and highest division art, least liable to fallacy, pure in itself, perpetual in its applicability, the worthy product of heaven, the sure source of fame on earth." Wise said: "The Hindus were the first scientific cultivators of the most important and essential of all the departments of medical knowledge, practical anatomy." There were several famous universities in ancient India, each university specializing in one branch of knowledge. The Taxila and Benares were the most famous and earliest universities, and their medical schools had distinguished teachers. There were 100 lecture rooms and around 10,000 students in the Nalanda University. There were six considerable blocks of residential buildings, each with four stories.

The birth of Buddha degraded the decency of the Hindu civilization. The Buddhist Period (600 B.C. to A.D. 600) became destructive for anatomy and consequently for surgery, discouraging anatomy and forbidding the dissection of animals because of ethics reasons. However, hospitals for men and animals were elevated and formed all over the country and the acquirement of research in medicine and chemistry achieved great promotion in the following centuries, as many famous astronomers, mathematicians, philosophers, religious leaders, poets and dramatists emerged. In this era, anesthetic drugs or curare were discovered which induced patients' sleep or deep stupor. At that time, A.D. 1550, a famous Hindu physician, Bhava Misra, an important teacher and writer issued a book related to the circulation of blood.

Hindu medicine gradually degraded and fell down, but did not die. Hindu scholars translated the great works of Charaka Sustruta from Sanskrit to Arabic and Persian. In Latin translations of Avicenna, Rhazes, and Serapion, the name of Charaka was frequently mentioned. We conclude this section of Hindu surgery with the words of Charaka: "Not for self, not the fulfillment of any earthly desire of gain, but solely for the good of suffering humanity, should you treat your patients, and so excel all."

Surgery in India reached creditable heights, and can be compared with the Greeks, exceeding Egyptian knowledge of surgery. In these countries, the connection between religion and medicine made it a struggle to adopt surgery as a profession. Surgery gradually succeeded, realized its apex, and finally declined. The one reason we can see is the slump and destruction of surgical science by religious authorities and dogma, and the growth of mysticism and conjecture which controlled the Middle Ages (Fishman 2005).

SURGERY IN CANADA

The history of medicine in Canada was initiated in September 1535 when Jacques Cartier built his castle on the bank of the St. Charles River facing the Indian village of Stadacona, which became sabotaged with scurvy. One colonist, "evidently possessing a knowledge of surgery," held a postmortem examination to try to learn the cause of death and thereby possible save the remaining members of the crew. This was the first autopsy performed in Canada.

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Quebec City was originally established by Champlain in 1608. Around 1660 Jean Martinet accepted his brother-in-law Paul Prudhomme as a pupil to teach him the basics of medicine and surgery. This was the first recorded medical system in Canada.

Duchess d'Aiguillon assigned several nuns from Dieppe, obtained a grant of land, and built the Hôtel-Dieu in Quebec City in 1639. Michael Sarrazin, who came to Canada, was a

Surgeon Major of French troops. He was on the staff of the hospital. Later he wrote a book, *Anatomy of Beaver* and enlisted his research concerning Canadian animals and plants.

The small community at Ville-Marie continuously developed into the modern city of Montreal. Originally, Montreal was constructed around the year 1631 as a religious community.A society was arranged with 6 members in the beginning. One of them, Madame de Bullion, granted 42,000 books devoted to a hospital for the new community.

An expedition consisting of a company of 100 soldiers under the command of Paul de Chomedy, Sieur de Maisonneuve, came to New France in 1641, followed by Mademoiselle Jeanne Mance who had the task to create a hospital in Montreal. Without any interruption from the Indians, the colony grew up and a hospital was established under Mademoiselle Mance in October 1644. Very soon, the first wounded patient arrived for treatment. The founder and manager of the hospital, Mille Mance had to have the patient treated by a surgeon.

Around 1773, an ostensibly new disease came to the attention of the Government of Lower Canada, perhaps brought by Scottish soldiers. Sadly the distressed patients wanted to keep their disease secret, afraid it might be syphilis. Medical treatment was far away, and thereafter the disease was epidemically spread when the Government learned about it. Governor-General Carleton immediately assigned a surgeon's colleague to bring mercurial therapy to treat the patients which was effective in many cases. Drs. John Bowman and Robert Jones in Montreal speculated that the disease had nothing to do with syphilis. At that time there was no university for medical education in Canada. The only way was to send Canadians to study abroad or be trained by former army surgeons, mostly Scotsmen and Englishmen. In 1750, this state of affairs was changed by Intendant Bigot, and every physician had to pass an examination. In 1788, the British Parliament decreed that within Lower Canada, and in the towns of Quebec and Montreal, physicians, surgeons and midwives could not practice without a license. The Act of 1847 united the physicians in Lower Canada under the title of "*The College of Physicians and Surgeons of Lower Canada*." In 1826, the first medical society was founded and the first medical journal was published.

As the French and English migration gradually increased, this enlargement of population lead to the spread of epidemic diseases like scurvy (which was a harmful influence to envelop Canada), and smallpox which caused a great number of deaths. The four epidemics of 1703, 1732, 1733, and 1755 were very harsh. Many attempts were made, but without much achievement, until Dr. Latham, a surgeon of the king, launched preventive vaccinations in 1768. Another disease probably was plague, occurring during three epidemics in 1711, 1718, and 1740. It was introduced by a ship coming from Thailand. There were no details of symptoms. Conditions were unhygienic at that time. Many epidemic diseases were liable to be spread.

For the treatment of typhoid fever, ice was used to reduce the temperature in the early 1780s. Asiatic cholera ran its five disasterous epidemics in the colonies (1832, 1834, 1849, 1852 and 1854), and in three months 2,215 people died in Quebec City alone.

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In 1815 in Montreal, after the war of 1812 was over, the population was increased to 20,000. It was the center of colonization and commerce. Meanwhile, there were plentiful sick and poor people needing medical care. At that time, the General Hospital of Montreal was established. In 1822, when lectures were presented by Drs. Holmes, Stephenson, Robertson and Caldwell, a medical school was contemplated. A year later, Montreal Medical Institution was established. Since 1829, this institution has existed as McGill University, named after the Hon. James McGill who facilitated the Royal Institution's upgrading of learning to found the university. In June 1829, the governors of "Burnside University of McGill College" had their first meeting to issue the Charter and the commencement of the university.

Quebec City as early as 1837, had a population of approximately 35,000 and Sir John Doratt, MD., inspector of General hospitals, proposed to establish a medical school in Quebec which was an important port at that time. There were numerous incoming vessels and sailors amenable to the transfer of infectious diseases and who suffered from accidents during their activities of loading and unloading. It was also not necessary to send medical students abroad. The curriculum in the new medical school included three years of course participation in the school, and two years of clinic commitment in the hospital.

When Lower Canada and Upper Canada became united in 1840, the medical science profession of Lower Canada already had a good influence and high standards, based on the experiences of the French and British universities, especially the University of Edinburgh and McGill Medical School. Up until 1855 McGill University was piloted and authorized by clergymen. When Sir Edmund Head became Governor-General of Canada, he assigned a physician, Dr. William Dawson as a new principal. Dr. Dawson was born in the town of Pictou, N. S., Canada in 1820. He had accomplished education in the schools of Nova Scotia and Edinburgh. His appointment marked the beginning of steady progress for the Canadian universities.

Lister's teachings on antisepsis were the example as Canadian disciples of Lister: Stewart of Halifax, Malloch of Hamilton, Grasset of Toronto, and Blanchard of Winnipeg. Dr. Chipman and Dr. Tait from Edinburgh taught students at McGill University. In 1884, William Osler, the most characterized alumnus of McGill, became Professor of Medicine at the University of Pennsylvania, later at Johns Hopkins Medical School, and was finally promoted to Professor of Medicine at Oxford.

There were only 25 students in the Montreal Medical Institute in 1823, but in 1920 there were around five hundred students. By then the contemporary McGill University was well established and famous in the world. In the early stages, it required a five-year course for students, and gradually the qualifications became much more ambitious, therefore McGill has always kept the highest possible standards in medicine and surgery (Tomnsend 2005).

SURGERY IN EIGHTEENTH CENTURY

France

During the eighteenth century, surgery had made more progress in France than in other countries. Paris became the outstanding medical and surgical center in Europe and so many medical students wished to study medicine there that the medical schools could not accept all

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of them. The barber surgeons were not recognized in the universities and they did not have the permission of institutions in which they were taught. In reality, they had learned in the battleground. It was disputed that such experience was valuable enough to train surgeons with their knowledge of anatomy and pathology. The main reason for the dominance of French surgery in the seventeenth and eighteenth centuries was the war undertaken by Louis XIV and his successors.

The leading surgeons in the early eighteenth century were Mareschal, Méry, Dionis, De La Peyronic and Petit. Georges Mareschal (1658−1736) was the chief surgeon to Louis XIV from 1703 and did surgery for many years at Charité Hospital. A serendipitous fistula bolstered by Louis XIV, which the surgeon Felix had cured, led the king to take a marked interest in surgery which at the commencement of the century was still at the beginning stage of medicine. Felix became the royal surgeon and was acceded by Mareschal. In 1724 Louis XV generated five professorial chairs of surgery at Saint-Côme.

When the Academy of Surgery was established by the king in 1731, due mainly to the efforts of Mareschal and La Peyronie, followed by the authorization of 1743, which clearly separated the surgeons from the barbers, surgery was granted the respectability and rank to which it had long been denied. After that, no one could be a master of surgery without first becoming skilled in arts.

The profession of surgery obtained its liberation in France mainly due to the work of Mareschal and François De La Peronie, the humanitarian surgeon who worked during the course of the eighteenth century to raise the status of his favorite art.

Jean Méry (1645−1722) was the chief surgeon at the Hôtel-Dieu who designed an operation of suprapubic puncture of the bladder. He also focused on the anatomy of the ear. Pierre Dionis trained in anatomy and later operative surgery on cadavers at the Jardin-du-Roiin Paris in the beginning of 1673. He published*L'anatomie de l'bomme, suivant la circulation du sang et les dernières découverts* (Paris, 1690); *Cours d'opérations de chirurgie demontrées au Jardin-du-Roi* (Paris, 1707); and *Traité général des accouchemens* (Paris, 1748). His books were very popular for 50 years, going into numerous editions and translations, even Chinese. He recommended using bands in amputations. Concerning lithotomy, he said the Marian operation was the safest and was perfect, but the suprapubic surgery could also be safe. To his knowledge, M. Bonnet had achieved the highest outstanding rate of operation at Hôtel-Dieu. His earlier work on anatomy was animated by Harvey's discovery of the circulation.

Francois de la Peyronie (1678−1747) was one of the most dominant French surgeons in this period. He was born in Montpellier in 1678 and studied in Paris. He later returned to his native city, where after certification, he soon became the chief surgeon in the Montpellier Hôtel-Dieu. He was requested in Paris in 1714 to treat the Due de Chaulnes whose case he treated very successfully. La Peyronie was hired as a surgeon in Paris. Some years later he became the chief surgeon of the Charité Hospital where he established an extremely large surgical practice. He was well equipped with funding from the Academy of Surgery in Paris in 1731 and the welfare of the Academy was always in his mind. The struggle between the physicians/surgeons and barbers ended in the complete separation of surgeons from barbers in 1743 when La Peyronie successfully made a convincing argument. Indeed he took every step in the first half of the eighteenth century which resulted in the greater well-being, esteem and greatness for the surgical profession. La Peyronie spent a large part of his affluence and

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prosperity on the Academy of Surgery of Paris which contributed to making Paris the surgical capital of the world.

The leading scientific surgeon of the first half of the eighteenth century was Jean Louis Petit (1674−1750). He was trained in Paris and in early 1700 he gave lectures in anatomy and surgery. Later he was promoted to director of the Academy of Surgery and was a surgical consultant in Poland and Spain. Petit enormously improved the surgical procedures of circular amputation by cutting the skin and muscle at different levels instead of the beheading method. He also improved how a herniotomy could be performed without opening the sac. Petit was one of the first to perform a cholecystotomy which was first introduced by FabriciusHildanus in 1618. However the operation was not performed with regularity until a century later. Petit abhorred: "How many people have died because this disease was not perceived of, because no operator could be found who would undertake to rid them of their disease by means of an operation." Petit was the first surgeon to open the mastoid and also invented the screw tourniquet. He published "*Traité des maladies des os*" in 1723 in Paris, and his notable; "*Traité des maladies chirurgicales et des operations*" in 1774 also in Paris.

Jean Baseilhac was born in 1703 of a surgeon's family, at Poyestruc, Department of Hautes-Pyrénees, France. He studied at Hôtel-Dieu at Lyons under the supervision of his uncle, also a surgeon. Two years later he went to Paris to enter the service of surgery at Paris Hôtel-Dieu as a regular undergraduate. In 1740, he was allowed to practice surgery among the working classes. He then proved extraordinary as a lithotomist and improved the surgical methods and devised surgical instruments. He was successful in establishing a special hospital for lithotomy patients in 1753 and stayed in active service until his death.

The Hôtel-Dieu in Paris was founded in 850 as a religious foundation similar to all hospitals in the Middle Ages. The English hospitals became international reformations, but they continued for a long time and their system is followed even now. The physicians and surgeons, like their regulations were for life time assignment and allowed their private practice. People could pray twice a day, and the hospital was provided with its own church.

Jean Astruc (1685−1766) was very experienced in syphilis and obstetrics. Barthélemy Saviard (1656−1702) was a good wound surgeon, who published "*Nouveau recueild'observationschirurgicales* (Paris, 1702). An English translation version appeared in 1740 in London. The author characterized the successful use of a tourniquet in the case of injury to the femoral artery needing ligation or digital constriction for the treatment of aneurysm.

René Jacques Croissant de Garengeot (1688−1759), born in Vetré in Bretagne, advanced almost all parts of operative surgery and was considered a very proficient anatomist. Hisworkswere: "*Traité des opérations de chirurgie*…. (Paris, 1731), and *L'operation de la taille par l'appareil lateral, ou la méthode de frère Jacques, corrigée de tous ses défants* (Paris, 1730). He improved many surgical procedures for strangulated hernia, hydrocele, hare-lip, lacrimal fistula and nasal polyps. He performed tracheotomies with a cannula, invented a special tourniquet and restored a nose which had been cut off and had lain for an appreciable time upon the ground.

Henri François le Dran (1685−1773), a surgeon in Paris, wrote many books on surgery and managed his anatomical school at the Charité Hospital of Paris. He was a distinguished teacher and his German students advanced his surgical methods throughout Germany. He was also a good lithotomist and a military surgeon.

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François-Sauveur Morand (1697−1773) was the first secretary of the Royal Academy of Surgery in Paris, and a very skilled surgeon even though he was not well educated. He performed disconnection of the upper arm and, in selected cases, disarticulation of the thigh. He practiced ovariotomy and removed urinary bladder stones. He was the first one to describe a case of osteomalacia in 1753. He used a hot iron to stop bleeding in very urgent cases of hemorrhage, or when the large number of vessels was difficult to ligate.

Claude Nicolas Le Cat (1700−1768) of Rouen, became the chief surgeon of the Hôtel-Dieu in 1729 and later a professor of anatomy and surgery in Rouen Medical School. He was famous as a lithotomist. He was a productive writer and received the "prize-essay writing."

Dominique Anel (1678−1725) of Toulouse was an experienced military surgeon. In 1710 he ligated the brachial artery of a priest in Rome because of traumatic aneurysm. This was regarded as a proud French surgery, earlier the method of John Hunter. In 1712, Anel was set up in Genoa, Italy where he designed his operation for lacrimal fistula with the Anel probe and syringe. Four years later, he returned to Paris practicing ophthalmology.

George Arnaud de Ronsil, a French surgeon practiced for many years in London before 1748. He wrote a valuable "*Dissertation on Hernia* (London, 1748)," and a two-volume work "*Memoiresde chirurgie* (London, 1762)." In 1732, he cut the caecum together with part of the terminal ileum and ascending colon in a case of strangulated hernia with necrosis of the intestines.

Alexis Littré (1658−1726) was the first to construe the type of hernia and the first to recommend the operation of colostomy (1710). His most beneficial publications were involved with pathological anatomy.

Nicolas Andry (1658−1742) of Lyons was the first to use the word: *orthopaedia,* now globally used. His work was designated: *L'orthopaedie, ou l'art de prévenir de corriger dans les enfans les difformités du corps* (Paris 1741). His second work was: *Suite d'orthopedia* (Paris 1742).

Pierre Brasdor (1721−1797), professor of anatomy and surgery in the Paris College of Surgeons, proposed that certain aneurysms be treated by distal ligation which was the first one performed in history. George de la Fave (1699−1781) removed the crystalline of the eye by simple pressure after making an incision with the cataract knife which he created. Furthermore, he transformed the Dran's operation for disarticulation of shoulder. His work "*Principles de chirurgie* (Paris, 1739) was very popular with medical students.

Antoine Louis (1723−1792) of Metz was the perpetual secretary of the Académie de Chirurgie. He organized the Academy activities which impacted the progress of French surgery. His main workswere: *Cours de chirurgie pratique sur les plaies d'armes à feu (Paris, 1746); Recueil d'anatomie et de chirurgie, pour servir de base à la théorie des plaies de têtes par contrecoup* (Paris, 1768).

Raphael Bienvenu Sabatier (1732−1811) was a professor at the Académie des Sciences. He was a lecturer on anatomy at the Royal College of Surgeons, Paris. He devoted himself to ophthalmology and surgery. His chief work was *De la méedecineoperatoire* (3 volumes, 1978−1810).

Jean Louis Belloq (1730−1807) was a professor of anatomy in Paris and designer of numerous surgical instruments including a cannula for plugging the posterior nares, still known by his name.

The most important French surgeon of the latter part of the eighteenth century was Pierre Joseph Desault (1744−1795). He had studied under Louis and Morand, and after contributing

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several years as a surgeon of the Charité (1782), Desault became a surgeon in Hôtal-Dieu in 1788. He had numerous surgical students and established the first surgical anatomy in France and improved the system of operations by designing new instruments, and creating an important surgical journal, the *Journal de Chirurgie* (1791−1792). His bandage for the fractured clavicle is well known even today. His student Bichat, detailed the materials of Desault's surgery in his *Oeuvres Chirurgicales* (3 volumes, 1798−1803).

J. Fr. Deschamps (1740−1824), a student from Moreau and a surgeon in France, wrote a history of lithotomy in 1796. Antoine Portal (1742−1832) was a professor of anatomy and the author of a seven-volume work on the history of anatomy and surgery.

Germany

German surgery in the eighteen century was represented by George Fischer in his exceptional treatise *ChirurgievorIooJahren* (Leipzig, 1876), by which one learns that surgery was proceeding there by itinerant charlatanry and the local barbers. The people themselves were grossly roving and irrational. They believed in charms and relics, and in the efficacies of the travelling stone-cutters, bone-setters and cataract-excision in spite of the opposition of the physicians and numerous commandments issued by their king.

Even the executioners competed with the surgeons. They were supposed to have special knowledge of the means of curing diseases considered to be due to witchcraft. A part of their business was to disjoint joints by the shelf or to break bones upon the wheel, and thus it was presumed that they had special skills in the repair of fractures or dislocations. Only much later do we find a real influence in that respect in Germany.

Lorenz Heister (1683−1758) was the first surgeon of significance of this era. Coming from Frankfort-on-Main, he started his studies in Giessen, but maintained his proper medical education in Leyden and Amsterdam. Under the guidance of Ruysch and Rau he extended and developed his competence in the army hospitals at Brabant and Flanders. He was appointed as professor of anatomy and surgery at the university. Heister started lectures in Latin, but his students were so naïve that he decided to print his text book of surgery first in the German language (Chirurgie, Nurenberg, 1718). The work was widely advanced and soon translated into Latin as *Institutions Chirurgicae* and then into English as *A General System of Surgery* (London, 1743). This book was adored: "The first complete systematic work on the science of surgery," and was still used as a text-book in Vienna as late as 1838. For one hundred and twenty years it served as an aid in education for medical students. Heister wrote around 200 different works. He was a good dentist, and in the field of eye diseases, he depicted that cataracts depend on the cloudiness of the lens.

Albrecht von Haller (1708−1777), of Bern, Switzerland, was a considerable physician in Germany. From 1736−1753 he was a professor of anatomy and surgery at the University of Gottingen. He exerted an excessive influence in Europe and pupils crowded to him from everywhere. Haller issued *Bibliotheca chirurgica* (two volumes, Bern, 1774−1775). Many famous professors respected him: "He placed on a firm foundation the experimental method of dealing with surgical problems which was so unsuccessfully employed by John Hunter, but which increased our knowledge during the present century."

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Johann Zacharias Platner (1694−1747) was a professor of anatomy and surgery at Leipzig from 1724 until his death. His *Institutioneschirurgae rationales tum medicae*, *etc.* (Leipzig, 1745) has been published in several editions and was translated into German and Dutch. Burchard David Mauchart (1696−1751), professor of anatomy and surgery at Tubingen (1726) worked chiefly on the anatomy and surgery of eyes.

To refresh the story of German army surgeons, the Friedrich Wilhelms Institute was founded in Berlin in 1724, and three years later, King Friedrich Wilhelm I established the Charité Hospital for the clinical training of medical and surgical students of the college. In 1748, a military, medical and surgical school was settled at Dresden; in 1795 the Friedrich Wilhelm Institute of Berlin was restructured into an entirely military medical school which was called the Pépinière.

The three most important German military surgeons of this era were Johann Ulrich Bilguer (1720−1796), Johann Lebrecht Schmucker (1712−1786), and Johnn Christian Anton Theden (1714−1797). Bilguer was born in Chur, Switzerland, studied in Basel and Paris, Schmucker, like Haller entered the army in 1741 and later became surgeon-general of the army. He wrote *De membrorumamputationerarissimeadministranda (1761)*, because of the too frequent use of amputation. Schmucker, also a Prussian surgeon-general, disagreed to trepanning. He started the therapy of skull injuries by cold poultices. He published *ChirurgischeWahrnehmungen* (2 volumes, 1774) and *VermischteChirurgischeSchriften* (3 volumes, Berlin, 1776−1782). Theden was a Prussian surgeon-general in 1786. He invented an elastic catheter and a covering of caoutchouc and wrote *NeueBemerkungen und ErfahrungenZurBereicherung der Wundarzneykunst und Medicin* (1771). Famous professors respected the surgical methods described by Theden as they were so remarkable in surgical practice.

In the latter half of the eighteenth century, the leading German surgeon was Carl Caspar von Siebold (1736−1807), the creator of the surgical school of Wurzburg. He was a pupil of Morand in Paris, and of Pott and Bromfield in London. He was the first surgeon in Germany to perform a symphyseotomy. In Wurzburg he taught as a professor of anatomy, surgery and obstetrics.

August Gottlifeb Richter (1742−1812) was a professor of surgery in Gottingen in 1766 and helped to found the scientific discipline of surgery in Germany. He toured widely and advised with the best French and English surgeons of that time. His work *Abbandlung von der Briichen* (1777−1779) mainly explained how the hernia was investigated and was the best monograph up to that time.

In the eighteenth century, surgery made little progress in Austria. In 1785, the Military Medico-Chirurgical Akademie was founded by Emperor Joseph II in Vienna. The courses were taught for two years and clinical instruction was accessible for one hundred students training with the patients of a twelve hundred bed hospital in Vienna. The graduates received Doctor of Surgery degrees and were assigned to army duty as soon as possible.

Giovanni Alessandro Brambilla (1728−1800), a native of Italy and a student of the University of Pavia, became a surgeon in the Austrian army and was an exceptional benefit to the Emperor. He wrote a book of guidance for the professors at the Military Academy (1784) and composed a large atlas of exploration made by the Italian anatomies and physicians.

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Great Britain

At the beginning of the eighteenth century, little adequate surgical instruction was available in England. The surgical student usually sought learning with a practicing surgeon. The convenience for studying anatomy was almost none. By order of Henry VIII, who broke up the abbey, the hospitals were taken from the Church by the government. St. Bartholomew's Hospital was re-built in 1544 and students and beginners were permitted to observe operations. It was not until 1763 that a series of systematic lectures on surgery were distributed there, first given by Percival Pott. At St. Thomas' Hospital in London, lectures on anatomy and surgery were launched by William Cheselden in 1720, and the Anatomical School was begun two years later. In 1785, the medical school was fully established. The Medical School of Guy's Hospital began in 1769 and soon the schools of this hospital and of St. Thomas were collaborating. The surgical lectures were given at St. Thomas and the medical courses at Guy's Hospital.

With the Act of 1540, the barbers and surgeons were combined into one company, but in 1745 the surgeons were able to separate themselves from barbers, and in 1800, George III issued a charter for the consolidation of the Royal College of Surgeons of London.

In Scotland in 1694, Archibald Pitcairn tried to start a medical school at Edinburgh. Only in 1726, was the medical school finally achieved chiefly through the services of John Monro, a retired army surgeon. William Cheselden (1688−1752) was the leading English surgeon in the first half of the eighteenth century. Gradually, the education system was improved and the requirements for better anatomical teaching through a system of private schools was established. William Cheselden was one of the earliest of those private teachers. At the age of twenty-three, he started a course of lectures on anatomy. He published "*Anatomy of the Human Body*" for thirteen editions. His practical point of was to treat it as applied anatomy. His awesome work "Osteographia" is perhaps the finest illustrated book of its kind. He was the best known surgeon to improve the operation of lithotomy.

Other surgeons of high dignity in London who followed Cheselden were: Samuel Sharp (1700−1778) at Guy's Hospital, Edward Nourse (1701−1761) at St. Bartholomew's Hospital; and William Bromfield (1712−1792) at St. George Hospital: all of whom gave courses in anatomy and surgery at their homes, and later moved to their hospitals. Samuel Sharp, a traditional surgeon with great intelligence, was the author of two books on surgery; "*A Critical Enquiry into the Present State of Surgery*" (London, 1750), and a "*Treatise on the Operations of Surgery*" (London, 1751); both of which were prevalent and went through many editions. Sharp devoted much to our present knowledge of the anatomy of hernia. He further designed the modern trephine used for craniotomies in brain surgery.

William Bromfield, also an authentic surgical author, published his "*Chirurgical Observations and Cases*" (two volumes, 1773) which consisted of important extensions to surgical techniques, especially concerning reciprocal lithotomy. William Beckett (1684−1738) of London wrote "*Practical Surgery Illustrated and Improved* (London, 1740). Benjamin Gooch, a surgeon of Norfolk and Norwich Hospital, published "*Cases and Practical Remarks in Surgery*" (London, 1758). He illustrated that in cases of wounds of arteries, ties must be above and below the wound.

James Douglas (1675−1742) was a Scottish physician and his brother, John Douglas, was a surgeon. James was a famous anatomist and obstetrician and his work was "*Description of the Peritoneum*" (London, 1730). John Douglas (1680−1742) discoursed on surgery and

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anatomy in London around 1720 and worked mainly as a lithotomist. His work entitled: "*LithotomiaDouglassiana*" (1720) described the operation of stone. Douglas was a student of Cheselden who was by far his superior.

During the middle of the eighteenth century, the most prominent English surgeon was Peacival Pott (1714−1788). In 1756, Pott was 42 years old, and fell from a horse and suffered an admixture fracture of his ankle. The term *Pott's fracture* has been applied to this kind of fracture since that time. Pott defined the fracture in 1769 in "*Some Few General Remarks on Fra*ctures." His "*Treatise on Ruptures*" was distributed at the end of the year. He then wrote at more frequent intervals, practical and succinct articles about hernia, hydrocele, fistula of lacrimal duct, head injuries, fractures and dislocations, chimney-sweep's cancer, paralysis due to disease of the vertebra, and others. The most accepted of his publications, "*Remarks on that kind of palsy of the lower limbs found to accompany a curvature of spine*" was published in 1779. These kinds of diseases of the vertebra had since been known as *Pott's disease*.

John Hunter (1728−1793), the greatest surgeon in the eighteenth century was equally famous with Paré and Lister as one of the three most dominant surgeons at that time, and the founder of experimental and surgical pathology. John Hunter came to London in 1748, two years after his brother William Hunter had begun to lecture there on anatomy and surgery. Hunter commenced surgery at St. Bartholomew's Hospital under Pott and Cheselden who with Pott and William Hunter were the principle surgical personalities in London in the eighteenth century. By 1754, John Hunter was a partner with his brother in the research of anatomy and physiology. During the two years from 1761 to 1763, Hunter served as a surgeon in the Spanish military campaign. He collected much valuable material for his later writing on gunshot wounds. By 1768 his surgical capability had allowed him to be elected as a surgeon in St. George's Hospital.

For the next twenty-five years Hunter worked enthusiastically in research of anatomy and pathology, and teaching his students. His work was accumulated as a great collection in the form of Hunter's museum of biological exhibits, which finally categorized 13,000 objects. He found that surgery was a science as well as an art. He studied and represented surgical circumstances such as surgical shock, phlebitis, pyemia, inflammatory processes, the lymphatics, and lacrimal ducts in the man. He was the first one to discriminate between hard and soft chancre, and the first one to study teeth experimentally. Hunter's considerable work was on the surgical pathology of the vascular system. He found that if the surgery was not applied to an aneurysm in its primeval state, amputation of a limb was the result for most patients. England became the surgical capital in the eighteenth century, attracting students and patients from many countries due to its excellent surgeons. Because of the great figures who influenced the surgical scene, all the outstanding surgeons were pupils of John Hunter, e.g., Abernethy, Cline, Astley Cooper, Blizard, Carlisle, Hey Leeds, Parkinson, Physick, Dorsey and the great physician Edward Jenner. John Hunter was always active and intelligent, and his interests were boundless.

One of the most famous Edinburgh surgeons of the eighteenth century was Benjamin Bell (1749−1806), a pupil of Munro and a surgeon at the Royal Infirmary. His *System of Surgery* (Edinburgh 1783−1787, 6 volumes) was issued in many editions, and was translated into French and German. He was one of the first surgeons to prevent and treat pain in surgical operations. He was also devoted to the treatment of infections after surgery. He published: "*On the Chirurgical Treatment of Inflammation.*" He also improved the methods of

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amputation "Save skin" was one of his surgical slogans, and he applied the principle during amputations and the cutting of tumors.

John Bell (1765−1820) became the pupil of Alexander Wood to whom he dedicated the first volume of "*Anatomy of the Human Body*" (1793). He became a Fellow of the Royal College of Surgeons (1786), lectured on anatomy and surgery for ten years in the extra-mural school at Surgeon's Square, and expanded into a leading consulting and operating surgeon in Edinburgh. He was a creative contributor to surgical literature. He wrote a three-volume work, *The Principles of Surgery* (London, 1801−1808), where chiseling was very attractive. He worked in defending the "doctrine of anastomosing arteries" in order to considerably reduce amputations due to the extensive injury of main arteries. In wounds and operations, he highlighted two points to be attended to: first, securing of arteries so that the patient may not be in danger from bleeding, and second, procuring a speedy adhesion by which pain, infection, waste of tissue and other adverse repercussion of wounds are prevented as much as possible. His brother was Sir Charles Bell, another famous anatomist and physiologist.

Prior to 1784, the eighteenth century had not made much progress in Ireland. The barbers and surgeons had a stabilized company which was created in 1446 when King Henry VI established a federation of barbers "for the promotion and exercise of the art of chirurgery." In 1572, Queen Elizabeth granted a second permit to the barbers and surgeons, and a third charter was given in 1687 by King James II. In 1784, the union between the barbers and the surgeons was dissolved and the Royal College of Surgeons of Ireland was constituted. Medical teaching began in Dublin in 1744 and perhaps the earliest Irish surgical author was William Dease (1752−1798), one of the founders of the Royal College of Surgeons in Dublin and an active lecturer and teacher. The second essential surgical author of Ireland was Sylvester O'Halloran (1728−1807), an outstanding surgeon from Limerick who had studied in Paris, London and Leyden. His work, *A Complete Treatise on Gangrene and Sphacelus, with a New Method of Amputation* was very famous at that time.

SURGERY IN NINETEENTH CENTURY

Great Britain

In the history of surgery in the nineteenth century, important advancements were the introduction of anesthetics, the establishment of aseptic and antiseptic surgery based upon the scientific foundation of the new science of bacteriology, the development of traditional surgery in the treatment of diseases and injuries of extremities with plastic surgery and orthopedic surgery, the rise and progress of abdominal and intracranial surgery, the entry of two nations, the United States and Russia into the fields of educating surgeons, the formation of surgical societies and associations, the removal of restrictions on the study of anatomy, the great progress made in pathology, the founding of museums like those of Hunter and Dupuytren,and the development of ophthalmology, otology, gynecology, dermatology, and laryngology into their present highly functional forms, all of which contributed a positive influence in the development of surgery.

"The Industrial Revolution" era had made more progress in surgery since 1800 than had taken place in the preceding eighteenth hundred years. Due mainly to the fiery effect of

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Hunterian teachings, London was one of the surgical capitals of the world in 1800. The great surgeons who operated and lectured there, were practically all advocates and adherents of Hunter.

Sir Astley Paston Cooper (1768−1841), more than any other of the surgeons who made the brightness of London glimmer so brilliantly, acquired the mantle of John Hunter.

Cooper came to London in 1784 from Norfolk. Young Cooper was first student of his uncle William, then a surgeon connected to Guy's Hospital. Cooper soon worked with Henry Cline, one of John Hunter's first pupils, who was then at St. Thomas'. Under Cline's supervision, Cooper began extremely hard work in anatomy. For the next two years he established the foundation for his complete knowledge of anatomy and surgical pathology. He appeared at Cline's lectures at St. Thomas', and also sat before Hunter himself at St. George's. He even went to Edinburgh in 1787 where he spent one year for his leading study in surgery. Back in London, Cooper worked under Cline again. Attending John Hunter's lectures, he also acquired much knowledge from work in Hunter's dissecting room. Cooper made so much progress in 1789 that he was assigned a demonstrator position in anatomy at St. Thomas' and then as assistant lecturer to his mentor, Henry Cline.

From that time forward, his progress was constant and he held the posts of lecturer in surgery and anatomy at St. Thomas, became professor of anatomy and finally in 1800 was appointed as a surgeon at Guy's Hospital. During these seven years he adhered forcefully to the great Hunterian principles of learning from anatomy and physiology what could be done in surgery. In 1800, he presented to the Royal Society his important surgical work, *Observation on the effects which take place from the destruction of the membrane Tympani of the Ear and Further Observation*, in which he showed that a breach of the ear drum did not always produce deafness and could, in cases of stoppage of the Eustachian tube, really improve hearing extremely by equalizing the pressure on both side of drum. The Royal Society awarded him the Copley Medal for this work.

Cooper's work, *Treatise on Hernia* (London, 1804, 1807) was the product of an ample bulk of surgical work on this problem performed both on cadavers and on living patients. Cooper was promoted as professor of comparative anatomy at the College of Surgeons (1813) and was then at the peak of his career. The amount of work he did in the lecture hall, in the operating room, and in the anatomy room was excessive. In 1816, Cooper performed his greatest operation to ligate the abdominal aorta, the greatest of the arterial vessels after performing the same style of a research operation in animals was satisfactory. Cooper received a baronetcy for his work in removing an infected sebaceous cyst from the scalp of King George IV.

Cooper, an irresistible personality, was greater as an operator than as a contributor to the science of surgery. His prominence at the height of his career was huge and the size of his practice truly astonishing. He was the greatest of Hunter's surgical pupils.

Sir William Lawrence (1783−1867) was exposed to anatomy for twelve years. In 1806, he was awarded a prize by the Royal College of Surgeons for a publication on hernia. He was an assistant surgeon in St. Bartholomew's hospital (1813), then he became professor of anatomy and physiology at the Royal College of Surgeons. In 1823 he achieved a position as a lecturer on surgery at St. Bartholomew's Hospital and was affiliated with the Aldersgate Medical School. His principal works were *Treatise on the disease of the eye* (1833), and his *lectures on Surgery* (1863).

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James Wardrop (1782−1869), who was born in Scotland and educated in Edinburgh, founded the West London Hospital of Surgery (1826) and instructed at the Aldersgate Street Medical School. In 1828 he became the surgeon of King George IV and seven years later began allocution in surgery at the Hunterian Medical School. Wardrop was the author of Morbid Anatomy of the Eye (Edinburgh 1808−1818, two volumes). He contributed to *Venesection* (1835) which was published in Philadelphia (1857), and was translated into Italian (Pisa, 1839) and German (Leipzig, 1840). Wardrop was an experienced surgeon, performing aneurysm surgery.

Joseph Constantine Carpue (1764−1846) was another important English surgeon in this era. He introduced the Indian operation for rhinoplasty and re-affirmed the value of suprapubic surgery for stone. Samuel Cooper (1780−1848) issued a highly popular *Surgical Dictionary,* (1809) which was the first detailed work in this field. Alexander Copland Hutchinson, a naval surgeon and author, published *Practical Observations on Surgery* (1816) and *Some Further Observations on the Subject of the Proper Period for Amputations in Gunshot Wounds* (1817).

Charles Aston Key (1793−1849) was a surgeon at Guy's Hospital when Cooper induced the authorities to found an autonomous medical school there and Key became a professor of Surgery in 1825. That year he ligated the subclavian artery and five years later he ligated the carotid artery because of an aneurysm. He also introduced some improvements in operations for hernia and lithotomy.

Sir Benjamin Collins Brodie (1783−1862) became Sir Everard Home's pupil at St. Grorge's Hospital in London. He lectured on anatomy at the Great Windmill Street School from 1805 to 1812. He was an assistant to Home, and was a professor of anatomy and surgery with the Royal College of Surgeons. From 1808 Brodie was on the surgical staff of St. George's and in 1822, he became a surgeon there. In 1832, upon the death of Home, he was assigned as one of the Sergeant-Surgeons to the King and later filled the same office for Queen Victoria. Brodie believed strongly in conservative surgery, chiefly as applied to orthopaedics in which he was a pioneer of the work of Liston and Fergusson. For the last thirty years of his life, he stood at the head of the medical profession in London. He was almost equally distinguished as a physiologist. In 1810, he delivered the Croonian lecture before the Royal Society. He did much experimental research on vegetable poisons. He also made admirable improvements in the science of surgery, e.g., his research was related to the diseases of the joints, un-united fracture of femur, and varicose veins of the legs. Brodie's greatest work was *On the Pathology and Surgery of the Disease of the Joints* (London, 1819), in which he presented his knowledge of diseases of articulation and differentiation between local and neurological disorders of the joints. Brodie was a pioneer in the subcutaneous surgery of the limbs, working on varicose veins as early as 1814.

Sir Charles Bell (1778−1842) of Edinburgh made his greatest honor as an anatomist physiologist. He worked as a surgeon and was the chief at the Middelsex Hospital. The following dissertations were contributed by him: *Anatomy of Expression* (1806); *System of Operative Surgery*; *Animal Mechanics* (1828); *Nervous System* (1830). He discovered distinct function of the nerves.

Benjamin Travers (1783−1858), professor of anatomy at Guy's Hospital, and a pupil of Astley Cooper, served as a surgeon at St. Thomas' hospital from 1815. His *Synopsis of the Diseases of the Eye* (1820) was admittedly the best book in English on this subject at that time. He wrote many treatises on the essential nature of soreness, and on the pathology of the

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nervous system and inflammation. In Edinburgh, there was no chair of surgery at the University until 1831. The professor of anatomy gave instructions for surgery at that time. The Royal College of Surgeons of Edinburgh authorized a chair of surgery in 1804.

John Thomson (1765−1846), the first chair had been a surgeon at the Royal Infirmary since 1800, and had given lectures in surgery. The chair of clinical surgery was created by King George III (1803), who bestowed it with a stipend of fifty pounds a year. James Russell (1755−1836), a surgeon of the Edinburgh Royal Infirmary was the first to hold this clinical chair. Russell had been one of six surgeons selected by the managers of the Royal Infirmary (1800). At the same time he had given up his position as an acting surgeon to the Royal Infirmary. Russell gave much useful information to well-attended classes. He resigned from the chair thirty years later at the age of eighty-one. Russell's practical essay on certain diseases of bones, termed necrosis (Edinburgh, 1794), created one of the first pursuits to give a thorough and detailed illustration of what must have been a common surgical issue.

When John Thomson resigned as the chair of military surgery in 1822, Sir George Ballingall (1786−1855) replaced him. At that time, the country was massively tired of war and was turning its attention to reconstruction and the economy for which a long and expensive operation was necessary. However, Ballingall had the experience in India, at Prince of Wales Island at Java, and with the army in France. He was assured of the need for a complete course of instruction, not only for the injurious incidents of warfare, but also on the diseases to which European troops were especially vulnerable abroad, and on the general hygiene of camps. The East India Company was very helpful, and in addition, the Royal College of Surgeons of Edinburgh modified their administration in military surgery instead of the one or two courses of surgery prescribed. This action of the college was abruptly followed by a corresponding movement on the part of heads of the medical departments of the navy, and the army.In response to Ballingall's efforts, his activities were strongly supported by *Lancet*. Ballingall contributed alot in teaching his subjects; *Outlines ofMilitary Surgery* and also his *Practical Observations on the Diseases of the European Troops in India* (Edinburgh 1823) which were highly admired at that time. Following his death, the special chair of military surgery was abrogated in 1856.

In Glasgow, the leading surgeon at the beginning of the nineteenth century was John Burns (1775−1850). He began his career as a lecturer on anatomy, and later became Professor of Surgery at the University of Glasgow. He wrote a book on surgery and on midwifery with ten editions. His brother, Allan Burns, began as a demonstrator in John Burns' anatomical school. He was the first to construe the liaison of the falciform process of fascia lata to femoral hernia. He was also the author of a book on the surgical anatomy of the head and neck. In his interesting work, he was the first who suggested the ligation of innominate artery.

The Edinburgh Medical School had been carried on by teachers who had no formal connections with the university until 1726. The Faculty of Medicine, however, consolidated professors as a number of "private lectures." The extra-mural school had been able to develop new branches of education. Thus, special courses and lectures were given by external lecturers in mental diseases, diseases of the ear, neurology, throat and nose, diseases of children, dermatology, applied anatomy, operative surgery, and tropical diseases for many years before a lectureship in these subjects was founded in the university. In 1895, the Extra-mural School was reconstructed and centralized as "The School of Medicine of the Royal College."

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France

The French Revolution played an extraordinary part in the development and reforming of their medicine system. In 1792−1793, all the medical faculties and societies in France were terminated. The great wars which began to involve Europe shortly thereafter made it critical however that a source of new medical and surgical experts be maintained. The army's needs came first. The surgical service program was executed into law in 1794, according to the essential requirement for military schools in Paris, Montpellier and Strassbourg. In 1804, the Paris School of Medicine was organized on a large scale, and was established as an institute of medical science which was helpful for solving national problems involving medicine. Soon in 1806, the Imperial University was established as a medical school branch.

French surgery of the nineteenth century is a flaring record of strong identities, men who were magnificent operators like Dupuytren, great organizers such as Baron Larrey, exceptional surgical historians and critics like Malgaigne, schemers of surgical knowledge like Boyer and developers of new growth like Civiale and Leroy.All the characters that have made France great as a nation and many that have increased her strength, can be found in surgeons.

The Napoleonic era was managed by such surgeons as Lassus, Pelletan, Lallement, Percy, Dubois, Boyer and Larrey. Pierre Lassus (1741−1807) was appointed as a professor of surgery in the Ecole de Santé and surgeon to Napoleon I. He published on the history of anatomy, on surgical pathology (1805−1806), and on operative surgery. Philippe Jean Pelletan (1747−1829), a student of Louis and of Sabatier, was promoted as a professor of clinical surgery when the Medical Faculty of Paris was established in 1804. He was also surgeon to Napoleon and chief surgeon at Hôtel-Dieu, after Desault. In 1815 he became a professor of operative surgery and wrote a three-volume work on clinical surgery (1810−1811).

André Marie Lallement (1750−1830) was a professor of surgery in the medical faculty of Paris and surgeon to the Salpetrière. Pierre François Percy (1754−1825) was a professor at the military medical school. Antoine Dubois (1756−1833) was a professor of anatomy at the Ecole de Santé. Alexis Boyer (1757−1833), a pupil of Desault and surgeon at the Charité, wrote a treatise on surgical diseases with 11 volumes (1814−1826) which was accepted as the most authoritative work on surgery in Paris for more than a generation.

Dominique Jean Larrey (1766−1842) played an important role in the growth of surgery. He was the leading military surgeon under Napoleon and an outstanding organizer. He was one of the earliest to perform the amputation at the hip-joint in 1803. Taking part in nearly all of Napoleon's battles, he created the famous "flying ambulances" (1792) to render immediate help to the wounded soldiers. The mortality rate of the French army was most remarkable. Larrey devoted his lifetime to surgery as expressed in his work entitled: "*Mémoires de medicine militaire* (4 volumes, Paris, 1812−1817). Napoleon considered Larrey the most honorable man he had ever known. He was truly the founder of modern military surgery.

Nineteenth century surgery in France was created and promoted by the major professors. Antoine Lembert (1802−1851), Dupuytren's pupil, found a method; the key to unlock the grave problem of successful intestinal suture. Lembert observed that in the approximation of serous tissue to serous, the mucosal tissue to mucosal lays the solution to the problem of intestinal anastomosis. "What is now known as Lembert's suture, which ensures that serous

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surface is applied to serous surface in suturing intestine is the bases of all modern gastric and intestinal surgery."Lembert defined his method in 1826.

Dupuytren's work was of importance also as the initial surgeon in the abscission of the lower jaw (1812) and in his successful ligation of external iliac (1815), and subclavian arteries (1819). He was the first surgeon to succeed in the treatment of arterial aneurysms by constriction. He was also the first operator to practice subcutaneous section of sterno-mastoid muscle in the treatment of wry-neck. Dupuytren was a surgical pathologist and his profound knowledge of surgical anatomy and physiology led to his brilliant work with enterotomy. His solid background in pathology led to such notable accomplishments such as his observations of fractures of the lower end of the fibula (Dupuytren's fracture, 1819), of connatural dislocation of the hip joint (1826), and retraction of the fingers from the embarrassment of palmar aponeurosis (1832).

Dupuytren's clinical lectures were interpreted and published by Brierre de Boismont and Buet (Paris, 4 volumes, 1932–1934), and very widely disseminated. Indeed, Dupuytren has hardly ever been exceeded as a surgical teacher. His clinics were popular during his operating career with surgical students from all parts of the world. His lectures related to injuries and diseases of the bone were translated by Le Gros Clark into English and issued by the Sydenham Society in 1847. Perhaps never in the history of surgery has such greatness as a surgeon been linked with such a deficiency of character as a man. Percy appropriately described him as the greatest of surgeons and the meanest of men.

Philibert-Joseph Roux (1780–1854) was a surgeon at the Charité in 1810 and a follower and disciple of Bichat. He was promoted as a professor of surgery in Paris from 1820 and acquired Dupuytren's position in the Hôtel-Dieu in 1835. He made various improvements to surgery, performing the first staphylorraphy in 1819 and was the first to suture the ruptured female perineum in 1832.

Jacques Lisfranc (1790–1847), surgeon at La Pitié, became famous for his surgical methods of the partial dismembering of the foot at the tarso-metatarsal joint in 1815 as well as his dislocation at the shoulder joint. In 1833, he had performed nine rectal cancer resections via the perineum with three post-operative deaths. He also did many surgeries of lithotomy and gynecology, especially amputation of the cervix uteri. As with Dupuytren, Lisfranc was of a very combative nature and became caustic about his surgical contemporaries.

Louis Joseph Sanson (1790–1883), a pupil of Dupuytren, became a surgeon of Hôtel-Dieu in 1825. After Dupuytren's death, he came to the chair of Clinical Surgery in 1836 as a winner of the position by *concours*. Jules Germain Cloquet (1790–1883) published a book on the Anatomy of Hernia (1817) and a classical work on human anatomy with brilliant plates.He succeeded Dubois as professor of clinical surgery (1833) in the Paris Faculty, having been previously a professor of surgical pathology for two years. He developed several new surgical instruments.

During this era, lithotomy and urology flourished in France, as they have always done. Among the leading characters in these fields were Souberbielle, Civiale, Heurteloup and Leroy d'Etiolles.

Joseph Souberbielle (1754–1846) became an experienced supera-pubic lithotomist, performing his operation on more than 1,200 cases. Jean Civiale (1792–1867), expert in urology and lithotrity, performed his first operation on a living patient in January 1824. He had his special "calculus" word at the Necker Hospital and an extensive urological practice. Charles Louis Stanislaus Heurteloup (1793–1864) devised the instruments to simplify them

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and improved lithotripsy. He reported *Principles of Lithotripsy* (1831). Jacques Mathieu Delpech (1777−1832), professor of surgery in 1812 was the primary specialist in orthopaedic surgery in France, performing for the first time a subcutaneous division of the Achillis tendon for club-foot (1816). He issued *Del'orthomorphic* (1828). In 1832, he was murdered by a patient who misunderstood that due to an operation for varicocele he had become impotent. Claude Francois Lallemand (1790−1853), professor of clinical surgery at Montpellier in 1819 was admittedly the leading surgeon in southern France. He designed the method of treating erectile tumors by needles. He also invented a method of autoplastic surgery by bending the flap without twisting, and thus eliminating all twisting of the flap.

The so-called third period (1835−1847) in the nineteenth century is as follows:

Charles Gabriel Pravaz (1791−1853) was extraordinary in orthopedics together with Jules Guerin. He was one of the first to set up this science on a stable foundation. He published on orthopedics and created the hypodermic syringe (1851) for the injection of tumors and varices.

Alfred-Armand-Louis-Marie Velpeau (1795−1867) who was a pupil of Brentonneau's, became a surgeon at various hospitals (1934−1967). His first work entitled: "*Traitéd'anatomiechirurgicale*" (1823) was decisive for surgeons. Velpeau also issued three volumes *Nouveau elements de medecineopératoire* which was the most extensive work on operative surgery in France at that time. It is beneficial historical material as it contains surgical procedures. In 1847 it was translated into English by Valentine Mott. *Velpeau's Diseases of the Breast* (1854), was the important reference for surgical clinics.

Jean-Zulema Amussat (1796−1856) started teaching individually even before he graduated from the Medical Faculty of Paris in 1826. Although not a professor, nor affiliated with a great hospital, Amussat made several notable contributions to surgery, especially his premier operation of a lumbar colostomy for obstruction of the colon in 1839. Amussat also created a lithotrite for urinary stones and a procedure for the torsion of arteries. For these contributions as well as for his studies on urethral stricture, he was awarded various prizes by the Paris Academy of Medicine.

Joseph Gensoul (1797−1858) became a chief-surgeon at the Hôtel-Dieu in Lyons (1826). During that year he was the first to cut the whole maxilla and the following year, he was the first one in France to dissect half of the lower jaw. In the same year, he had totally removed the parotid gland. Philipp Frédéric Blandin (1798−1849) continued after Richerand in 1841 as a professor of operative surgery of the Paris faculty and was also a surgeon in the Hôtel-Dieu. Raoul Henri Joseph Scoutettin (1799−1871), a remarkable military surgeon, was a professor at Metz (1836) and later in the military hospital at Strassburg (1840). During the Crimean War, he was the chief of the military hospital in Constantinople. Antoine Joseph Jobert de Lamballe (1799−1867) received Roux as a professor of surgery in the Medical Faculty in Paris in 1854. Jobert's main work was in gynecological surgery, especially in the treatment of vesico-vaginal fistulas, and particularly in plastic surgery.

Stanislaus Laugier (1799−1872), a pupil of Dupuytren, became professor of clinical surgery in the Paris Faculty of Medicine (1848) and succeeded Roux at the Hôtel-Dieu (1854). He was the first to encourage the suturing of divided nerves and the first to notice the desertion of cerebro-spinal fluid from the ear in some cases of fissured skull. Laugier was an interpreter of surgery. Amédée Bonnet (1802−1858) was a surgeon of the Hôtel-Dieu in Lyons who became famous for his special treatment of diseases of joints by fixation with immobile bandages. He also performed construction of the bulb without removing the

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muscles of the eye. His essays on the treatment of joint diseases (1845, 1853) were his best works. AugusteBérard (1802−1846) was the successor of Sanson as professor of clinical surgery at the Medical Faculty of Paris. His work in surgery included contraption on staphylorrhaphy, wound watering, varices and elevated tumors and fracture reductions. Jean Gasoard Blaise Goyrand (1803−1866), a chief surgeon of the hospital made distinguishable contributions on the termination of the tongue, amputations, urethral fistula, fractures of the lower end of the radius Goyrand, and was one of the dominant French provincial surgeons at that time.

Auguste Théodor Vidal de Cassis (1803−1856) wrote *Traité de pathologieexterneet de médecineopératoire* (5 volumes, 1838−1841), a widely used manual of surgery which reached a fifth edition in 1860, and was transcribed into German by Bardeleben. He was the first to inject a solution of silver nitrate into the uterus for the treatment of syphilis. Jean Baptiste Lucien Baudens (1804−1857) was a military surgeon and served in Africa from 1830 to 1837. In 1838, he became a professor in the hospital at Lille, and four years later, professor at Val de Grâce. His methods of abscission of the shoulder and amputation of foot were highly recognized, as well as his ice-treatment of wounds. Jules Roux (1807−1877) was a professor in the Medical School of Toulon in 1842. He created a beneficial modification of the technique for disconnections of the foot and was the first to inject iodine into diseased shoulder-joints.

The most important French surgeons of the period 1847−1870 were Malgaigne, Denonvilliers, Gosselin, Richet, Nélaton, Chassaignac, Follin, Broca, Trelat and Dolbeau.

Joseph-François Malgaigne (1806−1865) graduated from Paris in 1831 and then graduated through the various surgical grades. For many years he was a surgeon at the Hospital Saint Louis and later at La Charité. He continued after Blandin in the chair of operative surgery at Paris. Malgaigne's greatest contribution to surgery was his unique manner of evaluating surgical techniques and contraptions by which the new methods of statistical calculation were associated with actual surgical experiments.

A surgeon of outstanding perception and ability as well as a brilliant stylist, his *Histoire de Chirurgie* (Paris, 1840) and his edition of *Ambrose Paré* with a biography of the latter (Paris, 1840), are rich mines for historians as well as for practicing surgeons. Malgaigne's *Manuel de médecineopératoire* (1834) was widely read, and published over seven editions. It was translated into various European languages and even into Arabic. His tremendous surgical work was his *Traité des fractures et des luxations* (Paris 1847−1855), in which he correlated the fruits of his own work in this field with those of his pioneers and contemporaries.

Edouard-Pierre-Marie Chassaignac (1804−1879) devised the ecraseur for stopping hemorrhaging and recommended modification in the treatment of abscesses and infected wounds by drainage. He also protected wounds by complete occlusion. The carotid tubercle is known as *Chassaignac's tubercle.*

A great French surgeon in this period was August Nélaton (1807−1873), a Parisian who studied under Dupuytren at Hôtel-Dieu, as well as at the Foundling Hospital. Nélaton was promoted as a professor of clinical surgery at Paris in 1851 and was a surgeon for many years at Hospital Saint Louis, together with Malgaigne; the two started their significant careers at the same time. The fact that Nélaton was a humble and exceedingly kind man who was friendly to all surgeons and never made an enemy, perhaps inclined the conditions in his favor over Dupuytren. Nélaton was one of the competent clinicians, lecturers and operators of the

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great French in the nineteenth century, when France prevailed in surgery in the world. He wrote *Eléments de pathologiechirurgicle* (Paris 5 volumes, 1844−1860). His clinical lectures were translated by an American surgeon, Walter Franklin Atlee, who studied under Nélaton and published them in English (1855). They show the bright precision of a surgical teacher and the innovative methods which Nélaton was constantly putting into practice. Nélaton proposed a flexible rubber catheter and invented the porcelain-headed round probe for bullet wounds. He also emphasized that severed arteries be ligated at both ends within the wound, and brought this creditable practice into clinical use in France. He was also a pioneer of ovariotomy in his country.

Charles Pierre Denonvilliers (1808−1872) became chief of the school of constructive anatomy of the Hôtel-Dieu (1842) and professor of surgery in the same hospital (1856), and later inspector-general of public teaching for medicine. *Denonvilliers' fascia* was named after him. Athanase Léon Gosselin (1815−1887) became professor of surgery of the Paris Medical Faculty (1858), and surgeon to the Charité nine years later. He devoted his work to surgical journals in two volumes (Paris 1873). Louis Alfred Richet (1816−1891) was promoted as professor of clinical surgery at the Paris Medical Faculty (1864). His chief work was the *Traitépratiqued'anatomiemédico-chirurgicale* (Paris, 1857). EugèneFollin (1823−1867), a pupil of Velpeau, an adequate diagnostician and anatomist, died before he accomplished his six-volume work *Traitéelémentaire de pathologieexterne*, which was completed and published by Duplay.

Paul Broca (1824−1880) was the prominent pioneer in modern surgery of the brain. He was a surgeon at St. Antoine, La Pitié and finally at the Hospital Necker. He initiated craniometric methods and localized the third frontal contortion of the left brain as the speech center (*Broca's area*) and laid the groundwork for cranial geography. Broca designed the method of diagnosing the site of cerebral tumors by the limitation of the disrupted function.

UlysseTrélat (1828−1880) became professor of surgical pathology of the Paris Medical Faculty and surgeon to various hospitals, including La Pitié and La Charité. Trélat was one of the first in France to comprehend the importance of Lister's antibiotic method in surgery. He specialized in surgery of the face and neck and produced two clinical treatises in 1877 and 1891.

Henri Ferdinand Dolbeau (1830−1877) succeeded Jobert de Lamballe at the Hôtel-Dieu (1865), after service at Children's Hospital and the Necker. Three years later, he was nominated as a professor of the Medical Faculty of Paris. His important scholarly improvements are on the lithotrity via the perineal route, epispadias, club-foot, chondromatous masses, and spinal bifida.

Other important French surgeons of this period, though less well-known, were Giraldès, Petrequin, Guersant, Guerin, Sédillot, Pozzi and Richard. Paul Louis Benoît Guersant (1800−1870) was a surgeon at Children's Hospital from 1833 to 1860 and wrote a treatise on pediatric surgery (1864−1867), which was translated into English. Jules Réné Guerin (1801−1886) was a student of Boyer and Roux. He was the designer of the *Gatte Médicale de Paris* which was published for forty years. He specialized in orthopedics, practicing mainly in his private hospital. He wrote broadly on osseous abnormality from 1838.

Charles Emmanuel Sédillot studied at Paris and at the Val de Grace and graduated in 1829. He was a pupil of Boyer and Roux. He was promoted as a professor of operative surgery at Val de Grace in 1836 and later was nominated as a professor of surgery at Strassbourg. He was the first to properly amputate at the hip joint in Paris in 1840. He was the

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first to perform gastrostomy which was nominated by his name. He also contributed greatly in the surgery of lingual carcinoma, plastic surgery and orthopedics.

Joachim Albin Cardozo Cazado Giraldès (1807−1855), a Portuguese, became a surgeon at the Children's Hospital and at the Central Bureau in Paris. He issued his *Clinical lessons on the surgical diseases of children* in 1869. Joseph Pierre Eleanor Petrequin (1809−1876) became a professor of surgery at Lyons in 1855. He wrote on medical, surgical and topographical anatomy, and on clinical surgery as practiced at the Hôtel-Dieu in Lyons. He distributed two important volumes on the *Chirurgied'Hippocrate* in 1878 among other publications. Felix Adolphe Richard (1822−1872) was an assistant to Nélaton and a surgeon at the Paris Central Bureau (1852). He published his *PratiqueJournaliere de la Chirurgie* in 1868.

Italy

The leading Italian surgeons during the first half of the nineteenth century were Palletta, Scarpa, Monteggia, Vacca-Berling-hieri, Porta and Rizzoli. Giovani Battista Palletta (1747−1832) instructed anatomy and clinical surgery when he was the chief surgeon of l'Ospedale Maggiore. Giovanni Battista Monteggia (1762−1815) became co-author with Professor Antonio Scarpa of the five volumes *Istituzioni di Chirurgia* (1802) which extended to seven volumes during its fourth edition in 1830. In 1795 Monteggia became a professor of anatomy and surgery in Milan. Andrea VaccaBerlinghieri (1772−1826) was recognized to the chair of surgery in Pisa in 1801. He remained as an important surgical center. Luigi Porta (1800−1875) studied for three years in Vienna after the fulfillment of his medical courses in Pavia. In 1832, he was appointed as a professor of surgery in Pavia as the successor of Scarpa and achieved international prominence for his special expertise in operations on the vascular system and on the thyroid gland. Francesco Rizzoli (1909−1980) was a professor of surgery and obstetrics at Bologna from 1840. He was identified as the father of Italian orthopedics and established the Orthopedic Institute there which carries his name.

Antonio Scarpa (1752−1832), a pupil of Morgagni at Padua was promoted as a professor of anatomy at Modena and later at Pavia, where he was also a professor of clinical surgery. As an anatomist, he characterized the round window of the ear, Scarpa's triangle of the thigh, Scarpa's fascia, the naso-palaine nerve, and Scarpa's membrane. He published on the surgical treatment of inguinal hernia, cataract and aneurysm. Scarpa was the most famous Italian anatomist and surgeon at that time.

Spain

In Spain, Antonio Gimbernat (1734−1816) detailed (1768) a new operation for femoral hernia together with the discovery of the ligamentous structure in imperative arch which was nominated by his name. Gimbernat (1768) also discovered the Cloquet ganglion in the femoral ring, which he exposed to John Hunter in 1775, and defined totally in 1793. Hunter liked the new method and promised to detail it in his lectures and to use it when he had the opportunity to operate on patients.

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Holland

In the Netherlands, the leading surgeons of the first part of the nineteenth century were Hendriksz, Onsensoort, Tilanus and Ranke. Pieter Hendriksz (1779−1845) was a professor of surgery at Leyden and moved to Amsterdam from 1828 to 1832. Anthony Gerard van Onsensoort (1782−1841) was a professor at Utrecht and a dominator on military surgery, on which he produced a treatise in 1832. Christian Bernard Tilanus (1796−1883) studied under Dupuytren and Listranc in Paris and was the first to instruct clinical courses in surgery at the Children's Hospital in Amsterdam where he was employed as the chair of surgery and obstetrics. Hans Rudolph Ranke (1849−1887) studied under von Volkmann and was appointed as a professor of surgery at Groningen in 1876.

Germany

The dominant surgeons in southern Germany and Austria during the first part of the nineteenth century were Franz X. Rudtozffer (1760−1833), professor of surgery at Vienna from 1810 to 1821; Christoph B. Zang (1772−1835), military surgeon; Joseph von Wattmann (1779−1866); and Vincenz Sebastian von Kern (1766−1829), professor of surgery at Laibach (1797) and at Vienna from 1805 to 1824, who exerted an energetic influence on the advancement of surgery and surgical teaching in Germany and northern Italy.

The best-known surgeons in northern Germany during this age were Mursinna, Hasselbach (1759−1816), Langenbeck, von Walther, von Graefe, Dieffenbach and Chelius. Christian L. Mursinna (1744−1823) was appointed as a professor at the Military Medical School in Berlin and was a practicing surgeon at Charité Hospital. Conrad J. M. Langenbeck (1776−1851) instructed anatomy and surgery at Gottingen, was surgeon general of the Hanoverian army in 1814 and professor of anatomy and surgery at Gottingen. He created the operation of iridodesis for an artificial pupil in 1817. His treatise on lithotomy was issued in 1802 and four years later, developed the first volume of his *Bibliothek fur die Chirurgie* which was definitely completed in eight volumes in 1828. A five-volume treatise on the diagnosis and therapeutics of surgical diseases was published during the years 1822−1850.

Philipp F. von Walther (1782−1849) studied at Heidelberg and Vienna and was appointed as a professor and surgeon at Bamberg Hospital when he was 21 years old. The following year, he was promoted as a professor of surgery and physiology at Landshut. In 1818, he was promoted as a professor of surgery at the newly created University of Bonn and in 1830, he held the chair of surgery at Munich. He was recognized as the founder of modern surgery in Bavaria and one of the first to counterpart physiological principles with those of surgery. He produced numerous treatises, the most important being System der Chirurgie. In 1845, he properly construed corneal darkness.

Carl Ferdinand von Graefe (1787−1840) was one of the few tremendous surgeons in a period when the surgical capitals of Europe were Paris and London. Von Graefe, Dieffenbach and Langenback were the pioneers of modern German surgery.

Von Graefe was the founder of modern plastic surgery, developing new methods for the design of artificial noses (rhinoplasty) and eyelids (blepharoplasty). He also guided the palatine suture for the treatment of congenital cleft palate. He was the first in Germany to cut

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off the lower jaw, and the primary German surgeon to ligate the innominate artery. He was a professor of surgery and director of the surgical clinic at Berlin University from 1810 to 1840.

Johann Friedrich Dieffenbach (1792−1847), a graduate of Wurzburg, who had also studied under Dupuytren and Delpech, inherited von Graefe's position as director of the surgical department of the University of Berlin (1840). He did extensive improvements in the field of plastic surgery, and later also in orthopedics and internal operations. In 1836, Dieffenbach performed the first successful end-to-end anastomosis of small intestine using Lembert's suture. His myotomies and tenotomies became famous. He cured 140 cases of club-foot by tenotomy, and also made extraordinary progress in strabismus by resecting the tendons of ocular muscles.

Max Joseph von Chelius (1794−1876), a pupil of von Walther, was promoted as a professor of surgery at Heidelberg (1819) and published a handbook on surgery in 1823 which became a classic text book all over Germany. The manual continued as the most widely used textbook in surgery for over twenty-five years, and was translated into many European languages and were continued with eight German editions (1823−1857).

Other great German surgeons of this era were: Karl August Kuhl (1774−1840), professor of surgery at Leipzig, who properly ligated both carotids, the innominate, and subclavian arteries in patients. Kajetan von Textor (1782−1860) a pupil of von Walther, Boyer and Scarpa, was appointed as a professor at Wurzburg and a specialist in resections. Carl Wilhelm Wutzer (1789−1863) was a professor of surgery at Halle and later at Bonn. He designed a special operation for the radical cure of inguinal hernia. Gustav Benedict (1785−1862) was a famous ophthalmologist at Breslau. John Carl George Fricke (1790−1841), a pupil of von Graefe, was a surgeon of Hamburg General Hospital, invented special forceps and a vaginal speculum. Michael Jaeger (1795−1838), wrote decisively on resections and on the diseases of bones and joints. Friedrich August von Ammon (1799−1861) was an eye surgeon, and the author of important treatises on plastic surgery, orthopedics, and ophthalmology. George Mojsi-sovis (1799−1860), was a surgeon at Vienna General Hospital. Leopold Grossheim (1799−1844) published a three volume textbook on operative surgery (1830−1835). Ernst Blasius (1802−1875) was a well known author, and Adolph Wernher (1809−1883) who wrote a four-volume handbook on general and regional surgery that became a very popular and valuable work.

The next sequence of important German surgeons were Johann F. Heyfelder (1798−1869), who wrote an important dissertation on resections and amputations in 1854 and other works. Wilhelm Baum (1799−1883), Gustav B. Gunther (1801−1866), a professor of surgery at Kiel and Franz Schuh (1804−1865), a professor of surgery at Vienna.

Georg Friedrich Louis Stromeyer (1804−1876) was surgeon-general at Hanover and a professor at the Universities of Erlangen, Munich, Freiburg and Kiel. He was recognized as the father of modern German military surgery. He published his extensive *Maximen der Kriegsbeilkunst* in 1855 and was devoted to orthopedic surgery by his procedure of subcutaneous tenotomy for injuredlimbs. He also wrote about the resection of joints.

Franz von Pitha (1810−1875) together with Thedor Billroth published a handbook of general and regional surgery, and was considered an excellent diagnostician.

Bernhard Rudolph Konrad von Langenbeck (1810−1887) was one of the greatest surgeons of all times and a graduate of Gottingen. He followed Dieffenbach at Berlin in 1847. He practiced every branch of surgery, devising no less than 21 major operations, mainly in the difficult field of joint excision, including the ankle, hip, knee, shoulder and elbow. He

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launched the German Surgical Society and the Archiv fur klinische Chirurgie, the outstanding European surgical journal. Many of the major surgeons in Europe were Langenbeck's pupils, most remarkably Brillron and Gurlt. As a teacher he was unsurpassed and unique.

Victor von Bruns (1812−1883) of the University of Tubingen was one of the founders of laryngology, being the first one to remove a laryngeal tumor via natural transition. Wenzel von Linhart (1821−1877) was an anatomist, surgeon and author. Albrecht Theodor Middeldorpf (1824−1868) was a professor at Breslau and recommended galvano-cautery. He made various noticeable improvementson the subject of fractures and dislocations.He was the first one to operate for gastric fistula (1859) and oesophageal tumors. Busch (1826−1881), a pupil of Langenbek, was promoted as a professor of surgery at Bonn and wrote considerably on hernia, plastic surgery, orthopedics and gunshot wounds. He also wrote a two-volume textbook on surgery (1857−1869). Carl Ernst Albrecht Wagner (1827−1870), a pupil of Langenbeck, and professor of surgery at Konigsberg, was an experienced operator and a good teacher.

Other important surgeons in themid-nineteenth century were: Jacob von Heine (1800−1879), planner of famous orthopedic hospital at Cannstatt and remarkable author on orthopedic surgery, who produced a famous treatise on poliomyelitis in 1840, a disease which he accurately characterized as a spinal lesion for the first time. Hermann Demme (1802−1867) was a professor of surgery at Berne. August Burow (1809−1874), a pupil of Dieffenbach and a famous plastic surgeon, ophthalmologist and author. Edward Zeis (1807−1868), a professor of surgery at Marbury and later chief surgeon at the City Hospital of Dresden, published important works on plastic surgery. Benedikt Stilling (1810−1879) of Cassell, the first ovariotomist in Germany, performing the operation via the extraperitoneal route in 1837, he also wrote considerable articles on the treatment of stricture by internal urethrotomy. In 1840 he was the first to use the term "Vasomotor," using it as a "hypothetic designation of nerve thread supplying the blood-vessels." Joseph Blazina (1812−1885) was appointed as a professor of surgery at Prague. Hermann Julius Paul (1824−1877) of Breslau was the author of works on traditional and regional surgery. Renst Ludwig Schillbach (1825−1898) of Jena was the author of a monograph on the resection of bones. August Gustav Herrmann (1831−1874) of Prague was the author of relevant works on military surgery, and Carl Wilhelm von Heine (1838−1877), a professor of surgery at Innsbruck (1869) contributed academicly on gangrene and on military surgery.

The most decisive German surgeons of the latter part of the nineteenth century include: Johann Dumreicher (1815−1880) of Vienna; Gotteried F. F. Loeffler (1815−1874), an important military surgeon and author; C. J. F. L. Gustav Simon (1824−1876), medical director of the Hessian army and professor of surgery at Rostock (1861) and later at Heidelberg (1867) founded a private hospital. In 1853, he repaired of vesicovaginal fistulae, gynecology and urological surgery, and he was the first one in Europe to perform a nephrectomy. He was one of the inventors of modern urologic surgery. Gustav Simon published many monographs including treatises on splenectomy and plastic surgery at the Bethanien Hospital (1862); Georg Albert Lucke (1827−1894) became assistant to Blasius at Halle (1854) and later, assistant to Langenbeck at Berlin. He was promoted as a professor of surgery (1865) at Berne and later he obtained the same position at Strassburg. Theodor Billroth (1829−1894) of the island of Rugen, was Langenbeck's tremendous pupil at Berlin (1854). He was a professor of surgery at Zurich (1860−1867) and Vienna (1867−1894). Billroth was the builder and pioneer of modern abdominal surgery, making possible

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procedures within peritoneum as Syme, Dupuytren and Langenbeck performed on the extremities. He completed the first resection of the esophagus (1872); first excision from the larynx of cancer (1873), and executed the first resection of pylorus for cancer (1881). He operated numerous intestinal resections and enterorrhaphies. He was an affectionate friend of the famous German composer, Johannes Brahms.

Richard von Volkmann (1830−1889) of Leipzig, a professor of surgery at Halle (1867−1889) was the first to execute the excision of rectum cancer (1878). He furthermore detailed cancer in paraffin-workers and the ischemic compression or paralyses. Von Volkmann was the foremost German defender of Listerian antisepsis. He was the founder of the *Sammlung Klinischer Vortrage* in 1870.

Other influential German surgeons were: Adolf G. J. von Thaden of Holstein, a pupil of Esmarch and surgeon at the city hospital at Altona; Carl Hueter (1838−1882) attended post-graduate courses in Vienna, London and Paris after graduation in 1859. Later he became a private-docent in Berlin where he assisted Langenbeck. He was nominated as a professor of surgery (1868) at Rostock as a successor of Gustav Simon, and the following year he was employed as the chair of surgery at Greifswald. He was a skilled bacteriologist and pathologist as well as a master surgeon and who created much advancement in the technique of excision of the rectum, tracheotomy, resection, etc. He was a creative writer of surgical books. Hermann Mass (1842−1886) became professor of surgery at Freiberg (1877) and he wrote an imperative work on military surgery.

Robert Gersuny (1844−1924) of Teplitz-Schonau, a pupil of Billroth was an innovator of many operative methods in the surgical and gynecological fields. In plastic surgery his name was distinguished for his Paraffin method. His book *Arzt und Patient* was transcribed into English and he wrote *Theodor Billroth* (1922), to honor his teacher. Gersuny's Klebe symptom was named after him.

Friendrich Trendelenburg (1844−1924) of Berlin, a pupil of Langenback, was principally interested in strictures of trachea and esophagus. He recommended his tampon-cannula in 1869, and his gastrostomy operation for the therapy of strictures of the esophagus (1877). In the operations on the viscera, he used the high pelvic posture (position), now internationally known as "Trendelenburg's position." He was the first to suture the patella in Germany (1878). He helped in establishing the German Surgical Society (1872), and wrote its history.

The tremendous German historian of surgery was definitely Ernst Julius Gurlt (1825−1899) of Berlin, who was a professor of surgery at Berlin University in 1862. Gurlt engaged in extensive research in the history of modern surgery, which blossomed in his truly great work, *Geschichte der Chirurgie* (3 volumes, Berlin, 1898). In this history, he described the development of the art and science of surgery from its commencement to the Renaissance with a propensity of detail work and an unsurpassed degree of truthfulness. The work was translated in total into English.

Friedrich von Esmarch (1823−1908) was the leading military surgeon of Germany during the last half of the nineteenth century. Esmarch was a professor of surgery at Kiel from 1857 to 1899. He provided service in all the wars and made important improvements to the art of military surgery. He recommended the first aid bandage on the battlefield in the wars of 1869 to 1870. A most valuable contribution was the so called "Esmarch bandage." The benefit was to control hemorrhaging before ligation, which saved many lives on the battlefield. Valuable work by Esmarch was also done in the field of abscission after gunshot wounds.

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Paul Kraske (1851−1930) was an assistant under von Volkmann. From 1883 to 1919, he was director of the Chirurgische Klinik of the University of Freiburg. He became brilliant because he discovered a method to remove high location of rectum cancer which bears his name.

Fedor Krause (1857−1927), another prominent German surgeon of Friedland, Silesia, studied under Julius Hirscherg, Robert Koch, C. Friedlaender, Weigert and Volkmann. He was the principle of Augusta Hospital. His main direction was to treat tuberculosis of bones and joints. He was also involved in the therapeutics of neuralgia of facial nerves and in the surgery of brain and spinal cord. His distributed, excellent thesis "*Lebrbuch der Chirurgischen Operationen*" (Berlin and Wien, 1912−1914, was translated into English, Russian and Spanish. His surgical method of destruction of the gasserian ganglion carried his name.

Karl Thiersch (1822−1895) of Munich was a pupil of Stromeyer. He was appointed as a professor of surgery at Erlangen and Leipzig. He became known for his work in skin-grafting. He also published *Der Epithelialkrebs* (Leipzig, 1865) on epithelial cancer, healing of wounds, and phosphorus necrosis.

Ernst von Bergmann (1836−1907) was the successor to von Langenbeck in the chair of surgery at the University of Berlin. He studied at the University of Dorpat. During the crusades of 1870 to 1877, he had great possibilities to expand his knowledge of surgery. He was assigned to the professorship at Wurzburg in 1878 and in 1882 he developed in Berlin as Langenbeck's successor. He was the outstanding German surgeon in the field of head injury and brain surgery. He reported on fatty embolism, diseases of lymph glands, surgery of the joints, ligation of femoral vein, etc. He was the first to recommend sterilization by steam in surgery (1886). The highly illustrated pattern of operating room asepsis was settled by von Bergmann.

Vincenz Czerny (1842−1916) and Anton Wolfler (1850−1917) were two immense surgeons in Germany. Czerny, as a professor first at Freiburg, and later in Heidelberg (1887), he was the first to remove sub-peritoneal uterine fibroids by way of the vagina (1881). He was a pupil of Billroth's and worked in the fields of extirpation of larynx, the esophagus, the kidney, and general visceral surgery. He was the creator of the Institute for Experimental Cancer Research and the Heidelberg Samariterhaus (1906). At Wolfler of Kopezen near Kladrau, Wolfer operated the first gastroenterostomy in 1881. His improvement to surgery of the tongue and kidneys were very beneficial. Together with Kocher he worked and wrote about the dangers of total removal of the thyroid gland. He wrote about struma, erysipelas, amputations and foreign bodies in the stomach and intestines of the man.

Russia

The original Russian surgery began long ago, and early sources of the history of Russian medicine saw the same primitive folk therapies and superstitions which are found in ancient people. In Richter's work *Geschichte der Medizin in Russland* (3 volumes, Moscow, 1813−1817), it was expressed that the early Russians were so healthy and strong that they did not need much medical treatment. But doubtless, they wanted it even though there was a lack of physicians. In the fourth century B.C., after initiation of the Christian religion, the Byzantine priests and hermits took care of the sick people. Prior to the period of the Tatar

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aggression and dominance (1223−1370), the tzars and dignitaries had started importing physicians from Western Europe. But the ruthless treatment of the Tatar rules soon kept foreign physicians away from Russia. For instance, a physician Leo, a Venetian Jew, who failed to treat a Muscovite prince for a foot sickness, and Anton, a German physician, who along with Leo was sentenced to death because of failure to cure the disease.

During the following centuries, epidemic diseases and poverty, leprosy and syphilis, repeatedly imperiled all of Russia, killing large percentages of the population because of the lack of medical treatment. In the sixteenth century, Ivan the Terrible (1533−1584) tried to bring in foreign physicians. This had already been tried by his forbearer, who had authorized a German, Hans Schlitte, living in Moscow, to bring German creative people and mechanics into Russia. Schlitte had already selected more than one hundred German artists, physicians, operative surgeons, barber-surgeons, surgical assistants and druggists. But the Hanseatic League and the Livonian Order, for bureaucratic reasons, put most of the party in prison. Eventually, only a few foreign physicians were brought in. At the end of the 16th century, the first Russian medical book was published and the first juridical autopsy was performed in 1623.

The Romanov Empire tried to enhance and institutionalize living conditions. Tzar Feodor built a hospital, which had a staff consisting of physicians and surgeons who gave courses in medicine and surgery to students, who needed to pass an examination after five years of study to get their medical degree.

It was not late in the seventeenth century before real improvement was made, when Peter the Great, whose regime from 1694 to 1724, tried in any way to build up westernized industry and science in Russia. During his European travels in Germany, Holland, England and Austria, he personally studied the art of ship-building and was even taught some medical and surgical training, whereby he performed quite a few of surgeries after he returned to Russia. Couching cataracts, abscess draining and tapping dropsies were performed by him, although not always successfully. He built the first hospital in 1706 and the first medical school in Russia in 1707. In the following years, he controlled many hospitals which had been founded. He also induced many German and Dutch physicians to serve in his army and navy. Regrettably, his female subordinates did not care about medical advancement and social welfare. During the Seven Years War, a large number of wounded soldiers died for lack of adequate medical care, because all hospitals built by Peter the Great had long since crashed and no new hospitals had been set up.

Catherine II (1762−1796), the Great lover, who reigned 34 years, had some permissive propensity, and Russia opened some new hospitals and other welfare institutions. Catherine was induced by Voltaire to accept a vaccination against smallpox together with her son, Grand Duke Paul Thomas Dimsdale. The physician who performed this service, was compensated with the title of Baron, along with $60,000 and a pension for life of $2,500. During Catherine's reign (1769), Ambodik recommended the use of obstetric forceps.

When Catherine died, her son Paul (1796−1801) proved to be an atrocious and weak-minded ruler, but some developments were made during his reign. The Collegium Medicum (1763) was rebuilt as the Medico-Chirurgical Academy (1800) with Sir James Wylie (1768−1854) as the first president. Wylie, an Aberdeen graduate, was the physician to Tzar Paul (1798−1801). Wylie (1808) became Inspector General of the Army Medical Board of Health (1812), and first director of the Medical Department of the Military of War. Medical

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teaching up to the middle of the nineteenth century was rather insufficient. The old textbooks of the eighteenth century were used to teach surgery, pathology and chemistry.

In the first half of the nineteenth century, Matvei Mundrov contributed a lot to Russian medical history. He published the first Russian manual in the field of surgery and lectured on military hygiene. The Military Medical Academy of St. Petersburg (1835) became very influential in the improvement of Russian surgery. Conrad Frederick Uden was appointed as the first professor of special pathology and therapy. Ivan Feodorovich Busch (1771−1843) issued the first Russian manual of surgery (1807) and was a distinguished teacher. Salomon, one of his pupils, operated the first lithotripsy and the first ligation of the internal iliac artery in Russia.

Other important surgeons in Russian history were: Simon Zybelin who was promoted as professor of anatomy and surgery in the University of Moscow in 1868; Joseph Czekierski (1777−1826) of Warsaw, who was appointed as professor of surgery and also wrote a four-volume treatise on surgery (1817−1818); Andreas Sydoratzky (1788−1815), a famous lecturer on surgery at the University of Moscow; Elias Bujalski (1789−1864), who wrote *Tabulae Anatomico-Chirurgical,* distributed in 1828 and reprinted in 1852, was also an anatomist in the Military Medico-chirurgical College at St. Petersburg; Karl Daniel von Haart-Man (1792−1877), the author of Casus Chirurgici (1815), was a professor of obstetrics and surgery at the University of Helsingfors (New Finland) which was newly founded (1833). Leo Nagumowitsch (1792−1815) was a well-known military surgeon and author of an essential manual on the treatment of gunshot wounds (1832, Russian). Christian Salomon was appointed as a professor of surgery at St. Petersburg and wrote a two-volume textbook on operative surgery (1840); Ivan Rklisky (1805−1861) wrote a Russian treatise on operative surgery (1847) and was a clinical professor of surgery at St. Petersburg.

Under Alexander II (1855−1881) a powerful pursuit was made to improve and modernize Russian medicine. Most Russians had never seen a physician at all and only military medicine was available. The conditions in abundant rural districts were so poor that there was no chance at all for a physician to ever practice. The tzar, a liberator, tried to solve the internal problems of his country by creating a great network of district assemblies or Zemstvo governments. The plan was excessive and could be carried through only partly. By 1890, there were 1,422 medical centres in 359 districts with 1,068 hospitals and 414 clinics, with a total personnel of 1,805 physicians, 8,046 assistants and 2,454 midwives.

The greatest Russian surgeon and head of social welfare was Nikolai Ivanovich Pirogoff (1810−1881), who graduated from the University of Moscow at 22 years of age and who produced a monograph on the ligation of the abdominal aorta (1832). He then studied two years in Berlin and Gottingen. Returning to Russia (1835), he lectured for five years at Dorpat and then was promoted to professor of surgery in the Military Academy at St. Petersburg. During the Crimean War (1854−1855), he coordinated female nurses to take care of the wounded. He was well known as the first surgeon in Europe, together with James Syme of Edinburg and Robert Liston to apply ether anesthesia (1847). He made 800 post investigations of cholera victims (1848), and taught many pupils. He had extensive opportunities to study from serial wars with abominable torture for the wounded soldiers and people, such as pyemia, erysipelas, hospital gangrene, and purulent edema. He was strongly demanding of medical support from the incompetent government. He had to completely resign his professorship. Pirogoff operated his method of entire osteoplastic amputation of the foot (1854). He was known for his great atlas of 220 topographic, anatomical, delineated

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plates (1851−1854) based on frozen sections in three planes. Also for his treatise on military surgery (1864), and for advocating small, barrack-like pavilions to prevent the spread of epidemic diseases. He did his absolute best to promote medical education in his country, and was respected by the Russians as the greatest surgeon in medical history.

An important Military surgeon after Pirogoff was Nikolai Alexandrovich Velyaminov (1855−1920), the pioneer of the first Russian surgical journal (1885), who regulated the Russian Red Cross during the Russo-Japanese War (1904), and was surgical inspector on the staff of Grand Duke Nicholas (1914). Nikolai Vassilievich Skliffosovsky (1936−1904) earlier boosted and recommended antisepsis. He created the Russian clamp for suturing bones in the operative treatment of false joints. In cases of oesophageal narrowing, he successfully operated gastrostomy, and in goitre patients, he ligated the jugular vein. Vladimir Dmitrievich Vladimirov (1837−1903) was the first to use osteoplastic resection of tarsus (1872), a method later improved by Mikulicz (1881). Sergiei Petrovich Kolomnin (1842−1886) was a promoter of ignipuncture. Vladmir Feodorovich Snegirev (1847−1916), a well-known gynecologist, introduced vaporization of the uterus (1898) and published about uterine bleeding in 1885. Alexander Alexievich Bobbroy (1805−1904) created an osteoplastic method of operation for spinal bifida (1892), and was famous for his surgical treatment of liver hydatids (1896−1898). Paul Petrovich Pelechin (1842−) introduced antisepsis into Russia (1868). Peter Ivanovich Dyakonov (1855−1908) in Orel, a professor of surgery at the university, started the annual congress of Russian surgeons (1888), published a book on death from chloroform (1890), on antiseptic wound therapy (1895), and was the editor of *Kirurgiya* (Moscow 1897−1908).

Alexiei Andrevevich Vvemensky (1856−1900) at Tomsk issued papers on lithotrity in children (1887), on calculus in women (1893), and on the topography of the female pelvis (1893). Anton Yakovlvich Krassovski (1823−1898) recommended ovariotomy in Russia (1862) and published a treatise on operative obstetrics (1885). Ivan Petrovich Pavlov (1849−1936) of Ryazan was devoted to pharmacology and especially to physiology. He became (1890) director of the Oldenburg Institute of Experimental Medicine, and the first laboratory of physiological surgery was built under his guidance (1891). Pavlov, who explored the secretory nerves of the pancreas and so-called psychic secretion, received the Nobel Prize (1904) for his research work on the digestive glands. He was honored by the tzar when he died in 1936.

In modern Soviet surgery, there were several physicians who did very interesting research in blood transfusions in dogs. During the World War, the citrate method was introduced and it was the first time blood could be kept for a few days before transfusion. At that time, blood banks showed quite a few impairments, e.g., loss of potassium by the red cells. In blood preserved for 10 days, the concentration of potassium could become dangerous, and also the prothrombin concentration in the blood dropped rapidly. At that time scientists realized that a whole blood transfusion is preferable, but there were many shortcomings needing to be solved.

American Surgery

America Surgery came at last in this age. The first period or colonial period, was recorded by Hunterian principles. Most American surgeons were trained and graduated from Scottish, and English schools of medicine. This period may be extended to 1820. There were

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many heroic operations like aneurysms and amputations. The most crucial surgeons in this period were the two Warrens, father and son in Boston. Physick, often called “The Father of America Surgery.” Wright Post in New York, Dorsey, the nephew of Physick in Philadelphia, and Nathan Smith in Yale.

The second period, when American surgeons studied in American medical schools, saw the predominant influence of Hunter Wane who graduated from the enormous schools of Paris, which at that time (1820−1850) was the surgical center of the world, and which fascinated many American surgeons. The pioneer of American surgeons was Valentine Mott in New York who did extraordinary work in the field of vascular and osseous surgery. This period also saw the era-making establishment of ether general anesthesia invented by John Collins Warren, Henry Bigelow and George Hayward. In this period, the ovariotomy became a fundamental part of surgery.

In the third period, dating from 1850 to 1900, American surgery progressed rapidly to perfection. Marion Sims made gynecology a science with the help of his pupils Emmet and Thomas. Their practice increasingly developed the capacity of operative surgery, and surgical peculiarities were established. When Langenbeck and Billroth reigned in Europe and when Virchow established cellular pathology, it was very charming for American surgeons to attend German medical schools. American surgeons now went to Berlin, Vienna and Leipzig for their post-doctoral training. In this period, Samul Gross developed surgical pathology securely in America. In this epoch, Johns Hopkins University, undergraduate and graduate schools were well-established. The Mayo Clinic in Rochester, Minnesota, influenced by the two great operators, the Mayo brothers. The patron of American surgery, John B. Murphy of Chicago, managed the surgery of the Mid-West and put every surgeon in America under his control. At Murphy’s side there was the figure of the celebrated Swiss-American surgeon, Albert Ochsner.

From 1900 onwards, American surgery gradually became independent. Surgical centers grew and spread through the American continent. Surgery advanced and generated in every direction. With the help of Rontgen rays, the methods of blood transfusion were clarified, general and local anesthesia were gradually well established which made surgery much more successful. Creative methods to overcome surgical shock were developed and many brilliant surgeons moved from America to international surgical centers. In 1930 a Nobel Prize was awarded to an America physician, which encouraged and enhanced American surgery very much. Colossal adversities were overcome and the tiny seed which was sown hopefully was being producing an enjoyable harvest.

Bleeding, infection and pain were the major barriers of surgery before the industrial revolution. During the entire history of medicine and surgery, there is a constant repeatable sad record. The massive and dreaded phenomenon of lethal infection was the main subject running its detrimental epidemic diseases all over the Europe. Many patients died after surgery because of septicemia, pyemia, pueral fever, gangrene, and in almost all cases, the results were the same, death. Up to the end of the eighteenth century the mortality rate of 80% was truly appalling. Thankfully, the mortality rate dropped to 50% in the nineteenth century.

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PREVENTION OF INFECTION

Two men were revolutionary for smashing the monster of infection as it obsessed the surgeon. Louis Pasteur (1822−1895), a native of the Jura district was trained as a chemist. He did the evaporation research to induce lactic acid organisms. Their growth could be paused or constricted and even damaged by controlled heat. From this, *Pasteurization* started in the modern food industry and the seed of antisepsis lies within it. At that time, surgeons knew that pus came from infection, and if infection could be prevented there would no putridness.

Luckily, Pasteur's work on lactic acid fermentation attracted the attention of Joseph Lister (1827−1912) during the early 1860's. Lister snatched the key factor and saw immediately that infection could be terminated at the start. The question was how. The Pasteurian method of heat sterilization could not be applied to living tissues. According to the good results obtained at Carlisle where excrement was treated with carbolic acid, Lister began experiments with solutions of carbolic acid of varying concentrations as disinfectant agents. He advised to spray carbolic acid solution in the operating room because he believed that the pathogenic organisms were active in the air. In 1865, Lister started using antisepsis in surgical patients. Lister submitted two era-making articles in the Lancet (1867). The first one was "*On a new method of treating compound fracture, abscesses, etc., with observations on the condition of suppuration,*" and the second one, "*On the antiseptic principle in the practise of surgery.*" Robert Koch in Munich in his *Cause of infection in wounds* (1877) illustrated for the first time that many different types of organisms could cause different type of infection, and that each had clear distinctive properties which caused different symptoms.

It became clear that surgical scientists understood that antisepsis was half of the solution to the problem of infection. Ernst von Bergmann and his assistant Schimmelbusch in 1886 expanded the embellished doctrine of sterilization by steam for surgical instruments, gauzes, tissues and dressings which have been taken up throughout the world. To include the operating surgeon's and his assistants' hands (1890) in the category, Halsted recommended the use of rubber gloves. Ten years later, William Hunter at Charing Cross Hospital first used the gauze mask over the face of the operator. This, like rubber gloves, is now universally applied.

Shortly after the effectiveness of antisepsis was established, Lister showed his mortality rate in amputations fell from 43% to 15%. Lister also created catgut ligatures of blood vessels (1880). This is one of the most influential improvements in the history of hemostasis and was widely used in surgery after 1880.

Joseph Lister and Jame Syme of Edinburgh (1869), eight years later became professors of surgery at King's College where Lister continued until his retirement in 1896. He was a respected surgeon in his university. He was of utmost modesty and kindness, and shared his honors with others. Lister had an ample scientific generosity, hospitality and unselfishness, not so common in science.

In the First World War, especially with the great carnage, asepsis was impossible for the primary treatment of the traumas of war. With the widest possible use of antisepsis, coupled with preventive vaccinations against tetanus, the military surgeon could handle the huge disaster lists.The use of sulfa medicines had remarkably reduced infections, and had greatly aided in the battle against wound infections since the First World War. The use of sulfa drugs kept septicemias, gas gangrenes and other fatal infections to a minimum.

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Antibiotics have been used for thousands of years to treat infections, and until the last century many people did not know that infections were caused by bacteria, such as pneumonia and diarrhoea which caused lots of human death in the developed world. It was in the late nineteenth century that scientists commenced researching antibacterial chemicals. Paul Ehrlich, a German physician, determined that a chemical named arsphenamine definitely treated syphilis in 1909. This became the first modern antibiotic which was used for over 30 years by Ukrainian-American innovator and microbiologist Selman Waksman, who in his lifetime invented over 20 antibiotics (The Nobel Foundation 1945; Cruickshank 1955). Alexander Fleming (1881−1955) was a Scottish biologist, pharmacologist and biotechnologist. His best discoveries are the enzyme lysozyme in 1923 and the world's first antibiotic benzylpenicillin (Penicillin G) from the mold *Penicilliumnotatum* in 1928, for which he shared the Nobel Prize in Physiology or Medicine in 1945 with Howard Florey and Ernst Boris Chain (Wikipedia contributors 2017; History of Antibody 2015). During World War II, penicillin was widely used to treat troops throughout Europe, and had saved a lot of lives (History of antibiotics 2017).

Paul Ehrlich and his colleagues developed a systematic screening approach which became the cornerstone of drug research strategies in the pharmaceutical industry that resulted in thousands of drugs identified and translated into clinical practice. During the earliest antibiotics research, this process led to the invention of sulfa drugs which treated numerous diseases with its antibacterial activity (Domagk 1935; Hugh 2002) from World War I to present clinical practice.

The discovery of the first three antimicrobials, Salvarsan, Prontosil, and penicillin was the example for identifying further drugs. The period between the 1950s and 1970s was the golden era of discovery of novel antibiotics. Therefore, with the decline of the discovery rate, scientists tried to struggle with the counteraction of pathogens against antibiotics which were transformed by existing antibiotics (Chopra 2002). Combination therapy of coupling antibiotics may increase the efficacy of bacteriophages, and diminish the side effects of antibiotics (Lu and Collins 2009).

ANESTHESIA

The enormous improvement of surgery is due to the revelations and advancement of all methods of anesthesia. Because of the discovery of nitrous oxide, ether and chloroform, the original surgeons performed surgery using these tools. Subsequently the simple drop method of anesthesia intake was upgraded, and the "gas-ether" method progressed. A simple method was to put the patient under nitrous oxide first, then gradually take over by ether, totally without severe side-effects. Thomas L. Bennett in New York City, learned the anesthesia method in England from Hewitt in 1895, and therefore built up the new devices which spread everywhere (Wallin 1972).

In 1858, Albert Niemann used the analgesic chemicals of cocaine, when placed on the tongue. Carl Koller in 1884 recognized that it had the same efficacy on the eyes. Spinal anesthesia, especially for surgery below the diaphragm, came into clinical utilization mainly due to Halsted's initial work. Einhorn found that novocaine and its clinical use by Braun in 1905, brought local anesthesia briskly into clinical utilization. Novocaine was a great

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improvement. It was 7 times less toxic than cocaine and it could be sterilized by boiling and kept for a long time without degeneration. Combined with adrenalin its efficacy could be boosted and protracted. Its analogs were less toxic and better than novocaine. In Germany, Karl Ludwig Schleich in 1892 used subcutaneous injections of diminished solutions of cocaine. August Bier and Stovain in 1906 recommended spinal anesthesia. Halsted and Matas made valuable improvements to this technique.

In 1920, a method of combined local and consumption anesthesia was proposed by Crile and Lower in Cleveland which could extremely diminish operative neuroses shock and nausea. Local spinal and regional anesthesia was extensively used in clinic operations. Paravertebral anesthesia was originally designed by Sellheim in 1905, and it was first practiced in urologic surgery by Kappis in 1912 and was pursued by Heinrich Braun's parasacral anesthesia in 1914. Caudal block anesthesia was reported by Stoeckel in 1909, and trans-sacral anesthesia was developed and improved by Danis in 1914. Ethylene anesthesia in genito-urinary operations was recommended by Kretschmer in 1911. Penthosal sodium was applied in the clinics and this improvement was announced by Lundy in 1935.

Oxygen use was started in a compound with nitrous oxide in first stage anesthesia. Cyclopropane was reported as its first clinical application by Neff, Rovenstine and Waters at the University of Wisconsin in 1930. It is the most flammable and fiery of all gases used in anesthesia. Oil-ether colonic anesthesia was recommended in 1913. Other methods were still in experimental stages by brilliant specialists and scientists.

Sevoflurane is a more recent extension to inhalational anesthetics which had been started in clinical use. Compared with isoflurane or halothane, it is less dissoluble in the blood. It has more rapid absorption and introduction than other inhalational agents, an enhanced depth of anaesthesia and faster destruction and rehabilitation. This is distinctly favorable in paediatric and adult anaesthesia. Sevolurane firstly reported in 1972, was approved for clinical application in Japan in 1990, in Germany in 1995, and then in the USA in 1996. It has low blood solubility. Only desflurane and nitrous oxide are lower and produce a rapid wash-in and wash-out in the blood. This may result in a more rapid recovery from anaesthesia in correlation with conventional inhalational anaesthetics, enflurane, halothane and isoflurane. There have been no case reports internationally of sevolurane-associated renal failure. Sevofluane is a great, safe anesthesia medicine in clinics (Frink 1993; Eger 1994; Young 1995; Conzen 1996; Patel 1996; Wallin 1975).

The third great significant addition to modern surgery was the Rountgen Rays (X-ray) by Wilhem Konrad Rontgen (1845−1923) in 1895. Firstly, in cases with imagined foreign bodies or in fractures, an X-ray was helpful for the diagnosis. At that time they composed a diagnosable procedure in medicine and surgery. The examination of internal organs by X-rays followed, and finally the rays were reversed to a curative application. Rontgen's discovery was preceded by Crookes tube. Hertz and Lenard did a bright experiment on electromagnetic circumstance, Rontgen advanced a series experiments until he discovered the elemental nature of the phenomenon. He then presented his dissertation "*On a New Kind of Rays*" to the physical Medical Society of Wurzburg. In the beginning, scientists did not know that X-rays have an unhealthy effect and can burn the skin. Many workers with X-rays lost their limbs even their lives. After the danger was identified, the beneficial methods were well established to prevent X-rays side-effects. At present, X-rays are very safe for the diagnosis and treatment of patients.

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Conclusion

A surgeon is a doctor who can do surgery. To be a surgeon is to be a member of one of the world's most attractive professions. Research in surgery has never been determinate. In hospitals and laboratories throughout the world, scientists are challenging the unknown, uninterrupted. Surgery around the world, from primitive times to the present day, a completed history of the world of surgery, its origins, progression and celebration owes tremendous gratitude to the man who devoted their lives to the never ending search for new techniques in the treatment of cancer, brain tumors, heart abnormalities, organ transplantations, thoracoplasty, lung or liver lobectomy, vascular surgery, robotic surgery and other dramatic surgical innovations (Alter 2009).

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Chapter 2

THE DISCOVERY, DEVELOPMENT AND PATENT PROTECTION OF NEW DRUGS IN EXPERIMENTAL AND CLINICAL RESEARCH

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ABSTRACT

Inventing novel medical drugs and improving the quality of medical care are becoming more and more important; there is a demand to focus on patients' needs, especially in the current economic climate. However, current drug research and development (R&D) is more complex and challenging, because development of a new drug is under increasing pressure that is very costly, time-consuming, with a high risk and with low success rate. To develop new and effective drugs, to reduce side effects and hospitalizations, and to extend the life of patients, continuous pharmaceutical innovation is one of the gravest responsibilities of pharmaceutical industries. It takes an average of 12 years for a potential new medicine to develop from basic laboratory research to clinical treatment. It includes the early-phase basic and preclinical research, which is the process of discovering and identifying an investigational drug and performing initial experiments in animals; the middle-phase clinical trials to evaluate the candidate medicine safety, efficacy and overall relationship between benefits and risks; and the late-phase post-market safety monitoring. It also includes very important steps to obtain regulatory approval with a new drug application and patent protection.

This chapter describes the current state of pharmaceutical research and development; provides a basic understanding of working on new drug development in basic, preclinical and clinical research; indicates the relationship between patent protection and trademark application for new drugs; considers how well markets are working to deliver new drugs; and discusses how to increase the commercial life of a new drug by extension of the

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patent term, so as to accomplish our mission with honesty and science, focusing on patient needs, and developing new drugs and therapies for medical diseases.

Keywords: clinical trials, FDA, investigational new drug, patent protection, pharmaceuticals, research and development

ABBREVIATIONS

ADMET	Absorption, distribution, metabolism, excretion, and toxicity
BLA	Biologics license application
ED50	Median effective dose
FDA	U.S. Food and Drug Administration
GCP	Good clinical practice
GLP	Good laboratory practice
GMP	Good manufacturing practice
HTS	High-throughput screening techniques
IEC	Independent ethics committee
IND	Investigational new drug
IP	Intellectual property
LD50	Median lethal dose
NDA	New drug application
NSF	National Science Foundation
PTO	U.S. Patent and Trademark Office
PhRMA	Pharmaceutical research and manufacturers of America
PD	Pharmacodynamics
PK	Pharmacokinetics
R&D	Research and development
SOP	Standard operating procedure
UHTS	Ultra-high-throughput screening

INTRODUCTION

In humans, drugs of plant or animal origin have been used to treat diseases for more than 3,000 years. They were already recorded in China around 1100 BCE (Before Common Era), and at least 1,900 different remedies were used for people by the end of the 16th century [1, 2]. Since the 18th century, herbal practitioners had been identified in many indigenous populations around the world, but the use of herbal medicines was based on what practitioners knew and what worked, not why or how [3–5]. In the 1780s, William Withering (1741–1799), an English botanist, chemist, physician, and the discoverer of digitalis, was the first person to study the active ingredient in an herbal remedy, to isolate digitalis from the foxglove, and to describe its extraction from various parts of the plants. He published *An Account of the Foxglove and some of its Medical Uses*, which contained records on clinical trials and notes on digitalis's effects and toxicity [6–8]. During the early 19th century, chemists believed that compounds from living organisms were endowed with a

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vital force and distinguished them from inorganic compounds [1]. In 1828, Friedrich Wöhler (1800–1882), a German chemist, was best known as the first to isolate several chemical elements and describe the organic chemistry of urea [9, 10]. In the late 19th century, pharmacology began to emerge. Oswald Schmiedeberg (1838–1921), professor of pharmacology at the University of Strasbourg in France, studied the pharmacology of chloroform and chloral hydrate. He is now recognized as the founder of modern pharmacology and is referred to as the "Father of Modern Pharmacology." He published the classic text *Outline of Pharmacology* in 1878 (Figure 2.1) [11–13].

In the 1920s and 1930s, a number of major advances were made by the modern pharmaceutical industry as both penicillin and insulin were identified and manufactured, and are still produced for patients now. During the post-war period from the 1950s to the 1990s, new antibiotics and analgesics became major advances in drug development. Since the middle of the 19th century, beta blockers, ACE inhibitors, benzodiazepines and a wide range of novel anti-cancer medicines have made massive contributions. In the late period of the 19th century, new classes of biopharmaceutical medicines were developed and attained significant success in the clinical treatment of patients [14].

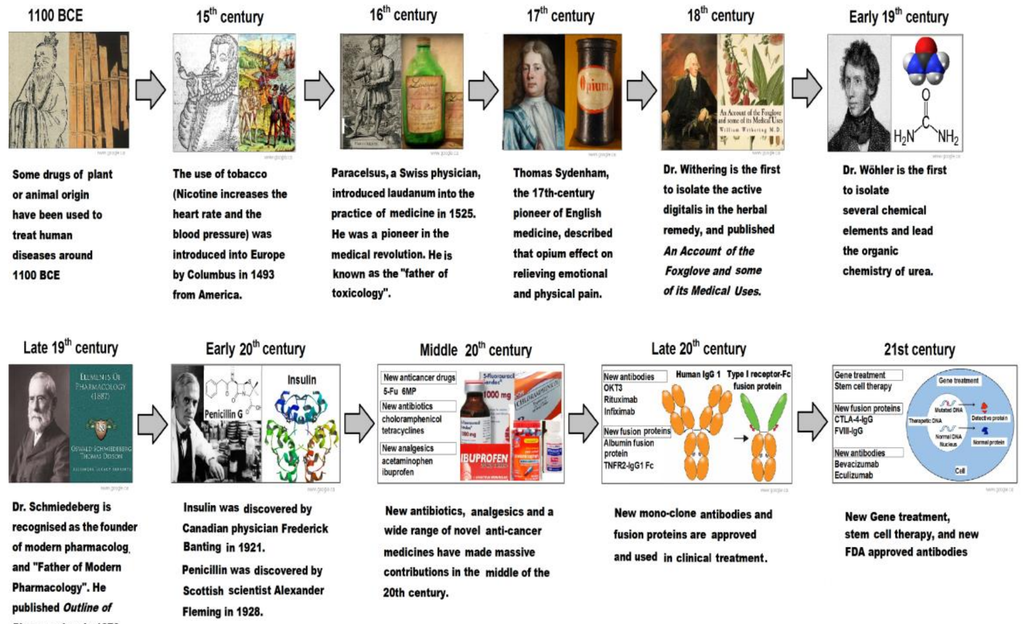

Figure 2.1. A brief history of chemical and biological medicine development. The timeline shows selected evolutionary landmarks in chemical and biological medicine discovery and development.

PHARMACEUTICALS

Pharmaceutical Medicine

Pharmaceutical medicine is the medical and scientific discipline concerned with the discovery, development, evaluation, production, registration, monitoring and marketing of

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new medicines for the benefit of patients and the healthcare community [15]. The core discipline of pharmaceutical companies is subject to a variety of laws and regulations that regulate the patents, testing, safety, efficacy and marketing of drugs. The largest pharmaceutical companies in the world, such as Novartis, Pfizer, Merck, Johnson & Johnson, GlaxoSmithKline, Eli Lilly, Bayer, Roche, and AstraZeneca, had previously developed natural medical products, such as morphine, quinine and strychnine, and moved into large-scale production of drugs in the middle of the 19th century [16]. Aspirin, a chemically modified version of salicylic acid, showed a much improved efficacy, and this product is still in widespread use today [1]. The pharmaceutical companies may deal in generic and brand name medications, are expected to generate 1 trillion USD in revenue and make up one of the most prominent sectors of the worldwide economy [17].

However, today's pharmaceutical industry is facing troubles that are partly caused by the patent system. The large pharmaceutical companies have invested huge funds to discover new drugs, but they are meeting huge amounts of competition with generic pharmaceutical companies in the marketing. When their drugs are no longer protected by patents that ensure the innovating companies to retain exclusive rights to sell the drugs, the newer generic pharmaceutical companies produce generic drugs and enter the market [1, 18, 19]. Some of the very well-known big companies represent only 40% of the pharmaceutical market, as they have to correspond to only a small fraction of the industry as a whole, with 90% of generic companies which produce the vast majority of all pharmaceuticals sold. The data of Figure 2.2 show that some larger pharmaceutical companies lost revenue because of initial patent expiry in 2010–2012 [16, 20, 21].

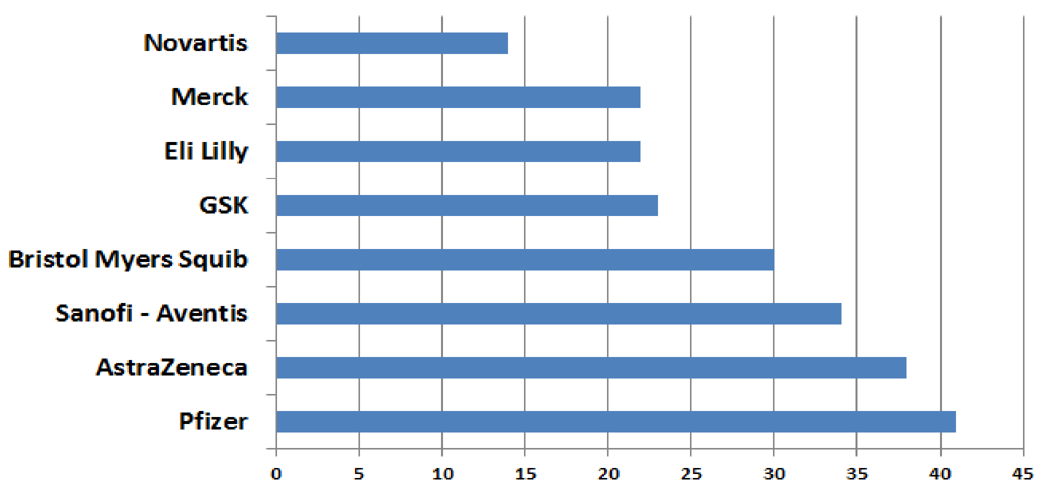

Figure 2.2. Patent expiry and revenue loss. Some larger pharmaceutical companies lost revenue because of initial patent expiry and low-priced generic drug competition (2010–2012).

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Brand Name Drugs and Generic Drugs

Brand name drugs, also called innovator drugs, are drugs that have trade names and have been protected by a patent; they can be produced and sold only by the patent holding company.

A generic drug is a copy of a brand name drug. When the initial patent protection for a brand name drug has expired, generic versions of the drug can be offered for sale after getting the approval from the United States Food and Drug Administration (FDA). Generic drugs are equivalent to a brand name product in dosage, strength, route of administration, performance, and quality [22]. Once the generic drug is manufactured and introduced into the market, the monopoly of the patent holder is removed. This condition will encourage competition in the pharmaceutical market, and this competition results in a significant drop of drug costs. The initial patent company will have to renew the patent by forming a new version of the drug that is significantly changed compared to its original compound. However, the new version of the compound not only needs new clinical trials, but also has to compete with the original generic molecule in the market, unless drug administration regulators find faults and remove the original from the market. The main difference between the branded drugs and the generic drugs is that the branded drugs are produced by a company which holds the patent for 20 years. However, the generic drugs are made available at the market by the pharmaceutical companies only after the patent protection period expires (after 20 years). Other major differences include cost and risk. Table 2.1 shows major similarities and differences between brand name drugs and generic drugs. The brand name drugs are far more expensive than the generic drugs.

Table 2.1. Similarities and differences between brand name and generic drugs

Brand name Drugs	Generic Drugs
Innovator drugs	Copies of brand name drugs
First version sold by the innovator company	Produced after the original patent expires
Similar in safety	Similar in safety
Similar in efficacy	Similar in efficacy
Similar in active compositions	Similar in active compositions
Similar in dosage and administration	Similar in dosage and administration
Difference in the color and shape	Difference in the color and shape
Difference in inactive ingredients	Difference in the inactive ingredients
Difference in the cost (higher price $$$)	Difference in the cost (lower price $)

Biologic Drugs and Biosimilar Drugs

Biologic drugs are drugs that come from living organisms or their cells, and aree often made by using biotechnology. A biologic drug is a large, complex, protein-based molecule with a trade name that is protected by a patent.

A biosimilar drug is almost a copy of an original biologic product that is manufactured by a different generic company [23]. Biosimilars can be manufactured when the original biologic drug patent expires [24]

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Generics vs. Biosimilars

There are differences between biosimilars and generic drugs in size, molecular weight, structure, complexity, manufacturing, stability, and approval requirements (Table 2.2) [25–31].

Table 2.2. Generic drugs *vs.* biosimilar drugs

Properties	Generics	Biosimilars
Size	Small	Large
Molecular weight	~ 150 Daltons	~ 150,000 Daltons
Structure	Simple and well-defined	Complex with potential Structural variations
Manufacturing	Predictable chemical process to make identical copy	Specialized biological process To make similar copy
Complexity	Easy to fully characterize	Difficult to characterize
Stability	Relatively stable	Sensitive to storage and handing conditions
Adverse immune reaction	Lower potential	Higher potential
Manufacturing quality tests	≤ 50	≥ 250
Approval requirements	Small clinical trials in healthy volunteers	Large clinical trials in patients

Generic Pharmaceutical Companies and Innovative Pharmaceutical Companies

Generic pharmaceutical companies are free to manufacture and sell what are now termed generic drugs, when the original biologic drug patent expires. Generic pharmaceutical companies should submit a drug manufacturer application, an Abbreviated New Drug Application (ANDA), with the FDA before manufacture. Before the FDA approves the application, the generic drug must meet the same standards that the original brand name drug has. They are low in cost and low in risk business, with no need to invest in any research costs; thus, generic pharmaceutical companies never have an unsuccessful product. The products that companies choose to manufacture and sell have shown to be valuable and commercially successful in the market.

Table 2.3. "Pay for delay" agreement between patent holder companies and generic companies

Patent Status	**Cost Price**	**Scrip Price**	**Annual Profit/1000 scrips**	
			Patent Holder	**Generic Company**
Patent Protection	$1	$10	$9000	$0
Patent Expired		$2	$1000	$1000
Patent holder pays generic company $2000/1000 prescriptions to delay manufacture and sale			$7000	$2000

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By comparison, the innovative pharmaceutical companies (initial patent companies) operate under a completely different business model. They invest huge funds in R&D and bring the new drug to the market. It is a long process which is very expensive, time consuming, and involves extremely high risks, particularly in the clinical trials where the success rate through clinical trials is extremely low (<1% of candidate drugs). Recently, an agreement known as "pay for delay" between the patent holder companies and generic companies has come into play. When a drug patent expires, the patent holder company pays the generic company not to manufacture this product. Then, both the patent holder and the generic company gain higher financial benefits, but the legality of these deals is under question (Table 2.3) [1, 16].

Cost and Time for New Drug Discovery and Development

Drug discovery and development is the process of bringing a new pharmaceutical drug to the market once a lead compound has been identified through the whole process. It includes pre-clinical research on microorganisms and animals, filing some applications to conduct clinical trials on humans, such as via the United States Food and Drug Administration (FDA) for an investigational new drug (IND) application, and via the U.S. Patent and Trademark Office (PTO) for patent and trademark application, and may include the steps for obtaining regulatory approval with a new drug application (NDA) to the market. As a result, the process for developing new medicines is growing in difficulty and length. On average, it takes more than 10–12 years for a new medicine to complete the journey from basic discovery to the market, including clinical trials of six to seven years on average alone [32–34]. The average cost to each successful drug was estimated to be about $6 billion in 1980, according to the data from National Science Foundation (NSF). By 2004, the cost had grown to more than $17 billion with an average increase of 5 percent per year. The pharmaceutical industry's trade association, Pharmaceutical Research and Manufacturers of America (PhRMA), reported larger expenditures and faster growth on new drug discovery and development. Figure 2.3 depicts that the average cost of each successful drug was estimated to be $4 billion in 1970, and it had grown to more than $60 billion in 2015. These data are estimated by both PhRMA and NSF. The curve A was reported by PhRMA, and shows annual expenditures for drug research and development profile (1970–2015). Unlike the NSF data, PhRMA's estimates include research and development performed outside the United States as well as further research and development that occur after a drug has gone to the market; the curve B is from the NSF report and shows a data series from 1980 to 2014. It includes only research and development conducted in the United States on drugs that have not yet reached the market [35]. Data from 1980 to 1998 come from the NSF report "Company and Other Funds for Industrial R&D Performance, by Industry and by Size of Company: 1953–98," available at www.nsf.gov/statistics/iris/search_hist.cfm? indx=10; [36]; data from 1999 to 2014 come from the NSF Division of Science Resources Statistics's annual "Research and Development in Industry," available at www.nsf.gov/statistics/industry [37].

These data incorporate the cost of failures. The thousand millions of compounds may be screened in the R&D process, only a few of which will ultimately receive FDA approval. Less than 12 percent of the candidate medicines will be approved by the FDA and enter a phase I clinical study. A study result was reported by the Tufts Center for the Study of Drug

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Development that showed 21.5 percent of drugs that started phase I trials were eventually approved for marketing during the period of 1980 to 1990, however, in the time period from 2006 to 2015, the success rate was only 9.6% [38, 39]. During drug development, to avoid costly failures, intelligent clinical trial program design is very important and can prevent false negative results. Likewise, success requires a vast amount of teamwork from excellent scientific minds, highly sophisticated technologies, complex project management, gold standard procedures, careful decision-making, and also luck.

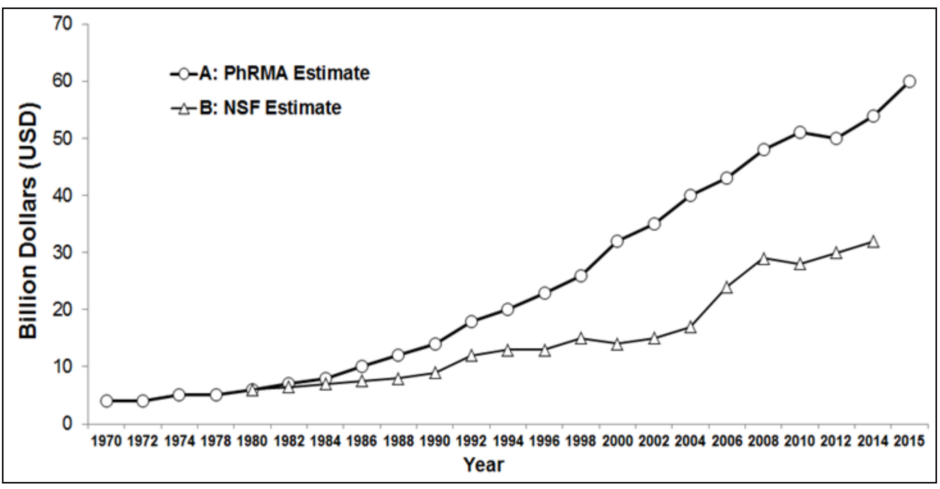

Figure 2.3. Estimates of annual spending on research and development. The curve A is from PhRMA and shows annual expenditures for drug research and development profiles (1970–2015); the curve B is from NSF and shows data that was only conducted in the United States on drugs that have not yet reached the market (1980–2014).

RESEARCH TEAMS AND GOOD LABORATORY PRACTICES

Research Teams

The excellence of pharmaceutical science and healthcare related research has been recognized internationally. It spans the complete continuum from basic research to translational science and clinical practice. Designing and running experimental or clinical research require the skills of many different types of experts. Each team may be set up at different sites; every research team member and his/her responsibilities should include the following steps:

Principal Investigator (PI)

The principal investigators are responsible for all experimental or pre-clinical and clinical studies, such as to develop the concept of the study, to design the protocol, to submit the application for FDA and PTO approval, and to supervise data collection, analysis, interpretation, and presentation.

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Pharmaceutical Chemical and Biological Scientists

Modern drug research and development requires the teamwork of pharmaceutical chemical and biological scientists. Pharmaceutical chemical scientists can perform research to study a company's current products to find out if they can be improved. They also may discover and develop new chemical compounds and determine their effects with other chemicals. Their duties include maintaining reports, documents and files regarding testing.

Pharmaceutical biological scientists can perform research to gain a better understanding of human diseases; they often work to evaluate new potential active chemical or biological products, determine their effects on biological systems, and improve drug development.

Data Managers

Data managers are responsible for managing the collection of data throughout the course of experimental or clinical research, input the data, work with the PI to identify what data will be tracked, provide data to monitoring agencies, and prepare summaries for interim and final data analysis.

Staff Surgeons, Physicians and Nurses

The surgeons, physicians, nurses, and other staff members are responsible for treating patients according to the clinical trial protocol, assessing and recording how patients respond to the drug in-treatment and the drug side effects they may have, helping to take care of the patients during a clinical trial, working with the PI to report trends of how patients are doing on the treatment, and providing each patient's medical care.

Patent Agents

The patent lawyers and attorneys have the specialized qualification necessary for representing clients in obtaining patents and acting in all matters and procedures relating to patent law and practice. The patent attorneys and trademark agents also work in some jurisdictions and approach many legal questions during drug development.

Good Laboratory Practices (GLP)

In the preclinical and clinical studies, FDA requires researchers to use good laboratory practices (GLP) that specifically refer to a quality system of management controls for laboratories and organizations to ensure high quality in uniformity, consistency, reliability, reproducibility, safety, animals and their uses. The principles of GLP set the minimum basic requirements for study conduct, personnel, facilities, equipment, written protocols, operating procedures, study reports, and a system of quality assurance (QA) for each study that helps to assure the safety of any FDA-regulated product. GLP regulations set the minimum basic requirements as stated below [40]:

GLP Performance

All study protocols should be performed according to the principles of GLP.

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GLP Standard Operating Procedures (SOPs)

Standard Operating Procedures are instructions that are written in detail to achieve uniformity of the performance of a specific function, and help maximum safety and operational efficiency for the fields of pharmaceutical and clinical research. A well-written SOP can be used to satisfy several compliance requirements. The procedures may be useful to operationalize documents such as plans, regulation, compliance, and policies. SOPs should be used by all staff members in their work environment. All high-class processes and procedures should be put on in the standard operating procedures.

GLP Test Systems

All physical, chemical, biochemical and biological studies should precisely follow the SOP procedures.

GLP Responsibilities

The organization of the study should involve management responsibilities, sponsor responsibilities, director responsibilities, PI responsibilities, and study personnel responsibilities.

GLP Facilities and Equipment

All facilities and equipment that are now being used for the study should match GLP guidance. Any significant changes to the equipment should be identified. GLP quality equipment systems should have a comprehensive, up-to-date list of all equipment used in the facilities, and should establish a maintenance and calibration program for equipment. Equipment procedures should include the frequency of calibration and maintenance, routine inspection, cleaning, and testing.

GLP Records and Reports

All study reports, specimens, raw data, and other study-related documents should be kept in the archives.

GLP Quality Assurance Program

A system of quality assurance (QA) oversight for each study should assure the safety of an FDA-regulated product.

To evaluate drug safety, the GLP compliant scientists conduct pre-clinical studies, including single dose toxicity, repeated dose toxicity (acute and chronic), reproductive toxicity, mutagenic potential, carcinogenic potential, PK/PD studies, and local tolerance studies [41–43]. These pre-clinical results must be provided in detail before clinical trials. After preclinical testing, researchers with PI review their results and decide whether the drug should be tested in humans.

Pharmaceutical Research and Development Process

Following well-designed plans and established models, the process of new drug development makes sure whether or not a potential new medicine is safe and effective before it reaches the market. The whole process involves six steps: drug discovery, pre-clinical

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research, patent and an investigational new drug (IND) application, clinical research, FDA review of the new drug application (NDA), and post-market safety monitoring (Figure 2.4).

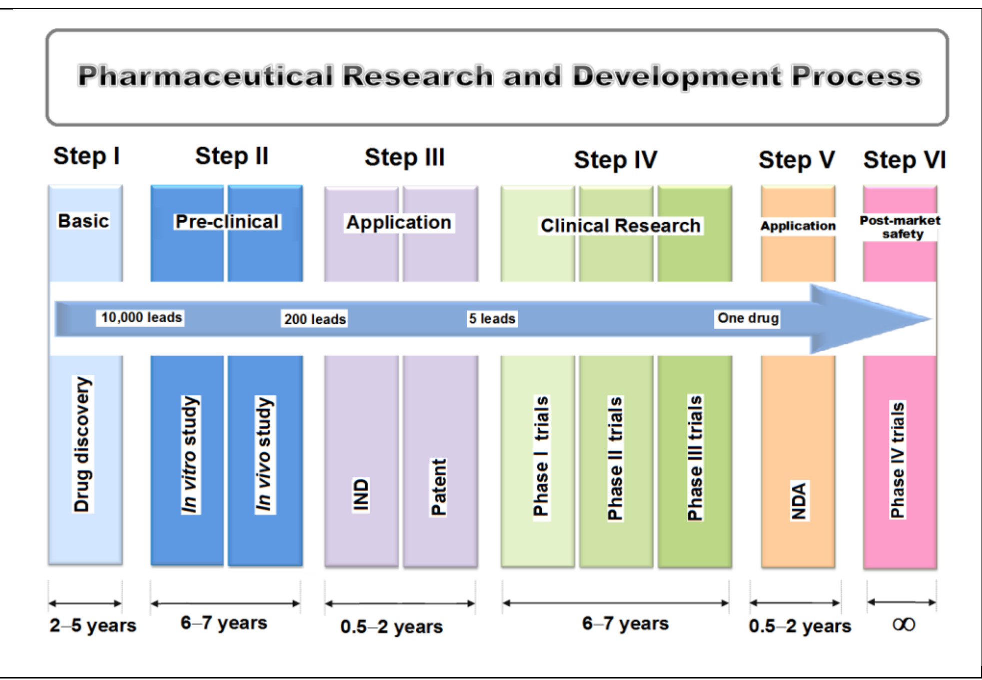

Figure 2.4. The pharmaceutical research and development process for new drugs. The six steps of the whole process for new drug development: drug discovery in the laboratories, pre-clinical research, patent and investigational new drug (IND) application, clinical research, FDA review of new drug application (NDA), and post-market safety monitoring.

STEP I. Drug Discovery

Lead discovery is the first major stage of the R&D process, including the early basic studies, which are designed to identify and evaluate investigational new drugs in the laboratories. Researchers and pharmaceutical companies design, discover, and synthesize chemical or biological molecular compounds to stop or reverse the negative effects of the diseases by blocking signal pathways and disease mechanisms; or by multiple screening testing to find possible beneficial effects against diseases; or by existing drug treatments to find their new unanticipated effects. These drug targets can be a wide variety of things, such as a particular cell type, enzyme, gene, or signal pathway. Recently, the selection for a potential candidate compound within these vast chemical or biological libraries has been simplified by the introduction of High Throughput Screening Techniques (HTS) and Ultra High Throughput Screening (UHTS), which use advances in robotics, automation, miniaturisation and data handling. During this stage, thousands of compounds may be potential candidates for development. Scientists take approximately two to five years to identify and select some promising drug candidates for further development. After this stage of research, there are only a few compounds which look promising and will be studied in the next stage.

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STEP II. Pre-Clinical Studies

Lead identification is the second major stage of the R&D process, and marks the transition from laboratory basic research into further pre-clinical development. Once promising compounds are discovered, identified and selected, the development process will begin and conduct serial experiments to collect further information. The drug evaluation includes the compound's best dosage and best route to give in animals; the compound's absorption, distribution, metabolism, excretion, and toxicity (ADME-T) testing; the compound's pharmacodynamics (PD, what the drug does to the body) and pharmacokinetics (PK, what the body does to the drug) studies; the compound's potential benefits and mechanisms of action; the compound's effectiveness as compared with similar medicine products; the compound's stability in chemical, light, and moisture conditions; and the compound's side effects in animals, including which organs are targeted by this compound, as well as whether there are any long-term carcinogenic effects or toxic effects on mammalian reproduction (Figure 2.5).

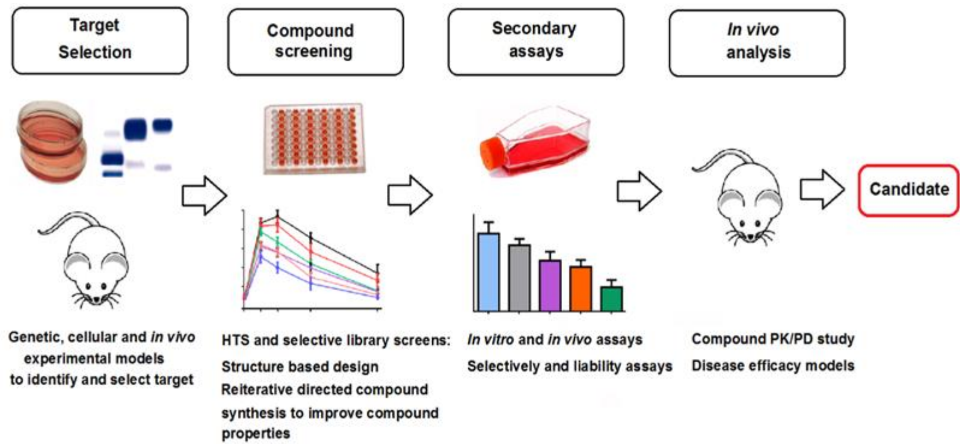

Figure 2.5. Preclinical research. Preclinical research should evaluate efficacy and toxicity of new drugs *in vitro* and *in vivo* .

Lead optimisation is the third stage of the R&D process. In order to increase the efficacy of a drug while decreasing any potential side effects, some attempts will be made to modify the molecular structure in various ways. It may take another six to seven years of research time via *in vitro* and *in vivo* experiments in the pre-clinical study before entering clinical research phase.

During this stage, there are *in vitro* and *in vivo* studies that are carried out to determine whether these candidate compounds are ready to be studied in human research. *In vitro* experiments are conducted in the laboratory on living cells or tissue cultures and *in vivo* studies focus on animal models. Through these studies, it will be understood how the drug works and what the potential side effects on humans might be. Also, it will be determined how to make large enough quantities of the drug to use in the next set of clinical trials. It may not be easy to make large amounts of compounds to use in preclinical stage. The most important point is that safety evaluation should begin in the early stages in order to provide a preliminary assessment of drug safety before its use in humans.

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In vitro Study

An *in vitro* (Latin: in glass) study is performed using components of an organism that have been isolated from their usual biological surroundings, such as microorganisms and cells which can be studied in an artificial model. They all are called test tube experiments [44, 45]. *In vitro* studies can be miniaturized and automated by yielding high-throughput screening methods for testing molecules in pharmacology and toxicology [46]. Another advantage of *in vitro* methods is that human cells can be studied without extrapolation from an experimental animal's cellular responses [47].

In vivo Study

An *in vivo* (Latin: within the living) study is conducted in living beings, such as microorganisms, animals, humans, or whole plants. An animal study is the main method of *in vivo* studies [44, 45]. *In vivo* studies are often used and preferred over *in vitro* studies because they are better suited for observing the overall effects of an experiment or a compound on a living subject. There are many examples of *in vivo* investigations, such as purified bacterial toxins, therapeutic antibodies, antibiotics, antiviral drugs, and other new protein compounds. In drug discovery, the verification of efficacy *in vivo* is very crucial; sometimes, *in vitro* studies show the potential results of the new drug, but they may be misleading or produce false conclusions [48, 49].

ADME-T Study

In order to know how the body processes the investigational compound, absorption, distribution, metabolism, excretion, and toxicity (ADME-T) testing should be performed in *in vitro* (cell culture) and *in vivo* (animal) models (Figure 2.6) [50–54].

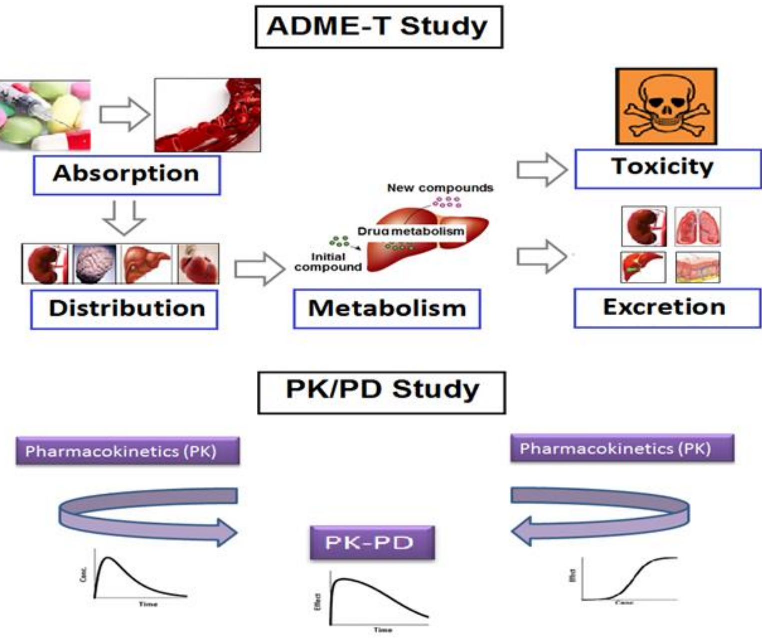

Figure 2.6. An ADME-T study and a PK/PD study. ADME-T study and PK/PD studies are performed to evaluate the toxicity and efficacy of new drugs.

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ADME Study

Absorption Study

Absorption studies aim to understand how a drug enters the body and reaches the blood, plasma and sera. Drugs may be introduced into the body by several routes. They may be taken by mouth (orally); given by injection (intravenously, intramuscularly, or subcutaneously); sprayed into the mouth, nose, and lungs (nasally or inhalation); or applied to the skin (cutaneously). Some factors can reduce drug absorption after drug administration, such as poor compound solubility, gastric emptying time, intestinal transit time, chemical instability in the stomach, and the inability to permeate the intestinal wall.

Distribution Study

It is intended to understand where a drug distributes throughout the body after it has been absorbed. The compound needs to be carried to its effector site by the blood. After entry into the systemic circulation, the drug is subjected to numerous distribution processes that tend to lower its plasma concentration. Some factors may affect drug distribution, such as regional blood flow rates, molecular size, binding to serum proteins, and complex forming. Distribution can be a serious problem at some natural barriers like the blood brain barrier.

Metabolism Study

Its purpose is to understand how a drug is changed by the body. A compound begins to break down as soon as it enters the body. The majority of the small molecule drug metabolism is carried out in the liver by redox enzymes. As metabolism occurs, the initial compound is converted into new compounds called metabolites. When metabolites are pharmacologically inert, the metabolism deactivates the administered dose of the parent drug and this usually reduces the drug effects on the body.

Excretion Study

It is aimed to understand how a compound and its metabolites are removed from the body via excretion. There are three main sites where the drug is excreted.

Urine excretion: The kidneys are the most important excretion pathway for the glomerular filtration of an unbound drug; it passes out through the urine.

Biliary and fecal excretion: Biliary and fecal excretion is another pathway used; the liver passes the drug to the gut until the drug is finally excreted along with waste products or feces.

Breathing and sweat excretion: Excretion via lungs and skin is also another important elimination pathway.

Toxicity Study

The aim of the toxicity study is to identify adverse effects of an investigational compound on humans and animals. Adverse effects depend on two main factors, routes of absorption (oral, inhalation, dermal, and blood) and dose (duration and concentration of exposure). Generally, both acute and chronic toxicity models should be used in a toxicity study. A toxicity study can also be conducted by an *in vivo* study that is performed on non-human animals [55].

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Types of Preclinical Toxicity Study

Safety Pharmacology

It is used to determine the effects of the drug on specialized organs (e.g., cardiovascular, respiratory, neurological, etc.).

Chronic Toxicity/Carcinogenicity

It is used to determine the effects of long-term exposure to the drug, including the ability to induce cancer.

Reproductive Toxicity/Teratogenicity

It is used to evaluate effects on reproductive function and the ability to produce birth defects.

Results from preclinical toxicology studies should indicate a minimum and safe starting dose for clinical studies; provide information on a drug treatment regimen that would produce the least amount of toxicity; assess target organ toxicity and reversibility; and provide insight into biomarkers for clinical screening, diagnosis, and monitoring of disease progression.

Factors Influencing of Toxicity Study

There are some factors which may influence results of a toxicity study, such as dosage (acute and chronic exposures), ways of exposure (inhalation, oral, skin and blood absorption), and other factors, such as animal species, age, sex, health, environment, and individual characteristics.

The Dose-Effect Relationship/the Dose-Response Relationship

It is essential to determine dose-effect and dose-response relationships in a toxicity study [56].

The dose-effect relationship is the relationship between dose and effect at the individual level. An increased dose may increase a more severe effect. A dose-effect curve may be obtained at the levels of the whole organism, the cells or the target molecules.

The dose-response relationship is the relationship between the dose and the percentage of individuals showing a specific effect. With increasing dose, a greater number of individuals in the exposed population will usually be affected. The dose-response curve for most toxic effects has a sigmoid shape. There is usually a low dose range where there is no response detected; as the dose increases, the response follows an ascending curve that will usually reach a plateau at a 100% response. The dose-response curve reflects the variations among individuals in a population.

There are some factors to be used to evaluate dose-effect and dose-response relationships.

Dose may be expressed in different ways, such as tissue dose, target dose, and exposure and absorbed dose. The tissue dose is the amount of substance in a specific tissue. The target *dose* is the amount of substance bound to the critical molecule and is more exactly associated with the toxic effect. The exposure dose is the air concentration of a pollutant inhaled during the period of a certain time. The absorbed dose is the amount present in the body at a certain time during or after exposure.

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Median Lethal Dose (50% lethal dose, LD50) is the dose which causes the death of 50% (one half) of test animals. LD50 is used mainly to determine acute toxicity. The larger the LD50 value, the lower the toxicity.

Median Lethal Concentration (50% lethal concentration, LC50) is the concentration that kills 50% of the test animals during the observation period.

Median Effective Dose (ED50) is the dose that produces a therapeutic response or desired effect in 50% of the population. It is commonly used as a measure of the reasonable expectancy of a drug effect.

Dose Threshold is a dose level below which no observable effect occurs. Thresholds are thought to exist for certain effects, like acute toxic effects, but not for others, like carcinogenic effects.

Time is often included in the dose concept; it is usually more important for understanding repeated exposures and chronic effects than for single exposures and acute effects.

Effects

Additive effect of drugs occurs in two substances used in combination that produces the same total effect as the sum of the individual effects.

Antagonistic effect of drugs occurs in two substances used in combination that causes a decrease in the effects of both drugs through their interaction.

Synergistic effect of drugs occurs in two substances used in combination that causes an increase in the effects of both drugs via their interaction.

No-Observed-Adverse-Effect-Level (NOAEL) is no adverse effect level, or the highest dose that does not cause a toxic effect, and is an important part of the non-clinical risk assessment.

A successful drug must be absorbed into the bloodstream; distributed to the proper sites of action in the body; metabolized efficiently and effectively; be successfully excreted from the body; and shown to be non-toxic in the tests. The candidate drug will undergo years of further testing and analysis before potentially being reviewed for approval by the U.S. Food and Drug Administration (FDA). During this period of time, 30% of all drug candidates crash at this step due to toxicity profiles and side effects.

PK/PD Study

Pharmacokinetic (PK)/pharmacodynamics (PD) modeling is a technique that combines the two classical pharmacologic disciplines of PK and PD. PK is the study of how an organism affects a drug, whereas PD is the study of how the drug affects the organism. Both together are evaluated in the PK/PD model. A major goal of PK/PD in clinical pharmacology is the quantitative prediction of drug effects [57, 58]. Before clinical testing, it is important to compare the PK/PD properties of candidate molecules for dose, concentration, efficacy, and toxicity. There are five types of PK/PD modeling: direct PK/PD model, indirect link PK/PD model, indirect response PK/PD model, cell lifespan model, and complex response model. There are the most common factors to be used to evaluate PK/PD study [59].

Dose: The dose is the amount of a drug taken at any one time. This can be expressed as the weight of the drug (e.g., 1 mg), volume of the drug solution (e.g., 1 mL), and the number of dosage forms (e.g., 1 capsule).

Dosing interval time: It is time between drug dose administrations.

Cmax: It is the highest plasma concentration of a drug after administration.

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Cmin: It is the lowest (trough) concentration that a drug reaches before the next administration.

Tmax: The amount of time that a drug is present at the maximum concentration in plasma or serum.

Volume of distribution: The apparent volume in which a drug is distributed, such as the parameter related to drug concentration and the drug amount in the body.

Concentration: It is the amount of a drug in a given volume of plasma or serum.

Elimination half-life: It is the time required for the concentration of the drug to reach half of its original value.

Elimination rate constant: The rate at which a drug is removed from the body.

Infusion rate: It is the rate of infusion required to balance the elimination.

Area under the curve: It is the integral part of the concentration-time curve after a single dose or in a steady state.

Clearance: It is the volume of plasma or serum cleared of the drug per unit time.

Bioavailability: It is a term used in pharmacology and refers to the degree and rate at which an administered drug is absorbed by the body's circulatory system. In clinical trials, the bioavailability of a drug is a key factor to be measured in phase I and phase II trials.

Fluctuation: It is the peak trough fluctuation within one dosing interval at a steady state.

Animal Study

Animal testing, known as *in vivo* testing, is the use of non-human animals in experiments, and seeks to control the variables that affect the behavior or biological system under study. Animals are similar to human beings in many ways, e.g., chimpanzees share 99% of their DNA with humans, and mice are 98% genetically similar to humans. Therefore, they are appropriate research subjects, and have contributed to many life-saving cures and medical treatments [60]. In addition, because of the short life cycles of animals, they become better research subjects than human beings. For example, laboratory rodents live for only two to three years, so they are particularly well-suited to long-term cancer research because of their short lifespans [61].

Choice of Animals

Naive

All animals should be *naïve* in all study groups. They did not encounter an antigen/antibody, pathogen, protein, or drug, and did not have a particular experience or were not subject to a particular experiment [62]. According to Animal Rule (October 2015 edition) from the guidelines of the Food and Drug Administration, Center for Drug Evaluation and Research (CDER), U.S. Department of Health and Human Services, Center for Biologics Evaluation and Research (CBER), The Canadian Council on Animal Care (CCAC) and other similar international and regional authorities, any prior research treatment, even as control group animals, may have the potential infection which can cause stress and alter animal's physiological or biological responses [63]. In addition, according to the guidelines of Institutional Animal Care and Use Committee (IACUC), multiple survival surgical procedures on a single animal are also discouraged [64].

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Species

Drug toxicity testing in animals usually requires at least two mammalian species, including one rodent (e.g., rat, mouse) species and one non-rodent (e.g., dog, non-human primate) species prior to human trials [65]. Other species (e.g., rabbit, ferret, hamster, and pig) may be used for special cases, such as vaccine studies.

Number

The number of animals should be determined to ensure scientifically valid results. Well-designed experiments use a sufficient number of animals to achieve the scientific objective, include the necessary control groups and incorporate appropriate statistical analyses.

Sex

The male/female animal composition should be justified in the study groups.

Animal Models

The proper preclinical models which can efficiently predict drug behavior in humans is essential prior to testing a drug in humans. Qualification of an animal model should be under The Animal Model Qualification Program (AMQP) that is supported by CDER and used in drug development under the Animal Rule [66].

Ethical Issues

All proposals to use animals for research must be approved by an Institutional Animal Care and Use Committee (IACUC) set up by each research facility before pursuing animals. Humane treatment is enforced by each facility's IACUC. The most major research institutions' programs are voluntarily reviewed for humane practices by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC) [67, 68].

Three Rs

The moral and ethical questions raised by performing experiments on animals are subject to debate, and viewpoints have shifted significantly over the 20th century [69]. Animal testing in the research has been reduced in recent years for both ethical and cost reasons. The rule of the Three Rs (replacement, reduction, and refinement), first described by W. Russell and R. Burch in 1959, is usually employed to capitalize the first letter of each of the three 'R' principles (Figure 2.7) [70, 71].

Replacement refers to methods that prefer the use of non-animal methods over animal methods whenever it is possible to achieve the same scientific goals. Replacement methods include the use of:

Animal tissues and cells
Computer models
Established cell lines
Immature forms of vertebrates (worms)

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Reduction refers to methods that make researchers obtain comparable levels of information from fewer numbers of animals, or to obtain more information from the same number of animals.

Refinement refers to methods that minimize potential pain, suffering or distress, and enhance animal welfare for the animals used.

Today, the 3Rs are guiding principles for a more ethical use of animals in research, and are becoming a framework for conducting high quality science in the academic and industrial sectors with more focus on developing alternative approaches, which avoid the use of animals [72, 73]. There are a number of reasons for this including the need for better models and tools that more closely reflect human biology and predict the efficacy and safety of new medicines.

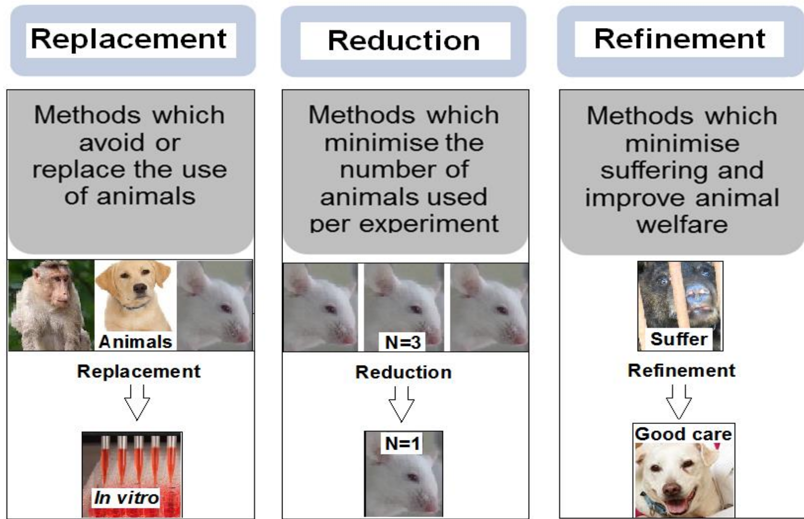

Figure 2.7. The rule of replacement, reduction and refinement in an animal study. The rule of the 3Rs refers to the use of non-animal methods over animal methods; the use of fewer the numbers of animals to obtain more information from the same number of animals; and to improve animal welfare.

STEP III. Two Important Applications for Investigational New Drug and Patent before Clinical Studies

Investigational New Drug and Investigational New Drug Application

After a new candidate drug has been screened for pharmacological activity and toxicity in a pre-clinical study, and has been identified to be a viable candidate for further development, the sponsors should collect all data and information about this product, and ensure that it has no unreasonable risks when it will be used in the next early clinical study in humans. Before clinical trials, all sponsor biotechnology and pharmaceutical companies must submit two important applications, an investigational new drug (IND) application and patent application; both of these applications must be approved respectively by the U.S. Food and Drug Administration (FDA) and U.S. Patent and Trademark Office (PTO). The FDA approval of an IND product is necessary before the drug can be marketed; and the PTO approves that the IND patent can protect the inventions of the pharmaceutical company. These approval processes are lengthy, and sponsor pharmaceutical companies should minimize the time spent on the process as part of a profit maximizing strategy [74].

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IND Type

There are three IND types (Figure 2.8) [75, 76].

An Investigator IND is submitted by a physician who both initiates and conducts an investigation, and under whose immediate direction the investigational drug is administered or dispensed. A physician might submit a research IND to propose studying an unapproved drug, or an approved product for new indication or in a new patient population.

An Emergency Use IND allows the FDA to authorize the use of an experimental drug in an emergency situation that does not allow time for submission of an IND.

A Treatment IND is submitted for experimental drugs showing promise in clinical testing for serious or immediately life-threatening conditions while the final clinical work is conducted and the FDA review takes place.

IND Application with FDA

Generally, IND application should contain data and information in four broad areas [77]:

All Results of Pharmacology and Toxicology Studies

The information should show that all preclinical data ensure that the product is reasonably safe for initial study in human clinical trials. For studies of marketed drugs in new indications, this section should contain data from animal models to support the utility of the drug in the new indication.

Manufacturing Information

The information should ensure that the manufacturing company can adequately produce and supply consistent batches of the drug.

Clinical Trial Plans and Protocols

The information should show that detailed protocols for clinical trials are assessed and whether the initial phase trials will expose subjects to unnecessary risks.

Information About the Investigator

The information should provide the qualifications of clinical investigators – professionals (generally physicians) who oversee the administration of the experimental compound – to assess whether they are qualified to fulfill their clinical trial duties.

IND Review Team

An IND review team is a group of specialists in different scientific fields. Each member has different responsibilities.

Project Managers coordinate all team member activities throughout the review process as the primary contact for the sponsor.

Medical Officers review all clinical study information and data.

Chemists and Biologists evaluate drug stability, quality control, continuity, and the presence of impurities, and review the data to assess responses across different classes of microbes or animals.

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*Pharmacokineticists e*valuate the data of drug absorption, distribution, metabolism, excretion and toxicity studies, and interpret blood level data at different time intervals from clinical trials, as a way to assess drug dosages and administration schedules.

Statisticians interpret clinical trial designs and data, and work closely with the medical officers to evaluate protocols, safety, and efficacy data.

IND Approval

Once the IND application is submitted, the FDA review will be a 30 calendar dayprocessing time. The process protects volunteers who participate in clinical trials from unreasonable and significant risk in clinical trials. The FDA responds to IND applicants in one of two ways:

IND approval: The candidate drug will move into phase I clinical trial.

Clinical hold: The candidate drug will be delayed or stopped for the investigation. The FDA can place a clinical hold for specific reasons, including investigators not being qualified; materials for the volunteer participants are misleading; the candidate drug can expose patients to unreasonable or significant risks; and the IND application does not include enough information about the trial risks.

At this step, approximately 85% of all drugs for IND applications are permitted to undergo clinical trials. Figure 8 depicts the application process of an IND:

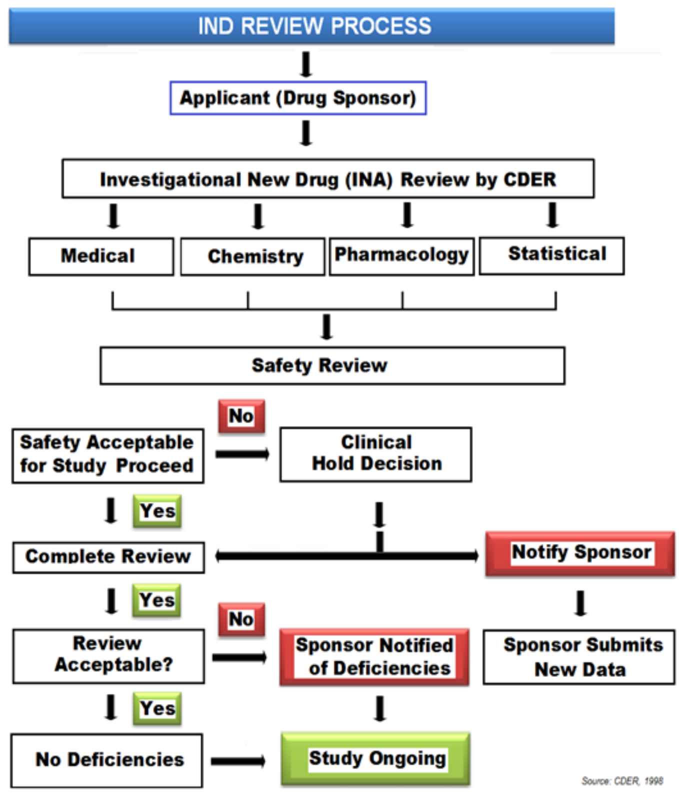

Figure 2.8. The investigational new drug application review process. The graphic depicts the application process of an IND. It is a 30 calendar day processing time from FDA review a complete IND application to make decision.

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Patents Protection for Intellectual Property and Patent Application

Intellectual Property

Intellectual property (IP) rights are the protections in law and are granted to the creators of intellectual property. IP rights include patents, trademarks, trade dress, copyright, industrial design rights, and geographical indication which enable people to earn recognition or financially benefit from what they invent or create [78, 79].

In drug development, IP protection provides incentives for investing companies who make the long and costly investments that lead to medical advances. Strong IP protection, in the form of patents and exclusivity, provides the opportunity for companies to potentially recoup investments made to develop new medicines and to fund future research [80].

Patent and Market Exclusivity

A patent is a limited legal monopoly granted to the owner of an invention that prevents others from making, using, importing or selling the invention without owner permission. It can be a product, a new method, or a technical improvement on how certain objects work [74]. A patent is an exclusive right to grant an inventor market exclusivity over a new invention or medication. Market exclusivity means tremendous economic rewards for the patent holder because it provides the inventor with a monopoly over the invention for the 20 years patent term.

Today, in the field of novel drug development, pharmaceutical companies are facing increased costs for drug development and aggressive competition from generic drug companies, therefore patent protection and the market exclusivity help to ensure a return on investment [81]. It allows the inventor to prevent others from commercially using ideas or inventions without the creator's permission during the life of the patent [18, 79, 82, 83]. Patent protection is granted for a limited period, generally 20 years from the filing date of the application. The patented drugs are temporarily safe from the competition of generic companies. Obtaining a patent and retaining market exclusivity can be a treacherous process, especially in the arena of pharmaceutical patents. In the USA, 74% of all new drug sales tend to occur in the 5 year exclusivity window following drug approval, with an additional 15% of sales realized in the 3 years following the loss of exclusivity when cheaper generic versions enter the market [84, 85].

Patent Application with PTO

The innovative pharmaceutical organizations should file a patent application to PTO on a new biotechnology and pharmaceutical drug before clinical trials. In this way, the innovating companies can obtain an exclusive right with respect to their inventions without others being able to copy and sell it for a set period of time [86].

The patent application should be filed before FDA approval of IND, because patents are important IP safeguards. There are some reasons for filing a patent application before an IND application with the FDA: i) If pharmaceutical organizations begin the IND application before the patent application, it is possible to lose royalties, market exclusivity, and company value in the process, because other companies may get the patent before them. They will have to license the pharmaceutical from the other companies or abandon the FDA process, and lose millions that have been spent in research and development; ii) the patent application has a

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lower safety standard than the FDA application, because the patent applicant is not required to provide any clinical evidence of drug safety; iii) the FDA approval may be accelerated for a patented compound; and iv) a patented new drug will attract the interest of potential investors who can provide the capital to support the FDA approval of clinical trials. Application, creating and protecting for a new compound patent requires a very close collaboration between pharmaceutical scientists and lawyers because scientific, legal, and practical considerations must be carefully weighed to best protect an inventor's rights [87, 88].

Patent Problems

When a pharmaceutical company first develops a new patented drug that is used in clinical treatment, it has the exclusive right to be sold under a brand name by which the clinicians can prescribe the drug for patients. The drug is covered under patent protection, which means that only the pharmaceutical company that holds the patent is allowed to manufacture and market the drug, and eventually make a profit from it. Although a drug patent is awarded for around 20 years, in most cases, the lifetime of the patent is no more than 10 years after the drug has finally received FDA approval, because the company application for a new drug patent needs time before the clinical trials. During this short period, pharmaceutical organizations have to recoup all the costs in drug development, together with the manufacturing and marketing costs. After a patent expires, generic drug competition will lead to a dramatic reduction in price and major loss of market share. Thus, it is not surprising that research companies try to extend patent life as much as possible [16, 90].

STEP IV. Clinical Trials Studies

While preclinical research answers basic questions about a new drug’s safety, innovating pharmaceutical companies send the data to the FDA for IND approval as soon as possible. Once the FDA has approved, clinical research of new drugs can begin in human trials. Before a clinical trial begins, researchers should review all prior information about the drug to answer clinical research questions and develop clinical research objectives. They should follow good clinical practice (GCP) standards in clinical research.

Good Clinical Practice (GCP) Standards

Clinical trials in humans involve a wide range of practical and ethical problems. Each phase of the trial must be approved by an ethics committee, and all patients must give their prior informed consent to participate. Human clinical trials must be subject to good clinical practice (GCP) standards. GCP is an international ethical and scientific quality standard that is provided by the International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH) for designing, conducting, recording and reporting clinical trials that involve the participation of human subjects. Regardless of whether a clinical trial is a large, multi-center study in patients, or a small clinical pharmacological study in healthy subjects, the relevant ICH-GCP standard should be followed by the sponsoring companies, the investigators, the ethics committees, and any clinical research organizations [91–94].

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The GCP principles provide assurance that the rights, safety and well-being of a clinical trial subjects are protected. They also ensure that the research yields quality scientific data. It is very important to understand the background of the formation of the ICH-GCP guidelines. There are thirteen core principles of ICH-GCP [93, 95–100].

Ethical Principles

Clinical trials should be conducted in accordance with the ethical principles that have their origin in the Declaration of Helsinki (DoH) and that are consistent with GCP and applicable regulatory requirements.

Favourable Benefits vs. Risks

Before a clinical trial is initiated, foreseeable risks and inconveniences should be weighed against the anticipated benefit for the individual trial's subject and society. A clinical trial should be initiated and continued only if the anticipated benefits justify the risks.

Subject Rights

The rights, safety and well-being of the trial subjects override the interests of science and society.

Adequate Supporting Data

The available non-clinical and clinical information on an investigational product should be adequate to support the proposed clinical trial.

Scientifically Sound Protocol

Clinical trials should be scientifically sound and described in a clear, detailed protocol.

Independent Ethics Committee Oversight

A trial should be conducted in compliance with the protocol that has received a prior institutional review board (IRB)/independent ethics committee (IEC) approval or favorable opinion.

Medical Care by Qualified Investigator

The medical care given to subjects, and the medical decisions made on their behalf, should always be the responsibility of a qualified physician or, when appropriate, a qualified dentist.

Qualified Personnel

Each individual involved in conducting a clinical trial should be qualified by education, training and experience to do their respective tasks.

Informed Consent

Freely given informed consent should be obtained from every subject prior to participation in the clinical trial.

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Record Keeping

All clinical trial information should be recorded, handled and stored in a way that allows its accurate reporting, interpretation and verification.

Subject Confidentiality

The confidentiality of records that could identify subjects should be protected, respecting the privacy and confidentiality rules in accordance with the applicable regulatory requirements.

GMP Manufacturing of the Investigational Product

Investigational products should be manufactured, handled and stored in accordance with an applicable Good Manufacturing Practice (GMP). They should be used in accordance with the approved protocol.

Quality Assurance and Monitoring

Systems with procedures that assure the quality of every aspect of the clinical trial should be implemented.

Four Distinct Phases of Clinical Studies in Humans

Before a clinical trial begins, researchers should review prior information about the drug questions and objectives. They should focus on two essential questions: does the drug work in patients? Is it safe in humans? Additionally, they should also answer other questions, such as how long the study will last; how many people will be part of the study; who qualifies to participate; whether there will be a control group and other ways to limit research bias; by which way the drug will be given to patients and at what dosage; what assessments will be conducted, when, and what data will be collected, and how the data will be reviewed and analyzed.

A clinical trial is a typical series of research from early-stage, small scale studies to late-stage, large scale studies (Table 2.4) [101]. Clinical trials take place in four distinct phases; the first three phases (I, II, and III) start before the drug is marketed, and the fourth phase (IV) begins once the drug has been licensed and prescribed. Every phase is considered a separate trial. After completion of a phase, investigators are required to submit all data for FDA approval before continuing to the next phase. Individual trials may encompass more than one phase. A common example of this is combined phase I/II or phase II/III trials. Therefore, it may be easier to think of early-phase and late-phase studies. The drug development process normally proceeds through all four phases over many years. If the drug successfully passes through phases I, II, and III, it will usually be approved by the national regulatory authority for use in patients. In order to reduce or eliminate bias, all trials will be blinded, and the patients receiving the candidate drug will be unaware of whether they are part of the treatment group or the control group. Also, many trials are double blind; none, neither patients nor physicians, will be aware of group information [102, 103].

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Table 2.4. Four distinct phases of clinical studies in humans

Phase	Goal	Length	Dose	Monitor	Number	Success
Pre-clinical	Efficacy, toxicity, PK/PD in non-human subjects	~ 4 years	Unrestricted dose	Researchers	*In vitro* *in vivo*	
Phase 0	PK/PD in healthy volunteers	Several months to 1 years	Very low dose	Clinical researchers	10 people	
Phase I	Dose-ranging in healthy volunteers	Several months	Sub-therapeutic dose	Clinical researchers	20-100 healthy volunteers	70%
Phase II	Efficacy and side effects in patients	Several months to 2 years	Therapeutic dose	Clinical researchers	100-300 patients	33%
Phase III	Efficacy, effectiveness and safety in patients	1 to 4 years	Therapeutic dose	Clinical researchers	300-3,000 patients	25-30%
Phase IV	Post-marketing surveillance		Therapeutic dose	Physicians	Anyone seeking treatment	N/A

Phase I. Initial Safety Testing in a Small Group of Healthy People

The main goal of a phase I study is to confirm that the results derived from the preclinical study are replicated in human subjects. The studies are usually conducted with a small number of healthy volunteers, generally 20 to 100 healthy volunteers or people with the disease exposed to very low amounts of the candidate drug for short periods (several months) under carefully controlled and monitored conditions. Researchers monitor the pharmacokinetics of a drug which is absorbed, metabolized and eliminated from the body, and also study the drug's pharmacodynamics, which are related to side effects.

Main Goal of Phase I Studies

How the new drug should be given (such as by mouth or injection) and how often?

What are the safest dosages and the highest dose that a person can tolerate (maximum tolerated dose)?

What effects does the drug have on the people taking the drug?

What side effects does the drug have on the people taking the drug?

Phase I Studies Can Be Further Divided

Phase Ia studies (Single ascending dose)

In single dose studies, small groups of patients are given a single dose of the drug while they are observed and tested for a period of time to confirm drug safety. If they do not exhibit any adverse side effects, a new group of a higher dose is then given the drug. If an additional unacceptable toxicity is observed, then the dose escalation is terminated and that dose is declared to be the maximally tolerated dose [104–106].

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Phase Ib studies (Multiple ascending dose)

Multiple dose studies investigate the PK and PD of the drug, and evaluate its safety and tolerability. During the studies, patient samples are collected at various time points and analyzed to acquire information on how the drug is processed within the body [105–107].

Data from this phase I studies will be compared with data from the pre-clinical studies to ensure that the drug is working as anticipated. Despite the care and preparation taken, phase I trials are the first time for the drug to appear in human experiments, and the unexpected can happen. In this stage, it will be approximately 70% of tested drugs that will move to the next phase [107].

Phase II. Assess Safety and Efficacy in a Relatively Small Group of Patients

In phase II studies, the primary purpose is to evaluate whether a candidate drug has effectiveness or has side effects in a relative number (up to a few hundred) of patient volunteers with the disease, and with the study length of several months to two years. Many phase II studies evaluate patients receiving the drug compared with patients receiving a different drug treatment, either an inactive substance (placebo), or a different drug that is usually considered the standard of care for the disease. Researchers also analyze optimal dose and schedules for using the drug, and examine the possible short-term side effects and risks associated with the drug. If the drug continues to show promise, researchers should prepare and design a protocol for the next and larger phase III studies. In this stage, it will be approximately 33% of tested drugs which will move on to the next phase [106].

Phase III. Demonstrate Safety and Efficacy in a Large Group of Patients

The main goal of the phase III studies is to generate statistically significant data about the safety, efficacy and the overall benefit-risk relationship of the investigational medicine. Phase III trials may enroll 300 to 3,000 patients with length time of 1 to 4 years, and are the costliest and longest studies, often encompassing hundreds of study sites at hospitals and centers around the world. The companies should ensure high quality production of the medicine for use in this trial, as well as planning for the full-scale production of the medicine after FDA approval. The innovating pharmaceutical company also coordinates closely with research staffs at each trial site to monitor the study; and works with some clinical research organizations (CRO) to aid in recruitment and day-to-day operations of the trials.

A candidate drug may take from six to ten years to complete the first three phases of clinical studies. The time taken is determined by the duration of the disease that is being treated and by the extended time that it can sometimes take to assemble sufficient patients for the trials [1]. People are often surprised that drug development takes such a long time, even though the vast amount of results that have been generated on the candidate drug before phase III studies fail at this phase with a high failure rate. As of 2010, about 50% of drug candidates either fail during the phase III studies or are rejected by the national regulatory agency [107]. It will be only approximately 25% of the drugs which will move on to the next phase study [106].

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STEP V. New Drug Application (NDA) to the FDA for the Approval of Marketing

Once a compound has passed its early-phase preclinical trials and late-phase clinical trials, all results and data analysis demonstrates and confirms that the experimental drug is both safe and effective for its intended use; the sponsoring company will submit a New Drug Application (NDA) or Biologics License Application (BLA) to the FDA requesting the approval to market the drug [108].

New Drug Application (NDA)

The purpose of New Drug Application (NDA) is to demonstrate that a drug is safe and effective for its intended use in the human population studies. These applications contain all the results and data analysis from the entire clinical studies, as well as the earlier preclinical results and proposals for the manufacturing and labeling of the new medicine. Pharmaceutical companies must provide information including the development of a safety update report (DSUR), proposed labeling, drug abuse information, patent information, any data from studies that may have been conducted in the world, institutional review board compliance information, and directions for use. It will run up to 100,000 pages or more [106, 109].

FDA Review

Once the FDA receives NDA application, the review team will examine firstly whether the application is complete or not. If it is not complete, the review team can refuse to file the NDA approval. If it is complete, The FDA review members, including scientists, physicians and statisticians, thoroughly examine and evaluate all submitted data and results about the drug to determine whether the benefits outweigh the risks; what information must be included in the drug label; whether the proposed manufacturing process is adequate; and if there is any need for certain prescription criteria or special physician training. The review team has 6 to 10 months to make a decision on whether to approve this drug. The FDA review process includes four steps below:

The FDA review team conducts a full review of the application. The medical officers and the statisticians review clinical data, while pharmacologists review the data from animal studies.

The FDA inspectors travel to clinical multi-center sites around the world to conduct a routine inspection. The Agency looks for evidence of fabrication, manipulation, or withholding of data.

The project managers assemble all individual reviews, other documents, and get together to form an action package. These documents become the record for the FDA review.

The FDA finally makes a decision to approve or not to approve the NAD application. It requires a 6 to 10 month processing time [106, 110].

FDA Approval

When the FDA determines the new drug to be safe and effective for its intended use in humans, it is necessary to work with the applicants to develop and refine prescribing information. Labeling accurately and objectively describes the basis for approval and how best to use the drug [106].

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Manufacturing

Once FDA and NAD approval is obtained, the pharmaceutical company should – in accordance with the FDA Good Manufacturing Practices (GMP) regulations – produce the highest standard to ensure drug safety and quality in each step of the manufacturing process, to ensure that the drug is available to patients for many years [106].

STEP VI. Post-Market Safety Monitoring (Phase IV trials)

Despite clinical trials providing important information on the efficacy and safety of a new drug, even when the drug has been launched in the market, it is impossible to have complete information about the safety of a drug after the time of FDA approval. Sometimes, the FDA requires additional studies on the already approved drug; this is known as *phase IV studies*. Phase IV trials can be set up to evaluate the long-term safety of the new medicine. As a much larger number of patients start taking the drug, the company must continue to monitor it carefully for newly found adverse effects of the drug. Periodic reports to the FDA should be submitted on a quarterly basis for the first 3 years, and annually thereafter. The FDA reviews reports of problems with prescription and over-the-counter drugs, and decides to add cautions to the dosage or usage information, as well as other measures for more serious issues [111–114].

CONCLUSION

The research and development of a new medicine is a long, costly, complex and rigorous process, which needs savvy, strategic, organizational, and managerial decisions. Every step should be focused on ensuring the highest level of drug safety in humans. Once a new and effective medicine is identified through the process of drug development, it will be brought to the market as early as possible. In this chapter, we presented a comprehensive overview of the process of drug discovery and development from an early-phase of basic research to final marketing. We discussed basic knowledge about new drug discovery and development; how patent intellectual property protections are established; and how the interplay between the U.S. Patent and Trademark Office and the Food and Drug Administration applications affects the patent approval process.

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Chapter 3

ETHICAL, LEGAL AND EDUCATIONAL ISSUES IN EXPERIMENTAL SURGERY

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ABSTRACT

Experimental surgery is an acceptable procedure using large or small laboratory animals necessary to acquire technical skills crucial to the structured learning of surgery. Use of animal experiments in research and training has given important contributions to surgical progress with a number of important discoveries, which have not only saved millions of human and non-human lives, but also allowed for the increase of human life expectancy, which in some developed countries doubled and continues to rise today. The use of animal models remains indispensable in the foreseeable future and shall comply with minimal ethical standards set out for animal welfare.

The use of non-animals as alternative subjects in experimental surgery also helped to improve good technical skills, although they are being used with limited evidence-based data to support their validity and reliability. Skill acquisition and maintenance of surgical ability should be preserved by active and operative practice, research, and training laboratories. In this chapter, regulations that protect animals and manage their use in experimental surgery are outlined, and some alternative methods used in experimental surgery training and research, such as surgical simulators to help students or trainees to develop skills before practice on real patients are summarized.

Keywords: animal model, experimental surgery, education, ethic, regulations, surgical simulators

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ABBREVIATIONS

CCAC	Canadian council on animal care
CIOMS	Council for International Organizations of Medical Sciences.
CT	Computer Tomography
dV-Trainer	da Vinci-Trainer
dV-SS	da Vinci Skill Stimulator
IACUC	Institutional Animal Care and Use Committees
IRB	Institutional Review Board
MIST-VR	Minimally Invasive Surgery Trainer Virtual Reality
MRI	Magnetic Resonance Imagery
RAS	Robot-Assisted Surgery
ROSS	Robotic Surgical Simulator
VRS	Virtual Reality simulators
VIPAR	Virtual Interactive Presence and Augmented Reality

INTRODUCTION

In the year 500 B.C., Alcmaeon from Greece and other scientists performed dissections on animals in order to know more about their anatomies (Geller 2007; von Staden 1989). In the 16th century, anatomists and others successful artists like Leonardo de Vinci (1452–1519) were given permission to dissect human corpses and pursued anatomical investigations of muscle and bone structure (Guerrini 2003). An English physician, William Harvey (1578–1657) described completely in detail the circulation of blood via his experiments on live deer (Shackelford 2003; Gregory 2001). Over the centuries, surgical techniques like dissecting living animals were developed and improved (Guerrini 2003; Prioreschi 1998; Maehle 1987). These experiments on living animals have profoundly affected all sectors of medicine, particularly in surgery (Porter 1995; McMullen 1995). Up to the early nineteenth century, there was a significant increase in the use of animals in scientific experiments (Pearce 1912; Davis 1903; Ernst 1902). Innovated surgical procedures and treatments based on research using living animal models were discovered, and continued to develop (Bertoloni 2013; Elliot 1987). Specific examples include a kidney transplantation which was previously performed and perfected in dogs led to the first effective human transplant of a kidney in the mid-1900s (Murray 1955). Heart valve replacement, artificial heart valves as well as the artificial hearts now being used in humans would not have been possible without experimental surgery research using animal models. Techniques of sewing blood vessels together was developed through surgeries on dogs and cats by Alexis Carrel, for which he was even awarded a Nobel Prize in 1912 (Balner 1981; Lee 1961); these research studieson laboratory animals also led to the understanding of organ rejection and how to overcome it (Stuart 1968; Hamburger 1981, Petrini 2013).

In experimental surgery, the principal and obvious purpose is to develop satisfactory surgical techniques; to test the validity and safety of new surgical techniques and also allow for the study of special pathophysiological aspects. This involves the discovery of the causes and conditions which make surgical interference a necessity (Jensen 1996; de la Garza-Rodea

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2007). This implicates the interest in understanding physiology, biology, biochemistry, biophysics, etc. in terms of their relationship to general applications in the body and also to comparative aspects (Felts 2000; Dear 2006; Van der Worp 2010). Even though animals differ from people in many ways, they are also very similar to people in many ways. Animals develop many of the same diseases as people, including hemophilia, diabetes, and epilepsy (Swindle 1988). Animals are also susceptible to many of the same bacteria and viruses as people, such as anthrax, smallpox, and malaria (Gay 1986a and 1986b; Gay 1989; Bertoloni 2013). An animal is chosen as an "animal model" for research only if it shares characteristics with people that are relevant to the research. Animal model testing continues to be a natural specific research model, specifically for experimental surgery, where biologic and extremely complex physiologic systems are interrelated, making it a perfect model available for exploring, explaining or predicting the course of diseases or the effects of possible treatments by observing the entire living system (Gay 1986a and 1986b; Gay 1989; Rollin 2007).

Although experiments on living animals led to medical progress with a number of important discoveries, which have not only saved millions of lives human and non-human alike, but also allowed for the increase of human life expectancy (Billingham 1977; Bennett 1990; Pound 2004), they have also contributed to the emergence of many protestation movements between scientific inquiry and public concern. The use of animals for experimentation in research has been debated, defended, and protested by both individuals and organizations and individuals at various levels (Aldhous1999; Dikshit 2012). Attitudes in the continuum relationship between humans and animals spans amongst those who support no regulation of the human use of animals and those who advocate absolute animal liberation from all human use (Festing 2007; Crettaz von Roten 2012). Each year in the world, an estimation of nearly half a million animals are used for teaching exercises or research in surgery (Badyal 2009; Ringach 2010). In conditions of the non-availability of alternative subjects, the researcher or surgeon must be guided at all times by the principles of humane and rigorous experimentation, as well as a sense of moral and legal responsibility for an animal's welfare during their period of care (Festing 1989; Orlans1998; Dolan 2007).

Alternative methods such as computer models and other new teaching approaches such as surgical simulators and training in virtual environments have been used in ways to refine experimental techniques, to reduce the number of animals needed to obtain valid results or to replace animals with other feasible medical research methods (Fanua 2001; Furka 2006). Although these alternative methods are in most cases limited for technology transfer, few difficulties are reproducible (Gruber 2004).

Furthermore, experimental surgery represents an essential stage in the training of surgeons. It's most important aim is the improvement of human health. Balance between observance of the law and respect for the animals should be found and carefully observed in the exercise of this field. At present, it is impossible to advance surgical research without the use of animal models for some aspects of research. This chapter will outline some regulations that protect animals and manage their use in experimental surgery. The developments and advances in surgery are a continuing phenomenon; therefore, there still remains the need to teach practitioners new skills. Experimental surgery research and teaching, using animals is increasingly being questioned concerning the forms of animal use, maintaining animal costs, etc. Thus, a reflection is necessary on the rise in interest of alternatives to the use of live or dead animals, such as the use of surgical simulators, interactive 3D computer models and digital surgery programs.

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EDUCATION ISSUES IN EXPERIMENTAL SURGERY

Experimental surgery has been acknowledged as fundamental to as surgeon's education. It simplifies surgical teaching and promotes the surgeon's scientific reasoning (de Montbrun 2012; Palter 2010).

Traditional Teaching Methods in Experimental Surgery

In general, these methods offer the benefits of seeing and learning surgical techniques, an opportunity to refine or improve proficiency in older surgical techniques, and also to help learn and practice the utilization of new surgical equipment. Specifically, animal models, humans or animal cadavers have been employed in these methods because of their analogy in anatomy and physiology compared with males and within species, which is suitable for all surgical and experimental purposes (Dewhurst 2011; Wilson-Sanders 2011; Dewhurst 2012).

Dissection and Vivisection

These practices provide better quality education, offer the best preparation for scientific careers, and help to familiarize trainees with basic operative skills with the hope of developing or improving their manipulative skill and dexterity (Elliot 1987; Connor 1997). However, this practice involves unnecessary pain, suffering, and the unnecessary killing of animals; it undermines conservation efforts and ignores welfare standards during animal capture, preparation, and shipping; it focuses only on descriptive biology to the detriment of creative scientific thinking and research; it causes some students to abandon further science education or careers; and it weakens the respect for life and the humane treatment of animals.

Surgery on Living Animal Model

This form of training is an effective form of a surgical teaching method because its share many of the same features as human surgery, thus replicating human surgery with high-fidelity (Tan 2011, Roberts 2006). As a result, trainees can practice every element of an operation, including not only the technical skills involved in a procedure, but also the avoidance of complications and their management when they arise. With this method, *in vivo* porcine and canine models have been used extensively in endoscopic, laparoscopic and other forms of training, including endoscopic submucosal dissection, cholecystectomy and coronary bypass (Tan 2011). *Ex vivo* animal tissue has also been occasionally used, but offers lower-fidelity simulation than live animals.

Use of this method has caused some concern like structural differences between human and animal anatomy, some ethical concerns, and its expensive and complex requirement in order to monitor hemostatic changes. Although efforts to minimize the need for animal use continues in the estimable future, it appears that animal experimentation is still essential to enable scientists to have a more precise understanding of human disease, including its

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diagnosis, prognosis and therapeutic intervention (Kehinde 2013). A featured role in courses on animal ethics, animal welfare, and animal rights at all educational levels and fields should be to seriously consider the ceasing of unethical animal use (Balcombe 2000; Tanaka 2006).

Cadavers Obtained from Ethical Sources

Many body donation programs allow willing individuals to donate deceased, ethically-sourced cadavers for students or researchers to study, offering the chance to gain a deeper understanding of anatomy, physiology, pathology etc., and improve skills (Prioreschi 1998; Chambers 2015). Fresh cadaveric tissue is the gold standard for surgical simulation because of its approximation to living tissue and its anatomic structures, which are encountered in the operating room (Carey 2014). Although this form of method uses dead tissue and thus cannot faithfully emulate all physiological conditions, some cadaveric surgical courses have utilized pressurized systems to perfuse cadaveric tissues with blood, creating high-fidelity models for vascular, microvascular and trauma surgery (Carey 2014; Garret 2001). This method has been used for training flap coverage techniques as well as various endoscopic and laparoscopic operations (Carey 2015; Imakuma 2016).

Unfortunately, the use of human cadavers has caused some concerns (like the poor tissue compliance caused by embalming), thus making some surgeries difficult (Reznick 2006). The limited availability of human cadavers and their costs have restricted their widespread distribution and use (Anastakis 1999). Additionally, they require regular maintenance and special facilities, and are not reusable following certain procedures.

Application of Microsurgery

Microsurgery has expanded the scope of many surgical specialties and is evolving into an integral part of medical training programs. The complexity of microsurgery requires considerable time and resources for adequate training and practice (Chan 2007; Singh 2014). Microsurgery techniques are the only possibility permitted by law to execute experimental surgery research in rodents. These techniques can be performed many times and in a short time, thus improving dexterously surgical techniques and skills (Lahiri 2005; Mucke 2013; Yadav 2016). It is an indispensable tool in virtually all fields of surgery with a promising future. It offers several advantages over the conventional surgery such as cleaner and smaller incisions, lesser hemorrhage, minimal tissue handling and a closer wound approximation. It is a technically demanding procedure and has a steep learning curve. To set up an establishment is costly, and this also includes a restricted area of vision, loss of depth perception and loss of visual reference points.

Experimental microsurgery has the cross-disciplinary features of the sciences and techniques for the growth of medicine, pharmacology, veterinary studies, engineering, etc. Training for vascular anastomosis led to rodent transplantation models. These models were used for immunology and immunosuppressant research. Also, microsurgical techniques led to the master catheter technique for injecting various drugs and gene vectors (Kobayashi 2016).

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Alternatives Teaching Methods in Experimental Surgery

In recent years, many scientists were devoted to the progress of animal welfare and development of alternatives to animal use in the life sciences. Starting in 1980, a remarkable decline in the number of animals used in research has been registered, although there has been an increase in the number of biomedical publications (Mukerjee 1997; Stephens 2001 and 2012); and the rise in the use of genetically modified animals has also led to this downward trend to continue (Taylor 2005; Ormandy 2009). However, this data is limited to the Western world, as statistics on animal use in emerging countries are unavailable (Kulpa-Eddy 2007; Rowan 2007).

These non-animal or alternative methods allow researchers or students to repeat the procedure until it can be proficiently and confidently understood. Also, this impacts budgets by dramatically reducing experimental costs (Schöffl 2003; Kurosawa 2008). Studies show that these alternative models reduced the numbers of live animals used by 80–100%. In most developed countries, these replacement alternatives have to meet the essential needs of humans, and have to be considered before animal experiments are proposed while they are also expected to provide satisfactory results (Liebsch 2011).

Changes occurring in healthcare systems around the world and patient safety have further been limited to the training experience of novice surgeons; consequently, these have resulted in extraordinary innovations in surgical simulations (Hutter 2006; Erel 2008; Wignall 2008). Advancements in computing and graphical capabilities offered new innovations in simulator technology. This rapidly incorporated surgical simulation technologies and offered an increasing potential to more closely mimic reality and present entire operations, allowing for the practice of surgeons to develop new skills and maintain those they already possess.

Surgical Simulators Teaching Models

With these teaching methods, trainees repeatedly practice techniques and manage complications in performing the simulated operation until they achieve expertise. This also aids the trainees in the development of critical psychomotor, technical, judgment skills (Gallager 2005); itwill also help in reducing operative times, lower complication rates and improve patient outcomes (Seymour 2008; Zevin 2014).

Surgical simulators originated over 2,500 years ago and have undergone an enormous amount of transformation since the early 1990s, evolving from mannequins of human cadaver models, live animal models (Cooper 2004), and plastic bench-top kits to 3D printing and patient-specific VR systems (Sarker 2007; Owen 2012; Valentine 2016). In recent years, a large number of surgical simulators have emerged that are unique to different surgical specialties, procedures and procedural variations. For example, different bench-top and VR simulators exist for the practice of endoscopic foreign body removal, laparoscopic common bile duct exploration, cleft palate repair and intestinal anastomosis among many others (Satava 2008). Specific simulators also exist for unique complications of a specific surgery, such as a sheep-based simulator for managing vascular emergencies during skull base surgery (Badash 2016). The following is a list of some of the most popular types of surgical simulators in use.

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Bench-top Simularors

These are synthetic stand-alone simulation models that allow for the practice and assessment of surgical skills. They were judged effective and simple, and are commonly used by educators to assess the proficiency of novice surgeons. Some bench-top simulators offer basic surgical techniques with low-fidelity like knot-tying and suturing (Sarker 2007); others combine both synthetic and animal parts with high-fidelity (Valentine 2016), and are able to replicate and train in complete operations, such as fracture fixation, joint replacement and aneurysm repair (Sarker 2007) and other minimally invasive surgeries (de Montbrun 2012).

Virtual Similators Models

These simulators are computer-based systems which allow for the practice of surgical techniques on a computer; the surgical trainee uses tools to manipulate a series of computerized images, thus performing surgery in a virtual environment (Tan 2011). Modern VR simulators create realistic environments that capture minute anatomical details with high accuracy. Therefore, high-fidelity and anatomically correct simulations are offered that are entirely reusable (de Visser 2011). They are designed to teach laparoscopic and endoscopic procedures (de Montbrun 2012), for instance, the MIST-VR system, which is a low-fidelity system designed to teach basic laparoscopic skills, suturing and knot-tying (Wilson 1997). High-fidelity VR systems include LapSim, Lap Mentor, and NeuroTouch. The Lap Mentor is a particularly inclusive system that includes over 65 cases in the fields of general surgery, gynecology, urology, and bariatric surgery. VR simulation has been found to reduce the operative time of surgical trainees and to improve the efficacy of their operative performance (Alaker 2016; Seymour 2008; Kundhal 2009; Hyltander 2002). It has been recommended that these simulators be formally included in surgical curricula (Zevin 2014; Tan 2011). The downside of VR simulations includes high costs, lack of force-feedback, and limited realism of some simulation models (Palter 2010).

Robot-Assisted Simulators Models

Recent developments in surgical simulations have involved the creation of simulation programs for the robot-assisted surgical system, da Vinci. The Robotic Surgical Simulator (RoSS) is a stand-alone device that teaches novice surgeons the skills required for performing robot-assisted surgery (RAS) (Rehman 2013; Liu 2015).

Robot-assisted surgery (RAS) simulators represent a relatively new development in the field of surgical simulation. Currently, there are 4 widely used RAS simulators for the da Vinci System: the SEP-Robot, RoSS, dV-Trainer, and the da Vinci Skills Simulator (Liu 2015). The RoSS and dV-Trainer are stand-alone devices with controls resembling those of the da Vinci system (Liu 2015). The da Vinci Skills Simulator is a hardware pack which loads a VR simulator onto the actual da Vinci device (Lyons 2013).

The da Vinci surgical system was first introduced in the United States in 1999. It involves a surgeon using foot pedals, dual hand controls and a controllable 3D camera to guide a robot through surgical procedures (Liu 2015). The surgeon views a virtual environment rather than

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a live endoscopic feed through the user interface. The da Vinci skills simulators are low-fidelity and thus only allow for the practice of individual surgical tasks, like testing hand-eye coordination, tissue manipulation, suturing and knot-tying (Abboudi 2013). They also produce metrics of performance based on completion time, error measures, and motion analysis (Liu 2015).

3D Rapid Prototyping VR Simulators

These technologies involve the use of medical imaging, fused filament deposit, stereo-lithography, scintigraphy, MRI and 3D printers to build synthetic models of patient-specific organs and vasculature, and to create patient-specific 3D models that enable the planning of various operations (Vakharia 2016). Some of the models produced by rapid prototyping are able to replicate actual patients' anatomical structures with remarkable realism (Anderson 2016; Vakharia 2016; Khan 2014).

In the field of neurosurgical simulation, 3D printers are used to create reliable models of patient-specific cerebro-vascular pathology from information provided by CT angiograms (Ryo 2001). When printed with the surrounding bony structures, these models allow the surgeon to plan the trajectory of approach to aneurysms and to test different aneurysm clips for the appropriate size and shape (Vakharia 2016; Kimura 2009). In cardiac surgery, 3D-printed heart models rendered from cross-sectional patient images have been used in simulations to train staff on postoperative critical care (Olivieri 2016).

Patient-Specific VR Simulators

With these simulators, patient CT data is captured and reproduced on a 3D virtual simulator (Endo 2014; Makiyama 2015). This allows the surgeon to practice laparoscopic procedures preoperatively in a virtual environment with accurate renditions of the patient's anatomical variations. The overall accuracy of the simulator is high, capturing structures such as tumors, ureters, and renal arteries and veins with 95–100% accuracy (Makiyama 2015); and time needed for all simulation is relatively short, taking around 2.5 hours for hepatectomy and pancreatectomy simulators. These patient-specific simulators have emerged for use in pancreatectomies, hepatectomies, renal surgery, and hand surgery (Endo 2014; Makiyama 2015), and may be effective tools for the preoperative planning of complicated procedures. Unlike 3D printers, these simulators are also readily reusable and do not consume resources, further supporting their clinical utility.

Virtual Interactive Presence and Augmented Reality (VIPAR)

The VIPAR system utilizes augmented reality technology to enable audiovisual collaboration over the internet with just a 760 ms delay. The visual field of a surgeon in one location is converted into a simulation that is projected to a surgeon elsewhere. As a result, the operating surgeon can be guided in real-time by a more experienced surgeon or collaborate to identify anatomical structures, guide surgical maneuvers, and discuss an overall

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surgical approach (Shenai 2011; Shenai 2014; Ponce 2014). VIPAR, known as telementoring, has been used in orthopedic surgery and in neurosurgery for training.

RESEARCH ISSUES IN EXPERIMENTAL SURGERY

Surgical techniques or treatment innovation is usually a result of frequently historical observations or experimentation that brings gradual improvements to existing techniques. Experimental surgery research is very indispensable and fundamental forresearch methods for making progress in surgery and medicine in general (Souba 2001; Javed 2005; de la Garza-Rodea 2007) (Table 3.1). In the last three decades, transplant surgery has obtained better results, undoubtedly because of the use of new drugs for immunosuppression perfusion, and organ storage, which were initiated from microsurgical transplantation models in rodents (Di Cataldo 2001) (Table 3.2).

Experimental surgical research faces two major problems in funding due to inflexible opposition of public opinion.

1. The law forbids the excessive use of large animals, instead of the use of small animals, which require special training of microsurgical techniques.
2. The use of living animals for experimental surgery should be limited to the minimum compatible with the pursuit of legitimate scientific ends (Di Cataldo 2001; Schnaider 2003; Schanaider 2004).

A new process in making decisions regarding resources, strategies to improve extramural funding, and new approaches for selecting research foci are needed to meet the issues and challenges in experimental research (Smythe 2010)

Ethical Issues and Experimental Surgery

Over the centuries, animal experiments were being increasingly prompted by existing clinical problems and carried out with the ultimate goal of developing new therapeutic approaches to tackle these issues. In the secondhalf of twentieth century, the scientific world witnessed considerable progress in the development and acknowledgment of the Three Rs (replacement, reduction, and refinement). This principle has become the overarching principle of several legislative documents regulating animal use in science (Louhimies 2012). Biomedical researchers in both industry and academia have also acknowledged the central importance of the Three Rs and the need for more transparency regarding animal use in biomedical research (Carlsson 2004; Olsson 2011).

Refinement, Reduction and Replacement Principles

Ethical considerations make the relationship with animals very important, especially as communication between both researchers and animals is limited.

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Table 3.1. Advantages and Disadvantages of Experimental Surgery Research

Advantages	Disadvantages
This research aids in controlling independent variables for the experiment's aim to remove extraneous and unwanted variables. The control over the irrelevant variables is higher compared to other research types or methods.	Experiments cannot be carried out at times because you cannot manipulate independent variables either due to ethical or practical reasons. Taking for instance a situation wherein you are enthusiastic about the effects of an individual's culture or the tendency to help strangers, you cannot do the experiment. The reason for this is simply because you are not capable of manipulating the individual's culture.
Easy Determination of Cause and Effect Relationship The experimental design of this type of research includes manipulating independent variables to easily determine the cause and effect relationship.	This controls irrelevant variables at times, and this also means creating situations that are somehow artificial.
Due to the control set up by experimenter and the strict conditions, better results can be achieved. Another good thing about experimental research is that experiments can be repeated and results can be checked again. Better results that have been obtained can also give the researcher greater confidence regarding the results.	Affect the efficiency of the results
Experimental research is gaining insights to instruction methods, performing experiments and combining methods for rigidity, determining the best for the population and providing greater transferability.	Results applied to one situation that can only be applied to one situation and may be hard to replicate and lastly difficulty in measuring human response
Surgical training is a multifaceted process that should produce not only a good clinician and technician, but also a good communicator, health advocate and professional.	Results may not be generalized into real-life situations
	Experimental designs are frequently contrived scenarios that do not often mimic the things that happen in the real world.
	Experimental research helps with internal validity; however, this is at the expense of the external validity.
	This research can be time consuming and expensive
	Several areas wherein experiments cannot be utilized due to ethical and practical considerations.
	The degree on which results can be generalized all over situations and real world applications are limited.
	Personal biases
	Unreliable samples

Source: https://occupytheory.org/advantages-and-disadvantages-of-experiments research.

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Table 3.2. Medical Advances Depended on Experimental Surgery Research

Year	Animal Model	Discovery	Authors
1930s		Modern anesthetics for surgery	
1940s		Heart-lung machine for open-heart surgery	
1950s		Kidney transplants. Cardiac pacemakers and replacement heart valves. Hip replacement surgery	
1960s		Corneal transplants Coronary bypass operations Heart transplants	
1970s		Improved sutures and other surgical techniques	
1980s		Immunosuppressant drugs for organ transplants CAT scanning for improved diagnosis Life-support systems for premature babies	
1990s		Laparoscopic surgical techniques	
2000s		Cell therapy	

Source: Giddens S, Giddens O. Future Techniques in Surgery. 2003; books.google.com.

An important ethical principle of animal use in experimental surgery education or research is that alternatives to live animals should be used whenever possible. One concept of alternatives has been outlined in the book *The Principles of Humane Animal Experimental Techniques*, Charles Thomas, Springfield, IL, by Russell and Burch in 1959, with the principle of 3Rs. This concept constituted a continuing effort to more efficiently use laboratory animals. It has been adopted by a number of scientists and many animal advocacy organizations, and is internationally accepted as criteria for humane animal use in research and testing (Inoue 2007; Russell 2005; Nuremberg Code). This concept established the care that must be taken to conduct research with animals, and according to these principles:

- The number of animals used in an experiment or procedure must be reduced in order to increase the methodological quality and enhance the statistical analysis of data.
- Refinement of the techniques used must have the purpose to decrease the incidence or amount of animal pain and distress, and provide their well-being based on their behavioral needs.
- Replacing experimented animals with *in vitro* and in silico models that could be alternatives in teaching and research. Alternately, live animals may be replaced with non-animal models, such as dummies for an introduction to dissection for teaching the structure of the animal or the human body, mechanical or computer models, audiovisual aids, or *in vitro* modeling. However, there are no alternative scientific methods that allow for the full replacement of animals in experimental surgery education or research studies. Disadvantages for replacement mostly stem from the fact that any models are dependent on pre-existing information. Live organisms are a complex system; some of the variables in physiology and pathology are unknown. Thus, any research study on new biological processes must utilize a living organism at some point. Advantages concerning replacement include the use of pre-existing knowledge for teaching, applying known principles to new systems to look for similarities, and using less expensive animals or models to screen large numbers of agents for toxicity or mutagenicity (Guhad 2005; Mardas 2013).

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The concern within the scientific community and in the general public over the use of animals in research and education has put a system for protecting research subjects under increasing pressure. Under the currently dominant regulatory ethics paradigm, experimental surgery protocols for research or teaching issues must be reviewed and approved by an institutional review board (IRB) or equivalent, to protect study subjects and investigators or researchers from the inherent conflicts of interest (Moore 2000; Akelina 2001; Richmond 2002).

In experimental surgery research, reducing the number of animals should not compromise the detection of biological effects nor lead to the repetition of experiments. The refinement is influenced by a study design and sample size calculation, the control of variation, the statistical hypothesis, and the choice of the statistical test used for data analysis and interpretation. Intra-sample variation can be reduced by using animals that are genetically and sanitarily homogenous, in addition to controlling environmental variables (Monamy 2000; Mohammad 2013).

Ethical consideration in experimental surgical studies must consider the suitability of the selected model, management of the animal throughout the study or training, and euthanasia after the study/training; this is all done for an attempt to eliminate or reduce to a minimum the discomfort and pain of the animal model (Snow 2008).

Much still remains to be done to establish total management for animal experiments. Improvement of the effective utilization of these animal experiments is also required from both economical and ethical points of view (Akelina 2001; Ramsey 2007). Before starting surgical training, sufficient education concerning animal ethics and dry laboratory training should be completed; the point of departure in the existing legislation on animal experimentation is the principle of responsible use (Inoue 2007; Hendriksen 2006; Tanaka 2006; Orlans 1996).

REGULATIONS ISSUES AND EXPERIMENTAL SURGERY

Since the beginning of the 21st century, much effort has been dedicated to animal welfare throughout world (Table 3.3). Animal rights refer to the moral right of animals to be treated with respect and without exploitation. The rights of animals under captivity or domestication have been universally declared as follows: The right to be free from hunger, the right to be free from discomfort, the right to be free from fear and distress, and the freedom to express normal behavior (Table 3.4).

In scientific studies or training, whereas there are no definite national laws guiding or regulating the use of animals, several laws have been in existence to regulate the action of humans towards animals. These laws have guided how, when and the manner in which to conduct trainings as well as scientific researches on animals. Several institutions and universities have put in place guidelines for the use of animals in scientific studies and trainings (Richmond 2002; Dolan 2007).

For animal use in experimental surgery, legislation required that all live animals operated on for research, teaching or testing purposes must be regulated by laws and regulations clearly described in the Guide for the Care and Use of Laboratory Animals (CCAC 1984); regulations also established the oversight system of the Institutional Animal Care and Use

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Committees (IACUCs) to evaluate research protocols, review care programs and inspect laboratory facilities, assess and educate laboratory personnel, and investigate complaints about the misuse or mistreatment of animals (Keune 2014).

Proposals of animal use are reviewed based on the potential for learning new information, or for teaching skills or concepts that cannot be obtained using an alternative (Keune 2014). Also, there must be a provisions ensuring that animal surgeries are performed in as humane a manner as possible, minimizing pain, distress or discomfort. Provisions also include that all personnel with animal contact be trained and be skilled in any experimental procedures that will be performed at each research or educational institution. In addition, analgesics, anesthetics and sedatives must be used when needed (Hampshire 2007). Finally, basic husbandry requirements must be specified, ensuring that pre- and post-operative care are provided in an optimal manner (Flecknell 1987; Wall 2014).

In several countries, legal precautions guiding the use of animals for research vary in the level of protection given to animals. In response to these worldwide variations, the Council for International Organizations of Medical Sciences published in 1985 a set of International Guiding Principles for Biomedical Research Involving Animals, which were intended to provide a "conceptual and ethical framework" for countries with no legislation. In the United States, the Animal Welfare Act operates as the main federal law relating to laboratory animals (Nuno 2013) (Table 3.3; Table 3.4).

Table 3.3. Chronology of Law and Regulations/Worldwide

Year	Law/Regulations	Country
1822	"An Act to Prevent the Cruel and Improper Treatment of Cattle" "Martin's Act" or "The Act of 1822"	United Kingdom
1824	Society for the Prevention of Cruelty to Animals (SPCA)	United Kingdom
1828	America's first animal protection act, outlawing cruelty against cattle, sheep and horses	USA
1840	Royal Society for the Prevention of Cruelty to Animals (RSPCA)	United Kingdom
1846	The societe protectrice des animaux (Animal Protection Society)	France
1866	American Society for the Prevention of Cruelty to Animals (ASPCA)	USA
1875	Cruelty to Animals Act of 1876	UK
1876	Victoria Street Society for the Protection of Animals from Vivisection	UK
1877	American Humane Association (AHA)	USA
1883	American Antivivisection Society (AAVS)	USA
1898	British Union for the Abolition of Vivisection (BUAV)	United Kingdom
1954	Humane Society of the United States (HSUS)	USA
1954 - 1957	National Catholic Society for Animal Rights, which is later extended beyond its denominational origins and renamed the International Society for the Animal Rights (ISAR)	USA
1966	Animal Welfare act (Laboratory Animal Welfare Act)	USA
1992	Congress enacts the Animal Enterprise protection Act	USA
2000		

Source: Bickenbach JE. Ethics, law, and policy. 2012; books.google.com.

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Table 3.4. Universal Declaration of the Right of Animals

This declaration hereby proclaims that:
Article 1: All animals are born equal due to life and the same rights to existence.
Article 2: 1. All animals are entitled to respect. 2. Man as an animal shall not arrogate to himself the right to exterminate or inhumanely exploit other animals. 3. All animals have a right to the attention, care and protection of Man.
Article 3: No animal shall be ill-treated or subjected to “animal acts.” If an animal must be killed, this should be instantaneous and without distress.
Article 4: Wild animals have a right to liberty in their environment where they should be allowed to procreate. Deprivation of freedom even for educational purposes is an infringement of this right
Article 5: Animals living traditionally in a human environment have a right to live and grow in the rhythm and under the conditions of life and freedom peculiar to their species. Any interference by Man with this rhythm or conditions for purpose of gain is an infringement of this right.
Article 6: All companion animals have the right to complete their natural life span. Abandonment of an animal is a cruel and degrading act.
Article 7: All working animals are entitled to a reasonable limitation of the duration andintensity of work to the necessary nourishment and rest.
Article 8: Animal experimentation, involving physical or psychological suffering is incompatible with the right of animals, whether it be for scientific, medical or commercial or any other form of research.Two replacement methods must be used and developed.
Article 9: Where animals are used in the food industry, they shall be reared, transported, lairaged and killed without the infliction of suffering.
Article 10: 1. No animal shall be exploited to the amusement of Man. 2. Exhibitions and spectacles involving animals are incompatible with their dignity.
Article 11: Any act involving the wanton killing of an animal is biocide, which is a crime against life.
Article 12: 1. Any act involving the mass killing of wild animals is genocide that is a crime against the species. 2. Pollution or destruction of the natural environment leads to genocide.
Article 13: 1. Dead animals shall be treated with respect. 2. Scenes of violence involving animals shall be banned from cinemas and television except if for humane education.
Article 14: Representatives of movements that defend animal rights should have an effective voice at all levels of government. The rights of animals, like human rights, should enjoy the protection of law. To conscientize the scientific community to this rights, the 24th of April has been observed as the day of laboratory animal protection.

Source: Wise S. Rattling the cage: Toward legal rights for animals. 2014; books. google.com.

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CHALLENGES IN EXPERIMENTAL SURGICAL EDUCATION

The Choice of the Animal Model: In experimental surgery research or education training, the choice of an animal model which suits all fields of application is almost impossible. Anatomic considerations such as size of organs, accessibility of the organs and tissues of interest are the primary determinants for model selection.

Outcome Issues: Experimental results should be published, regardless of statistical significance, to avoid conducting redundant studies; medical literature reviews advance that the number of laboratory animals used in past experiments could have been diminished while still obtaining the same statistically valid results.

In some cases, application of one alternative concept may have an adverse effect in another area (i.e., using a “lower” animal, minimizing pain, or distress may require using more animals) (Remie 2001).

The limitations resulting from interspecies differences, the distortion of outcomes arising from experimental environments and protocols, and the poor methodological quality of many animal experiments are likely to be technically and theoretically impossible to overcome.

Ethical Challenge: Industrially farmed animals (like pigs) are less prone to ethical considerations. These animals are more convenient for their fecundity and also are defined by the development of inbred major histocompatibility complex (Bhogal 2006). There is still a lack of communication between researchers and the population, leading to misunderstandings and making researchers unpopular (Plomer 2005).

Surgical Innovation: There are no clear regulations governing innovative surgery. Surgical innovations raise challenging ethical issues since the risks of the novel operation may not be known (Frader 1998; Ergina 2009; Johnson 2012). In addition, even if the risks are known by the innovator, the actual risks to experimental animals when learning the new techniques are unknown (Angelos 2013; Broekman 2016; Geiger 2015; Tan 2011; Reitsma 2002).

CONCLUSION

World health challenges allow surgery as a science to become complex and technically demanding. Experimental surgery as an integral part of surgical training programs to equip generations of surgeons with acquired skills and to meet the challenges of the future.

Training and research in surgical techniques have historically relied on the use of live animal models. These animal experiments have played a vital role in surgery progression and remain indispensable in the foreseeable future. Focusing on the continuous improvement of these experiments animals’ well-being, as well as further development of replacement alternatives for these models are still important.

The recent evolution in technology has brought about extraordinary innovations in surgery training, such as surgical simulation as an alternative to previous methods. Surgical simulation-based training is widely used in surgery education, helping students and trainees to develop good technical skills before practicing on real patients. However, various forms of surgical simulators are often being used with limited evidence-based data to support their validity and reliability. Several factors such as budget constraints, medicolegal concerns, etc.

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have to be overcome prior to the effective use of the surgical simulators as alternative methods in experimental surgery research and training.

To meet the challenges that occur within healthcare systems around the world remains important; experimental surgery proved to be a cornerstone in advancing surgical processes and improving surgical innovations. Principal goals in experimental surgery education should include using animal models, simulation technology and efforts to standardize technical evaluation techniques. In research, continuous strategies to improve funding and build upon new approaches to differentiatethe strengths related to surgical practice should be supported.

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Chapter 4

VETERINARY CARE AND MANAGEMENT OF RODENT AND LARGE ANIMAL MODELS IN TRANSPLANTATION RESEARCH

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ABSTRACT

Transplantation research cannot be accomplished without the use of animal models. This chapter aims to provide the reader with husbandry, medical, regulatory and ethical considerations when working with animals undergoing transplantation from a veterinary perspective. Successful implementation of novel pre-clinical transplant treatments/approaches, require close collaboration between research and veterinary teams. Here we provide a review of the regulatory guidelines and medical and husbandry care of small rodents (rats and mice) and large animals (swine and non-human primates).

Keyword: rodents, swine, dogs, nonhuman primates, husbandry, medical, regulatory and ethical considerations

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ABBREVIATIONS

AAALAC	Association for Assessment and Accreditation of Laboratory Animal Care
AWA	Animal Welfare Act
CMV	cytomegalovirus
EBV	Epstein Bar Virus
GVHD	graft-versus-host disease
HCT	hematopoietic cell transplant
IACUC	Institutional Animal Care and Use Committee
NHP	non-human primates
NSG	NOD/SCID common gamma chain receptor knockout
PTLD	post-transplant proliferative disease
USDA	United States Dept. of Agriculture

INTRODUCTION

The importance animals have had in advancements in biomedical research cannot be overemphasized. Their use, whether mammalian or non-mammalian, has been required for meaningful cures as no *in vitro* approach can recapitulate biological responses with the same fidelity as *in vivo* systems. In the field of transplantation research and immunology, *in vivo* experimentation has been pivotal for progress in achieving treatments that have saved innumerable patient's lives. Currently, no novel transplant approach may advance to clinical trials unless proof of principle has been demonstrated first in small (mainly mouse) and then large (dog/pig or non-human primate or NHPs) animal models. This chapter is intended to help the transplant biologist, scientist or clinician (regardless of their level of training) understand the important role of the regulatory, ethical, husbandry and medical care required when working with animals in transplantation from the veterinary point of view.

REGULATIONS GUIDING ANIMAL USE IN BIOMEDICAL RESEARCH

Research centers are tasked with adhering to important rules/regulations placed to protect animals used in research. To some degree these regulations may seem an overburden, and sometimes may delay research, however they were originally placed for the prevention of the unethical use of animals and the maintenance of high quality research. Transplant investigators are directly, indirectly (or both) impacted by these regulations mandated by the United States, Department of Agriculture (USDA) and the Public Health Service (PHS). Here we provide a synopsis of such regulations. The goal is to aid the personnel using animals in transplantation better understand what veterinarians, animal welfare offices (IACUCs), and principal investigators have to comply with.

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The Guide for the Care and Use of Laboratory Animals

(The Guide) provides multiple in-depth performance measures for evaluating an institution's animal care program, including the veterinary care and surgical program. The *Guide*, denotes an entire section dedicated to adequate veterinary care, addressing procurement, transportation, disease control, medical management, emergency care, and record keeping. In addition, performance measures for surgery are described such as training, pre-surgical planning, surgical facilities, asepsis, intra-operative monitoring, post-operative care, pain and distress, and euthanasia (National Research, 2011). Some important key points are needed for providing adequate veterinary care; (i) oversight of animal care and use to ensure that the program meets applicable standards for animal health and welfare; (ii) knowledge of the current and proposed use of animals in the institution's research, testing, teaching, and production programs; (iii) application of appropriate treatment or control measures, including euthanasia if indicated, following the diagnosis of an animal disease or injury; (iv) consultation with researchers on animal methodologies, surgery, and peri-surgical care; and (v) delivery of competent professional judgment to select the most appropriate agents to alleviate pain or distress in order to assure humane treatment of animals while avoiding undue interference with experimental goals.

Although the *Guide* provides detailed indices for minimal standards regarding animal welfare, practices, and animal care, additional references and veterinary professional association position statements and guidance documents should be reviewed and incorporated. The overall responsibility for veterinary care relies upon the attending veterinarian to provide direction to investigative staff and personnel involved in the care and welfare of animals and for guidance on best practices for the institution's surgical programs. Guidance from the attending veterinarian is a necessary component to ensure an institution's primary goal, which should be to establish a program with systematic internal review and self-oversight in order to maximize humane care, animal welfare, and scientific integrity.

The Animal Welfare Act and Public Health Service Policy

The two main regulatory documents governing animal use in biomedical research are the Animal Welfare Act (AWA), enforced by the USDA, and the Public Health Service Policy, administered by the Office of Laboratory Animal Welfare at the National Institutes of Health. The main difference between these is the species which are covered. The AWA, originally the Laboratory Animal Welfare Act of 1966, regulates all mammals used in research, including NHP and swine, but excludes rats of the genus *Rattus,* and mice of the genus *Mus,* that are bred for use in research. The Public Health Service Policy covers all live vertebrate animals used or intended for use in research, research training, experimentation, or biological testing at institutions supported by Public Health Service funds. Both documents mandate that: 1) institutions have an Institutional Animal Care and Use Committee (IACUC); 2) provide adequate veterinary care; 3) provide adequate training for all personnel involved in research; 4) primary investigators have considered alternatives to painful procedures; 5) a veterinarian is consulted about appropriate use of anesthetics and analgesics for surgical or potentially painful procedures. For researchers involved in transplantation medicine, regulations specifically addressing surgical procedures include appropriate provision for pre-operative

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and post-operative care of the animals in accordance with established veterinary medical and nursing practices; all survival surgery is performed using aseptic procedures, including surgical gloves, masks, sterile instruments, and aseptic techniques in well maintained surgical suites. Use of neuromuscular blockers in animal research is strictly regulated and only permitted with specific scientific justification. Any protocol requiring multiple major surgical procedures on an animal (surgical intervention that penetrates and exposes a body cavity or produces substantial impairment of physical or physiologic functions) requires special consideration and approval by the IACUC.

AAALAC international (previously known as the Association for Assessment and Accreditation of Laboratory Animal Care) is a private, nonprofit agency that provides voluntary accreditation and assessment of animal care programs with the goal to promote animal welfare. In the United States, AAALAC utilizes the *Guide for the Care and Use of Laboratory Animals and the Guide for the Care and Use of Agricultural Animals in Research and Teaching* (*Ag Guide)(Guide for the Care and Use of Agricultural Animals in Research and Teaching, 2010)* as primary references for programmatic assessments. The process to achieve accreditation is multi-factorial. Institutions must first draft a program description and submit for review. Afterwards, a site visit assessment will occur consisting of AAALAC Council on Accreditation members and ad hoc consultants. The number of site evaluators and days spent for the site visit will vary depending upon the size and complexity of the institution's program. Large academic or industry settings with multiple satellite facilities may require an entire business week for the site visit. The site visit report will be reviewed by the entire AAALAC Council on Accreditation to determine if the institution meets requirements for accreditation. According to the AAALAC International website there are greater than 950 organizations in 41 countries that have received AAALAC International accreditation ("AAALAC International," 2017). For centers in which transplantation studies are being performed and which require NIH funding, AAALAC accreditation is highly desired and often expected.

COMMON ANIMAL MODELS USED IN TRANSPLANTATION RESEARCH

Rodents in Transplantation Research

Both the rat and mouse have made significant contributions in the field of transplant medicine. Recently, however, the mouse has replaced the rat as the workhorse of biomedical research, for many reasons. Its small size and ability to breed rapidly enables researchers to house large populations easily. Specifically, production of genetically modified animals can be done in months, while large animal species requires years (Betthauser 2000; Cozzi 1995). Further, other than humans, there are more biological reagents available to test mouse and rat proteins and chemicals than any other species. Lastly, and not trivially, current USDA Animal Welfare regulations (as previously mentioned) do not cover mice and rats of the genus *Mus* or *Rattus* breed for research, greatly easing the regulatory burden placed on the researcher.

These advantages for the use of the mouse also extend to the field of transplantation medicine, where the work with the mouse forms the launching pad for many future investigations, both in animals and humans. Overcoming immune rejection of allografts is

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one of the major limitations in the field of transplantation. In addition to using rodents to test potential pharmacological methods for immunosuppression and graft acceptance, there are two other factors that have enabled rodents to play a valuable role in transplantation medicine by bypassing the risk of tissue/organ rejection. First, many strains of mice (and some rat strains) are genetically inbred to the point of being genetically identical (Fox 2007). This requires over 20 generations of inbreeding, having large populations of animals which are essentially genetically identical twins, eliminates much of the risk of rejection in these animals. One limitation a researcher must be aware of when using inbred strains of mice is genetic drift, which is the gradual accumulation of mutations within an inbred strain of mice. It is important to mention that mice of the same strain from different vendors are likely no longer genetically identical due to genetic drift (Fox 2007). Genetic drift may lead to gain or loss of function of genes which may impact transplant outcomes (by adding or removing major/minor immunostimulatory antigens). Despite this, the ability to freely perform syngeneic and allogeneic transplants (please refer to Chapter 27, GVHD) within mice has been a valuable research tool for transplantation scientists.

Over the last 50 years, there have been dramatic improvements in the ability to generate genetically modified immunosuppressed mice. The first immunosuppressed mouse was the nude mouse, which resulted from a spontaneous mutation of the FOXN1 gene resulting in the elimination of the thymus and subsequently T-lymphocyte-mediated cellular immunity. Shortly after this, the SCID (mutation of the protein kinase, DNA-activated, catalytic polypeptide gene) and RAG (mutation of the recombination activating gene 1 or 2) mice were developed, which were able to eliminate both the B- and T- lymphocytes (Croy 2001). A myriad of other proteins in the immune system have been eliminated and combined in mice, further impairing or eliminating key cell populations responsible for rejection. Currently, the most advanced form of this is the NOD/Scid common gamma chain knockout mouse (NSG) (Racki 2010), which also lacks NK cells, has laid the cornerstone for the generation of the humanized mouse (Kalscheuer 2012; Li 2014). Further refinement of this model has included the addition of human transgenes such as major histocompatibility factors and human cytokines which have further “humanized” these animals.

Using these severely immunocompromised models has allowed for further “humanizing” the mouse by allowing the engraftment human hematopoietic stem cells (mainly bone marrow stem cells). Other organs have been co-transplanted to aid in the maturation of such human stem cells (human thymus and fetal liver) (Shultz 2003; Shultz 2012; Shultz 2007). Forced expression of human cytokines within the mouse has enhanced the level of engraftment of the transferred human stem cells into these mice. Tolerance to tissues (such as skin) can be assessed after a human immune system has been developed in the mouse. These mice, though extremely useful for transplant studies, continue to be met with significant limitations. Mainly, the co-existence of human and mouse cells within the mouse is an artificial biological system which has often not translated well into the clinic (Shultz 2010; Yeoman 1993).

Non-Human Primates in Transplantation Research

Old world monkeys, mainly *rhesus macaques (Macaca mulatta), cynomolgus macaques (Macaca fascicularis)* and Hymadryas baboons (*Papio hymadryas)* are some of the most utilized non-human primate animal models in transplantation research. Monkeys have a

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unique role because of their close phylogenetic relationship to humans. Their susceptibility to human infectious agents and response to experimentally induced diseases are critically important to the advancement of medicine. Baboons are preferred for studies of xeno-transplantation because they are larger and considered to be hardier compared to macaques (Mohiuddin 2015). In transplantation many of the human-specific monoclonal antibodies and immunosuppressive agents cross-react (Kawai 2000; Kawai 1999) with NHPs. Despite these similarities to humans, due to ethical concerns, NHPs are among the least utilized animal models used in research.

Some notable clinically relevant differences between rodents and NHP models are briefly described. Mouse pre-transplant preparatory regimens do not follow the protocols used in humans, where gradual immunosuppressive therapies are given over days/ weeks in the clinic. In addition, it is difficult to evaluate clinically relevant treatment-related complications in rodent models. As an example, cytomegalovirus (CMV) observed in monkeys during transplant closely mimics CMV reactivation in humans (Han 2010). Furthermore, the fact that mice are kept in an extremely clean environment does not immunologically mimic what occurs post-transplant in outbred (non-SPF) species and humans. Toll-like receptors and T cell memory has been shown to interfere with rejection/tolerance studies (Yamada 2012). Thus, for some studies, the ideal pre-clinical model is one which can address the applicability of murine findings with responses to conditioning regimens that approximate humans. Hence, non-human primate models have the obvious advantage of similarity to humans.

Development of monkey models with specific ancestry and with known genetics is important for transplantation. Monkeys have one offspring (when compared to other species such as swine which can have 8−10) and their gestation is long (five and a half months) compared to other commonly used species such as swine (a little under 4 months), hence developing a colony is time consuming and costly. In this aspect, pigs have a clear advantage as they are easier to breed as previously mentioned. There is currently available a colony of *cynomolgus macaques* in the Mauritius Islands that have been naturally inbred. These animals were transported five hundred years ago to the Islands in ships by settlers. These few founder monkeys have over the past five centuries expanded their colony and currently are the best (natural) genetically characterized colony of macaques (Greene 2008; O'Connor 2007), making them attractive for studies of allogeneic transplantation(Duran-Struuck 2016; Zitsman 2016). There are other colonies commercially available, but their genetics is not as well described.

Swine in Transplantation Research

Swine have been increasingly used in biomedical research both as general large-animal biological models in teaching and research, and for the study of specific disease conditions due to their anatomic and physiologic characteristics.

As previously alluded, murine studies have been critical for determining immunological and molecular mechanisms of disease and therapy. However, results obtained in murine models with respect to studies of hematopoietic cell transplant (HCT) or solid organ often cannot be extrapolated to large animals or man (Storb, 2003; van Bekkum 1984). In addition, and as mentioned, it is difficult to evaluate clinically relevant HCT treatment-related complications in rodent models. The swine model has the most extensive panel of reagents

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available for any mammalian species (other than human and mouse) (Haverson 2001; Lunney1986; Pescovitz 1994; Pescovitz 1998; Pescovitz 1990; Pescovitz 1985; Saalmuller, 1996; Saalmuller 1994; Saalmuller1994a, 1994b; Saalmuller 1994; Saalmuller 1994; Saalmuller 1989; Saalmüller 1989; Zuckermann 1998). The ideal pre-clinical model in which to address applicability of murine findings would involve a large animal with responses to conditioning regimens and to HCT that resemble those of humans. Primate HCT models have the obvious advantage of similarity to humans (as discussed), but they are extremely expensive and the animals are difficult to breed. Dog models are more practical and have been widely used (Cain 1989; Ladiges 1990). Studies of hematopoietic and solid organ transplantation have been extended to miniature swine as a unique preclinical model of HCT (Sakamoto 1988; Duran-Struuck 2015). A selective breeding program to develop and maintain miniature swine with defined MHC genes as a large animal model for studies of transplantation biology was initiated over thirty years ago by Sachs (Sachs 1976). At present, there are three different well-described MHC haplotypes commercially available, swine leukocyte antigen $(SLA)^a$, SLA^c, and SLA^d. They provide most of the transplantation combinations relevant to humans and have been the swine most utilized in transplantation research.

BIOLOGY AND HUSBANDRY

Biology and Husbandry of Rodents

The research mouse (*Mus musculus*) and rat (*Rattus norvigicus*) have been bred and developed for more than 80 years and subsequently have been well-characterized. They belong to the order rodentia, family Muridae, subfamily murinae. The rat is in the genus *Rattus*, while the mouse in the genus *Mus*. The rodents are characterized by continuously growing incisors and otherwise are widely adapted between the species. Both mice and rats are considered to be nocturnal, omnivorous, social and intelligent species capable of being trained for experimental purposes. In addition, both can be domesticated well and can develop positive social relations with people (Fox 2007; Fox 2015).

The care and housing of mice and rats has become quite refined over the last 60 years. There are several commercially available feeds which are nutritionally complete for most strains of mice which have not been genetically modified. Care must be exercised with some strains of mice which have been adapted to have special dietary requirements. Mice and rats used in transplantation will be immunosuppressed either genetically (SCID, Nude, NSG etc.), chemically (i.e., cyclophosphamide, etc.), or by gamma irradiation (Duran-Struuck 2009; Duran-Struuck 2008). These mice are highly susceptible to Pseudomonas infections, transmitted through the water (Duran-Struuck 2009). These normally benign infections can be highly lethal in immunocompromised mice. Therefore, mice housed in biomedical research facilities typically receive, either hyperchlorinated, acidified or reverse osmosis treated water, which has been shown to be effective at preventing these organisms from colonizing the mice. In some instances, antibiotics are added to the water. Studies have shown that each of these water treatments is able to support normal growth and activity in mice (Hall 1980; Les 1968).

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Because of the extensive use of immunosuppressed rodents in transplant studies, a significant effort is invested to control normally benign, but often lethal diseases in such animals. Strict husbandry, handling and quarantine procedures are necessary to prevent infections. Even with the strictest of controls, occasional outbreaks occur in facilities. These outbreaks can be devastating to a research program, particularly depending on the time of the outbreak and mice involved (Fox 2007; Jacoby 1998). The importance of strict adherence to an institution biosecurity procedure cannot be overstated.

Biology and Husbandry of Non-Human Primates

There are specific regulatory requirements pertaining to space and enrichment required for each non-human primate species (*Guide for the care and use of laboratory animals*, 1985). Here, we will focus on a few important aspects pertaining to Old World monkeys.

Monkeys live in troops and are social animals. Research centers must have a plan to address the social needs of these NHP species. Hence, individual animals that are overaggressive or debilitated should be individually housed and must be appropriately monitored. Since they are a social species, single housing must be documented and justified as per the AWA. When arriving at a new institution, the macaques should be quarantined for a period of time to allow them to acclimate to the new environment and allow for testing of potentially infectious agents, including TB, to prevent the introduction of an agent to the established colony.

In general, macaques and baboons adapt well to the research environment. The temperatures required for housing are relatively broad (60−85°F). Their light cycle can be set at 12 hour light-12 hour dark to permit for a normal biorhythm. Macaques are relatively small (max of 8−10kg) and baboons are larger (20−30 kg) with males being larger than females. Their anatomic similarities with humans are evident, however their size may sometimes be a limiting factor for use in human-specific procedures (i.e., leukapheresis) (Pathiraja 2013).

Old World monkeys are frugivorous. In the wild they mostly eat fruit and their diet is supplemented with roots, bark, grass and some small mammals. Baboons, being larger than the macaques, may eat larger prey. In the laboratory they adapt to biscuit diet with relative ease. Commercial vendors guarantee a balanced diet (i.e., Lab Diet). Old World Monkeys require to be provided with vitamin C, and special attention should be focused at the expiration date to ensure vitamin C's full viability. Monkeys also require daily fruits, vegetables, legumes and foraging substrates (seeds, hay, etc.) to encourage species-specific behaviors.

NHPs can harbor dangerous zoonotic diseases (such as cercopithecine herpes virus 1, aka Herpes B) (Elmore 2008) that can be lethal to humans. These diseases, mostly viral, can become reactivated and shed during periods of stress (captivity/shipping) and immunosuppression (peri-transplant periods). Specific pathogen-free (SPF) production colonies are rapidly being developed and will become the standard. These SPF animals will be useful in minimizing zoonotic exposure of unwanted pathogens to laboratory and veterinary personnel (Yee 2016). In addition, minimizing herpetic diseases (not-necessarily zoonotic) which kill animals during their period of immunosuppression (i.e., gamma-herpes virus inducing lymphomas) or non-herpetic infections such as cytomegalovirus (CMV) (Burwitz 2016; Duran-Struuck 2016; Russell 2016), may decrease the number of animals lost

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post-transplant due to infection/viral reactivation. Currently animals suspected of having a contagious disease must be isolated from other healthy animals in the colony. Transplanted and immunosuppressed animals are already isolated (in HEPA-filtered cages when possible) to prevent them from acquiring (any) infections from the environment or conspecifics. Of note, such animals should always have visual and hearing company to minimize stress (which is detrimental for the animal's well-being). It is important that selection of companionship should be consulted with the veterinary team who will take the proper heath and psychological considerations.

Biology and Husbandry of Swine

There are many breeds of swine; however, miniature swine are very popular as laboratory animals. Swine bred for the food industry are often too big to be used in biomedical research. The connotation of "miniature" does not denote them being small when compared to humans. They are small compared to commercially used pigs (which weigh >500 kg). Miniature swine are of human size (50−110 kg). Some of the most commonly used breeds include Yucatan, Hanford, Sinclair, Hormel, and Gottingen.

Swine, like mice and NHPs have some important husbandry requirements. Newly received pigs to the facility should be given a minimum of 72 hours to adjust to the new environment. Physical exams and screening tests for parasites can be performed during the quarantine. Diet changes should be gradual over several days, with fiber increased if stress-induced diarrhea develops.

Shipping fever (stress-induced viral infections/reactivations and secondary bacterial infections) is not uncommon, and fecal assessment for parasites must be performed to minimize introducing (or reintroducing) pathogenic agents into the herd to be used for transplant.

Housing of animals is important. In general, swine are best housed in pens. These may be constructed of chain-link fencing or stainless steel or aluminum. Wood should be avoided because of pigs' ability to chew it and its costly sanitation. From a welfare perspective, wood shavings is beneficial as it allows for rooting, a normal swine behavior. However, when animals are to undergo anesthesia and require to be fasted (at least overnight), access to wood shavings is often not favored due to the risk of intussusceptions secondary to the ingestion of flooring material.

Flooring for swine is of great importance. Smooth flooring (epoxy or similar) should be avoided in order to minimize injury. Swine are hooved animals and have significant difficulties ambulating in smooth floors, particularly when floors are wet. In general, floors should provide some traction. This can be done by mixing sand with the epoxy or cement to maintain it rough/granular. More importantly, the use of abrasive surfaces is very beneficial for animals that are kept for long-term (as often occurs in tolerant animals used in pre-clinical transplantation). This allows for normal wear of the hooves and minimizes sedation for hoof trimming. Pigs with overgrown hooves can develop musculoskeletal conditions and be more prone to injuries. Raised flooring has been found to be useful as a means to keep excreta away from pigs, which aids in sanitation and can be valuable when working with immunosuppressed pigs. The use of slatted fiberglass floors with grit to provide hoof wear is generally ideal in these situations. These flooring options are expensive; however, they allow

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for good sanitation and since they are lightweight and easy to remove from pens, also minimize the risk of injury to animal care workers.

Continuous access to water is necessary and very important as swine are susceptible to salt poisoning following dehydration. Acute sodium (and water imbalances) can lead to significant brain swelling and herniation resulting in a neurologic syndrome that frequently leads to death. The use of water buckets is discouraged as they are prone to being turned and difficulties in drinking due to neck anatomy/conformation. Auto-watering systems are preferred, however these can be dangerous when animals run against them (potential of penetrating trauma), especially during periods of recovery post-anesthesia. From a practical standpoint and for transplantation studies, the availability of metabolic cages for large animals permits easy quantification of feces/urine. The level of dehydration can be difficult to assess by physical exam, but indication of dehydration can be detected using eyelid, axillary or inguinal skin tenting and confirmed through blood analysis.

Swine have unique biological attributes which make them well-positioned for biomedical research. Their cardiovascular system is similar to that of humans (Swindle 2012). They have a predominant right-sided dominance of the coronary arterial supply to the myocardium (like in humans). They are the premier species targeted to be used for xenotransplantation (swine into the man). This is due, in part, to the similarities they share with humans and the ethical acceptance of their use in contrast to NHPs.

There have been numerous studies describing the similarities and differences in skin histology between swine and humans. Both have well-defined dermal papillae and rete ridges (Laber 2009). The size, distribution and orientation of blood vessels in the dermis of the pig are similar to those found in human skin. The epidermal turnover time, type of keratinous proteins found within the skin and lipid composition of the stratum corneum is very similar between humans and swine (Lavker 1991; Miller 1998; Nicolaides 1968). This makes them unique for skin and composite tissue transplant studies and graft-versus-host disease (GVHD) (Duran-Struuck 2015; Horner 2009; Leonard 2014). As an allogeneic model of transplantation, swine have been an excellent model for the study of GVHD, a side effect of allogeneic bone marrow transplantation. A clinical GVHD scoring system has been developed in swine (Duran-Struuck 2015) that mimics exactly what is performed in hospitals.

Histologically, the stomach of both humans and pigs has a glandular epithelium, although pigs have a muscular and mucoid glandular structure called the torus pyloricus, which is located near the pyloric sphincter. The pig GI tract is very long, extending as much as fifteen times the length of the body (Laber 2009). The anatomy of the colon is significantly different. It has a centrifugal and centripetal loop, thus coiling within itself and occupying a significant part of the abdominal cavity. Physiologically, pigs and humans are both omnivores, and they share similar characteristics with regards to digestion and intestinal transport. Indeed, this likeness may also help to explain their similarity in liver metabolism as will be discussed next.

The pig has been readily utilized as an animal model for hepatic studies (Wolf 1997). Interestingly, some studies claim that the metabolic function of porcine liver may be more similar to humans than other non-human primate species (Drougas 1996). Physiologically, one important function of the liver in both humans and swine is the synthesis of albumin, the most abundant protein in plasma. There is 65% similarity between human and porcine albumin, although serum albumin concentration is lower in pigs than in primates (Hammer 1998; Platt 2000). Bilirubin can be used as a measure of functional capacity of the liver.

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Serum bilirubin concentrations are closely monitored and used for GVHD scoring in the clinic (Ferrara 2009).

The lymphoid organs, mainly the lymph nodes, spleen, thymus and bone marrow are targets of GVHD and are also the locations where allogeneic immune responses are primed (Ferrara 2009). The most important gross and histopathological differences between swine and humans are in the characteristics of the lymph nodes and thymus. The lymph nodes of swine display a unique histological characteristic: the typical cortex and medulla are reversed with germinal centers located in the interior of the gland (Laber 2009).

Lastly, and of interest in transplant immunology studies, the pig has a very large thymus, part of which extends out of the thoracic cavity. As a result, thymus biopsies are very easy to perform and are minimally invasive as there is no need to enter the thoracic cavity.

Anesthesia

There are no absolute regulations in the United States that require a veterinarian or licensed veterinary technician to provide or oversee anesthesia for complex surgical procedures within biomedical research. The primary source of guidance, as mandated by Public Health Service Policy on Humane Care and Use of Laboratory Animals (and utilized by AAALAC International for institutional program assessment) is the *Guide for the Care and Use of Laboratory Animals (Guide)*. The *Guide* indicates that research teams should be provided appropriate education and training to ensure that they have the necessary knowledge and expertise for the specific animal procedures proposed, including aseptic surgical technique, anesthesia, and analgesia. Additional guidance regarding surgical and anesthesia training is published by the Academy of Surgical Research ("Guidelines for training in surgical research with animals. Academy of Surgical Research," 2009).

Although most veterinarians and veterinary technicians receive anesthesia training as part of their curriculum, the type of training may not have been specific or advanced enough to adequately perform anesthesia for complex transplantation surgeries. As a result, veterinary personnel may not be fully proficient to perform anesthesia for complex surgical procedures until additional training or experience is acquired. Each institution should develop a surgical and anesthesia training program, to ensure that surgical procedures including anesthesia are performed in such a manner to maximize animal health and welfare, to minimize distress or adverse effects and to meet scientific goals. To assist with training requirements there are currently multiple organizations that offer training material such as the American Association for Laboratory Animal Science (AALAS), (https://www.aalas.org), Laboratory Animal Welfare Training Exchange (LAWTE), (http://www.lawte.org), and the Academy of Surgical Research (ASR), (http://surgicalresearch.org) which also offers anesthesia-related certification (Surgical Research Anesthetist-SRA). In addition, advanced training may also be acquired through collaborations with research institutions currently performing complex transplantation surgery. Board certified veterinarians from the American College of Veterinary Anesthesia and Analgesia (ACVAA) and American College of Laboratory Animal Medicine (ACLAM) or anesthesia medical personnel within human hospitals associated with biomedical research institutions can be consulted regarding best anesthesia practices for complex transplant surgeries.

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A thorough review on veterinary anesthesia including pharmacology, equipment, and monitoring is published within the veterinary literature (Beckman 2013; Fish 2008; P.A. 2016; Grimm 2015; Murphy 2012). However, the basics of anesthesia and pain management will be reviewed in this chapter by species. Significant overlap between NHPs and swine naturally occurs based on their size and close physiology. Rodents, on the other hand, and based on their small size and metabolism, require alternative approaches. We subsequently discuss anesthesia approaches in these commonly used species in transplantation.

Anesthesia of Mice and Rats

The small size and high metabolic rate of mice and rats makes anesthesia a unique challenge in this species. Routes of anesthesia and anesthetic monitoring used in other species are all available in the mouse, but many are technically very difficult or challenging, making them unacceptable/unusable/impractical to routine mouse transplantation surgery. Despite this, there is a strong growing body of literature which is advancing the field of murine anesthesia, improving the efficacy and assessment of a surgical plane of anesthesia, prediction of impending anesthetic complications and potential clinical interventions (Erickson 2016; Jaber 2014). This section will also address the most commonly used anesthetic protocols in mice, including practical anesthetic support and monitoring procedures.

In order to safely perform surgery on any animal, transplant or otherwise, the animal must be safely anesthetized to a plane where it is unconscious (and therefore unable to experience pain) and does not move in response to a noxious stimulus. Animals will reach a plane of unconsciousness before losing the withdrawal response to a noxious stimulus, however, confirming this plane of anesthesia in animals is difficult to do reliably. The need to maintain immobility during surgery mandates that animals be anesthetized until they do not move in response to the noxious stimulus. When an animal is anesthetized to the loss of consciousness and has lost the withdrawal response to a noxious stimulus (i.e., toe pinch), it is referred to as being at a surgical plane of anesthesia (Campagna 2003; Fish 2008; Tranquilli 2007). Ideally, the animal will not be anesthetized (also referred as "deep") enough as to have lost its autonomic reflexes, but that is difficult to assess in rodents, particularly in mice, as we discuss below.

Inhalant vs. Injectable Anesthesia

The current recommendation for all rodent surgeries is the use of inhalant isoflurane (Fish 2008). This anesthetic has been shown to reliably induce (and maintain) a surgical plane of anesthesia in both mice and rats. The inhalant anesthetics, across species, have been demonstrated to have a very steep dose response curve, meaning that almost 100% of animals can be maintained at a specific plane of anesthesia, in the case of transplant studies, a surgical plane of anesthesia. This is beneficial as it prevents animals to migrate towards a deeper, potentially more dangerous plane of anesthesia (Campagna 2003).

The use of bolus injectable anesthetics has a much more variable response. This variable response to the anesthetic bolus results in unreliable outcomes. Often this approach may result in animals not reaching an adequate plane of anesthesia for surgery or becoming too deep and dying during the procedure. These deaths are attributed to responses to the anesthetic regimen, independent of the surgical intervention(s). This scenario is rarely observed with

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inhalant anesthetics. An important reason pertains to the mechanisms of clearance of the inhalant anesthetics. Inhalants are rapidly cleared through the lungs. Hence, the anesthetic dose can rapidly be titrated in all animals which are adequately ventilating (Tranquilli 2007).

Injectable anesthesia is an important part of anesthesia for most veterinary species, however this is done with intravenous access, where the dose administered can be carefully titrated to the animal's response. While intravenous access is possible in the mouse, it is technically difficult to establish and maintain, requiring bolus, intraperitoneal dosing. The disadvantage of this is that with the variable response to injectable anesthetics, discussed above, it may result in some animals receiving an anesthetic overdose, and others receiving an inadequate dose to achieve a surgical plane of anesthesia. To date, there is very little information on the appropriate re-dosing of animals which fail to reach the desired surgical plane of anesthesia. Most information is based on anecdotal, untested procedures.

The delivery of isoflurane does require specialized anesthetic equipment, including an anesthetic gas vaporizer, induction chamber and waste anesthetic gas scavenger system (Herrmann 2013) It is beyond the scope of this chapter to go into a detailed discussion of these components, except to say that there are a variety of portable rodent anesthesia setups which can be used to effectively and safely anesthetize mice with inhalant anesthetics.

The most significant side effect associated with isoflurane anesthesia is a profound, dose-dependent hypotension. For routine transplant surgeries, where little data collection occurs, it can be a potential variable which will confound results. Especially for heart, gastrointestinal and other solid organ models that require good perfusion for the survival of such organ. Generally, the largest complication that may occur is a consequence of inadequate monitoring of the plane of anesthesia. Hypotension may result in a normal compensatory increase in heart rate, which may be interpreted as an inadequate plane of anesthesia. Fluid support may be able to help address this complication (addressed below). The newer inhalant anesthetics, sevoflurane and desflurane, have been successfully used in mice, however, there is much less data on the use of these inhalants when compared with isoflurane (Herrmann 2013).

Common Injectable Anesthetic Protocols for Mice

The most commonly used injectable anesthetic protocols in rodents are ketamine in combination with either xylazine or xylazine and acepromazine. Generally, studies testing the effects of the different anesthetic protocols in mice report that the addition of acepromazine to the ketamine/xylazine combination dramatically increases the efficacy of the anesthetic protocol. This combination has been shown to provide effective anesthesia for both mouse and rat liver transplantation surgeries (He 2010). Researchers must keep in mind the following factors as they initiate a surgical protocol is: (i) the profound variability in dosing requirements; (ii) the species and strain of animal being used; (iii) the condition of the animal at the time of surgery and; (iv)the skill of the surgeon. Therefore, initial dosing recommendations must be used only as starting guidelines and the final anesthetic protocol will need to be adjusted to the specific surgical conditions. The ketamine/xylazine/acepromazine protocol is a good example of multimodal anesthesia, with the combination of the three different classes of drugs limiting the dose required to achieve the anesthetic goals of the surgery, thereby minimizing the side-effects associated with each individual drug (Table 4.1). The role of monitoring in the side-effect prevention is addressed later in this chapter. Injectable drugs undergo hepatic and/or renal excretion, (He 2010) meaning that transplantation involving these organs may require significant adjustments of their dosing.

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Other older injectable anesthetic protocols have been used in the past, including barbiturates, but these are less utilized in transplantation compared to the ketamine-based protocols.

Table 4.1. Anesthetics for Rodents

Drug	**Classification**	**Post op Analgesia**	**Main Side Effect**
Ketamine	Dissociative Anesthetic	Yes	Respiratory Suppression at high doses
Xylazine	Alpha adrenergic Agonist	No	Bradycardia and hypotension
Acepromazine	Phenothiazine	No	Hypotension
Isolfurane	Inhalant	No	Hypotension
Common Anesthetic protocols for mice to achieve a surgical plane anesthesia			
Isoflurane (Recommended): 3-4% for induction 1.5-2.5% for maintenance of anesthesia			
Ketamine 80-120 mg/kg, Xylazine 8-10 mg/kg, acepromazine 1-2 mg/kg Intraperitoneal. Provides approximately 30-40 minutes of surgical plane anesthesia.			

Doses from:

1. Erickson, R. L. *et al.* Intraperitoneal Continuous-Rate Infusion for the Maintenance of Anesthesia in Laboratory Mice (Mus musculus). *Journal of the American Association for Laboratory Animal Science: JAALAS* **55**, 548-557 (2016).
2. Arras, M., Rettich, A., Cinelli, P., Kasermann, H. P. & Burki, K. Assessment of post-laparotomy pain in laboratory mice by telemetric recording of heart rate and heart rate variability. *BMC Vet Res* **3**, 16, doi:10.1186/1746-6148-3-16 (2007).
3. Buitrago, S., Martin, T. E., Tetens-Woodring, J., Belicha-Villanueva, A. & Wilding, G. E. Safety and efficacy of various combinations of injectable anesthetics in BALB/c mice. *Journal of the American Association for Laboratory Animal Science: JAALAS* **47**, 11-17 (2008).

Anesthetic Support and Monitoring

The small size and high metabolic rate of mice makes routine monitoring of mice particularly challenging. The resting mouse heart rate ranges between 500−700 beats per minute and even under anesthesia will range from 250−450 beats per minute, depending on the anesthetic protocol (Erickson 2016). Further, many common ECG machines and pulse oximeters are unable to accurately monitor such high heart rates. Specialized, potentially expensive monitoring equipment is therefore required. Monitoring of blood pressure in mice can be achieved in conscious mice using tail plethysmography, however this technique requires the mice be kept at relatively high temperatures to ensure adequate blood flow to the tail, which is impractical in mice. The blood pressure can be accurately measured in anesthetized mice following carotid catheterization. This would entail an additional surgical procedure during the murine transplant surgery. Placement of central arterial pressure measurements would be appropriate if the information provided would lead to medical treatments. Hence, these approaches though medically appropriate may be impractical in these species as they would prolong the anesthetic time and would further risk anesthetic complications.

The four most practical methods to monitor mouse anesthesia are: 1) body temperature; 2) respiratory rate and pattern; 3) skin/mucus membrane color; and 4) response to a noxious stimulus. The body temperature also needs to be closely monitored under anesthesia because

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the mouse rapidly loses heat due to the high ratio of surface area to body volume. Studies have shown that mice can rapidly have the body temperature drop to below 30º C in less than 20 minutes. Rats are more resistant to this drop in body temperature, but they too will experience hypothermia if they do not receive supplemental heat (Malkinson 1993). The most obvious complication associated with hypothermia is a delayed recovery from anesthesia, but in other species, anesthetic-induced hypothermia is associated with cardiac arrhythmias, increased risk of infection and coagulopathies (Tranquilli 2007), all of which could complicate the results of any transplant surgery. When providing supplemental heat to the animal, it is important to use a circulating water blanket, as opposed to an electric heating pad, as these pads may have hot spots which can do thermal injury to the animal.

Respiratory rate of rodents is easily monitored under general anesthesia and will depend on the anesthetic being administered. Mice being maintained with a ketamine/ xylazine/acepromazine protocol will normally have respiratory rates of +/-150 breaths per minute and will be at significant risk of anesthetic arrest when the rate goes below 80 to 120 breaths per minute. Adverse outcomes are strain-dependent (Marx JO unpublished- personal experience), (Jaber 2014). Mice maintained under isoflurane will have respiratory rates in the area of 60 breaths per minute (Erickson 2016). Further, the depth of anesthesia can be closely monitored by respiratory rate, with changes in respiratory rate correlating to changes in depth of anesthesia.

The color of the skin on the feet and nose and mucus membranes can be used as an indicator of peripheral perfusion of rodents and potentially hypoxia. This finding may be difficult to detect in some rodents (if high skin pigmentation) and marked changes in the color of the skin and mucus membranes should be interpreted as profound changes in the animal's condition.

Lastly, the test most commonly used to assess if the animal has lost its movement in response to a noxious stimulus is the toe pinch, in which a firm pinch is applied to the foot of the mouse. This should be performed before beginning any surgical procedure. Care must be taken to apply a strong stimulus, while realizing that it is easy to injure the foot with a pinch if too aggressive. The 300 gram Touch Test Device (North Coast Medical, Gilroy, CA) can be used to deliver a uniform, non-injurious noxious stimulus to the foot (Erickson 2016). It must be remembered that this noxious stimulus is a fairly mild stimulus and more anesthetic may be required for more painful procedures.

The larger size of rats makes monitoring anesthesia much easier than in mice. The heart rate of 330−450 beats per minute and respiratory rate of 85 breathes per minute can be easily monitored with many pulse oximeters and by visual monitoring. Monitoring the temperature of rats is also important, although they will lose heat much more slowly than mice, due to the larger relative body volume: surface area ratio.

Anesthesia of Swine and Non-Human Primates in Transplantation

NHP Anesthesia Basics

Inhalant anesthetics are favored over injectable ones for their safety as mentioned earlier. Rebreathing anesthesia machines may be preferred over non-rebreathing circuits in order to

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minimize waste gas exposure from higher flow rates required with non-rebreathing circuits. This is also beneficial for warming of gases to prevent hypothermia. Ideally the rebreathing bag should be five times the animal's estimated tidal volume and the anesthesia system should be pressure checked for leaks (e.g., soda lime canister connection, breathing tubing, one way valves, or rebreathing bag) prior to use. The anesthetic plan including pre-operative and post-operative care should be reviewed with veterinary personnel prior to transplantation surgery. For personnel and animal safety concerns, most macaques and baboons are immobilized first with a dissociative such as ketamine or the dissociative-benzodiazepine combination, tiletamine-zolazepam (Telazol®) (Murphy 2012). Ketamine can also be combined with a benzodiazepine such as midazolam or diazepam or an alpha-2 agonist such as dexmedetomidine or xylazine for additional sedation and muscle relaxation (Lee 2010; Flynt 2010; Haag 2010; Taylor 2010; Theriault 2008; Reed 2008; Niekrasz 2008). Non-ketamine neuroleptanalgesic combinations have also been described for brief immobilization of macaques (Kimura 2007; Ochi 2014; Votava 2011). After immobilization, the NHP can be fitted with a facemask to provide supplemental oxygen while an intravenous catheter is placed for fluid and drug administration. At this point, the NHP may be induced for endotracheal intubation using an inhalant such as isoflurane or sevoflurane by face-mask. Alternatively, the NHP may be induced with an intravenous bolus of a sedative-hypnotic such as propofol (Authier 2006; Fowler 2001) or alphaxalone and subsequently intubated. Both propofol and alphaxalone can cause apnea so careful titration to promote intubation is warranted. Once intubated, the NHP can be maintained on an inhalant anesthetic with oxygen or in combination with an intravenous continuous infusion of a full mu agonist opioid (e.g., fentanyl, alfentanil, sufentanil, or remifentanil), benzodiazepine (e.g., midazolam), sedative-hypnotic (e.g., propofol or alphaxalone) or a combination for a balanced anesthesia approach (Bauer 2007; Casoni 2015; Valverde 2000). This approach minimizes adverse alterations in cardiac output, blood pressure, and respiratory functions typically associated with higher anesthetic doses, and provides additional muscle relaxation and analgesia. Furthermore, studies have demonstrated that the addition of NMDA antagonists such as ketamine during surgery and prior to intense surgical stimuli may reduce the development of central sensitization and potential post-operative hyperalgesia (Pozzi 2006; Wagner 2002). Total intravenous anesthesia such as a continuous infusion of propofol and a short acting mu agonist opioid is commonly used for neurosurgical anesthesia and may be an alternative to inhalant anesthesia especially for shorter transplantation surgical procedures. Advantages include not having to utilize an anesthetic machine and reductions in waste anesthetic gases. However, unlike inhalant anesthetics that can be rapidly titrated to defined MAC level targets and monitored via expired concentrations, intravenous anesthetics must be titrated to predetermined pharmacodynamic parameters which may vary between species and may not be defined for NHPs. In addition, NHPs receiving continuous infusions of intravenous anesthetics should be intubated and supplemented with oxygen to ensure proper hemoglobin saturation and to protect the airway against possible regurgitation during surgery. For these reasons, a balanced anesthetic regimen consisting of a combination of an inhalant with additional analgesics and/or sedatives is preferred for NHP transplantation surgical anesthesia until additional studies are performed outlining the use of total intravenous anesthesia in research animals involving transplantation. If a full mu agonist opioid is utilized as a premedicant or during the intra-operative period, a partial mu opioid such as buprenorphine should be withheld until the post-operative recovery period, as it may reverse the analgesic

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efficacy of the full mu agonist. Sedative, anesthetic, and analgesic doses for non-human primates are outlined in Tables 4.2 and 4.3.

Table 4.2. Post-operative Analgesics for Rodents [98, 129]

Drug	Classification	Dose in mice
Buprenorphine	Partial mu opiate agonist	0.05-0.1 mg/kg
Buprenorphine Sustained Release	Partial mu opiate agonist	Mice 0.5-1.0 mg/kg SC Rats 1.0-1.2 mg/kg SC
Meloxicam	NSAID	Mouse: 5 mg/kg PO or SC Rat: 1-2 mg/kg PO or SC
Lidocaine	Local anesthetic short acting	10 mg/kg locally
Bupivacaine	Local anesthetic long acting	6 mg/kg locally

Doses from:

1. Flecknell, P. A. Analgesia of small mammals. *The veterinary clinics of North America. Exotic animal practice* **4**, 47-56, vi (2001).
2. Hawk, C., Leary, S. & Morris, T. (Blackwell Pub., Ames, IA, 2005

Anesthetic monitoring should be based upon direct assessment of the animal including jaw tone, eye position, palpebral-corneal reflexes, heart rate, pulse quality, respiratory rate and depth, temperature, mucous membrane color and capillary refill time. Additional monitoring equipment that is useful for complex transplantation surgery includes pulse oximetry for hemoglobin saturation, capnography to assess ventilation and alterations in cardiac output, blood pressure (direct arterial, oscillometric, or doppler), arterial blood gas analyzer to assess acid-base status and electrolytes, electrocardiography, and hematology analysis such as hematocrit and total protein to assess dilution of plasma proteins secondary to fluid administration ("American College of Laboratory Animal Medicine Recommendations for Monitoring Anesthetized Veterinary Patients," 2009; Duke-Novakovski 2015; Flegal 2009; Fox 2008; Fox 2009; Yeung 2014). A more detailed description of nonhuman anesthesia is provided by Murphy et al. (Murphy 2012).

Since post-operative care may involve multiple drugs, blood sampling, monitoring, or fluid administration requiring intravenous or arterial access, pre-surgical planning and additional equipment such as jacketed tethering systems and infusion pumps may be warranted. Since transplant surgeries can be very challenging, a dedicated research area with well-trained staff and coordination with veterinary care is required to minimize complications associated with surgery and immunosuppressive therapy, enhance monitoring, and maximize animal welfare. Cooper and Wagner, provide a review of NHP xenotransplantation (Cooper 2012).

Anesthesia in Swine

Anesthesia for transplant surgery in swine is very similar to techniques used for non-human primate anesthesia. Swindle at al. provides a thorough review of swine anesthesia,

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Table 4.3. Non-Human Primate (Macaque & Baboon)
Sedative - Immobilization Combinations and Intravenous Anesthesia

DRUG OR DRUG COMBINATION	DOSE
Ketamine	5 - 15 mg/kg IM
Ketamine: Xylazine	10 mg/kg: 0.15 - 0.25 mg/kg IM
Ketamine: Diazepam	15 mg/kg: 0.3 - 1 mg/kg IM
Ketamine: Midazolam	5 - 15 mg/kg: 0.05 - 0.15 mg/kg IM
Ketamine: Dexmedetomidine	5 -10 mg/kg: 10 - 30 mcg/kg IM
Midazolam: Dexmedetomidine	3 mg/kg: 0.015 mg/kg IM
Tiletamine - Zolazepam (Telazol®)	2 - 6 mg/kg IM
Alphaxalone: Dexmedetomidine[a]	5 mg/kg: 10 mcg/kg IM
Alphaxalone: Diazepam[b]	5 mg/kg: 0.5 mg/kg IM
INTRAVENOUS ANESTHESIA (generally preceded by ketamine +/- sedative)	
Propofol	2 - 8 mg/kg IV to effect 18 - 24 mg/kg/h continuous intravenous infusion
Alphaxalone	1-3 mg/kg IV bolus 0.01 - 0.13 mg/kg/min continuous intravenous infusion
Propofol: Fentanyl	Propofol: 2.4 - 7.2 mg/kg/h Fentanyl: 10-25 mcg/kg/h continuous intravenous infusion
INHALANT ANESTHETICS	
Isoflurane	3-5% induction 0.5 - 3% Maintenance
Sevoflurane	4-8% Induction 1.25 - 4% Maintenance

Drug dosages adapted from the following: Murphy KL, Baxter MG, Flecknell PA. Anesthesia and Analgesia in Nonhuman Primates. In: Abee CR, Mansfield K, Tardif S, Morris T, eds. *Nonhuman Primates in Biomedical Research*: Academic Press, an imprint of Elsevier; 2012:403-435.

analgesia, and post-operative monitoring (Swindle 2016). Solid food is generally removed 6−8 hours prior to surgery and longer for lower gastrointestinal surgery. Water is provided until the time of surgery and may be supplemented with nutritional fluids or glucose for prolonged fasting to prevent hypoglycemia (Swindle 2016a; Swindle 2016b). Swine are generally immobilized and sedated with a combination of ketamine and xylazine, ketamine and dexmedetomidine, or the combination of tiletamine-zolazepam (Telazol®). Tiletamine-zolazepam is preferred in larger pigs due to smaller injection volumes and diminished stress with injection. However, if cardiac transplantation surgery will be performed, alpha-2 agonists (e.g., xylazine or dexmedetomidine) and tiletamine-zolazepam should be used with caution or avoided due to cardiac arrhythmias and cardiac depressant effects. In these cases ketamine and midazolam can be used for pre-induction sedation. Swine should be pre-oxygenated prior to intubation, which can be performed in dorsal or sternal decumbency. Endotracheal intubation can be challenging in swine as the larynx is angled relative to the trachea. Standard veterinary laryngoscope blades may need extensions (3−5 cm) for swine >

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50 kg (Swindle 2016b). After sedation and venous access is achieved, swine can be induced with propofol or a combination of propofol and fentanyl with titration to avoid apnea. Anticholinergic agents such as atropine or glycopyrrolate are generally not needed for routine use, but can be used to decrease secretions or treat bradycardia associated with alpha-2 agonists and mu opioids. Anesthesia should be maintained with an inhalant such as isoflurane or sevoflurane with oxygen as a carrier gas. Similar to non-human primate anesthesia, inhalants can be used in conjunction with a continuous intravenous infusion of a mu opioid such as fentanyl, sufentanil, or remifentanil to provide additional analgesia, or in combination with propotol or midazolam for a balanced anesthesia approach. In order to achieve adequate steady-state plasma levels, opioids such as fentanyl, sufentanil, or remifentanil should be administered with a loading dose bolus (during induction or immediately afterwards) prior to initiating a continuous intravenous infusion. The use of a balanced anesthesia approach allows for lower doses of each individual anesthetic agent if used alone thus decreasing overall cardiodepressant effects and hypotension. Hypotension is primarily treated by correcting fluid losses, titrating anesthetic depth, and the use of intravenous inotropes such as dobutamine (2−10 mcg/kg/min), dopamine (2−10 mcg/kg/min), or epinephrine (1−10 mcg/min) titrated to response. Thermal support (e.g., warm intravenous fluids, circulating warm-water blanket, and/or forced-air warming) should be provided during anesthesia to prevent hypothermia. Sedative, anesthetic, and analgesic doses for swine are outlined in Tables 4.4 and 4.5.

Anesthetic monitoring should include electrocardiogram, capnography, pulse oximetry, blood pressure monitoring, be it peripheral or invasive, body temperature, and assessment of muscular tone and/or withdraw reflexes. For many transplant procedures, attention should also be given to periodic blood gas monitoring. Bispectral index (BIS) monitoring via electroencephalogram has been evaluated in swine in conjunction with various anesthetic regimens (Jaber 2015; Martin-Cancho 2006; Martin-Cancho 2004) and surgical models (Baars 2013; Kurita 2012). Overall, its utility is limited to the delineation of light versus deep anesthetic planes and cannot be used as the sole mechanism for anesthetic plane determination.

FLUID SUPPORT

Rodents

In addition to warming anesthetized rodents, providing fluid support is very beneficial to helping maintain the animal's vitals in a healthy range. In both humans and most routine veterinary species, the administration of intravenous fluids is considered essential to maintain blood pressure and normal renal and cardiac function (Tranquilli 2007). Unfortunately, and as discussed above, intravenous access is difficult/limited in most rodents, meaning that routine fluid administration is done either into the subcutaneous space or intraperitoneally. While these routes of administration are far inferior to intravenous administration, anecdotal evidence suggests that they will help maintain the animal during a transplant surgical procedure, particularly in prolonged surgeries (Tranquilli 2007). One mL of warmed, isotonic crystalloid fluids (LRS) is frequently administered intraperitoneally after induction of anesthesia. These fluids will need to be given subcutaneously for intra-abdominal surgeries.

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Non-Human Primates

Published reviews of fluid therapy for small animals can be utilized as guidelines for fluid therapy in NHPs and swine in a surgical transplantation research setting (Boller 2015; Byers 2016; Mazzaferro 2013). The goals or choice of fluid therapy should be made based upon the types of fluids lost, comorbid disease processes, hydration and electrolyte status. Isotonic crystalloids (e.g., 0.9% sodium chloride, lactated ringers, Normosol-R) are the mainstay of therapy for replacing fluid losses during surgery or dehydration in the perioperative setting. Maintenance fluids comprised of lower sodium concentrations (e.g., 0.45% sodium chloride) are generally used to replace sensible and insensible fluid losses once dehydration has been corrected. Maintenance fluid rates can be calculated based upon resting energy expenditures. The formula for resting energy expenditure (REE), (ml H_20 per 24 hours) = [(30 × body weight kg) +70] can be used as a starting point, yet body weight, serum osmolality, biochemical chemistries, electrolytes and urine-specific gravity should also be monitored and evaluated. Intraoperative fluid rates are generally 5−10ml/kg/h to replace sensible and insensible losses. Fluid losses during surgery can be corrected with isotonic crystalloids using 2−3 times the volume loss to replace intravascular fluid deficits. Treatment of shock relies upon the blood volume of the animal. When treating hypovolemic shock, isotonic crystalloids should be administered at ¼ total shock dose increments. Afterwards, blood pressure, central venous pressure, mucous membrane color, capillary refill time, heart rate, pulse pressure, and serum lactate can be monitored to assess perfusion (Boller 2015). Additional ¼ total blood volume crystalloid boluses may be repeated one to two times or until perfusion parameters have stabilized (Mazzaferro 2013). Hypertonic saline, synthetic colloids (hydroxyethyl starches or dextrans), or blood products can be used in combination with crystalloids to enhance and prolong intravascular volume expansion. Crystalloids to replace the fluids drawn into the intravascular space from the interstitial compartment should follow the use of hypertonic saline or colloids (Mazzaferro 2013). Since the volume expansion due to crystalloid therapy is relatively short (20−30 minutes), colloids may be considered for longer surgical procedures in order to provide sustained expansion of the intravascular space and decrease the potential to dilute coagulation factors and plasma proteins(Boller 2015). Adverse effects of synthetic colloid therapy include volume overload and coagulopathies with higher doses. The use of synthetic colloids such as hydroxyethyl starch for intravenous fluid resuscitation has been questioned due to recent reports of kidney failure and coagulopathies in critically ill human patients, yet additional research is needed to fully assess their impact in veterinary medicine (Cazzolli 2015).

Swine

Perioperative care of swine undergoing transplantation requires the standard of care typical of any surgical event (Swindle 2013): vascular access for fluid therapy and pharmaceutical administration, preemptive and sustained analgesic therapy, normothermic maintenance, and proper anesthetic monitoring. Vascular access can be easily achieved via percutaneous catheter placement in a superficial peripheral vessel, such as the auricular or cephalic veins. The Seldinger technique, with or without ultrasound guidance, has gained popularity for use in swine for the percutaneous catheterization of the external jugular or

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femoral veins to avoid surgical subcutaneous cutdown procedures which have been classically favored for catheterization of these vessels (Flournoy 2009; Larsson 2015; Izer 2016). Standard fluid therapy rates are utilized intraoperatively to maintain blood pressure at 60−70 mmHg for adequate perfusion. Placing the fluid lines through a fluid warmer immediately prior to administration will also assist in maintaining adequate body temperature during the procedure. Serum and hematological parameters can be taken from the catheters and continued physical exams can be performed without sedating the pig when awake.

Pain Management

Pain can be described as an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage (Merskey 1994). In addition, it is generally assumed that events or procedures noted to be painful in humans will also be painful in animals. Although mandated or highly recommended by regulations and the *Guide*, pain associated with transplantation surgeries, procedures, or immunosuppressive therapy should be treated and addressed from an ethical and a scientific perspective to reduce confounding research variability secondary to stress or adverse physiologic effects of untreated or poorly treated pain. In addition, the American College of Laboratory Animal Medicine, American Animal Hospital Association, and the American College of Veterinary Anesthesiologists have published position statements indicating the need to address pain in companion and research animals ("American College of Laboratory Animal Medicine Position Statement on Pain and Distress in Research Animals," 2016; "American College of Veterinary Anesthesiologists' Position Paper on the Treatment of Pain in Animals," 2006; Epstein 2015). Hence, managing pain in animals undergoing surgery is of key importance for both animal welfare and experimental outcomes. Below we discuss some approaches to the management of pain in rodents, NHPs and swine.

Analgesia in Rodents

Assessment of Pain

Proper administration of analgesics is complicated by two factors in rodents, 1) the difficulty in assessing pain and 2) the rapid metabolism of drugs, including analgesics, requiring an increase in dosing frequencies compared to other species (Brown 2004; Flecknell 2001). Rodents, like many other prey species, have evolutionary adapted to mask overt signs of pain until the pain is extreme that becomes debilitating, at which point becomes visually clear. Studies have shown that training and practice in identifying the subtle signs of pain can significantly improve the ability of trained professionals to recognize pain. Cases of pain may be identified by non-specific signs of disease, such as decreased movement, poor hair coat, and loss of body condition (Flecknell 2011). However, it must be remembered that these are non-specific signs of pain and may be indicative of other health issues with the animals. Rodents can exhibit signs typical of other species, such as lameness, abnormal gait and increased aggression which are easily recognized as being due to pain. Recent publications have reported ***facial grimace scoring*** for both the mouse and the rat. They utilize a series of

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changes in the facial appearance of the animals to pain and appear to be a valuable tool in pain assessment of rodents (Leach 2012; Matsumiya 2012). In addition, subtle signs such as abdominal stenting and stretching are indicative of abdominal pain (Flecknell 2001).

When pain is either identified or suspected, analgesic therapy is indicated, however this must be done within the context of the experimental goals of the project. Non-steroidal anti-inflammatory drugs (NSAIDs) may be unacceptable for studies focusing on the immune systems response to a challenge. Critical evaluation of this conflict in transplant studies is critical as many analgesics, in addition to the NSAIDs, may impact the immune system. Therefore, discussions with the veterinary team should be started early during the design of the study in order to avoid administration of drugs that can affect the results of the transplant studies. As a team, veterinarians and researchers should work together to find the proper analgesic regimen. Typically, there are three main pharmaceutical approaches to post-surgical pain in rodents. These are local anesthetics, NSAIDs and opiates (Table 4.2).

Local anesthetic/analgesia therapy consists primarily of either lidocaine or bupivacaine, both working by blocking sodium influx into the nerve cells, preventing nerve activation (Lemke 2000). Lidocaine has a more rapid mechanism of action and shorter duration, while bupivacaine has a longer onset to action, but significantly longer duration of action. Studies combining the two drugs, to optimize the beneficial effects of each, yield inconsistent results (de Jong 1981; Ribotsky1996).

The systemic analgesics Meloxicam and Carprofen are currently the most commonly used NSAID in rodents (Fish 2008). Its reported duration of action is 12 to 24 hours, however many times this appears to be an over estimate of its duration of action. Meloxicam has the advantage of being an oral and injectable drug. While these drugs are associated with minimal side effects (Ratsep 2013), they are unlikely to be adequate alone for the control of pain in more invasive surgical procedures (Roughan 2016). Opiate analgesia is an important part of any post-operative analgesic plan (Lemke 2000). While not without side effects and regulatory control, their efficacy warrants their inclusion in most rodent surgical models. Currently, the partial mu agonist buprenorphine is the most commonly used post-operative analgesic in mice. There are two commonly used formulations, a regular product, which is reported to provide relief for 4−8 hours, and a sustained release product, which although labeled for 72 hour dosing intervals, studies indicate that its duration of action may be variable depending on the nature of the painful stimulus (Guarnieri 2012; Healy 2014; Kendall 2014; Kendall 2016; Seymour 2016).

In summary, it cannot be overemphasized that pain management needs to be tailored to each specific experiment and the investigator needs to communicate with the veterinary staff in order for the team to understand how these agents may impact the experimental outcomes.

Analgesia in NHPs

There is limited information regarding validated pain scoring systems for NHPs and pain is most commonly based upon physiologic or behavioral indices. In the wild, NHPs and swine generally mask signs of pain or distress as a means of adaptation and survival. This makes the recognition of pain or distress especially mild to moderate pain in the laboratory setting more challenging to assess. Although there are multiple publications and reviews describing pain assessment and analgesic therapy in laboratory research animals, (Fish 2008;

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Flecknell 2016; Morton 1985) additional information is still warranted especially correlating analgesic plasma concentrations to clinical analgesia and specific pain indices. Multimodal therapy consisting of analgesics with different mechanisms of action that target different aspects of the pain pathway is generally recommended for post-surgical pain (Lamont 2008). Commonly used agents consist of local anesthetics prior to surgical closure with the addition of a systemic NSAID, opioid or both. Ideally, analgesics should be administered preemptively prior to surgery to reduce potential central sensitization and development of chronic pain (Wagner 2002). A thorough review of analgesic therapy is beyond the scope of this chapter and reviews of analgesic therapy for NHPs have been published (Murphy 2012). The primary NSAIDs used in NHPs are meloxicam and carprofen. Allison, et al. demonstrated that carprofen 2.2 mg/kg administered intramuscularly (IM) as a solo agent or in combination with buprenorphine 0.01 mg/kg IM to baboons before laparotomy surgery provided sufficient post-operative analgesia (Allison 2007). Bauer et al., explored the utility of three meloxicam formulations, oral, IM, and a sustained release based upon a biodegradable polymer matrix (Meloxicam SR®, 10 mg/ml ZooPharm, Fort Collins, CO) in maintaining therapeutic analgesic plasma concentrations. Mild to severe skin lesions were reported with the original formulation of meloxicam SR, yet were not noted in a modified formulation. Results from the study indicated that a single dose of the sustained release formulation provided therapeutic plasma levels for 72 hours compared to 12−24 hours with a single IM dose and very low concentrations associated with oral dosing (Bauer 2014).

The pharmacokinetics of a buprenorphine sustained release product based upon the same biodegradable polymer formulation as Meloxicam SR® have also been researched in macaques. Nunamaker et al. reported that the sustained release buprenorphine formulation-Buprenorphine SR® (ZooPharm, Fort Collins, CO) 0.2 mg/kg subcutaneously provided plasma serum concentrations greater than the human therapeutic threshold (0.1 ng/ml) for up to five days (Nunamaker 2013). Immediate release buprenorphine 0.03 mg/kg IM was best administered every 12 hours to ensure plasma levels exceeding the human theoretical threshold. Injection reactions were reported in 40% of macaques with Buprenorphine SR® and reactions ranged from mild erythema to a raised plaque (Nunamaker 2013). Kelly et al. demonstrated consistent buprenorphine plasma concentrations exceeding the human theoretical threshold (0.1 ng/ml) for over 12 hours when administered at a dose of 0.03 mg/kg IM to rhesus macaques (Kelly 2014). The results of these studies with sustained release analgesics are promising as there is a clinical need to develop sustained acting analgesic therapy in NHPs in order to minimize the stress of restraint and pain associated with multiple injections. However, in the absence of a sustained release formulation, if immediate release buprenorphine will be used for post-surgical analgesia, the authors recommend dosing at 0.03 mg/kg at a minimum frequency of every 12 hours preferably in combination with a NSAID for multimodal anesthesia. Sedative, anesthetic, and analgesic doses for non-human primates are outlined in Tables 4.3 and 4.4.

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Table 4.4. Non-Human Primate (Macaque & Baboon), Sedative - Premedication and Analgesic Agents Immobilization Combinations and Intravenous Anesthesia

Drug	Dose
OPIOIDS	
Buprenorphine	0.005 - 0.03 mg/kg SQ/IM/IV Q6-12h
Morphine	0.1 - 2 mg/kg SQ/IM q3-6h
Fentanyl	5 - 10 mcg/kg IV bolus followed by 10-25 mcg/kg/h continuous intravenous infusion
NONSTEROIDAL ANTI-INFLAMMATORIES	
Carprofen	2 - 4 mg/kg SQ/IM Daily
Meloxicam	0.2 mg/kg SQ Daily
LOCAL ANESTHETICS	
Lidocaine	1 - 4 mg/kg SQ infiltration
Bupivacaine	1 - 2 mg/kg SQ infiltration

Drug dosages adapted from the following: Murphy KL, Baxter MG, Flecknell PA. Anesthesia and Analgesia in Nonhuman Primates. In: Abee CR, Mansfield K, Tardif S, Morris T, eds. *Nonhuman Primates in Biomedical Research*: Academic Press, an imprint of Elsevier; 2012:403-435.

The *Guide* indicates that preemptive analgesia should be utilized to enhance surgical stability and to optimize postoperative care and pain management (National Research Council 2011). In these efforts, Carlson, et al., evaluated the pharmacokinetics and plasma concentrations of two fentanyl transdermal formulations, a transdermal patch (25 mcg/h) commonly used in human patients and companion animals, and a new transdermal fentanyl solution (Recuvyra®, Elanco, Greenfield IN) for use in cynomolgus macaques. Unlike with the fentanyl patch, macaques dosed with the fentanyl solution at 2.6 mg/kg demonstrated adverse effects such as bradycardia, severe respiratory depression, and hypothermia. However, a dose of 1.95 mg/kg was tolerated without adverse effects. Although the transdermal fentanyl solution provided higher plasma levels, significant variations in plasma concentrations and pharmacokinetic parameters were noted with the fentanyl solution groups (Carlson 2016). Advantages of both fentanyl formulations include chronic steady-state plasma levels for continuous analgesia. Major disadvantages for using transdermal patches in primates include variability and time for absorption (patches should be placed 12 hours in advance), requirement for jacket to protect patch from removal, and accidental overdose if ingested. Major disadvantages with the fentanyl solution include rapid absorption and potential overdose once applied, requiring the need for continuous reversal with an opioid antagonist until plasma concentrations are reduced below adverse effect levels. Additional studies are warranted before transdermal fentanyl solutions can be routinely used in NHPs.

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Analgesia in Swine

Postoperative pain assessments must be conducted and the analgesic regimen adjusted based on the outcome. Evaluation of pain in swine is challenging but has been reviewed (Ison 2016). The evaluator must be familiar with normal swine behavior in the housing environment and the animals should be acclimated to personnel and handling prior to the postoperative period. Evaluations should include assessment of spontaneous behaviors (e.g., ambulation, rooting behavior, posture), interactive behaviors (e.g., approaches personnel, seeks solitude when personnel enter enclosure), and a clinical assessment encompassing vital parameters, incisional palpation, and nutritional status. Example postoperative evaluations are available in published literature (Castel 2014; Ison 2016; Swindle 2015) but development of model-specific assessment systems is ideal. Scoring systems are commonly used as they provide an objective outcome for these subjective assessments and may be as simple as a visual analog scale or as complex as a variable rating scale (Ison 2016). Consultation with a veterinarian during development and implementation of postoperative pain assessments is strongly encouraged.

Administration of analgesics prior to a surgical procedure, or preemptively, is considered appropriate veterinary care and must be part of the perioperative preparations. Analgesic administration prior to the painful stimulus prevents the initial neuron signaling cascade (i.e., pain reflex) that establishes central sensitization and hyperalgesia post-surgery. Multimodal analgesic therapy, as with non-human primates, is comprised of pharmacologic agents with different mechanisms of action and is preferred for the reasons previously mentioned. This most typically involves an opioid derivative (e.g., fentanyl, oxymorphone, buprenorphine) combined with a NSAID (e.g., carprofen, meloxicam, ketoprofen) and/or a regional/local nerve block (e.g., lidocaine, bupivacaine), for which multi-holed wound soaker catheters, with or without an elastomeric pump, have become popular.

Continuous rate infusion systems should be considered peri- and post-operatively to maintain steady and efficacious analgesic plasma concentrations, tailored to the individual animal. As maintenance of exteriorized vascular catheters can be challenging in swine, sustained-release formulations of various analgesics available as FDA-approved human pharmaceuticals have been proposed for use in this species. Several transdermal patch delivery systems, including fentanyl and buprenorphine, have been evaluated in swine (Thiede 2014; Wilkinson 2001). Swine skin is similar to human skin (Swindle 2012) but swine have distinct differences in skin pH, body temperature, and subcutaneous adipose depots which may affect the pharmacokinetics in swine compared to humans. Overall, literature and experience indicate breed and inter-individual variability in drug absorption with transdermal delivery systems, resulting in the potential of under- or over-dosing the patient. Combined with a lack of information regarding the therapeutic analgesic threshold in swine for many analgesics, the utility of sustained release formulations in swine pain management may be limited. Sedative, anesthetic, and analgesic doses for swine are outlined in Tables 4.5 and 4.6.

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Table 4.5. Swine: Premedication and Analgesic Agents

Drug	Dose
PHENOTHIAZINES	
Acepromazine	0.2 - 1 mg/kg SQ/IM
ANTICHOLINERGICS	
Atropine	0.05 mg/kg IM
Glycopyrrolate	0.004 - 0.01 mg/kg SQ/IM
BENZODIAZIPINES	
Midazolam	0.1 - 0.5 mg/kg SQ/IM/IV
Diazepam	0.5 - 10 mg/kg SQ/IM
OPIOIDS ***Most opioids are relatively short acting in swine, require frequent dosing or continuous rate infusions***	
Buprenorphine	0.01 - 0.1 mg/kg SQ/IM Q6-12h
Oxymorphone	0.15 mg/kg SQ/IM QID
Meperidine	2 - 10 mg/kg SQ/IM QID
Fentanyl*	0.02 - 0.05 mg/kg SQ/IM q2h or IV bolus followed by 30 - 100mcg/kg/h continuous intravenous infusion
Fentanyl patch*	5 mcg/kg/h Topically q72h
Sufentanil	5 - 10 mcg/kg IM q2h or IV bolus followed by 10 - 30 mcg/kg/h continuous intravenous infusion
Remifentanil	30 - 60 mcg/kg/h
NONSTEROIDAL ANTI-INFLAMMATORIES	
Carprofen	2 mg/kg SQ/IM/PO BID
Meloxicam	0.4 mg/kg SQ/IM Daily
Ketoprofen	1 - 3 mg/kg SQ/IM/PO BID
LOCAL ANESTHETICS	
Lidocaine	1 - 4 mg/kg SQ infiltration
Bupivacaine	1 - 2 mg/kg SQ infiltration

Drug dosages adapted from the following: Swindle MM, Sistino J. Anesthesia, Analgesia, and Perioperative Care. In: Swindle MM, Smith AC, eds. *Swine in the Laboratory, Surgery, Anesthesia, Imaging, and Experimental Techniques*. 3rd ed. Boca Raton: CRC Press; 2016:39-87; Swindle, Michael M. "Anesthesia & Analgesia in Swine." *Sinclair Bio-resources*. Website. Accessed 17 Jan. 2017. <http://www.sinclairresearch.com/assets/sites/2/Anesthesia-Analgesia-Small.pdf>.

*Not recommended as single agents for post–operative analgesia since absorption and efficacy is highly variable.

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Table 4.6. Swine: Sedative - Immobilization Combinations and Intravenous Anesthesia

DRUG COMBINATION	DOSE
Ketamine: Xylazine	15 - 20 mg/kg: 2 mg/kg SQ/IM
Ketamine: Midazolam	33 mg/kg: 0.5 mg/kg SQ/IM
Ketamine: Dexmedetomidine	10 mg/kg: 10 mcg/kg SQ/IM
Tiletamine - Zolazepam (Telazol®)	2 - 8.8 mg/kg SQ/IM
Tiletamine - Zolazepam (Telazol®): Xylazine	4 - 6 mg/kg: 2.2 mg/kg SQ/IM
Alphaxalone: Dexmedetomidine[a]	5 mg/kg: 10 mcg/kg IM
Alphaxalone: Diazepam[b]	5 mg/kg: 0.5 mg/kg IM
INTRAVENOUS ANESTHESIA	
Propofol	4 - 20 mg/kg IV to effect 12 - 20 mg/kg/h continuous intravenous infusion
Propofol: Midazolam: Fentanyl	Propofol: 2 - 4.4 mg/kg/h Midazolam: 0.4 - 0.7 mg/kg/h Fentanyl: 0.003 - 0.005 mg/kg/h continuous intravenous infusion

Drug dosages adapted from the following: Swindle MM, Sistino J. Anesthesia, Analgesia, and Perioperative Care. In: Swindle MM, Smith AC, eds. *Swine in the Laboratory, Surgery, Anesthesia, Imaging, and Experimental Techniques*. 3rd ed. Boca Raton: CRC Press; 2016:39-87; Swindle, Michael M. "Anesthesia & Analgesia in Swine." *Sinclair Bio-resources*. Website. Accessed 17 Jan. 2017. <http://www.sinclairresearch.com/assets/sites/2/Anesthesia-Analgesia-Small.pdf>.

[a] Santos M, Bertran de Lis BT, Tendillo FJ. Effects of intramuscular dexmedetomidine in combination with ketamine or alfaxalone in swine. *Veterinary anaesthesia and analgesia.* Jan 2016;43(1):81-85.

[b] Santos Gonzalez M, Bertran de Lis BT, Tendillo Cortijo FJ. Effects of intramuscular alfaxalone alone or in combination with diazepam in swine. *Veterinary anaesthesia and analgesia.* Jul 2013;40(4):399-402.

EUTHANASIA

Rodents

The American Veterinary Medical Association periodically publishes a well-researched guide on acceptable methods of euthanasia for all animals (Cima 2013). Achieving the most humane method of euthanasia has been extensively researched. The most common method of rodent euthanasia is performed by carbon dioxide administration. Current research studying this method has focused on minimizing the time the animals experience pain and distress during the period of hypoxia, hypercarbia and the production of carbonic acid in the lungs and mucosa. Hypercarbia in the brain leads to unconsciousness (Conlee, Stephens, Rowan, & King, 2005). The rate of chamber displacement with carbon dioxide is an important area of study in the field of laboratory animal medicine. The effects of carbon dioxide and isoflurane in neonatal mice and rats are limited. Hence, they often require extremely long periods of time in the carbon dioxide chamber to attain death (Pritchett-Corning 2009). Mice and rats

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may also be euthanized by injections of pentobarbital or by cervical dislocation or decapitation, but general anesthesia is required for these techniques to be employed, unless a scientific justification is given for their usage.

NHPs and Swine

Methods of euthanasia for non-human primates and swine are outlined in the American Veterinary Medical Association Guidelines for the Euthanasia of Animals: 2013 edition (Leary 2013). Intravenous administration of a barbiturate euthanasia solution (e.g., concentrated sodium pentobarbital) is an acceptable method for euthanasia for both non-human primates and swine. Non-human primates and swine should be sedated and immobilized prior to euthanasia to minimize stress and facilitate placement of an intravenous catheter. Euthanasia may also be performed at the end of a non-recovery transplantation surgery while the animal is anesthetized for surgery.

POST-TRANSPLANT MEDICAL COMPLICATIONS IN ANIMALS

There are many side effects related to transplantation. Here we briefly mention some of them which are life-threatening, and encountered in mice, swine and non-human primates.

Graft-versus-Host Disease Post-Hematopoietic Cell Transplantation

Graft-versus-host disease (GVHD) occurs mainly due to activation of alloreactive T-cells in the donor hematopoietic cell (HC) inoculum. This is an aggressive immune driven reaction where all epithelial and lymphohematopoietic tissues of the host are attacked by the donor T cells that are present in the stem cell graft. Briefly, the pathophysiology of GVHD can be summarized as a three step process. In phase one, the conditioning regimen (irradiation, chemotherapy) causes the damage and activation of host tissues. In this phase, the gut is especially affected. Translocation of bacteria and bacterial toxins (lipopolysaccharide or LPS) through the gut mucosa stimulates the secretion of inflammatory cytokines. Donor T cell activation occurs in phase two. In phase three, the effector functions from mononuclear phagocytes induced by LPS and other stimulatory molecules that leak through the intestinal mucosa during phase one and two, induces the damage to target host tissues. Activated macrophages along with cytotoxic T lymphocytes secrete inflammatory cytokines which cause further target damage through cell apoptosis. Most therapeutic approaches to reduce GVHD rely on immunosuppression and/or *ex vivo* removal of donor T-cells from the donor bone marrow graft. Immunosuppression is required to avoid this condition. Unfortunately, sometimes animals (and humans) are refractory to high doses of steroids. This is an area of intense research. Please refer to Chapter 6, Volume 2 for more details.

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Infections

Bacterial/Protozoal

Animals are most at risk of infection during their neutropenic period (especially when neutrophils are under 500 cells/uL). Continued monitoring of their white blood cell counts is often required, particularly in protocols that are severely myeloablative. Often, antibiotic therapy needs to be used aggressively. Close collaboration with the veterinarian in order to choose the right antibiotic, dose and route for compliance (in each species) helps minimize side effects and antibiotic resistance. Often, animals may develop veterinary-specific diseases with clinical symptoms that overlap expected transplantation side effects (Crepeau 2012). Swine and monkeys may develop respiratory fungal infections. Placement of animals in HEPA-filtered cages or Bio-Bubbles is encouraged to minimize unwanted air-borne illnesses. In severely immunosuppressed animals, fungal pneumonias can translocate to the blood leading to life-threatening fungemias. Mice are usually in barrier rooms and housed in micro-isolator cages where their environment is controlled. This setup minimizes fungal/protozoal infections. Similar arrangements for larger animals are not as standard and can be costly for researchers. Despite being able to control the environment, mice (and large animals) can still become sick after the peri-transplant regimen by pathogens that enter the bloodstream via other routes (skin, gut). Close monitoring of experimental and control animals may lead the investigator to rule in/out potential causes (Duran-Struuck 2009).

Viral Diseases

Viral diseases often appear when patients have deficiencies in their T cell responses. Hence, these can occur in patients that have normal neutrophil counts, but which are receiving (or have received) immunosuppressive regimens that impede the function or deplete T cells. We discuss briefly two of the most common viral diseases which are observed in immunosuppressed animals.

i. **Epstein Bar Virus (NHPs)/Porcine lymphotropic herpesvirus (in swine**). EBV and PLHV are common herpetic diseases that can reactivate post-transplant. These lead to a condition known as post-transplant lymphoproliferative disease (PTLD). In PTLD the B cell population aggressively expands as a result of a primary herpesviral infection (or reactivation) of B cells that are no longer kept "in check" by CD8 cytotoxic T cells. Therefore, immunocompromised transplant patients with depressed T cells (which include CD8 T cells) are at the highest risk. The variability of the patient population, both clinically and pathologically, complicates the ability to study this disease (Duran-Struuck 2015).

   Swine and monkeys undergoing HCT or solid organ transplantation develop PTLD with a presentation resembling human PTLD. Supportive markers suggestive of PTLD (in addition to an acute increase of lymphocytes) include elevations of LDH, a serum marker shown to be increased in humans and pigs (Duran-Struuck 2015; Matar 2015) with PTLD (it has yet not been documented in NHPs). Increases in LDH in general precede (24−48 hours) the increase of the B cell population (Matar 2015). Like in humans, leukocytosis and generalized lymphadenopathy is commonly observed in NHPs and swine.

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Treatment is centered on the ability to regain T cell immunity. There are three general approaches. First, decreasing the level of immunosuppression is a first line of defense by harnessing the cytotoxic cells of the host. This approach however risks the rejection of the graft rejection or GVHD. Second, infusion of rituximab (an anti-CD20 monoclonal antibody which targets CD20 on the surface of B cells) is commonly utilized in humans and has shown to be very successful (Tobinai 1998). Rituximab works in NHPs but it does not work in pigs as the anti-CD20 antibody does not cross-react with swine B cells. A third, and more novel approach consists in exploiting cellular immunotherapies. The least refined, but previously widely used approach included doing donor leukocyte infusions (DLIs) from EBV+ donors (usually from the same graft donor). The approach comes with significant risk, mainly GVHD. A cleaner cellular approach is the expansion of anti-EBV (or anti-PLHV in swine) cytotoxic T cells *in vitro* prior to infusion. Interestingly, limited GVHD has been observed with this approach (Bollard 2004) and are currently favored over bulk DLIs. More recently, the development of genetically engineered T cells by modifying their T cell receptors or creating a chimeric antigen receptor (CAR) have shown to be promising (Till 2012).

ii. **Cytomegalovirus:** Cytomegaloviruses (CMV) are betaherpesviruses that are commonly found in all species including human, NHPs and swine. The incidence is high but CMV rarely causes overt disease in immunocompetent individuals (Nakamae 2009). CMV is transmitted horizontally and is clinically associated with immunosuppression. Immunodeficient monkeys or swine that are infected with CMV develop necrotizing encephalitis, enteritis, lymphadenitis and/or interstitial pneumonia. CMV, therefore, poses great threat to animal models (and patients) undergoing immunosuppressive therapies**.** Animals at risk of developing CMV need to have their serum assessed for viremias and treated with antivirals. It is the authors experience that gancyclovir at 12.5 mg/kg IV BID works best in cynomolgus macaques. These doses are higher than what is given to humans (5 mg/kg) but human doses do not clear cynomolgus CMV. Antivirals (in general) have significant side-effects. Notably, bone marrow (BM) suppression is one of the most serious ones. Foscarnet or Cidofivir can also be used as adjunctive therapy, but are BM and nephrotoxic respectively. Failure to treat CMV in T cell-deficient animals can lead to death (Komanduri 1998). In essence, balancing the benefits and drawbacks of antivirals makes treatment of CMV difficult. Similar to EBV, discontinuation of immunosuppression and infusion of anti-CMV T cells is becoming more common (Zhou 2009). However, as previously mentioned, careful assessment of the side-effects encountered with T cell therapies must be thoughfully considered.

ETHICS OF TRANSPLANTATION RESEARCH FROM THE VETERINARY PERSPECTIVE

Animal research has had a critical role in scientific and medical advances during the past century. However, the use of animals has been a subject of heated debate. As with most ethical issues, there is a wide spectrum of views on the use of animals in science and

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medicine, and stewardship toward animals in general, ranging from absolutists concerned with the natural rights of animals to those who view animals only as an exploitable resource. These viewpoints have contributed to the development of ethical principles for animal use, which in turn have shaped regulations by the United States Department of Agriculture and the Public Health Service, and supported by organizations such as the American Association of Laboratory Animal Science (AALAS) AAALAC, and the AVMA.

Evolutionary theory purports that a hierarchy of moral standing exists among animals. In other words, the most complex animals, mammals, are at the top, progressing backward through the tree of life past the less complex vertebrates - birds, then reptiles, amphibians and fish - followed by the invertebrates, which lack any sort of regulatory care due to their relatively primitive nervous systems. A wealth of scientific research clearly illustrates that animals with advanced nervous systems, such as NHPs and marine mammals, have demonstrated abilities that humans can relate to and value, such as advanced social behavior, the ability to react to both positive and negative stimuli, intelligence and even self-awareness. What once seemed to be a clear cognitive distinction between humans and other animals has become less apparent; with that, the fading historical assumption of a clear moral distinction between humans and animals suggests that gradations in moral value is wise to apply to animals. Thus, scientists and medical professionals who work with animals have adopted several practical ethical principles to justify their work, and the most predominant among these is utility.

Modern principled thought has shifted from whether animals have moral status to how much moral status they have and what rights come with that status. It is important to note that there have been hundreds of years of philosophical argument surrounding the consequentialist theory that correlates with the principle of utility, namely utilitarianism. This theory was initially developed by the philosophers, Jeremy Benthem and John Stuart Mills, during the 19th and 20th centuries. The arguments, and frequently misunderstandings, that surround the theory of utilitarianism focus on the idea that the benefits of the research to people outweigh the costs to the animals; said in another far less popular way, “the ends justify the means.” However, despite the fact that this phrase suggests a clear lack of compassion is contradictory; in reality the sentiment is quite the opposite. When working alongside the scientists and medical professionals doing transplant work, for what they believe to be a “greater good,” it is clear that they feel sincere compassion for the animals in their care.

The utilitarian argument claims that moral status is derived from an animal’s capacity to suffer, along with the experience of enjoying its life. In respect to these facets of life experience, many animals are no different than humans. They can certainly feel pain, as well as experience pleasure. Therefore, per one utilitarian position, they should have the same moral status as humans and deserve equal treatment. However, as previously mentioned, a different utilitarian position held by many advocates of animal research claims that animals cannot be considered morally equal to humans. This claim states that the benefits to humans from animal experimentation outweigh the harm done to animals.

Since most animals do not have the cognitive capabilities of humans and do not possess complete autonomy, such as the rational choice to pursue specific life goals, their moral life experience does not compare with that of human beings. This exclusion of animals from the same level of human moral experience supports the claim that the potential human benefits of the research outweigh the harm done to animals. But for many people this begs the question, what about the fact that infants and mentally handicapped individuals frequently lack

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complex cognitive capacities, full autonomy, or even both traits? The reply by animal research advocates is that the infants will, in time, meet the complete criteria of an adult human; moreover, the utility of animals in research may lead to treatments for individuals with these various cognitive deficits. An important caveat to this premise, of course, is that these innovative methods to correct aberrant genetic mutations in humans, such as genetic engineering with zinc-finger nucleases and CRISPr-Cas9 remain a separate and rather challenging relatively current ethical issue.

Thus, proponents of a more centrist position usually advocate a few basic principles that must always be followed when utilizing animals in scientific research and medicine. These are commonly known as the three *Rs: replacement, reduction, and refinement*. The first principle, *replacement*, calls for the preferential use of less complex organisms whenever possible. For example, bacteria and yeast, invertebrates such as nematode worms and fruit flies, and representatives of each of the other four groups of vertebrates, are all commonly used model organisms, and all are preferred over mammals for discovery science as well as medicine, *to a point*. Scientists and medical professionals pursue alternatives to using live animals, such as replacement with non-animal models like physical or digital models during a practical introduction to Anatomy and Physiology. A related example discussed in science education involves the question of reducing the use of surplus animals in high school and undergraduate school biology labs. In educational settings such as these, living animals (or at one time free-living animals) may not have worth relative to alternatives in a costs/benefits argument. Additionally, mechanical or computer models, audiovisual student aids, and *in vitro* modeling have proven useful for certain learning outcomes allowing for reduced use of living organisms. In fact, each Institutional Animal Care and Use Committee (IACUC) protocol requires referenced information about the alternatives that have been considered, why they were rejected, and how the Principal Investigator searched for them.

That said, disadvantages to replacement can be significant. In a system as complex as a live organism, many variables in physiology and pathophysiology remain unknown. This is critical in the argument for the use of animals in research. It must be recognized that treatments, be they surgical, drug-based, or another and aimed to being used in humans, will be significantly safer and most likely superior when first tested in animal models; indeed, there are no closer models to human beings than mammals, and large-animal mammalian models in particular.

The second principle, *reduction*, requires minimizing the number of animals needed to perform an experiment or teach a concept. This alternative is also required in IACUC protocols, where justification of the species to be used and the numbers needed for each experimental group are required. By examining these parameters, the IACUC can determine if thoughtful experimental design was employed to minimize overall animal use.

The third principle, *refinement*, requires constantly improving upon experimental protocols to minimize pain or distress of animals being used for scientific and medical purposes whenever possible. This expectation is addressed in numerous questions throughout the IACUC protocol as well. Examples of refinement include setting the earliest possible endpoint for experiments using animals, ensuring that all those who work with animals, both directly and indirectly, receive excellent training before performing any procedure (as previously discussed), and penalizing any person or institution failing to follow federally mandated guidelines, such as improper drug dosing and numerous other potential problems.

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The reality is that the fields of science and medicine have felt entitled to perform invasive procedures on animals, including all forms of vertebrates, for the sake of improving the lives of other people; and animals certainly have suffered in various ways inside the facilities required to care for them as humanely as possible. For example, animals feel fear and anxiety, they react to disturbances in their natural routines, are separated from family members and prevented from play opportunities, sometimes are placed in abnormal housing with individuals of the same species, they can be affected by unnatural diets and food acquisition, reduced movement, and so forth. Therefore, it is crucial for bothdiscovery science and applied science investigators, as well as animal care staff, to do everything possible to minimize the discomfort and maximize the enrichment of each individual animal that lives its life for the benefit of humanity.

CONCLUSION

In essence, animals will continue to be key players in transplantation research. We hope that the reader has now a better understanding of the husbandry, medical, regulatory and ethical considerations that need to be taken into consideration when working with research animals. We have established the basis for successful veterinary, anesthetic, analgesic and ethical arguments that support pre-clinical studies in pursuit of advances in transplantation biology and medicine.

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Chapter 5

PRE-OPERATIVE AND POSTOPERATIVE CARE IN EXPERIMENTAL SURGERY

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ABSTRACT

Experimental animal surgery is currently practised in research, drug/device/procedure testing, teaching, and production in the fields of medical science, biology and veterinary medicine. The main purpose for experimenting on animals is to replace human body by animal models in the studies of pharmaceutical and biological research and industry. An experimental animal is a biological system, and most of the experimental surgeries on animals are complicated processes, especially survival surgeries. In addition to improve surgical techniques, preoperative/postoperative care is essential to guarantee surgery success and recovery after surgery so as to avoid unfavorable responses and anomalous experimental results. With the objective of contributing to research in experimental surgery, this chapter presents the principal regulations of pre- and postoperative care in experimental surgery which are adhered for animal welfare. In order to achieve reliable, reproducible and precise results, in compliance with animal welfare regulations, pre- and postoperative cares including quarantine, restraint, acclimatization, health inspection, necessary fasting, recovery from anesthesia, fluid/electrolyte balance, anti-infection, and pain relief, have to be adopted in practicing successful and humane experimental surgery.

Keywords: pre-postoperative care, quarantine, analgesia, infection

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INTRODUCTION

Experimental animal surgery should focus on all procedure of the perioperative care of the animal, including pre- and postoperative management, which cannot be arbitrarily divided. Proper management of animal greatly increases the success rate of surgery. It improves animal well-being during the recovery period, alleviates the sufferings of the animal and enhances validity of the study. The term pre-postoperative care refers to the activities before and after a surgical procedure, which ensure to provide the best possible care for an animal selected for experimental surgery. It is the responsibility of the research or teaching institution to ensure that all personnel are qualified and trained to conduct surgical procedures. In this chapter a brief description of the fundamental concepts and management of pre- and postoperative care of experimental animal surgery is given.

PRE-OPERATIVE MANAGEMENT

Animal Identification

Properly identify the animals prior to surgery. Obtain all information needed to specifically identify individual animal from animal suppliers, including sex, age, weight, colony history, and health records. In some instances, medical and vaccination histories can also be helpful in evaluating their health status.

Acclimatization

Always acclimatize the animals to the facility for a period of adaptation prior to surgery (Obernier 2006). The effects of stress on the animal have the potential for influencing experimental outcomes and jeopardizing the animal welfare. An animal stress-reduction plan should be available. Proper restraint and handling will help to prevent injury and minimize stress to the animal and ensure the validity of experimental results. It is important to provide a period of adaptation prior to undergoing surgical procedures in which the animals can adjust their physiologic and metabolic systems to stabilize to a new environment and adapt to an alternate form of restraint or frequent handling. This acclimatization period will increase the reliability of the results obtained from the experimental protocol which are not to be compromised by uncontrolled environmental influences. The length of time for acclimation will depend on the type and duration of animal transportation, the species, and the intended use of the animals. It has been reported in the literature the proper time for acclimation for mice, rats, guinea pigs, nonhuman primates, and goats, and for other species as well (Conour 2006; Landi 1982; Prasad 1978; Tuli 1995; Sanhouri 1989; Kagira 2007).

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Animal Health Status Evaluation

The experimental animals are living animals with characteristics and health issues that may influence or be of concern while conducting a study. An appropriate protocol for the experiments using animals is primarily based on exhaustive evaluation of clinical conditions, including influences on the research and the welfare of the animals (Burkholder 2012).

It is essential to obtain sufficient, detailed and reliable health information from animal suppliers to establish the length of quarantine, define the potential risks to personnel and animals, and determine whether therapy is required before animals are released from quarantine (Butler 1995). The animal health status should be evaluated before undergoing a surgical procedure, usually during a quarantine period when the animals are kept separate from conditioned animals of known health status. Information from suppliers about animal quality should be sufficient. Only animals exhibiting good health and normal behavior should be included in a study. Pre-existing health conditions may negatively affect the success of the surgical procedures. Experimental animal surgery usually can be scheduled after performing the physical examinations appropriate to the species and necessary laboratory examinations to determine the presence of abnormalities or conditions which could affect the outcome of the surgical procedure and impact the study results (Lerche 2008; Roberts 2008).

Physical examination: It is important to develop a standardized physical exam process according to animal species. In general, it should include the following.

A. General information
B. Visual inspection and behavioral status assessment: The animals should be alert and behaving normally and should have clear eyes and a smooth coat without trauma.
C. Physical exam from head to tail. If necessary, include vital signs monitoring, depending on the feasibility and experimental design.

Laboratory examination: The necessity of laboratory examination is determined by the research protocol, animal species, animal health history and the duration of the studies. Laboratory evaluation of blood, urine, and feces are frequently-used laboratory examinations in assessing animal health status. The medical image-aided diagnosis, such as radiography, ultrasonic imaging, computed tomographic imaging, nuclear magnetic resonance, and endoscopy technique may be needed especially by the research protocols. In many animal species, viral infections are common without clinical signs of disease, but may cause clinical diseases when the animals are subjected to surgery. The infections also interfere in getting true results when conducting immune-response experiments, as the pre-existing viral infections modify the animal immune-system. Serologic testing is convenient and useful for detecting viral infections without clinical signs of disease (Ward 2006).

Proper Handling and Restraint

Use proper handling and restraint methods to avoid animal and personnel injuries. Acclimating animals to restrain and frequent handling prior to surgical procedures reduces stress in animals and also minimizes stress and fear for personnel during handling (Flecknell 1991). Before handling and restraint, information about the various animal behaviours is of

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considerable importance. All the procedures should be careful, accurate, rapid but without being rough. To initially restrain a rat, for example, the handler should gently grasp it around the shoulders. The handler's thumb can then be placed under the rat's mandible, to prevent bites, and the rat's hind limbs can be supported with the other hand. Restraint should be firm but not too tight as this will impede the animal's respiration.

Antimicrobial Control

Indiscriminate use of antibiotics should be avoided. Antibiotics may interfere with an experimental protocol, either by a direct interaction or by influencing pharmacokinetics/ metabolism of the compounds under investigation. The indications for antibiotics in a particular animal surgery should be critically reviewed. Antibiotics might be indicated prophylactically for certain surgical protocols or when known breaks in sterility occur, but should not be used as a substitute for proper aseptic techniques.

POSTOPERATIVE CARE

Postoperative care begins as the animal is recovering from anesthesia. Common general postoperative complications include postoperative fever, pain, atelectasis, wound infection, deep vein thrombosis and subsequent embolism, due to anaesthesia and surgical wound. They may either be general or specific to the type of surgery, and can be dangerous to animal life such as deep vein thrombosis. For survival animal surgery, an effective postoperative plan and its implementation can minimise surgical complications by improving its physiologic status and enhance the animal's recovery. It should be tailored to the procedure and individualized for the well-being of each animal. The research staff and the animal care staff both contribute to the well-being of the animal. Specific surgical procedures requiring intensive postoperative care should be scheduled by considering the availability of experienced personnel to be on duty. Careful observation is necessary for postoperative animals with detailed daily record, for keeping all personnel involved in understanding the animal recovery process (Field 2007). For most animals, comfort care is beneficial but in some small rodents, excessive care will disturb and stress the animals.

Recovery from Anesthesia

The animal should be kept under observation during recovery from anesthesia until that animal is able to hold itself in a sternal position (able to hold its head up) without risk of injury. Since anesthetized rodents and rabbits can rest on their chest steadily, they must be surveilled until regaining consciousness upon seeing their ambulation. The duration of the recovery depends on the anesthetic protocol and on administration time of the last dose. In general, when using injectable agents, the animals need longer recovery period than the animals using inhalational anaesthesia. The animal must be able to maintain normal physiology. Heart rate, respiration, and hydration should be stable and within normal limits for the species.

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To avoid being hurt from cage mates, animals should recover from anesthesia in individual cage. Provide a clean, warm, quiet, and comfortable space/cage without sharp corners or projections. Water bowl should be removed from the cage to prevent accidental drowning. To prevent vomiting, gastric contents reflux and aspiration pneumonia, we should preserve the airway by keeping the animal intubated until appearance of swallowing reflex or cough. Preoperative fasting can reduce postoperative vomiting. Rodents intended for surgery may be fasted or fed as indicated by the protocol; as postoperative vomiting does not occur in rodents, fasting is not required. But short fasting can enhance the effect of certain anesthetic agents (Toth 2000). The body position adopted postoperatively is designed with gravity assistance to prevent suffocation through obstruction of the airway, both mechanical obstruction caused mostly by the tongue to fall to the back of the pharynx, and fluid obstruction, usually by vomit. Most animal species are placed in sternal recumbency. In ruminants it prevents the aspiration of rumen contents but also promotes release of rumen gases. Lying on either side postoperative is adopted for rabbits, dogs and cats. For animals taking longer to recover, turn the animal from side to side periodically to prevent development of hypostatic pneumonia.

When vomiting occurs, lower animal’s head will be than the body to prevent aspiration of gastric fluid. Severe retching or coughing during recovery may indicate aspiration of blood, fluids or vomitus. If an aspiration event occurs, the animal’s upper airway should be cleared by vacuum extractor or syringe. Other management of an aspiration includes observation, oxygen, antibiotics and corticosteroids therapy.

Postoperative respiratory depression is often associated with anesthesia, and it can be limited by choice of appropriate anesthesia protocol. For timely detection of respiratory depression, one should carefully observe the animal's respiratory rate and breathing pattern. If respiration is shallow or infrequent, a respiratory stimulant such as doxapram (which is a central respiratory stimulant with a brief duration of action) can be administered (Martindale 1996). Continuous postoperative oxygenation is beneficial for most animals. The small animals waking up in the incubator can get continued oxygen supply directly through chamber. For large animals, place the tail end of an oxygen catheter near the nares.

Hypothermia

Perioperative hypothermia is one of the major unpredictable *causes* of *perioperative* death. It is common in the early postoperative period which is associated with physiological derangements, due to direct thermoregulation inhibition by anesthetics. Exposed body cavities during surgical procedures might cause further heat loss. Small animals are particularly more susceptible because of the higher surface area-to-volume ratio, which facilitates heat loss through the skin (Brown 1969).

It is advocated that postoperative animals should be placed in a quiet recovery room with soft lighting where the room temperature should be about 24 °C, and certain facilities like resuscitation equipment must be ready to be used by competent personnel. In most cases, small animals can be placed in the ordinary cage free of airflow (Archibald 1968). Surgery table with metal surface can be insulated with mats. Mechanical sources of heat, such as hair dryers, heating pads and infra-red lamps, are dangerous. Supplementary heating sources should be used carefully to avoid serious burns. Circulating warm-water blankets are ideal for

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unconscious animals. Using a towel or blanket as the cage padding is preferable rather than using the bedding such as wood chips or shavings, which may stick to the animal's mouth, eyes, nose or wound (Heine 1998; Ruys 1991).

Infection

Postoperative infection is a common phenomenon in experimental surgery. It is inevitable to use antibiotics, but use of antibiotics sometimes will seriously interfere with the experiment.

Strict aseptic operation and good tissue handling techniques during surgery are essential to prevent postoperative wound infection. Antibiotics should not be used in place of surgical asepsis. If antibiotics are being used, for example when the operation involves a contaminated viscus, they should be administered before surgery to ensure that they reach to effective concentration locally. However, antibiotics should also be used postoperatively when possible contaminations have occurred during surgical operation procedure, to prevent further spreading of infection. Careful attention to body temperature increasing within 72 hours after surgery might indicate bacterial infection or antibiotic resistance.

An appropriate antibiotic should be selected carefully, based on potential organism and probable sensitivity, and also should be administered at an adequate dose at the recommended frequency to minimize the development of resistance. Except for undesirable interference by antibiotics with an experiment, some other areas are also considered: dosage (also extrapolation of dose information from other species), antibiotic toxicity, routes of administration, and use of combinations of antibiotics, etc.

Fluid/Electrolyte Balance

Dehydration is a feature commonly observed after operation. Body fluids must be maintained within physiologic limits postoperatively. In most cases, weight loss represents a lack of fluid; by monitoring preoperative and postoperative weight changes, one can better guide the amount of fluid. Other signs like color of mucous membranes, heart rate, and blood pressure may also be the indications of dehydration. Liquid supplementation after surgery is important to prevent postoperative dehydration. If the animal is completely awake, give the liquid by oral administration. In the situation of fasted animals required by experiment protocol, or when the animals may not be able to eat or drink, fluids must be administrated by subcutaneous, intraperitoneal or intravenous injection of glucose saline. A regular evaluation of body fluid volume, electrolytes, and pH is needed before parenteral administration.

Pain Relief

It should be assumed that a surgical procedure can cause pain not only in people but also in the animal. As a subjective emotion, pain can be experienced even in the absence of physical tissue damage, and the level of feeling can be modified by other emotions including fear, memory and stress (Nolan 2016). Different levels of postoperative pain and distress

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should be expected after surgery but it can be difficult to detect because of individual and species variation. As many animals may tolerate pain without demonstrating obvious signs, the animal must be closely observed to recognize the signs of pain and evaluate the severity of pain. Each species shows pain in different ways and criteria for assessing pain in various species also differ (Morton 1985). Reducing mobility or restlessness, abnormal posture, screaming, lack of eating food and drink, and signs of depression are the common signals of the animals feeling pain (Roughan 2009; Bateson 1991; Carstens 2000; Hawkins 2002; Holton 1998; Hughes 1983; Hughes 2008; Martini 2000; Roughan 2000; 2003; 2004; Sneddon 2006). If there is doubt about the presence of pain, an analgesic should be administered. Pain can be assumed to have been present if administration of analgesics causes amelioration of these signs and thus analgesia should be continued.

Analgesic drugs can be classified into two categories: non-steroidal anti-inflammatory drugs which alleviate pain by reducing local inflammatory responses, such as aspirin and acetaminophen (paracetamol); the opioid analgesics (narcotic agonists) such as morphine, pethidine, and buprenorphine, which act on the brain for controlling neurologic pain with high potency and long duration of action. The dosage and the period of the analgesic administration depend on how invasive the surgery is and how severe is the pain (Mathews 2008). For those surgeries such as laparotomy, thoracotomy, craniotomy, the opioid analgesics should be used for 24–72h postoperative. For the use of analgesics, one should consider the possible side effects, mainly respiratory and/ or cardiovascular depression and other interactions. Analgesics use should be balanced by potential beneficial effects and possible detrimental effects.

In addition to clinical side effects, the use of analgesics has the risk of interference with experimental results and might confound data obtained from the study.

CONCLUSION

Perioperative care is the care that is given before, during and after surgery. It is a systemic procedure which requires integrated planning. This period is used to prepare the animal both physically and psychologically for the surgical procedure and after surgery. In experimental animal science, cares of animals before and after operation are very important, which will directly affect the results of the experiment. It has been stated that caring for an experimental animal is like caring of a sick child (Mathews 1968). The process begins with animal health status evaluation. Using animals with pre-existing health conditions may not affect the operation itself, but pre-existing diseases might affect the whole experimental results. Surgeons should not only consider that they are conducting operations but they also have to ensure that the animals are well housed, well fed, and free of unnecessary pain. Recent advances in anesthesia, monitoring, pain assessment and analgesia provide many opportunities for improving perioperative quality, and promoting high standards of animal welfare.

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Chapter 6

FLUID, ELECTROLYTES, AND ACID-BASE BALANCE IN EXPERIMENTAL SURGERY

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ABSTRACT

During surgery, disturbances of body water distribution, electrolyte homeostasis, and acid-base physiology may be secondary to trauma, preexisting medical conditions which alter normal physiology, or the nature of the surgery. Understanding normal physiology and pathophysiology related to these abnormalities is imperatively important in experimental surgery as these parameters are of critical significance for surgical experiment animals.

A normal animal's body weight is composed of about 60% water, which is distributed throughout the intracellular and extracellular compartments. These two major compartments are separated by specialized membranes that are semipermeable to allow water to equilibrate across the membrane according to the osmotic-pressure gradient. Changes in both fluid volume and electrolyte composition, acid-base balance occur preoperatively, intraoperatively, and post operatively, as well as in response to trauma and sepsis. The evaluation of these changes commonly requires a focused history and physical examination, followed by interpretation of biochemical studies of experimental animals.

Fluids are administered to experimental animals to replace fluid losses (e.g., vomiting, blood loss, water loss from the respiratory system) to correct electrolyte abnormalities, promote kidney diuresis, and maintain the tissue or organ perfusion rate before and during surgery or while undergoing anesthesia. Factors considered for fluid therapy are types of surgery and severity of conditions, degree of dehydration, conditions of experiment animals, organic functions of experiment animals and type of electrolyte imbalance.

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Keywords: acid-base imbalance, electrolytes disorders, fluid therapy, experiment animals

ABBREVIATIONS

HCO_3^-:	Ion Bicarbonate
ADH:	Antidiuretic hormone
ATP:	Adenosine triphosphate
BUN:	Blood urea nitrogen
D5W:	5% Dextrose in water
ECF:	Extra cellular fluid
EN:	Enteral nutrition
GFR:	Glomerular filter rate
GIT:	Gastro intestinal tract
iCa:	Ionized calcium
Pi:	Inorganic phosphate
ICF:	Intra cellular fluid
LRS:	Lactated ringer's solution
mEq/L:	Milliequivalents per litre
PN:	Parenteral nutrition
PPN:	Partial parenteral nutrition
PTH:	Parathyroid hormone
RTA:	Renal tubular acidosis
TBF:	Total body fluid
TBW:	Total body water
UA^-:	Unmeasured anions

INTRODUCTION

The organic system and organs take part in harmonizing and regulating fluid, electrolytes and acid-base balance. During surgical procedures, those harmonization and regulation usually go on some changes which can be prevented or corrected so either their effects be minimized during surgical procedures.

In most animals, about 60% to 70% of body weight is water. This is much higher in neonates and can have as much as 80% of their body weight. Aged and obese animals tend to have lower percentage of water (Naylor 1990; Rose 2001). The total body fluid (TBF) is distributed in two major compartments: 55–75% is intracellular fluid (ICF) and 25–45% is extracellular fluid (ECF). The extracellular compartment is further divided into intravascular fluids (blood plasma), extravascular fluids (interstitial fluid, lymph), inaccessible bone fluid (water trapped in deep layers of bone not readily exchangeable) and transcellular fluids (secretions of glandular tissues, gastro-intestinal tract (GIT) fluids, respiratory fluids, aqueous humour, peritoneal fluid, cerebrospinal fluid, etc.) (Constable 2003; Rose 2001; Halperin 1999; Naylor 1990). It has estimated that total body water of a healthy dog, is approximately 534–660 ml/kg; total intravascular fluid volume has been estimated as 80–90 ml/kg in dogs and cats.

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Osmolality. The intravascular space contains only 8% of total body water (TBW). Water is held in specific fluid compartments by ionic, osmotic, and oncotic particles that are distributed uniquely among these spaces. Serum osmolality is determined by the number of these particles, osmotically active in the solution. The primary extracellular cations, sodium and smaller amounts of other cations (potassium, calcium, magnesium, and other organic cations) with electro neutrally balanced anions (e.g., chloride, bicarbonate, phosphate, sulfate, and other organic anions) plus glucose and urea; generate osmotic forces that maintain fluid in the extracellular space. These extracellular cations, anions, and other osmotic substances result in an extracellular osmolality of 280 to 300 mOsm/L. In most animals, it is maintained between 270–310 mOsm/L (Oh 1995; Rose 2001). This osmolality can be measured or calculated by following formula:

$$Osmolality\ (mOsm/liter) = 2((Na^{+}) + (K^{+})) + (Glucose)/18 + (BUN)/2.8^{-1}$$

(The glucose and blood urea nitrogen (BUN) are measured in mg/dl rather than in mmol/L, so it must be converted respectively by 18 and 2.8, for the units to be consistent).

The primary intracellular cation, potassium, and smaller amounts of others cations (e.g., sodium, magnesium, calcium) balanced electro neutrally with intracellular anions (i.e., organic phosphates and smaller amounts of ionic proteins, chloride, bicarbonate) create tissue-specific intracellular osmotic forces. Fluids movements between ECF and ICF occur freely across most of cell membranes, keeping the total concentration of the solutes or osmoles in equilibrium (Halperin 1999; Rose 2001).

Dehydration, hypovolemia and hypervolemia. In surgical experiment animals, hypotonic fluid loss is characterized by a loss of fluid in excess of solute and can result in a relative increase in serum osmolality. Isotonic fluid loss refers to a loss of fluid that has the same osmolality as plasma, and hypertonic fluid loss occur when solutes are lost from body. Dehydration in experiment animals refers to a decrease in TBW. It depends usually on the type of fluid lost (Rose 2001; Dibartola 2006a). Hypovolemia refers to inadequate circulating volume, usually result in hypovolemic shock from excessive hemorrhage such as that observed with a bleeding splenic mass, arterial laceration, etc. it can also occur due to a severe fluid loss, end-stage renal failure in most surgical experiment animals (Halperin 1999; Rose 2001; Dibartola 2006a). Hypervolemia in surgical experiment animal can be due to heart failure, renal failure, and/or iatrogenic fluid overload. Hypertension is not an indicator of hypervolemia (Halperin 1999; Rose 2001; Dibartola 2006a).

Electrolytes homeostasis. Electrolytes are essential cofactors in enzyme mediated biochemical reactions. Differentials electrolyte composition on both inner and outer side of cellular membrane establishes the electrical potentials which make nerve and muscular contraction, and metabolic reactions possible. ECF contains mainly Na+, Cl-, HCO3- & HPO4’2-. ICF contains mainly K+, HCO3-, PO4’3-, SO4’2- and citrate. Kidneys and circulating blood plays a prime importance role in maintaining electrolyte balance. In general, sodium is highly conserved; only very little is excreted in the urine. Substantial quantities of potassium and calcium are excreted on a daily basis, particularly due to the fact that dietary intake of potassium and calcium tends to be high (Halperin 1999; Rose 2001).

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Water balance. The kidneys have also a primary role in water balance through their concentrating and diluting functions. The osmolality of the glomerular filtrate as it gets in the proximal tubule is identical to that of plasma. In this segment of the tubule, the bulk of the urinary solutes (e.g., Na^+ and other electrolytes, amino acids, glucose) and water is reabsorbed isosmotically. As the filtrate traverses the descending limb of the loop of Henle, water diffuses from the tubule lumen to the hypertonic medullary interstitium. The tubular fluid then becomes progressively more dilute as it transits through the ascending limb of the loop of Henle. This diluting process occurs because active Na^+ reabsorption is unaccompanied by water in this segment. The fluid is hypoosmotic to plasma on its arrival in the distal tubule. Antidiuretic hormone (ADH) stimulates reabsorption of sodium chloride in the ascending limb and thus facilitates generation of the renal medullary solute gradient necessary for urinary concentration (Blumenfeld 2002). Hormonal action on the collecting ducts determines the final concentration of urine produced. In a state of antidiuresis, ADH increases water permeability and augments aldosterone-stimulated Na^+ reabsorption, thereby promoting water movement from the tubule lumen to the interstitium for reclamation. In a state of water diuresis, when ADH is not present, the collecting duct remains impermeable to water, and dilute urine is produced. Also, in the distal collecting duct, urea permeability is relatively high and is increased in the presence of ADH; thus, urea moves back into the renal medullary interstitium for maintenance of the medullary gradient (Anderson 1982; Brater 1998; Rose 2001).

The balance between acidity and alkalinity in animal body is referred as acid base balance and it is important homeostatic mechanism determined mainly by H^+ ion concentration in various body fluids (Rose 2001).

Disorders of body fluids, electrolytes, and acid-base balance are common in surgical experiment animals; and have frequently occurring etiologies (e.g., vomiting, diarrhea, diabetic ketoacidosis) and infrequently occurring ones (e.g., inorganic acid ingestion, diabetes insipidus, mineralocorticoid excess). These disorders can disrupt cellular and vital organ function in otherwise healthy experiment animals. Theirs evaluation typically requires a focused history and physical examination, followed by interpretation of biochemical factors. A prompt diagnosis and therapeutic interventions are respectively required as well to avoid the consequences of vital organ hypoperfusion and electrolyte imbalance (Halperin 1999).

In this chapter, an overview of fluids, electrolytes and acid-base abnormalities is shortly described. Due to a similarity in physiology among different surgical experiment animals, the extrapolation of this described information should be permissible to most laboratory animal species used in medical research, during and after experimental surgery.

ELECTROLYTES DISORDERS

Disorders of sodium and water. Sodium is the major extracellular cation. In humans, normal value of ECF is 140 mEq/L, and 10 mEq/L in its ICF, approximately. The maintenance of volume and tonicity of body fluids depends on regulation of sodium and water balance (Halperin 1999; DiBartola 2006a). Sodium is regulated by the kidneys in which the volume and constitution of filtrate reaching the collecting ducts is dependent on glomerular filter rate (GFR), sympathetic tone and angiotensin II acting via the effects of

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ADH and aldosterone to conserve water and sodium. Normothermic extra-renal losses are minimal (±10 mmol/day).

Hyonatraemia. (Dogs serum conc of $Na^+ < 140$ mEq/L) In small animal, the etiology of hyponatraemia is usually caused by hypoadrenocorticism, post obstructive diuresis, diuretic treatment, congestive heart failure, severe liver disease and nephritic syndrome. In Large animal, hyponatraemia is usually caused by acute diarrhea, bladder rupture in new born foals, chronic wasting disease, intrinsic kidney disease and diuresis, gastrointestinal fistula, severe haemorrhage and excessive sweating (Decaux 2003; Wellman 2012).

Generally, treatment of this sodium and water disorders include 5% sodium bicarbonate, Lactated Ringers solution as a precursor of bicarbonate, normal 5% saline that could be avoided if acidosis is present. The calculation of Na^+ requirement for replacement therapy in mEq=140 – measured plasma sodium × weight in Kgs (Adrogue 2000a; Rose 2001). The time of development of hyponatremia and the presence of symptoms should dictate the management of hyponatremia. Correction of plasma [Na^+] should be undertaken without delay in symptomatic experiments animals, particularly those experiencing seizures.

In humans, development of hyponatremia in less than 48 hours and the presence of symptoms strongly suggest that the benefit of treating acute cerebral edema outweighs the risk of treatment-associated adverse effects. Hypertonic sodium chloride with or without a loop diuretic is usually started at a rate of 1–2 ml/kg/hour to raise sodium concentration by 1–2 mEq/L/hr. This rapid correction of hyponatremia should be limited to the initial phase of management. The overall correction of [Na^+] should not exceed 8–12 mEq/L for 24 hours, as the risk of osmotic demyelination rises above this limit (DiBartola 2006a; Fried 1997).

In humans and experiment animal, the rate of correction in chronic hyponatremia has been a major concern. Refers to a guideline in humans, the rate of sodium correction should not exceed 0.5 to 1.0 mEqNa^+/L/hour to avoid induction of neurologic disease (Adrogue 1997; Sterns 1990). The optimal period for correction of chronic hyponatremia remains unclear. A period of 72 hours to several days has been proposed refering to some previous studies (Angelos 1999); fluid therapy should be anyway discontinued, and the serum sodium concentration measured, in case of neurologic signs. In dogs, gradual resolution of neurologic signs has been reported, treatment with nonspecific supportive care (O'Brien 1994).

Hypernatraemia. (Dogs $Na^+ > 155$ mEq/L; Cats $Na^+ > 160$ mEq/L) In large animals, its etiology includes prolonged exposure to dry heat, respiratory loss with fever, low intake of water, excessive salt intake with adequate water. In small animals, major causes include pure water loss, diabetes insipidus, hypertonic $NaHCO_3$ administration, cardiac arrest, feline urethral obstruction, acute renal failure (Wickstrom 2008; Wellman 2012).

Treatment of different types of hypernatraemia as hypervolaemic, hypovolaemic and isovolaemic include the intake of fresh water in sufficient quantities, 5% dextrose or maintenance fluid intravenously, salt poisoning and loop diuretics (Adrogue 2000b; DiBartola 2006a; Palevsky 1998). In case of acute hypernatremia (< 48 hours), it was shown that the brain cells do not develop idiogenic osmoles, that explain the success of a rapid correction of hypernatremia using either isotonic or polyionic fluid therapy; regardless of this, medical and veterinary literature favors a gradual correction of hypernatremia with careful monitoring of neurologic status (Adrogue 2000b; James 2007; Lien 2007). In chronic hypernatremia, the potential exists for ICF of brain cells to contain idiogenic osmoles and increased sodium concentration. Slow correction of hypernatremia is required to avoid brain edema (Angelos 1999).

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Disorders of chloride. Chloride constitutes the major anion filtered by the glomeruli and reabsorbed in the renal tubules. It plays an important role in preserving osmolality and partaking acid-base regulation. Its approximately 2/3 of the anions in plasma (approximately 110 mEq/L in dogs and 120 mEq/L in cats). Its intracellular concentration is much lower than its plasma concentration and dependent on the resting membrane potential of the cell. It is important in maintaining a normal acid–base state, normal renal tubular function and in the formation of gastric acid. Chloride loss is mainly from the stomach, bile, pancreatic and intestinal secretions (Hobbs 2002; Rose 2001).

Regulation of chloride is quietly related to sodium and inversely related to plasma bicarbonate. In the renal proximal tubule, chloride is excreted with ammonium ions to eliminate hydrogen ions in exchange for sodium and result in the production of acid urine (Yunos 2010).

Hypochloremia. Usually associated with excessive loss of fluids and occurs as a result of an increase in the net loss of electrolyte in the intestinal tract in acute intestinal obstruction, dilatation and impaction and torsion of the abomasum and enteritis. Its symptoms include anorexia, weight loss, lethargy, mild polydipsia and polyurea (Yunos 2010, Rose 2001).

Hyperchloremia. It is defined as a serum level of chloride >117 mmol/L in dogs. The Causes of hyperchloremia may include: loss of body fluids from prolonged vomiting, diarrhea, sweating or high fever (dehydration), high levels of blood sodium, kidney failure, or kidney disorders, diabetes insipidus or diabetic coma, drugs such as: androgens, corticosteroids, estrogens, and certain diuretics (Yunos 2010; Rose 2001). Treatment usually requires intravenous fluid therapy, but caution should be exercised to avoid sudden cerebral edema (Piperisova 2009).

Disorders of potassium. Potassium is the major intracellular cation in mammalian cells. Potassium and sodium have a reversed relationship in ECF and ICF. Serum potassium concentration was shown to be a poor indicator of potassium status in animals. Determination of intracellular potassium concentration in erythrocytes or muscle cells is a more accurate way to assess potassium depletion. In experimental studies of dogs, control values for ICF sodium and potassium concentrations in skeletal muscle were 8.4 to 13.7 and 139 to 142 mEq/L, respectively. In cats, total body potassium is approximately 55 mEg/kg body weight and almost all of this potassium is readily exchangeable. In a study of potassium depletion in dogs, the control value for total exchangeable potassium as determined by dilution was 47.1 mEq/kg body weights (range, 39.8 to 61.1 mEq/kg) (Wickstrom 2008; Wellman 2012).

Intracellular potassium is important for maintenance of normal cell volume and growth as it is required for the normal function of enzymes responsible for nucleic acid, glycogen, and protein synthesis. Studies also proved that 95% or more of total body potassium is located within cells, with muscle containing 60% to 75% of this potassium. Remaining 5% of the body's potassium is located in the ECF; maintaining the ECF potassium concentration within narrow limits is critical to avoid the life-threatening effects of hyperkalemia on cardiac conduction.

The kidneys are responsible of a mostly amount of potassium's excretion which is stimulated further by aldosterone activity, increased delivery of sodium to aldosterone-sensitive renal reabsorption sites, and high urinary flows. Potassium levels also can be lowered by excretion of smaller amount through the gastrointestinal tract or when moves out of the circulation and into cells, a process that is facilitated by insulin, alkalosis, and beta-adrenergic stimulants.

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Hypokalemia (Serum K+ conc < 3.5 mEq/L). It occurs as a result of decreased dietary intake, increased renal excretion, abomasal stasis, intestinal obstruction and enteritis, the prolonged use of potassium-free solutions in fluid therapy for diarrhoeic animals may result in excessive renal excretion of potassium. Hypokalemia effects on myocytes may predispose the animals to atrial and ventricular tachyarrhythmias, atrioventricular dissociation, and ventricular fibrillation. In addition, hypokalemia can predispose the heart to digitalis-induced arrhythmias and cause myocardial cells to be refractory to class I antiarrhythmic drugs.

Treatment of hypokalemia proposes to prevent or correct cardiac electrical disturbances and serious neuromuscular weakness. Usually, this treatment includes potassium chloride intravenously or orally potassium bicarbonate, potassium citrate orally (Gennari 1998; Mandal 1997). Animals may be refractory to correction of hypokalemia when hypomagnesemia and hypocalcemia exist. It is crucially important to correct all concurrent electrolyte abnormalities to attain normal neuromuscular function. Also, care must be taken not to create hyperkalemia when treating hypokalemia. Frequent monitoring (q 6–8 hours) of serum potassium concentration is recommended when aggressive replenishment is required. In diabetic patients with hypokalemia, insulin or sodium bicarbonate administration should be delayed until serum potassium concentrations have been replenished. The long-term treatment concerns to return total body potassium to normal levels and identify and correct the primary underlying disease (Halperin 1999; Rose 2001).

Hyperkalemia (serum K^+ conc > 6 mEq/L). Usually, the increase of potassium levels occurs with renal potassium retention, especially with low urine flow or when potassium is extruded from cells during acidosis and alpha-adrenergic activity. Pathologic increases in potassium levels may occur with acute tissue destruction and when the kidney cannot excrete potassium due to acute or chronic renal dysfunction. In small animals rapid infusion of potassium salts, high dose of potassium, penicillin G, oliguric acute renal failure, Terminal stages of chronic renal failure, urethral obstruction, lower urinary tract rupture, metabolic acidosis and hypoadrenocorticism. Hyperkalemia has a profound effect on cardiac function. There is usually marked bradycardia and arrhythmia and sudden cardiac arrest. Changes in the cardiac conduction system are usually evidenced by ECG changes, which may indicate potentially life-threatening arrhythmias. Classic ECG changes include tented T waves, widened QRS complexes, atrio-ventricular conduction blocks, accelerated idio-ventricular rhythm, sino-atrial block, ventricular fibrillation, and asystole.

No absolute level of serum potassium concentration is associated with a particular ECG abnormality; the ECG may be normal with life-threatening hyperkalemia and ECG abnormalities need not be present for emergency treatment of severe hyperkalemia. Bradycardia is commonly seen with hyperkalemia as a result of prolonged de- and repolarization of the myocardial conduction system. Hyperkalemia may also produce mild hyperchloremic metabolic acidosis.

Treatment of hyperkalemia is proposed to prevent adverse cardiac complications, and is aimed at one of following mechanisms: direct antagonism of hyperkalemia on cell membrane polarization, redistribution of extracellular potassium into the intracellular compartment and removal of potassium from the body. Studies shown that acute changes in serum potassium concentrations dispose to produce potentially life-threatening clinical effects than are produced by chronic changes. Animals with acute serum potassium concentrations greater than 6.5 mEq/L or with ECG changes suggestive of hyperkalemia are typically treated. An administration of calcium gluconate is usually given to temporarily alleviate the effects of

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hyperkalemia on heart. To correct acidosis, an administration of $NaHCO_3$ is given intravenously and shifts K^+ to the intracellular component (Mandal 1997).

Disorders of calcium. Calcium is required for many vital intracellular and extracellular functions, as well as for skeletal support. Ionized calcium (iCa) is required for enzymatic reactions, membrane transport and stability, blood coagulation, nerve conduction, neuromuscular transmission, muscle contraction, vascular smooth muscle tone, hormone secretion, bone formation and resorption, control of hepatic glycogen metabolism, and cell growth and division (Bushinsky 1998).

Intracellular iCa serves as an ionic messenger, conveying signals received at the cell surface to the inside of the cell. In the extracellular fluid, it regulates cell function in many organs, including the parathyroid gland, kidneys, and thyroid cells by binding to a newly identified cell membrane-bound calcium-sensing receptor. Almost all of the body's calcium is found in bone. A small amount of total bone calcium serves as a dynamic source of calcium resorption and deposition under hormonal control to keep an appropriate physiologic calcium concentration in the intracellular and extracellular fluids.

Hypocalcaemia (total serum Ca^{++} < 10–11 mg/dl dogs). It has diverse etiology and can occur immediately after parturition, hypoprotenemia, hypoparathyroid condition and in acute or chronic renal failure, puerperal tetany, ethylene glycol intoxication and inappropriate administration of a hypertonic phosphate enema (Wellman 2012). Treatment include Cattle 40% calcium borogluconate intravenously; Calcium chloride/calcium gluconate iv; maintenance dose = 10 ml of 10% Ca gluconate added to 500 ml of isotonic normal saline 0.9% Nacl solution (Rose 2001; Hobbs 2002).

Hypercalcaemia. Its occurs most frequently as a result of primary hyperparathyroidism, which is an excess production of PTH caused by generalized parathyroid hyperplasia, a solitary parathyroid adenoma, or as a part of multiple endocrine neoplasia type I or IIA. It can also occur when there is malignancy-induced destruction of bone resulting in release of calcium into the circulation, or when a tumor produces a PTH-like substance, most occurs commonly in adenocarcinomas of the kidney, ovary, or endometrium, and in squamous cancers of the lung or head and neck structures. Treatment involves therapy for the hypercalcemia and associated symptoms depending on severity; and the treatment of the underlying cause (Stewart 2005).

Disorders of phosphorus. Phosphorus plays an important role in metabolic activity such as carbohydrate and energy metabolism that naturally depends on the capacity to phosphorylate intermediate metabolites and to store energy released during oxidation in high-energy phosphate bonds such as ATP or phosphocreatine. Phosphorus is an integral component of 2, 3-DPG, a compound that regulates oxygen discharge from hemoglobin and then it is critical for oxygen delivery to tissues. In the body, phosphorus is present as a stable inorganic phosphate (Pi), an organic phosphate ester, or a phospholipid. The largest fraction of the body phosphorus (~85% of total body phosphorus) is incorporated into bone in an insoluble inorganic phosphate form (dihydroxyapatite); largely located in ICF (~14%), while < 1% of the total body phosphorus is found in ECF, which includes blood serum or plasma. In the ECF, phosphorus is present either as Pi, forming the metabolically relevant fraction, or as phospholipids. The extracellular Pi fraction is largely (~85%) ionized (either $H_2PO_4^-$ or HPO_4^{2-}), while ~10% is protein bound and 5% is complexed with other minerals such as calcium or magnesium. The concentration of Pi in the ECF and thus in serum is dictated by the equilibrium between Pi uptake from the digestive tract, Pi excretion in urine (monogastric

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species), saliva (ruminants), and milk; the uptake or release of Pi from bone; and compartmental Pi shifts between the ECF and ICF (Tatted 1997).

Hypophosphatemia (post-parturient Haemoglobinuria). In cattle, it occurs in under conditions similar to those of hypocalcaemia such as a decrease in feed intake or alimentary tract stasis. Treatment includes rapid administration of sodium phosphate salt solutions is commonly practiced. Mono- or dibasic phosphate salts (either Na_2HPO_4 or NaH_2PO_4) infused IV rapidly increase the serum Pi concentration. No effective approach to prevent hypophosphatemia and phosphorus depletion at the onset of lactation is known (Wickstrom 2008; Wellman 2012).

Hyperphosphatemia. It is closely associated with a decreased renal phosphate excretion, which occurs with chronic renal failure, hypoparathyroidism and hypomagnesemia. It's also caused by excessive intracellular phosphate movement to the extracellular fluid, as seen in tumor lysis, tumor necrosis, acute hemolysis, and rarely, acute metabolic acidosis due to chronic renal disease. The mainstay of therapy is reduction and restriction of dietary phosphate (Tatted 1997; Wickstrom 2008).

Disorders of magnesium. Magnesium is distributed intracellularly in bone with smaller amounts found in muscle and other soft tissue (50% – 60% of total body Mg are distributed in bone, 40%–50% in soft tissues, and <1% in the extracellular fluid). Intracellular Mg is required for activation of enzymes involving phosphate compounds such as ATPases, kinases, and phosphatases; and for synthesis of RNA, DNA, and protein. Magnesium is a cofactor for >300 enzymatic reactions involving ATP, including glycolysis and oxidative phosphorylation. It is also important in the function of the Na^+/K^+-ATPase pump, membrane stabilization, nerve conduction, ion transportation, and calcium channel activity. It also regulates the movement of calcium into smooth muscle cells, giving it a pivotal role in cardiac contractile strength and peripheral vascular tone. Low ionized Mg concentrations accelerate the transmission of nerve impulses.

Magnesium in bone is not readily available to support intracellular and extracellular phosphate levels, thus making dietary magnesium and renal magnesium reabsorption and excretion an important component of acute magnesium homeostasis. Renal reabsorption of most of the filtered magnesium occurs at the loop of Henle and distal tubular sites, which makes renal magnesium wasting possible when distal tubule function is disturbed by use of diuretics (Halperin 1999; Rose 2001).

Hypomagnesemia. Magnesium deficit (Plasma Mg^{++} < 1.5–2.5 mEq/l). It due to inadequate energy intake while grazing lush pasture low in magnesium, starvation, anorexia, low dietary content of magnesium, diarrhea and hypothyroidism. Clinical manifestations of severe hypomagnesemia include muscle weakness, muscle fasciculations, ventricular arrhythmias, seizures, ataxia, and coma.

Treatment of hypomagnesemia include magnesium sulphate 10% intravenously, usually concurrent administration of calcium is advisable; magnesium salts may be administered as a 20% solution in 5% dextrose. Less acute and complicated forms of hypomagnesemia can be treated with oral magnesium chloride. Potassium-sparing diuretics are helpful in treating magnesium deficiency created by chronic diuretic use (Rose 2001; Wellman 2012).

Hypermagnesemia. Magnesium excess (plasma Mg concentration >2 mg/dL [1.1 mmol/L]) is a rare condition reported only in monogastric animals. In normal renal function, hypermagnesemia is an uncommon clinical event because the kidneys efficiently excrete excess magnesium. Hypermagnesemia is most commonly caused by severe renal failure.

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Other causes of hypermagnesemia include adrenal insufficiency and hypothyroidism. Hypermagnesemia has been reported in cats with renal failure that were receiving IV fluid therapy.

Studies have reported that as plasma Mg concentrations exceed 2.5 mmol/L, there may be ECG changes with prolongation of the PR interval; at 5 mmol/L, deep tendon reflexes disappear, followed by hypotension and respiratory depression. Cardiac arrest may occur with blood Mg levels > 6.0–7.5 mmol/L.

The treatment includes avoiding magnesium-containing compounds when the renal function is compromised; in situation with cardiac or respiratory effects of hypermagnesemia, intravenous calcium gluconate should be requiring to antagonize the effect of magnesium (Rose 2001; Wellman 2012).

ACID-BASE DISORDERS

Acid-base homeostasis can be assessing by measuring the bicarbonate (HCO_3^-) concentration and PCO_2, along with the extracellular hydrogen ion concentration (blood pH). Normal ranges for HCO_3^- and PCO_2 are respectively 21 to 30 mEq/l and 35 to 45 mm Hg. pH is in the normal range of 7.35 to 7.45 when HCO_3^- and PCO_2 are at normal range. Although these values can represent a combination of abnormal acid-base events (Halperin 1999; Gordon 2004).

Acid-base disorders usually begin with an initial respiratory or renal event, altering the PCO_2 or [HCO_3^-], respectively. It is measured by arterial blood gases with pH as the key parameter; plasma pH is deviated out of the normal range (7.35–7.45). Acidosis corresponds to pH of arterial blood <7.35; Alkalosis corresponds to pH of arterial blood > 7.45. Both, acidosis and alkalosis refers to the pathophysiologic processes that cause net accumulation of acid or alkali in the body, whereas acidemia and alkalemia refers specifically to the pH of extracellular fluid. In acidemia, the extracellular fluid pH is less than normal and the [H^+] is higher than normal. In alkalemia, the extracellular fluid pH is higher than normal and the [H^+] is lower than normal. Due to the effectiveness of compensatory mechanisms, animals can have acidosis or alkalosis but not acidemia or alkalemia. i.e., a dog with chronic respiratory alkalosis may have a blood pH that is within the normal range. Such an animal has alkalosis, but does not have alkalemia (DeMorais 2006). The primary acid base disorders are divided into metabolic and respiratory disturbances: metabolic acidosis, metabolic alkalosis, respiratory acidosis, and respiratory alkalosis. The kidneys have been considered responsible for regulation of the metabolic component (blood bicarbonate concentration, [HCO_3^-]) and the lungs for regulation of the respiratory component (partial pressure of CO_2, [pCO_2]). Each primary (metabolic or respiratory) acid base disturbance is accompanied by a secondary opposing response in the other system (respiratory or metabolic) (Dibartola 2006b). A combined respiratory and metabolic acidosis suggests a mixed acid-base disorder (Hood 1998).

The presence of an acid-base disturbance may also appear as a quantitative alteration in serum chloride and serum bicarbonate concentrations on a basic electrolyte panel. The acid-base-induced changes in serum chloride and bicarbonate concentration are reciprocal and equivalent unless there is an excess of unmeasured anions (i.e, anions other than chloride or bicarbonate). Such unmeasured anions (e.g., beta-hydroxy-butyrate in diabetic ketoacidosis,

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lactic acid in lactic acidosis, or various toxins) can be detected by calculating the anion gap, which is measured by the following formula: {($[Na^+]$ + $[K^+]$) - ($[HCO_3^-]$ + $[Cl^-]$)} where $[Na^+]$ = sodium concentration; $[K^+]$ = potassium concentration; and $[Cl^-]$ = chloride concentration, for which the normal value is 8 to 16 mEq/l. This anion gap can also be measure as {($[Na^+]$ - $[Cl^-]$) + ($[HCO_3^-]$)}, for which the normal value is 6 to 12 mEq/L. Either way, the presence of an elevated anion gap indicates metabolic acidosis due to an excessive amount of intrinsic or unmeasured anions produced by the addition of extrinsic or the excess production of fixed acids containing anions other than chloride (e.g., ethylene glycol ingestion and lactic acidosis, respectively).

Metabolic Acid-Base Disorders

Metabolic acidosis. It is the most common acid-base disturbance encountered in sick small animals, horses and camelids. It is identified by a decreased bicarbonate (HCO_3^-) and base excess on a blood gas analysis, and a decreased HCO_3^- on the chemistry panel. Main cause in animals include: an increased accumulation of hydrogen, typically caused by the addition, retention, or excess production of acids. Initially, the excess acid is buffered by bicarbonate content, and eventually, this buffering consumes the bicarbonate reserves and decrease bicarbonate level. Metabolic acidosis also occurs by loss of bicarbonate, leaving the body unable to buffer normally produced acid (Hopper 2012; Constable 2014). Metabolic acidosis can present with a normal or increased anion gap. With high anion gap, the cause typically is addition of an acid, such as beta-hydroxybutyric acid and acetoacetic acid in diabetic ketoacidosis, lactic acid in sepsis, uremic toxin in renal failure, or ingested toxins. In a situation with normal anion gap, the cause typically is the inability to excrete acid via the kidneys (type 1 renal tubular acidosis), gastrointestinal HCO_3^- loss due to diarrhea, or renal dysfunction in type 2 renal tubular acidosis. Symptoms of metabolic acidosis include diarrhea, polydipsia and polyuria, tinnitus, vision loss, urolithiasis symptoms, etc. (Dubose 1997; Hobbs 2002). To evaluate metabolic acidosis, following tests are useful.

Anion Gap: Occurs when metabolic acidosis is associated with normal anion gap, hypobicarbonatemia and hyperchloremia; usually caused by HCO_3^- loss in diarrhea or renal tubular acidosis (type 2). Also, when metabolic acidosis is accompanied by a high anion gap caused by the addition, retention, or production of fixed acids containing unmeasured anions.

Osmolar Gap: The serum osmolar gap (i.e., the difference between measured and calculated serum osmolality, which is normally less than 10 mOsm/kg) is useful in evaluating metabolic acidosis, and should be measured in all cases when the anion gap is large and toxic ingestion is suspected.

Potassium: Metabolic acidosis causes extracellular hyperkalemia. This occurs by removal of hydrogen ions $[H^+]$ from the extracellular fluid into cells, process that results in reciprocal movement of potassium out of cells and into the extracellular fluid. Absence of acidosis-induced hyperkalemia suggests severe total body potassium depletion (Mandal 1997; Lee 2013).

Urine pH and urinary anion gap: Hypokalemic non anion-gap metabolic acidosis with suboptimal urinary acidification (i.e., pH greater than 5) and a positive urinary anion gap ($[U_{Na+} + U_{K+}]$ - $[UCl^-]$) suggests distal renal tubular acidosis (RTA) (type I) caused by decreased renal acid excretion. The same events occur in proximal RTA (type II) when

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[HCO_3^-] is below the reduced bicarbonate excretion threshold above which bicarbonate is excreted producing a urinary pH greater than 5. A negative urinary anion gap also is consistent with hyperchloremic metabolic acidosis caused by diarrhea, with normal renal [H^+] excretion capacity, produces a urinary pH of less than 5 (Ishihara 1998).

The essential treatment of metabolic acidosis is to stop the excess acid production, addition, or retention or to terminate the bicarbonate loss. Restoration of effective circulating volume is essential to terminating the production of organic acids related to poor perfusion (e.g., lactic acidosis) and to promoting delivery of hydrogen ions to the distal renal tubule for excretion. Hypokalemia also should be corrected to restore renal concentrating capacity and improve urinary acidification ability.

Titration metabolic acidosis. Also called high anion gap acidosis is a primary acid-base disorder (i.e., it does not occur in compensation to a primary respiratory acid-base disorder). The titration or consumption of bicarbonate by a non-volatile non-chloride containing acid results in a high anion gap metabolic acidosis. Main causes of this titration metabolic acidosis in almost species animals include L-lactate, a decreased excretion of normally filtered acids due to kidney. In Small animals, the main causes include ketoacidosis, toxic metabolites (e.g., ethylene glycol, salicylates). In cattle, causes include Ketoacidosis, D-lactate acidosis. In the camelids, ketoacidosis and L-lactate are major causes (Dibartola 2006b; Rose 2001; Constable 2014).

Bicarbonate loss metabolic acidosis: Intestinal loss from secretory diarrhea is the most common cause of this type of primary acid-base disturbance and is the most frequent cause of a bicarbonate loss acidosis in ruminants, particularly calves. In the kidney, loss of bicarbonate is accompanied by retention of hydrogen with chloride in excess of sodium. In this type of acid-base disorder, body maintains electro neutrality by increasing or retaining Cl^-, with hydrogen, since HCO_3^- is an anion. Thus, an acidosis due to HCO_3^- loss is usually accompanied by a corrected hyperchloremia, with a normal anion gap (Rose 2001; Constable 2014).

Gain of a chloride-containing non-volatile acid metabolic acidosis: This primary hyperchloremic metabolic acidosis occurs with distal renal tubular acidosis, when the proton pump (H-ATPase) in the distal nephron cannot pump out hydrogen with chloride passively following. Thus, hydrogen with chloride is retained resulting in a primary hyperchloremic normal anion gap metabolic acidosis.

In humans, it occurs with inherited defects in the H-ATPase in the distal nephron that causes excretion of hydrogen into the urine. The kidney can create a hyperchloremic normal anion gap metabolic acidosis as compensation for a primary respiratory alkalosis or as correction for a primary metabolic alkalosis, through a decreased ammoniagenesis in the proximal renal tubules primarily so hydrogen is no longer excreted as NH_4Cl, resulting in concomitant loss of bicarbonate; a decreased H-ATPase activity in the collecting tubule primarily, so hydrogen and chloride are retained. Both of these processes results in retention of hydrogen and chloride (in excess of sodium), leading to a compensatory or corrective hyperchloremic normal anion gap metabolic acidosis (Rose 2001; Dibartola 2006b).

Gain of chloride without hydrogen as the proton: In animals, particularly cattle, an acidifying effect can occur and cause urinary acidification as a corrective response, via strong ion principles, when calcium chloride or diets with negative cation are given to anion balance (Constable 2014).

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Hyperchloremic metabolic acidosis. In animals, the presence of a hyperchloremic normal anion gap metabolic acidosis (low bicarbonate, high Cl^{-}_{corr}) does not mean the acidosis is a primary disorder. Clinical assessment of animals is required and knowledge of the underlying disease prior to determine whether a hyperchloremic metabolic acidosis is primary or secondary to a respiratory acidosis. In situation of a primary respiratory alkalosis with a compensatory hyperchloremic metabolic acidosis, they should be a presence of a clinical disease or condition causing hyperventilation, the blood pH will be more alkaline than acidic and the pCO_2 will be quite low. Kidney function should also be normal for an animal to be able to compensate for a primary respiratory alkalosis. In small animals, the main causes of hyperchloremic metabolic acidosis include proximal or distal renal tubular acidosis, vomiting of intestinal contents secretory diarrhea, compensation for a primary respiratory alkalosis, loss of bicarbonate in saliva.

Metabolic alkalosis. A metabolic alkalosis is identified by an increased HCO_3^- and base excess on a blood gas analysis, and an increased HCO_3^- and/or decreased Cl^{-}_{corr} on the chemistry panel. Metabolic alkalosis may be caused by Loss of a chloride-containing non-volatile acid (e.g., HCl, NH_4Cl) causes loss of Cl^- without concomitant loss of Na^+. These types of metabolic alkalosis are chloride-responsive. In rare cases, renal losses of hydrogen (e.g., stimulation of the H-ATPase in the collecting tubules) can cause acid loss without much chloride loss, e.g., primary hyperaldosteronism. This latter type of metabolic alkalosis is chloride-unresponsiveness. Metabolic alkalosis may also be caused by a gain of base or bicarbonate; but this is a far less common.

Metabolic alkalosis due to acid loss. In small and large animals, this type of alkalosis is caused by compensation for a primary respiratory acidosis or correction for a primary metabolic acidosis, vomiting of gastric contents, excessive sweating, sequestration of abomasal contents, etc. This type of alkalosis usually responds to chloride supplementation.

Metabolic alkalosis due to base gain. This type of metabolic alkalosis is very uncommon and usually iatrogenic. Administration of $NaHCO_3$ (e.g., treatment of metabolic acidosis) or organic anions (which are metabolized to HCO_3^-, e.g., citrate in massive blood transfusions), may cause a metabolic alkalosis, particularly under conditions of volume depletion or renal dysfunction.

The presence of a metabolic alkalosis (high bicarbonate, low Cl^{-}_{corr}) does not mean the metabolic alkalosis is a primary disorder. Clinical assessment of the patient and knowledge of the underlying disease are required to determine whether a metabolic alkalosis is primary or secondary to a respiratory acidosis (Constable 2014).

Respiratory Acid-Base Disorders

This acidosis is usually due to inefficient ventilation caused by central nervous system respiratory center inhibition, disorders of the respiratory and chest wall muscles, obstruction to ventilation, and pulmonary perfusion and/or diffusion dysfunction. Decreased ventilation leads to hypercapnia (an increased level of $P_{CO2)}$, which stimulates compensatory renal retention of HCO3. Renal compensation HCO_3^- retention is always slow in providing substantial or immediate protection against increasing systemic H^+. It occurs at a rate of 1 mEq/L or 3.5 mEq/L for every 10-mm Hg increase in PCO_2, respectively, up to a limit of 30 mEq/L for acute respiratory acidosis and 45 mEq/L for chronic respiratory acidosis.

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Symptoms are always due to an underlying pulmonary disease and commonly include shortness of breath. Treatment is directed to the primary cause of poor ventilation to increase the effectiveness of ventilation and pulmonary gas exchange. Oxygen administration may be essential in case of hypoxemia caused by respiratory depression (Dibartola 2006b, Rose 2001).

Respiratory acidosis. It is identified by an increased pCO_2 and low pH or tendency towards a low pH on a blood gas analysis. Chemistry panel usually not provide any information on the respiratory component of acid-base status. It is caused by decreased ventilation or gas exchange in the alveoli, which can be secondary to neurologic, musculoskeletal, pulmonary, and cardiac disorders. In animals, most common causes are primary pulmonary disease, ranging from upper airway obstruction to pneumonia. Diseases or drugs that inhibit the medullary respiratory center can also produce a profound respiratory acidosis, e.g., general anesthesia (Rose 2001; Dibartola 2006b).

Respiratory alkalosis. It is caused by situations or events that increase volume and rate of ventilation, resulting in a reduction in the level of PCO_2, which decreased cellular production and stimulates compensatory renal excretion of HCO_3^-, leading to a compensatory reduction of serum HCO_3^-. The immediate cellular response against decreasing systemic H^+, is usually slow in respiratory alkalosis. In primary acute and chronic respiratory alkalosis, the compensatory serum HCO_3^- reduction occurs at a rate of 2 mEq/L or 4 mEq/L for every 10-mm Hg decrease in the PCO_2 level, respectively. The limits of the metabolic compensations in acute and chronic respiratory alkalosis are serum HCO_3^- levels of 18 mEq/L and 12 mEq/L, respectively. If the compensatory HCO_3^- loss is less than or greater than predicted, a mixed acid-base disorder may be present (Hobbs 2002; Laffey 2002).

Respiratory alkalosis is induced by processes that lead to hyperventilation which include pulmonary diseases with hypoxemia; psychogenic disorders, such as anxiety, fever. Symptoms occur with respiratory alkalosis and acute hypocapnia include circumoral and digital paresthesia, light-headedness, carpopedal spasm, and tetany. Treatment is directed to the underlying cause.

Mixed Acid-Base Disorders. It's typically detected when a primary acid-base disturbance is not associated with the expected compensation. This failure of compensation concludes the presence of a respiratory and metabolic acid-base event arising concurrently with the primary event. In animals, it might result from two or rarely three separate primary disturbances present simultaneously. Four disorders cannot coexist in one animal because hyperventilation which refers to respiratory alkalosis and hypoventilation which refers to respiratory acidosis cannot occur at the same time in one animal (Dibartola 2006b). A normal pH with an abnormal serum HCO_3^- and PCO_2 typically also indicates the presence of a mixed acid-base disorder (Hobbs 2002).

In small and large animals, a mixed acid-base disturbance should be suspected in following situations: normal pH normal with an abnormal pCO_2 and/or bicarbonate, when change in pH is greater than can be attributed to one disorder alone, when pCO_2 and HCO_3^- change is in opposite directions, when the degree of change in acid-base results is not proportional.

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Anaesthesia, Surgery and Fluid Balance

During perioperative period, many experiment animals are dehydrated prior surgery by prolonged fasting, use of purgatives or diuretic therapy. In addition, intraoperative losses of body's fluid are frequently underestimated and excess losses, both surgical and third-space losses, persist into the early postoperative period. Consequently, a tendency towards hypovolaemia is usually present leading to thirst and vasopressin secretion. During anaesthesia period, the most important response is sodium and water retention. Number of factors may contribute to this including: the effects of anaesthetic agents on renal blood flow and GFR; effects of intraoperative hypotension or hypovolaemia on renal function; increased sympathetic tone and circulating catecholamines causing renal vasoconstriction; the salt and water retaining effects of increased plasma cortisol and aldosterone levels in response to the stress of surgery; and increased ADH activity which may increase 50–100-fold during surgery and falls at the end of surgery, without return to normal for 3–5 days (similar to the period of post-operative oliguria) (Desborough 2000, Aitkenhead 2001). During surgery, changes also occur in capillary membrane porosity as a result of the cytokine-mediated responses to tissue injury and bacteraemia. Despite a considerable increase in lymphatic drainage, fluid accumulates in previously 'dry' tissues. The situation often exists where circulatory hypovolaemia is significant enough to threaten organ perfusion whilst these 'third-space' tissues are waterlogged. More fluid is usually required in this situation to maintain circulatory volume and adequate organ perfusion (Dobb 1984).

Fluid Therapy for Maintenance and Replacement during Surgery

Fluid therapy serves to correct dehydration, acidosis and alkalosis, electrolyte deficiencies, nutrition and calorie. This therapy should be individualized and fitted to each case and constantly re-evaluated and reformulated according to changes in status (Schott 2006). Fluid selection is prescribed by the animal's needs, including volume, rate, and fluid composition required, as well as location the fluid is needed (interstitial versus intravascular) (Naylor 1990; Kirby 2009). Factors to consider include the following: acute versus chronic conditions, animal pathology (e.g., acid-base balance, oncotic pressure and electrolyte abnormalities), comorbid conditions (DiBartola 2006b; Hallowell 2009).

A variety of conditions can be effectively managed using three types of fluids: a balanced isotonic electrolyte (e.g., a crystalloid such as lactated Ringer's solution (LRS)); a hypotonic solution (e.g., a crystalloid such as 5% dextrose in water (D5W)); and a synthetic colloid (e.g., a hydroxyethyl starch) (Table 6.1).

Fluid therapy often begins with the maintenance rate, which is the amount of fluid estimated to maintain normal patient fluid balance. This Maintenance fluid therapy is indicated for animals that are not eating or drinking, but do not have volume depletion, hypotension, or ongoing losses. Replacement fluids are proposed to replace lost body fluids and electrolytes (Anderson 1982; Wellman 2012).

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Table 6.1: Available Fluids Used in Experimental Surgery

Commercially available fluids	
(Electrolyte Solutions) Isolyte, Premolyte, Dextroselyte	Daily water and electrolyte maintenance and replacement of lose.
Dextrose Solutions	Used in prophylaxis and treatment of ketosis in starvation, diarrhea, vomiting or high fever.
Plasma Volume Expander Dextran in 0.9% Sodium Chloride	Indicated in shock due to decreased effective blood volume, severe dehydration and surgical procedures and anesthesia.

Using replacement solutions for short-term maintenance fluid therapy typically does not alter electrolyte balance; however, electrolyte imbalances can occur in patients with renal disease or in those receiving long-term administration of replacement solutions for maintenance (Hobbs 2002). In most case, isotonic polyionic replacement crystalloid such as LRS is used as either replacement or as maintenance fluids. When it's administering for maintenance, it may predispose the animals to hypernatremia and hypokalemia because this solution contains more sodium (Na) and less potassium (K) than the animals normally loses. Animals with normal renal function and well hydrated are typically able to excrete excess Na and thus do not develop hypernatremia. Animals that receive replacement solutions for maintenance fluid therapy may develop Hypokalemia, in case of anorexia, vomiting or diarrhea because the kidneys do not conserve potassium very well. Monitoring of serum electrolytes is required periodically (e.g., q 24 h) (Macintire 2004; Schott 2006; Macintire 2012).

Enteral nutrition. Previous studies reported that in number of animal species, enteral fluids administered alone, or in conjunction with intravenous fluids, are useful for the treatment of dehydration and electrolyte loss associated with diarrhoea (Lopes 2003; Kirby 2009). It's very suitable for treatment of mild to moderately dehydrated animals with some intact intestinal epithelium and motile small intestine. It is contraindicated in animals that are severely dehydrated and /or in hypovolaemic shock when used alone. If used in conjunction with intravenous fluids, enteral nutrition (EN) may ameliorate the effects of villous atrophy and malnutrition (Rainger 2006; Amstrong 1988).

The species of animal, the underlying condition, and the constituents of the fluid, usually influence the choice of the enteral fluid. Available enteral fluids have following components: sodium, chloride and carbohydrates, the amounts of ions and other ingredients such as potassium, alkalising agents, amino acids and short chain fatty acids which may vary (Rainger 2006).

Parenteral nutrition. It's an important feeding modality for patients unable to receive adequate enteral nutrition. Although, it's effectiveness in animals has not been proven, human studies have shown that using parenteral nutrition (PN) in appropriately selected cases can improve clinical outcome, reduce hospitalization time, and even reduce the overall cost of patient care (Chan 2002; Koneru 2010).

PN formulations for animals are readily available through market, and it involves intravenous administration of nutrients to patients that require nutritional support and are anorectic despite correction of dehydration and other metabolic derangements. PN solutions components are usually mixtures of dextrose solutions, lipid emulsions, and amino acid

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solutions that variably contain electrolytes, vitamins, and mineral supplements. A mixture of dextrose, lipids, and amino acids together in a PN formulation, resulting in a solution called a three-in-one solution or total nutrient admixture. Three-in-one solution is well tolerated by animals, and easy to administer; it provides an animal's short-term amino acid, glucose, and energy needs in one solution (Rombeau 2001; Hallowell 2009; Koneru 2010). Solution of dextrose and amino acid solutions without lipids is given to animals as well. This solution contains fewer kilocalories per milliliter, require substantially greater volumes of delivery to meet caloric needs, and do not need a daily replacement as with lipid containing admixtures. Its use can help to avoid negative side effects of lipid administration.

An alternative to the total nutrient admixtures with lipids, dextrose, and amino acids is the use of partial parenteral nutrition (PPN) solutions with only amino acids and/or dextrose (Lippert 1989; Plunkett 2002). Available solution such as a mixture of 3% amino acids, glycerol, and electrolytes can be administered continuously for longer than 24 hours because it does not contain lipids, which deteriorate over time.

Careful monitoring of experiment animals receiving PN is important to identify and rectify metabolic abnormalities that may develop (Remillard 1989; Macintire 2005). Vital signs (i.e., temperature, pulse and respiratory rates, animal attitude) should be seriously monitored every 4 to 6 hours for the first 2 to 3 days and at a decreasing frequency thereafter. Body weight should be measured every 12 to 24 hours. Blood and urine glucose should also be evaluated at least every 12 hours for the first 2 to 3 days for evidence of hyperglycemic complications (Pyle 2004; Chan 2002; Amstrong 1988).

PN has a disadvantages to be economically cost, and could induced mechanical complications, metabolic complications and infections which occurs secondary to contamination and growth of bacteria and fungi in a PN bag, nosocomial bacterial or fungal contamination during administration, or bacterial translocation from the animal's own body (specifically the gastrointestinal tract or skin at the catheter site). Contamination of the PN bag during compounding and nosocomial infection introduced during administration can be controlled by careful preparation of PN and aseptic handling of the intravenous tubing (Kelly 1996; Koneru 2010).

Refeeding syndrome. It's usually described as the potentially fatal shifts in fluids and electrolytes that may occur in malnourished patients receiving enterally or parenterally refeeding. These shifts result from hormonal and metabolic changes, caused by rapid initiation of refeeding after a period of undernutrition (Kelly 1996; Kirby 2009). The hallmark biochemical feature of this syndrome is hypophosphatemia (Marinella 2003). However, the syndrome is complex and may also feature abnormal sodium and fluid balance; changes in glucose, protein, and fat metabolism; thiamine deficiency; hypokalemia; and hypomagnesaemia. Understanding of refeeding syndrome and identification of animals at risk is essentially important as the condition is preventable and the metabolic complications are avoidable. Experiment animals at high risk include undernourished animals and those who have had little or no energy intake for more than 10 days. Refeeding should be started at a low level of energy replacement. Vitamin supplementation should also be started with refeeding and continued for at least 10 days. The adjustment of electrolyte and fluid imbalances before feeding is not necessary; it should be done in company with feeding (Miller 2000; Bartges 2001; Tsai 2003).

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CONCLUSION

Fluid, electrolyte, and acid-base abnormalities can lead to fatal consequences in surgical experiment animals. Understanding of knowledge about fluid and electrolyte homeostasis, acid-base balance and the underlying pathophysiology related to their respective disorders, are of crucial importance to provide optimal management required during and after experiments surgery. In addition, fluid therapy, which is important for many medical conditions in experimental surgery, should be administered depending on experiment animals needs, according to a well-defined fluid administration rates.

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Chapter 7

IMAGERY TECHNIQUES IN EXPERIMENTAL SURGERY

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ABSTRACT

Imaging technologies have been widely applied in animal disease models established by experimental surgery. Imaging technologies can be used to explore the mechanism of human diseases at the molecular level and evaluate the effect of new diagnostic and therapeutic agents on human diseases. In this chapter, the updated application of imaging technologies for studying human diseases in animal models will be reviewed.

Keywords: imagery techniques, experimental surgery

ABBREVIATIONS

US:	Ultrasound
Micro-CT:	Micro-computed tomography
OI:	Optical imaging
Micro-SPECT:	Micro-single photon emission computed tomography
Micro-PET:	Micro-positron emission tomography
MRI:	Magnetic resonance imaging
UBM:	Ultrasound biomicroscopy
TEE:	Transesophageal echocardiography
2D:	Two-dimensional

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CEUS:	Contrast-enhanced US
SCI:	Spinal cord injury
SCBF:	Spinal cord blood flow
IVC:	Inferior vena cava
3D:	Three-Dimensional
ECG:	Electrocardiogram
CCD:	Charged-coupled detector
CMOS:	Complementary metal oxide semiconductor
TFT:	Thin-film transistor
PCXD:	Photon counting X-ray detectors
MI:	Myocardial infarction
LV:	Left ventricular
GFP:	Green fluorescent protein
DOT:	Diffuse optical tomography
PAT:	Photo-acoustic tomography
FLI:	Fluorescence imaging
OPAL:	Optical projection of acquired luminescence
BLS:	Bright-light surgery
FGS:	Fluorescence-guided surgery
FOV:	Field of view
CZT:	Cadmium zinc telluride
PMT:	Photomultiplier tubes
AD:	Alzheimer's disease
rCBF:	Regional cerebral blood flow
FDG:	Fluoro-2-deoxy-D-glucose
EDA:	Esophagoduodenal anastomosis
EGFR:	Epidermal growth factor receptor
GK:	Goto-Kakizaki
DWI:	Diffusion-weighted imaging
fMRI:	functional MRI
DCE–MRI:	Dynamic contrast-enhanced imaging
ASL:	Arterial spin labeling
BMSCs:	Bone mesenchymal stem cells
EPCs:	Endothelial progenitor cells
OA:	Osteoarthritis
ROIs:	Regions of interest
MT.ThC:	Medial tibial cartilage thickness
MS:	Multiple sclerosis
EAE:	Experimental autoimmune encephalomyelitis
CAA:	Cerebral amyloid angiopathies
MRA:	Magnetic resonance angiography
VSI:	Vessel size imaging
μMRI:	microscopic magnetic resonance imaging
3R:	Replacement, reduction and refinement

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INTRODUCTION

Translational research is changing the medical practice and the way to solve health problems. The application of translational research is the basis for science-based medicine and is the proper future orientation since it benefits from multidisciplinary, multiphase, and multisectorial projects to obtain solutions for health problems (Milne 2009). The application of experimental surgery animal models has an important role in preclinical study. The experimental surgery animal models establish a bridge between molecular discovery and clinical application. The *in vitro* effect of a molecule needs to be explored *in vivo* for its molecular interactions (Franc 2008). Imaging technologies can be used to explore disease in real time, in a quantitative way and at the molecular level. Disease progression and therapeutic effect can be repeatedly and non-invasively monitored by the imaging technologies (Cunha 2014). The advantages of preclinical imaging methods include 1) the decrease of biological variability; 2) unique information in distinct forms, repeated or modulated as needed; 3) reduction in the number of animals for study (Cunha 2014). The imaging techniques for preclinical study include ultrasound (US), micro-computed tomography (micro-CT), optical imaging (OI), micro-single photon emission computed tomography (micro-SPECT), micro-positron emission tomography (micro-PET) and magnetic resonance imaging (MRI). Each technique has its own advantages and limitations. Multimodality devices are under investigation to overcome the limitations and acquire complementary pathophysiological information.

The combination of high-resolution techniques (micro-CT or MRI) with highly sensitive techniques (micro-PET or micro-SPECT) will expand the horizons of different research fields. It will help to explore the underlying mechanisms of disease and provide efficient and unique tools for assessing new chemical entities and candidate drugs. The added advantage of animal imaging techniques has prompted their growing application. The combination of experimental surgery animal models of disease and highly developed imaging tools can shorten the project length and improve the level of confidence in the obtained results. Moreover, the preclinical validation of drug target is driving the use of animal *in vivo* imaging technologies for drug development (Franc 2008; Pomper 2005).

This chapter will review the imaging techniques for experimental surgery animal models. The contribution and relative importance of each technique, including its advantages and limitations, is outlined.

IMAGING TECHNIQUES

Imaging techniques are non-invasive which can be used to screen the whole disease process, from disease onset to progression and therapy monitoring. The significant advantages of imaging techniques are the reduction of biological variability and considerable decrease in the number of animals required for a particular study (Zanzonico 2011). Furthermore, the study of complex interactions between the physiological process and biochemical process in biological systems can only be performed with intact animals. It is impossible to fully assess such interactions with cell- or tissue culture-based methods or in *ex vivo* systems. As most imaging techniques are the same as those used in the clinical setting, results are easily translatable to humans (Zanzonico 2011; Weissleder 2001).

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Nevertheless, several challenges need to be resolved when imaging mouse as compared with human. These challenges include the subject size, the total volume, the spatial resolution and the total time spent for images (Weissleder 2001). The crucial challenge is to acquire high signal-to-noise ratio and to localize precisely the imaging probe, while keeping a good temporal resolution with the minimum amount of molecular probe (Massoud 2003).

Nuclear medicine-based techniques (PET and SPECT), OI, CT, MRI and US are the most acceptable for non-invasive *in vivo* imaging in genomics, proteomics, and nanotechnologies (Grassi 2008). Imaging equipment performance has been improved by the advance of imaging software and hardware (Zanzonico 2011). The existing imaging technologies differ basically in their physical basis and in the type of information provided (Table 7.1).

Table 7.1. Summary of general properties of diagnostic imaging techniques

Imaging technique	Physical basis	Information provided
US	Sound reflection of high-frequency sound waves	Internal movements and flows, differences of Tissues
CT	X-rays	Tissue density
OI	Light emissions	Probe uptake
SPECT	Gamma-radiation	Tracer uptake
PET	Gamma-radiation	Tracer uptake
MRI	Magnetic properties	Tissue composition

Adopted from Cunha 2014.

ULTRASOUND (US)

US refers to sound waves with frequencies higher than 20,000 cycles/s (Hz) that are not detectable by the human ear (Liang 2003; Coatney 2001). The principle of US is to produce images by the application of the interaction of sound waves with living tissues/organs (Coatney 2001). Diagnostic US usually utilize frequencies between 2 and 15 MHz (Coatney 2001). Preclinical systems utilize higher frequencies (20–50 MHz) to produce images with higher spatial resolution and a sufficient penetration for anatomical and functional real-time information of the animal models (Tremoleda 2012).

US can provide many specialized imaging formats and techniques that are applied in preclinical study. US biomicroscopy (UBM), transesophageal echocardiography (TEE) and contrast-enhanced imaging are three specialized ultrasound imaging methods for rodents (Coatney 2001). Doppler-based models can detect dynamic and real-time images to decide the speed of a moving tissue and acquire quantitative structural and functional information (Coatney 2001). The effect of Doppler is according to the change of sound-wave frequency when a sound wave is reflected by a moving target. The larger the Doppler shift, the larger the speed of the blood flow (Coatney 2001). Contrast agents with acoustic properties that differ from the target tissues can be used to enhance image quality (Liang 2003). Molecular-targeted contrast agents may be used to estimate biological processes at the molecular level (Deshpande 2010). The most common US contrast agents are microbubbles, which are small and stabilized gas-encapsulated micro-particles (<10 μm). After intravenous injection, they

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act comparably to the red blood cells in the circulation and supply a strong reflective blood/gas interface (Greco 2012).

UBM can obtain images using two-dimensional (2D) brightness-mode, pulsed and continuous wave Doppler and color flow Doppler (Comley 2011; Greco 2012). This imaging technique has been applied for the embryonic evaluation and eye development (Golden 2012; Cheung 2007), murine models of myocardial infarction and left ventricular remodeling (Kaufmann 2007), and mouse model of colorectal cancer (Alves 2013). The advantages of US over other imaging techniques include high spatial and temporal resolution, a rapid frame rate, real-time and quantitative imaging, no ionizing radiation, less expensive and extensive availability (Liang 2003; Deshpande 2010). However, US may cause bioeffects such as tissue heating and shock waves (Liang 2003). Skilled operator is needed to acquire accurate, repeatable, and high-quality images (Coatney 2001). US cannot be commonly applied in the brain, spinal cord, and lung due to limitations in imaging bone and gas-filled structures (Greco 2012).

US can be utilized to evaluate muscle atrophy after nerve injury for longitudinal analysis of nerve regeneration in rats (Hundepool 2015). Dynamic contrast-enhanced US (CEUS) changes may supply the angiogenesis and hemodynamic information of rabbit hepatic tumors (Zhou 2015). CEUS was used to evaluate the effects of peripheral sympathetic denervation in common iliac arteries on peripheral microperfusion of the biceps femoris in pigs (Steinberg 2015).

Microbubbles ameliorate the recognition and interpretation of tumor progression, inflammatory processes and cardiovascular diseases. US molecular imaging can be perceived by binding targeted moieties, peptides and antibodies to the surfaces of microbubble due to the excellent sensitivity and specificity. The targets of molecular microbubbles include integrins, selectins, intercellular adhesion molecule 1, vascular cell adhesion molecule 1 and vascular endothelial growth factor receptor-2. These molecular microbubbles were produced and were shown in preclinical experiments to be able to selectively bind to tumor blood vessels and atherosclerotic plaques (Kiessling 2012).

Guided high mechanical index-induced microbubble cavitation from a diagnostic US transducer added to low-dose tissue plasminogen activator (tPA) can quickly ameliorate regional function and decrease infarct size in acute ST segment elevation myocardial infarction (Wu 2015).

Several antibody- or peptide-targeted microbubbles have been favorably evaluated for imaging receptors overexpressed on tumor blood vessels and on atherosclerotic plaques in experimental animal study. The first molecularly targeted microbubble was used in clinical trials to diagnose and localize prostate cancer (Kiessling 2012). Furthermore, microbubbles have been applied for thrombolysis, for increasing drug delivery across biologic barriers, and for integrating disease diagnosis and therapy. Additionally, US with microbubbles supply an adaptable tool for molecular imaging and therapy. Real-time CEUS imaging was used to evaluate post-spinal cord injury (SCI) and spinal cord blood flow (SCBF) changes in a rat contusion model of traumatic spinal cord injury (Dubory 2015). US imaging indicated unequal blood flow along the intact spinal cord in the uninjured animal. Critical ischemia at the level of the epicenter was detected 15 min after SCI. SCBF was not affected in the more remote intact areas. SCBF was markedly decreased in the regions close to the epicenter. The result was similar to the previously reported “ischemic penumbra zone”. This technique is of

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great interest for evaluating the treatment effect on limiting ischemia and tissue necrosis subsequent to SCI.

Molecular US imaging was applied to evaluate the early inflammatory effects following 30 min of renal ischemia in animal model (Hoyt 2015). It may determine the extent of injury and observe the tissue throughout disease progression. Molecular US imaging was performed at different time points after carotid artery denudation in atherogenic diet fed mice to monitor accurately the reendothelialization of vessels (Curaj 2015). The results indicate that molecular US imaging is encouraging to evaluate vascular damage and to observe endothelial recovery after arterial interventions. US molecular imaging with a dual P- and E-selectin-targeted contrast agent can accurately detect acute inflammation in a murine model of chronic inflammatory bowel disease (Machtaler 2015). US was used to monitor the improvement of regional and global LV function in rat model of acute myocardial infarction after 5 escalating doses of c-kit (pos) cardiac stem cells were administered (Tang 2015).

US has often been used in the evaluation of venous thrombosis in the clinical setting and can be applied to evaluate thrombus formation and its resolution in the experimental setting. US technique can also be utilized to supply surrogate measures of thrombus composition. This technique has been applied to precisely assess clot age in a rat inferior vena cava (IVC) stasis model (Emelianov 2002). High-frequency US only enabled 2D data acquisition in either length or cross-sectional area for assessments of thrombus size. Three-dimensional (3D) acquisition systems often operated to quantify subcutaneous tumor development could be employed to reconstruct the thrombus and supply a better measure of thrombus burden (Ingram 2013). Fibrin and platelet integrin αIIbβ3 was able to be distinguished by antibody-targeted microbubble contrast agents at sites of arterial thrombus formation in a mouse xenograft model of human pancreatic cancer. These agents could be used for the evaluation of venous thrombosis (Wang 2012). Other agents are available to measure the activation of endothelial cell during atherogenesis (Kaufmann 2010; Weller 2003).

Measurements of blood flow in the vessel lumen have been employed in the evaluation of venous thrombus formation. Blood flow can be measured in the thrombosed segment utilizing an ultrasonic flow probe over a period of 30 to 40 minutes in mouse vascular injury model and mouse xenograft model of human pancreatic cancer (Cardenas 2011; Wang 2012). Time to occlusion of the vessel can be applied as a measurement of thrombus formation. Nevertheless, calculation of time to occlusion may be complicated by residual flow through the thrombosed vessel. Also, blood flow can be measured at fixed time points to monitor the degree of vessel stenosis. The technique has proven powerful to the effects of residual blood flow of the arterial system in murine model of ferric chloride-induced vena cava thrombosis (Wang 2006). Change in blood flow can be used as a measure of vessel stenosis although it cannot provide a direct measure of thrombus size. Ultrasonic blood flow measurement cannot be used in longitudinal studies of the same animal due to the invasive and terminal nature of this model.

MICRO-CT

The application of micro-CT imaging has increased in many preclinical studies over the last decade. The advantages of micro-CT include scanning efficiency, high resolution and

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relatively low cost compared to other imaging methods. Micro-CT scanner is according to the same physical principles as a clinical CT scanner and it is produced for higher resolution imaging. Micro-CT usually makes 3D tomographic data at microscopic resolution (voxel size $\leq$ 100 μm^3) by catching several hundred, 2D cone-beam projections from multiple angles around the animal (Holdsworth 2002). The raw projection data are kept on computer before image reconstruction is performed using dark current and flat-field images. Micro-CT is a morphological imaging technique where the differences in tissue densities are measured and compared. It is performed at an almost microscopic level compared to a clinical CT system for human imaging (Cavanaugh 2004). A typical micro-CT system includes an X-ray detector and an X-ray tube assembled in opposing positions in a rotating gantry. Micro-CT requires images with higher resolution although the physical principles are the same for both preclinical and clinical scanners. Special elements had to be presented, such as detectors with small components and X-ray tubes with smaller focal spots, to generate sharp images. Micro-CT supplies 3D tomographic data at microscopic spatial resolution (50 µm) and sub-second temporal resolution (50 ms) (Ritman 2011) of bone structures and soft tissues by catching hundreds of 2D projections from different angles around the animal (Cavanaugh 2004; Badea 2008; de Kemp 2010; Kagadis 2010).

X-ray Sources

The choice of the X-ray source greatly influences micro-CT system performance. Most X-ray tubes with micro-focus or mini-focus tubes (focal spot diameter < ~50 µm) work with extremely low photon output (on the order of 100-times lower) compared with the high-power tubes of clinical CT scanner due to the tradeoff between thermal loading of the source's metallic anode and focal spot size (Badea 2004). This fact causes a considerable increase in scan time for micro-CT to get within the noise level seen in clinical CT of about 5–10 HU. The most applied X-ray sources for micro-CT are micro-focus, fixed tungsten anode tubes working in continuous mode, with anode currents in the range of 50–1000 µA and voltages in the range of 20–100 kV. *In vivo* micro-CT systems working at low magnification frequently utilize pulsed X-ray sources with wider focal spots and higher power. The application of pulsed X-ray sources is particularly important for prospective electrocardiogram (ECG) gating (Badea 2008). This kind of imaging is also able to be performed with sources operating in continuous mode by utilizing external shutters; nevertheless, the total quantity of photons emitted during each pulse is extremely low when using shutters and the X-ray transmission through the closed shutter should be considered for appropriate raw data calibration. Additional X-ray tube models have been suggested. The application of a compact field-emission micro-focus X-ray source based on carbon nanotubes has been examined (Cao 2010; Cao 2009).

Open-type sources with thin-target spread anodes conquer the restrictions, supplying radiation beams with angular apertures in the range of 140°–160° with the feasibility of positioning the object almost in contact with the focus. However, the back of transmission targets cannot be chilled with liquid, decreasing the maximum possible power per unit area compared to reflection anodes. The spatial resolution available with this kind of source can be < 1 micron, which is similar to synchrotron-based imaging. However, synchrotron-based nano-CT and micro-CT are still superior to laboratory-level micro- and nano-CT systems

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concerning signal-to-noise ratio and contrast resolution because synchrotrons supply monochromatic, tunable radiation, avoiding beam hardening artifacts, and much higher photon fluxes, decreasing noise (Stolz 2011).

X-ray Detectors

The X-ray detector is a crucial part of a micro-CT scanner. Most of micro-CT systems hitherto apply digital flat-surface 2D detectors bringing to a cone beam scanning geometry. High-resolution CT was used to reconstruct a rat kidney from the digitization of multiple film radiographs (Kujoory 1980). Early prototype micro-CT systems used X-ray image intensifiers read by charge-coupled devices (CCDs) (Feldkamp 1989; Holdsworth 1993; Boone 1993). Combined recognition systems including scintillator screens coupled to CCDs via fiber-optic bundles, with various de-magnifying ratios, became the standard for micro-CT imaging (Paulus 2000; Goertzen 2004). More recently, advances in complementary metal oxide semiconductor (CMOS) technology caused to produce large area detectors with high frame rates which are the most broadly utilized systems for animal imaging (Lee 2003; Kalender 2007).

Because of their very low dark noise with respect to flat-panel CMOS detectors, cooled CCDs are still used, especially in applications involving low X-ray fluences. The application of single-pixel or small area detectors operating in photon counting mode (Paulus 2000) and direct conversion detectors coupled to thin-film transistor (TFT) arrays (Goertzen 2004) for animal micro-CT was reported. Energy discerning photon counting X-ray detectors (PCXD) are the subject of concentrated study and pledge to make spectral CT possible. Actually, micro-CT is the experimental stage for spectral CT using PCXD (Taguchi 2013). PCXDs with energy binning can enhance CT performance by counting and binning each X-ray detected into a number of energy bins identical to the number of energy thresholds per pixel. PCXDs enable for the removal of dark noise in the image by refusing all counts under the signal and also enable for spectral segregation. The technology is at its beginning stage and will grow rapidly. Sensor materials for PCXDs comprise silicon, gallium arsenide, and cadmium telluride with pixel sizes as small as 55 μm (Taguchi 2013). The spectral detectors cannot immediately replace the conventional, energy-integrating detectors for biomedical CT applications due to technical limitations (Taguchi 2011).

System Geometry

Two common system geometries for micro-CT are rotating gantry and rotating specimen. The majority of *in vivo* micro-CT systems apply a rotating gantry geometry in which detector and the X-ray tube are assembled in a single gantry. The gantry rotates around a central axis while the specimen or the animal reclines stationary on a table between detector and the tube. Most *ex vivo* micro-CT systems use rotating specimen geometry where the X-ray source and detector are immobile. It is more feasible to modify the position of the specimen and alter magnification for rotating specimen systems than in rotating gantry systems. In producing micro-CT systems with either geometry, a balance must be reached between the required spatial resolution, the system dimensions, the focal spot size of the X-ray source, and the

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photon fluence created by the X-ray source. Placing the specimen farther from the detector and closer to the X-ray source increases geometric magnification, enhancing spatial resolution while decreasing the field of view covered by the detector. The magnification can be increased up to the limits established by penumbra blurring or the system dimensions. Penumbra blurring illustrates the X-ray projection blur depending on the finite size of the system configuration and the X-ray tube focal spot (Badea 2008). If source to specimen distance and the specimen to detector distance are similar, as in nearly all of rotating gantry systems, penumbra blurring is comparable to the focal spot size.

To enable the use of a high fluence X-ray source with a large focal spot, the specimen can be put close to the detector, limiting spatial resolution, but allowing extremely short exposures for rodent cardio-respiratory imaging. The configuration has been indicated for dynamic, *in vivo* micro-CT imaging in rotating specimen geometry (Badea 2005). Regardless of system geometry, geometric calibration is an extremely vital need for successful image reconstruction (Johnston 2008). Zoom-in capability and variable geometry is provided in some scanners (Panetta 2012). With such changes, geometric calibration is needed to boost spatial resolution for reconstructed image data. Automated calibration techniques have been established which assess geometric parameters depending on projections of a generic object (Panetta 2008) and by reconstruction-based optimization of image sharpness metrics (Wicklein 2012).

Contrast Agents

The poor contrast sensitivity in biological soft tissue is one of the major challenges for CT imaging. Different staining techniques increased soft tissue contrast for *ex vivo* specimens scanned by micro-CT. Osmium staining has been shown successfully in several micro-CT applications (Johnson 2006). Inorganic iodine and phosphotungstic acid staining are simpler to manipulate with much less toxicity compared to osmium. Inorganic iodine and phosphotungstic acid produce high-contrast X-ray images of many soft tissues (Metscher 2009).

Vascular contrast agents for *in vivo* micro-CT imaging include low molecular weight and blood pool. Iodine-based, low molecular weight contrast agents utilized for clinical CT imaging applications can be applied for preclinical micro-CT in rodents. But such agents remove from mouse vasculature within seconds (Lin 2008). Iodine-based, blood pool contrast agents (e.g., Fenestra from ART, eXIA from Binitio Biomedical) supply stable enhancement over the course of minutes to an hour which facilitates translation of clinical imaging applications to rodent model systems (Ashton 2014). Larger nanoparticle contrast agents dependent on variety of high atomic number elements display great potential for functional and molecular CT imaging applications and supply fine control over vascular half-life. Nanoparticle contrast agents are applied to reduce quick renal clearance (Ashton 2015).

Technological advances in the field of CT have promoted the progress of high-resolution micro-CT imaging platforms suitable for preclinical application. The applications of micro-CT include the assessment of skeletal and lung abnormalities, heart function, and tumor growth. Micro-CT is a powerful technique for the quantitative assessment of angiogenesis related to solid tumors or ischemia (Kagadis 2010, Badea 2008). CT is the favorite technique for lung imaging due to the high contrast yielded by air/tissue differences in the preclinical

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situation; imaging of such small and moving structures has been challenging (Badea 2008). Respiratory gating devices were developed recently to reduce breathing motion-related artifacts (de Kemp 2010). Micro-CT was able to dynamically evaluate the topographic distribution and progression of lung fibrosis in small animal models (Zhou 2015). CT is totally dependent on the quantification of X-ray attenuation by the tissues, meaning that fine differences are very hard to differentiate due to almost indistinguishable attenuation characteristics. In an attempt to defeat this limitation, contrast agents might be used to increase CT sensitivity by highlighting either the vascular tree or specific areas of organs or tissues. The most commonly utilized CT contrast agents are iodinated water-soluble compounds (Lee 2013).

Contrast-enhanced micro-CT has been used widely in the study of murine models of cardiovascular diseases, including critical limb ischemia (Takeda 2011), abdominal aortic aneurysms (Nahrendorf 2011), and myocardial infarction (Nahrendorf 2007). Visualization of the vasculature by micro-CT needs intravenous administration of high molecular-weight blood-pool contrast agents (Nebuloni 2013). Contrast-enhanced micro-CT is able to longitudinally measure thrombus resolution as shown in a murine model of IVC stenosis (Grover 2015). Contrast-enhanced micro-CT needs segmentation of thrombus from surrounding tissue for 3D reconstruction and extraction of volumetric data. The strength of this technique is the ability to get high-resolution images with reconstructed voxel dimensions in the range of 20 to 60 μm (Grover 2015). Limitations of this technique for thrombus imaging include the cost of the imaging time, the application of expensive and potentially nephrotoxic contrast agents and the high doses of ionizing radiation needed. Furthermore, contrast-enhanced micro-CT supplies only anatomical data. It cannot provide information on thrombus composition as can be acquired by MRI.

Cardiac Imaging

Micro-CT was used for cardiac morphological and functional imaging in mice and rats (Badea 2007; Nahrendorf 2007; Badea 2008; Detombe 2008). Contrast agents are used to differentiate between the myocardium and blood. Cardiac functional metrics such as cardiac output and ejection fraction was determined by 4D data obtained after blood pool contrast agent is used in most cardiac micro-CT studies. The exactness of these measurements was assessed as a role of contrast agent volume and projections number applied for reconstruction (Badea 2008). Deduction of the volume of contrast agent decreased hypervolemia effects on the hemodynamics and reduction in the number of projections directly reduced radiation dose. The total quantity of contrast agents injected may be further decreased for blood pool contrast agents with higher iodine concentration. eXIA 160 is a contrast agent with a high iodine concentration (160 mg I/ml). The injection quantity was 0.1 ml per 20 gram in a mouse for cardiac micro-CT (Detombe 2012).

Both global cardiac functional metrics (myocardial mass, ejection fraction, cardiac output and stroke volume) and localized dynamic metric (wall mall motion) can be computed using 4D cardiac micro-CT data sets. Numerous software packages (Yushkevich 2006) and algorithmic techniques (Suri 2000; Frangi 2001) are available for semi- and fully-automated cardiac segmentation. Efficient, fully-automated cardiac segmentation is extremely useful for

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structural and functional phenotyping practices. Micro-CT was able to illustrate cardiac anatomy and function in a genetic model of dilated cardiomyopathy (Badea 2007).

Micro-CT was first applied for weekly imaging in mouse models of myocardial infarction (MI) to monitor left ventricular (LV) remodeling post-infarction (Detombe 2008). 3D cine micro-CT reliably and rapidly quantified infarct size and evaluated murine anatomy and physiology after coronary ligation using delayed contrast enhancement by combining a low molecular weight contrast agent and the blood pool contrast agent Fenestra VC (Nahrendorf 2007). This efficient imaging tool is a precious supplement to the current phenotyping apparatus and will enable rapid testing of novel drugs and cell-based interventions in murine models. The dose administered to the animal needs to be assessed for *in vivo* micro-CT. 4D micro-CT techniques with better image reconstruction algorithms have reduced the dose to 0.2 Gy (Badea 2011; Sawall 2011; Armitage 2012) compared to 1 Gy in PG-based cardiac micro-CT (Badea 2005).

Lung Imaging

Micro-CT is very practical for the lung imaging without extra contrast agents due to density difference between soft tissues and air-filled lungs. Although CT is a powerful technique for lung imaging, micro-CT lung imaging in animal models has been challenging because of their small size and rapid respiratory motion. A voxel size on the order of 75 μm is needed to supply anatomic resolution in the mouse similar to that acquired in CT for humans (Ritman 2005). Different gating strategies were performed to obtain each projection at the same point during each respiratory cycle. To control the respiration by mechanical ventilation and intubate the animal is a successful technique of respiratory gating (Hedlund 2002; Namati 2006). Projections can be obtained at precisely identical point in every respiratory cycle (Badea 2004). Micro-CT with respiratory gating has been applied to investigate lots of lung diseases. Micro-CT can be utilized to continuously observe lung metastases in mice (Li 2006) and monitor the lung tumor growth (Hori 2008; Namati 2010; Rudyanto 2013; Li 2013). Micro-CT was used to evaluate the effect of radiation therapy (Kirsch 2010; Perez 2013) or chemotherapy (Ueno 2012) on mice lung tumor model and pulmonary damage caused by radiation therapy (Saito 2012).

Micro-CT can be applied favorably to investigate different pulmonary disease animal models, such as emphysema and fibrosis. An emphysema model was induced in mice by using intratracheal instillation of pancreatic elastase and imaged with micro-CT (Postnov 2005). Emphysemic regions have indicated lower HU values compared to the normal lung and the lung volume in emphysemic animals was enlarged. Fibrosis models also have been induced in mice by instillation of bleomycin and monitored longitudinally with micro-CT during disease progression (Shofer 2007). Micro-CT can detect the fibrotic lung regions by the resulting increase in HU. Moreover, micro-CT can evaluate lung compliance in small animals by executing breath-holding over a range of specified pressures and determining lung volumes from the corresponding images to make a pressure-volume curve (Shofer 2007; Guerrero 2006). The loss of pulmonary compliance was evaluated in a study of irradiated mice (Guerrero 2007).

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Micro-CT was used to monitor the progression of emphysema in elastase-induced emphysema mice model (Artaechevarria 2011), cigarette smoke-induced pulmonary inflammation mice model (D'hulst 2005), cigarette smoke-induced emphysema mice model (Shan 2014; Sasaki 2015) and lipopolysaccharide-induced emphysema mice model (Kobayashi 2013; Chen 2016).

Liver Imaging

Micro-CT with liver-specific contrast agent was used to identify liver metastasis in an experimental model of animal pheochromocytoma (Martiniova 2010). Contrast-enhanced micro-CT measurements of hepatic fractional extracellular space and macromolecular contrast material uptake were used to evaluate the degree of early hepatic fibrosis in a rat model (Varenika 2013). Micro-CT was used to evaluate the effect of partial hepatectomy on the hepatic hemodynamics in a rat model (Debbaut 2012). Micro-CT can be used for the first time to observe the liver regeneration of transplanted hepatocytes in a mouse model (Hickey 2015).

Micro-CT with the contrast agent ExiTron nano6000 was used to determine three mouse models of liver injury caused by alcohol, lipopolysaccharide/D-galactosamine and bile duct ligation (Hua 2015). Micro-CT was used to scan isolated rat livers injected with the contrast polymer Microfil (®) which detected hepatic arteriolo-portal venular shunts (Kline 2014). Contrast-enhanced micro-CT was able to precisely observe angiogenesis and antiangiogenic therapy effects in chronic hepatic injury in mice induced by carbon tetrachloride- or bile duct ligation (Ehling 2014).

Bone Imaging

Micro-CT is very useful for high-resolution, non-invasive bone imaging due to the natural contrast between soft tissues and bone. Micro-CT was shown as successful technique for exploring the development of rodent knee longitudinally in a rodent surgical model of osteoarthritis (Appleton 2007; McErlain 2008). Study in critical sized rat calvarial defects suggested that micro-CT was a useful supplement to other techniques for objective assessment of bone tissue engineering and regeneration (Cowan 2007). Micro-CT is able to precisely measure different bone parameters, such as trabecular thickness, cortical thickness, bone mineral density, bone volume, bone surface ratio, and cross-sectional area (Bouxsein 2010). Parameters such as trabecular thickness, bone surface ratio, bone volume ratio and volumetric bone mineral density were measured by micro-CT in the study of bone architecture changes over time (Waarsing 2005). These micro-CT bone structural parameters were applied to quantify the results of stem cell treatments (Lee 2009) and vascular endothelial growth factor (Li 2009) in bone loss, cortical thickening and bone healing in total body irradiation mice with bone marrow transplantation (Dumas 2009).

Micro-CT was used to estimate the short- and long-term effects of estrogen deficiency on trabecular bone of ovariectomized rats (Laib 2001). The calvarial defect animal model is often used to study wound healing and bone regeneration. Micro-CT supplies a precise and rapid method to measure the bone mineral content within calvarial defects in the rat. The

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advantages of micro-CT analysis comprise highly quantitative measurements, small coefficient of variation and the ability for serial measurements in the same living animal (Umoh 2009). Micro-CT is very useful to precisely calculate the 3D structure of engineered bone scaffolds, with especial emphasis on connectivity and porosity (Lin 2005; Ho 2006).

Neovascularization is important for the regeneration of fracture bone. First study using micro-CT for microvascular morphology in an osteoporotic critical size defect in the clinically relevant localization of the femur metaphysis was reported (Kampschulte 2015). Neovascularization indicated notably different morphological feature when compared to the non-injured side. The description of microvascularization may be useful for the further assessment of biomaterials and their effect on angiogenesis and their influence on improved bone repair in animal model. Micro-CT scan performed 3 months after vascularized elbow allotransplantation in the rat to verify union in rats treated with cyclosporine and show the joint destruction in the nonimmunosuppressed rats (Tang 2015). Micro-CT was used to monitor the quantification of fracture healing response in a standardized mouse model for combined injury of traumatic brain injury and femoral osteotomy (Tsitsilonis 2015).

Micro-CT supplies a dependable platform for preclinical imaging that is complementary to other animal imaging techniques for many morphological and functional imaging applications. Recently developed contrast agents and innovative acquisition and reconstruction strategies display remarkable promise in overcoming the limitations including low contrast and radiation dose. Combined with powerful new opportunities in spectral and phase contrast imaging, these advances will surely continue to enlarge the applications of micro-CT in experimental surgery.

Vascular Imaging

Micro-CT vascular imaging was performed essentially with blood pool contrast agents. Micro-CT scan was longer than clinical CT scan because of higher resolution need. Low molecular weight contrast agents were not able to be utilized for vascular imaging with early micro-CT scanners due to long scan time. Micro-CT scan can be completed in minutes now. Low molecular weight contrast agents were utilized for vascular imaging of squamous cell carcinomas nude mice model (Kiessling 2004) and mice models of subarachnoid hemorrhage, stroke and cancer (Schambach 2010). But, the contrast agents needed to be continuously or repeatedly provided during the scan to reach a constant level of vascular enhancement. Blood pool contrast agents were very effective for different vascular imaging, such as vascular permeability measurement in rat sepsis model (Langheinrich 2006), vasculature imaging in mice liver metastasis model (Graham 2008), lung vasculature imaging in rats and mice models of pulmonary disease (Johnson, 2007), liver vasculature imaging in mice liver ischemia and reperfusion model (Chouker 2008) and assessments of vascular morphology, diameter, and branching in mouse vascular corrosion cast model (Vandeghinste 2011).

Tumor Imaging

Micro-CT has been confirmed to be functional in tumor perception and tracking, as well as in imaging tumor angiogenesis. Micro-CT was able to detect murine lung nodules with a

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minimum volume of 0.63 mm3 (Cody 2005). Cancer imaging with micro-CT is closely connected to the administration of contrast agents. Dynamic micro-CT has been used for the direct measurement of perfusion in tumors following the administration of conventional contrast agent (Kan 2005; Phongkitkarun 2004). The rapid scans frequently need a decrease in spatial resolution in the longitudinal direction to reach sufficient coverage in the longitudinal direction. CT perfusion measurements allow the quantitative assessment of functional parameters, such as permeability-surface area product, blood volume and blood flow. Blood pool and nanoparticle contrast agents are favored for cancer imaging with micro-CT.

Micro-CT with Fenestra VC had the best capability for determining vessel diameter, tortuosity, and density for alveolar rhabdomyosarcomas in a transgenic mouse model. But instrument with a higher resolution was required to visualize tumor capillaries (Kindlmann 2005). Micro-CT scan was used to monitor nude mice bearing STC-1 tumors 15 and 30 days after grafting (Almajdub 2007). Micro-CT scan was performed at 2 and 4 hours after animal received the Fenestra LC injection. Live tumors (0.3–1.5 mm) at day 30 were detected by micro-CT scan with Fenestra LC. Micro-CT scan enabled quantitative follow-up of tumors both in the liver and the spleen up to 15 days after a single Fenestra LC injection.

The main advantages of micro-CT over other imaging modalities are short time required for scanning, detailed morphological information and high spatial resolution images. However, the radiation load related with CT imaging, and the volume of contrast agents (used to enhance the contrast resolution) that may be injected into a single animal, are possible limitations of which researchers should be aware. Normally, based on the desired image contrast and resolution, modern scanners supply short acquisition times with radiation doses in the range of 10–50 mGy and resolution levels are below 100 μm (Kalender 2011). Balance between animal irradiation and image quality needs to be considered in order to avoid unnecessary radiation exposure.

Micro-CT was used to monitor the development of lung tumors in a mouse model of lung adenocarcinoma and dynamic changes in response to treatment. The accuracy of micro-CT was validated with end-point histological quantification (Hegab 2016). Contrast-enhanced micro-CT was used to monitor tumor growth of orthotopic glioblastoma multiforme in nude mice (Yahyanejad 2014). Tumor size detected by micro-CT was well correlated with tumor size acquired by histologic analysis. The precision of micro-CT with contrast agent for mice glioma xenografts was evaluated (Kirschner 2015). Longitudinal micro-CT imaging of brain tumor growth in mice was practicable with high sensitivity and the radiation effects were able to be observed.

The function of macrophages in prostate cancer skeletal metastasis was evaluated by micro-CT in macrophage-ablated mice (Soki 2015). Skeletal tumor growth in macrophage-ablated mice was markedly reduced compared to control mice. Micro-CT results suggested that bone volume was increased in mice treated with clodronate.

Micro-CT with contrast agent exitron nano 12000 enabled automatic analysis of tumor lesions for evaluation of cancer size in mouse hepatocellular carcinoma (Rothe 2015).

Micro-CT localized and determined the volume of liver tumors after intrahepatic or intrasplenic graft of Hep55.1C cells in mice (Bour 2014). The robotized intratumoral injection of low amount of Doxorubicin considerably decreased tumor growth by about 60% after 3 weeks, whereas manual intraperitoneal administration of the same amount of drug had no notable effect on tumor growth.

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Brain Imaging

The new Micro-CT blood-brain barrier imaging method with the intra-arterial infusion of iopromide enabled serial quantitative visualization of post-stroke blood-brain barrier dysfunction in mice, with high resolution and in a sensitive manner (Park 2014). Micro-CT with iodine-based contrast was used to differentiate the total differences in the overall mice brain shape caused by experimental treatment (Anderson 2015). Micro-CT was performed to confirm effective blood delivery into the cisterna magna in murine single-blood-injection subarachnoid hemorrhage model (Kamp 2014). Micro-CT was able to recognize most major cerebral vessels and their minor branches in mouse models (Ghanavati 2014).

OI

OI contains different techniques that have in common the application of a set of respective sensing devices and light sources to capture the resulting photon distribution (Bremer 2003). These techniques are classified according to the type of source-detector setting and the contrast mechanism involved. The activity of the chosen molecular targets was able to be measured after targeted fluorescent or activatable probes were developed. Imaging of such probes needs the excitement of the probe at a definite wavelength and identification of the specific signal emission at a significantly different wavelength. The most applicable OI techniques include bioluminescence, fluorescence, and near-infrared fluorescence imaging (Bremer 2003). For image acquisition, the animal needs to be placed in a light-tight imaging enclosure where the emitted light is subsequently caught by a CCD. Extremely sensitive detectors have been made in order to expose visible to near infra-red light emitted from the body. CCD detectors were made of silicon crystals sliced into thin portions for assembly into integrated circuits (Massoud 2003). CCD cameras operated by converting light photons at wavelengths between 400 and 1,000 nm, that strike a CCD pixel with energy of only 2–3 eV (Massoud 2003). The principle of bioluminescence imaging involved the emission of visible photons at specific wavelengths depending on reactions catalyzed by enzymes (luciferases) existing in many organisms as protists, fungi, insects, bacteria, among other species (Wilson 1988). Luciferases activated the oxidation of luciferins (substrate) to generate non-reactive oxyluciferins and release photons of light (Greer 2002). Advances in molecular techniques have caused the isolation of many luciferase genes that have been applied to build DNA vectors (Wilson 1988). The most frequently applied varieties of luciferase–luciferin are the firefly (Fluc) (Greer 2002).

Highly sensitive imaging systems enabled the quantitative identification of small numbers of cells or organisms that expressed luciferase as a transgene (Weissleder 2001). This technique required the target cells to have been previously genetically transduced *ex vivo* in order to express the luciferase gene. The luciferase substrate (luciferin) is then orderly supplied to achieve the target cells. Then it is oxidized by luciferase and emitted photons that are captured by CCD cameras (Bremer 2003).

A bioluminescence image is frequently displayed as a color image that is overlapped on a gray-scale photographic image of the small animal using overlay and image analysis software. To discover very low levels of signal is the main advantage of optical

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bioluminescence imaging. The principle of fluorescence imaging is dissimilar to that of bioluminescence. Cells can be either genetically transduced to express a fluorescent probe like a fluorescence-labeled antibody or a fluorescence molecule such as the green fluorescent protein (GFP) (Massoud 2003). In near-infrared fluorescence imaging, light in the 700- to 900-nm range is used to boost tissue penetration and reduce autofluorescence from non-target tissues (Weissleder 2002).

Diffuse optical tomography (DOT) showed better imaging performance as multiple source-detector pairs were applied to build 3D images with good spatial resolution, high sensitivity, accurate quantification and volumetric localization (Culver 2008). However, effective resolution of bioluminescence, fluorescence, or NIR imaging is still substandard and the resulting images are not quantitative. Because factors such as attenuation, the scattering, and dispersion of the emitted light as it penetrates tissue layers mean the captured signal is mostly based on the depth of the tissue of origin (Weissleder 2001). A more recent technique to fluorescence imaging of deeper structures utilized fluorescence mediated tomography (Ntziachristos 2003) and applies multiple projections and quantifies light around the boundaries of the object. Reconstructed images could be acquired with a fluorochrome detection threshold in the nanomolar range and a resolution of 1–2 mm after mathematical processing (Massoud 2003). Contrary to planar images, these 3D images were quantitative, as the signal intensity had direct connection with the local concentration of fluorochrome (Ntziachristos 2003).

The photo-acoustic tomography (PAT) is an imaging technique that benefits from the values of pure OI or ultrasound imaging, without the major disadvantages of each technique. It was performed by illuminating the sample with short pulses of laser beams and collecting the ultrasound waves produced by the photo-acoustic effect. The target tissue absorbed a fraction of incident light pulse energy and converted into heat to cause a rise in the temperature and a thermal expansion of the object. This increase in pressure propagated as a sound wave, which was able to be discovered externally as an acoustic signal (Wang 2012). PAT has achieved enormous development in the past decade and its main preclinical applications include imaging of tumor microenvironments, drug response, angiogenesis, and biomarkers (Wang 2012).

OI established on high-energy beta particle-emitting radionuclides is the more recently discovered variant of this imaging modality (Robertson 2009). It displayed that positron-emitting (β+) radionuclides can supply perceptible light, compatible with Cerenkov radiation, with a continuous spectrum that is weighted towards blue bands of the electromagnetic spectrum and the ultraviolet. Charged particles moving at very high speeds can produce Cerenkov radiation. In certain media, the emission of optical photons can be caught by CCD cameras. Cerenkov radiation is made in a persistent spectrum from the near ultraviolet through the observable spectrum (Robertson 2009). Recent works (Liu 2010; Robertson 2009) showed that images may be produced with ordinary OI equipment by the low-energy window of light (1.2–3.1 eV, 400–1,000 nm) based on beta particle-emitting (β^+ and β^-) radionuclides. The radionuclide agents that have already been evaluated contain 18F-FDG, Na18F, Na131I, 90YCl3, and several 90Y-labeled peptides. The superiorities of radionuclide OI/Cerenkov imaging over the traditional OI techniques basically associate to the fact that imaging probes are the same as those applied in the clinical context, showing a broad emission spectrum, enabling to track the radioactive probe at different wavelengths without excitation light. Nevertheless, some limitations of this technique, including limited tissue

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penetration and comparatively bad quantification ability compared with PET and SPECT are common to other OI techniques (Liu 2010).

The most powerful advantages of OI include the feasibility of simultaneous imaging of six anesthetized mice, determination of physiological and pathophysiological processes at the molecular and cellular level *in vivo* with high specificity, low cost, short acquisition time, relatively high throughput and the high sensitivity (Massoud 2003; Vooijs 2002). An extra advantage of OI is to produce multichannel imaging using several probes with different spectral characteristics. Fluorescence imaging (FLI) produces high-resolution, real-time mapping with the application of a contrast agent and can considerably intensify intraoperative imaging. Optical projection of acquired luminescence (OPAL) provided increased visualization for lymph nodes from every anatomical location in pigs with intradermal injection of indocyanine green (Ringhausen 2015). FLI was used to evaluate the blood perfusion of the animal with implantation of a bone autograft and a blood-borne fluorescent agent. Bone formation was measured by FLI using a hydroxyapatite-targeted probe and μCT analysis (Cohn Yakubovich 2015).

Combination of bright-light surgery (BLS) and fluorescence-guided surgery (FGS) considerably reduced the residual fibrosarcoma volume and ameliorated disease-free survival in retroperitoneal-implanted human fibrosarcoma nude mice (Uehara 2015). FGS notably decreased recurrence occurring in multiple sites including the lung, liver and other organs compared to BLS in an experimental colorectal liver metastasis nude mouse model. Furthermore, FGS markedly extended disease-free survival and overall survival compared to BLS (Murakami 2015).

MICRO-SPECT

The selection of the collimator for SPECT has an important effect on the resolution and sensitivity of the system (Van Audenhaege 2015). Pinhole collimators are utilized to acquire submillimeter resolution and multiple pinholes are usually integrated to raise sensitivity for small animal imaging. Detectors with better essential spatial resolution and current collimator production methods are improving collimation.

SPECT is based on the tracer principle which requires the injection of tiny amounts of radioactive tracers and the outside evaluation of their bio-distribution by proper detectors. Tracer molecules less than 10^{-10} molar can be discovered by SPECT *in vivo* with submillimeter (0.5–0.7 mm) resolution, allowing the measurement of the molecular processes in which radioactive tracers are involved (Meikle 2005). The spatial resolution and system sensitivity have been greatly improved after the application of multi-pinhole collimation has become the standard in most preclinical SPECT systems (Peterson 2012). The increase of the number of pinholes and/or the angular sampling for a fixed field of view (FOV) can ameliorate the sensitivity (Kagadis 2010; Khalil 2011). The added pinholes may reduce the sensitivity and reconstruction artifacts may finally appear (Peterson 2012). Thus, the application of a high-sensitivity, non-multiplexing, multi-pinhole approach might be considered a step forward.

Advances in electronics have not only increased sensitivity, but also greatly reduced the whole equipment size and cost (Kagadis 2010; Peterson 2012; Khalil 2011). Some preclinical

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SPECT systems included semi-conductor materials, such as cadmium zinc telluride (CZT) or silicon and directly transformed gamma rays to electric signals (Franc 2008; Peterson 2011). These detectors provided excellent spatial resolution as low as 0.38 mm and energy resolution, especially valuable for low-energy radionuclides (as iodine-125) or dual isotope applications (Franc 2008).

The application of scintillation crystals coupled to Photomultiplier tubes (PMT) is very usual. In recent years, position evaluation based on anger logic principles has indicated that hardware progress is the basis of a substantial growth in available computational power. This has enabled the inauguration of more sophisticated algorithms in data processing, basically iterative algorithms, as the maximum-likelihood evaluation and resolution recovery advanced solutions, thus enhancing global performance of systems (Peterson 2012).

SPECT emitters are well-suited for radiolabeling different molecules, including endogenous biomolecules, such as selectins, hormones, peptides and antibodies. These biomolecules are comparatively large and blood clearance is low. This favored the use of radionuclides with longer half-lives in order to widen the temporal window of observation, in contrast to positron emitters (Meikle 2005).

MI could be well imagined using Na [18F] F PET/CT (Han 2015). Na [18F] F uptake was approximately connected with myocardial apoptosis but not with calcification. Na [18F] F PET/CT was positive at the perfusion deficit area in [99mTc] MIBI SPECT/CT and showed almost the same signal distribution with [99mTc] HMDP SPECT/CT. The results suggested that Na [18F] F PET/CT could be a promising hot-spot imaging modality for MI. Na [18F] F PET/CT had few limitations for the early identification of MI, because the highest uptake of Na [18F] F manifested as late as 1 day after MI (Han 2015).

MICRO-PET

PET imaging is dependent on discovering two time-concurring high-energy photons from the emission of a positron emitting radioisotope. The physics of the emission, and the recognition of the concurring photons, yield PET imaging special abilities for both extremely high sensitivity and precise evaluation of the *in vivo* radiotracer concentration (Vaquero 2015). PET imaging has been broadly applied for clinical neurology, oncology and cardiology investigation. PET imaging has been used as a vital technique in preclinical investigations, especially for evaluating animal models of disease.

PET presents a very important development in the efforts to ameliorate the knowledge of neurodegenerative disorders such as Alzheimer's disease (AD). PET produces the most dependable perceptible biomarkers for the clinical diagnosis (Shimojo 2015). PET is a well-recognized clinical and preclinical imaging technology which depends on the use of compounds labeled with positron-emitting radioisotopes to image and to quantify several biochemical and physiological processes (Phelps 2000). It is widely accepted as a fundamental tool for biomedical research because of its molecular, non-invasive, non-destructive intrinsic nature and the ability to study many biochemical/biological processes *in vivo* (Phelps 2000). PET is used in different fields from oncology to neurology and cardiology. The most commonly used positron emitters include 18F, 15O, 13N, and 11C, but other isotopes such as 124I, 94mTc, 89Zr, 76Br, 68Ga, 60, 61, 64 Cu are gaining increasing

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attention. In fact, peptides labeled with 68Ga have already indicated very promising results in the assessment of neuroendocrine tumors (Kowalski 2003). 89Zr is well-suited for the labeling of monoclonal antibodies due to more time for tumor targeting and optimal biodistribution (Holland 2009). Comparably, 124I has shown higher efficacy in lesion recognition and the ability to supply lesion-specific dosimetry in the context of differentiated thyroid cancer (Basu 2011). These radionuclides are difficult to acquire for many research groups due to their production method, the relative complexity and low yield, although these radionuclides enable the study of many physiological and biochemical processes (Fass 2008). Furthermore, their relatively short physical half-life makes distribution over long distances unfeasible or very expensive. Actually, the application of this imaging technique may need in situ radioisotope production and compound labeling because of the short physical half-lives of most positron emitters. Therefore, the existence at close distance of a multidisciplinary and highly skilled/specialized team is needed.

Actually, lots of radiotracers can only be utilized if they are made locally. So it is now considered ideal for a preclinical facility to be near a radiopharmaceutical production facility. Nevertheless, recent progresses in cyclotron technologies might overcome those aspects in the future. A perfect PET scanner designed for animal imaging would have the following characteristics: high spatial resolution (sub-millimeter range), high sensitivity, detector ring with a FOV optimized to the specific targeted animal size range, good temporal resolution, and multimodality imaging capability (Levin 2007; Yao 2012). Many commercially accessible micro-PET scanners have resulted from previously developed prototypes in academia.

Micro-PET detector blocks include inorganic scintillators, such as lutetium oxyorthosilicate, lutetium-yttrium oxyorthosilicate, or gadolinium orthosilicate, which have substituted the old bismuth germanate scintillators with their more suitable speed, light output, and detection efficiency (Levin 2007; Hutchins 2008). Micro-PET instruments produce much better sensitivity and spatial resolution compared to clinical scanners. These scanners can reach spatial resolution values of around 1.0 mm in reconstructed images, but only conjugating the use of radioisotopes with low energy positrons (from 18F, for instance) with a state-of-the-art equipment that could achieve pixel size less than 1.2 mm and an intrinsic resolution of 0.7 mm (Lecomte 2004). Over the years, PET has been inaugurated as a powerful and reliable instrument for biomedical research. The application of PET is most determined by the presence of an 'on site solution' for radiopharmaceutical production.

Micro-PET application has been increasing significantly in industrialized countries, although it has these relevant potential limitations (Comley 2011). (13)N-NH3-micro-PET scan was used to evaluate the cardiac and renal perfusion in myocardial infarction (MI) rats induced by permanent coronary artery ligation. Cardiac perfusion was obviously decreased in MI group, directly associated with ejection fraction and inversely related to MI size. Renal perfusion exhibited non-significant decrease in MI group compared to sham group. (13)N-NH3-microPET could be a potential tool for assessment of cardiac and renal functional and perfusion changes in rat MI models (Juárez-Orozco 2015).

PET detects the tumor according to the metabolism difference between tumor and normal tissue. The metabolism of the transplanted B16 melanoma in the lung of mice was detected by micro PET/CT with [(18) F] fluoro-2-deoxy-D-glucose (FDG). It was difficult to detect the tumor smaller than 1 mm by micro PET/CT. The lung cancer had a notably higher metabolic rate compared with the normal lung tissue (Qiao 2014). Micro-PET with 4-[18F]-ADAM (a

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serotonin transporter imaging agent) was able to be applied to detect serotonergic neuron loss, monitor the progress of Parkinson's disease and evaluate the effectiveness of therapy in a Parkinsonian rat model (Weng 2013). PET was a valid method for tracking the inflammatory response in an experimental model of acute lung injury (Zambelli 2012).

Regional cerebral blood flow (rCBF) measurement in rats can obtain the pathophysiologic information of the cerebral circulation. The AV-shunt method enabled less invasive, quantitative and duplicable assessment of rCBF in [15O] H_2O PET studies in rats than direct blood sampling and radioassay (Ose 2012).

The esophageal lesions detected by the micro-PET scan with (18) F- FDG were consistent with the changes found in endoscopic observation and histopathology in esophageal mucosa in rat model of esophagoduodenal anastomosis (EDA) (Schiffman 2010). The epidermal growth factor receptor (EGFR) was the target of Erlotinib (Tarceva); Erlotinib was frequently overexpressed in lung cancer and was able to be labeled with [(11) C] by reacting the normethyl precursor with [(11) C]-methyl iodide. Dynamic micro-PET with [11C]-labeled Erlotinib distinguished Erlotinib-sensitive lung cancer in mice with lung tumor xenografts (Memon 2009).

The Goto-Kakizaki (GK) rat, a type 2 diabetic rat model, is preferred for evaluating the surgical effect on diabetes. [11C]-Dihydrotetrabenazine micro-PET scanning is a practicable technique for evaluating the beta-cell mass in GK rodents after metabolic surgical procedures and could prove to be an important modality for assessing beta-cell performance in type 2 diabetes (Inabnet 2009).

MRI

MRI is a non-ionizing 3D imaging technique that employs tissues magnetic properties and their interplays with strong outside magnetic fields. Hydrogen nucleus (1H) from water molecules is most applied in MRI imaging because of its paramagnetic properties and its ubiquitous body distribution. In short, the underlying principle is that, when a specimen in a magnetic field is undergone to a radio-frequency pulse, its protons consume energy and make a measurable signal during the relaxation phase that can be numerically encoded through magnetic field gradients to make digital images. The power of the signal is a function of the number of protons. The dissimilarities of those protons between tissues determine the demonstration of the image (Lewis 2002; Pomper 2005; Dufort 2010; Tremoleda 2011). Water owns a lot of biophysical magnetic signatures in organs and tissues, and the way to achieve goals in different experiments is optimizing experimental parameters and methods in order to increase contrast between normal and abnormal tissue (Lewis 2002).

MRI produces morphological images with excellent contrast and spatial resolution, moreover, information about metabolism, tissue elasticity, tissue composition, oxygenation, perfusion, and identification of molecular probes is obtained in a single acquisition without radiation exposure (Koba 2011). These attributes have increased the widespread utilization of MRI (Comley 2011). Preclinical MRI scanners need a tenfold increase in spatial resolution in each dimension compared with clinical scanners, resulting in signal decreases of at least 1,000. Stronger gradient sets, specific receiver coils, radiofrequency receiver chains, and stronger magnets were established in order to conquer this challenge (Jakob 2011). Animal

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MRI scanners should be ideally higher than 7.0 T since the signal-to-noise ratio increases with the magnetic field. Actually, committed small bore MR devices can run between 4.7 and 21 T. If requirements regarding spatial and temporal resolution are not especially strict, clinical scanners may be an acceptable alternative as long as optimized pulse sequences and special radiofrequency coils are used (Jakob 2011; Brockmann 2007).

Several MRI techniques are available for the investigation of many biological processes. Diffusion-weighted imaging (DWI) is able to describe the movement of protons in tissues which are mainly from the water molecule during a period of time. Molecular diffusion is the consequence of the interplay of molecules with different 'barriers' such as fibers, membranes, cellular organelles and other molecules. The information about tissue architecture and cellularity can be obtained by the molecular diffusion patterns. The higher the cell density, the lower the diffusion will be (Leroy-Willig 2007). DWI has been applied for tumor characterization in non-moving structures such as bones and the brain (Weber 2011), being extremely sensitive for the early recognition of brain ischemia (Schaefer 2000).

Functional MRI (fMRI) is new MRI technique which is dependent on the conjunction of both neuronal activity and brain hemodynamics. fMRI basically quantifies changes in blood oxygenation, which translate into changes in the magnetic field. Deoxyhemoglobin and oxyhemoglobin has dissimilar magnetic properties to induce inhomogeneities into the surrounding magnetic field. Deoxyhemoglobin concentration decrease will increase image intensity. fMRI is frequently performed for brain activation studies (Heeger 2002).

Perfusion measurements are possible using two different MRI methods: the dynamic contrast-enhanced imaging (DCE–MRI) with an intravenous bolus injection of a contrast agent during its first passage through the organs and (ii) arterial spin labeling (ASL) with the arterial blood water magnetization as an internal contrast agent (Leroy-Willig 2007). The contrast agent size in DCE–MRI will measure its washout from the vascular space to determine vascular permeability (Leroy-Willig 2007). The ASL technique acts better at high magnetic fields and highly irrigated organs, such as kidneys, muscles, heart and brain. The advantages of ASL technique include no need of contrast injection and repeated measurements (Leroy-Willig 2007; Goetti 2013; Cutajar 2012; Thomas 2011; Kazan 2010).

MRI is now a recognized modality for imaging the cardiovascular system to provide precious information concerning structure, function, and perfusion at cellular/molecular levels in animal models of cardiovascular disease. It is also an important technique for the assessment of new contrast agents and the verification of the cardiovascular phenotypes of genetically modified mice (Tsui 2009; de Kemp 2010). MRI showed high signals of the aortic walls in mice with atherosclerosis confirmed by histology (Qiu 2010). 7T MRI could monitor labeled rat bone mesenchymal stem cells (BMSCs) in injured common carotid artery after allograft transplantation (Cao 2009). New techniques involving casting (*ex vivo*), markers (both *in vivo* and *ex vivo*) and MRI (*ex vivo*; 7 Tesla) were developed that allowed correlation of changes in local and longitudinal wall shear stress with the temporal variations of the diameter and intima-media thickening in the venous segment of arteriovenous fistulas in pig model (Rajabi-Jagahrgh 2014).

In oncology, MRI has been applied widely for tumor growth and development study in a wide variety of animal models. Due to its sensitivity to dynamic processes, it is an outstanding tool to image tumor perfusion/angiogenesis and oxygenation. Moreover, MRI is especially favorable in observing *in vivo* tumor growth and ablation in response to treatment due to the non-invasive and non-destructive nature. It can serially follow individual subjects

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of a given cohort for long term (Lewis 2002; Dufort 2010). MRI can explore endothelial progenitor cells (EPCs) involvement in tumor neovasculature due to excellent biocompatibility and sensitivity (Chen 2014).

Micro-MRI has also been successfully used in the evaluation of musculoskeletal tissue structures (Tremoleda 2011). Micro-MRI was applied to evaluate joint inflammation in a rat surgical osteoarthritis (OA) (Jones 2010). Increased and diffuse water signal in the epiphyses of untreated OA rats was detected by micro-MRI 8 weeks after surgery which indicated a bone marrow lesion-like stimulus. Micro-MRI scan was used to quantify wear after tribological simulation of the medial compartmental bovine knee *in vitro*. Knees assessed with the intact meniscus did not reveal change in surface roughness and detectable cartilage loss or deformation. However, the meniscus removal increased contact stress and frictional coefficient which induced immediate surface fibrillation, biomechanical wear and permanent deformation of cartilage (McCann 2009).

Currently X-ray and CT are applied for assessing the spinal fusion process. But both imaging techniques have limitations in discernment of the early stages of this fusion process, because they can only visualize mineralized bone. A practicability study was carried out in a goat spinal fusion model to assess the detection capacity of different tissues with MRI (Uffen 2008). Micro-MRI was used to monitor 6- and 12-months follow-up specimens. The degradable cage material and different types of tissue were determined in the fusion zone and selected as regions of interest (ROIs). Then, the ROIs were ascertained and average signal intensities of every individual ROI were quantified. An outstanding match was found between MRI images and the histological results. Measurable differences were revealed by MRI images in signal intensity between fibrocartilage, fibrous tissue, degradable implant material, bone with hematopoietic marrow and bone with adipose marrow. In time the signal intensity of fibrous tissue, bone with hematopoietic red marrow and bone with adipose marrow stayed comparatively consistent. Additionally, the signal intensity of the degradable implant material and the fibrocartilage modified notably in time and indicated change of structure and composition. MRI supplies detailed information about the early fusion process and enables early diagnosis of non-union (Uffen 2008).

Quantitative 3D high resolution microMRI enabled for non-invasive longitudinal medial tibial cartilage thickness (MT.ThC) assessments in four different locations in both the control and rabbits with anterior cruciate ligament transection. The MT.ThC measurement in the minimum interbone distance area demonstrated the severity of the disease which was the most efficacious to monitor the progress of the medial tibial cartilage destruction (Boulocher 2007). MRI confirmed the phenotypes in rodent models of abdominal wall defects (Carnaghan 2013).

The applications of cryogenic radiofrequency coils MRI in animal models improved image quality and decreased measurement time (Niendorf 2015). MRI is the most sensitive assessment to identify and determine multiple sclerosis (MS) lesions (Milo 2014). Experimental autoimmune encephalomyelitis (EAE) is a MS animal model.

Cryogenically-cooled RF coils to produce microscopic magnetic resonance imaging (µMRI) *in vivo* disclose brain pathology in mice EAE model (Waiczies 2012). The brain inflammation in different areas was detected at the early stage of EAE. µMRI showed obviously the pathology of EAE even without the contrast agent application, and displayed outstanding similarity with conventional histology. High resolution images were obtained

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within short scan times. So μMRI can continuously monitor the dynamics and kinetics of immune cell infiltration.

Cerebral amyloid angiopathies (CAA) are neurodegenerative pathologies regarding cerebrovascular dysfunction which constitute a considerable part of the pathophysiology of AD (Biffi 2011). MRI was confirmed to be especially useful for evaluating the effect of amyloid deposition and vascular dysfunction in the pathophysiology and treatment of CAA and AD (Klohs 2014). MRI was able to detect vascular remodeling of hemodynamic alterations which was vital for investigating the consequence of CAA on vascular integrity and function (Salat 2014). Magnetic resonance angiography (MRA) techniques can be utilized to evaluate the cerebral vascular tree. The signal in time-of-flight (TOF)-MRA is determined by the blood flow and is helpful for ascertaining variations of big blood vessels and decreased blood flow in animal models of AD and CAA (Niendorf 2015). The increased resolution of TOF-MRA by cryogenically-cooled coil system is extremely important to ameliorate the recognition of smaller vessel and determine the pathologies associated with neurovascular disease (Niendorf 2015). Contrast-enhanced micro-MRA with cryogenically-cooled coil system and a spatial resolution of (60 × 60 × 61) μm3 was able to detect age-dependent remodeling of the cerebral micro vasculature in CAA mice (Klohs 2012). The periphery vessels were able to be identified by higher spatial resolution contrast-enhanced micro-MRA (Figueiredo 2012).

Cryogenic radiofrequency coils MRI with an in-plane spatial resolution of 51mm was able to detect the tumor borders in an animal model of high grade glioma (Ku 2013; Vinnakota 2013). Glioma mouse model was evaluated by cryogenically-cooled RF coil system combined with vessel size imaging (VSI) and DCE-MRI. A gradient echo spin echo sequence with gadolinium-based contrast agent was used to monitor VSI, vasculature permeability and vasculature perfusion at the same time in a mouse orthotopic glioma model with an antiangiogenic agent treatment (Kording 2014).

EchoMRI™ devices utilize a nuclear magnetic resonance technique to monitor the masses of fat, lean tissues, free water, and total water in a whole body for live animals (Kovner 2010). Fat and lean mass were measured by EchoMRI-700 (Houston, TX) in rat (Ghislain 2016) and mice (Fontés 2015, Plante 2015).

CONCLUSION

Imaging has driven experimental surgery to a higher-level and is believed to be high throughput techniques with immanent enormous advantages over the more conventional *ex vivo* techniques. Its emphasis is on real-time monitoring, being non-destructive and noninvasive. It also allows the provision of multiple time points for longitudinal studies. Multilevel information from cellular/molecular to organ/organism can be acquired. The development and dissemination of animal imaging equipment and techniques have contributed to a very significant decrease in the number of animals needed for preclinical research, fully complying with 3R (Replacement, Reduction and Refinement) policies and strategies. Each imaging technique shows innate advantages and limitations, supplying divergent kinds of information, most often complementary. While nuclear techniques are appropriate for delivering basically molecular and functional information, CTs and MRIs

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produce images with excellent spatial resolution. The combination of the functional with morphological imaging modalities has proven very powerful, contributing more precise and practical information. PET and SPECT are by far the most sensitive modality and can detect labeled molecules in picomolar concentrations. Preclinical PET is more often combined with PET-CT and PET-MRI, respectively (Comley 2011). The combination of imaging techniques with high-resolution and highly sensitive techniques will acquire functional information and expand the horizons of research. It will not only enlarge the understanding of the underlying mechanisms of disease, but also supply efficient tools for assessing new therapeutic agents. The added value of multimodality or hybrid imaging techniques has achieved its broad acceptance and widespread application over the last 10 years. New hardware and software will further broaden the range of applications and image quality. The radiolabeling chemistry of SPECT is often simple and easy. The radionuclides have a variety of photonic energies and half-lives. The contemporaneous acquisition of several probes labeled with divergent radionuclides may evaluate the discrete molecular events. The user should strictly comply with the standard operating procedure about biosafety, radiation protection, and animal comfort. Well trained staffs are important to efficiently work with different animal models and the equipment/devices.

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Chapter 8

TELEMETRY APPLICATIONS IN EXPERIMENTAL ANIMALS FOR STUDYING HUMAN DISEASES

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ABSTRACT

Radio telemetry technique has considerably improved in the last 15 years and has been applied to monitor different physiological signals in conscious and free moving laboratory animals. Implantable radio telemetry can be used to measure physiological parameters including blood pressure (BP), heart rate (HR), right ventricular pressure (RVP), left ventricular pressure (LVP), electrocardiogram (ECG), respiratory rate (RR), intrapleural pressure (IPP), intra-abdominal pressure (IAP), gastric pH, stomach temperature, gastric slow wave, intestine slow wave, intrauterine pressure (IUP), bladder pressure, renal pelvic pressure (RPP), intra-cavernous pressure (ICP1), intracranial pressure (ICP2), electroencephalogram (EEG), electromyography (EMG), sympathetic nerve activity, single neuron activity, intraocular pressure (IOP), activity, body temperature (BT) and arterial blood glucose. The aim of this chapter is to present an update on the current developments of radio telemetry for studying human diseases in animal models established by experimental surgery.

Keywords: radiotelemetry, physiologic signals, laboratory animals, experimental surgery

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ABBREVIATIONS

BP	Blood pressure
HR	Heart rate
RVP	Right ventricular pressure
LVP	Left ventricular pressure
ECG	Electrocardiogram
RR	Respiratory rate
IPP	Intrapleural pressure
IAP	Intraabdominal pressure
IUP	Intrauterine pressure
RPP	Renal pelvic pressure
ICP1	Intracavernous pressure
ICP2	Intracranial pressure
EEG	Electroencephalogram
EMG	Electromyography
IOP	Intraocular pressure
BT	Body temperature
DSI	Data science international
AAAs	Abdominal aortic aneurysms
LV	Left ventricular
RV	Right ventricle
PH	Pulmonary hypertension
PAB	Pulmonary artery banding
PA	Pulmonary artery
NHP	Non-human primate
IM	Intramuscular
IV	Intravenously
GI	Gastrointestinal
GRTs	Gastric residence times
WMC	Wireless motility capsule
CPEs	Carbon paste electrodes
WT	Wild-type
PTL	Preterm labor
OT	Oxytocin
CSP	Corpus spongiosum of the penis
SCI	Spinal cord injury
ISP	Intraspongiosal pressure
PTL	Preterm labor
UI	Urinary incontinence
LUTD	Lower urinary tract dysfunction
TBI	Traumatic brain injury
RSNA	Renal sympathetic nerve activity
IVBG	Intravenous blood glucose

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INTRODUCTION

Physiologic signals monitoring of laboratory animals has an important role in modern biomedical research. Telemetry is a promising technology to measure various physiological parameters in conscious and freely moving animals. Telemetry is the transmission of measured physiological parameters to remote receivers via an antenna in conscious and freely moving animals. The receiver transforms the analog frequency signal into a digital signal in a data acquisition system. The quality of physiological signals obtained from conscious, freely moving animals is preferable, because the data are recorded under the normal state of the animals. Telemetry systems are classified as three types including wire telemetry system, radio telemetry system, and fiber-optic telemetry system according to the signal transmission medium. Radio telemetry application has been dramatically increased since the first affordable, reliable, and user friendly commercial telemetry implantable devices for physiological signals measurements from conscious and freely moving animals was developed (Brockway 1993). The components of the radio telemetry system include implantable transmitter, telemetry receiver, data exchange matrix, ambient barometric pressure and data acquisition/analysis system. Radio telemetry unites tiny sensors and transmitters to discover and transmit physiologic signals in animals to telemetry receivers. The receivers are connected to a data exchange matrix that inputs to a software system designed for acquisition and analysis. The implantable transmitter can directly measure the physiologic signals and telemeters it digitally from within the animal. The telemetry receiver converts the analog frequency signal into a digital signal to a computer with data acquisition system. Ambient barometric pressure is also monitored and deducted from the telemetered pressure by data acquisition software to correct changes in atmospheric pressure (Mills 2000). The data acquisition system can keep, handle, format, arrange, and output the data according to the user's instructions.

The advantages of implantable radio telemetry in biomedical research include elimination of artifact from restraint stress, a humane method for monitoring physiologic parameters in conscious animals, reduction of animal use in research, elimination of infection risk from exit sites, automated data collection around-the-clock, unrestricted continuous data collection from days to months, availability for all laboratory species, data quality improvement compared to conventional measurement techniques and utility of implanted animals for successive studies (Kramer 2001; Kramer 2003). The disadvantages of implantable radio telemetry include the cost to acquire the requisite equipment, specialized training/certifications for surgically prepare and study animals, large amounts of data generated by continuous or regular scheduled sampling and dedicated space within the animal facility to conduct studies and possibility of long-term drift (Kramer 2001; Kramer 2003).

Radio telemetry is an important tool for collection of a considerable number of physiological parameters including BP (Mills 2000; Kramer 2001; Kramer 2003; Braga 2009; Sivarajah 2010; Ingram-Ross 2012), HR (Mills 2000; Kramer 2001; Kramer 2003; Braga 2009; Sivarajah 2010; Ingram-Ross 2012), RVP (Oommen 2015; Handoko 2008; Haushalter 2008), LVP (Lujan 2012; Cools 2014; Holdsworth 2014), ECG (Kramer 2001; Kramer 2003; Ingram-Ross 2012), RR (Kramer 2003; Ingram-Ross 2012), IPP (Ednick 2007; Bhatia 2012; Murphy 2001), IAP (Rauch 2012; Goineau 2013), gastric pH (Rauch 2012; Sagawa 2009), stomach temperature (Austin 2006; Sauvé 2014), gastric slow wave (Farajidavar 2012),

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intestine slow wave (Woo 2010), IUP (Pierce 2010; Chellman 2004; Rada 2015), bladder pressure (Noël 2013; McCafferty 2009; Djurhuus 1990), RPP (Djurhuus 1990), ICP1 (Bernabé 1999; Bernabé 1995), ICP2 (Guild 2015; Hiploylee 2014; Kawoos 2014; Silasi 2009), EEG (Kramer 2003; Weiergräber 2005; Bassett 2014), EMG (Kramer 2003; Weiergräber 2005; Bassett 2014), sympathetic nerve activity (Stocker 2013; Hamza 2012; Robinson 2015; Burke 2011), single neuron activity (Korshunov 2006; Lei 2004; Roy 2012), IOP (Gouws 2007; Schnell 1996; Dinslage 1998; Downs 2011), activity (Johansson 1997; Hoffmann 2012), BT (Kramer 2001; Johansson 1997; Hoffmann 2012) and arterial blood glucose (Brockway 2015).

Implantable radiotelemetry is now a feasible and commercially available alternative to conventional measurement methods for physiological signals in biomedical research. Implant telemetry devices are available from different manufacturers including Data science international (DSI, St-Paul, MN, USA), *The Millar Telemetry System (*Houston, Texas USA*),* Stellar Telemetry (Homburg Germany), Emka TECHNOLOGIES (Paris, France) and EndoGear® I (Ithaca, NY USA), Konigsberg Instruments (Pasadena, CA, USA), Mini Mitter (Sunriver, OR, USA); Remo Technologies Ltd (Salisbury, UK); and Telemetronics Biomedical BV (Wageningen, The Netherlands).

TELEMETRY APPLICATIONS IN EXPERIMENTAL SURGERY FOR CARDIOVASCULAR DISEASES

Hypertension is an important contributor to the world disease burden. Lots of evidences demonstrate that hypertension is the main risk factor for cardiovascular mortality and morbidity. Furthermore, it can also cause several illnesses, such as renal failure, stroke, or aneurysm.

It is better to perform the study of cardiovascular physiology and pathophysiology in conscious animals to avoid the complications caused by anesthesia. Anesthesia can desperately inhibit the cardiovascular system and undermine the ability to regulate body temperature. Highly reliable data can be acquired by radio telemetry in animals under physiological conditions. Radio telemetry systems for monitoring cardiovascular parameters, including ECG, HR and BP were originally produced for pharmaceutical companies (Butz 2001; Mills 2000), in response to prerequisites by the FDA to present continuous monitoring of vital functions with each new drug application. Together with BT and activity indices (Mills 2000), they have been the most repeatedly described physiological signals monitored via radio telemetry, especially in rodents.

BP Measurement

BP is usually monitored to evaluate the reactivity of the cardiovascular system to environmental factors (salt, diet or stress) and genetic factors. The techniques for monitoring BP in experimental animals have refined greatly over the past decade, and several techniques are accessible that enable routine measurement of BP during day and night over prolonged periods of time in unrestrained, unstressed and conscious animals. Although indirect

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techniques that allow only intermittent recording of BP may be useful for some studies, techniques for directly monitoring BP are normally preferred because of their ability to measure the highly dynamic nature of BP in a comprehensive fashion. The technique selection should eventually be guided by the study objectives to ensure that the technique chosen is suitable for the type of animal experiment.

BP monitoring in animals study is a very practical tool for finding fundamental mechanisms of cardiovascular diseases such as heart failure (Oommen 2015), hypertension (Kramer 2003; Handoko 2008), and pre-eclampsia (Hoffmann 2008; Falcao 2009). Furthermore, BP measurement in experimental animals is often used to investigate the long-term effect of pharmacological agents for treating hypertension.

BP measurement methods for animals include indirect methods and direct methods. The tail-cuff plethysmography is the most frequently used indirect method for BP measurement in animals. American Heart Association recommended the tail-cuff plethysmography for noninvasive measurement of systolic BP changes in large numbers of animals, substantial group differences in systolic BP, substantial changes in systolic BP over time and frank systolic hypertension (Kurtz 2005). The tail-cuff plethysmography is often performed during the day and cannot provide BP information during the night when rodents are more active. The tail-cuff plethysmography measurement of BP in mice and rats induces substantial amounts of thermal and restraint stress which affects BP, heart rate, and stress hormones. BP results measured by indirect methods have displayed poor agreement with BP results simultaneously monitored by direct methods (Reddy 2003; Jamieson 1997). Systolic BP measured by tail-cuff has revealed sizable differences with systolic pressure monitored by direct methods in some cases (Reddy 2003). Furthermore, continuous measurement of BP cannot be performed with the tail-cuff method.

BP can be directly recorded via radio telemetry methods or by indwelling catheters connected to externally mounted transducers. American Heart Association recommended direct BP measurement methods for continuous BP measurement, monitoring BP in free moving animals, studying BP effects of different interventions or variables (drugs, diet, genotype), quantifying relationships between BP and other variables (target organ damage), identifying intermittent or subtle forms of hypertension and quantifying the magnitude of hypertension (Kurtz 2005). 24-hour BP and HR can be measured by indwelling catheters and animals needed to be tethered to an outside transducer (Li 1999).

The use of genetically modified rodent models is increased for investigating the causes of cardiovascular diseases and potential treatments for cardiovascular diseases. Mouse models are frequently used for the study of hypertension, heart failure, atherothrombotic disease and abdominal aortic aneurysms (AAAs) (Zaragoza 2011).

Mice BP Implant Telemetry Surgery

There are two methods for telemetric transmitter implantation including the abdominal aorta implantation and carotid artery implantation.

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Abdominal Aorta Implant Telemetry Surgery

Before implantation of the TA11PA-C20 BP device (DSI), the catheters of the probes were filled with an antithrombotic gel. The telemetry probes were calibrated by the data acquisition system and the pressure pre offset was checked. Then the probes were sterilized overnight in 2% glutaraldehyde following instructions provided by the vendor. The transmitters were washed and soaked in sterile 0.9% saline immediately prior to implantation (Gross 2000; Carlson 2000). Mice were anaesthetized with a mixture of ketamine/xylacine. The abdominal surgical areas were shaved and denuded by a depilatory cream. The mice were put on a heating pad to maintain body temperature during surgery and in the recovery period. The aorta was separated from the surrounding tissues through abdominal incision. The aorta was separated from the vena cava below the left renal artery and a suture (4–0) was placed around the aorta. The abdominal aorta was rinsed with 2% lidocaine before the transmitter catheter was inserted. The catheter was inserted into the aorta from a 25 gauge needle and advanced 5−6 mm after the aorta was blocked. Subsequently, the catheter was fixed by Vetbond tissue adhesive and the aorta was rinsed with 2% lidocaine to reduce aorta spasms. The incision was closed after transmitter was fixed on the abdominal wall. Lower-extremity paralysis was encountered in a large number of animals which subsequently died or were euthanized (Gross 2000). The success rate was less than 20% (Carlson 2000; Reddy 2003).

Carotid Artery Implant Telemetry Surgery

Abdominal aorta implantation had a high mortality rate and a general inability to perform studies reliably in most transgenic mice. Carotid artery implantation technique was subsequently established (Carlson 2000; Reddy 2003). Radio-telemetry transmitter was prepared according to the method described in mice abdominal aorta implantation. The mice were anesthetized and maintained as described in mice abdominal aorta implantation. Neck was prepared by shaving and a depilatory cream. All surgical procedures were done according to aseptic surgical methods. A vertical incision was then made on the neck and a subcutaneous pouch in the right flank was prepared for implant body placement. The left common carotid artery was separated and ligated with the first suture (7–0 silk) at the site of bifurcation into the internal and external branches (Wu 2012; Luo 2012). The suture was then retracted to the head side. The second suture was put around the common carotid artery about 10 mm below the bifurcation, and blood flow was blocked by retracting this suture to the tail. The tip of the telemetry probe catheter was gently held by vessel cannulation forceps, and the artery lumen was inserted near the carotid bifurcation using a 26-G needle on which the tip had been bent at a 90-degree angle. The tip of the catheter was inserted into the carotid artery lumen and advanced to the point of the occlusion suture by the 26-G needle. The catheter was fixed with suture and a drop of Vetbond. The incision of the neck was closed with suture (6–0 silk). BP was measured after one week of recovery.

The success rate of the carotid artery implantation was more than 90% compared with less than 20% success rate with the abdominal aorta implantation method reported by the same study (Carlson 2000). The carotid placement did not block blood flow to any major area. The results showed that occlusion of the right carotid artery induced only a transient rise in arterial pressure and no significant chronic effect was observed. In contrast, the abdominal

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aorta implantation considerably blocked blood flow to the hindquarters and caused animal death within 24 to 48 hours. The catheter tip is quite big compared to the abdominal aorta of the adult mouse and may damage blood flow to the lower extremities causing high rates of morbidity and mortality (Carlson 2000; Butz 2001). Thus, implantation of the catheter into the thoracic aorta via the common carotid artery is an often applied approach in mice, although some investigators have also developed femoral artery implantation method (Vecchione 2002). The carotid artery implantation has been verified to be a very reliable and successful method for acquiring high-fidelity measurements over weeks and months in average size mice (Carlson 2000; Butz 2001; Wu 2012; Luo 2012). Exteriorization of the probe and perturbation of the wound were shown when the transmitter body was placed at mid-scapular position (Carlson 2000; Butz 2001). Keeping the transmitter body subcutaneously along the inner flank of the mouse has proven quite effective. The implants keep relatively undisturbed over the months of implantation because this region is not easily reached by the mouse (Butz 2001). Furthermore, the procedure for this approach is less invasive by avoiding abdominal surgery. Animals generally need at least 7 days to recover before BP is measured after circadian rhythms and basal BP levels return to normal (Butz 2001). The measurement can be either intermittent or continuous according to the purpose of the study.

Mice LVP Implant Telemetry Surgery

The mouse has numerous advantages over other animal models for the molecular study of left ventricular (LV) function. Worldwide efforts are now in the process to knock out specific genes in the offspring of mice and to knock in genes with specific mutations and/or to selectively express or silence gene products in different tissues to understand, prevent, and treat cardiovascular diseases (Manis 2007).

Radio telemetry transmitter was prepared according to the method described in mice abdominal aorta implantation. All surgical procedures were done according to aseptic surgical methods. A ventilator was intubated to support the animal after anesthesia was maintained. A left thoracotomy through the 3rd intercostal space was performed to expose the heart. A loose purse-string 8–0 silk suture was put around the apex after the apex was exposed. The myocardium was inserted by 21-gauge needle in the center of the purse-string suture. The catheter of a telemetry implant TA11PA-C10 (DSI) was routed through the 6th intercostal space and inserted into the LV through the apical stab wound for continuous measurement of LVP in free moving animal. The LVP signal was checked to confirm the proper placement of catheter. The purse string suture was closed after the LVP signal was verified. The chest and the skin were closed. The transmitter body was kept subcutaneously on the left flank (Ishizaka 2004; Joho 2007; Lujan 2012). The animals recovered after at least one week of the surgery.

Mice RVP Implant Telemetry Surgery

Pulmonary hypertension (PH) has a poor prognosis which causes right heart dysfunction, right heart failure and death. It is characterized by abnormal vessel wall cell proliferation and

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abnormal vascular remodeling and increased pulmonary vascular resistance (Humbert 2004). Mice PH model can be induced by hypoxia (Weissmann 2014) or pulmonary artery banding (PAB) (Oommen 2015). Radio telemetry transmitter was prepared according to the method described in mice abdominal aorta implantation. All surgical procedures were done according to aseptic surgical methods. The telemetry implant TA11PA-C10 was implanted in PH mice model. The catheter was inserted in the right ventricle (RV) via the jugular vein under ultrasound control. The catheter position was confirmed by displaying the pressure amplitude of the RV on the computer monitor (Weissmann 2014).

Rat BP Implant Telemetry Surgery

Rat Femoral Artery Implant Telemetry Surgery

Radio telemetry transmitter was prepared according to the method described in mice abdominal aorta implantation. All surgical procedures were done according to aseptic surgical methods. The abdominal cavity was opened using the ventral midline incision after anesthesia was induced and maintained. The catheter of radio telemetry transmitter TA11PA-C40 was tunneled through the abdominal wall to the incision of the left femoral artery. Then it was inserted centrally to a distance of 3 cm and secured in place. Five ml sterile saline was supplied by IP injection to each rat to compensate for loss of fluids before the abdominal wound was closed. The transmitter body was then kept in the abdomen and fixed to abdominal muscles (Hill 2002; Dutil 2001). The transmitter body also can be placed subcutaneously along the flank to avoid intraperitoneal invasion (Kramer 2001).

Abdominal Aorta Implant Telemetry Surgery for BP and ECG Measurement

Radio telemetry transmitter TL11M2-C50-PXT (DSI) was prepared according to the method described in mice abdominal aorta implantation. All surgical procedures were done according to aseptic surgical methods. Surgery was performed under isoflurane anesthesia. Carprofen (5 mg/kg, SC) was administered prior to surgery to the anesthetized rats. The descending aorta was carefully isolated by sterile cotton applicators and blunt dissection after an abdominal incision (4–5 cm) was made. A 2–0 occlusion silk was inserted between the aorta and the vena cava to block backflow. The aorta was punctured as the method described in mice using a 21 g needle bent 90° at the beveled end. The tip of the catheter was inserted guided by the needle. Surgical glue Vetbond was applied to the puncture site to secure the catheter. The transmitter was secured in the abdomen and the skin was closed. The rat was allowed to recover on a heating pad before it was returned to the cage (Greene 2007).

Mouse telemetry transmitter TA11PA-C10 can be implanted to rats with reliable blood pressure and heart rate data similar to those acquired by using the rat transmitter TA11PA-C40. Reducing the size of the transmitter with accuracy in data acquisition will enable researchers to acquire reliable cardiovascular data (Braga 2009). The approach of introducing the catheter directly into the aorta of the rat with Vetbond to secure it in place has been mainly replaced by a simpler introduction of the catheter from the femoral artery and

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positioning of the transmitter body subcutaneously along the flank without intraperitoneal invasion.

Rat RVP Implant Telemetry Surgery

Radio telemetry transmitter TA11PA-C40 was prepared according to the method described in mice abdominal aorta implantation. All surgical procedures were done according to aseptic surgical methods. The pressure was measured continuously during surgery to verify proper position of the tip of the catheter. The trans-thoracic approach and trans-diaphragm approach were reported (Handoko 2008).

Trans-Thoracic Approach

The abdominal cavity was opened by midline laparotomy after anesthesia was induced and maintained. The transmitter was kept in the peritoneal cavity. The heart was exposed by a left thoracotomy conducted at the sixth intercostal space and mid-clavicular line. The catheter of the implant was subsequently tunneled subcutaneously to the thorax cavity. Heart was exposed after the ribs were retracted by small hooks. The pericardium was then opened. A superficial purse string (6–0 Prolene, Ethicon) was put on the RV free wall close to the apex. Through the purse string, a 19 G syringe needle was inserted into the RV in the direction of the RV outflow tract to avoid any coronary vessel. The site was wiped with a sterile cotton stick after the needle was removed. The tip of the catheter was then inserted into the RV by a vessel cannulation forceps. Live pressure waveform trace reaffirmed proper RV catheterization and RV systolic/diastolic pressures are usually about 25/1 mmHg. The pressure catheter was subsequently approached for about 2 cm and the tip of the catheter was left beyond the pulmonary valves. Moreover, live pressure waveform trace verified correct positioning of the pressure catheter in the pulmonary artery where PA systolic/diastolic pressures are commonly about 25/10 mmHg. Eventually, the catheter was secured by the purse string and tissue adhesive Vetbond at the site of insertion. Full expansion of the lungs was promoted by using a small burst of positive pressure (maximally 10 cm H_2O for 1 s). A chest tube (18 G) was put as air outlet and was removed after proper thorax excursions were found. The ribs were closed and the chest muscles were laid back in layers. The transmitter was sutured to the abdominal muscle. The abdominal wall was closed. The skin of the chest and abdomen was closed.

Trans-Diaphragm Approach

A 5-cm midline laparotomy was made and the abdominal cavity was exposed by two hooks. The xiphoid process was lifted by a retraction suture to expose the diaphragm during the whole procedure. Wet gazes were used to cover the visceral organs temporarily and a blade holder was applied to push down the liver gently. A small midline incision in the diaphragm was next performed. The heart was well exposed by two retraction sutures in two sides of the diaphragm. The RV catheterization was completed similarly as mentioned above. Blood clots in the thorax were eliminated after catheterization. A running suture (5–0 Vicryl) was used to close the diaphragm. The pressure catheter was protruded the diaphragm at the dorsal end of the incision. The retraction suture from the xiphoid process was discarded. Full

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expansion of the lungs was performed as described above. The chest tube was put between the sutures of the diaphragm until proper thorax excursions were observed. One drop of Vetbond was used to further fix the closure of the diaphragm. The abdomen was closed after the transmitter was secured to the abdominal wall.

Five ml warm sterile saline was supplied by IP injection to all animals at the end of the procedure. The animals were housed in individual cages for recovery. The animals were monitored and received post-surgical analgesia buprenorphine for the first two days after surgery. Drinking gel pads and softened rat chow were given until animals completely recovered from surgery.

Rat LVP Implant Telemetry Surgery

Radio telemetry transmitter TA11PA-C40 was prepared according to the method described in mice abdominal aorta implantation. All surgical procedures were done according to aseptic surgical methods. After anesthesia was induced and maintained, all animals were intubated and supported on a ventilator. A left thoracotomy through the 4th intercostal space was performed to expose the heart. A loose purse-string suture (6–0 silk) was put around the apex. 18-gauge needle was used to puncture the myocardium in the center of the purse-string suture. Then, the catheter of a telemetry device was inserted into the LV by the 6th intercostal space. The proper placement of pressure catheter was confirmed by LVP measurement. The purse string suture was closed after the proper position was verified. The chest was closed in layers and the skin was closed. The transmitter body was kept in the abdominal cavity (Lujan 2012).

Dog BP, LVP and ECG Implant Telemetry Surgery

Assessment of cardiovascular parameters, including ECG and BP, is needed by the regulatory guidelines. Implantable radio telemetry devices are commonly performed chronically in non-rodent species for safety pharmacology studies. High-quality BP and ECGs signals are crucial for the identification of potentially important changes in ECG and hemodynamic parameters. Proper catheter and lead placement are needed to acquire high-quality data. The detailed surgery procedures were described (Takahara 2001). In short, radio telemetry transmitter TL11M3-D70-PCP (DSI) was prepared according to the method described in mice abdominal aorta implantation. All surgical procedures were done according to aseptic surgical methods. Beagle dogs were originally anesthetized with thiopental sodium (30 mg/kg, IV). 1.0% halothane vaporized with 100% oxygen was inhaled with a volume-limited ventilator after intubation with a cuffed endotracheal tube. The chest incision was performed at the 6th intercostal space to expose the heart. A pressure catheter of the implantable transmitter unit was inserted into the LV of the heart through its apex for recording the LVP. Subcutaneous pouch was made in the left flank before closing the chest. The unit's tabs were sutured in the underlying tissue. Another pressure catheter was tunneled subcutaneously from the flank to the groin where the left femoral artery was isolated. The catheter tip was sent to the abdominal aorta from the femoral artery and secured with silk ligature. One lead of ECG was routed under the skin close to the right axilla and the other was

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fixed in the area of the lower left abdomen to monitor the lead II ECG. All incisions were closed and antibiotic prophylaxis was administered for 10 days. BP was measured 4 weeks after the surgery.

Non-Human Primate (NHP) BP and ECG Implant Telemetry Surgery

NHP is always an important preclinical cardiovascular disease model. The accurate BP measurement in NHP was utilized to evaluate the efficacy of test agents for cardiovascular diseases. Telemetry is becoming more extensively utilized in preclinical testing for large animals. NHP has an important role as a large animal model to assess the cardiovascular efficacy of novel test agents.

Multiple model systems are available to monitor BP in conscious NHP from restrained to telemeterized animals. The affordable commercial telemetry systems have resulted in a significant increase in primate telemetry application.

NHP were anesthetized with an intramuscular (IM) injection of acepromazine (10 mg/ml, 0.14 mg/kg) and ketamine (100 mg/ml, 13.6 mg/kg). 10% lidocaine spray was provided onto the arytenoids prior to endotracheal intubation by laryngoscopy. A sterile ophthalmic ointment was given to both eyes to prevent drying of the cornea. Animals were then kept on a heating pad and inhaled a mixture of 1.0 L/min oxygen (O_2) and 2.5% isoflurane. RR was kept between 10 and 12 breaths/min with an inspiratory airway pressure between 18 and 20 cm H_2O using a mechanical ventilator. HR and pulsatile hemoglobin saturation in O_2 were monitored during anesthesia. Prophylactic antibiotic therapy (Cefazolin injectable, 25 mg/kg) was supplied by IM injection at least 1 h before surgery, at the end of surgery and every 8 h for 24 h after surgery. Analgesic (buprenorphine 0.05 mg/kg) was provided by IM injection upon completion of the surgery and was given every 8 h for 24 h post-surgery. Fluid therapy was administered intravenously (IV) throughout anesthesia using sterile Lactated Ringer's solution at a rate of 10 ml/kg/h. The surgical region was shaved and aseptically prepared using isopropyl alcohol and 4% chlorhexidine gluconate (Authier 2007).

Radio telemetry transmitter D70-PCT™ (DSI) was prepared similarly to the method described in mice abdominal aorta implantation. All surgical procedures were done according to aseptic surgical methods. The transmitter D70-PCT™ (DSI) was inserted between the abdominal internal oblique and the abdominal transverse muscles from a longitudinal abdominal incision. The right inguinal incision was made to expose the right femoral artery. The transmitter catheter was tunneled subcutaneously to the right inguinal incision using a trocar. The catheter was inserted into the femoral artery. Skin incisions were performed on the left lateral aspect of the thorax close to the last rib and on the right side of the thorax in the area of the thoracic inlet to enable ECG lead placement. The skin incisions were closed with interrupted intradermal absorbable sutures (Polyglactin 3-0). All monkeys were equipped with tether system and a jacket after surgery. The monkeys were returned to their cage after the rectal BT reached at least 37.0°C (Authier 2007).

The surgery was conducted using aseptic technique, under general anesthesia with intermittent positive pressure ventilation. A dorsoventral incision through the skin was done on the left flank posterior to the last rib. A pocket was prepared between internal and the external abdominal oblique muscle layers to keep the transmitter body. A left thoracotomy was completed through the 4th intercostal space. The biopotential leads, LV and arterial

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pressure catheters were entered the thoracic cavity through the intercostal space caudal to the thoracotomy. The LV catheter was put in the apex of the heart through an incision of the left ventricle and fixed with a non-absorbable purse string suture. The positive biopotential electrode was fixed to the apical end of the left ventricle, near the pressure catheter. The negative electrode was put near the base of the heart, cranial to the aortic arch. The arterial pressure catheter was put in the descending thoracic aorta through a stab incision and secured by a preplaced non absorbable purse string suture. Thoracotomy incision was closed in several layers with absorbable suture. Air was evacuated from the thorax with the lungs fully inflated before final closure. The animals were maintained by the respirator until surgery was finished and then slowly removed the respirator. The animals were observed for normal breathing and monitored for signs of abnormal breathing. Post-operative analgesics were given and the animals recovered from anesthesia. Animals needed at least 4 weeks to recover after surgery.

Subcutaneous Lead Placement via Laparotomy

The surgery was performed with aseptic technique in 8 monkeys, using general anesthesia plus intermittent positive pressure ventilation. A midline laparotomy incision was made to keep the transmitter body inside the abdomen. The arterial pressure catheter was inserted into the femoral artery. The catheter was secured via ligatures around the artery. The biopotential leads were subcutaneously secured in an approximate Lead II configuration. The positive lead was kept on the left side of the caudal thorax. The negative lead was put near the thoracic inlet on the right side of the thorax (Henriques 2010). The study indicated that placing leads directly on the epicardium remarkably decreases signal interruption due to room disruptions and subsequent animal exhilaration. This surgical technique showed adequate sensitivity to monitor changes in ECG parameters, specifically QTc interval prolongation in monkey.

Non-Human Primate (NHP) LVP Implant Telemetry Surgery

ECG leads were placed in a base-apex epicardial arrangement according to a general method previously reported in the non-human primate to optimize the consistency and amplitude of ECG component waveforms (Moddrelle 2012).

A left thoracotomy from the 5th intercostal space was done and the heart cradled in the pericardium. The bio-potential leads were inserted to the 7th intercostal space, entered the thorax, and trimmed and tied to expose a loop of bare wire. The positive biopotential lead was attached to the epicardial surface of the LV near the ventricular apex with non-absorbable suture. The negative bio-potential lead was kept in the neighborhood of pericardial fat tissue near the mediastinal aspect of the heart/base of the right atrium by non-absorbable suture. TL11-M3-D70-PCTP (DSI) LVP catheter was introduced via the LV apex using an intermedullary pin, and secured within the LV using non-absorbable suture. Telemetry (D70-PCTP) signals were continuously evaluated during surgery to confirm functionality of the telemetry probes and optimize the ECG based on lead arrangement. Telemetry data were collected two weeks later. Ventricular rhythm variants was significantly bigger in animals with LV catheters (62/67; 93%) compared to animals with epicardial leads only (21/55; 38.2%) and surgically naive animals (9/18; 50%) (Holdsworth 2014). The catheter/transducer

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implantation surgery causes inflammation, damage and cardiac tissue scar in the vicinity of the ventricular apex which is usually for catheterization (Henriques 2010). Myocardial damage caused by acute puncture injury through the ventricular apex and catheter placement was able to induce ventricular arrhythmias (Sarazan 2011; Sarazan 2012), the continued interaction of the catheter with adjacent transmural and endocardial tissues with potential associated foreign body response (Anderson 2008; Baird 2013).

TELEMETRY APPLICATIONS IN EXPERIMENTAL SURGERY FOR RESPIRATORY DISEASES

Respiration is an important vital sign, especially for patients suffering from sleep apnea syndrome, asthma and chronic obstructive pulmonary disease. Many parameters, such as respiratory CO_2 emission, respiratory airflow, respiratory sounds and the kinematics of chest or abdomen associated with respiratory activity, can be used for respiration monitoring. The respiratory-related chest or abdominal movements are the most common measured parameters.

The mechanical properties of the lung and ventilatory parameters (breathing patterns) are often monitored for evaluating respiratory function. The volume and flow changes associated with breathing patterns can be estimated directly by using a plethysmograph chamber or a pneumotachometer attached to a mask or a tracheal catheter. Changes in lung airflow can also be assessed by measuring thoracic displacement using inductive or impedance pneumography or sonomicrometry. Lots of methods have been adapted for application to conscious animals. IPP needs to be monitored to directly evaluate the mechanical properties of the lung.

IPP Measurements

IPP was monitored chronically in conscious monkeys after the catheter of radiotelemetry transmitter TL11M3-D70-PCP (DSI) was implanted beneath the serosal layer of the esophagus and within the thoracic cavity (Murphy 2001). This technique expedited the assessment of the effects of drugs, environmental agents, or disease on respiratory function by repeated measurements of respiratory function in a free moving nonhuman primate.

Flunixin meglumine (1 mg/kg IM) and acetylcholine (0.025 mg/kg IM) were given to monkeys. Anesthesia was induced by ketamine (10 mg/kg IM) and maintained by isoflurane (1–3%) delivered in 100% oxygen. A feeding tube was inserted to the stomach. The telemetry transmitter body was sutured to the abdominal wall after midline laparotomy was performed. Two retracting sutures were positioned in the stomach just below the cardia. The esophagus was found at the point where it enters the diaphragm and a suture was prepared at this site. An incision was performed through the serosal layer of the esophagus. A modified groove director was put between the muscularis and serosal layers and approached cranially through the diaphragm along the dorsolateral aspect of the esophagus guided by the feeding tube. The pressure catheter was advanced to the thoracic cavity. IPP was confirmed and a negative deflection was verified with each respiratory effort. The position of catheter was adjusted until a maximal change in pressure (>4 mmHg) was obtained. The catheter was fixed by the

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preplaced suture. The position of the catheter was verified by transesophageal ultrasound imaging.

The IPP measured by telemetry in rats was very inconsistent. Excessive struggling during the infusion periods caused the variability (Ewart 2010). Changes in IPP were measured by pressure sensitive tips of surgically implanted telemetry devices TA11PA-C40. Rats were anaesthetized using 2–4% isoflurane mixed in 3 L/min oxygen and the fur shaved from the inguinal area to the sternum. Rats were prepared under strict aseptic conditions with analgesia (0.01 mg/kg buprenorphine). The surgical area was then disinfected and a central abdominal incision was made. The lobes of the liver were carefully held back by moist cotton swabs and the esophagus was separated about 2 cm below the junction with the diaphragm. A trocar was inserted between the serosal and muscularis layers and tunneled cranially the thoracic cavity from the juncture with the diaphragm. The catheter of the telemetry implant was inserted into the channel after the trocar was removed. The pressure signal was monitored to confirm correct placement of the transmitter. The catheter of the transmitter was fixed in place at the entry point with Vetbond and a cellulose patch (DSI) after a pressure signal of maximal strength (amplitude of at least 20 cm H_2O) was reached. The body of the transmitter was kept in the peritoneal cavity. The wound was closed in layers.

Preclinical respiratory safety pharmacology studies are routinely performed in several animal models including rat, mouse, dog and monkey (Flandre 2003; Schierok 2000; Baldrick 2008; Murphy 2001). A new implantation device for large animals was designed at WIL Research Laboratories, LLC (WIL) in cooperation with DSI (Kearney 2010). The device joins an impedance-based sensor and lead set for the evaluation of respiratory function to the standard cardiovascular telemetry device used in large animals.

Beagle dogs were implanted with a prototype telemetry device similar to the TL11M2-D70-PCT transmitter (DSI), which can collect ECG (lead II configuration), BP, and BT in addition to respiratory data via impedance leads (Kearney 2010). The telemetry implant body was kept in the pleural cavity. Impedance leads were tunneled subcutaneously to a lateral midline incision. Impedance leads were fixed to the intercostal space at this position. Lead position was modified during each surgery in order to improve the signal quality and establish a standard procedure for this new surgery.

The results suggest the feasibility of further validation and application of implantable impedance in safety pharmacology studies, in order to obtain the advantage of chronic, simultaneous (time-matched) collection of cardiovascular and respiratory data in ambulatory setting, with the reduction of animal use and resources (Samson 2011). Identification of drug effectiveness using population based conversion factors promotes the potential application of this tool for fast screening of candidates and decrease of pre study positive control calibrations. Implantable impedance technique may also show advantages over externally placed devices through increased sensitivity and decrease in user error owing to less components. External systems are influenced by variation because of movement of bands following calibrations. No such concern is related to implanted devices, because the position of the impedance sensor should keep steady throughout the duration of the implant.

A new telemetry device was implanted in Göttingen minipigs to monitor transthoracic impedance to approximate respiratory tidal volume and rate (Willens 2014). The telemetry device was kept in a retroperitoneal pocket. Paired biopotential leads were secured intermuscularly at the position of the seventh rib midway between sternums bilaterally and

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spine to supply substitute data for respiratory function. Multiple cardiopulmonary endpoints were evaluated by telemetry in restrained, conscious rabbits (Horsmon 2016).

Telemetry Applications in Experimental Surgery for Digestive Diseases

Telemetry capsules were provided to monitor temperature, pH or pressure inside the gastrointestinal (GI) tract. These capsules were expected to substitute invasive techniques in the diagnosis of function disorders in the GI tract. Nevertheless, problems such as signal loss and uncertainty of the pills position restrained their application in a clinical setting.

The pH telemetry capsules (Bravo1 capsule) from Medtronic, Inc. (Minneapolis, MN) were calibrated ten minutes before dosing according to manufacturer's instructions (Sagawa 2009). Continuous pH measurements were monitored with Bravo capsules to explore the time course of gastric pH regarding gastric residence times (GRTs) and meals in dogs. Experiments were performed in home cages or study cages, and meals were provided at designated times. The dogs maintained fasted gastric pH acidic in both the study and home cages. The pH was usually increased for dogs in home cages and kept acidic for dogs in study cages when feeding time is close. The gastric pH maintained acidic during meal consumption and for at least 10 h after meals in both home cage and study cage. The GRTs between fasted (25 ± 32 min) and fed (686 ± 352 min) state was notably different with sizable inter- and intra-subject variability. Dogs had similar fasted gastric pH as monkey and human. But gastric pH of dogs remained acidic after meals. Dogs had shorter GRTs in fasted state and longer GRTS under fed conditions compared to humans (Mahar 2012).

A bidirectional telemetry system was designed and validated for measuring gastric slow wave activity (Farajidavar 2012). This device measured slow wave for the first time with an adequately high signal quality for recognizing individual slow wave activation times. The system was displayed to be extremely dependable and power efficient, and its small size made it portable and suitable for implantation.

A telemetry capsule system was produced and applied to record the slow wave activity of the small bowel which is a vital parameter for the diagnosis of gastric diseases (Woo 2010). The slow wave signal from the intraluminal electrodes was increased by the capsule which transferred the digitally sampled data by a radio frequency transmitter. The capsule (11×21 mm^2) was smaller than a capsule endoscope, and it kept active for at least 18 h.

An intra-abdominal monitoring implement, with a pressure sensor and electrodes within the gastric wall, enabled the continuous measurement of the intensity and frequency of contractions at the same time with an electromyogram (EMG) (Burger 2006). A representative inter-digestive mobility cycle was duplicatable in fasting dogs. The pressure curve increased considerably within 15 min of feeding and achieved its peak 30–45 min post-prandially. The peak frequency also obviously increased instantly after feeding and achieved the peak of 22 contractions per 5 min. Passive telemetry was confirmed to be an appropriate method for the long-term study of the physiological gastric motility and the effect of food additives in dogs.

For the abdominal implantation of the measurement device, the dogs were provided full anesthesia induced by the IV administration of thiopentone 20 mg/kg body weight. Then endotracheal intubation anesthesia was induced and kept with nitrous oxide and oxygen (2:1) and halothane (1–2%).

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The stomach was exteriorized for the placing of the pressure sensor from the abdominal incision. At the caudal side of the stomach, 2–3 cm from the pylorus and half way between the major and the minor curvatures of the stomach, a 1.5 cm long incision was performed through the muscle layer, so that the mucosal layer was protruding through the incision. The muscle layer was undermined within the submucosa with a forceps and the mushroom-like sensor head was put in this pocket in such a way that its diaphragm was lying on the mucosa and orientated towards the lumen of the stomach. The incision was then closed. The cable was also secured with a 5 cm long suture sling at the corpus area of the stomach to relieve the tension and the suture was 7 cm from the sensor head. The three electrodes were introduced into the muscle layer with a cannula and fixed with single sutures. EMG wires were also fixed with a long suture sling about 4 cm from the implantation site to reduce the tension. Sensor and EMG wires were connected by separate cables to the transponder case which was outside of the omentum major in the abdomen. The ring antenna was fixed onto a ring made of polyvinylchloride.

Continuous intra-abdominal pressure was monitored by a motility capsule in pig (Rauch 2012). The intra-vesical pressure varied from 3 to 15 mmHg recorded through the bladder. The intra-gastric pressure by the capsule fluctuated from 1 to 3 mmHg. Stomach pressure monitored by motility capsule underrated the intra-vesical pressure. Difference between intra-vesical pressure and gastric pressure could be produced by different position of the 2 devices to the zero reference point and gastric dilatation (Rauch 2012).

A new multi-channel wireless mapping system was designed to measure slow wave signals from the porcine gastric and intestinal serosa *in vivo* (Paskaranandavadivel 2015).

The device obtained high quality signals, and has the possibility to promote chronic measurement studies and clinical translation of spatiotemporal mapping.

The Smart Pill wireless motility capsule (WMC) system was approved by FDA to assess suspected delayed emptying in functional dyspepsia and gastroparesis. The device can monitor transit in the colon, small intestine and stomach by finding characteristic pH transitions, and evaluate pressure waves in each gut region (Hasler 2014). Accumulative benefits of WMC testing in patients with suspected gastroparesis comprise representation of pressure abnormalities, colonic and small intestinal transit delays. The effect of WMC methods in patients with upper gastrointestinal motor disorders and suspected gastroparesis will be validated in multicenter studies.

Telemetry Applications in Experimental Surgery for Obstetrics and Gynecology Diseases

About 20% of pregnant women are at high risk of preterm labor. 12.7% of babies born each year are premature in United States (Center for Disease Control and Prevention and National Center for Health Statistics data for 2005). Preterm labor explains 85% of infant mortality and 50% of infant neurological disorders (Callaghan 2006). The early detection of labor is very important for the treatment of preterm labor. Prediction of labor in normal pregnancies plays a crucial role in reducing unnecessary hospitalizations, interventions and expenses. Also, correct diagnosis of preterm labor will permit practitioners to begin therapy early in patients with true labor and avoid unnecessary treatment and hospitalization in patients who are not in true labor. Classification of pregnant patients and correct diagnosis of

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preterm labor are still crucial challenges in obstetrics. Uterine EMG has demonstrated considerable assurance for surveilling patients during pregnancy.

Genetically modified mouse models have been established to explore the signaling pathways crucial to labor-both preterm and term. Human and mouse are similar regarding many of the pathways involved to alter pregnancy outcome although there are some differences in parturition between these species (Mitchell 2009). Mouse models of modified parturition comprise those in which abnormal cervical ripening, progesterone withdrawal, myometrial contraction, or the circadian rhythm is affected (Ratajczak 2008). Modifications in individual genes can affect different pathways to parturition and finally cause a change in myometrial contraction. Although measurement of isometric tension can bring characterization of changes in uterine contraction associated with abnormal pregnancy in the mouse, they are limited to one stage of pregnancy rather than providing a comprehensive view of contractile and pressure changes over the course of pregnancy and delivery. Moreover, the animals need to be euthanized in this method. Therefore evaluation of the innate progression of labor or its initiation is impossible.

Rat IUP measurement by radiotelemetry was reported (Mackay 2009; Shi 2008). Telemetry transmitter (c50-pxt, DSI) was fixed to the abdominal wall with two wires placed in the myometrium for recording EMG activity and the catheter was passed into the uterine cavity for IUP measurement during labor. This method was useful for uterine EMG and IUP measurement, without adverse effects on delivery morbidity rates or mortality (Kothari 2008).

Radio telemetry transmitter was prepared according to the method described in mice abdominal aorta implantation. All surgical procedures were performed using aseptic surgical techniques. The abdominal cavity was opened using the ventral midline incision after anesthesia was induced and maintained. The uterus was carefully exposed and the total pups were counted. Catheter was inserted through a small incision at the top of one uterine horn. The pressure catheter was cautiously put in the uterine horn between the uterine wall and fetus by vessel cannulation forceps. The transmitter was carefully put in the lower portion of the abdominal cavity to avoid damage to the liver, and the implanted horn was kept in the body cavity. Soft food was given to animals from after surgery through to post-delivery to ameliorate maternal outcome.

The effect of telemetry was evaluated in both wild-type (WT) mice and defective parturition mice (SK3 channel-overexpressing mice) after telemetry implant was placed in the uterine horn. Uterine contractions were reduced and delivery was delayed in SK3 channel-overexpressing mice (Bond 2000; Pierce 2008). Continuous measurements from day 18 of pregnancy through delivery showed that WT mice usually delivered during the 12-h dark cycle after 19.5 days postcoitum. IUP slowly rose during this cycle to threefold higher than that recorded during the 12-h cycle before delivery in in WT mice. The SK3 channel-overexpressing mice displayed lower intrauterine pressure over the same period. These studies suggest that radiotelemetry can be applied efficiently to investigate uterine contractions, as part of the full characterization of genetic mouse models of disrupted labor.

Preterm labor (PTL) and uterine contractions in pregnant *cynomolgus* monkeys was assessed by a telemetric-based model (Chellman 2004). EMG and IUP were measured continuously as indicators of uterine activity. A pressure sensor was inserted into the amnion of pregnant monkeys on gestational day 120 ± 3 and biopotential sensors were fixed to the uterus. A telemetry transmitter was kept in a subcuticular pocket located in the flank. EMG and/or IUP were measured continuously post-operatively. IUP is a trustworthy parameter for

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recording intrauterine activity. A close relationship between IUP increase and bursts of activity in the EMG was observed. Basal uterine activity during the day with irregular contractions of < 10 mmHg was recorded in animals close to term. In the night, spontaneous contractions (10–40 mmHg) happened every 3–6 min. 5–60 mU oxytocin (OT) per kilogram per hour infusion caused artificial contractions of 15–40 mmHg that imitated preterm labor. These contractions kept unchanging for up to 14 h of constant infusion of OT and demonstrated a dose-dependent response to OT. Contractions went to baseline within 1 h after OT was removed. No desensitization of oxytocin-triggered contractions was shown when oxytocin was given daily for up to several weeks. This telemetric model typifies uterine contractions in NHP and supplies an outstanding method to assess pharmacological characteristics of drug candidates for PTL treatment (Chellman 2004).

Transgenic and knockout mouse are useful models in the investigation of genes affecting the timing and/or progression of labor contractions. Mice telemetry surgery methods for IUP measurement were shown (Pierce 2010; Rada 2015). IUP monitoring in the mouse from mid-pregnancy until delivery by telemetry was illustrated in detail (Rada 2015). This method may accelerate the development of therapeutics for myometrial disorders during pregnancy, including preterm labor (Rada 2015).

TELEMETRY APPLICATIONS IN EXPERIMENTAL SURGERY FOR UROLOGICAL DISEASES

RPP is important for the regulation of renal functions, such as sodium excretion, renin release and blood flow autoregulation. Different physiological controllers adjust renal function by their direct effects on intrinsic hemodynamics and renal tubular functions within the kidneys as well as indirect effects by amending RPP. Furthermore, many pathogenic factors may cause renal injury also through their direct destructive actions on the kidneys (RPP independent) and indirect destructive actions by modifying RPP that induces renal dysfunction (RPP dependent) (Cupples 2007, Mattson 2003; Mori 2006). To investigate the effect of RPP on renal function and dissect the pressure-independent and -dependent effects of different renal or cardiovascular controllers, an equipment to control the RPP is needed. Various servocontrol systems have been established for application in different animal models (Mori 2004; Nafz 1992). Nevertheless, these servocontrol systems were produced to utilize analog signals of BP acquired from indwelling arterial catheters and are difficult to establish and maintain.

An RPP servocontrol system with telemetry pressure device and LabVIEW computer software (National Instruments) was reported (Xia 2008). Lower abdominal aorta pressure in rats was measured by telemetry which reflected RPP. A computer program (LabVIEW) estimated the RPP data with a predetermined pressure setting and operated a bidirectional syringe pump to manage the inflation of a vascular occluder around the aorta above renal arteries.

The implantable carbon paste electrode (CPE)-telemetry system for PO2 assessment was described in detail (Koeners 2013). Briefly, reference electrodes and carbon paste were put in the renal medulla, while the auxiliary electrode was coiled and positioned on top of the kidney. Electrodes were kept to the renal capsule by a piece of surgical mesh glue and the

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leads were fixed on the aorta. The telemeter was placed in the abdomen. Each rat was put on a SmartPad that allowed transcutaneous recharging and continuous recording.

Vesico-urethral function can be assessed in animal by cystometry and urethral pressure profilometry or by electromyography under general anesthesia. Anesthesia can reduce bladder and urethral pressures and inhibit the micturition reflex. Urodynamic telemetry takes noteworthy benefits for urologic research and has been shown in pigs (Mills 2000), dogs (McCafferty 2009; Noël 2013) and monkey (Ghoniem 1996). Urodynamic telemetry can decrease stress to the animals because animal handling or chemical restraint is not needed. Anesthesia and catheters in bladder or urethra are known to change the micturition reflex.

A ventral midline celiotomy was made and the transmitter body was fixed subcutaneously to the left abdominal wall. The leads of the transmitter were entered the abdominal cavity from the abdominal wall. The first pressure catheter of transmitters TL11M3-D70-PCTP (DSI) was placed into the bladder through a hole made in the ventral bladder wall and was fixed to the bladder wall. The second pressure catheter was put free in a posterior intraabdominal position. The two EMG leads were kept in the smooth muscle part of the urethral wall at the level of the cranial third of the urethra, and fixed with simple interrupted sutures. The abdomen was routinely closed.

Long-term study of the lower urinary tract by telemetry should be performed during the night to obtain repeatable recordings due to circadian variations. The investigation of urethral smooth muscle function by EMG needle electrodes seems to be a practicable technique to evaluate the effects of drugs with urethral tropism.

Continuous measurements of pressures in the bulb of the corpus spongiosum of the penis (CSP) in awake, freely moving rats enables for simultaneous recording of erectile and micturition events (Nout 2007). Monitoring of CSP pressures can be conducted 8–12 weeks in conscious animals, and the methods have recently been verified in rats. New method to simultaneously monitor micturition and erections in rats by telemetry in the CSP was established (Nout 2007). The catheter of telemetry pressure implant was placed in the bulb of the CSP. CSP pressure was measured. Micturition was recorded by video to determine presence and volume simultaneously. Behavioral tests were performed to determine erection. Micturition duration measured by CSP pressure and volume calculated by urine weight were greatly matched. 100% of visually verified erectile events happened simultaneously with CSP pressure waveforms characteristic of erections during *ex* copula reflex erection tests. Telemetry found more erections during noncontact erection and mating tests compared to observation alone. Erections were shorter in duration and usually were characterized by a single suprasystolic CSP pressure peak. Quality of measurements kept stable for 8 weeks. Telemetric measurement of CSP pressure supplies a quantitative and qualitative evaluation of penile erections and micturition in freely behaving rats.

The major complications of spinal cord injury (SCI) include disruption of bladder function and sexual reflexes. Telemetric monitoring of CSP pressures was reported to evaluate micturition and erectile events following SCI in rats (Nout 2007). Pressure catheter was implanted in the bulb of the CSP rat. Telemetry measurement of CSP pressures in conscious rats is a precious and dependable technique for evaluating recovery of autonomic function following SCI (Nout 2007).

The advantages of telemetry measurement for intraspongiosal pressure (ISP) and ICP1 were summarized (Shamloul 2008). First, it enables for qualitative and quantitative evaluation of erectile responses in experimental animals, which is not possible by other techniques.

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Second, it also enables for monitoring of "real" ICP1 and ISP changes in conscious animals subjected to different sexual stimuli. Third, telemetric measurement can monitor intrapenile pressure changes at different time points. Thus the data collected are more reliable particularly in behavioral studies. Fourth, this technique takes the advantage of being able to analyze ICP1 changes in copulating male animals during actual sexual contacts with their females, supplying precious data on physiological changes occurring during mating. Fifth, extensive analysis of ICP1 and ISP changes during reflexive erection, non-contact erection and drug-induced erections can be only performed by telemetric recording. Sixth, ISP telemetry measurement in mice enabled to recognize a series of extended ISP peaks that easily corresponded to ejaculation (Soukhova-O'Hare 2007). Lastly, telemetry technique can be used to set ICP1 cut-off values for full erectile response to solve the issue of partial vs. full erectile response for behavioral evaluation of erectile responses. The application of telemetric measurement of ICP1 and ISP may become the gold standard method for evaluation of erectile responses.

The pressure of CSP was recorded by telemetry (Gulia 2008; Nout 2007; Salas 2007). In brief, the catheter tip of the telemetric transducer (TA11PA-C40 DSI) was inserted into the bulb through a slit made by a needle after the distal portion of the bulb of the CSP was gently exposed. The implant body was secured subcutaneously to the abdominal muscle. The catheter was fixed at entrance of the catheter by biological glue and was sutured to the fascia overlying the shaft of the penis.

Urinary incontinence (UI) is a frequent and disturbing problem which may affect the quality of life in the elderly, diabetics and autistic children. UI may also induce other complications including kidney failure and infections. Bladder sensors are needed for UI patients without sensation after bladder urine volume/pressure is over normal ranges. Pressure variations are recorded by a catheter-based sensor which is kept in bladder from the urethra. Disturbances in catheter lines can influence the accuracy of the measurement. Furthermore, infection can be induced by prolonged use of this method. Implantable telemetry sensors for bladder volume/pressure measurement would enable long-term measurement in the bladder and keeping the quality of life (Dakurah 2015).

A telecystometric implanted minipig model was established which can be used to explore the circadian behavior and physiological and pathological bladder function (Huppertz 2015).

Telemetry was used to monitor the systemic and urodynamic parameters in lower urinary tract dysfunction (LUTD) in rats induced by experimental traumatic brain injury (TBI) (Moody 2014). Telemetry measurement established that moderate TBI was a risk factor for neurogenic bladder disorder.

A new approach to cystometry by telemetry in conscious rats with cyclophosphamide-induced bladder dysfunction was reported (Monjotin 2016). Bladder pressure and voided volume are able to be measured in both anaesthetized and conscious animals by conventional cystometry. In conscious animals, bladder infusion cystometry can be influenced by several experimental biases which include the decreased movements of the animal, an under-estimation of the sensory element for triggering the micturition cycle and the compound of the infusion fluid. Radio-telemetry can continuously measure physiological parameters in fully unrestrained and more physiological conditions. A similar technique was established in beagle dogs (McCafferty 2009) or in minipigs (Huppertz 2015).

PA-C40 (DSI) telemetry implant was kept on the right side of the abdominal cavity and fixed on the abdominal wall in order to avoid contact with the bladder (Monjotin, 2016). The

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pressure catheter was put in the dome of the urinary bladder and sutured with 6-0 silk. The quality of traces and parameters recording monitored by telemetry were similar to those with conventional cystometry. This telemetry measurement is as precise as conventional cystometry. Compounds for modulating the voiding patterns may be assessed repeatedly by telemetry. Telemetry can be used to monitor the physiological and pathophysiological functions of the bladder. Telemetry will enable the pharmacological assessment of compounds for bladder function.

Renal sympathetic nerve activity (RSNA) and BP was measured by telemetry in two-kidney, one-clip rat model with renal denervation by cryoablation of the renal nerve to the clipped kidney (Rossi 2016). The catheter of the radio telemetry implant (TA11PA-C40; DSI) was inserted into the femoral artery and then advanced into the abdominal aorta after renal artery clipping was completed (Rossi 2016). The telemetry probe (Telemetry Research TR46S, Auckland, New Zealand), including BP and nerve electrode components, was implanted according to a modification of the technique (Stocker 2013). Briefly, left groin incision and left flank incision were made. A tunnel was made subcutaneously from the femoral area to the flank. The electrode wires from the transmitter were passed through this tunnel for subsequent placement. The catheter with BP telemetry probe was inserted into the left femoral artery and fixed. The left renal nerve was separated under a stereomicroscope. The nerve was carefully put onto the exposed ends of the electrode wires from the transmitter. The silicone casing of the proximal ends of the wires was then stabilized by anchoring it with 6-0 sutures to the adventitia of the aortic wall. The electrodes position and the nerve signal quality were determined by assessing the nerve sound by an audio monitor and confirmed by an oscilloscope. Then, the nerve and electrodes were covered with silicone gel (Kwik-Sil; World Precision Instruments, Sarasota, FL).

TELEMETRY APPLICATIONS IN EXPERIMENTAL SURGERY FOR NEUROLOGICAL DISEASES

Early diagnosis of shunt malfunction is crucial in preventing neurological morbidity and death in patients with hydrocephalus. Diagnostic techniques for shunt malfunction are limited. Implantable ICP2 monitor cannot be used to diagnose shunt malfunction for long term due to limited instrumentation lifespan, marked measurement drift and device complexity. Baric probe was produced to resolve these problems (Limbrick 2012). It includes a subdural fluid bladder and multichannel indicator that traces the location of an air-fluid interface. A handheld ultrasound probe is applied to examine the baric probe *in vivo* for noninvasive ICP2 measurement. Short- and long-term *in vivo* experiments were done in a porcine model with concurrent measurements of ICP2 by a fiber optic monitor. Baric probe, the long-term implantable ICP2 monitor produced to promote the prompt and accurate diagnosis of shunt malfunction. The baric probe demonstrated a constant linear relationship between ICP2 and the device's AFI in short- and long-term *in vivo* models.

Patients with head injury, idiopathic intracranial hypertension and hydrocephalus often need measurement of ICP2 and may require repeated recording for months or years. The gold standard for assessment of ICP2 is the external ventricular catheter. All the ICP2 recording tools in current clinical application need a physical connection between the brain and the

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external environment. The duration of measurement is limited due to infection. Telemetric recording implements have been produced but remarkable technical problems have prevented their application in routine clinical practice. All available ICP2 detectors are nonpermanent percutaneous implanted devices. Placement of these devices involved remarkable morbidity, especially infection. Repeated monitoring for patients needs several surgical procedures.

Precise telemetric ICP2 monitoring system can reduce the risk of infection in patients with severe head injury and keep its high reliability for a long time. Several neurosurgical conditions need careful control of ICP2 over several years to assure an acceptable outcome, such as idiopathic intracranial hypertension, hydrocephalus treated by endoscopic third ventriculostomy or a VP shunt. ICP2 is monitored by lumbar puncture tapping or by a ventricular access device for these situations. These invasive procedures, often necessary in children, have a risk of infection. Now ICP monitors display a notable zero drift of the sensor over time which excludes them from long-term use since the sensor of the implant cannot be recalibrated.

An implantable telemetric device would control many of the problems currently related to ICP2 measurement. Telemetry would enable the ICP to be recorded non-invasively at home; symptoms could then be easily assessed and the necessary adjustments to a programmable shunt, when present, could be done and confirmed. A fully implantable ICP2 sensor was designed for long-term use by utilizing micro- and nanotechnologies as well as advanced packaging technologies. The sensor implant includes an absolute capacitive pressure sensor and readout integrated circuit with ultralow power consumption and so an implanted battery does not need a power supply, which would otherwise limit the implant's lifetime. The implant is produced in a way so that it can be inserted as stated in the confirmed minimally invasive procedures known from current ICP monitors. The monitoring tip can be put either in the brain parenchyma or ventricle while the telemetry unit is placed outside the cranial bone directly under the skin for optimal linking to the external unit (Frischholz 2007).

EEG was measured by EEG telemetry transmitters of the 10–20 system in Cynomolgus monkey, Beagle dog and Sprague-Dawley rat (Bassett 2014). Pentylenetetrazol (PTZ) was given intravenously as a positive seizurogenic agent to decide seizure threshold. Premonitory clinical signs usually comprised hypersalivation, ataxia, emesis (except in rats), reduced physical activity, increased physiological tremors, and myoclonus.

The telemetry transmitter was kept between the aponeurosis of the transversus abdominis muscle and the internal abdominal oblique muscle for monkey. EEG electrodes were tunneled subcutaneously to a small skin incision in the neck. EEG leads (TL11M2-D70-EEE, DSI) were fixed on to the skull bones to measure three standard bipolar derivations (C3-O1, C4-O2 and Cz-Oz) by the 10–20 electrode system. A linear groove was performed in the cranial cortical bone to fix the electrodes with surgical glue Vetbond and acrylic. EMG recording was acquired by electrodes fixed to longitudinal muscles in the neck area and measured continuously with the telemetry transmitter. Measurement was performed three weeks after the surgery.

A telemetry transmitter (TL11M2-C50-PXT or F40-EET, DSI) for EEG measurement was used with one standard bipolar derivation (Fz-Oz) in rats. Penicillin G procaine (1.0 mL, 300 000 IU/mL) was given SC once daily for three days beginning on the day of surgery. Buprenorphine (0.04 mL, 0.3 mg/mL) was provided twice daily for three days. Local anesthetics (Bupivacaine, 0.25%, 0.1 mL; Lidocaine, 20 mg/mL, 0.1 mL) were given in 4 SC sites dispersed over the skull surgical site. The animal was put on a heating pad under the

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anesthesia of isoflurane. The telemetry transmitter was fixed in the abdominal cavity. The electrodes of EEG and EMG were tunneled subcutaneously to the neck skin incision. The abdominal skin incision was closed and the animal was kept in sternal recumbency to uncover the skull during the surgery. The EEG leads were fixed on the cranial bone to record one bipolar derivation while EMG leads were fixed to muscles of the neck. A linear groove was performed in the cranial cortical bone to fix the electrodes with surgical glue Vetbond and acrylic. Measurement was performed three weeks after the surgery.

Telemetry video-EEG for seizure liability studies was described in rats, dogs and NHP. Rats are a preferred model in seizure liability evaluations. Beagle dogs are commonly connected with overt susceptibility to seizure and are usually utilized in seizure liability investigations only if needed by regulators. NHP is a vital model in seizure liability evaluations, provided similarities to humans and a high translational potential.

A portable measurement system and techniques for acquiring chronic monitoring of single units and tracking rhesus monkey behavior in an open field was reported (Lei 2004). The techniques supply a new way to evaluate correlation between NHP behaviors and single neuron activity, and are also useful to investigate the cellular basis of NHP social behaviors.

Implantation of the device in masticatory muscles was able to evaluate the signals and ascertain the activity levels for daily muscle application (Langenbach 2002). Analysis of the transmitted EMG disclosed that the device can be utilized for prolonged *in vivo* EMG registration, identification of peak activity levels, and the evaluation of general muscle use by the time spent at different levels of activity.

Video-EEG measurement is a standard diagnostic method in humans. Its application in telemetered freely moving macaque monkeys for safety pharmacology study is limited. Rodents are usually used for proconvulsant risk assessment. NHP was used in toxicology and safety pharmacology. Telemetry implants were instrumented in cynomolgus monkeys (Authier 2009). EEG-video recording can be helpful to characterize neurological unfavorable effects with unforeseeable onset in NHP. Computerized video-EEG analysis was a precious method for safety pharmacology studies including proconvulsant risk evaluation, spectral analysis of frequency bands and sleep stage confirmation.

Pre-clinical animal models of human neurologic diseases can slowly develop the disease and be used to evaluate the interventions at various stages of the disease process. EEG continuous measurement in immature rodents models of seizures and other neurological disorders has a technical challenge due to the small physical size of immature rodents and their dependence on the dam prior to weaning (Zayachkivsky 2015).

The autonomic nervous system supervises involuntary control of different visceral organs such as the heart and blood vessel (Jänig 2006; Robertson 2012). The diagnostic methods for autonomic function evaluation have been established in laboratory animals. The limitations and usefulness of diagnostic methods was reviewed recently (Salman 2015). Telemetry was reported for the first time to simultaneously monitor BP and renal SNA in chronic renal dysfunction rat model (Salman 2015). The surgery details and techniques for monitoring renal, lumbar and splanchnic SNA in conscious rats was reviewed (Stocker 2013). Renal SNA and BP were able to be measured by telemetry in conscious mice (Hamza 2012). Brain tissue oxygen can be measured chronically by telemetry in conscious rats (Russell 2012).

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TELEMETRY APPLICATIONS IN EXPERIMENTAL SURGERY FOR GLAUCOMA

Glaucoma is one of the important reasons of blindness in the developed world (Quigley 2006). Ocular hypertension is an important risk factor for glaucoma. To decrease ocular hypertension is the only confirmed-effective therapy for glaucoma. IOP dynamics and its contribution to the disease progression are monitored by the tonometry methods usually used in clinical practice. IOP measurements which are taken at patient visits are not enough to characterize pressure variations due to disease and typical daily pressure variations.

Animal model for glaucoma research needs precise and duplicatable measurements of IOP. The pneumatonometer (PTG) is the typical technique used in pharmacology and eye research. The major disadvantages of this technique are that the animals must be restrained and human intervention is needed. Furthermore, a local anesthesia needs to be topically provided before IOP measurements by PTG are obtained. This may interfere with the absorption pattern or activity of the new drug in experiment. Radio telemetry system was first used in rabbit to characterize IOP in response to pharmacological agents (McLaren 1996). It was also used in rats (Lin 2010), mice (Li 2008), pigs (Leonardi 2009) and NHPs (Downs 2011).

Rabbits were anesthetized with IM ketamine (50 mg/kg) 20 minutes after IM xylazine (10 mg/kg). Anesthesia was maintained by providing additional doses of xylazine (5 mg/kg) or ketamine (50 mg/kg) as required. Animals were prepared according to aseptic surgery method. A 4-cm midline incision was performed on the dorsal neck. Muscle over the vertebral column, between the scapulae and the connective tissue were separated to make a pouch to carry the transducer capsule. The right eye was used in all experiments.

Slight head position changes modify the altitude of the eye relative to the telemetry transducer, which may cause big errors in the IOP measurement for animals with a large head and flexible neck (Downs 2015). IOP transducers should be kept in the orbit or eye to reduce head position artifact for NHPs and other large animals.

Implantation of Transmitters for Measurements of IOP

The pressure transmitters TA11PA-C40 were implanted subcutaneously under aseptic conditions. The rabbits were shaved in the scalp region and put on a warmed sterile operative field after the anesthesia was induced and maintained. A small scalp incision was performed and one subcutaneous air pocket was prepared in the neck region. The transmitter body was sutured in the neck pocket, and its pressure catheter was tunneled subcutaneously to an exit site in the superior conjunctival sac of the implanted eye. The telemetry catheter was subsequently inserted in the midvitreous of the eye which was 3 to 4 mm behind the corneoscleral junction. It was fixed at the site of entry with tissue adhesive Vetbond. The skin incision was closed. IOP, MAP, HR, and motor activity were able to be accurately and reliably measured by telemetry in conscious freely moving rabbits. This technique can be applied to evaluate the effects of drug treatment on the parameters over short or long time in conscious rabbits under physiological conditions.

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IOP Telemetry in NHPs

The benefits of the telemetry system from Konigsberg Instruments, Inc. (Pasadena, CA) included continuous measurement of IOP at a 500 Hz measurement frequency for about 24 months, low drift of < 3 mm Hg per month, direct calibration of implants by anterior chamber cannulation and manometry, and the transducer in the orbital wall adjacent to the eye (Downs 2011). The disadvantages of the system are cost, the surgery difficulty, and no replaceable battery (Downs 2011).

The IOP transducers is often kept under the skin in the nape of the neck or between the clavicles for rabbits, rodents, and other small animals whose eyes remain at about the same height as the pressure transducer during daily activity. For NHPs and other large animals, IOP transducers must be placed in the orbit or eye to minimize head position artifact (Downs 2015). IOP changes about 10 mm Hg from day to day and hour to hour in free moving nonhuman primates. It suggests that snapshot IOP recording may be insufficient to catch the true dynamic character of IOP (Downs 2011).

TELEMETRY APPLICATIONS IN EXPERIMENTAL SURGERY FOR ENDOCRINE SYSTEM DISEASES

Continuous blood glucose measurement is important for diabetic management because glycemic control can reduce the risk of diabetic complications such as heart diseases, diabetic retinopathy, stroke, kidney damage, and neuropathy. Various technical and biological challenges have limited chronic continuous glucose measurements for animal. New implantable glucose monitor HD-XG (DSI) was applied for continuous monitoring of plasma glucose levels in the arterial blood of rats (Brockway 2015). The catheter of HD-XG was inserted in abdominal aorta by the method as described for rat aorta BP implantation. Continuous measurements of glucose can last for 75 days or longer. Telemetry contributes significant advantages in the quantity and quality of data that can be obtained relative to existing methods. Telemetry gives the possibility to broaden the understanding of both glucose homeostasis and metabolism to improve treatment and cures for diabetes.

A new telemetry system for real-time simultaneous measurement of biosensor signals (brain glucose and lactate) and motion were reported (Rocchitta 2013). The device includes dual-channel, single-supply miniature potentiostat-I/V converter, a signal transmitter, a miniaturized microvibration sensor and a microcontroller unit. The device was validated and characterized before experiments. The biosensors were kept in the striatum of freely moving animals and the biotelemetric device was secured to the animal's head. The simultaneous monitoring of brain neurochemistry and motion could have an important effect in the study of motion-related disorders such as Parkinson's disease in freely moving animals treated with neurotoxins such as 6-hydroxydopamine, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine, and/or rotenone.

The intravenous blood glucose (IVBG) system was used to investigate the accuracy, reliability and safety in 100 patients at 6 US hospitals (Bochicchio 2015). The results suggest that the IVBG system is an automatic and practical glucose monitoring system. It provides accurate and reliable blood glucose measurement which ameliorates the safety and efficacy of

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insulin therapy and blood glucose control. Continuous glucose monitoring system can precisely determine blood glucose every few minutes (Joseph 2015).

TELEMETRY APPLICATIONS IN EXPERIMENTAL SURGERY FOR CANCER

Radiotherapy and/or chemotherapy with hyperthermia are encouraging strategies for cancer treatment. The key to success of this treatment is temperature control. Temperature measured by telemetry was valuable biomarker of disease progression in mice with lymphoma and could possibly be utilized more extensively to observe mice with other forms of cancer (Hunter 2014). The effects of therapeutic cancer vaccine CHP-NY-ESO-1 on the cardiovascular system were evaluated in dogs to monitor HR and BP by telemetry (Harada 2008). Cardiovascular system safety pharmacology study for resveratrol with cancer preventive activity was evaluated in dogs by telemetry (Johnson 2011).

In vivo sensors have been used to control the temperature changes in cancer cells during combined treatment (Kamal 2012). A smartphone was designed to manage temperature scheduling in *in vivo* sensors and communicate with local or remote clinicians to keep collaborative efforts for combined therapies against cancer.

Vomiting and nausea are usual side effects of cancer chemotherapy. Abdominal pressure measurement in ferret by telemetry is a trustworthy technique to observe emetic events which may accelerate the progress of anti-emetic treatment and new chemotherapeutic agents (Goineau 2013). Telemetry was used to evaluate the synergistic effects of ondansetron and aprepitant in cisplatin-induced emesis ferret model (Goineau 2014).

TELEMETRY APPLICATIONS IN EXPERIMENTAL SURGERY FOR ORGAN TRANSPLANTATION

Xenotransplantation is evaluated to resolve the crucial shortage of donor's organs with improvement of immunosuppressive strategies and the development of genetically modified organ-source pigs. Cardiac xenograft function assessment is hard and time consuming in a pig-to-baboon xenotransplant model. It has been traditionally achieved by palpation, ultrasound and biopsy. Frequent application of anesthesia to the baboon adds the chance of complications such as bleeding, infection, graft injury, morbidity and mortality rate. The animals can be monitored by radiotelemetry while they move freely within their cages. Telemetry has been reported to assess heterotopic cardiac allograft function (Chen 2000; Pirolo 1992; Groeneveld 1981; Koike 1988).

The reliable assessment of graft function provided clues for infection, rejection or loss of graft function. Frequent anesthesia of recipient baboons was avoided by the radio telemetry system to assess the cardiac function. BP, HR, EKG, activity and BT can be measured at the same time. Also, the radio telemetry system avoided manipulation by the primate recipients which were not able to endure external leads and recording devices well unless the animals were completely sedated or restrained. The technique supplied more humane treatment compared to the traditional methods (Chen 2000). Telemetric monitoring systems allowed

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non-invasive, trustworthy and continuous evaluations of cardiac xenograft function and contributed precious information for the improved understanding of transplantation pathophysiology. Importantly, telemetry system avoided repeated anesthesia of the animals and was able to measure without the effects of anesthetics.

The telemetry transmitter (TL11M3-D70-PCTP DSI) has two pressure catheters and two biopotential leads. One pressure catheter was inserted into the xenograft's left ventricle through the apex and fixed by a 6-0 purse-string suture with pledgets. The other pressure catheter was implanted into the aorta. The two biopotential leads were sutured and fixed onto the ventricular epicardium for ECG monitoring. The telemetry transmitter was put in a subcutaneous pocket in the chest wall before the thoracic cavity was closed. The parameters LVPSP, HR, ± dP/dtmax and QRSA recorded by telemetry are reliable indicators of myocardial damage related to graft rejection at an early stage after cardiac xenotransplantation. The application of the telemetry system might help to guide immunosuppressive therapy and further improve graft survival in future experiments (Fan 2013).

The advantages of this implantable telemetry system include the ability to continuously measure transplanted heart function and recipient temperature remotely and supply accurate measurements of graft survival and management of immunosuppression. The loss of graft function was reliably detected by the radio telemetry measurement of LVP. The complications of LVP probe include bleeding from the LVP probe insertion site and LV cavity thrombus formation. Electrical cardioversion for fibrillation was not able to use with telemetry system. Telemetry system needed to be removed if cardioversion was required (Horvath 2010).

Telemetry was reported to measure cardiovascular parameters in rats that performed a T4 spinal cord transection with or without grafting of embryonic brainstem-derived neural stem cells expressing GFP (Hou 2014). In addition, cardiovascular parameters can be compared before and after spinal cord injury in the same rat.

LVP evaluation by telemetry is a useful method to assess transplanted heart function in experimental heterotopic cardiac xenotransplantation (Horvath 2010). The telemetry was also helpful to detect early onset of fever in the recipients, thus intervene early and prevent potentially lethal septic complications. Continuous measurement of several parameters via telemetry enabled identification of changes related to early complications and rejection. Proper intervention and treatment was given to reverse rejection.

Continuous telemetry measurement found increased ventricular arrhythmias in coronary artery ligated rats with transplantation by intramyocardial injection (Narita 2013) and myocardial infarction mice with transplantations of cell-derived cardiomyocytes (Liao 2013).

CONCLUSION

Radio telemetry techniques have been successfully applied in animal models of human diseases from different systems. The physiologic signals can be monitored chronically in conscious animals for a long period of time. The data quality has been ameliorated compared with conventional methods. These techniques will play an important role to prompt the preclinical research for clinical application.

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Section II. Animal Surgery Models

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Chapter 9

SMALL ANIMALS AS EXPERIMENTAL MODELS

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ABSTRACT

Animal models can prove invaluable in predicting outcomes of human surgical procedures and medical interventions. Clearly the closer the anatomic and physiological similarity between the studied animal species and human, the more closely the animal model will mirror outcomes in people. Often non-human primates will most closely approximate results in humans. Nonetheless, ethical and economic concerns regarding these large animal models mean that small animal models retain strong utility. Generally speaking, large animal models are reserved for final pre-clinical toxicity and efficacy studies, with all preliminary studies conducted in small animal models. Small animals used in experimental surgery chiefly consist of mammals – rodents (mouse, rat, guinea pig and hamster) and rabbits. Mice are most commonly used because of their economy, rapid breeding cycle, ease of handling, and availability of inbred strains and genetically modified strains. The large animals (chiefly non-human primate, dog and pig) will be covered in next chapter.

The principles of the three R's: refine (less suffering,), reduce (lower animal numbers) and replace (alternative *in vitro* assays) must be applied in all small animal experiments.

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Well-designed animal model experiments have allowed us to develop the surgical techniques currently available for treating patients. In this chapter, we examine background to the common small animal models and practical aspects used in experimental surgery. We also look at the utility and limitations of small animal model surgery.

Keywords: animal model, surgery, rodent, mice, rat

INTRODUCTION

Use of animals in experimental surgery began many centuries ago. Galen used live animals in many of his early experiments for understanding anatomy in the second century AD. In 1100s, the Arabic surgeon Avenzoar would experiment on animals before trying new surgical techniques on humans. The Nobel laureate and surgeon Alexis Carrel used animals in his studies of vessel suturing in the 18th – 19th centuries. It was determined by the Nuremberg Code in 1947 that animal experimentation results must be the basis of conducting research on humans. This was later reinforced by the Helsinki declaration in 1975 (Calasans-MaiaI 2009). Animal models have been used extensively in the development of modern surgery and some models mirror specific diseases, such as the lipopolysaccharide (LPS) administration mouse model for septic shock. Animal models have been used to simulate all types of human transplant, common examples being heart and skin transplants (Chong 2013) and in surgical training e.g., pig laparoscopic cholecystectomy and nephrectomy.

HISTORY OF RODENT MODELS

In the early days of experimental animal surgery, the dog and pig models were most commonly used because of the technical ease of performing the larger vascular anastomoses. With the development of microsurgical techniques in the 1960s, rodents became more popular because of simplicity, favourable public and animal protective agencies opinion, and reduced costs (Chong 2013). The Rat genome was sequenced in 2004 and results revealed that the rat genome contains 2.75 billion base pairs (Gibbs 2004). This compares with 2.9 billion in the human genome and 2.6 billion in the mouse. Humans have 23 pairs of chromosomes, compared with 21 in rats and 20 in mice. In spite of this the three species contain overall a very similar number of genes, and most disease-associated genes are highly conserved between the species.

Mice have proven invaluable in the assessment of surgical disease (Chong 2013). They can be purchased inbred, with relatively reproducible characterised response to experimental protocols because of genetic similarity. The widespread availability of genetically modified mice, both transgenic and knockout, has made them invaluable assets in experimental models. Embryonic stem cells have been isolated and genetically manipulated in mice. In rats, eggs are sensitive to activation and do not tolerate genetic modification well, although this has been resolved within the last decade with cloning techniques allowing production of genetically modified rats (Doorschodt 2014). Interestingly, genes involved in detoxification show important differences between humans and rodents. Cytochrome P450 (CYP450) is

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important in metabolism of calcineurin inhibitors like cyclosporine, among other immunosuppressive drugs. The CYP450 subfamily member CYP2J has a single gene in humans, but four in rats and eight in mice (Uno 2009). It can be seen that although rodents bear similarities to humans and are vital to examine and test agents, there are important constraints in applicability.

The use of *in vivo* models facilitates the use of various measurements to determine efficacy of drug and others agents. Steroids when given to rabbits give similar results to humans. If dexamethasone is administered, neutrophilia is seen within a day. This is accompanied with lymphopenia. Lymphocyte numbers in the bone marrow are increased. Both lymphocyte and neutrophil function is suppressed, as measured by concanavalin-A proliferation and reactive oxygen species production respectively (Ulich 1988). Understanding of the complex effects that these agents have on the body could not be easily understood with *in vitro* models alone.

ANATOMY AND PHYSIOLOGY

Rodents belong to the order *Rodentia*, characterised by large incisor teeth, which are modified for gnawing. They have a life span of 1−3 years (Animal Care and Use Committee 2 2016; Animal Care and Use Committee 3 2016) and males weigh 300−500 g and females 250−300 g. It is important to note their differences in vitals from humans to ensure appropriate monitoring and treatment. Their heart rate range is 330−480 beats per minute (bpm) and respiratory rate is approximately 85 breaths per minute. Their temperature ranges from 35.9°C and 37.5°C and they are best kept at a room temperature of 18−26°C (Animal Care and Use Committee 1 2016). Male mice weigh 20−30 g and females 18−35 g. Their heart rate range is 310−840 bpm and respiratory rate is approximately 80−230 breaths per minute. Their temperature ranges from 36.5°C and 38°C and they are best kept at a room temperature of 18−26°C(Animal Care and Use Committee 2 2016).

Rat anatomy is similar in many respects to other mammals except that unlike humans they do not have a gall bladder. The circulatory system carries blood from the heart to the lungs, back to the heart and then to the rest of the body through the systemic circulation. The posterior portion of the body drains blood into the inferior vena cava, which delivers it to the sinus venosus, a quadrangular cavity. This is separated from the right atrial wall by a ridge of fibres. The superior vena cava, which also drains into the sinus venosus is made up of the right cranial vena cava, the left cranial vena cava and the two cardiac veins. As in humans, the ventricle walls are thicker and stronger than the atria and the left more so than the right. The average weight of the heart is 0.9−1.0 g in males and 0.67−0.8 g in females (Hu 2013). Due to the small size of the heart a transplant is generally conducted by end to side anastomosis of the thoracic aorta to the recipient’s abdominal aorta and the pulmonary Good haemostatic control is key as their circulating volume is 15−35 ml (Animal Care and Use Committee 2 2016).

SURGERY IN RODENT MODELS

Various surgical models are available in rodents, each with their own utility (Russell 1964). In general, mice and rats are the most commonly used species. Guinea pigs and

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Hamsters are used in xenotransplant experiments. Requirements for rodent surgery include; an animal preparation area, surgeon preparation area, surgical and recovery area.

In preparation for animal surgery, it is essential to secure prospective approval from the relevant ethical institution. These bodies vary between countries in structure, but all share the aim of eliminating cruelty to animals and ensuring that the 3 "Rs" are fulfilled. In USA, the ethical body is the institutional animal care and use committee (IACUC), which is typically based within each research institution at a local level. In UK in contrast, all animal experiments are approved by the central government (Home Office), which issues both a personal license to researchers and project license to the experiment. In practise this means that paperwork takes considerably longer in the UK, therefore advance planning is essential.

Once ethical approval is secured, animals are purchased. Animals are typically bought from large commercial designated suppliers such as Harlan© and Charles River©. These are typically inbred and genetically characterised. Alternatively, genetically modified bespoke animals may be moved between academic institutions, which may take time to ensure local vet certification for animal health status and exemption from pathogens. A record should be kept for each animal including its medical history, any abnormalities in appearance or behaviour, observations, examinations, tests and procedures.

Surgical instruments can be purchased from a wide variety of retailers, including Roboz©. A minimum set includes toothed and non-toothed forceps (e.g., DeBakey's), suture scissors, dissecting scissors (e.g., Metzenbaum/Iris/McIndoe), needle holder (called needle driver in USA), and diathermy (e.g., battery powered Bovie). For more delicate procedures, a micro needle holder (e.g., Castroviejo), and jeweler's forceps will be necessary. If possible these should be inspected before use with a magnifying lens to inspect for damage (Animal Care and Use Committee 1 2016). For survival procedures, expired items may be used if they are re-sterilized and their function is not compromised. All expired drugs and fluids cannot be used. For non-survival procedures all expired items may be used except for physiologically active drugs e.g., anaesthetics, analgesics (Animal research advisory committee 2015).

PRE-OPERATIVE PREPARATION

Ensure animals are of an appropriate age, weight and sex for the procedure in question. They should be acclimatised to the facility, which typically means they should have arrived at least five days before from the designated vendor or originating academic institution.

The operating area should always be clean and clear of clutter. It is important to be free from distractions, without foot traffic of other staff passing nearby. It should ideally be situated in a laminar flow hood, but in practise often a clean bench top is used for small animal work. The surgical area should be non-porous and durable. The bench top should be cleaned daily before use with disinfectant. Alcohols (70% ethyl alcohol or 85% isopropyl alcohol) should be left to disinfect for 15 minutes. Aldehydes, phenolics and chlorhexidine are other options. Ammonium compounds should be avoided as organic matter rapidly inactivates them (Animal Care and Use Committee 1 2016).

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The animal should be anaesthetised and shaved with electric clippers (♯40 blade), depilatory cream or shaved in an area separate from the surgical field. Depilatory creams are safe, effective, non-traumatic and non-toxic. They are also related with a significant reduction in skin surface bacteria and useful in areas that are difficult to use clippers (Animal Care and Use Committee 4 2016). Shaving is the least preferred method as it can cause microscopic defects, which may pre-dispose to surgical site infections. The prepared area should be approximately three times the size of the proposed incision (Pritchett-Corning 2011). The animal should be placed on a recirculating water heating pad if available (electrical heating pads should not be used during surgery) and sterilised using two or three applications of povidone iodine or chlorhexidine, with or without alcohol (Pritchett-Corning 2011). This should be from the proposed incision area outwards in a circular motion. The animal is then covered with sterile drapes and surgical instruments should be ideally placed in a hot bead steriliser. The drapes serve to improve sterility and minimise loss of heat and moisture. Rodents have a high surface area to mass ratio and rapidly lose heat and moisture. Hypothermia can predispose to complications such as infection and slow emergence from anaesthesia.

The surgeon should prepare by disinfecting the hands using a surgical scrub such as chlorhexidine. The hands are washed three times, and then dried with a sterile towel. A sterile gown and gloves are donned.

Anaesthesia

Starving the animals prior to surgery, as for human surgery, is not necessary because risk of regurgitation is low. Anaesthesia provides freedom from feelings. Analgesia, or freedom from pain, is particularly important, and may require additional drugs.

Stages of anaesthesia in terms of increasing depth are:

- Stage 1 — excitatory, disorientation, vocalization, urination, defecation.
- Stage 2 — loss of consciousness, many reflexes are intact but righting reflex is lost, rapid irregular breathing and rigidity.
- Stage 3 — surgical stage of anaesthesia, with loss of reflexes, muscle relaxation, deep and rhythmic breathing.
- Stage 4 — medullary paralysis with respiratory arrest, hypotension and imminent death. Cardio-pulmonary resuscitation and drugs to reverse anaesthesia must be given or animal will die (Animal Care and Use Committee 1 2016).

The depth of anaesthesia can be monitored using a foot, tail or abdominal skin pinch. There should not be twitching in response to this painful stimulus. In addition, respiratory rate, heart rate and temperature can be monitored. Additional equipment is necessary to monitor oxygen saturations (pulse oximetry) or carbon dioxide (capnometry) (Animal Care and Use Committee 1 2016). After induction of anaesthesia, ophthalmic ointment should be applied to the eyes to avoid corneal drying.

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Table 9.1. Injectable anaesthetics and adjuncts in laboratory animals adapted by use of materials from (Animal resource program 2016)

Drug(s)	Mouse	Rat	Rabbit	Comments
Ketamine: Xylazine	80−120 mg/kg: 5−10 mg/kg IP	75−100 mg/kg: 5−10mg/kg IP	20−40 mg/kg: 1−3 mg/kg IM	Provides 20−50 minutes of anaesthesia
Ketamine: Xylazine: Acepromazine	80−100 mg/kg IP: 10−20 mg/kg IP: 2−3 mg/kg IP	40 mg/kg: 5 mg/kg: 1 mg/kg IP	35 mg/kg: 5 mg/kg: 0.75 mg/kg IM	Duration of action: 30−45 minutes in rats and 45−75 minutes in rabbits
Ketamine: Diazepam (Valium)			5 mg/kg: 0.25 mg/kg IV	Short duration and light degree of anesthesia
Pentobarbital	40−60 mg/kg IP	40−50 mg/kg IP		Variable anesthetic depth. Duration 20−40 minutes in mice and 20−60 minutes in rats
Tribromoethanol (Avertin)	200−500 mg/kg IP			Duration of action: 15−45 minutes
Atipamezole	mg/kg IP	mg/kg IP	0.1−1.0 mg/kg IM, IP, SC or IV (depends on xylazine dose administered)	Xylazine reversal agent only
Glycopyrronium bromide			mg/kg IM, SC	Adjunct only: Decreases respiratory secretions and prevents bradycardia

IP = intra-peritoneal, S/C = subcutaneous, IM = intramuscular, IV = intravenous.

Anaesthesia can be intravenous, intramuscularly, intraperitoneal or inhalational. The dose of anaesthetic drug can be difficult to predict but should be based on the animal's body weight (which should be recorded on the day of the operation). Smaller animals often have a faster metabolic rate, and can often need higher dose per kg than larger animals. Differences in strain, sex, size all can affect amount of anaesthetic required. Hypothermic animals metabolise drugs more slowly and can need less anaesthetic. Likewise, very young or old animals may require a lower dose.

Hypothermia is a potential form of anaesthesia in neonatal rodents (up to 5 days old) with a similar potency to morphine 10 mg/kg s/c but may only last up to 30 minutes after removal from hypothermia and so is only suitable for short procedures. This can be induced by a cold water swim at 2−3°C or putting the neonate inside a glove finger and place in crushed ice for approximately 5−8 minutes until there is no response to pain. If this method is used, warming should be slow. And heating pads or lamps are avoided (Animal Care and Use Committee 1 2016). It should be noted that neonatal mice only open their eyes 10−12 days after birth (Animal Care and Use Committee 3 2016).

Injectable anaesthetics should be diluted as small volumes are used and if irritant, it should be injected at several sites. Intraperitoneally drugs commonly used are ketamine/xylazine or 1% pentobarbital. Ketamine/Xylazine is safe and efficacious but may cause hypotension, hypothermia and respiratory depression, it can be reversed by an alpha 2

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antagonist e.g., yohimbine or atipamezole. Its use is not as reliable in guinea pigs. Phenobarbital is rapid acting and easily injected but is a poor analgesic and progressively decreases blood pressure, heart rate and respiratory rate and causes acidosis, hypoxia and hypercarbia (Animal Care and Use Committee 1 2016). Fentanyl and midazolam may be considered as an adjunct as they provide analgesia, sedation and muscle relaxation. Neuromuscular agents should not be used alone as a method of anaesthesia (Animal research advisory committee 2016). Details of specific anaesthetic doses can be found in Table 9.1.

Inhalational anaesthesia allows greater operator control in terms of the depth and duration but risks gases inadvertently affecting the surgeon so a scavenging system should always be used. In rodents only non-rebreathing systems should be used. Isoflurane (Forane) is most commonly used as it permits rapid induction and recovery and minimally affects the cardiovascular and respiratory systems. However, a precision vaporizer is needed and some strains of rats (SHR and WKY) are more sensitive than normotensive (SD) rats. Halothane (Fluothane) is similar to isoflurane but is hepatotoxic and interferes with interferon stimulated natural killer cell activity in mice so is less favourable (Animal Care and Use Committee 1 2016). Ether is irritating to respiratory passages, is explosive and requires an open drop method where the rodent is dropped into a container that contains ether. It is unsafe in guinea pigs but has various complications in other rodents that can be fatal.

Intra-Operative Care

Knowledge of the anatomy dictates the most appropriate incision e.g., a v-shaped incision will open the anterior rib cage for wide exposure, while avoiding the mammary vessels. This is used when harvesting the heart for transplantation (Hu 2013). The operative field should be covered and wet by warm sterile saline.

Meticulous technique is necessary to minimise blood loss given the small circulating volumes of rodents as well as gentle handling of tissues to avoid trauma. Irrigating the surgical field with warm sterile saline will help reduce fluid loss. Instruments should be maintained as sterile as possible, e.g., by storing on the sterile drapes or in a beaker of alcohol. If the hot bead steriliser (260°C) is used, the instrument tips should be allowed to cool before contacting living tissues. Blood and soft tissue should be wiped off surgical instruments before placing in the hot bead sterilizer.

Microsurgery may be practised by suturing an incision on a latex glove under a microscope or on discarded cadavers of rodents. Typically for anastomosis, 8.0 to 10.0 non-cutting or round needle sutures may be used and 4.0 cutting or reverse cutting for abdominal closure for small animals (Hu 2013). Absorbable sutures are suited to soft tissues including blood vessels and non-absorbable should be used for the skin.

Post-Operative Care

When the operation is complete the animal should be placed in a cage with a clean absorbent paper base (not husbandry bedding) with half of the animals body on a heating pad. A warming lamp may alternatively be used. They should be observed and turned every 15

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minutes until ambulatory, then daily monitoring for one week. Food and water should be easily accessible. Rats consume 10−30 g/day of feed and drink 20−50 ml/day of water (Animal Care and Use Committee 2 2016) whereas mice consume less, 4−5 g/day of feed and 3−5 ml/day of water (Animal Care and Use Committee 3 2016). Antibiotic prophylaxis is not usually necessary. Abdominal wound clips and sutures should be removed 7−10 days post-op. Fluid loss may be addressed by administration directly to the abdominal cavity or warm subcutaneous saline (Hu 2013) at 3−5% of body weight (Animal Care and Use Committee 1 2016).

Specialised post-operative monitoring after cardiac surgery may use electrocardiogram. This is by using a 4 conductor shielded electrode cable which has hypodermic needles soldered to it. As the electrodes are placed subcutaneously, it is necessary to anaesthetise the animal in the supine position. There is only one precordial lead when investigating small animals. Leads 1, 2, 3, AvR, AvL and AvF will be produced (Hu 2013). As a rough guide of post-operative recovery and physiologic function, urine output in rats is 3.3 ml/100g/day and for mice is 0.5−1.0 ml/day (Animal Care and Use Committee 2 2016; Animal Care and Use Committee 3 2016).

After finishing surgery, all instruments should be thoroughly cleaned with soap, water and a brush. They should then be autoclaved where possible or soaked in disinfectant. Disinfectant should be rinsed off with sterile saline before operating. It is advisable to use a new sterile pack after 5 rodents (Animal Care and Use Committee 1 2016). The same cap and mask may continue to be worn between cases but sterile gloves and disposable gowns should be changed. Accurate records should be kept for surgical procedures. This should include the date, time, DOB, age, gender, name of surgeon, animal ID number, description of the operation, drugs used (name, dose, route) and intraoperative and post operative observations.

It is important to ensure animals are kept comfortable following surgery. Pre-emptive analgesic options are buprenorphine, morphine paracetamol and NSAIDs. Local anaesthetic to the wound site is also an option. Post-operative pain management is an important area to address for the humane treatment of these experimental models and will also aid their recovery.

Signs of pain in rodents include:

- Hunched up posture
- Shivering, blue extremities, hypothermia
- Unkempt, ruffled fur without proper grooming
- Anorexia, not eating and not passing faeces
- Dehydration, not drinking and ability to pinch skin
- Panting, rapid breathing

Both mice and rats have a harderian gland, which is horseshoe-shaped and found within the eyes orbit. It secretes a reddish-brown porphyrin pigment in differing quantities depending on the physiologic state, age, strain and sex of the mouse. During periods of stress i.e., pain or inadequate analgesia secretions increase and appears as ‘red crusts’ around the eyes and nostrils (Animal Care and Use Committee 2 2016; Animal Care and Use Committee 3 2016). If this is observed steps should be taken to address possible causes of stress (Table 9.2).

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Analgesics include opioids, non-steroidal anti-inflammatory drugs (NSAIDs) and paracetamol (acetaminophen). The opioids have an extensive first pass metabolism in the liver in rodents, somewhat limiting oral potency. Examples include morphine, fentanyl, carprofen and buprenorphine. Oxymorphone provides 2−3 hours of analgesia and is available in the form of duromoroh (Animal Care and Use Committee 1 2016). Pentazocine has similar analgesic effects but is less sedating. Naloxone may be used if necessary.

The most commonly used NSAIDs are ketoprofen, caprofen and ibuprofen. They are available in intravenous and intraperitoneal preparations. They are analgesic, anti-inflammatory and anti-pyretic. Paracetamol has limited analgesic properties in rodents, but does not cause intestinal haemorrhage like aspirin can (Table 9.3).

If the animal is euthanized or dies, their complete medical record should be retained and sent upon request to the IC Animal Program Director. All animal medical records should be kept for 3 years after the conclusion of the study (Animal research advisory committee 2014). The preferred process of euthanasia by veterinarians is sedation followed by injection of a barbiturate, preferably sodium pentobarbital (Calasans-MaiaI 2009).

Table 9.2. Possible signs associated with pain or distress in mice, rats and rabbits (Animal research advisory committee 2015)

Potential Signs	Mice	Rats	Rabbits
Decreased Food and Water Consumption	X	X	X
Weight loss	X	X	X
Self-imposed isolation/hiding	X	X	X
Self-mutilation, gnawing at limbs	X	X	X
Rapid Breathing	X	X	X
Opened-Mouth Breathing	X	X	X
Abdominal Breathing	X	X	X
Grinding Teeth		X	X
Biting/Growling/Aggression		X	X
Increased/Decreased Movement	X	X	X
Unkempt Appearance (Erected, Matted, or Dull Haircoat)	X	X	X
Abnormal Posture/Positioning (e.g., Head-pressing, Hunched Back)	X	X	X
Restless Sleep			X
Tearing (including Porphyria), Lack of Blinking Reflex		X	X
Dilated Pupils			X
Muscle Rigidity, Lack of Muscle Tone	X	X	X
Dehydration/Skin Tenting/Sunken Eyes	X	X	X
Twitching, trembling, tremor	X	X	X
Vocalization (Rare)	X	X	X
Redness or Swelling Around Surgical Site	X	X	X
Increased Salivation	X	X	X

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Table 9.3. Doses of analgesic drugs for mice, rats and rabbits (Animal resource program 2015)

Drug	Mouse	Rat	Rabbit	Comments
Bupivicaine 0.5% (Local anaesthetic)	Maximum 8 mg/kg S/C	Maximum 8 mg/kg S/C		Injected or dripped onto incision site. Duration 4−8 hours
Buprenorphine	0.05−0.1 mg/kg S/C, IP	0.01−0.05 mg/kg S/C, IP	0.01−0.05 mg/kg S/C, IM or IV	For mild to moderate pain Duration: 8−12 hours in mice and 6−12 hours in rats and rabbits
Butorphanol	1.0−5.0 mg/kg S/C	2.0 mg/kg S/C	0.1−0.5 mg/kg IM / IV	For mild to moderate pain Duration: 4 hours
Carprofen (NSAID)	4−5 mg/kg S/C	5−10 mg/kg S/C, PO	2−4 mg/kg S/C, 1.5 mg/kg PO	For mild to moderate pain Duration: 24 hours in mice/rats, 12−24 hours in rabbits
Flunixin meglumine (NSAID)	2.5 mg/kg S/C	2.5 mg/kg S/C	1.1 mg/kg S/C	Duration: 12 hours
Ketoprofen (NSAID)	5 mg/kg S/C	5 mg/kg S/C	3 mg/kg S/C	For mild pain Duration: 12−24 hours
Meloxicam (NSAID)	1−5 mg/kg S/C	1−2 mg/kg S/C, PO	0.2−0.3 mg/kg S/C	For mild pain Duration: 24 hours
Morphine	10 mg/kg S/C	10 mg/kg S/C	2−5 mg/kg S/C	For moderate to severe pain Duration: 2−3 hours in mice and rats, 2−4 hours in rabbits

S/C = subcutaneous, IM = intramuscular, IV = intravenous.

TRANSPLANT MODELS

The skin transplant was the first model developed and used by Sir Peter Medawar in the 1940s and 50s in seminal experiments, which led to the understanding of self and non-self (Brent 1976). This was the only model to study transplant immunology until the 1960s. The skin transplant model is technically straightforward. It involves excising a small square of full thickness skin from the donor animal and deep fat is dissected off. This is placed on a similar sized defect on the recipient abdominal wall and secured with glue or sutures. The graft is protected for 3−5 days with a dressing. This procedure will induce a rapid tempo of T cell-mediated rejection within 7−12 days for mice different at the major histocompatibility complex (MHC) locus (Mayer 1988).

The first rodent vascularized organ transplants were performed in the rat but now virtually all organ transplants performed in the rat are also performed in mice. Although technically more challenging (due to very small diameter of vessels), mouse models of transplantation have several advantages. First, many congenic, transgenic, and knockout strains are available. There are specific mouse strains available to study specific cell populations. Second, there are more reagents including monoclonal antibodies available for mice. Third, due to small body weight, mice need only about one tenth the quantity of drug that rats consume. The disadvantage of the mouse models is mainly technical, but it has been

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shown that spontaneous acceptance of both liver and kidney graft occurs more frequently in all mouse strains compared to rat (Qian 1994).

Heart transplants are the most popular vascularised allograft performed. The heart is transplanted in a heterotopic position, with the veins of the heart tied off. The donor aorta is anastomosed to recipient aorta and donor pulmonary artery anastomosed to recipient inferior vena cava. This makes a non-physiological model of circulation, and the heart is subject to a moderate level of chronic ischemia, so histological changes do not exactly mirror those seen in humans (Chong 2013). Graft function is monitored by palpation of heart beating, or by using electrocardiogram (Martins 2008).

Although rodent models of immunosuppression have provided useful data, large animal models mirror much more closely the events in humans. Laboratory mice are typically bred in specific pathogen-free conditions, so their immune systems are relatively naive and have not had alloantigen exposure sufficient to develop significant immunologic memory (Chong 2013). Rodents are also often transplanted very young, in the first few months of life, which further limits how well experiments can be extrapolated to humans, where recipients are mostly middle-aged.

CONCLUSION

In summary, it can be seen that small animal models provide a useful means of testing surgical techniques and drugs prior to use in humans. Limitations of these studies are an important constraint, and not all animal data can be extrapolated to humans. In particular, differences in the immune system between humans and rodents relate to the older age of human transplant recipients and greater exposure to pathogens. This creates more memory cells and therefore less dependence on co-stimulation and greater resistance to tolerance in humans. Additional differences in physiology and pharmacology limit extrapolation from small animal models to human studies. The small size of rodents creates technical anatomic constraint in developing surgical models. In spite of limitations, animal models remain the most useful predictor of surgical intervention utility.

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Chapter 10

LARGE AND TRANSLATIONAL ANIMAL MODELS IN EXPERIMENTAL SURGERY

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ABSTRACT

In the field of surgery, every discovery for improvement of human health must be translated into practical applications. Such discovery usually begins at "the bench" with basic research and then progresses to the clinical level. In experimental surgery, animal models play a very important role in the evaluation of efficacy and safety of new medical treatment or devices before their use in human clinical studies. Thus, we need an animal model that mimics aspects of human anatomy and physiology more closely, and provides practical and clinically relevant ways to study both the natural history and response to treatment. The development of such animal models require (i) to establish criteria for selection of the species most suitable as a model for the question under investigation; (ii) evaluate and validate standardized phenotyping protocols; (iii) create a database of existing models (v) scientific and ethical evaluation of experiments with large animals. This would help to make data from experimental surgery more reproducible and translatable to the clinic. An overview on a choice, evaluation and validation requirements of large and translational animal models is provided in this chapter, in order to facilitate the translation of experimental surgery to patients.

Keywords: large animal model, translational animal model, translational research

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ABBREVIATIONS

ACUC	Animal Care and Use Committee
FOSCC	Feline oral squamous cell carcinoma
GLP	Good laboratory practice
HNSCC	Head and neck squamous cell carcinoma
NHP	Non-Human Primate
RSV	Respiratory syncytial virus
TCC	Transitional cell carcinoma
LCL	Lateral Collateral Ligament

INTRODUCTION

Experimental surgery is tightly and viciously associated to animal experimentation in the popular imagination. Previous studies showed that the use of animal as experimental model is essential to the development of new and more effective methods of diagnosis and treatment of human diseases (Zerhouni 2005). Based on medical research literature, rodent models frequently fail to fully mimic clinical signs and significant pathologic hallmarks of human diseases (Boivin 2003; Evans 2015; Flisikowska 2012). In recent decades, interest within the scientific community in developing large animal models that more closely approximate the clinical and pathologic features of human disease has increased (Cibelli 2013).

In surgical research and training, large animal model-based studies remains relevant as well as the development of new surgical procedures are necessary. These models of experimentation have been judged as good surgical training or research models for many reasons. They are biologically similar to humans, therefore susceptible to many of the same health problems as humans (Brandacher 2012; Zerhouni 2005; Lumley 1990); i.e., chimpanzees share more that 99% of DNA with humans (Damy 2010). Additionally, a diversity of these animals provides very useful models for the study of diseases afflicting humans. According to medical literature information, it is estimated that between 25 and 30 million animals are used in medical research each year worldwide. Approximately 95% of these animals are rats and mice specifically bred for research and 4.25% of these animals include rabbits, guinea pigs, sheep, fish, frogs, insects, and other species. Most importantly, only 0.75% of the animals in research are cats, dogs, and primates (Bontrop 2001; Carlsson 2004). This wide range of animal species models gives choice for the most appropriate animal for the study design. Usually, the least phylogenetically developed animals available in sufficient quantity, is a preferred choice (Persidsky 2007; Liebschner 2004).

Experiments and procedures involving these animal models must be designed and performed with specific consideration of their pertinence to human or animal health, for the advancement of knowledge and for the benefit to society (Hassan 2005). Thus choice of an animal model should be phylogenetically similar to human beings to allow achievement of approximate information or result than what can be expected in human beings (Solinas 2014; Fagundes 2014). However, there has been a consistent outcry about unethical practices in the use of animals in biomedical research and training. The use of animal models in Experimental surgery research or training must be based on scientific, ethical, and legal principles

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(Hassan 2005). The three ethical aspects have to be kept in balance when carrying out surgical procedures to satisfy good quality, control and safety for both the trainees or researchers and the animals. Additionally, all animal research must be described in an Animal Care and Use Committee (ACUC) Protocol Form, which must be approved by the institutional ACUC prior any animal work being carried out.

Surgical animal models are usually divided into following two groups: small animal category requiring local animal ethical committee approval: rats, mice, Guinea pigs, rabbits (National Research Council 2011) and the large animal category requiring central animal ethical committee approval in addition to the local ethical committee: dogs, goats, pigs, primates (National Research Council 2011; Dougherty 2008). Mice, rats, rabbits and guinea pigs are universally accepted and specially bred for scientific research, despite a law governing the prevention of pain and performing euthanasia at the end of the study (Morton 1985). Among large animal models, non-human primates are only approved in special cases where the use of other animals is judged to be unreliable, and also in specific need because of their physiologically and anatomically closer features with man (Sughrue 2009). Species such as dogs and pigs share similar physiological and anatomic features with man and are conditionally approved. The use of ruminants is limited due to their varied and distinct physiology and anatomy structure. Mammals, birds, reptiles, amphibians and fish are used rarely (McGonigle 2014).

The common objective of basic science and biomedical research is to improve the health, although this goal has not been achieved as often as desired or needed (Begley 2011). Previous studies from medical literature have recognized translation as a vital component of research and a necessary process to reach health care improvements from basic science research (Fiscella 2008; Keramaris 2008). This process of translating basic scientific knowledge to patient care has been discussed for decades; its importance and difficulty involves applying insights, ideas, and discoveries generated through basic research for treatments or preventions of human diseases, and also taking insights, ideas, and discoveries from the clinic back to the basic experimental settings to identify underlying mechanisms; and this could be defined as a bridge between bench-to-bedside-and-back (Wolf 1974).

In surgery, translational research can be: studies discovering new therapeutic strategies, studies defining the effects of therapeutics in humans, studies providing basis for improving therapeutics, studies identifying clinically relevant biomarkers, and studies developing new drugs and procedures (Rubio 2010). It plays a critical role in helping to understand the mechanism of disease, to improve methods of early detection, and to identify and investigate potential treatment targets (Sung 2003; Wolf 2008).

The translation of surgical discoveries, particularly new surgical procedures or novel therapies of human diseases, requires development of suitable animal models. A translational animal model aids the translation of findings from basic science to practical applications that improve human health and well-being (Table 10.1). It supplies practical and clinically pertinent ways to study both the natural history and response to treatment of human diseases (Contopoulos-Ioannidis 2003). This animal model should not only necessitate replicating human diseases in etiopathogenesis and pathobiology but also required biomarkers development and toxicity prediction (van Meer 2015). Adequate design and conduct of experimental surgery research as well as further choice of appropriate animal translational models can offer invaluable information to the knowledge of surgery and medicine in general, including the discovery and development of new drugs (Westfall 2007).

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Table 10.1 Translational Model: From Bench to Bedside

SAFETY	EFFICACY	BIOMARKERS

Bench → **Bedside**

Proof of concept	Translational Model	Human Patients Pathophysiology

Source: Adapted from Sharing Advances on Large Animal Models. Gene Center, LMU Munich, December 15−17, 2014.

This chapter will help to understand the important role large animal models play in experimental surgery research and the benefits of translational animal models as an important research subject in bench-to-bedside-and-back cycle necessary for the human health care improvements.

Large Animal Models in Experimental Surgery

Historically, large animal models have played a critical role in the exploration and characterization of disease pathophysiology, target identification, and in the *in vivo* evaluation of novel therapeutic agents and treatments. In surgery, they are very advantageous and suitable models for surgical training, development of surgical techniques and research. Most of surgical conditions, specifically for the musculoskeletal, digestive, genitourinary, cardiovascular systems etc. are largely reproducible in the large animals because of their anatomical similarity to humans.

Choice and Experimental Values of Large Animal Models

Whatever the experimental model selected, critical analysis has to be made for the limitations imposed by the physical and anatomical differences from humans and of the pathology, therapies or surgical techniques that can potentially be investigated.

Criteria of large animal models have a different order of preference, according to the purpose of the animal's use. In surgical experiments, anatomic considerations such as size and accessibility of the organs and tissues of interest are the crucial primary determinants for model choice; i.e., for research based on models of human diseases, similarity in biology, both anatomical and physiological, is a primordial criterion of model choice (Al-Rakan 2014; Bugbee 2015); higher mammals, which are closer to humans, like cats, sheep, pigs, dogs and primates etc., which have the identical set of organs (heart, lungs and liver) like humans, constitute a better choice. Different species of large animals fulfil these necessities and offer numerous profitable characteristics (Lu 2014). For example, pigs share many anatomical

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structures (proportional organ sizes) (Gonzalez 2015) and physiological similarities (lifestyle: diurnal rhythms, omnivorous habits and propensity to sedentary behaviour) with humans than any other animal species. Therefore, it mimics human condition more accurately than other species, mainly with regards to skin, cardiovascular system, gastrointestinal tract, kidneys and spleen. Also, its convenient size and nature facilitate housing and adequate breeding, and maintenance of strains at any time of the year (early puberty, non-seasonal breeding, short weaning-to-mating interval and high prolificacy). Another outstandingly used model is sheep which is traditionally used as a model in orthopaedics, regenerative medicine, respiratory diseases, reproductive endocrinology and fertility therapies. Its size and temperament are convenient for housing and handling, and also facilitate sequential screening by non-invasive imaging and assessment of hormones and metabolites. It is especially interesting in developmental programming because of singleton pregnancy and similar developmental trajectory to humans; its screening of foetal and early postnatal development is also well-established. Swine also provides breeds and strains fitting for different surgical research objectives and its advantages as a model have been boosted by its availability of porcine genome data, which facilitates genetic modification and creation of genetically engineered strains for specific applications in translational research (Swindle 1994). The cow has proved to be an excellent animal model to investigate human reproduction with a comparable follicular growth length, cycle length and oocytes with the same size. In cows and women, metabolic stress syndromes, characterized by elevated levels of free fatty acids in the blood, have been related with reduced fertility. However, ethical issues prevent a proper experimental design with human reproductive material.

The genetic background such as congenic, isogenic, mutant, recombinant or transgenic characteristics have to be considered, once the experimental model has been chosen. As an example, rabbits (Oryctolagus cuniculus) and pigs (Sus scropha domesticus) breeds should be well defined. The white New Zealand rabbit is the breed most often used in experiments and the pig breeds most used are large white, landrace, Yorkshire, and Hampshire. Also, differences in physiology, genotype, phenotype and maturity at a given weight and age should all be taken into account (Albert 2012; Damy 2010). All animal species should also be sourced from the same supplier throughout an experiment, since even isogenic animals can vary from one animal house to another. Depending on feeding regime, Miniature pigs (usually from Brazil) weigh 0.5 kg at birth, 12−45 kg at 4 months of age and reach 45−100 kg when adults (Tanaka 2006). They should be recommended for longer experiments due to their small size and weight. Traditional pig breeds weight is increased rapidly from 1 kg at birth to 100 kg by 4 months, reaching around 150 kg at 9 months. Consequently, they should mostly be appropriate for acute experiments or experiments lasting a maximum of 3 weeks.

Available and Well Documented Large Animals Models in Experimental Surgery

Porcine

The features of the pig combined with an increasing availability of biological tools and reagents for use to study porcine tissue make the pig arguably the best model available for

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translational biomedical research. It is appreciated as a distinctly advantageous model for human beings in numerous fields of science and increasing number of articles and proceedings are being published in biomedical research using pigs (Swindle 2012) The pig has many fundamental anatomic, physiological, genomic, proteomic, immunologic, and nutritional similarities to human beings (Kirk 2003; Swindle 2012; Roura 2016; Rothkotter 2002; Bendixen 2010; Lunney 2007; Hart 2007; Ibrahim 2006) The pig also shows potential for interspecies transplantation work, as well as the ability to fulfill United States Food and Drug Administration requirements for pharmaceutical testing (Gonzalez 2015).

Dogs and Cats

Despite numerous advances in understanding cancer at the molecular level, timely and effective translation to clinical application of novel therapeutics in human cancer patients is lacking; most of failure of cancer therapeutic drugs were found to be closely associated with a result of toxicity or inefficacy not predicted by preclinical animal models (Tobinick 2009; Kola 2008). Dogs and cats as companion animals were found to be biologically relevant translational research model which allows spontaneous disease modeling of human cancer (Gardner 1996). These felines develop an oral squamous cell carcinoma (FOSCC) (Marretta 2015) which shares similar etiopathogenesis (tobacco and papillomavirus exposure) and molecular markers (EGFR, VEGF, and p53) with head and neck squamous cell carcinoma (HNSCC) (Tannehill-Gregg 2006), a common cancer with a poor prognosis and limited clinical advancements in humans. FOSCC and HNSCC share similar tumor biology, clinical outcome, treatment, and prognosis (Martin 2011; Rautava 2012; Bergkvist 2011).

Dog's naturally occurring bladder cancer very closely mimics human invasive bladder cancer, specifically high-grade invasive transitional cell carcinoma (TCC; also referred to as invasive urothelial carcinoma) in cellular and molecular features; biological behavior, including sites and frequency of metastasis and response to therapy (Knapp 2014). This model has been used to help define heritable risks (through very strong breed-associated risk) and environment risks and to evaluate prevention and treatment approaches that benefit humans as well as dogs. The preclinical treatment trials with this model are considered a win-win scenario by clinician scientists in improving detection of TCC and preneoplastic lesions, earlier intervention, better prediction of patient outcome, and more effective TCC management overall.

Caprine and Ovine Models

Previous studies proved caprine (goat) and ovine (sheep) models to be the most appropriate models for large-scale large animal studies in cartilage surface defect repair (Klein 2009; Kon 2010; Milano 2010; Jurgens 2013; Nukavarapu 2013), as the anatomy is closest to humans; they have similar biomechanics of their stifle joint to human knees (Proffen 2012) and they have an adequate cartilage thickness allowing for partial and full thickness defects (Patil 2014). Ovine stifle joint is used in experimental surgery training as device development model (Madry 2015; Ahern 2009; Allen 1998) because it has very similar cruciate ligaments to humans and large menisci as well as a similar lateral collateral

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ligament (LCL) complex, popliteus tendon and popliteofibular ligament. However, there are significant differences in their anatomy and biomechanics compared to humans; these differences must be recognised and considered in both study design and in comparing study outcomes, in order to enable the successful translation. For *in vivo* assessment of chondral and osteochondral defect repair (Chevrier 2015), this caprine model represents very a good and feasible option to conduct large animal studies to evaluate biological responses, durability, toxicology, lesion size and location analogous to human studies (Cook 2014).

Nonhuman Primates

Nonhuman primate (NHP) models in transplantation have been used as a variety of species, including baboons, macaques, and, rarely, chimpanzees-which are now eliminated as funded model (Hau 2006; Gluck 2003). NHP models have a high degree of homology with humans, and are predominantly used in highly targeted biologic- and antibody-based therapy studies. NHPs offer a rational basis for the translation of immunosuppressive and tolerance strategies from the bench to the bedside (Douglas 2013). Baboons have commonly been used in xenotransplantation studies, as their relatively large size better accommodates the porcine organs. Macaques (cynomolgus, rhesus, and pigtail) have defined major histocompatibility complex (MHC), and are sufficiently homologous with humans such that most molecular targets will cross-react, with the notable exception of CD3 (Adams 2002; Cendales 2005; Chen 2000). Practically all clinically used standard immunosuppressants have been found to be successful in prolonging graft survival in primate models first, particularly when adjustments for distribution and metabolism have been considered.

Experiments Design Strategy

Experiment protocols are different according to the selected species. In surgical research, a need for biological model, a choice of the species and a relevance of the proposed study have to be determined before deciding to initiate a project with large animals (Abdullahi 2014; Alam 2012). Knowledge of species used, their requirements, management and handling techniques have to be taken in consideration by experimental design to avoid wastage of animal lives.

The use of sensitive techniques for detecting biological differences and appropriate data collection and statistical analysis, are all prerequisites for reducing the number of animals used and for obtaining results with a high degree of acuity, reproducibility and precision (Puopolo 2004). All animal research should be described in an Animal Care and Use Committee Protocol Form, which should be approved by the institutional committee prior to any animal work being performed (Poole 1999).

In surgical research or training, experimental design should greatly consider an anatomic structure such as size of organs, accessibility of the organs and tissues which allow an easy understanding of the various physiological and pathological processes affecting human beings.

The use of large animals in research is exposed to intense societal and philosophical debate because of their psychosocial awareness and privileged status in the human

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community. Considerations driving species selection usually include scientific, ethical, legal and economic deliberations. Some large research animals such as dogs or non-human primates are highly regulated while others, such as pigs or goats receive less attention. Decisions related to the design and implementations of experiments have to be made in consultation with appropriate review groups such as the institutional animal care and use committee as well as the institution's appointed veterinarians (Kol 2015).

Indeed, ethical use and care of animals including the avoidance or minimization of distress, discomfort and pain have to be rigourously respected by careful protocol design outlining pre- and post-procedural use of analgesics, anesthesia, sedation and euthanasia. The subsequent recovery of animals from procedures must also be monitored to ensure their welfare (Graham 2015).

Welfare, Safety and Management

Regarding environmental standardization, several factors such as temperature, humidity, illumination, light/dark cycles and air quality have to be maintained within the experimental environment by automatic adjustments when housing; since their high variations could influence some metabolic processes, pathological changes and affect some biochemical parameters (Heine 1998). Furthermore, it was proven that good social aspect and environmental enrichment influence animal's physical and psychological welfare. In experimental surgery, individual housing was proven to be unavoidable (Ruys 1991). Specifications for the humane handling, care, treatment and transportation of these large animals have all been described in Animal Welfare Act and Animal Welfare Regulations (USDA 2013). Transportation from the supplier to the research center must be carried out in a manner that preserves both the health and welfare of the animals being transported. Acclimatization of animals to the experimentation area should be observed. Due to bioethical considerations, all procedures with animals should be carried out by trained professionals; they will be responsible for the development and education programs and contribute to the in-depth development of innovative concepts in the humane care and use of laboratory animals. Strict adherence to good laboratory practice (GLP) has to be applied to eliminate many sources of errors and uncertainties. Systematic errors and artifacts could be avoided through the application of technical validity and approved standardized operating procedures. In case of use of genetically-modified organisms, specifics directives have to be set out by technical committee on biosafety.

Challenges in Using Large Animal Model in Experimental Surgery

The practicalities of animal husbandry during treatment can only approximate the care human patients receive. Indwelling catheters and wound care after surgery are often difficult. In conscious animal models, monitoring and vascular access is challenging, often limiting the options for drug delivery and dosing schedules.

In transplantation, the immunologic diversity of large animals can lead to significant differences in outcome via heterologous immune interactions, an experimental parameter that is challenging to quantify and control for. An important issue in transplanted animals is the

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age of animals and survival time of grafts. For feasible reasons, adolescent animals are often used, and evidence suggests that these young animals, like young humans, have a predominately naïve immune system that will mature toward a memory phenotype as the animal ages (Rodriquez-Carreno 2002; Saalmüller 2002). Regarding graft survival in large animals, convention has established several years as indicative of acceptance. Based on medical literature, no data was supported that graft survival should be interpreted proportionally to lifespan. Data, should be interpreted for what it is, and not extrapolated to indicate longer survival in longer-lived animals (Kirk 2003).

TRANSLATIONAL ANIMAL MODELS IN EXPERIMENTAL SURGERY

Choice of Appropriate Translational Animal Models

Each animal model has specific advantages and disadvantages. The selection of an appropriate pre-clinical *in vivo* model is important in ensuring successful translation to the clinic. In order to choose an appropriate translational model, clinically pertinent question under investigation and hypothesis being tested are seriously important to be considered (Prabhakar 2012; Woolf 2008). Selection of species and strain of translational model has to be carefully made depending on the relevant research interest. The age, gender and health status of the translational animal model should be matched as closely as possible to the clinical condition under investigation; also very special conditions in the design need to be considered, depending on the pathology and the target of interest i.e., in a study with monoclonal antibody TGN1412, the anti T-cell co-stimulatory receptor CD28 antibody TGN1412 caused a severe cytokine storm in its first human volunteer trial (Hansen 2006; Stebbings 2007); but this effect was not observed in animal models using non-human primates during preclinical study. It was found that less activation of memory T-cells in non-human primates than in the human volunteers, despite a near 100% sequence identity of the target, could be the reason that might have afforded this contrariety (Attarwala 2010).

As it has been shown from medical research literature, information gathered from smaller animal translational model is usually less applicable to human. Larger animals offer the advantages of being a good translational animal model because of its closer anatomical similarity to human; easily submitted to some minimally invasive evaluation techniques (i.e., arthroscopy, CT scan, MRI, and biomarkers) without the need for a particular sacrifice (Bouchgua 2009; Hwang 2007; Garner 2011); allowing serial sampling of large amounts of blood and tissues; easy assessment of hormones and metabolites. Only disadvantages include cost and public perception (Mutter 2013; Kirk 2003).

In burn studies research for example, *in vitro* models are limited in their ability to capture all aspects of burn pathophysiology and the complex clinical features of human burn injury; but animal models are still essential for uncovering the molecular (Sayeed 2005; Williams 2009) and cellular (Marshall 2013) aspects that characterize human burn traumas. In clinic, the lack of a suitable animal model that captures all of the prominent features of burn trauma, is still a major limitation in searching for practical treatment options. Depending on heterogenous nature of burns trauma, specific valuable animal models are needed to uncover the post-burn pathological mechanisms and to test novel therapeutic approaches.

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Design and Conduction of Translational Experiments

Numerous factors in designing and conducting translational experiments have to be considered to yield conclusive data (Muschler 2010).

The number of animals to use is usually minimized for ethical and other reasons; this needs to be balanced with the statistical power required generate solid data in order to either verify, or to reject the experimental hypothesis (Feesting 2002).

The time course of therapeutic treatment or to evaluate the outcome should be taken in consideration for the translatability. In preclinical study, it was reported that the benefit of treatments might be greater in animal models because treatment is initiated at the time the injury is induced, before the development of disease, contrary to clinical trials where humans are treated much later in the disease process, normally after onset of symptoms and clear diagnosis.

Since subjective evaluations are generally very efficient way to score behavioral endpoints, it can be easier to create bias. High intra and inter-operator variation of most subjective measures have to be carefully controlled to avoid difficulty in standardization. Safety and quality of life endpoints (e.g., overall activity, body weight, food consumption) in animal efficacy studies should be standard with translational animal model in experimental surgery research.

Reproducibility of experimental animal results with slightly different parameters may produce different results (Crabbe 1999; Richter 2011; Wahlsten 2003). Desire of reporting experimental animal data with a positive or negative result is also inspirited to allow more reproducibility of experiments (Hooijmans 2011; Kilkenny 2010; Kilkenny 2012; Rice 2008).

Validation and prediction of translational animal models: By definition, a translational model is not a perfect replication of the clinical condition (Hackam 2006). Different typical criteria have to be taken in consideration for validation of translational animal model to fit for a specific clinical purpose or situation. Medical literature has shown that not all criteria of validation can be met by a single translational model. However, a combination of different models can eventually come closer to the clinical situation than a single or an even highly sophisticated model. Some authors like, Sams-Dodd (Sams Dodd 2006) has suggested a model validity scoring system in attempt to define an optimal combination of models. This scoring system uses five different important criteria (Table 10.2).

1. Species: Closer is a species to humans; more likely is the pathophysiology of the disease similar to humans (Akhtar 2015).
2. Complexity: more complex the involved test system is, the more probable that the relevant mechanisms are included; i.e., an *in vitro* ion-channel Test may detect the effect of a test compound on conductance of a cardiac ion-channel whereas an *ex vivo* or an *in vivo* test system can evaluate its effect on the overall cardiac effect e.g., on contractibility (Suzuki 2009).
3. Disease simulation: Translational animal models use different principles to induce the disease of interest. Simplest models usually do not attempt to induce a disease but easily adapt a measure in healthy individuals; other more complex model use drugs to induce disease symptoms. For many others disorders for which the etiology has not been fully elucidated, it is nearly impossible to truly simulate the disease symptoms. Infectious diseases may be one of the exceptions for the ease of

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replicating a disease simulation as shown for example by the development of a sophisticated neonatal lamb model for respiratory syncytial virus (RSV) infection (Derscheid 2012; Morton 1999).

4. Predictivity: The model should allow the comparison of different treatments and may help to decide as to whether an experimental treatment has a similar or even superior efficacy to the existing standard of care (Cook 2012).
5. Face validity: This criterion is usually differentiated depending on whether just one symptom of a disease is modeled or a set of symptoms. The scoring system (Table 10.2) can be used to assemble combination of models altogether for a maximal validity; i.e., the neonatal lamb model for RSV infection scores high for all criteria other than species and could thus be ideally complemented by an *ex vivo* human cell culture model (Villenave 2012).

Table 10.2. Proposed validity scoring system

Criterion	Value	Score
Species	Human	4
	Non-human primate	3
	Non-human mammal	2
	Non-mammal	1
Disease simulation	True	4
	Complex	3
	Pharmacological	2
	No	1
Face validity	>1 core symptom	4
	1 core symptom	3
	1 symptom	2
	No 1	1
Complexity	*In vivo*	4
	Tissue	3
	Cellular	2
	Sub-cellular/molecular	1
Predictivity	Graded for all pharmacology principles	4
	Graded for certain pharmacology principles	3
	All or none for certain pharmacology principles	2
	No or not shown	1

Source: Adapted from T. Denayer et al./New Horizons in Translational Medicine 2014; 2:5–11.

Summarily, following criteria are typically taken in consideration for general validation of translational animal model:

- Similarity in biology and symptoms between translational animal model and the human disease.
- Clinically effective interventions should demonstrate similar effect in the model.
- The target under investigation should have a similar role in the disease models as in the clinical situation; i.e., beta- 3 adrenergic receptor which has an important role in the energy metabolism of rodents but not in humans (Weyer 1999).

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Challenges and Limitations of Translational Animal Model

To bridge the translational gap between preclinical animal model and clinical trials, there is a need for making animal model testing more clinical trial-like. Several aspects need to be highlighted for improving the translational value of animal models:

1. Clinical trials endpoints like quality of life are not assessable in animal models. Quality of life is important issue in clinical trials, specifically for a variety of different chronic diseases. This endpoint is usually assessed by questionnaires, a methodology that obviously cannot be used in experimental animals (Kim 2010; Stasiak 2003). However, it was reported in a successful model of human brief pain inventory in canine (dogs) suffering from bone cancer pain (Brown 2009)
2. Requested endpoint is often limited in translational animal models than in clinical trials, some time for ethical reasons; i.e., in oncology, the tumor size is often the primary endpoint of preclinical tumor study models, while overall survival is targeted as endpoint in clinical trials (Marwick 2001).
3. Usefulness of translation and back translation. Back translation from clinical trials to animal models to clinical might help in understanding some clinical data in case of clinical trials failure (Alam 2012); it might be necessary for further improvement of predictive value for future research perspectives; i.e., Importance of this translation and back translation process in proteomics studies in order to improve the patient outcomes (Guest 2013).
4. Improve the quality of translational animal models. This involves following three aspects:

    a. Improving the performance quality of existing models. An adequate selection of animal models that fit for research purpose and a better ethical conduction of experiments, taking in consideration all translational study's factors are crucials aspects which could make preclinical data more reproducible and translatable to the clinic (Markides 2015; Mullane 2014).
    b. Ameliorate the way animal models are used in the decision making process.
    c. Promote the development of more clinically pertinent and predictable and alternative models. Several potential alternatives of animal models have been tested. Three of them are listed here:

        1) Humanized animal model: In this translational animal model, human cells or tissues are engrafted to the model depending on the human disease and question addressed (Ito 2012). This model is extremely useful as it permits functional research studies *in vivo* and hence supports clinical translation. It has helped to improve the clinical translation, especially in oncology and inflammatory diseases (Zhou 2014). Most commonly used are the human tumor xenograft models for study of cancer, and the humanized mouse models that mimic the human immune system (Siolas 2013; Petersen 2008). An important step will be the creation and use of humanized large animal models such as pigs and nonhuman primates, which will complement mice

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and may have greater predictive capability (Daadi 2014; Maisano 2015; Matsunari 2009).

2) Use of in silico modeling for reproducing disease mechanisms (Maxwell 2008; Schmidt 2013; Geerts 2013). This approach has been considered as an addition in the translational research armamentarium than a substitute for well-designed animal models.
3) Use of translational animal model in experimental surgery in establishing the safety of stem cell applications is very significant, since the dosages of biologics, the route of administration, treatment outcomes and the development of procedures and techniques, such as surgical and visualization technologies can be extrapolated readily to humans (Hare 2009; Casado 2012; Felfly 2014). Careful and rigorous selection of the best animal models for specific diseases is critical and represents a serious limitation because of the absence of models for disease conditions precisely recapitulating the human phenotype with comparable organ sizes and physiologies. Factors such as the cost, availability of animals and genetic tools, long-term monitoring and appropriate infrastructure may also represent limitation when choosing the optimal model. A need for further development of the regulatory requirements for translational large animal model is a must to ensure efficacy and safety of stem cell-based product applications for human therapeutics (Duda 2014).

CONCLUSION

The use of large animal models in experimental surgery research is scientifically justifiable because of important anatomic and physiological similarities with human beings. These models offer a number of marked translational advantages and play a key role in the development of novel treatments for human disease. Their requirements for larger, more specialized housing and surgical facilities, with higher expenses related to feed, veterinary care, and surgery costs have impeded their widespread use in medical research.

As translational animal models, they are essential to bridge the translational gap between basic research, preclinical research and clinical trials; and reversely, translated back from clinical findings that were not predicted and used to refine the animal models. The selection, evaluation and validation of these animal models should fit the purpose according to stringent criteria and being reproducible. Rational design of experiments should be a part of the translational plan and data from these animal models should be also essential in predicting the clinical outcome for development of specific research issues.

Ethical review, standardization and refinement of animal models by disease expert groups, elaboration of specific good laboratory practice and guidelines were reported to be effective in improving translational potential of these animal models. Acquiescence with high standards of care, refinement of research methodology, and husbandry techniques should be seriously considered before planning any experiments with these animals; and all of these factors should contribute to build the required safety data package valuable in development of the expected clinical performance of the treatment or others clinical issues.

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Continued innovation and refinement in these animal models as well as the need and value of more effective standardization are needed to develop an accurate and optimized guideline for such translational models. This could represent a potentially useful tool in experimental surgery that could help to achieve a greater success rate in translational studies.

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Chapter 11

CARDIOVASCULAR BIOMECHANICAL MODELS

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ABSTRACT

Computational and *in vitro* methods offer powerful means to model the cardiovascular system and investigate biomechanics related to blood flow, cardiovascular tissue, and medical devices. These models can be constructed to directly describe human anatomy and physiology, and can be more highly controlled compared to animal models. Low-order models composed of lumped-parameter elements and simplified descriptions of cardiac function can capture the global physiology, while high-order models exhibiting detailed 3D anatomy and dynamics can provide highly realistic replication of biomechanical interactions in a small region of the circulation. Multiscale models offer the freedom to capture biomechanics in different regions at the desired level of details. In this chapter we describe the fundamentals and the current state-of-the-art of model construction both in the computational and *in vitro* approaches. These models have been applied to understand the physiologic impacts of medical device implantations, predict surgical outcomes, and investigate hemodynamics in vascular diseases; we present several illustrative case studies here. Finally we examine the pros and cons of each type of models and discuss the considerations in proper model selection for a research study.

Keywords: lumped-parameter, image-based, patient-specific, CFD, *In vitro*, multiscale

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ABBREVIATIONS

LPM: lumped-parameter model
CFD: computational fluid dynamics
RCR: resistor-capacitor-resistor
VAD: ventricular assist device

INTRODUCTION

Surgical palliation of cardiovascular abnormalities and implementation of cardiovascular medical devices are intended to modify and improve patient hemodynamic parameters such as cardiac output, blood pressure, pulmonary perfusion, and blood flow patterns. Biomechanical parameters in the cardiovascular system such as 3D flow and pressure fields, as well as stress and strain in blood vessels, have direct effects on the progression of cardiovascular diseases (Caro 1971; Glagov 1988; Ku 1985; Malek 1999). The hemodynamic forces within blood vessels directly affect the biological adaptation of vessel diameters and wall thicknesses (Langille 1989; Wolinsky 1967). The design and evaluation of implantable devices require consideration of the interactions between the device and *in vivo* forces and motions. In this chapter we will examine several types of cardiovascular biomechanical models that can account for these important interactions in the investigation of cardiovascular devices and surgical procedures.

There are two inherent limitations to using animal models for studying human biomechanics: 1) the intrinsic variabilities in living organisms make highly controlled experiments difficult and 2) the physiological differences between the model animal and human make the implications of animal study results ambiguous. For these reasons, there is great motivation to employ non-living models that offer simplified, but well-controlled experiments that can be constructed based on human physiology and anatomy. Computational and *in vitro* models are two common approaches to constructing non-living biomechanical models. Models of different orders are used to capture the behavior of the cardiovascular system at different desired levels of details. Low-order models are simplified representations capable of encompassing large regions of the cardiovascular system; and high-order models can provide detailed 3D hemodynamic information for small regions of special interest.

Since the 1960s, lumped-parameter approaches have been used to construct low-order models of the circulatory system (Thiry 1976; Westerhof 2009; Westerhof 1969). While these models do not provide spatial information, they are capable of capturing complex vascular organizations and interactions, and providing realistic estimations of hemodynamics. Starting in the 1990s, the development of high-order models based on 3D medical imaging data allowed for biomechanical models to directly represent a small portion of patient-specific anatomy (Taylor 1998a; Taylor 1998b; Taylor 1999; Milner 1998; Steinman, 2002). Together, the current state-of-the-art involves combining models of different orders and constructing multiscale models to capture the relevant information of interest at the appropriate levels of details (Corsini 2013; Kung 2011b; Kung 2013; Vukicevic 2013; Schiavazzi 2015).

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In this chapter, we present the computational and *in vitro* approaches to both low-order and high-order biomechanical models of the cardiovascular system. We will discuss how these models can be coupled together to construct multiscale models; we will also look at several example applications of different model configurations. Lastly we will discuss the considerations for selecting an appropriate model suitable for a research study depending on the relevant physics and the types of parameters to be investigated.

LOW-ORDER MODELS

Low-order models are greatly simplified representations of the cardiovascular system typically used to capture the systemic behaviors of the entire circulation (or a large portion of the circulation). This approach can offer the "bird's eye view" of the system where the congregated behavior of each subcomponent is approximated without great amounts of detail. A very common type of low-order model for the cardiovascular system is the lumped-parameter model (LPM). In this approach, aspects of the cardiovascular system are represented by components analogical to electrical circuit elements. This type of model does not contain spatial information other than the structure of the vascular network represented as the corresponding circuit element organization in the model. Another type of cardiovascular low-order model is one that solves the 1D equations of blood flow (Hughes and Lubliner, 1973). This approach offers spatial information along a single dimension (down the length of blood vessels), and is a slightly more complex and computationally expensive type of low-order model. In this chapter, we will focus on the lumped-parameter approach to the low-order model.

In the vascular system, blood is driven through a vascular bed by arterial blood pressure. The vascular bed is typically considered passive in the sense that it does not generate pressure. The underlying physics governing the blood flow characteristics in a passive vascular bed can be modeled via 3 types of basic elements in the LPM: resistance, capacitance, and inductance. The resistance element describes the viscous dissipation of pressure energy as blood flows through vasculature. The compliance of blood vessels (mostly in large vessels) is modeled by the capacitance element in the LPM, which captures the effects of blood vessel elasticity damping pressure and flow pulsations. Lastly, inductance elements are used in the LPM to describe how the momentum of blood resists changes in flow. This aspect of blood flow is especially important for accurately modeling hemodynamics in regions close to the heart (i.e., flow through the aortic valve, in the abdominal aorta, etc) where the blood flow pulsation is significant. The capacitance and inductance are non-dissipative elements.

The active components in the LPM generate pressures to drive blood through the passive vascular beds. The main active component is the heart itself; the driving pressure generated by the heart is almost always included in an LPM. Pressures generated by other active components are selectively included in each specific model depending on their relative importance to the topic being investigated. These can include respiration, muscle pumps, and medical devices such as blood pumps.

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Computational Representation

Passive Components

The viscous flow resistance is modeled by the equation $P = QR$, where Q is the volumetric flow rate through the resistance, R is the resistance value, and P is the pressure drop across the resistance. In healthy situations, blood flow is laminar in most of the cardiovascular system. Small arterioles with little compliance (and thus little change in diameter over a cardiac cycle) contribute to most of vascular resistance. According to Poiseuille's solution for laminar flow in a tube, the viscous resistance of blood vessels is therefore relatively constant across the cardiac cycle at a given physiologic state. The viscous flow resistance in non-laminar flow regions, such as across heart valves or surgical shunts, are typically represented as flow-dependent resistances. The mathematical relationship between the flow and pressure drop in these elements is typically derived empirically (Migliavacca 2000).

Capacitance is modeled by the equation $Q=C\ dP/dt$, where C is the capacitance value, dP/dt is the time rate of change of the pressure across the capacitor, and Q is the volumetric flow rate into the capacitor. This equation describes the physical scenario where flow into a capacitor "charges up" the capacitor, much like how the internal pressure of an elastic balloon increases when fluid is pumped into the balloon.

Inductance is modeled by the equation $P = L\ dQ/dt$, where L is the inductance value, dQ/dt is the time rate of change of the volumetric flow rate through the inductor, and P is the pressure across the inductor. This relationship stems from Newton's second law and describes the physical scenario where a change in volumetric flow rate (acceleration) requires a pressure difference across the inductor (i.e., force applied to the fluid). A summary of the aforementioned passive components is provided in Figure 11.1.

Element	Symbol	Computational Model	Experimental Model
Resistance (models viscous dissipation)	(resistor symbol)	P = Q R	Parallel glass capillary tubes
Capacitance (models vessel compliance)	(capacitor symbol)	Q = C dP/dt	Trapped air pocket
Inductance (models blood momentum)	(inductor symbol)	P = L dQ/dt	Any tubing segment (parasitic)

Figure 11.1. Summary of common passive elements in the lumped-parameter model.

Heart Models

There are two common types of heart models for the computational LPM: the elastance function model and the active-passive function model. The first approach utilizes the normalized elastance function to model the ventricle. The elastance "E" is defined as the ratio between the pressure and relative volume inside a ventricle, and by the equation $P=E(t)\ (V-$

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Vo), where *E(t)* is the time-varying elastance, *V* is the blood volume inside the ventricle, *Vo* is the ventricle's reference volume, and *P* is the blood pressure in the ventricle. Conceptually, the elastance describes the "stiffness" of a ventricle at any point in time as it contracts and relaxes. The justification for using the normalized elastance function to model the ventricle is based on previous findings that the shape of the normalized (with respect to amplitude and timing of the peak) elastance waveform is relatively constant across people, and independent of ventricular load, contractile state, and various cardiac diseases (Suga 1973). Since the physiologic basis for the elastance function model is based on studies of the ventricle, this approach is typically not used for modeling the atria. In the implementation workflow, each elastance function model begins with the normalized elastance function (Figure 11.2a). This function is then "de-normalized" for each specific model simulation. The de-normalization involves stretching or shrinking the function in the x-axis according to the specific heart rate and ventricular activation to be simulated, scaling the function amplitude in the y-axis according to the contractility to be modeled, and offsetting the function in the y-axis to describe the appropriate passive filling characteristics of the ventricle (Kung 2014).

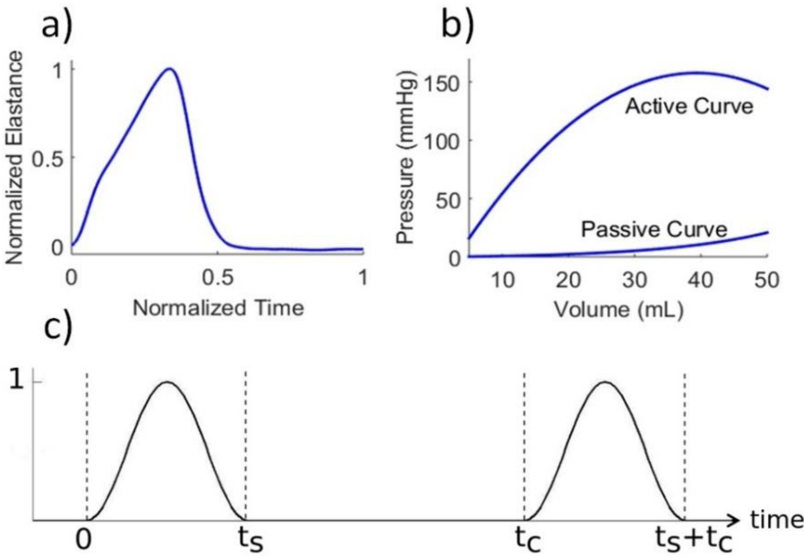

Figure 11.2. a) The normalized elastance function for modeling the ventricle b) An example active and passive curve for modeling a heart chamber (the ventricle of a pediatric patient is represented in this particular example) c) An example activation function to describe the cardiac contraction of each heartbeat. "tc" is the cardiac period and "ts" is the systolic length.

The active-passive function approach can be used to model both the atrium and ventricle. In the active-passive function model, two pressure-volume relationships (Figure 11.2b) are used to describe the active and passive behaviors of the heart chamber. The passive curve is defined based on the elastic properties of the chamber during relaxation, and the active curve is defined based on contractile characteristics. An activation function (Figure 11.2c) is used to describe the timing and duration of active contraction. Different shapes may be chosen for these curves depending on the design of the specific heart model. The pressure inside the heart chamber is then defined as $P=A*P_{act} + P_{pas}$, where A, P_{act}, and P_{pas} is the value of the

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activation function, active pressure, and passive pressure, respectively (Corsini 2013). Note that A is a function of time, and P_{act} and P_{pas} are functions of the heart chamber volume.

There are advantages and disadvantages to these two different heart modeling approaches. The elastance function approach is empirically-based; the normalized elastance function already encapsulates the congregated underlying mechanisms that result in the observed behavior of the ventricle. This model requires few parameters to be tuned for each modeling scenario, but only offers a somewhat “black box” approach to modeling the heart. The active-passive approach mathematically describes the underlying properties of heart tissue and contraction characteristics, and offers a lower-level mechanistic approach to capturing heart function. It requires the tuning of more parameters, each needing to be set appropriately in order for the model to accurately describe the specific heart to be simulated.

Other Active Components

Mathematical descriptions of other active components driving blood flow can be constructed and implemented into the computational LPM. Like the heart model, active components are generally implemented as pressure sources. The specific mathematical constructions to describe these components can vary greatly and the discussion is beyond the scope of this chapter. Common active components that are modeled besides the heart include respiration (Kung 2014; Baretta 2012) and ventricular assist devices (Giridharan 2002; Pekkan 2005; Schmidt 2016). Interested readers are encouraged to refer to the cited literature for further details.

Example Implementation

Here we present a simple example to illustrate how to use the various components discussed to perform calculations of pressure, flow, and volumes in a computational LPM. In this exercise we use an active-passive function model to describe an atrium, an elastance function model to describe a ventricle, and a few passive components to describe a simplified vascular bed. Note that the two ideal diodes included in this LPM represent heart valves and allow uni-directional flow with no added resistance (Figure 11.3).

Assume that based on atrial and ventricular characteristics, we have already defined $E(t)$, $P_{act}(Va)$, $P_{pas}(Va)$, and $A(t)$, which are the de-normalized elastance function of the ventricle, the atrial active and passive pressures as functions of atrial volume, and the activation function of the atrium, respectively. Assume that based on the scenario to be modeled, we have also already determined the resistance, capacitance, and inductance component values, the initial values of differential variables, and the ventricular reference volume “Vo.” The resulting system of equations describing this LPM is then:

$$Pv = E(t)(Vv - Vo) \quad \text{(Eqn 12.1)}$$

$$Pa = A(t)P_{act}(Va) + P_{pas}(Va) \quad \text{(Eqn 12.2)}$$

$$Q_{av} = \begin{cases} \frac{Pa-Pv}{R2} \; for \; Pa \geq Pv \\ 0 \; for \; Pv > Pa \end{cases} \quad \text{(Eqn 12.3)}$$

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$$\frac{dQ_L}{dt} = \begin{cases} 0 \; for \; Q_L \leq 0 \; and \; Pc > Pv \\ \frac{Pv-Pc}{L} \; else \end{cases} \quad \text{(Eqn 12.4)}$$

$$\frac{dPc}{dt} = \frac{Q_L - (\frac{Pc-Pa}{R1})}{C} \quad \text{(Eqn 12.5)}$$

$$\frac{dVa}{dt} = \frac{Pc-Pa}{R1} - Q_{av} \quad \text{(Eqn 12.6)}$$

$$\frac{dVv}{dt} = Q_{av} - Q_L \quad \text{(Eqn 12.7)}$$

This system of equations can be solved using a numerical time-stepping scheme, such as the 4th order Runge-Kutta method.

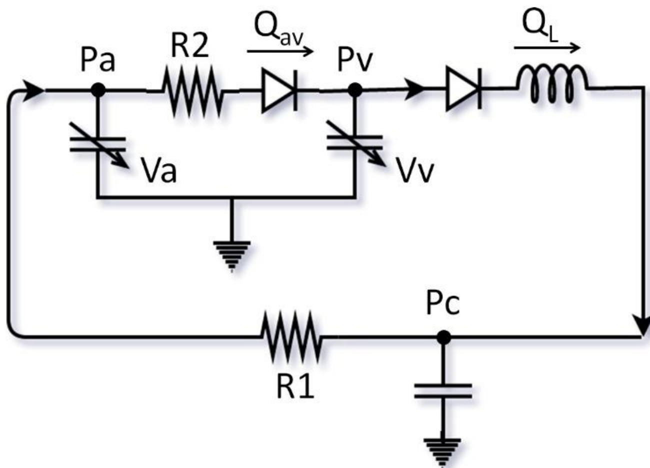

Figure 11.3. Schematic of the example LPM. “Va” and “Vv” are the atrial and ventricular volumes, respectively.

In vitro Representation

Passive Component: Resistance

According to the Poisuille solution, the viscous resistance of a flow conduit is a function of only the fluid property and the geometry of the conduit as expressed by equation 12.8. Note that this solution is only valid for laminar flow; in the turbulent flow regime, the resistance is a function of also the flow rate. Due to the fact that blood flow is laminar in the large number of parallel small blood vessels, under normal physiologic conditions the viscous resistance of a vascular bed behaves similar to an ideal resistor that is not a function of flow rate. In order to replicate this vascular resistance behavior, an *in vitro* resistance component should be constructed using a large number of parallel small channels. This is supported by examining the relationship $R \propto N^{3/2} \; Re \, / \, Q$ where R is the overall resistance of a resistor component, N is the number of parallel channels used to create the resistor, Re is the Reynolds number in each individual channel, and Q is the total flow rate through the resistor

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(Kung 2011). For a given resistance, in order to accommodate high enough (physiologic) flow rates while maintaining *Re* in the laminar flow regime, a large number of parallel channels should be used (Figure 11.4). A practical method to construct such a flow resistance component is by placing a number of glass capillary tubes in parallel inside a larger conduit as shown in Figure 11.1 (Kung 2011). The following equations for calculating the resistance value “R” and maximum laminar flow rate “Q_{max}”of the resistance component are useful for its design and construction:

$$R = \frac{8\,\mu\, l}{N\,\pi\, r^4} \quad \text{(Eqn 12.8)}$$

$$Q_{max} = 1050\,\pi\,\mu\, r\, N\,/\,\rho \quad \text{(Eqn 12.9)}$$

μ and ρ are the dynamic viscosity and density of the working fluid. *l, r,* and *N* are the length, inner radius, and total number of capillary tubes.

Alternatively, many studies choose to use a simple partially closed ball valve to create flow resistance in an experiment (Figliola 2010; Vukicevic 2013; Khoiy 2016; Timms 2011). This approach results in a resistance that is a direct function of flow rate (Figure 11.5) (Kung 2011). In experiments where the flow is fairly constant, the simple ball valve resistor may provide a good approximation of the desired resistance. In highly pulsatile flow scenarios, the approximation begins to deteriorate. While the ball valve flow resistor is very simple to construct, it is difficult to tune. Very small adjustments of the valve closure can result in significant changes in the resulting flow resistance. When employing a ball valve resistor in an experiment, it is important to completely avoid physical perturbation of the valve after tuning. Depending on the specific topic of study, the ball valve resistor may be an appropriate option to quickly provide a desired mean resistance.

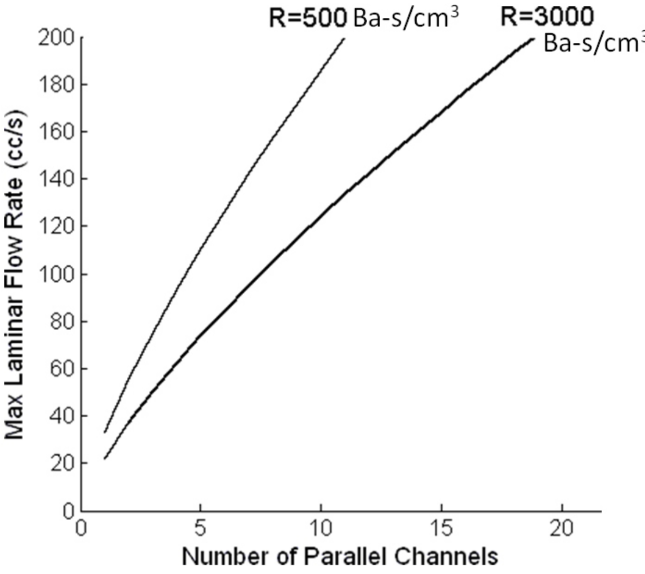

Figure 11.4. Maximum laminar flow rate versus number of parallel channels for the resistance component. In order to accommodate higher flow rates, a flow resistor needs to be constructed from an increasing number of parallel channels.

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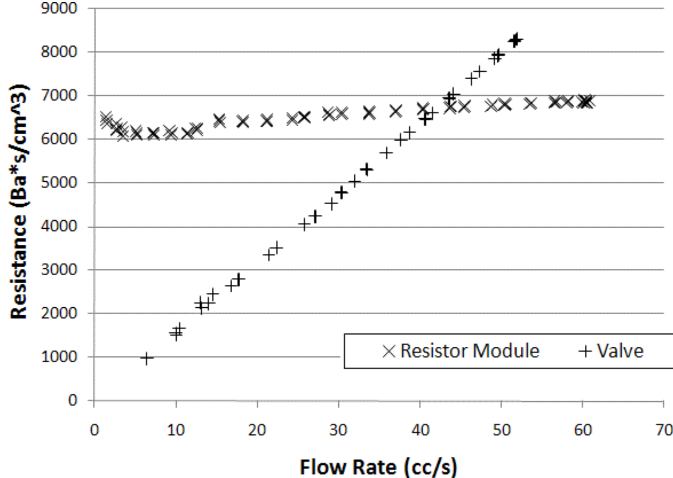

Figure 11.5. Resistance characteristics of a resistance component made from parallel capillary tubes, versus one made from a partially closed ball valve.

Passive Component: Capacitance

The most common method for constructing a flow capacitance is to use a chamber that traps a pocket of air, where liquid entering the chamber would compress the air pocket (Figure 11.1). By combining the mathematical definition of the capacitance and Boyle's law, we obtain that the capacitance of an air pocket due to compression is $C_c=V/P$, where V is the volume of the air and P is the absolute pressure of the air. For small perturbations in the air volume and pressure, the capacitance value stays fairly constant. Another contribution of capacitance that is a byproduct of the use of such a chamber is the changing height of the liquid-air interface as liquid enters and leaves the chamber. Using hydrostatic calculations we obtain that this capacitance is $C_h = A / \rho g$, where A is the horizontal cross-sectional area of the chamber, ρ is the density of the liquid, and g is gravity. C_c and C_h are two capacitances in series resulting from this setup. C_h is typically much larger than C_c and thus can be neglected with the two capacitances being in series. In the case where a very large capacitance is desired, a chamber that is open to the atmosphere can be used. In such a construction the capacitance is described by C_h alone (Kung 2011).

Passive Component: Inductance

The flow inductance describes how the acceleration of fluid results in a force which manifests as a pressure differential. Due to the fluid mass in flow conduits, the inductance is often a parasitic parameter in an *in vitro* experiment. The inductance "L" of a flow conduit can be calculated by $L = \rho\, l / A$ where l is the length and A the cross-sectional area of the conduit (Kung 2011). Inductance can be added to a flow system by simply putting in sections of long tubing. However, it is uncommon to insert tubing for the sole purpose of adding inductance, since the inherent inductance of existing connection tubing required in a flow system is often already sufficient or more than the desired vascular inductance to be modeled.

Active Components

The fluid flow in an *in vitro* experiment is typically driven using programmable flow pump. Commercial devices such as the 'Harvard pump' (Harvard Apparatus, Holliston. MA,

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USA) and the 'SuperPump' (Vivitro Labs, Victoria, BC, Canada) operate on a piston pump principle and are capable of generating outputs that resemble the flow through the aortic valve (containing regions of zero flow). The output of this type of pumps must contain a zero flow region due to the necessity for the piston cylinder to refill in each cardiac cycle. These pumps are relatively simple and inexpensive, and are ideal for *in vitro* experiments replicating flow conditions in regions near the heart. In order to create flow conditions corresponding to those at specific vascular regions further away from the heart, extensive downstream vascular simulator (i.e., an *in vitro* LPM) may be required to provide the necessary impedances (Groves 2014; Pahlevan 2013). The proper construction and tuning of the vascular simulator may be taxing.

A more sophisticated type of programmable flow pump utilizes a gear pump together with a stepper or servo motor to create output flow waveforms of an arbitrary shape. Custom built devices (Mechoor 2016) or commercial devices such as the 'CardioFlow' (Shelley Medical Imaging Technologies, London, ON, Canada) can generate flow waveforms precisely corresponding to those at specific anatomic locations. A feedback mechanism that adjusts motor input signal based on the actual flow output (Mechoor 2016) can be used to correct any errors in the output flow waveform due to varying downstream loading conditions. This type of programmable flow pump is the best option for re-creating precise flow waveform shapes without the need for complicated vascular simulator setup and tuning.

A programmable flow pump together with real-time pressure measurement feedback can construct an *in vitro* active component that approximates the Frank-Starling behavior of the heart, albeit without highly realistic end-systolic and end-diastolic pressure-volume characteristics and preload sensitivities (Baloa 2001; Gregory 2011; Gwak 2005; Timms 2011; Pantalos 2004; Ferrari 1998).

In the human body, the movement of the diaphragm due to respiration leads to dynamically changing pressures inside the thoracic and abdominal cavities, which then act on the blood vessels in those cavities to affect blood flow. An *in vitro* setup can capture this effect by applying an external pressure, typically by pneumatics, to flexible tubing that represent large blood vessels in those cavities.

Constructing the LPM

A vascular bed can typically be represented as a resistor-capacitor-resistor (RCR) block (Figure 11.6), with the proximal resistor "*Rp*" representing the combined resistance of the larger arteries, the capacitor "*C*" representing the combined compliance of the elastic vessels, and the distal resistor "*Rd*" representing the combined resistance of the arterioles and capillaries of the vascular bed. An additional capacitor and resistor are sometimes added to the RCR block to describe the venous compliance and resistance. RCR blocks representing different vascular beds are then organized together to represent the circulation to be modeled (Figure 11.7). Based on the goals of a specific research study and the particular regions of interest, the researcher must determine the appropriate amount of details when constructing different parts of the LPM. Inductors should be added to points of the LPM where flow is highly pulsatile. Active components such as the atria, ventricles, and respiration should be added as pertinent to the study.

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Once the structure of the LPM is determined, the component values and parameters of the LPM are often tuned based on clinical data acquired from the patients to be modeled. The averaged pressure drop and the flow across a vascular network together determine the total resistance of the network. The ratio of the proximal to the distal resistance, as well as the value of the capacitance both affect the shape of the flow and pressure waveforms. For example, given a specific inflow waveform to a vascular block, increasing the capacitor component value or decreasing the ratio of the proximal to distal resistance will result in decreased pressure waveform amplitude at the inlet of the block. An iterative process is often used to tune the values of the LPM components, as well as sometimes the structure of the LPM circuit, until the LPM produces hemodynamic conditions that match the corresponding clinical measurements.

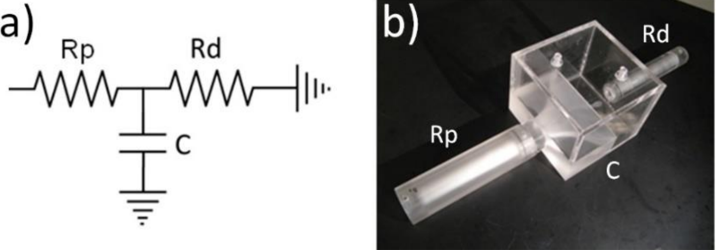

Figure 11.6. Basic lumped-parameter representation of a vascular bed using the resistor-capacitor-resistor block. a) Symbolic representation; b) *In vitro* physical module.

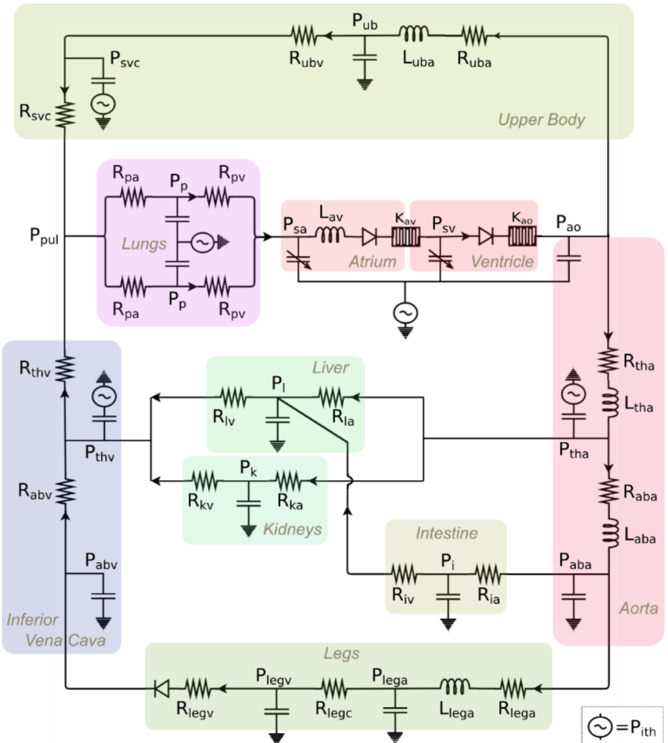

Figure 11.7. An example LPM describing the single-ventricle circulation. Pith represents the thoracic pressure. (Kung 2014).

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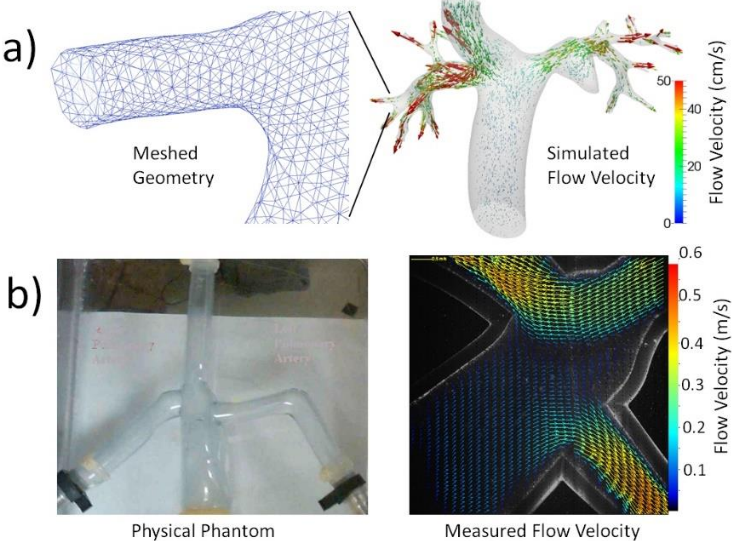

Figure 11.8. Analysis of blood flow patterns using a a) computational; and b) experimental[1] high-order model.

HIGH-ORDER MODELS

High-order models are capable of capturing detailed 3D dynamics that are dependent on anatomy and geometry (Figure 11.8). They are useful in situations where the details of these parameters have global effects, or are important to the topic of interest. For example, the design of the propeller in a blood pump or the proper closure of a heart valve has important impacts to the global hemodynamics (such as cardiac output and blood pressure). The blood flow pattern in an aneurysm revealing local areas of recirculation or low vessel wall shear stress may suggest thrombotic risks or biological responses that contribute to aneurysm enlargement. For these types of studies it is often necessary to use high-order models to capture the relevant dynamics.

The scope of high-order models is typically limited to small regions of the circulation, since the complexity and cost of high-order models make it currently impractical to model the entire circulation with them. 3D medical imaging data such as those from computed tomography or magnetic resonance imaging provide the geometrical basis for model construction. 3D segmentation based on image intensity and 2D segmentation along blood vessel centerlines are common methods for reconstructing high-order models that reflect patient-specific anatomy (Soler 2001; Les 2010). Using patient-specific high-order models, different surgical procedures can be attempted and evaluated via computational simulations or *in vitro* experiments. Models of vasculature with deformable walls are also capable of capturing important fluid-structure interactions in the cardiovascular system. Currently, one of the main challenges with utilizing deformable-wall models is the difficulty in prescribing relevant tissue properties, as this information is difficult to obtain from patients non-invasively.

[1] Courtesy Richard Figliola.

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Computational Representation

In a computational high-order model, the relevant geometry is discretized into a large number of entities (elements or control volumes) (Krause 1985; Peskin, 1972) using meshing software. Blood flow velocity and pressure are then computed using finite volume or finite element methods by solving the well-known mass conservation and incompressible Navier-Stokes equations. Newtonian fluid assumption is widely accepted for modeling hemodynamics in large blood vessels (Gijsen 1999). Non-Newtonian shear thinning behavior of blood needs to be considered in modeling scenarios such as capillary flow and flow near blood vessel walls. High-performance parallel computing and significant storage capacity are typically required for running high-order computational models.

To incorporate the effects of blood vessel wall deformation, the coupled momentum method (Figueroa 2006) was developed to be a computationally inexpensive method for capturing fluid-structure interactions. In this approach the blood vessel wall motion is prescribed as a velocity boundary condition to the fluid domain, while the fluid mesh remains fixed. This method is suitable in scenarios where the vessel deformation is small, and has been experimentally validated to produce reasonably accurate results for vessel-wall strains up to ~10% (Kung 2011a). For cases where the geometric deformation is large, the Arbitrary Lagrangian-Eulerian method (Long 2012; Bazilevs 2010) is often employed. In this method, the fluid mesh is moved in each computation step based on vessel wall motion and reconstructed when the geometrical deformation becomes large, resulting in a drastic increase in computational expense.

An alternative approach that does not require mesh reconstruction during large geometric deformation is the immersed boundary method (Peskin 1972; Zheng 2012). In this method the entire fluid domain is represented as a fixed Eulerian mesh with an arbitrary mobile boundary that represents the fluid-structure interface. This approach has been an active area of research especially for simulating heart valves (Griffith 2012; Wu 2016; Watton 2007; Borazjani 2013). One of the main limitations of the immersed boundary method is the lack of mesh density control with respect to the specific geometry to be modeled and thus difficulty in obtaining accurate shear stresses near fluid-structure interfaces.

There are various computational approaches for capturing the fluid-structure interaction involving the active myocardial wall motion. These can include the direct prescription of cardiac wall motion based on time-resolved imaging data (Lee 2013), and electromechanical models that simulate cardiac motion based on myocardial fiber orientation and activation (Trayanova 2011; Bayer 2012).

In vitro Representation

High-order *in vitro* models typically involve a physical replica of the anatomy, geometry, or medical device to be investigated. A physical model of the anatomy with which flow experiments can be performed is referred to as a “phantom.” Stereolithography (3D-printing) is a popular method for physically reconstructing patient specific anatomy. Commercial materials such as Somos® WaterShed plastic provide suitable options for 3D-printing rigid phantoms (Kung 2011b). However, there is currently no suitable material option for 3D-printing compliant phantoms with elastic properties that mimic real blood vessels. Rubber-

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like stereolithography materials such as TangoPlus (Stratasys Inc.) produce homogeneous compliant phantoms that are more visco-elastic than real vascular tissue. Phantoms made with silicon currently have the most realistic compliant properties, but cannot be 3D-printed. Highly uniform and sub-millimeter wall thickness is often desired in a compliant silicon vascular phantom. This can be achieved via a silicon dipping process for a phantom with trivial geometry (Kung 2011a). More complex phantom geometries need to be constructed using a silicon casting process involving an inner and outer mold; however, uniform and sub-millimeter wall thickness is difficult to achieve this way.

The selection of phantom material needs to account for compatibility with the desired mode of *in vitro* measurements. For example, phantoms for flow experiments intended in a particle imaging velocimetry study must have similar index of refraction as the working fluid. Studies employing ultrasound imaging should use phantoms with minimal acoustic attenuation and reflection. Phantoms used in magnetic resonance imaging studies must be made with materials that do not contain metal traces to avoid imaging artifacts.

A popular working fluid for *in vitro* models is 40% glycerol solution. This fluid possesses density and viscosity that are similar to those of blood and hence exhibits realistic fluid dynamic behaviors.

Coupled Multiscale Models

While the isolated use of a low-order model can have practical utilities (Schmidt 2016, Kung 2015), a high-order model is rarely used on its own since it only captures a very small region of the cardiovascular system. Much of the dynamics in a high-order model is determined by its boundary conditions. We can see from the computational simulation results in Figure 11.9 that the same geometric model with different outlet boundary condition prescriptions exhibits completely different pressure and flow behaviors. Proper boundary condition prescriptions in high-order models are essential for generating the correct flow split between multiple outlets and the proper pressure waveforms for producing accurate tissue movements in deformable models. In order to obtain realistic boundary conditions, a high-order model can be coupled to a low-order model, forming a multiscale model containing different domains. Figure 11.10 illustrates example multiscale models where a high-order anatomic model is coupled to a low-order LPM in order to reproduce local hemodynamics in a specific anatomic region within the context of the larger circulation.

In a multiscale model, the flow and pressure in each of the domains are coupled in a way such that they directly affect those in the other. The technical implementation of this coupling in the computational model involves passing flow and pressure information between the high-order and low-order models at each simulation time step. At each interface between the two domains, boundary condition is prescribed to the high-order model as either the Neumann or Dirichlet condition. The Neumann boundary condition is suitable when the most adjacent differential element in the low-order model near the interface is a capacitance. In this type of boundary condition, the low-order model provides pressure information to the high-order model, and the high-order model provides flow rate information to the low-order model. The Dirichlet boundary condition is suitable when the most adjacent differential element in the low-order model near the interface is an inductance. In this case, the low-order and high-order model provides flow and pressure information to the other, respectively. In an *in vitro*

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multiscale model, the coupling between the high and low order models can occur simply via direct physical connections (Figure 11.10b).

A coupled multiscale model can be categorized as either open-loop or closed-loop. The differentiation lies in whether the model is designed in a way such that the dynamic response of the heart is reflected. An open-loop model typically prescribes a fixed pressure or flow waveform as the active driver of the system, where a closed-loop model contains a heart model actively responding to factors such as preload and afterload. The open-loop model is simple to set up and suitable for studies focusing on the isolated dynamics of the high-order model. However, when the global physiological response is an important aspect of a study, the closed-loop model should be used.

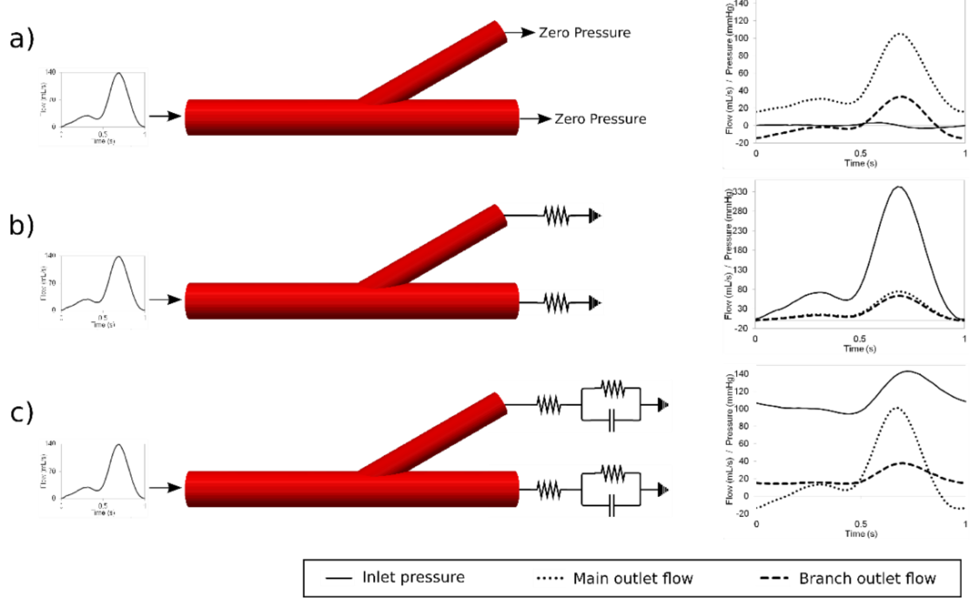

Figure 11.9. (a) Zero pressure; (b) resistance; and (c) RCR outlet boundary conditions prescribed to the same geometric model show that results in the high-order model are directly dependent on the prescribed boundary conditions. (Marsden 2015).

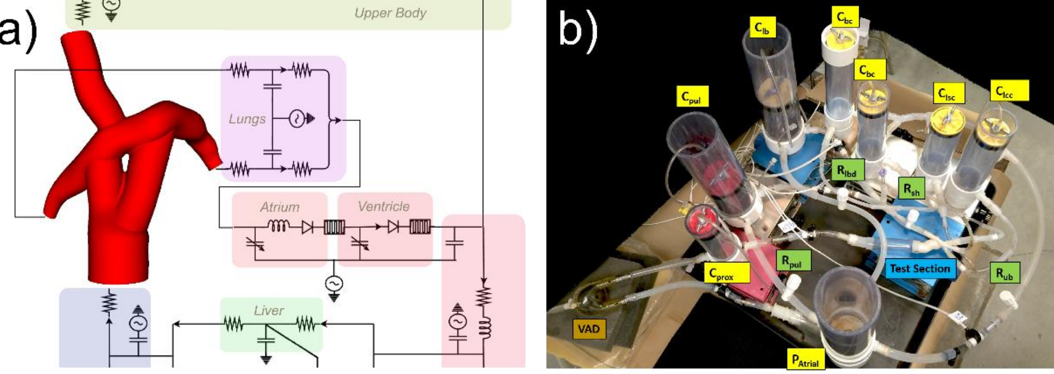

Figure 11.10. Example multiscale models of the single-ventricle circulation: a) Computational model of the stage 3 circulation with a high-order model representing the Fontan junction; and b) *In vitro* model of the stage 1 circulation with a high-order model representing the aortic arch and pulmonary shunt[a].

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EXAMPLE CASE STUDIES:

Ventricular Assist Device Implementation in Single Ventricle Infants: Low-Order Closed-Loop Computational Model

In this case study we examine the use of a low-order closed-loop computational model to investigate the physiologic impacts of ventricular assist device (VAD) implementation in single ventricle infants (Schmidt 2016). We will see how the model delineates mechanisms behind performance differences often observed clinically between steady and pulsatile VADs.

A VAD can be used as mechanical support in heart failure patients either as bridge-to-transplant, bridge-to-recovery, or destination therapy. VADs are used in various types of circulations including single ventricle circulations (Calvaruso 2007; Cardarelli 2009; Chu 2007) and normal bi-ventricular circulations (Hetzer 2006; Stiller 2003; Adachi 2011). The two main categories of these devices are the pulsatile or continuous flow VAD. A Pulsatile flow VAD (such as the Berlin Heart) drives blood via a pneumatically actuated membrane and one-way valves located at the inlet and outlet of the blood chamber, emulating the heart's distinct phases of diastole and systole. Some evidences suggest that pulsatile flow VAD may promote better ventricular unloading and more natural physiology (Cheng 2014; Drews 2008; Klotz 2004). Continuous flow VADs use spinning propellers to produce a pressure rise (Moazami 2013) and often have better reliability and smaller size, providing reduced risk of infection, bleeding, trauma, and thrombus (Cheng 2014; Drews 2008; Feller 2007; Kato 2011).

In this case study we use a closed-loop computational LPM (Figure 11.11) to investigate the physiologic response of stage 1 single ventricle patients to pulsatile and continuous flow VADs, and to identify mechanistic explanations for the differences in physiologic outcomes. Clinical data from six stage 1 single ventricle patients are used to tune the LPM to create six models each describing a unique patient. Ventricular contractility in each LPM is then diminished to simulate a heart failure condition. The HeartWare VAD and the Berlin Heart EXCOR VAD are each implemented in the LPM to describe the continuous and pulsatile VAD scenario, respectively. As the VAD attempts to draw blood from the ventricle, a negative ventricular pressure may result which could lead to ventricular tissue being drawn into the cannula and ventricular collapse (Salamonsen 2015). As part of the VAD implementation in the LPM, a ventricular suction equation is developed and incorporated to describe the suction resistance "R_{SUC}" due to ventricular collapse, thereby impeding VAD flow (Schmidt 2016).

The results of this modeling study show that the continuous VAD is capable of providing significantly higher cardiac output in all of the six patient physiologies examined (Table 11.1). Closer examination of the blood volume inside the pulsatile VAD reveals the underlying mechanism responsible for this difference. For the pulsatile VAD, cardiac output increases are achieved by means of increasing device heart rate or device size (i.e., maximum stroke volume). However, if a large pulsatile VAD is implemented in a stage 1 single ventricle patient, who typically is a small infant, ventricular suction tends to occur and the device is unable to fill completely during diastole as its pump rate increases (Figure 11.12). The decreased stroke volume at higher pump rates results in the inability to augment cardiac output.

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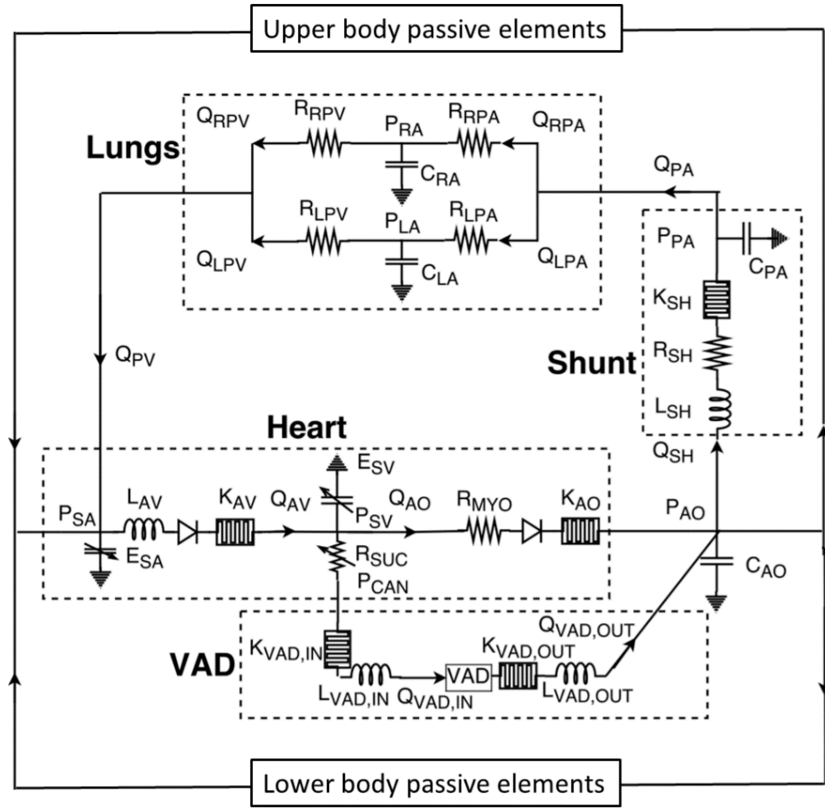

Figure 11.11. A low-order closed-loop computational model describing a ventricular assist device implemented in the stage 1 single-ventricle circulation.

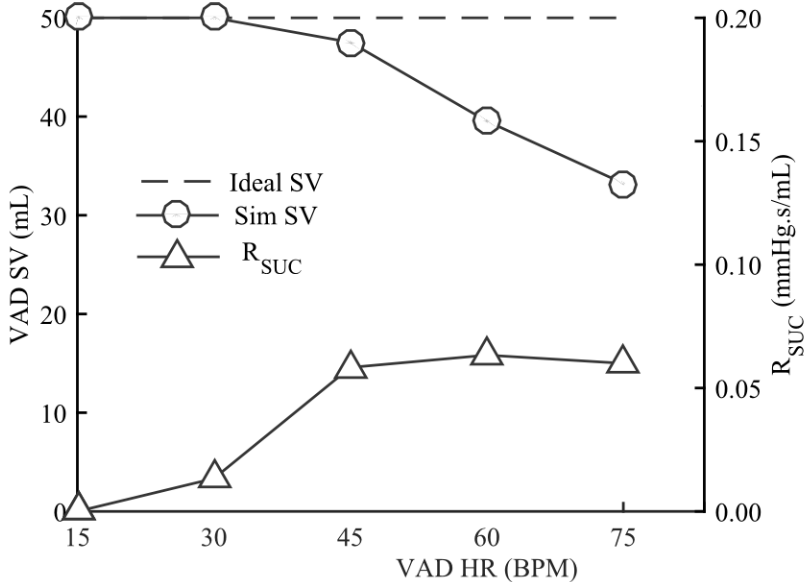

Figure 11.12. Stroke volume (SV) and suction resistance (R_{SUC}) versus VAD heart rate (HR) for the 50 mL Berlin Heart. Results show that the pulsatile VAD does not maintain the expected cardiac output at higher HR due to reduced SV. Data shown is the cohort mean across 6 patients.

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Hemodynamics in an Abdominal Aortic Aneurysm: Multiscale Open-Loop *In vitro* Model

Hemodynamic parameters such as the 3D blood flow and pressure fields have direct effects on the initiation and development of atherosclerosis and aneurysms (Taylor 1971; Glagov 1988; Ku 1985; Malek 1999). Modern imaging techniques such as phase-contrast magnetic resonance imaging can be used in an *in vitro* experiment to perform high-resolution direct measurements of flow velocity patterns in an anatomical phantom. In this case study, we demonstrate an *in vitro* model for studying the blood flow patterns in an abdominal aortic aneurysm (Kung 2011b).

A flow phantom made of MR-compatible resin (WaterShed® XC 11122, DSM Somos®, Elgin, IL) is 3D-printed according to a patient anatomical model constructed from a gadolinium-enhanced magnetic resonance angiography of the patient; anatomy of the abdominal aortic aneurysm and the renal and common iliac arteries are included in the flow phantom (Figure 11.13). Two RCR blocks are constructed to create downstream vascular impedances for the renal and aortic outlets of the phantom. The values of the resistor and capacitor components are set to produce physiologically realistic pressure and flow split waveforms under a physiologically realistic inlet flow waveform (Table 11.2). The resistor components are constructed by placing a large number of thin-walled glass capillary tubes (Sutter Instrument, CA) in parallel inside a plexiglass cylinder. The capacitor components have a smooth contour for the inlet in order to minimize flow turbulences and thus avoid parasitic resistances.

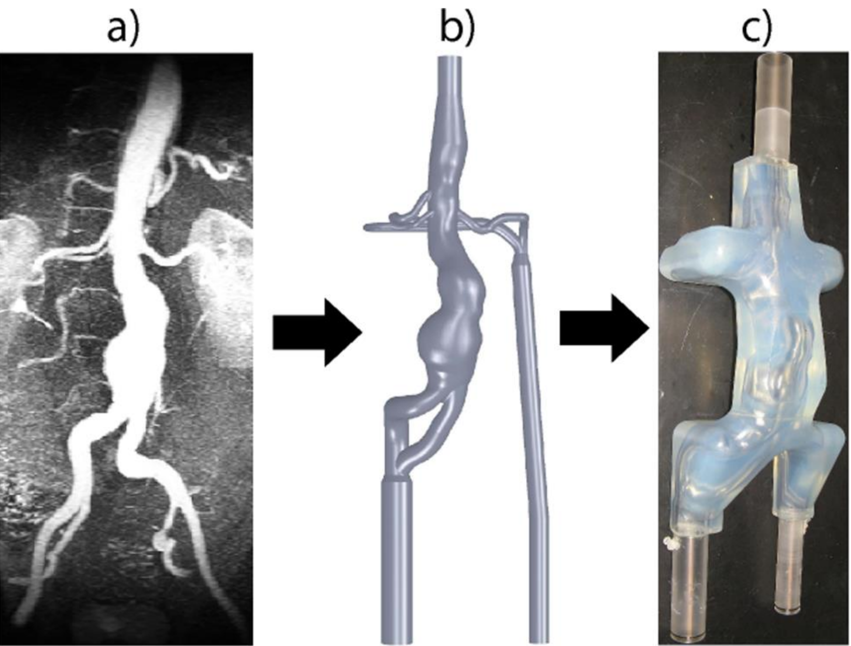

Figure 11.13. Anatomical phantom construction: a) MR Imaging data from an abdominal aortic aneurysm patient. b) 3D computer model constructed based on patient imaging data. c) Physical phantom constructed from 3D computer model (Kung 2011b).

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We connect the RCR blocks to the anatomic flow phantom and place them in a flow system (Figure 11.14). Based on the flow waveforms measured in the patient (Bax 2005; Les 2010), we use a custom-built, MR-compatible, and computer-controlled pulsatile pump (Ku 2005) in parallel with a steady flow pump (Model 3-MD-HC, Little Giant Pump Co., OK) to reproduce the supra-renal aortic flow waveforms as the input flow to the phantom. The phantom input flow is monitored using a MR-compatible ultrasonic transit-time flow sensor (8PXL, Transonic Systems, NY).

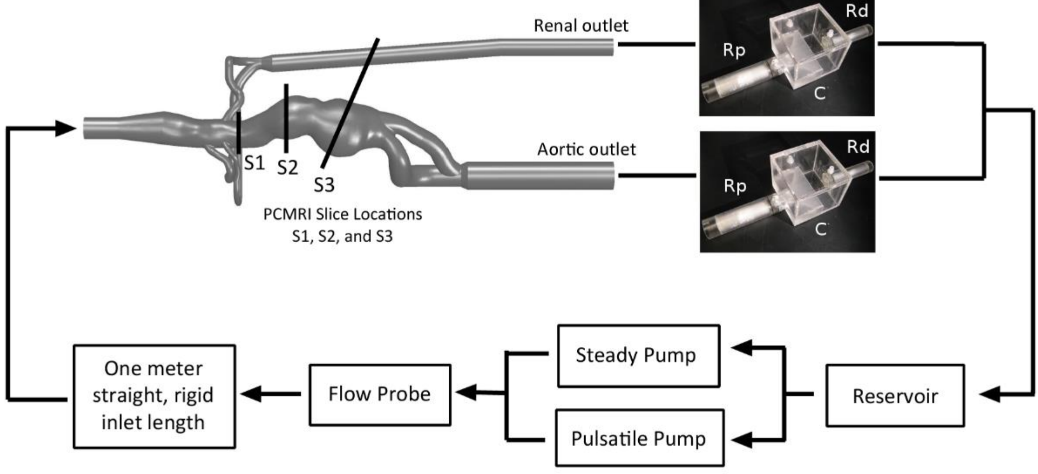

Figure 11.14. Flow system setup for the abdominal aortic aneurysm *in vitro* model.

Table 11.1. Maximum cardiac output (L/min) achieved in each patient without VAD, with pulsatile VAD, and with continuous VAD

	Patient A	Patient B	Patient C	Patient D	Patient E	Patient F
Pre-VAD	1.29	1.55	1.42	1.60	1.87	1.75
Pulsatile VAD	1.92	2.30	2.14	2.24	2.21	2.79
Continuous VAD	3.11	3.37	3.36	3.51	3.31	4.09

Table 11.2. Values of lumped-parameter components in the RCR blocks of the abdominal aortic aneurysm *in vitro* model. Rp, C, and Rd are the proximal resistance, capacitance, and distal resistance, respectively

	Aortic outlet	Renal outlet
Rp (dynes-s/cm^5)	549	3053
C (cm^5/dynes)	3.25e-4	1.64e-4
Rd (dynes-s/cm^5)	7132	5951

Using a cardiac-gated 2D 3-component cine PCMRI sequence in a 1.5T GE MR scanner (Signa, GE Medical Systems, Waukesha, WI) and an 8-channel cardiac coil, we acquire flow velocity measurements at different slice locations within the phantom. The slice locations represent the mid-aneurysm location for each lobe of the bilobed aneurysm anatomy and the

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location directly downstream of the renal branches where flow is likely to be complex. As we examine the velocity imaging results at different time points in the flow cycle (Figure 11.15), it is easy to see the prominent swirling of blood and the stagnant regions in the large aneurysm. This observed flow pattern may provide information regarding the endothelial health in the aneurysm. Such an *in vitro* setup can also serve as a testbed to investigate the hemodynamic effects of various surgical procedures or vascular device implantations.

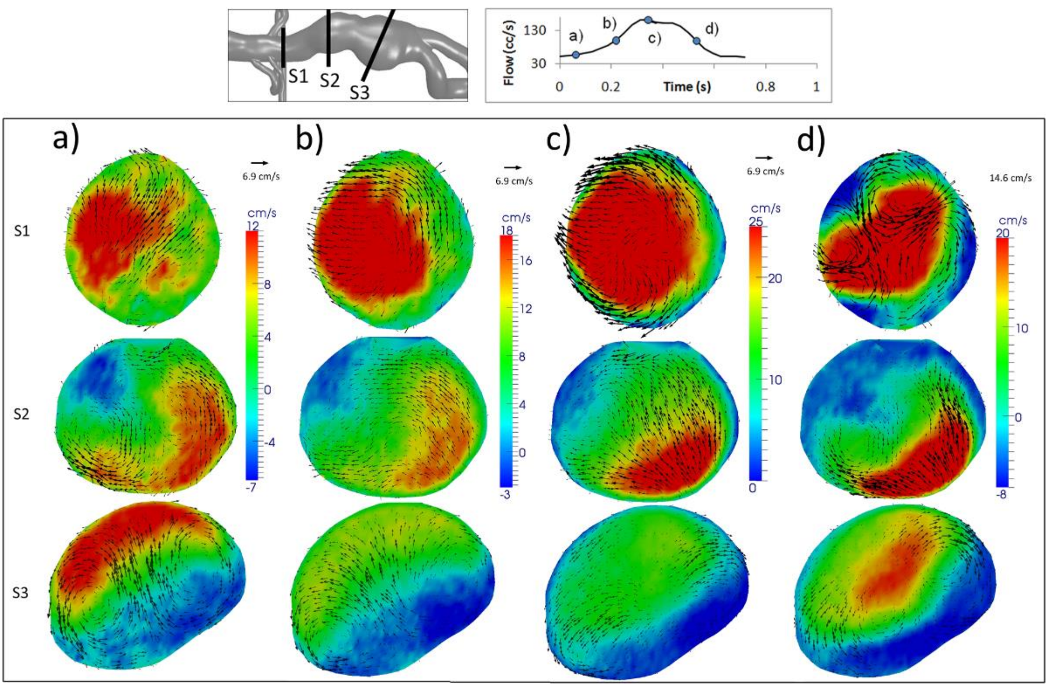

Figure 11.15. Phase-contrast magnetic resonance imaging measured flow velocities in the abdominal aortic aneurysm *in vitro* phantom experiment.

Virtual Surgery of the Superior Vena Cava to Pulmonary Artery Anastomosis: Multiscale Closed-Loop Computational Model

In this case study we demonstrate the use of a multiscale closed-loop computational model to predict and compare surgical outcomes of the single ventricle stage 2 palliation procedure. In this surgical procedure, the superior vena cava is connected to the pulmonary artery via two options: the “Glenn” or the “Hemi-Fontan” procedure (Norwood 1991). The Glenn procedure severs the superior vena cava from the right atrium and connects it to the right pulmonary artery, where the Hemi-Fontan procedure connects the top half of the right atrium to the pulmonary artery while leaving the superior vena cava attached to the atrium. Various degrees of left pulmonary stenosis are common among these patients, and part of the consideration of the stage 2 procedure is whether to perform the additional surgical steps to remove the stenosis, if present. Using a multiscale computational model, we investigate the hemodynamic differences between the Glenn and the Hemi-Fontan surgical options, as well as the physiologic impacts of a left pulmonary stenosis.

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From 3D imaging data of a patient, we construct a high-order computational model that is a representation of the patient's pre-operative anatomy. We then perform "virtual" Glenn or Hemi-Fontan surgeries and add a "virtual" left pulmonary stenosis of various severities by computationally modifying the pre-operative anatomic geometry (Figure 11.16). Using the pre-operative clinical data from the same patient, we tune an LPM (the low-order model) to represent the global circulation of the patient and couple the high and low order models to complete a multiscale simulation setup (Figure 11.17). The coupled multiscale simulation is run using open source software Simvascular (www.simtk.org) on a super computing cluster.

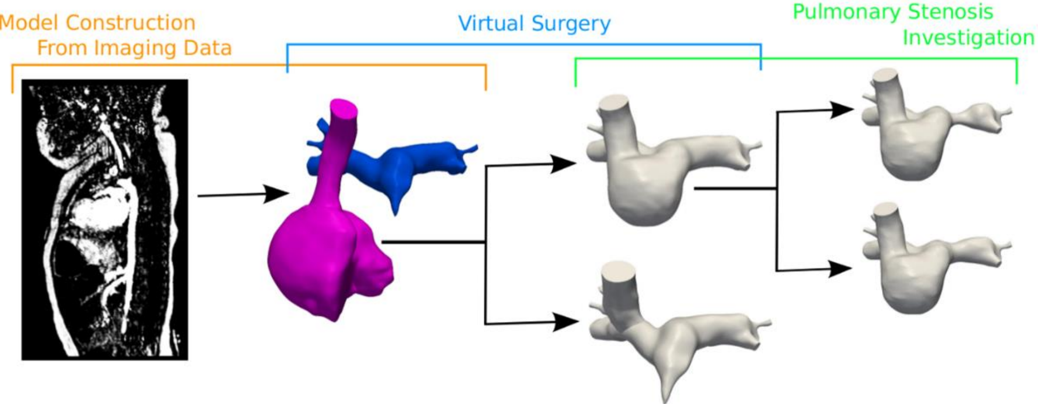

Figure 11.16. Virtual surgery and virtual pulmonary stenosis investigation from preoperative 3D anatomical model constructed based on patient imaging data. (Marsden 2015).

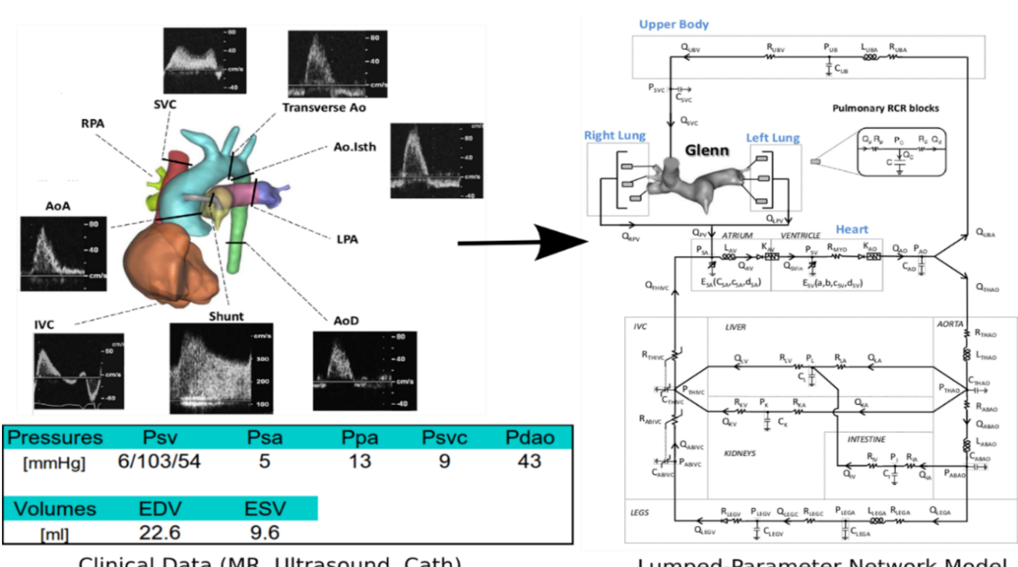

Figure 11.17. Preoperative clinical data supplies parameter tuning information for the closed-loop multiscale model consisting of a LPM coupled to a 3D anatomical model. (Marsden 2015).

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Clinically relevant information such as the 3D pressure and flow in the high-order model (Figure 11.18), time tracings of physiologic parameters in the LPM (Figure 11.19), and power loss in the surgical junction relative to other systemic powers (Figure 11.20) can be extracted from the multiscale simulation results. This modeling investigation reveals that while flow patterns within the surgical junction are affected by different surgical options, physiology at the global level is not. In this particular case study, a 50% or 85% area stenosis also does not produce a significant impact to the global physiology. This is due to the fact that compared to the systemic power losses, the power loss in the surgical junction is relatively small. The differences in the 3D flow patterns between the surgical options, however, may provide information for assessing clinical considerations such as thrombotic risk.

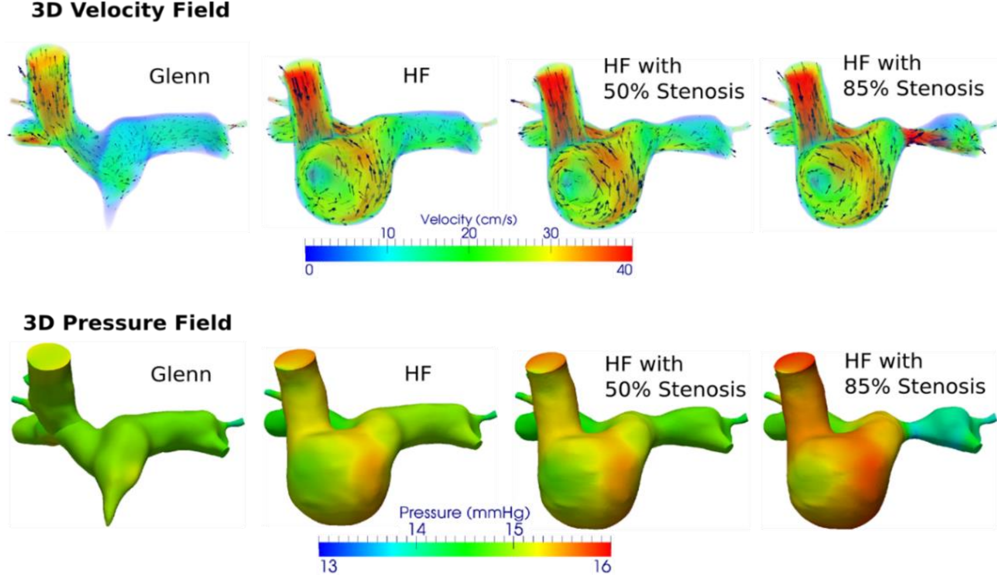

Figure 11.18. Simulated 3D pressure and flow patterns in the high-order model (Marsden 2015).

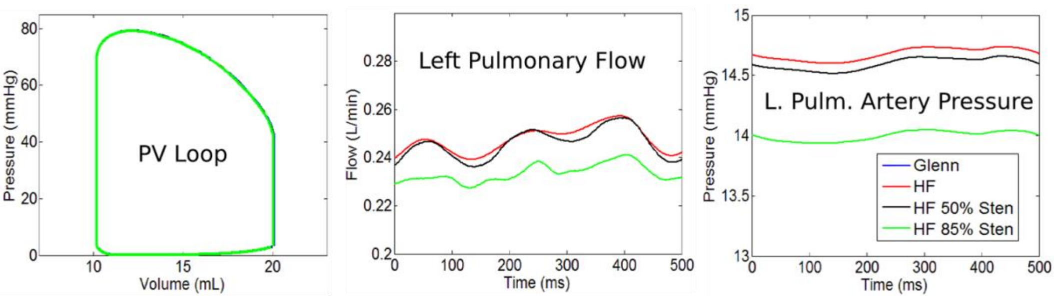

Figure 11.19. 0D physiologic parameters extracted from the low-order model (Marsden 2015).

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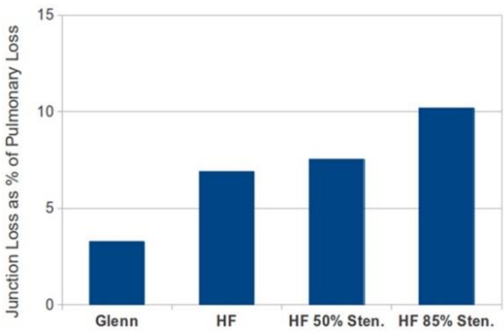

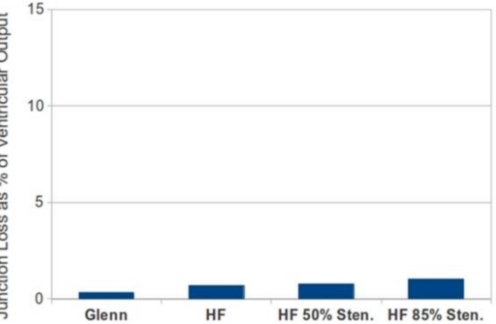

Figure 11.20. Power loss in the surgical junction as a fraction of other systemic powers (Marsden 2015).

MODEL SELECTION

Designing a cardiovascular model that is appropriate for the topic of study is essential for obtaining meaningful results. The choices between high versus low order models and computational versus *in vitro* models should depend on the input information available, the output information desired, and the aspects of biomechanics important to the study.

Low-Order versus High-Order

The main advantage of low-order models is that they are relatively low-cost to perform. Low-order models are suitable for simulating overall physiology and examining pressure and flow waveforms at different parts of the circulation. Since detailed geometric data is not required for model construction, it is also simpler (compared to high-order models) to create new implementations to describe complex scenarios such as the operation of active medical devices (i.e., the first example case study) as long as enough data is available to construct the required mathematical descriptions. The limitation of low-order models is their inability to account for or capture 3D parameters; therefore in the cases where the effects of these 3D quantities are important to the topic of study, high-order models should be employed. High-order models require detailed geometric input information and are labour-intensive to construct. Simulations of high-order models are also computationally expensive. In the choice between high or low order models, one should determine whether geometry or 3D dynamics affect the quantities of interest or whether the 3D parameters themselves are the quantities of interest. If the answer is yes to either of these questions, a high-order model is suitable. The use of high-order models should be limited to regions of the circulation that require them. The rest of the circulation should be represented using low-order models in order to achieve the most resource-efficient multiscale modeling approach for a research study.

Computational versus *In vitro*

Computational models are typically less costly and quicker to construct than *in vitro* models. A computational low-order model can be set up within minutes with a few lines of

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code while an *in vitro* model could require hours of machining and physically connecting several components in a flow loop. In research studies that investigate systematic changes in a model, a computational setup can be automated to require minimal manual involvement. Extraction of output parameters is also more straight-forward in a computational model, as the desired information can be directly accessed from the computation data. In an *in vitro* model, measurements of the desired parameters require different sensor implementations and/or imaging setup and are subject to measurement noise and limited resolution. Measurements in an *in vitro* model with magnetic resonance, ultrasound, or particle imaging velocimetry often bring challenging technical requirements for phantom construction and experiment setup. For example, all of the devices in an *in vitro* setup for magnetic resonance imaging must be MR compatible to ensure safety and to avoid signal interference and imaging artifacts. This often means a large physical setup since long tubing and cables are required to keep the flow pump and pressure measurement equipment at a safe distance from the MR scanner. In an experiment for particle imaging velocimetry, index of refraction must be matched between the phantom and the working fluid, which significantly complicates phantom construction and often results in the necessity of adding corrosive chemicals such as sodium iodide in the working fluid. The strict geometrical setup criteria of particle imaging velocimetry also limit data acquisition to very few imaging planes. Lastly, the ability for *in vitro* models to describe the closed-loop response of the heart to changing preload and afterload is currently primitive, presenting a significant limitation compared to computational models.

On the other hand, computational models may not capture as many aspects of the relevant physics compared to *in vitro* models, especially in the area of fluid-structure interactions. Computational simulations of fluid-structure interactions are technically challenging, and reliable simulation techniques may not exist for the scenarios of interest. For example, current computational capabilities for simulating heart valve dynamics are primitive and very few groups have successfully performed simulations able to model leaflet contact (Wu 2016). Transient analysis of 3D computational models involving rotary blood pumps is extremely computationally expensive, therefore most studies are limited to steady-state analyses without accounting for realistic pulsatile flow scenarios (Fraser 2011). For the situations where computational methods face significant limitations, an *in vitro* model where the fluid-structure interactions are directly replicated in a physical setup may be more suitable. *In vitro* high-order models are often suitable for medical device studies, since the device to be investigated can be implemented directly into the anatomical phantom under realistic flow and pressure conditions. Measurements of the resulting hemodynamics can reveal detailed 3D mechanical interactions between device operation and patient physiology and anatomy.

CONCLUSION AND FUTURE PERSPECTIVES

Computational and *in vitro* cardiovascular biomechanical models provide highly-controlled approaches for studying the cardiovascular system. This chapter provides an overview of several major types of models, the pros and cons of each, and a few examples of model application. The proper selection of high-order versus low-order and computational versus *in vitro* models for each research topic is important for capturing the relevant dynamics

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and minimizing resource usage. When employed properly, the types of biomechanical models presented in this chapter can bring significant progress to a research project at lower costs of resource and time compared to the use of animal models.

There are several areas of current development towards broadening the scope and usefulness of cardiovascular biomechanics models. The first is the incorporation of cellular biomechanics and biochemistry to capture the interactions and effects of biological responses at the microscale. Computationally, this would involve coupling cellular kinetics models with macroscale hemodynamic models (i.e., those presented in this chapter). Experimentally, tissue engineered constructs containing live cells can be implemented into an anatomical vascular simulator and allowed to directly interact with the macroscale hemodynamics. Second, there is a need for *in vitro* models to accurately capture closed-loop responses of the cardiovascular system, which is important for accounting for physiologic feedback and assessing physiologic impacts. A method to address this need is currently being developed in the author's research group and referred to as the "Physiology Simulation Coupled Experiment." This approach is similar to the hardware-in-the-loop concept commonly used in the aviation industry and couples a high-order *in vitro* model to a low-order computational physiology model. Finally, there is a pressing need for the validation of model predictions against clinical data. Due to the limitations in clinical data acquisition and lack of clinical database for modeling purposes, reliable large-scale validation of cardiovascular biomechanical models is rare. Close coordination between clinicians and engineers is essential for bridging this gap and bringing the much-needed validation to justify application of these biomechanical models towards clinical use.

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Chapter 12

ISOLATED KIDNEY PERFUSION MODELS

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ABSTRACT

The isolated perfused kidney (IPK) model emerged in the 1970s and has since been extensively used to study renal drug disposition. The model allows to elucidate mechanisms of drug excretion, screen for drug-drug interactions, study renal drug metabolism, and to correlate renal drug disposition to kidney function. Over the last decade, IPK has been particularly useful in the development of preservation techniques for deceased donor kidneys before transplantation. To increase the donor pool, kidneys retrieved from extended criteria donors (ECD) and donation after circulatory death (DCD) donors are increasingly utilized. ECD organs might particularly benefit from hypothermic machine perfusion (HMP), which has been hypothesized to protect deceased donor kidneys from ischemia-reperfusion (I/R) injury. Conflicting data exist on the benefit of HMP for DCD kidneys. DCD kidneys are known to suffer extensive ischemic damage and avoidance of cold-induced injury in sub-normothermic machine perfusion (SMP) might be beneficial for these organs. In addition, higher temperatures reduce vascular stiffness resulting in less endothelial and vascular impairment. Normothermic machine perfusion (NMP) mimics the physiological environment and maintains cellular metabolism during preservation. Numerous cold preservation solutions exist and are still being developed, but the University of Wisconsin solution is still the gold standard in renal preservation. Today, most research focuses on *ex vivo* treatment of grafts before transplantation.

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Keywords: isolated kidney perfusion, *ex vivo* perfusion, renal physiology, drug disposition, machine perfusion, solutions, preservation, and kidney transplantation

ABBREVIATIONS

ATC	apricitabine
BPAR	first biopsy proven acute rejection
BSA	bovine serum albumin
CI	confidence interval
CIT	cold ischemia time
COR	controlled oxygenated rewarming
CSA	cyclosporine A
DBD	donation after brain death
DCD	donation after cardiac death
DGF	delayed graft function
DME	dimethylaminoethanol
ECD	extended criteria donors
GFR	glomerular filtration rate
GST	glutathione S-transferase
HES	hydroxyethyl starch
H-FABP	heart-type fatty acid binding protein
HMP	hypothermic machine perfusion
HOC	hyperosmolar citrate
HOPE	hypothermic oxygenated machine perfusion
HTK	histidine-tryptophan-ketoglutarate
IGF	initial graft function
I/R	ischemia-reperfusion
IGL-1	Institut Georges Lopez-1
IPK	Isolated perfused kidney
KHS	Krebs-Henseleit bicarbonate
L-FABP	liver-type fatty acid binding protein
MP	machine perfusion
MSC	mesenchymal stem cell therapy
MTP	metoprolol
MTX	methotrexate
NHB	non-heart beating
NMP	normothermic machine perfusion
NSX	N4-acetylsulfisoxazole
OR	odds ratio
PAH	para-aminohippuric acid
PBS	phosphate-buffered sucrose
PEG	polyethylene glycol
PFC	perfluorcarbon
PHP	pyridoxalated haemoglobin polyoxythylene

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Pn	prednisolone
PNF	primary non-function
RCT	randomized controlled trial
RR	renovascular resistance
SA	salicylic acid
SCS	static cold storage
siRNA	small interfering RNA
SMA	superior mesenteric artery
SMP	sub-normothermic machine perfusion
SU	salicyluric acid
SX	sulfisoxazole
UW	University of Wisconsin
WIT	warm ischemia time

INTRODUCTION

The isolated kidney perfusion model (IPK) was first established in 1876, when Bunge and Schmeideberg reported the synthesis of hippurate from glycine and benzoic acid in isolated perfused dog kidneys (Chang 2013). The model was originally used to investigate physiological and biochemical aspects of renal function, but soon became a useful tool in drug disposition studies. In the 1970s, Bowman and Bekersky using a rat model demonstrated the utility of IPK for drug disposition analysis. Hence the model was used to elucidate mechanisms of drug excretion, screen for drug-drug interactions, study renal drug metabolism, and to correlate renal drug disposition to kidney function (Bowman 1975; Bekersky 1984; Bekersky 1983; Bekersky 1980; Bekersky 1980). Over the last decade, *ex vivo* isolated kidney perfusion has been particularly useful in the establishing of preservation techniques for deceased donor kidneys before transplantation. Although cheaper and more practical, the perfusion of small laboratory animal kidneys has limited value in testing novel preservation techniques since differences in renal function largely differ from humans. Therefore, porcine kidney systems are commonly used to mimic the situation in humans. In this chapter, characteristics of hypo-, normo-, and subnormothermic kidney machine perfusion will be described.

While the potential applications of IPK experimentation are enormous, several disadvantages are encountered with this technique. Abnormalities in renal hemodynamics, urinary concentration and dilution ability, and excretion of fluid and electrolytes are prominent defects of the model. High renal perfusate flow due to perfusates with low-viscosity, and diminished distal tubular functions are the proposed sources of these model flaws. Glomerular and proximal tubular functions are well preserved however. Lastly, the surgical procedure involved in IPK requires considerable training and experience to perfect (David 2004).

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The Isolated Perfused Kidney System

Surgical Procedure (Retrieval of the Kidney)

Many different retrieval techniques similar to human nephrectomy could be used. We therefore describe below the preferred technique for explantation of the right kidney from a living donor animal. After the animal is anesthetized a midline incision is made from the bladder to sternum. Major anatomical features (right kidney, right renal artery and vein, aorta, superior mesenteric artery, right ureter) are identified and cleared of overlying connective tissue and fat. First the right ureter is cannulated. Three loose ligatures are made around the right renal artery and proximal and distal superior mesenteric artery, respectively. Prior to insertion of an arterial cannula, the distal ligature on the superior mesenteric artery is tied. This is followed by a small incision made proximal to the tied distal ligature on the mesenteric artery and a cannula is inserted into the artery. The cannula is then threaded across the aorta into the right renal artery. The other ligatures around the mesenteric artery and renal artery are then tied to secure the cannula in place. Finally, the right kidney and its associated vessels and ureter are excised en bloc. Figure 12.1 shows a schematic drawing of the surgical field for cannulation of the renal artery with major anatomic landmarks and placement of ligatures.

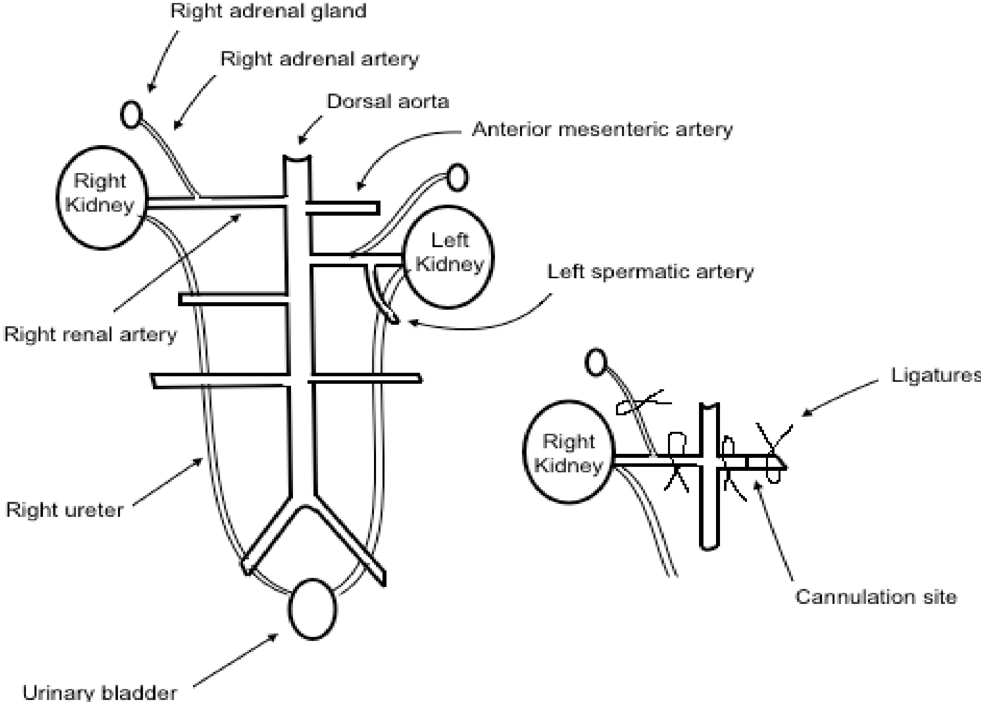

Figure 12.1. Schematic drawing of the surgical field for cannulation of the renal artery with major landmarks and placement of ligatures.

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Perfusion Apparatus

A schematic representation of an experimental IPK model is shown in Figure 12.2. The perfusion apparatus consists of a pump system to deliver the perfusion solution to the renal artery at variable speeds to achieve the desired arterial pressure. Especially for small animal IPK systems roller pumps are used. The perfusion flow produced by the roller pump is high with values up to 35 ml/min (Wang 2004). The high perfusion flow compared to *in vivo* measurements is due to lower viscosity of the perfusion solution with respect to blood, and it is essential to maintain the adequate oxygenation of renal tissue (Bekersky 1983).

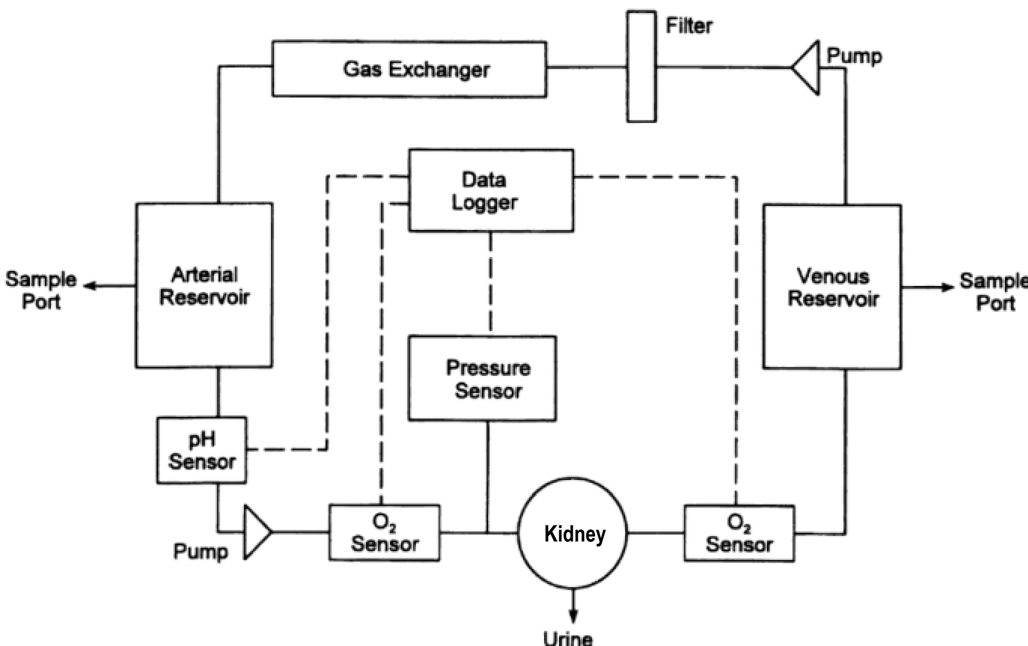

Figure 12.2. Schematic representation of an experimental isolated perfused kidney model.

Oxygenation of the perfusate can be achieved by bubbling an oxygen gas mixture directly into the arterial perfusate reservoir through a fine bubble diffuser. Foaming of the perfusate can be avoided by passing the perfusate through a semi-permeable membrane such as thin walled silicone tubing. An experimental IPK set-up is shown in Figure 12.3.

There are several key indices of kidney function that require close monitoring during isolated perfused kidney experiments. These include glomerular filtration rate (GFR), fractional reabsorption of glucose (to asses proximal tubule function), and fractional reabsorption of sodium ions (to asses distal tubule function). In addition, urine flow rate, urine pH and perfusion pressure are also monitored. Generally, GFR values are lower in IPK compared to *in vivo* values. A lower GFR leads to a decrease in filtered load and, occasionally, to lower absolute reabsorption with respect to a kidney with normal GFR (Maack 1986). Also, urine is almost isosmolar with the perfusion solution and the renal ability to concentration and dilute is severely interrupted.

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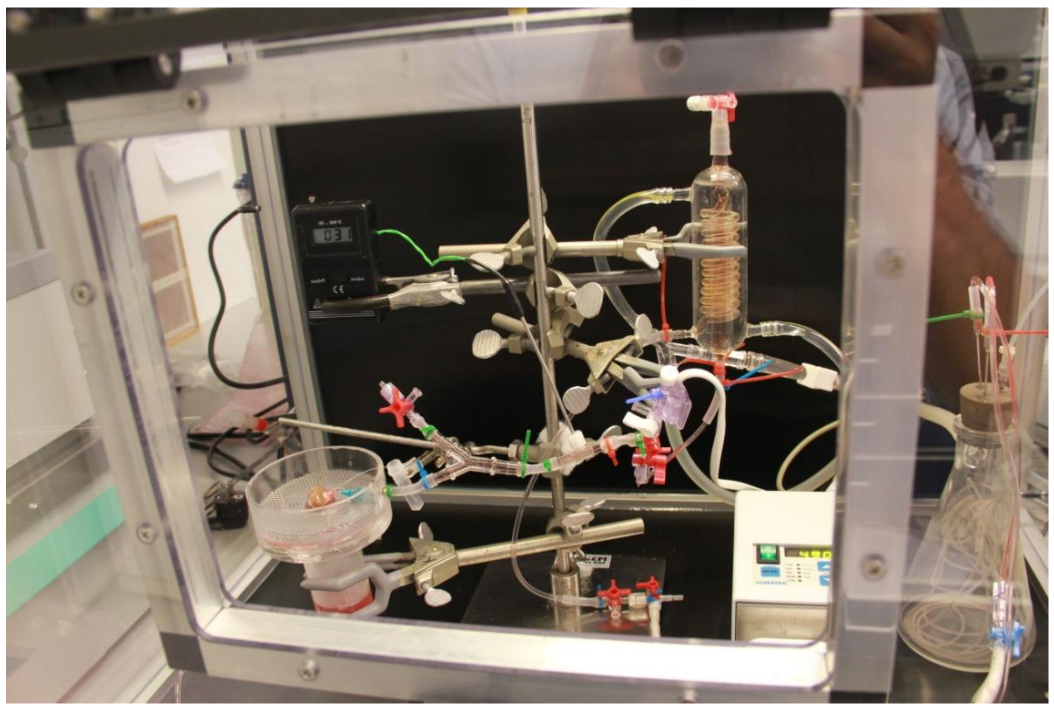

Figure 12.3. Experimental kidney perfusion model.

Perfusion Solutions

The main criteria considered when selecting an artificial perfusate medium, is to make the perfusate analogous to plasma. The basis component of IPK perfusion solution is Krebs-Henseleit bicarbonate (KHS) buffer. KHS solution contains a mixture of amino acids, electrolytes and other salts comparable to those measured *in vivo* . Glucose is added as an energy source and to support optimal GFR, cellular metabolism, and fractional reabsorption of sodium and potassium ions. Bovine serum albumin (BSA) or dextran is most commonly used as an oncotic agent in the perfusion medium. The oncotic agent generates a 'physiological' coloidosmotic pressure and allows for assessment of the relation between drug-protein binding and renal excretion. During experimental studies, the concentration of BSA can be varied in a controlled manner to allow variations in drug free fraction in the perfusate. When BSA concentration is low (< 6%), dextran should be added in order to maintain the necessary oncotic pressure within the renal vasculature (David 2004). Noteworthy, GFR values obtained with BSA as an oncotic agent are lower than those found *in vivo* . In contrast to albumin, dextran does not bind to other compounds, which makes dextran more useful for pharmacokinetic studies. The most commonly used albumin concentration is 60–65 g/l. The molecular weight of dextran must be ranged between 60,000–90,000 Da (Bekersky 1983; Maack 1986).

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Renal Disposition of Drugs

The IPK model provides an excellent tool to elucidate more precise information on the underlying mechanisms in pharmacokinetics and renal clearance of a drug. The binding of drugs to plasma proteins can have many effects on the processes that contribute to metabolism, distribution and excretion of drugs. An extensive list of drug disposition studies using the IPK model can be found in the literature, with Bekersky being one of the pioneers in the research field. An overview of drug disposition studies over the years can be found in Table 12.1.

Table 12.1. Drug disposition studies using the IPK model

Researchers	Compound	Findings
(Engbersen et al., 2012)	Sulfonylurea drugs	Sulfonylurea drugs exert differential effects on vascular smooth muscle channels. Glibenclamide and glimepiride will interact with these channels at therapeutic concentrations
(Arnaud-Batista et al., 2012)	Bufadienolides	The study shows direct diuretic, natriuretic, and kaliuretic effects of bufalin in IPK and the relevance of Na^{+}-K^{+}-ATPase-mediated signal transduction
(El-Gowilly, 2011)	Metorpolol (MTP) and cyclosporine A (CSA)	MTP abrogates the hypertensive and nephrotoxic effects of CSA
(Wang, Evans, Knights, & Miners, 2011)	4-methylumbelliferone and 4-methylumbelliferyl glucoronide	Data confirmed an important role for the kidney in metabolic clearance of xenobiotics via glucuronidation
(Fonseca-Magalhães et al., 2011)	Sertraline	Sertraline had antispasmodic effects probably caused by a direct action on vascular smooth muscle cells
(Babayeva, Cox, White, & Taft, 2011)	Apricitabine (ATC)	This study showed that IPK is more sensitive to secretory inhibition as compared to *in vivo* . The study generated important information on renal handling of ATC to support its development and commercialization
(Ajavon, Bonate, & Taft, 2010)	Clofarabine	Clofarabine is a substrate for a cimetidine-sensitive organic cation transporter system in the kidney. The magnitude of cimetidine-clofarabine interaction was similar *in vivo* and in IPK
(Tamhane, Chakilam, Jayaraj, Thakkar, & Taft, 2010)	VX-702 (a novel p38 MAPK inhibitor) and methotrexate	Drug-drug interaction between VX-702 and methotrexate would be unlikely
(Poola, Kalis, Plakogiannis, & Taft, 2003)	Pentamidine and tetraethylammonium	Potential drug-drug interactions between pentamidine and organic cations exist
(Zarzuelo, Lanao, López, & Sánchez-Navarro, 2002)	Netilmicin	The apparent partition coefficients in renal cortex and medulla show higher values for slower perfusion rates of netilmicin
(Katayama, Yasuhara, & Hori, 1999)	Cefoperazone	Plasma clearance of unbound cefoperazone is decreased in acute renal failure

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Table 12.1. (Continued)

Researchers	Compound	Findings
(Shanahan, Evans, & Nation, 1997)	Morphine	The results suggest that tubular secretion of morphine is not saturated over a wide range of concentrations (0.2–200 microM)
(Masereeuw, Moons, & Russel, 1997)	Hydrochlorothiazide	The diuretic effect of hydrochlorothiazide is restricted by saturable accumulation and secretion
(Kugler, Olson, & Smith, 1995)	Quinapril and quinaprilat	The clearance ratio of quinapril represented extensive tubular secretion. Following quinaprilat administration, a net secretion process for renal elimination was indicated
(Boom, Moons, & Russel, 1994)	Cimetidine	Renal handling of cimetidine in the IPK is concentration-dependant and is determined by glomerular filtration, active tubular secretion, and a substantial flow- and pH-dependent passive reabsorption
(Statkevich, Fournier, & Sweeney, 1993)	Methotrexate, indomethacin and flurbiprofen	The secretory component was significantly inhibited by indomethacin and flurbiprofen after concomitant administration of oncolytic doses of methotrexate
(Redegeld, Hofman, van de Loo, Koster, & Noordhoek, 1991)	2-methyl-1, 4-naphtoquinone (gluthathione conjugate of menadione)	Thiodione-mediated toxicity can be linked to cellular uptake by anionic transport systems and metabolism by gamma-glutamyltranspeptidase
(Cox, Moons, Russel, & van Ginneken, 1990)	Naproxen	Relatively low doses of naproxen exert a specific stereoselective effect on kidney function caused by inhibition of prostaglandin E2 synthesis. High doses exert a non-stereoselective effect on kidney function.
(Lohr & Acara, 1990)	Dimethylaminoethanol (DME) and choline	DME is an effective inhibitor of betaine production and may be an important agent for the study of osmoregulation
(Somogyi, Hovens, Muirhead, & Bochner, 1989)	Amiloride and cimetidine	Cimetidine inhibits renal tubular secretion of amiloride. In humans, gastrointestinal absorption of amiloride and cimetidine appear to be reduced by each other
(Bekersky & Popick, 1986)	Bumetanide and furosemide	Diuresis was produced by both drugs. Furosemide was dependant on high clearance from filtration and secretion. Bumetanide was filtered and partially absorbed.
(Bekersky, Popick, & Colburn, 1984)	Sulfisoxazole (SX) and N4-acetylsulfisoxazole (NSX)	The metabolism od SX to NSX is reversible and influenced by protein binding. Tubular excretion of SX is a function of total drug in the perfusate
(Bekersky, Popick, & Colburn, 1983)	Gentamicin	Addition of gentamicin-specific antiserum decreased renal clearance and tissue accumulation of gentamicin
(Rocci, Szefler, Acara, & Jusko, 1981)	Prednisolone (Pn)	The extent of urinary clearance of Pn and prednisone is related to glomerular filtration and passive tubular reabsorption
(Bekersky, Colburn, Fishman, & Kaplan, 1980)	Salicylic acid (SA) and salicyluric acid (SU)	SA/SU metabolism was found to be reversible and a larger renal contribution to overall salicylate disposition is suggested

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During IPK studies, renal excretion mechanisms can be studied by dose-escalation studies or interaction experiments. By comparing renal disposition of a certain drug over a range of doses, nonlinear excretion behavior can be identified. Likewise, the disposition of cimetidine was studied over a range of several doses. The study demonstrated that net tubular secretion was observed at lower doses, progressing to net tubular reabsorption with increasing dose (Boom 1994). In addition, specific mechanisms of renal excretion can be identified through co-administration of known inhibitors of the organic anion and organic cation transport systems. Two commonly used inhibitors are probenecid and cimetidine. Subsequently, probenecid has shown to reduce renal excretion of the anionic compound bumetanide (Bekersky 1986). Cimetidine, a common probe for renal cation transport, has been utilized to study the renal disposition of amiloride (Somogyi 1989). Reduced clearance in the presence of potential interactants demonstrates a role of these transport systems in drug excretion.

Continuous monitoring of kidney function during IPK experiments offers the potential for correlating renal drug excretion with pharmacodynamic effects. This is particularly valuable for diuretic drugs. For example, the IPK model was used to correlate the relationship among perfusate drug concentration, kidney accumulation, and diuretic response following hydrochlorothiazide administration (Masereeuw 1997). The model has also been used to characterize the renal disposition and pharmacodynamic effects of the diuretic agents bumetanide and furosemide (Bekersky 1986).

In addition to studying pharmacokinetic-pharmacodynamic relationships between drugs, the IPK model has been an effective tool to study mechanisms of nephrotoxicity. In an early study by Bekersky et al., gentamicin appeared to have a significant effect on renal potassium handling making it potentially nephrotoxic (Bekersky 1983). More recently, the anti-fungal agent pentamidine was found to be associated with a high incidence of kidney toxicity (Poola 2003).

Although the liver is the main organ responsible for drug biotransformation, the kidney contains many drug-metabolizing enzymes and contributes to renal metabolism of a number of medications. Biotransformation has been demonstrated in a number of medications in the IPK, like prednisolone, sulfisoxazole and glutathione (Rocci 1981; Bekersky 1984; Redegeld 1991). A distinct advantage of drug metabolism studies in the isolated kidney compared to *in vitro* experiments is that the overall contributions of transport mechanisms and biotransformation by the kidney can be elucidated. Also, mechanisms of excretion of the formed metabolites can be monitored.

Drug-drug interaction studies are routinely used in IPK studies for two reasons: to determine/confirm pathways contributing to drug excretion and to identify medications that may display clinically significant interactions when co-administered to patients (Taft 2004). Antiviral medications are primarily excreted by renal mechanisms. Since these agents are commonly part of a multi-drug regimen, the potential exists for clinically significant drug interactions to occur. The IPK model allows for assessment of possible drug-drug interactions in a controlled manner. A study affirmed how the interaction between methotrexate (MTX) and non-steroidal anti-inflammatory drugs can be toxic. The kidney is the primary route of excretion for MTX, a dihydrofolate reductase inhibitor used in treatment of cancer, psoriasis, and arthritis. They demonstrated that secretory transport was significantly inhibited by indomethacin and flurbiprofen (Statkevich 1993).

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Over the past years, advances in scientific technology have significantly impacted the drug development process. Demonstrated correlations between IPK data and clinical outcomes make the model a potentially useful tool for drug discovery and evaluation in humans.

Other Potential Applications of the IPK Model

Besides studying drug dispositions, the IPK model can be useful in several other areas of interest. There are studies that identify the effect of aging (Savant 2001; Awe 2007) and gender (Taft 2006) on drug disposition. The excretion of para-aminohippuric acid (PAH), an indicator of renal plasma flow, was significantly affected by aging. The study demonstrated an age-dependent reduction in PAH excretion. However, the correlation between PAH clearance and GFR was limited, which contradicts the 'Whole Nephron Hypothesis' (each nephron is either a fully functional unit or does not function) (Savant 2001). Ageing was also found to decrease alpha1-adrenoceptor-mediated vasoconstriction of rat kidneys (Awe 2007). The IPK model provided supportive evidence that gender-dependent transport differences exist at the basolateral and luminal membrane of the kidney. Renal clearance of PAH and furosemide was higher in male rat perfusion experiments compared to females. Gender differences in transporter expression and function may result in differences in systemic exposure of narrow therapeutic range medications and risks to nephrotoxic agents. Therefore, disparities in drug disposition between males and females are an emerging issue for drug development (Taft 2006). Another important and successful application of the IPK model is as a potential preservation technique before kidney transplantation.

The IPK Model as a Preservation Technique

In the 1960s, Belzer et al. started experiments studying hypothermic machine perfusion (HMP) in canine kidneys and the first successful human transplantation of a hypothermically machine perfused kidney took place in 1968 (Belzer 1967). After the definition of irreversible coma in 1968, the concept of brain death became acceptable in the early 1970s. Consequently, the shift to recover organs from heart beating instead of non-heart-beating (NHB), nowadays named donation after circulatory death (DCD) donors, increased success rates and the majority of transplant centers abandoned the practice of DCD donation. Initially, only young and mainly trauma patients were considered suitable for donation and these organs were preserved equally well by either static cold storage (SCS) or HMP. Over the last few decades, more marginal donors, as well as DCD donors are accepted to overcome organ shortage and machine perfusion regained worldwide interest. Substantial experimental work contributed to improved clinical results and a recent randomized controlled trial (RCT) proved clinical efficacy short- (Moers 2009) and long-term (Moers 2012). Therefore, machine perfusion is the present method of choice to obtain good long-term preservation and for preservation of marginal kidneys

In the U.S. by 2008, nearly half of the kidney transplants from extended criteria donors (ECD) and 70% of those from DCD donors were machine perfused indicating the common

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belief that these organs require better preservation techniques. In other parts of the world machine perfusion is slowly increasing as well. During perfusion after organ retrieval, the preservation is continuously pumped through the organ via the vasculature. A heat exchanger regulates the perfusate temperature at hypothermic (0−4ºC), sub-normothermic (20−30ºC) or normothermic (37ºC) temperatures. If required, the perfusate can be oxygenated via a membrane oxygenator.

Machine perfusion perfuses the organ *ex situ,* after it has been procured, cannulated and connected to a pump (Jochmans 2016). Continuous perfusion (from procurement to implantation) or pre-implantation perfusion (after a period of SCS and just before transplantation) is most commonly used. During perfusion, the kidney can be supplied with nutrients and oxygen, have waste products removed, and drugs or other therapeutic agents supplied as needed. Challenges are however: understanding the metabolic requirement of the *ex vivo* perfused organ, development of viability biomarkers, and maintenance of endothelial cell integrity (Belzer 1980).

Hypothermic Machine Perfusion

HMP is based on a controlled continuous circulation of a cold perfusate (0−4ºC), which has been hypothesized to protect the deceased donor kidneys from injuries related to ischemia and reperfusion. Immediately after procurement, the organ is attached to the perfusion system via the renal artery. Circulation of the perfusate is achieved through a device that generates either a continuous or pulsatile flow by a roller pump or centrifugal pump. The interest in HMP decreased in 1969, after the introduction of SCS by Collins, because this preservation technique was more simple and economic and allowed preservation up to much longer times (> 30 hours) (Catena 2013). However, in recent years the interest in HMP regained attention due to the greater number of organs from marginal donors, which are predisposed to primary graft non-function (PNF) and delayed graft function (DGF). HMP maintains the hemodynamic stimulation of the vasculature, which plays a critical role under normal physiologic conditions. Evidence supporting the hypothesis that HMP may reduce ischemia-reperfusion (I/R) injury was published by Wszola et al. Kidneys preserved by SCS showed elevated levels of genes that are correlated with I/R injury compared to hypothermic machine perfused kidneys (Wszola 2014). Zhang et al. observed more apoptotic cells in cold stored kidneys versus HMP kidneys in a canine DCD model. In parallel, phospho-AKT and ezrin expression was induced in the HMP group, implying that cell proliferation, turnover, and renewal are positively influenced when kidneys are subjected to HMP instead of SCS (Zhang 2016). A meta-analysis conducted between 1971 and 2001 showed that HMP is associated with a relative risk of DGF of 0.80 (95%CI = 0.67–0.96) compared to SCS (Jochmans 2015). More recently, a large RCT comparing continuous HMP with SCS of 336 kidney pairs using the LifePort™ pump was conducted in 2009. This MP-trial showed an overall reduced risk of DGF in HMP kidneys compared to SCS kidneys (20.8% vs. 26.5%, $p = 0.05$). One-year graft survival significantly increased from 90% in SCS kidneys to 94% in HMP kidneys ($p = 0.04$) (Moers 2009). Also on longer term the protective effects of machine perfusion were evident (Moers 2012)

Figures 12.4, 12.5 show machine-perfused kidneys in the clinical setting.

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Figure 12.4. Kidney preservation by machine perfusion.

Figure 12.5. Inside the kidney preservation machine.

Extended Criteria Donor Kidneys

As mentioned earlier, more marginal, or ECD, kidneys are used to overcome the problem of increasing organ shortage. ECD kidneys might particularly benefit from HMP, since they are more susceptible to preservation-induced injury, DGF and PNF. A study compared ECD kidneys that were cold stored during transport followed by HMP to kidneys that were cold stored throughout the preservation period. The HMP organs showed markedly reduced expression of all analyzed cytokines as well as soluble intracellular adhesion molecular type 1, compared to constant or even elevated levels in the cold stored group (Tozzi 2013). In a study on 91 randomized ECD kidney pairs, HMP significantly reduced the risk of DGF compared to SCS (adjusted odds ratio (OR) = 0.46; 95% CI = 0.21–0.99). Also, PNF was

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lower and 1-year graft survival was higher in hypothermic machine perfused kidneys (3% vs. 12%, $p = 0.04$ and 92.3% vs. 80.2%, $p = 0.02$, respectively) (Treckmann 2011).

One category of ECD donors is the DCD donor (previously referred to as non-heart-beating). In these donors circulatory arrest occurs before organ procurement. DCD kidneys are exposed to a period of warm ischemia during the period of circulatory arrest, which makes these organs particularly vulnerable to the development of DGF. The literature reveals conflicting data on HMP of DCD kidneys. Some studies have suggested that HMP of DCD kidneys leads to better outcomes, but other studies could not find a difference in DGF or graft survival. In the multi-center Machine Perfusion trial in the Eurotransplant region on 82 DCD kidney pairs, continuous HMP showed a reduced risk of DGF compared to SCS kidneys (53.7% vs. 69.5% respectively, $p = 0.025$) (Jochmans 2010). In 2010, Watson et al. published results on a RCT in the U.K. comparing HMP with SCS for 45 DCD kidney pairs. Their results did not show a beneficial effect on 1- or 3-year graft survival or DGF for hypothermic machine perfused kidneys (Watson 2010). The contradicting outcomes of these currently largest RCTs might be related to the setting in which HMP is performed. Hosgood et al., showed in a porcine kidney transplant model that the beneficial effect of MP disappears when kidneys are not pumped immediately after procurement (Hosgood 2011). Following this, in the Machine Perfusion trial, kidneys were pumped immediately after retrieval at the donor center, whereas in the U.K. trial kidneys were cold stored during transfer and then HMP was started.

Preservation Solution

Many preservation solutions exist, all with different compositions aimed to prevent I/R injury and maximizing graft survival and function. Early preservation solution consisted of diluted blood and ringers lactate solution. After the introduction of SCS by Collins in 1969, prolonged kidney preservation became feasible. Currently, the most commonly used preservation solutions are University of Wisconsin (UW) and histidine-tryptophan-ketoglurate (HTK) followed by smaller use of Celsior, Institute George-Lopez (IGL-1), Polysol, Euro-Collins, hypertonic citrate or Marshall solution (HOC), and phosphate-buffered sucrose (PBS).

In 1987, Belzer and colleagues developed the University of Wisconsin (UW) solution (also known as Belzer's solution and produced by Organ Recovery Systems as KPS-1) and this has become the gold standard among organ preservation solutions today. In the European Multicenter Trial the use of UW versus EuroCollins solution significantly reduced the rate of DGF (10% vs. 23%) (Ploeg 1992). UW shows good outcome of short and long-term kidney preservation, but the main disadvantage is its high viscosity mainly due to hydroxyl ethyl starch (HES) (Catena 2013). For HMP, UW-G is used, in which a more extracellular solution is achieved through replacing lactobionate by gluconate and reducing the potassium level (Catena 2013). Although UW solution is considered the ideal solution for kidney preservation, it does not completely prevent I/R induced injury.

Some institutions favor the low-viscosity HTK solution to preserve DCD kidneys as opposed to the assumed less favorable washout facilitated with the high-viscosity UW solution. HTK was introduced in 1980 by Bretschneider and is may be used in HMP at low pressures and high perfusion volumes. More recently, Rauen and De Groot developed Custodiol-N solution as improved modification of the former HTK solution. The solution is particularly suitable, because the iron chelators inhibit cold-induced free radical mediated

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injury and have already shown to be effective to protect isolated rat livers during HMP (Stegemann 2010). Minor et al. using a porcine transplant model provided *in vivo* evidence for the sustainability of Custodiol-N as an effective perfusate for renal machine perfusion. HMP with Custodiol-N resulted in less acute tubular injury and lower serum creatinine levels compared to KPS-1. Also, lower expression of endothelin-1 and of Toll-like receptor 4, suggests less endothelial stress in the Custodiol-N perfused kidneys (Minor 2015).

Developed in 1994, Celsior solution is a colloid and combines the osmotic efficacy of UW (lactobionate and mannitol) with the potent buffering ability of HTK (histidine). It is particularly beneficial to prevent tissue edema caused by hydrostatic pressure of HMP, because Celsior contains macromolecules that do not pass the cellular membrane (Catena 2013).

The fairly new preservation solution IGL-1 combines the advantages of UW and Celsior and is characterized by high sodium concentration and polyethylene glycol (PEG, 35 kDa), substituting HES. A non-randomized prospective multi-center study showed the same effectiveness for IGL-1 versus UW solution, but lower costs for the former (Codas 2009). Polysol, a new low-viscosity preservation solution, is introduced to facilitate transplantation of ischemically damaged organs. Amino acids, vitamins, potent buffers, and anti-oxidants have been added to support metabolism during HMP (Bessems 2005). One of the main components is PEG 35, a low-molecular-weight colloid that does not increase viscosity as seen with HES-containing solutions. Polysol is currently used in experimental settings only, but recent reports seem promising. A study in a porcine auto-transplant model reports better preservation of microcirculation and renal function after 20-hour SCS preservation compared to UW (Schreinemachers 2009).

HOC or Marshall's solution is extensively used for clinical transplantation in the U.K. and Australia. HOC is an intracellular solution with high potassium content, mannitol as the impermanent ion, and citrate as its buffer. The hypertonic character of HOC prevents entry of fluid into the cells and has shown to be effective for 72-hour canine kidney preservation (Kay 2009; Hosgood 2009; Bagul 2009; Nicholson 2009).

Role of Oxygenation

In HMP oxygenation plays a central role of importance for reconditioning after ischemic storage. Lack of oxygen leads to ATP decrease, which can be as large as 80% for kidneys (Jassem 2004). Ischemic damage can be minimized by various preservation solutions. In some settings, perfusion per se was not sufficient to protect the organ from injury and failed to provide functional improvement upon reperfusion. Continuous oxygenation might be useful to support the remaining metabolic demand of the organ during preservation. On the other hand, high concentrations of oxygen are believed to favor the production of oxygen free radicals, thus promoting adverse effects on tissue during long-term oxygenated preservation (Stegemann 2010; Rauen 2004). However, several studies strongly suggest oxygen to be a constitutive adjunct in organ preservation by HMP, improving functional outcome of the graft. Hoyer and colleagues showed that HMP with 100% oxygen results in higher flow and lower renal resistance (RR) during perfusion. In addition, kidneys that were exposed to 100% oxygen during perfusion had a significantly higher graft function than kidneys that did not receive any oxygen during HMP (Hoyer 2014). The positive results after HMP with oxygen were confirmed by studies with a 3-month post-transplant follow-up by Thuillier et al. Oxygenated kidneys had lower serum creatinine peak and significantly lower levels of several

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injury markers. The positive short-term effects were followed by lower serum creatinine, less proteinuria and less chronic kidney fibrosis post-transplant (Thuillier 2013). The efficacy of oxygenation in DCD kidneys is currently investigated in a RCT executed in Europe (www.cope.eu).

Kron et al. investigated the effects of 1-hour hypothermic oxygenated perfusion (HOPE) before transplantation after kidneys were cold stored. HOPE-treated DCD kidneys showed dramatically better function after transplantation compared to SCS grafts in terms of nuclear injury, macrophage activation, endothelium activation, tubulus damage, and graft function (Kron 2016). Grafts that have been exposed to a period of warm ischemia seem to particularly benefit from oxygenated perfusion. A study by Buchs et al. found that levels of ATP were restored in DCD porcine kidneys, but no added benefit of oxygenation was found in kidneys without injury (Buchs 2011).

Perfusion Parameters

Machine perfusion provides the opportunity to determine perfusion parameters including perfusion pressure, perfusion flow, and renovascular resistance. Flow and resistance are often used to assess suitability for transplantation. Kidneys with low flow (< 100 mL/min) and high RR (> 0.4 mmHg/mL/min) are believed to be higher-risk organs (Mozes 2005). However, the literature is inconsistent. Several studies proposed that there is no significant relation between low flows during preservation and post-transplant outcomes. Mozes et al. analyzed 336 ECD kidneys and showed similar outcomes between ‘poor’ (RR 0.4–0.6 mmHg/mL/min) perfusion parameters and ‘good’ perfusion parameters (Mozes 2005). Likewise, acceptable short- and long-term results were reported in a small series of deceased donor kidneys with ‘poor’ perfusion parameters (flow < 80 mL/min and RR > 0.4 mmHg/mL/min) (Guarrera 2010). On the contrary, Jochmans et al., showed in a multivariate analysis of 302 kidneys that RR at the end of HMP was an independent risk factor for the development of DGF and 1-year graft failure (Jochmans 2011). Matsuno and colleagues found that MP flow is a reliable indicator of graft viability based on the rate of PNF and immediate renal allograft function, especially in marginal donors. The highest rate of PNF (25.7%) was found in kidneys that were perfused with a flow of 0.45–0.65 mL/min/g, compared to 0% in kidneys that were preserved with a flow of > 0.9 mL/min/g (Matsuno 2006). In a large database study, pumped ECD kidneys (n = 2,351) with high resistance at the end of MP (> 0.25 mmHg/ml/min) were associated with higher odds of discard. ECD kidneys with low resistance (< 0.25 mmHg/mL/min) were discarded 14.0% of the time and kidneys with high pressure (> 0.25 mmHg/mL/min) had a discard rate of 25.7%. Those with resistances >0.38 mmHg/mL/min even had a discard rate of 53.1% (p < 0.0001) (Sung 2008). This suggests that RR is an important tool to be used in kidney graft quality assessment and can be helpful for clinicians in the postoperative management of recipients of these organs. Nevertheless, it cannot be used as a stand-alone viability parameter to accept or discard a given kidney.

The average flow rate for a kidney is achieved at a mean of 2 hours, but the organ does not seem to be much affected by longer pump times (> 24 hours) (Patel 2012). A study on 339 deceased donor recipients showed that DGF rates were unaffected by length of pump time, short (< 24 hours) or long (> 24 hours) (Ciancio 2010). There are also studies that favor a longer pump time. It has been suggested that a significantly lower rate of first biopsy proven acute rejection (BPAR) was seen among recipients with longer pump times, even though there was no trend found for improved graft survival (Ciancio 2010). A study on 66 pairs of

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recipients showed that longer pump times were not associated with any unfavorable effect on DGF, graft and patient survival, or impaired renal function. In fact, they offered a significantly favorable protection from BPAR with pump time > 24 hours (Ciancio 2012). An explanation for the favorable effect of MP on rejection is currently unknown. One theory is a better flushing of immunogenic cells (passenger leukocytes) from the deceased kidney. Surprisingly, BPAR occurrence is known to be associated with an increased risk of graft failure, but no associations between longer pump times and decreased rates of graft failure were found. More recently, a large registry analysis (90,000 kidneys) has shown that HMP reduced the risk of DGF compared with SCS, regardless of very short or very long cold ischemia time (CIT) (Gill, Dong, Eng, Landsberg, & Gill, 2014). Nevertheless, because CIT is a well-established predictor of DGF, a balance between minimizing CIT and any potential benefits of HMP is required and the ideal pumping time has not been established yet.

Viability Assessment

One key question during organ preservation is whether the likelihood of a renal graft to resume function can be predicted. Accurate evaluation of graft function is essential to prevent unnecessary discard and to plan appropriate postoperative care. Machine perfusion offers the possibility to assess organ quality during perfusion and identify differences in metabolomic profile between immediate or delayed graft function. The assessment of organ viability is especially important in marginal kidneys with their increased risk of developing DGF and PNF. Perfusate biomarkers are promising tools to accept or discard kidneys for transplantation. However, the value of perfusate biomarkers to predict organ viability is not or hardly known. Overall, the literature remains mixed about the predictive utility of these physical measurements with regard to kidney viability and allograft outcomes.

One of the most promising biomarkers and frequently used for viability testing is glutathione S-transferase (GST), an enzyme localized in the renal tubulus. Warm ischemia predominantly damages proximal tubule cells; therefore, the appearance of GST in the perfusate may reflect the number of proximal tubule cells that are damaged (Daemen 1997). Several studies demonstrated that αGST strongly correlates with warm ischemia time (WIT) and is hence able to discriminate viable from non-viable DCD kidneys (Daemen 1997; Kievit 1997). In a subset of 111 kidneys from the Machine Perfusion trial, GST levels at the end of HMP were higher in kidneys that developed DGF, but not after multivariable adjustment (Kozaki 2000). Hoogland et al. analyzed perfusate samples of 335 DCD kidneys and found that total GST, heart-type fatty acid binding protein (H-FABP), redox-active iron and neutrophil gelatinase-associated lipocalin (NGAL) were not associated with PNF or 1-year graft failure. They did show an association of LDH and IL-18 concentrations with PNF (OR = 1.001; 95%CI = 1.000–1.002). The association might be more apparent in DCD kidneys compared to DBD donors. The reason behind this is as follows; LDH measured in machine perfusate is released either by the perfused kidney or by hemolytic erythrocytes from the capillaries (Cohen 1998); DCD kidneys sustain a period of warm ischemia with stasis of blood on the organs and therefore, may have a relatively high concentration of erythrocytes and blood clots in the capillaries after poor initial flush out after cardiac arrest (Hoogland 2013).

Redox-active iron is released during I/R injury and is believed to catalyze the formation of oxygen-free radicals, which are known to induce apoptotic and necrotic cell death and subsequent inflammatory responses (de Vries 2004). Also, cold ischemia has been indicated

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to induce tissue accumulation of redox-active iron, which is considered a critical step in cold storage-induced injury. A study by de Vries et al. showed that redox-active iron concentrations were significantly higher in ischemically injured NHB donor kidneys compared to heart beating donor kidneys that were not subjected to warm ischemia (3.9 ± 1.1 vs. 2.8 ± 1.0 µmol/L, $p = 0.001$) (de Vries 2006). Moreover, redox-active iron concentration was an independent predictor of post-transplant graft viability (OR 1.68, $p = 0.01$).

IL-18 is a proinflammatory cytokine produced by macrophages and other cell types present in the kidney during I/R injury and contributes to inflammatory reactions. IL-18 was shown to be significantly associated with PNF in multivariate analysis on 335 transplanted DCD donor kidneys (OR 1.001; 95% CI 1.000−1.002, $p = 0.003$) (Hoogland 2013). Parikh et al. found higher IL-18 values at the end of perfusion in DGF kidneys (13.4 pg/mL vs. 11.8 pg/mL, $p = 0.005$) (Parikh 2015).

In addition, significantly lower levels of glucose in DGF kidneys compared to immediate graft function (IGF) organs (7.78 vs. 9.46, $p = 0.006$) were observed by Guy et al. The same group observed significantly different concentrations of inosine and leucine (0.002 vs. 0.013 mM, $p = 0.009$ and 0.011 vs. 0.006 mM, $p = 0.036$), as well as gluconate levels (49.10 vs. 59.51, $p = 0.009$) (Guy 2015).

Currently, NGAL and liver-type fatty acid-binding protein (L-FABP) are approved biomarkers of kidney injury in Europe and clinically used in Japan. Nonetheless, a recent study in 671 recipients by Parikh et al. only found modest associations for perfusate NGAL and L-FABP at the end of HMP with allograft function, indicating that these biomarkers have poor prognostic utility (Parikh 2016). Median perfusate concentrations of NGAL, kidney injury molecule 1, IL-18 and L-FABP were either lower or not significantly different in discarded kidneys compared to transplanted kidneys. Notably, recipients of transplanted kidneys with 'undesirable' biomarker levels experienced acceptable 6-month allograft function, suggesting that these characteristics should not be used in isolation for discard decisions. Additional studies must confirm the utility of biomarkers to assess kidney quality.

Devices

As HMP is gaining recognition, more perfusion machines are becoming available (Figure 13.6). The most commonly used device is the pressure-driven LifePort Kidney Transporter (Organ Recovery Systems, Itasca, IL, USA). Another commonly used device is the flow-driven Waters Medical RM3 (Birmingham, AL, USA). In both systems, the operator can modify the systolic perfusion pressure, with flow and resistance recorded over time. Relevant differences between the systems are related to the way both devices generate and control flow and pressure. The RM3 uses a flow-driven system, which requires 50% higher systolic pressures (45 vs. 30 mmHg) to obtain similar flow rates. A comparative study on both systems demonstrated minimal differences in early kidney recovery, but significantly higher levels of post-transplant fibrosis in kidneys preserved by RM3 compared to LifePort (Wszola 2013). Another study showed favorable results for the LifePort device compared to RM3 with shorter duration of DGF after transplant, lower incidences of interstitial fibrosis and tubular atrophy in biopsies taken within 1-year post transplant, and lower renal resistance during perfusion (Wszola 2013). Other devices currently on the market are the oxygenated MP system from Organ-Assist bv and the Waves machine (Waters).

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Figure 13.6. Kidney machine perfusion devices. A: Waters Medical RM3, B: LifePort Kidney Transporter, C: Kidney Assist Transporter.

Sub-Normothermic Machine Perfusion

The interest in sub-normothermic machine perfusion (SMP) has increased after more became known on hypothermia-induced organ injury caused by organ cooling during preservation. Avoidance of cold-induced injury might be particularly favorable for DCD organs, which are known to suffer extensive ischemic damage. In a porcine model of renal I/R injury, oxygenated SMP demonstrated better protection of the organ compared to cold storage. SMP grafts showed nearly twofold better values of creatinine clearances compared to oxygenated HMP (Hoyer 2014). Another benefit is that SMP allows higher pressures to be used without risk of endothelial and vascular impairment as higher temperature reduces vascular stiffness (Hoyer 2014). Higher temperatures also lead to higher metabolic demands requiring active oxygenation and sufficient perfusion flow. Clinical use of SMP in clinical kidney transplantation has not yet been reported.

Gradual Rewarming

One cause of ischemic damage could be the abrupt temperature shift from hypo- to normothermia, leading to mitochondrial dysfunction and proapoptotic signal transduction (Minor 2013). Therefore, recent studies have investigated the effect of the promising new aspect of controlled oxygenated rewarming (COR). The protective effect of controlled tissue rewarming after cold storage was first shown in a pig liver perfusion model, where end-ischemic MP with gradual rewarming resulted in superior functional recovery compared to hypo- or subnormothermic perfusion prior to reperfusion (Minor 2013). Recently, this evidence was supported in a pig liver perfusion model comparing COR up to sub-normothermic temperatures to NMP. COR resulted in lower mitochondrial caspase-9-activity. Significantly lower enzyme leakage and higher bile production were also observed during reperfusion (Hoyer 2016). Schopp et al. reported a nearly twofold increase in renal clearances of creatinine and urea after COR of pig kidneys compared to controls. This was accompanied by significantly improved renal oxygen consumption and less cellular apoptosis (Schopp 2015). Similarly, Mahboub et al. demonstrated less renal parenchymal injury, tubular injury and better endothelial preservation in the gradual rewarming groups versus the control group. Perfusate AST and LDH were less increased in the gradual rewarmed kidneys and

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sodium re-absorption was improved. Heat shock protein 70, intracellular adhesion molecule 1 and vascular cell adhesion molecule 1 mRNA expressions were decreased in gradually rewarmed kidneys (Mahboub 2015). These data suggest that a gradual increase in temperature results in better energetic recovery, less cellular injury, and ultimately superior function of kidneys subjected to COR. The first clinical application of COR has recently been applied in a first clinical series of 6 liver transplant patients. All treated recipients had normal liver function after 6-month follow-up (Hoyer 2016).

Normothermic Machine Perfusion

In contrast to cold preservation, the concept of NMP is to replicate the physiological environment and maintain cellular metabolism during preservation. Aerobic metabolism can be restored allowing the kidney to regain function; this can minimize or avoid the cold ischemic insult and is particularly beneficial for DCD and ECD kidneys. The mechanistic effect of *ex vivo* NMP has not yet been thoroughly investigated. Nonetheless, in addition to replenishing ATP, studies have shown that repair mechanisms are up regulated during NMP. The expression of heat shock protein 70 has been shown to increase after NMP, which ensures effective recovery and prevents oxidative damage (Hosgood 2013). On the contrary, the act of rewarming may aggravate inflammatory processes and increase graft injury. Proinflammatory cytokines, such as IL-6, are upregulated in response to ischemic injury and were released in the kidney during reperfusion after NMP (Hosgood 2013). However, IL-6 can also have anti-inflammatory properties and is capable of resolving the tissue damage after its initial inflammatory response (Nechemia-Arbely 2008).

Ex vivo NMP can be used throughout the preservation interval or for shorter periods. The most practical approach is to combine normothermic and hypothermic techniques. Subsequently, 1- or 2-hour periods of NMP have been performed to restore function and replenish ATP after warm and cold ischemic injury in porcine kidneys (Bagul 2008; Hosgood 2013; Hosgood 2011). The kidneys had less tubular injury, improved blood flow and oxygenation and were metabolically more stable than the cold stored kidneys. In 2011, Hosgood et al. reported the first case of successful kidney transplantation in man after the organ was cold stored for 11 hours, followed by 35 min of *ex vivo* NMP. The 55-year old recipient had slow graft function, but the patient remained dialysis independent (Hosgood 2011). A pilot clinical study compared 18 ECD kidneys that received 1 hour of pre-implantation blood-based NMP with 47 matched SCS controls. Significantly lower DGF rates were observed with pre-implantation NMP (5.6% vs. 36.2%, $p = 0.014$) (Nicholson 2013).

Since DCD and ECD organs poorly tolerate the effects of hypothermia in SCS, a more novel approach of NMP totally avoids storage in hypothermic conditions. A study by Kaths et al. demonstrated that NMP with an erythrocyte-based solution could replace hypothermic storage techniques and thus avoid its harmful effects. NMP kidneys showed similar serum creatinine levels and creatinine clearance post-transplant compared to SCS grafts. After 10 days follow-up, NMP animals had serum creatinine and blood urea nitrogen values comparable to basal levels, whereas SCS grafts showed elevated values (Kaths 2016). Recently, Hosgood et al. reported the successful transplantation of a pair of human kidneys that were deemed unsuitable for transplantation due to inadequate perfusion, but subsequently transplanted after perfusion and assessment using *ex vivo* NMP (Hosgood 2016).

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Preservation Solution

Continuous perfusion with an oxygenated solution to support aerobic metabolism is key under normothermic conditions. During NMP the perfusate can be based on either a natural oxygen carrier such as blood or an artificial-based medium. Early preservation solutions consisted of diluted blood and Ringers lactate solution. From 1985 until 1993 Collins or Euro-Collins solution was the common used solution, and from 1993 onwards this shifted to UW, which is still the gold standard for abdominal organ preservation today (Wight 2003).

Brasile et al. were the first to develop an acellular normothermic solution based on perfluorocarbon (PFC) emulsion and enriched tissue culture-like medium containing essential and non-essential amino acids, lipids and carbohydrates (Brasile 1994). PFCs are inert solutions that have a high capacity for dissolving oxygen. New, more stable, PFCs are now being developed mainly as blood substitutes. Humphreys et al. recently used a commercially made PFC to provide oxygenation and reduce ischemic injury to the kidney during warm ischemia by retrograde infusion through the urinary system (Humphreys 2006). These new generation PFCs could potentially be developed as normothermic preservation solutions. However, due to complexity of manufacturing they are very expensive. A more stable pyridoxalated haemoglobin polyoxyethylene (PHP) solution has proven to be more successful and Brasile et al. have since replaced the PFC with this solution (Stubenitsky 2000). Other solutions such as Lifor (an artificial preservation medium containing a non-protein oxygen carrier, nutrients and a growth factors) have also been used during NMP. Some studies have shown higher flow rates in Lifor perfused kidneys compared to UW (Gage 2009; Olschewski 2010). The most newly reported acellular solution is Hemarina-M101, extracellular hemoglobin derived from a marine invertebrate. It has been formulated into an oxygen carrier mainly as a blood substitute. M101 has been used in SCS to deliver oxygen with favorable results and functions over a wide range of temperatures (4−37ºC) (Thuillier 2011).

A blood-based solution during NMP was previously considered to have their limitations because of hemolysis, platelet activation, and detoriation in the oxygen-carrying capacity of red blood cells, high intra-renal resistance and tissue edema (Thiara 2007). However, much has been learnt and they provide a more natural environment for the kidney compared with artificial-based solutions. The early results of an isolated perfusion system that perfused kidneys with one unit of compatible packed red blood cells mixed with a similar priming solution with added protective agents are promising. In a series of 18 ECD kidneys DGF was 5.6% compared to 36% in similarly matched recipients of a kidney undergoing SCS (Nicholson 2013). The exact composition of the perfusion solution will be subject to investigation.

Viability Assessment

In contrast to HMP, kidney function can be evaluated during NMP by assessing macroscopic appearance of blood perfusion, renal blood flow and urine output. Recently, Hosgood et al. used *ex vivo* NMP to develop a novel scoring system for pre-transplant assessment of marginal kidneys (Table 12.2) (Hosgood 2015). Based on assessment scores from their clinical series, it became clear that kidneys with a score of 1 to 4 could be transplanted successfully. Kidneys with a score of 5 would be considered very high-risk and unlikely to be suitable for transplantation. In their study on 36 kidneys, grafts with an assessment score of 3 had a significantly higher incidence of DGF (38%) compared to kidneys with a score of 1 (6%) or 2 (0%) ($p = 0.024$).

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Table 12.2. ***Ex vivo*** **normothermic perfusion assessment score (Hosgood 2015)**

	Score
Macroscopic assessment	
Grade I: excellent perfusion (global pink appearance)	1
Grade II: moderate perfusion (patchy appearance)	2
Grade III: poor perfusion (global mottled and purple/black appearance)	3
Renal blood flow (ml per min per 100g)	
Threshold ≥ 50	0
Threshold <50	1
Total urine output	
Threshold ≥ 43	0
Threshold <43	1

Therapies

Preventing I/R injury is an ultimate goal for researchers and extensive research is currently carried out to find ways of preventing ischemic injury and improving renal function during machine perfusion. NMP also provides the opportunity to add therapies to a functioning organ to directly manipulate and improve its condition. One way of achieving this is through recently developed gene therapies. During NMP, genes are administered with a viral, non-viral or cell based vector. RNA interference uses small interfering RNA (siRNA) to modulate and protect the kidney. A study by Zheng et al. observed reduced levels of blood urea nitrogen, serum creatinine, cell apoptosis, histological changes and expression of proinflammatory cytokines after administration of a siRNA solution. They also reported prolonged graft survival compared to control groups (Zheng 2016). Gene silencing through siRNA injection can be of great potential in clinical organ transplantation.

The administration of gaseous molecules, such as nitric oxide in the form of soluble carbon monoxide-releasing molecules enhances renal blood flow and has been shown to be protective against reperfusion injury in porcine kidneys (Hosgood 2008). Over the last decade, mesenchymal stem cell therapy (MSC) has emerged as a potential therapeutic strategy. The ability of stem cells to differentiate and self-renew make them ideally suited for cellular therapy. Not everything is known about the mechanism of MSC therapy, but most likely the paracrine effect of the secreted growth factor re-established kidney tissue leading to clinical improvement (Wang 2013). Vascular endothelial growth factor was a critical factor in MSCs induced renoprotection in a rat model of ischemia-reperfusion injury (Tögel 2005). To date, the mechanism of MSC is understood as follows: therapeutic infusion of MSCs into an impaired kidney with existing host cells; engraftment and differentiation of MSCs into the host tissue or organ, followed by release of paracrine and/or endocrine signals. NMP can facilitate MSC delivery directly to the kidney. MSCs have appeared to stimulate renal recovery through engraftment and differentiation into renal cells among several kinds of acute kidney diseases (Wang 2013). These constantly developing therapies provide enormous scope in the treatment of reperfusion injury, rejection or to prolong graft survival.

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CONCLUSION

The IPK model is an effective tool to study renal drug disposition. When the experimental design is appropriate and the results are sensibly interpreted, it can be utilized as an important experimental tool in the study of renal physiology and pathophysiology. More recently, kidney machine perfusion has emerged as a successful method for organ preservation before transplantation. Hypothermic, normothermic and sub-normothermic machine perfusion preservations are all potential methods to increase the exsisting donor pool. The ability to recover marginal kidneys can be particularly beneficial.

Conflicts of interest: The authors of this chapter have no conflicts of interest to declare with any machine perfusion device.

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Chapter 13

ISOLATED LIVER PERFUSION SYSTEMS

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ABSTRACT

The *ex vivo* liver perfusion model was first described in the 1850s. Over the years isolated hepatic perfusion (IHP) has been used to investigate hepatobiliary function, toxicity due to drug-drug interactions, to treat malignancies, and as a potential method of organ preservation for transplantation. The liver is frequently targeted by primary cancer and is often affected by metastases from various malignancies. The unique hepatic vascular anatomy allows vascular isolation of the liver to deliver high doses of cytotoxic agents with minimal system toxicity. This way, hepatic metastases can be treated using the IHP technique. More recently, IHP emerged as a promising technique to increase the organ donor pool, particularly for livers retrieved from extended criteria donors (ECD) and donation after circulatory death (DCD) donors. Hypothermic machine perfusion (HMP) is currently the simplest approach of liver machine perfusion, and therefore with high practicability and relatively low costs. It is an attractive method to avoid further warm ischemic injury and minimize graft metabolic requirements, while providing oxygenation and metabolic support. Numerous *ex vivo* HMP experiments have been published in the last 2 decades and they demonstrated beneficial results compared to static cold storage (SCS). On the contrary, it has been hypothesized that a more gradual rewarming course may relieve the cold ischemic insult by employing a stepwise normalization of temperature and metabolic demand. Recent studies have shown promising effects of the new aspect of controlled oxygenated rewarming (COR). Normothermic machine perfusion (NMP) enables prolonged preservation without time-

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dependent damage that is caused by cold preservation, the ability of the organ to recover from injury, and the opportunity to measure the function of the organ during preservation in order to predict post-transplant outcomes. Following kidney preservation, liver machine perfusion is close to becoming standard clinical practice.

Keywords: isolated perfusion, malignancies, drug disposition, machine perfusion, DCD

ABBREVIATIONS

ALT	alanine aminotransferase
AST	aspartate aminotransferase
ATP	adenosine triphosphate
ADP	adenosine diphosphate
BCKA	branched-chain keto acids
COR	controlled oxygenated rewarming
CS	cold storage
CyA	cyclosporine A
DBD	donation after brain death
DGF	delayed graft function
DCD	donation after circulatory death
ECD	extended criteria donor
HAT	hepatic artery thrombosis
HMP	hypothermic machine perfusion
HTK	histidine-tryptophan-ketoglurate
HOPE	hypothermic oxygenated machine perfusion
IGL	Institut George Lopez
IHP	isolated hepatic perfusion
I/R	ischemia-reperfusion
ITBL	ischemic-type biliary lesions
IVC	inferior vena cava
KPS-1	kidney machine perfusion 1
LDH	lactate dehydrogenase
NMP	normothermic machine perfusion
PNF	primary non-function
PVP	peribiliary vascular plexus
SCS	static cold storage
SMP	subnormothermic machine perfusion
TNFα	tumor necrosis factor alpha
UW	University of Wisconsin
VAS	Vasosol

INTRODUCTION

In the 1850s, Claude Bernard was the first to describe an *ex vivo* liver perfusion model. Shortly after, an isolated hepatic perfusion (IHP) model perfused with blood was used to

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identify the role of the liver in synthesis of plasma proteins (Miller 1951). Over the years IHP has been used to investigate hepatobiliary function, toxicity due to drug-drug interactions, to treat malignancies, and as a potential method to preserve organs for transplantation.

A significant advantage of the IHP system is that hepatobiliary function can be assessed in the absence of extrahepatic factors, such as circulating hormones and changes in cardiovascular function. Furthermore, extrahepatic clearance of biomarkers is eliminated and experimental conditions are carefully controlled. By measuring influent and effluent concentrations of biomarker compounds infused into the portal vein as well as biliary concentrations, rates of hepatocellular uptake in addition to biliary excretion can be determined. In addition, accurate determinations of the rate of bile acid-dependent and -independent bile flow are possible in the isolated perfused liver (Chazouillères 1991). Since the IHP model avoids neural and hormonal interferences and excludes influences from absorption and non-hepatic elimination routes, it also provides a relatively clean hepatic system to study drug-drug interactions and pharmacokinetics.

The liver is frequently affected by cancer. Primary liver cancer is the sixth most common cancer in the world and also the third cause of cancer-related death. Surgical resection offers the best chance on long-term survival, but for 80% of patients with liver metastases this is not feasible due to the extent or location of the disease (Tzeng 2013). Liver malignancies have a dominant or exclusive vascular supply from the hepatic artery, whereas the majority of the blood supply of non-tumorous liver parenchyma is derived from the portal vein (Breedis 1954). The unique hepatic anatomy allows vascular isolation of the liver to deliver high doses of cytotoxic agents with minimal system toxicity. This way, various malignancies with metastases to the liver can be treated using the IHP technique. This section will describe the surgical procedure of IHP and an overview of malignancies treated with IHP.

Currently the fastest developing use of IHP is as a potential method to recover marginal organs for transplantation. Ever since Thomas Starz performed the first human liver transplant in 1963, liver transplantation is now fully established as the standard treatment for patients with end-stage liver failure. Since the early days of transplantation, static cold storage (SCS) (a bag inside an ice bucket) has been the standard preservation of organs. In the early era of transplantation there were attempts to preserve livers with machine perfusion. However, after the first unsuccessful attempts of hypothermic perfusion (HMP) of human livers, the clinical use and development of human liver perfusion devices did not move forward.

Unfortunately, the current disparity between supply and demand of liver grafts has dramatically increased the waiting time and mortality on patients in the waiting list. Consequently, there has been an increase in the use of marginal, or so-called extended criteria donor (ECD) grafts over the past decades. In particular, donation after circulatory death (DCD) liver grafts has become increasingly important as these grafts make up an increasing portion of the donor pool. DCD grafts are known to be more susceptible to ischemia-reperfusion (I/R) injury, leading to higher rates of delayed graft function (DGF), primary non-function (PNF) and biliary complications post-transplant (Pezzati 2015; Attia 2008). Biliary complications in DCD organs are a major source of morbidity, graft loss, and even mortality long-term after liver transplantation. They are much more common in DCD grafts compared to livers from brain dead donors (20–40% vs. 5% respectively). The most troublesome biliary complication is the ischemic-type biliary lesions (ITBL), also called ischemic cholangiopathy. The risk of ischemic cholangiopathy is 10 times higher in DCD donors compared to brain

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dead donors (Jay 2011). Machine perfusion allows the opportunity to repair damage of warm ischemia and cold storage, reawaken liver metabolic function, and measure viability of a liver graft prior to transplantation. However, the simplicity and lower costs of traditional SCS have kept this method as the gold standard for transplantation centers today. Growing data supports the use of machine perfusion preservation, but the variability of systems, techniques, settings, and costs have interfered with standardization and global use. Many innovative approaches have been developed to improve the quality of preservation, assess the quality of the organ during preservation and even allow repair injury of the donor organ prior to transplantation. In this context, machine perfusion of the liver at various temperatures has emerged as a prominent method to achieve these goals. Characteristics of machine perfusion at different temperatures will be discussed later in this chapter.

PHARMACOKINETIC AND PHARMACODYNAMIC STUDIES USING THE ISOLATED HEPATIC PERFUSION

Hepatobiliary function is complex and numerous techniques have been developed to evaluate the hepatic physiology and effect of toxic substances on hepatobiliary function. In 1951, Miller et al. described an isolated perfused liver model to identify roles of the liver in synthesis of plasma proteins. It was not until the 1960s however, that this technique was applied to study metabolism and pharmacokinetics (Liu 2004). For decades researchers have studied cultured isolated cells, but it has become clear that the spatiotemporal organization of different cell types in a tissue requires studies using models of the intact organ or tissue. This applies particularly to the liver as the major organ of metabolic transformation and activity. A 3-hour perfusion period has usually been applied to study hepatic clearance and metabolism, but the perfused liver can be viable up to 8 hours. Based on the amounts of dose recovered in perfusate, bile, and liver, the drug distribution and mass balance can be accurately calculated. In addition, the IHP allows for plasma and bile samples to be easily collected for identification of metabolites (Liu 2004).

Many compounds are present in blood as a plasma protein bound form, but only the unbound fractions are believed to be taken up and cleared by the liver. The extent of plasma protein binding may have a major impact on the rate at which a compound is cleared from plasma and enters the hepatocytes. The IHP model provides an excellent tool to study the effect of plasma protein binding on hepatic uptake and clearance. Subsequently, a study showed that defenestration of the liver sinusoidal endothelium in old age results in reduced hepatic extraction of highly protein-bound drugs, like diazepam (Mitchell 2012). The difference in hepatic clearance and the presence and absence of plasma proteins simply determines the effect of plasma protein binding.

Drug Disposition Studies

The IHP has been extensively used as an intact organ model for determining hepatic clearance and drug metabolism. Since IHP avoids neural and hormonal interferences and excludes influences from absorption and non-hepatic elimination routes, it provides a relatively clean hepatic system to study metabolism and pharmacokinetics. Table 13.1 provides an overview of drug disposition studies that have been done using the IHP model.

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Table 13.1. Drug disposition studies using the isolated hepatic perfusion model

Researchers	Compound	Findings
Khezrian 2015	Etoposide and cyclosporine A	It was proven that CyA led to significant in hepatic excretion, hepatic clearance, and half-life of etoposide in a dose-dependent manner.
Pfeifer 2013	Rosuvastatin (RSV)	The data confirmed the significant role of basolateral efflux clearance (CLBL) in RSV hepatic elimination, and demonstrated that both CLBL and RSV biliary clearance influence RSV hepatic and systemic exposure.
Lavasani 2013	Tramadol	The pharmacokinetic of tramadol and its three metabolites are influenced by diabetes.
Zhang 2012	Piper methysticum (kavalactone)	Liver macrophages may be a factor in liver injury induced by Piper methysticum. Characterization and modulation of the liver macrophage response may enable the development of strategies to avoid these hepatic side effects.
Mitchell 2012	Diazepam	The results highlight the importance of the liver sinusoidal endothelium in the ultrafiltration of highly protein-bound drugs, and may also provide an additional mechanism for reduced hepatic clearance of diazepam in conditions associated with defenestration.
Mitchell 2011	Paracetamol	Poloxamer 407 treatment resulted in increased recovery and decreased permeability-surface area product of paracetamol following a single pass through the isolated perfused liver.
Miranda 2008	Silymarin	The data indicate a primary role for multidrug resistance-associated protein 2 in the biliary elimination of silymarin flavonolignan conjugates.
Mehvar 2004	Cyclosporine A	The tissue binding of cyclosporine A is substantial, slowly reversible, and gender-dependent in isolated perfused rat livers.
Holecek 2003	Glutamine	Decreased delivery of glutamine to hepatic tissue activates glutamine synthesis, decreases resynthesis of essential branched-chain amino acid from branched-chain keto acids (BCKA), increases catabolism of BCKA, and has a significant effect on protein turnover in hepatic tissue.
Dewar 2002	Nicotine	The data support the hypothesis that inhibition of glycolysis by nicotine increases oxygen uptake due to an ADP-dependent increase in mitochondrial respiration.
Farabos 2001	Irinotecan	The data indicate that the hepatic disposition of irinotecan may vary at high dose, both at the level of biliary excretion and of activation to the antineoplastic drug SN-38.
Beaufort 2001	Vecuronium	Hypothermia significantly and reversibly reduced the net uptake up vecuronium.
Roumi 1997	Hexarelin (HEX)	Hepatic extraction of HEX is low, allowing researchers to predict that its hepatic clearance may be limited upon HEX protein binding.
Wilhelm 1996	Aluminum	It was concluded that by using the isolated perfused rat changes of liver functions occur only at very high aluminum concentrations in the perfusate and that only negligible amounts of aluminum are eliminated by the liver.
Takakura 1996	Oligonucleotides	The hepatic uptake process of phosphodiester oligonucleotides greatly depends on their types.
Chou 1993	Barbituric acids	The similarity between the dispersion number for different barbiturates and that for reference markers suggests that the relative axial spreading of these barbiturates is determined primarily by the heterogeneity of the hepatic vasculatory system.
Mets 1993	Lidocaine	Lidocaine metabolism may be an early indicator of severe hepatic hypoxia.

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Table 13.1. (Continued)

Researchers	Compound	Findings
Hussein 1993	Diclofenac	Additional parameters influence the relationship between availability and fraction unbound for diclofenac.
Díaz-García 1992	Diazepam	The dispersion of diazepam in the perfused rat liver is determined primarily by the architecture of the hepatic microvasculature.
Mol 1992	Steroidal muscle relaxants	Results indicate that the pharmacokinetic analysis of the hepatic disposition of steroidal muscle relaxants may be used to evaluate actual transport phenomena participating in the hepatic disposition of these drugs.
Lenzen 1991	Antiarrhythmic drugs	The present results indicate that antiarrhythmic drugs produce cholestasis in the isolated perfused rat liver independently of their adverse effect on hepatic hemodynamics.
De Bandt 1990	Norepinephrine	The results suggest that norepinephrine modulates hepatic protein balance.
Ballet 1987	Doxorubicin (DX)	In the isolated perfused rat liver, DXC has a low extraction ratio, is poorly metabolized and extensively excreted into bile.
Jones 1984	Propranolol	The data confirm precisely to the predictions of the venous equilibrium model and are incompatible with the sinusoidal model. The apparently 'unphysiological' venous equilibrium model represents a valid description of the hepatic elimination of propranolol.
Avner 1982	Protoporphyrin	It may be inferred that if plasma protoporphyrin concentration is in excess of that predicted from total protoporphyrin excretion, a defect in hepatic protoporphyrin clearance exists.
Meijer 1976	d-tubocurarine (d-TC)	The results support the idea that the balance of hydrophilic and hydrophobic properties is an important factor determining hepatic transport of organic compounds.

Drug transporters play a significant role in drug disposition and response. Considering the function of efflux transporters, the inhibition of these drug transporters by inhibitors like cyclosporine A (CyA), may lead to better responses after drug treatment (Khezrian 2015). There have been several studies focusing on the effect of CyA on pharmacokinetic behavior of etoposide (an anticancer agent). However, hepatic clearance has thus far not been described. The IHP model makes it possible to evaluate the etoposide interactions with different transporters and its hepatic disposition during co-administration with different doses of CyA. Subsequently, it was proven that CyA led to significant changes in hepatic excretion, hepatic clearance, and half-life of etoposide in a dose-dependent manner (Khezrian 2015). In addition, the tissue binding of CyA was shown to be substantial, slowly reversible, and gender-dependent in isolated perfused rat livers (Mehvar 2004).

In addition, the IHP is an effective tool to study liver toxicity. Hepatic toxicity is observed as a side effect in some herbal treatments, including the anxiolytic crop Piper methysticum. Extensive damage to the hepatic sinusoids was displayed after IHP with Piper methysticum (Zhang 2012). Exposure of the isolated liver to aluminum can accumulate this metal in the organ, which can be toxic to the hepatic tissue at high concentrations. By isolated perfused rat livers, changes of liver function were shown to occur only at high aluminum concentrations in the perfusate and only negligible amounts of aluminum were eliminated by the liver (Wilhelm 1996).

Hepatic biotransformation of pharmaceutical agents may be altered by different physical states. An isolated perfusion model using diabetic rat livers showed that the pharmacokinetic

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effect of tramadol (a pain reliever) was influenced by diabetes (Lavasani 2013). Pharmacodynamics of muscle relaxants may be influenced by hypothermia. A study showed that hypothermia reduced hepatic net uptake and hepatic metabolism of vecoronium. The IHP model allowed the researchers to study the influence of hypothermia on just the liver, because other organs that are important for the distribution or elimination of vecoronium are absent (Beaufort 2001). Likewise, glutamine deficiency, a common finding in severe illness, has a negative influence on hepatic metabolism. This evidence was supported using an isolated perfused rat liver, which showed that decreased delivery of glutamine to hepatic tissue activates glutamine synthesis, decreases resynthesis of essential branched-chain amino acid from branched-chain keto acids (BCKA), increases catabolism of BCKA, and has a significant effect on protein turnover in hepatic tissue (Holecek 2003).

The enzyme glycyrrhizinate was shown to reduce portal hypertension in isolated perfused rat livers with chronic hepatitis (Zhao 2013). Xu et al. showed how glycyrrhizin increases hepatic glutathione content possibly through inhibition of multidrug resistance associated protein-2, which then reduced the biliary excretion of glutathione (Xu 2012).

Gene Therapy

The liver is one of the major target organs for gene therapy of inherited metabolic disorders. The objective of *in vivo* gene delivery is to achieve genetic modification of the target organ. A major concern here is that the vector spreads to other areas than the target organ and insufficient transduction efficacy *in vivo* can be a reason for treatment failure. IHP allows for specific *in vivo* gene delivery and local administration of the vector or the tissue of interest (Kinoshita 2010; de Roos 1997). It has been demonstrated that *in vivo* IHP is effective for targeted delivery of a transgene to the liver with recombinant adenoviruses (de Roos 1997). Toxic effects related to the exposure of hepatocytes to the adenoviruses were not observed and this procedure resulted in a significantly higher expression of the marker gene compared to infusion of the vector into the portal vein. Shigeo et al. described a procedure for IHP that enables reproducible and tissue-specific gene delivery by Sendai virus vectors. Direct intravenous injection of Sendai virus vectors is otherwise not feasible because of their hemagglutinating and hemolytic activities. Marker genes were expressed in most parenchymal hepatocytes and undetectable in non-hepatic tissues, indicating that this approach offers good clinical potential for human gene therapy (Fujita 2006). Moreover, van Etten et al. performed IHP in rats with a recombinant adenoviral vector and demonstrated a decreased neutralizing antibody formation and high transduction efficacy compared to systemic intravenous treatment (van Etten 2004).

Figure 13.1 shows a schematic representation of an experimental liver machine perfusion set-up. The rat liver is placed in an organ chamber (A) after which tubes enabled perfusion fluid to flow to the arterial and portal side. Two tubular membrane oxygenators provide oxygen to the perfusion solution, as well as removal of CO_2 (B). Roller pumps on both sides provide a continuous flow to the portal vein and a pulsatile or continuous flow to the hepatic artery (C). Elastic tubing and a bubble trapper (E) allow elimination of the pulses in the portal flow. For the hepatic artery, the bubble trapper can be removed for a pulsatile flow and replaced to create a continuous flow (E). The temperature of the perfusion solution can be manually maintained by 2 heat exchangers connected to water baths (D). Two inline sensors

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are used to measure temperature (F). Flow and pressure (G) are detected by in-line sensors, and data is analyzed and displayed in real time on a computer. Several bubble traps can be used to eliminate air bubbles in the perfusion solution (H). Bile is collected in Eppendorf tubes (I).

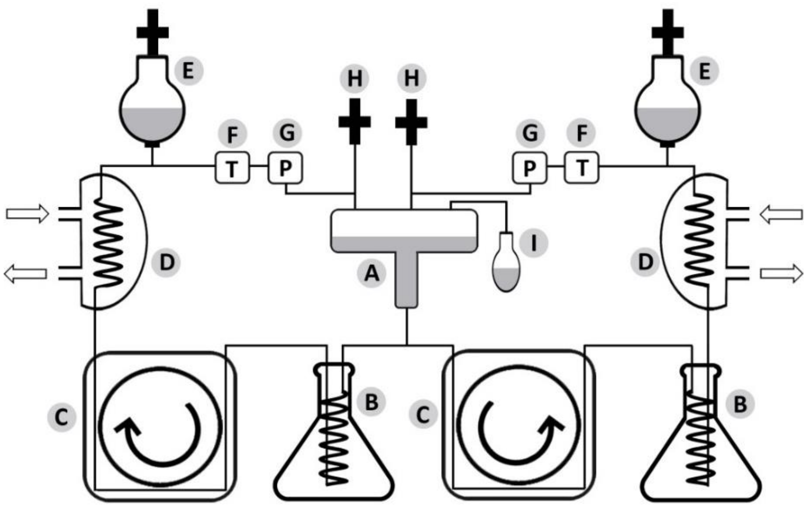

Figure 13.1. Schematic set-up of a liver machine perfusion system.

Isolated Liver Perfusion as a Treatment for Malignancies

For several decades, hepatic perfusion via the operative (isolated hepatic perfusion) or percutaneous (percutaneous hepatic perfusion) technique has been tested clinically in patients with diffuse isolated liver metastases from various solid organ cancers. Dr. Robert Ausman was the first to describe IHP to treat malignancies over 50 years ago. The technique was first refined in a canine model and later he reported outcomes of 5 patients treated with 60-minute IHP. Toxicity was considerable and there was no long-term follow up, but there was evidence of antitumor activity in 2 out of 5 of his patients, so a therapeutic effect of IHP was possible (Ausman, 1961). Two decades thereafter, there were scattered reports from several centers around the world describing IHP in small numbers of patients. The procedure was generally felt to be risky and thus far no clear benefits had been shown. However, in 1992, the IHP model regained interest after Drs. Lienard and Lejeune reported a 90% complete response rate in 29 patients with a high-grade unresectable extremity sarcoma or in-transit melanoma (Lienard 1992). They combined chemotherapy with the toxic tumor necrosis factor alpha (TNFα), which placed emphasis on ensuring complete vascular liver isolation to make sure that no systemic leaks occurred during perfusion.

One of the more important aspects of IHP treatment is monitoring for perfusate leaks during perfusion. A significant leak into the systemic circulation of a patient can be fatal depending on the regimen used. Inadequate ligation of the small IVC vessels is a typical predisposition for systemic leaks. To monitor intraoperative leaking, a system has been designed using human serum albumin labeled with ‘I’ (iodine-131). A 10 times smaller amount of I is injected into the systemic circulation compared to the perfusion circuit. A

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gamma-counter is placed over the centrifugal pump housing to monitor for radioactivity in the systemic circulation. A 10% leak would result in a doubling of the radioactive counts in the systemic circulation. Leaks will be detected immediately and the perfusion can be stopped (Boone 2012).

Surgical Procedure

The unique hepatic anatomy allows vascular isolation of the liver to deliver high doses of cytotoxic agents with minimal system toxicity (Burgmans 2016). After induction of general anesthesia, a laparotomy is performed. The inferior vena cava (IVC) is exposed by performing a generous Kocher maneuver of the duodenum. Then, ligation of all venous tributaries to the IVC is performed. Following heparinization, the portal vein structures are isolated and the arterial cannula is placed into the proper hepatic artery via the gastroduodenal artery. Venous cannulas are placed in the saphenous, portal and axillary veins and the patient is placed on veno-veno bypass to maintain systemic venous return. Hereafter, the IVC is clamped with supra- and infra-hepatic clamps, while a retrohepatic cannula is inserted to collect the venous outflow (Figure 13.2). The drug is infused via the arterial cannula. Upon completion of the infusion, the liver is flushed and venotomies are repaired. Physiologic hepatic blood flow can then be promptly restored (Yamamoto 2014; Reddy 2014).

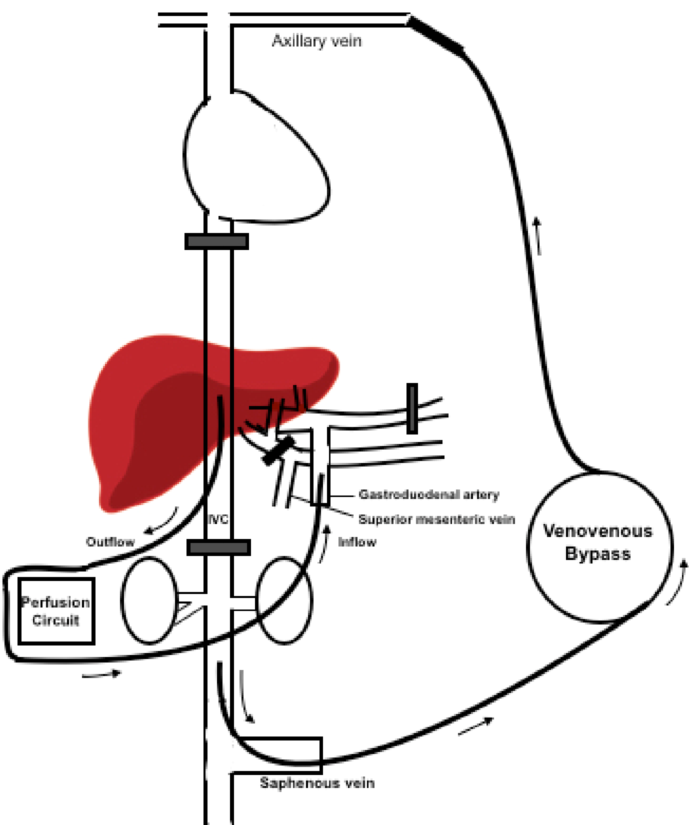

Figure 13.2. Illustration showing the isolated hepatic perfusion circuit.

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Uveal Melanoma Liver Metastases

Approximately one third of patients with uveal melanoma develop liver metastases. The prognosis is poor with a median survival of 6 months (Diener-West 2005). For these patients IHP offers an aggressive regional therapy to control disease progression in the liver. Several studies showed response rates between 30–60% with a median survival of 10–12 months when treating their patients with IHP (Table 13.2). The results of IHP are better than those obtained with systemic therapy alone and a research group in Sweden reported a 14-month survival benefit with IHP versus the treatment administered to the longest surviving patients with uveal melanoma (Olofsson 2014). Recently, the same group described 68 patients treated with IHP for uveal melanoma metastases and they reported an overall response rate of 67% (Ben-Shabat 2016). Median survival was 22 months and 20% of the patients had a complete response. In addition to cancer treatment, IHP offers a unique possibility to study exosomes from the liver of patients with uveal melanoma liver metastases. Eldh et al. showed that specific melanoma signatures could be seen in circulating exosomes from patients with advanced melanoma. They also showed that the amount of protein per exosomes increased with the disease, which indicates a potential role of exosomes as diagnostic markers (Eldh 2014).

Table 13.2. Isolated hepatic perfusion for uveal melanoma liver metastases

Author	Year	*N*	Mortality *n (%)*	Median survival (mo)	Median progression (mo)
Ben-Shabat 2016	2016	68	5 (7%)	22	10
Rizell 2008	2008	20 (+ 7 cutaneous and anal melanoma)	6 (22%)	12.6	-
van Iersel 2007	2008	12	0 (0%)	10	6.6
van Etten 2009	2003	8	0 (0%)	11	6
Noter 2004	2003	8	0 (0%)	9.9	6.7
Alexander 2003	2003	29	0 (0%)	12.1	8
Alexander 2000	2000	22	1 (5%)	11	9

Colorectal Cancer Liver Metastases

For colorectal cancer liver metastases, second-line chemotherapy response rates are less than 25% and median survival is less than 15 months (Giantonio 2007; Reddy 2014). IHP treatment in colorectal cancer patients has shown to be controversial. In a case-control study, van Iersel and colleagues showed no beneficial effect when comparing IHP with melphalan (n = 99) to systemic chemotherapy (n = 111) in patients with unresectable colorectal cancer liver metastases. Median overall survival was 25.0 months for IHP and 21.7 months for systemic treatment. Treatment-related mortality was 2% for systemic treatment and 6% for IHP (van Iersel 2010). One of the major drawbacks is its hepatoxicity with associated morbidity, mainly attributable to veno-occlusive disease (van Iersel 2007). Impressive response rates for systemic chemotherapy may render IHP unjustifiable as a first-line therapy, but it could be

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reserved as a second-line therapy. Table 13.3 lists data from prospective Phase I/II clinical trials of IHP for colorectal cancer liver metastases.

Current research focuses on additional chemotherapeutic agents that can be used to improve response rates in IHP. Also, identification of the ideal patient population, perfusate, and combination of regional-systemic therapy will be important in defining the role of IHP and its acceptance as a recognized modality in the treatment of patients with unresectable metastatic solid tumors.

Table 13.3. Isolated hepatic perfusion for colorectal liver metastases

Author	Year	*N*	Mortality n (%)	Median survival (mo)	Median progression (mo)	Radiographic response	Complete response
Zeh 2009	2009	13	1 (8%)	25	15	66%	8%
van Iersel 2007	2007	30	1 (3%)	16.9	11.5	41%	0%
Rothbarth 2003	2003	73	4 (6%)	28.8	7.7	59%	5%
Bartlett 2001	2000	51	1 (2%)	16	8.5	76%	0%
Vahrmeijer 2000	2000	24	2 (8%)	19		29%	6%

MACHINE PERFUSION FOR LIVER PRESERVATION

Donor shortage has led to increased mortality rates of patients on the waiting list. To expand the donor pool of suitable organs for transplantation, there is an increased interest in utilizing ECD organs. These organs comprise, for example, livers with varying degrees of steatosis and livers that were retrieved after circulatory arrest of the donor (the so-called DCD donors). ECD livers have a greater susceptibility to ischemia reperfusion injury and a higher risk of poor post-transplant outcomes. *Ex vivo* machine perfusion has shown to be a promising new modality in the organ preservation field to reduce I/R injury and recover ECD liver grafts. In addition, the model allows assessment of viability and function of grafts prior to transplantation. Based on different perfusion temperatures, three machine perfusion variants are currently explored: hypothermic machine perfusion (HMP) (temperatures around 5°C), sub-normothermic machine perfusion (SMP) (20–30°C), and normothermic machine perfusion (NMP) (35–37°C).

Figure 13.3 shows a human liver in a closed perfusion circuit. The liver is placed in the chamber with a blood-based solution. The organ is perfused through both the portal vein and hepatic artery. Circulation of the perfusion fluid is driven by the two roller pumps on the floor. Lastly, two bubble trappers make sure no air bubbles circulate in the perfusion system.

Devices

To date, no liver machine perfusion devices have been approved by the Food and Drug Administration. Four devices are currently used in clinical trials: OrganOx *metra*® Liver Assist, TransMedics OCS™ Liver, LifePort Liver Transporter (Figure 13.4).

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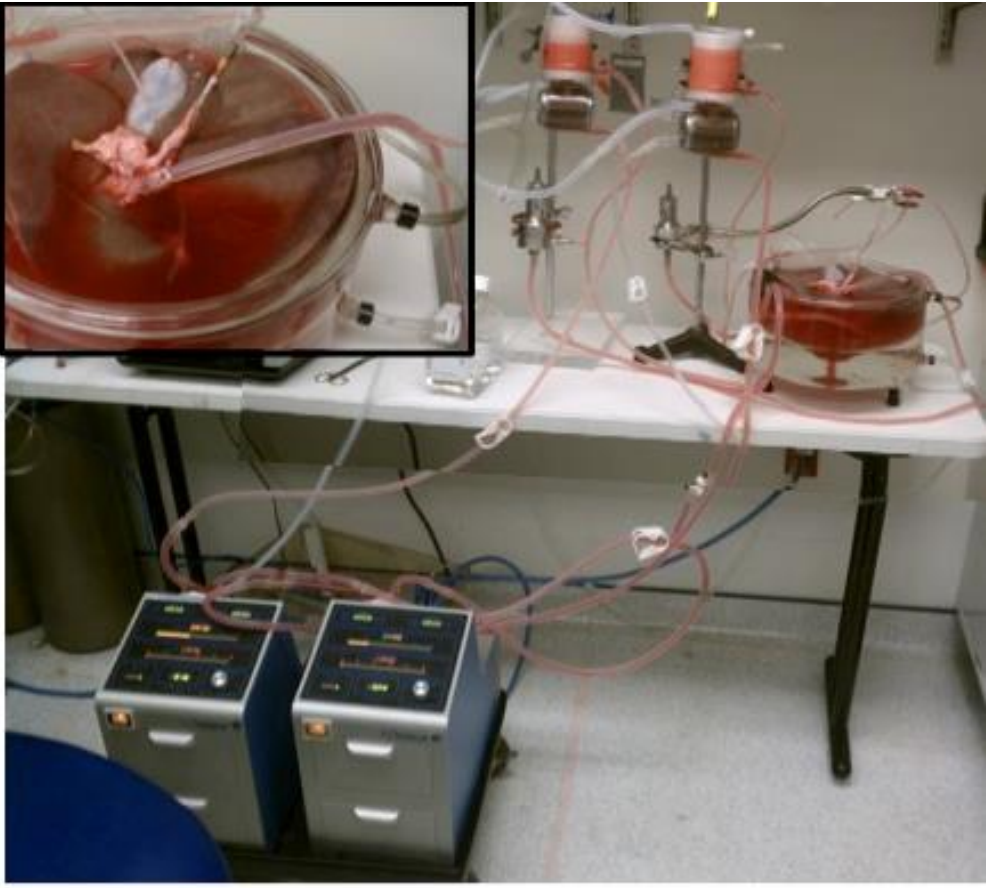

Figure 13.3. Human liver in a closed perfusion circuit.

Hypothermic Machine Perfusion

Since the early days of preservation, SCS has been the standard preservation of organs (Figure 13.5). In the early era of transplantation there were attempts to preserve livers with machine perfusion. However, after the first unsuccessful attempts of HMP of human livers by Starzl et al. in 1967, the clinical use and development of human liver perfusion devices did not move forward (Starzl 1967). After a landmark paper was published in 2009 on the improved preservation of human kidney grafts with machine preservation, it has become a very hot topic with great clinical relevance for transplantation of all organs (Moers 2009). The first successful experiments using HMP were documented by the Wisconsin group in 1985, first by D'Alessandro et al. (D'Alessandro 1986) and later by Pienaar et al. (Pienaar 1990). Both managed to preserve and transplant good-quality canine livers after 72-hr HMP in a canine model. HMP is currently the simplest approach of liver machine perfusion, with therefore high practicability and at relatively low costs. It is an attractive method to avoid further warm ischemic injury and minimize graft metabolic requirements, while providing oxygenation and metabolic support. Numerous *ex vivo* liver HMP experiments have been published in the last 2 decades and they demonstrated beneficial results compared to SCS.

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Guarerra et al. were the first to report on hypothermic dual (portal vein and hepatic artery) perfusion applied after SCS. The twenty patients that received machine-perfused livers showed significantly less peak enzyme release and a shorter hospital stay, as well as less early graft dysfunction compared to the control group (Guarrera 2010). In 2015, the same group showed less biliary complications after application of hypothermic perfusion to 31 ECD organs that were declined by the originating United Network for Organ Sharing region (Guarrera 2015). The Groningen group applied end-ischemic hypothermic oxygenated machine perfusion (HOPE) in DCD livers. The results confirm a greater than 10-fold adenosine triphosphate (ATP) increases by HOPE and less non-anastomotic biliary strictures in transplant recipients (Westerkamp 2015). Recently, the same group applied 2 hrs of HOPE after traditional cold storage and showed a great than 15-fold increase in ATP levels compared to livers that were normothermically perfused after SCS. Cumulative bile production and biliary secretion of bilirubin and bicarbonate were significantly higher after HOPE, but no differences in hepatobiliary injury markers were found (Westerkamp 2016). Dutkowski et al. were the first to compare between standard-preserved (n = 50) and HOPE-treated (n = 25) DCD liver transplants, demonstrating important benefits in favor of the HOPE approach. Their study showed less I/R injury and better graft function with a lower incidence of later intrahepatic biliary complications. Most importantly, overall and cholangiopathy-free graft survival after 1-year post transplant was 90% in HOPE-treated livers compared to 69% in unperfused organs (Dutkowski 2015). The full potential of end-ischemic HOPE should be discovered in the upcoming years.

Figure 13.4. Liver machine perfusion devices currently used in clinical trials. A: OrganOx *metra*®, B: Liver Assist, C: TransMedics OCS™ Liver, D: LifePort Liver Transporter.

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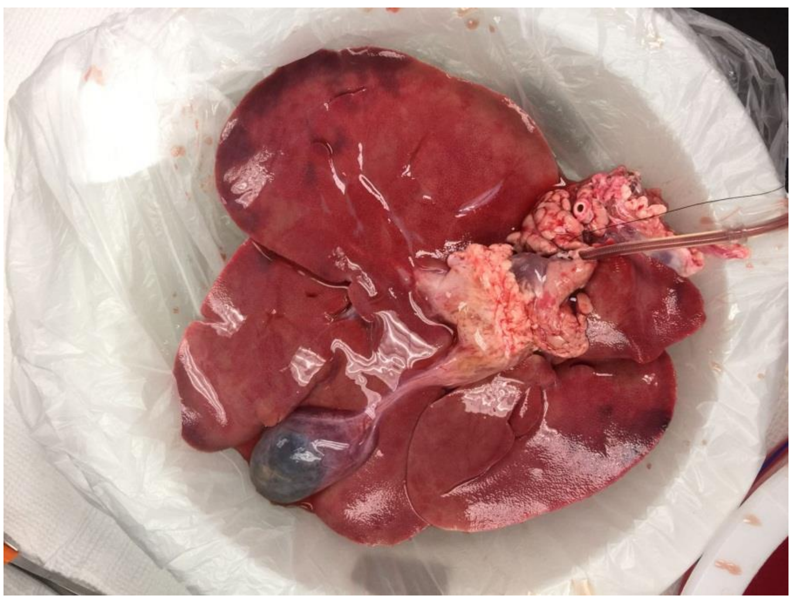

Figure 13.5. Liver prepared for static cold storage.

Perfusion Solution for Machine Preservation

The most commonly used perfusion medium during HMP is the original or modified University of Wisconsin (UW) solution or UW machine perfusion (UW-MP) solution. In addition, studies have used Institut George Lopez (IGL), Celsior or Histidine-Tryptophan-Ketoglurate (HTK) solutions. Polysol is a more recently developed preservation solution and was shown to decrease aminotransferases compared to the modified UW solution in rat livers. Also, machine perfusion using Polysol resulted in increased perfusate flow, bile production, and ammonia clearance (Bessems 2005).

The new generation solution Vasosol (VAS) is a machine perfusion solution based on Organ Recovery Systems kidney machine perfusion solution 1 (KPS-1) that is enhanced with antioxidants, metabolic intermediates and vasodilators. The solution has been shown to decrease transaminases and proinflammatory cytokines, and shortened hospital stays in human liver transplant recipients (Bae 2014). With the addition of a-tocopherol, the benefits can be further improved. Bae et al. found a reduced level of alanine aminotransferase (ALT), as well as reduced levels of inflammatory cytokines during reperfusion in a DCD rodent liver model (Bae 2014). Human application of HMP is currently done using Belzer's machine perfusate or its modifications, including ketoglutarate, nitroglycerine, i-arginine, n-acetylcysteine, and prostaglandin E1. A low potassium concentration decreases vascular resistance in hypothermic conditions and the presence of starch increases viscosity. Therefore, solutions with low potassium and without starch appear advantageous (Schlegel 2015). However, unlike the UW solution in kidney perfusion, no gold standard has yet emerged for liver preservation.

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Is Oxygenation Necessary during HMP?

Oxygenation of the preservation solution has been a neglected aspect of HMP. However, attention is desired since oxygen is necessary for ATP synthesis, but could also result in an increase of toxic reactive oxygen species. Previous studies have shown successful HMP of livers without any additional oxygenation (Guarrera 2005). On the other hand, the efficiency of HMP to preserve functional and structural liver integrity may be markedly increased by additional oxygenation of the perfusate during machine perfusion. ‘t Hart et al. showed significantly higher ATP content in 21% and 95% oxygen saturation groups compared to non-oxygenated livers. In addition, livers preserved without additional oxygen showed significantly higher aspartate aminotransferase (AST), ALT and lactate dehydrogenase (LDH) levels probably related to a decreased energy state preventing homeostasis of the cell. In the 0% and 95% oxygenated groups an increase of reactive-oxygen species was found after cold storage in UW-MP solution. Therefore, saturation of UW-MP with 21% oxygen may provide optimal preservation results (‘t Hart 2005). Contrary to this, Lüer et al. demonstrated efficient HMP by addition of 100% oxygen. HMP with 100% oxygen resulted in reduced oxygen free radical-mediated lipid peroxidation after reperfusion compared to livers oxygenated with air (20% oxygen). Also, a nearly 2-fold increase in bile production was observed in 100% oxygenated perfused livers compared to 20% or 0% oxygenated organs ($p < 0.05$) (Lüer 2010).

Perfusion Pressure and Flow

In contrast to kidney preservation, data on HMP of livers are scarce and there is still controversy when it comes to ideal parameters and settings during machine preservation. Various perfusion pressure and flow settings have been used in different studies, all aimed to minimize ischemic injury and recover the organ before transplantation. HMP settings are by definition not particularly physiologic with perfusate temperatures most commonly defined as 4°C. A critical target of injury by hypothermic preservation is the sinusoidal endothelial cell. ‘t Hart et al. found that increased perfusion pressures during HMP in rats resulted in more complete perfusion, but increased endothelial damage. They concluded that 25% of the physiological perfusion pressure gave the best results (‘t Hart 2007). In line with this, high flow rates in pigs were shown to induce detrimental effects due to sinusoidal endothelial injury through over-expression of von Willebrand factor and TNF with subsequent activation of Kupffer- and endothelial cells (Fondevila 2012). Also, dilation of the sinusoids appears to be more pronounced in livers perfused with high flows. Excessive dilation may lead to shear stress, endothelial cell lining disruption, and eventually suboptimal parenchymal preservation (Vekemans 2007). A human study by Dutkowski et al. confirmed that a low portal perfusion pressure of 3 mmHg results in complete perfusion without evidence of sinusoidal impairment (Dutkowski 2014). Generally, most studies prefer low portal vein (3−5 mmHg) and arterial (20−30 mmHg) pressures.

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Is Portal Perfusion Alone Effective in Hypothermic Conditions?

Several different perfusion routes have been advocated for machine liver perfusion and the best perfusion method has been discussed widely. Perfusion through the portal vein alone is commonly used in rat liver perfusion experiments. Arguments for perfusion of the hepatic artery include perhaps superior oxygen supply to the peribiliary vascular plexus (PVP) (Brüggenwirth 2016). Slieker has shown that the biliary tree receives blood supply from the portal vein (Slieker 2012). However, the biliary tract is mainly supplied with arterial blood through the PVP and therefore, any injury to the PVP may contribute the ischemic death of biliary epithelial cells after transplantation (Nishida 2006). The importance of hepatic artery perfusion is also illustrated by the high incidence of biliary necrosis and strictures after hepatic artery thrombosis (HAT) in patients after transplantation (Mourad 2014). Additionally, during early reperfusion the oxygen delivery is heavily dependent on arterial flow and as a result, Foley et al. showed superior bile production and output of phospholipid, cholesterol and bilirubin in dual perfused livers (Foley 1999). Combined arterial and portal machine perfusion of DCD porcine livers also results in less arteriolonecrosis and better preservation of the PVP (Op den Dries 2014). On the contrary, single portal vein perfusion adds simplicity to liver machine perfusion and authors have described portal perfusion to be sufficient to prevent I/R injury of the large bile ducts (Schlegel 2016). Hepatic artery perfusion alone seems to be less beneficial than single portal vein or dual perfusion (Compagnon 2001). Figures 13.6 and 13.7 show an experimental set-up of a dual perfused rat liver.

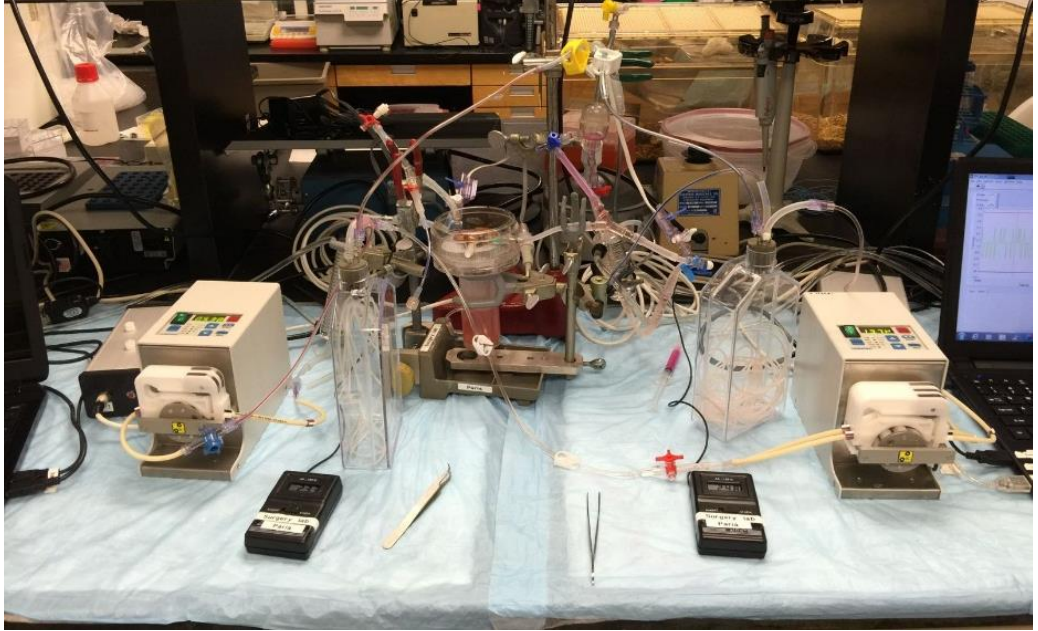

Figure 13.6. Dual perfused rat liver.

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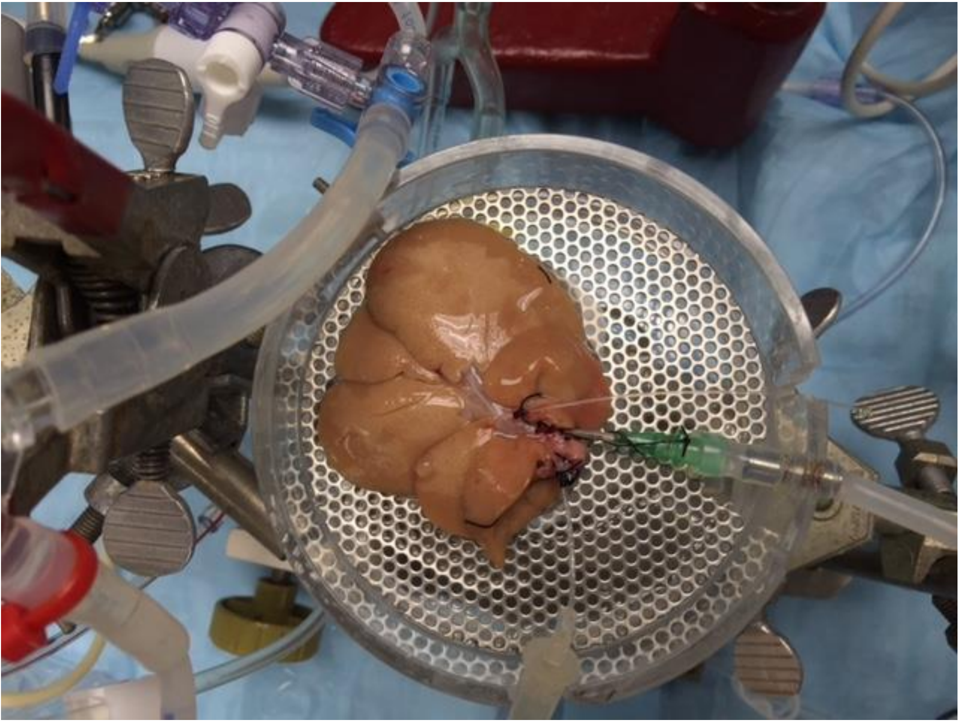

Figure 13.7. Dual perfused rat liver close-up.

Sub-Normothermic Machine Perfusion

Warm perfused organ preservation regained interest after more became known about the detrimental effects of hypothermic storage, particularly for ECD livers. It has been hypothesized that a more gradual rewarming course may relieve the cold ischemic insult by employing a stepwise normalization of temperature and metabolic demand. SMP systems have been investigated and might benefit from a lower metabolic demand compared to NMP, while still maintaining sufficient metabolism for viability testing and improvement of graft function. Bruinsma et al. showed a considerable recovery of energy metabolism during SMP. Most notable were an increase in ATP and other adenine nucleotide ratios (Bruinsma 2016). The same study showed great promise for the use of metabolomic analysis during machine perfusion of the liver. Severely ischemic and steatotic DCD livers had a significantly higher release of injury markers. Subsequently, metabolic profiling was able to cluster livers with similar metabolomic patterns based on the degree of injury. Moreover, perfusion parameters combined with differences in metabolic factors suggest variable mechanisms that result in poor energy recovery in injured livers (Bruinsma 2016). The clinical application of metabolomics remains underexplored, but emerging techniques such as rapid evaporative ionization mass spectrometry will enable near-real-time metabolic profiling in the future.

Recently, Bruinsma and colleagues applied 3 hours of oxygenated SMP to human livers that were discarded for transplantation. Beneficial results were observed regarding improved oxygen uptake, lactate levels, and ATP content, as well as increased bile production (Bruinsma 2014). This evidence is proven by previous experiments using an SMP model in

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animal experiments. Vairretti et al. showed improved cell survival and hepatic preservation of normal and steatotic livers that were machine perfused at 20 degrees compared to SCS livers (Vairetti 2009; Vairetti 2007). Likewise, Berendsen et al. were able to effectively regenerate ischemically damaged rat livers using 3 hr SMP without oxygen carriers (Berendsen 2012). Lastly, Gringeri et al. confirmed that SMP was able to resuscitate liver grafts from DCD pig livers. The SMP group showed better histopathologic results with significantly less hepatic damage compared to SCS livers (Gringeri 2012). Unfortunately, no clinically controlled studies have been executed to date and future studies are required to determine whether SMP translates to better post-transplant outcomes.

Controlled Oxygenated Rewarming

One cause of ischemic damage could be the abrupt temperature shift from hypo- to normothermia, leading to mitochondrial dysfunction and proapoptotic signal transduction. Therefore, recent studies have investigated the effect of the promising new aspect of controlled oxygenated rewarming (COR). The protective effect of controlled tissue rewarming after cold storage was first shown in a pig model, where end-ischemic MP with gradual rewarming resulted in superior functional recovery compared to hypo- or subnormothermic perfusion prior to reperfusion (Minor 013). Hoyer et al. supported this evidence in a porcine model comparing COR up to sub-normothermic temperatures to NMP. COR resulted in lower mitochondrial caspase-9-activity. Significantly lower enzyme leakage and higher bile production were observed during reperfusion (Hoyer 2016). The same group was the first to successfully apply COR in a clinical series of 6 patients. Graft survival rates after 6 months were 100% in the COR group and 80.9% in matched controls (p = 0.24). This first clinical application suggests that controlled graft rewarming after cold storage is a feasible and safe method in clinical practice and might become an adjunct in organ preservation (Hoyer 2016).

Normothermic Machine Perfusion

The concept underlying NMP is the recreation of a physiological environment during preservation. NMP enables prolonged preservation without time-dependent damage that is caused by cold preservation, the ability of the organ to recover from injury, and the opportunity to measure the function of the organ during preservation in order to predict post-transplant outcomes. NMP was first described in the 1930s, by Alexis Carrel and Charles Lindbergh (Carrel 1935). Several animal experiments followed, but with marginal results (Kestens 1966; Brettschneider 1968; Brettschneider 1968) Later, Brettschneider et al. were able to preserve 7 human livers for 4–7 hours and all recipients survived the first postoperative week. The introduction of Collins solution shortly after, and the ease of SCS moved the emphasis away from machine perfusion (Collins 1969). However, the increasing pressure to expand the donor pool with high-risk organs leads to regained interest in machine perfusion. A landmark paper by Schön et al. in 2001 demonstrated in a porcine model that NMP is a feasible preservation method and enabled successful transplantation of donor livers that had been subjected to warm ischemia (Schön 2001). Around the same time, the potential

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benefits of NMP regarding synthetic and metabolic function were demonstrated (Friend 2001; Imber 2002). There has been ongoing debate on whether the benefits of NMP can be delivered by a brief period of perfusion after SCS, or requires complete avoidance of cooling. The Oxford group investigated the effect of 5 hours and 20 hours preservation times in a DBD and DCD porcine liver transplantation model. They showed no difference between SCS and NMP in the 5 hours preserved DBD livers. However, in the 20 hours preserved DBD livers 6 out of 7 normothermically perfused livers survived compared to 2 out of 7 SCS transplants. In the DCD group, none of the SCS transplanted animals survived compared to 4 out of 5 NMP transplanted livers. This suggests that NMP might be able to resuscitate livers that experienced warm ischemic injury prior to retrieval that would otherwise not have been viable with cold preservation alone (Brockmann 2009). With promising results from large animal models, NMP moved into discarded human liver experiments. Op den Dries et al. have reported results on 4 discarded DCD human liver grafts after 6 hr of NMP using the Liver Assist (OrganAssist) followed by cold preservation. This demonstrated feasibility of NMP in human livers with well-perfused livers, continuous bile production, reduced lactate levels and preservation of liver histology and histological examination (op den Dries 2013). Recently, Mergental et al. transplanted 4 DCD livers that were rejected due to prolonged warm ischemic times, and 1 liver from a DBD donor that was declined for high liver function tests. All livers were exposed to a variable period of SCS prior to commencing NMP. The transplant procedure was uneventful in every recipient and all grafts showed immediate graft function (Mergental 2016).

In addition, it has been hypothesized that NMP might be beneficial for the recovery of steatotic livers since cooling of these organs considerably alters the compliance of hepatic tissue. Also, NMP reduces I/R injury for which steatotic livers are particularly sensitive, and allows mobilization and reduction of intracellular fat (Jamieson 2011). NMP is of great potential to increase to existing donor pool available for liver transplantation. The ability of this new technology to push back boundaries of organ acceptance is paramount. If NMP is to succeed, its value will need to be shown in the context of improved outcomes of ECD livers.

Viability Assessment

Several groups have demonstrated that NMP provides a tool to assess organ viability pre-transplantation as the liver is maintained in a physiological metabolic state (op den Dries 2013; Adham 1997). Sutton et al. recently suggested that bile output and other metabolic parameters may differentiate viable from non-viable livers (Sutton 2014). The same group demonstrated that a rising bicarbonate level in the perfusate indicated a viable liver. In addition, the ability of the liver to maintain acid-base homeostasis has been demonstrated to be good predictor of post-operative outcome (St Peter 2003). When comparing SCS to NMP preserved porcine livers, the latter group was able to correct the pH, while SCS livers were unable to reverse the acidosis (St Peter 2003). Lastly, non-viable grafts were noted to have portal pressures significantly greater than those that functioned, with portal pressures maintained at physiological values (Brockmann 2009).

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CONCLUSION

The IHP model is an effective tool to study pharmacokinetic and pharmacodynamic effects on the liver. Besides, IHP allows clinicians to administer chemotherapeutic agents to treat various malignancies with hepatic metastases. The technique is still developing to yield the improved response rates in the future. Over the last decades, a lot of research has focused on liver machine perfusion as a preservation technique prior to transplantation. It has not been implemented as the gold standard for liver preservation today, but hypothermic-, normothermic- and sub-normothermic machine perfusion preservations are all potential methods to increase the existing donor pool.

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Section III. Experimental Repair, Regeneration and Transplantation Models

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Chapter 14

EXPERIMENTAL MODELS OF CARTILAGE REPAIR

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ABSTRACT

Cartilage focal injuries resulting from trauma and sports can lead to disability and osteoarthritis (OA). The World Health Organization estimates that 9.6% of the male and 18% of the female population over 60 suffers from OA worldwide. Chronic OA greatly limits movement and day-to-day activities. The progressive degeneration of hyaline cartilage is caused by physical stress and its inability to regenerate spontaneously due to its avascular nature and hypocellularity. It is, therefore, necessary to develop and test new surgical and pharmacological cartilage repair methods and materials in animal models mimicking clinical scenarios. Rodents, rabbit, dog, pig, Göttingen minipig, horse, cow, nonhuman primates (NHPs) and human cartilage have been utilized to model *in vitro* (monolayer-, suspension-, pellet-, micromass cultures), *ex vivo* (explants) and *in vivo* repair after cartilage loss. Current surgical cartilage repair approaches involve bone marrow stimulation by microfracturing, mosaicplasty, autologous chondrocyte implantation (ACI) and allograft transplantation. Chondrocyte or chondrogenic stem cell (adult, reprogrammed iPSCs) therapy with scaffolds has been especially promising for cartilage repair owing to the regenerative, anti-inflammatory and immunomodulatory properties of stem cells. It is also possible to conduct selective differentiation into only articular chondrocytes. The quality of repaired cartilage can be analyzed by micro-CT, MRI and immunohistochemical techniques using various (OARSI, ICRS) scoring systems. Furthermore, the development of new delivery systems for both the pharmacological (chondrogenic growth factors, small molecules, chondroprotective agents) and cell-based therapies is also essential to their proper translation in clinic. Despite these hurdles, this field warrants the attention of surgeons, researchers and the public due to the prevalence of individuals afflicted with cartilage injuries, chronic

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rheumatic conditions, ageing populations and the advent of many novel treatment options.

Keywords: cartilage, chondrocytes, mesenchymal stem cells, growth factors, scaffolds

ABBREVIATIONS

ACI	Autologous chondrocyte implantation
ADSCs	Adipose-derived stem cells
BMP	Bone morphogenetic protein
BMSCs	Bone marrow-derived mesenchymal stem cells
COX2	Cyclooxygenase-2
DMM	Destabilization of medial meniscus
ECM	Extracellular matrix
FGF	Fibroblast growth factor
GDF	Growth differentiation factor
HA	Hyaluronic acid
ICRS	International Cartilage Repair Society
IGF	Insulin-like growth factor
iPSCs	Induced pluripotent stem cells
MMPs	Matrix metalloproteinases
MRI	Magnetic resonance imaging
MSCs	Mesenchymal stem cells
NHPs	Nonhuman primates
NSAIDs	Non-steroidal anti-inflammatory drugs
OA	Osteoarthritis
OARSI	Osteoarthritis Research Society International
PBMCs	Peripheral blood mononuclear cells
PRP	Platelet-rich plasma
Rac1	Ras-related C3 botulinum toxin substrate 1
Sox9	SRY (sex determining region Y)-box 9
TGF-β	Transforming growth factor beta

INTRODUCTION

Animals contain different types of cartilage tissues. Some examples include the hyaline cartilage of the nasal septum, tracheal rings, ribs, the elastic cartilages of the ear and epiglottis and the fibrocartilages of the intervertebral discs, temporomandibular joint disc, and knee meniscus (Huey 2012). Articular cartilage is an important joint tissue that provides a friction-free gliding surface, absorbs shocks and distributes weight, thus enabling diverse and complex human movements. Hyaline cartilage includes articular cartilage which consists of distinct superficial, transitional, radial and calcified zones. However, cartilage is frequently injured by traumatic work- and sports-related activities creating focal chondral or

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osteochondral injuries/defects which lead to common joint degenerative disease, osteoarthritis (OA) over the long term, causing a great amount of pain (Blaney Davidson 2017), disability and an economic burden. Disc herniation and ruptured annulus fibrosus is frequent cause of back pain requiring lumber discectomy (Guterl 2013). Although mostly associated with older people, increasing physical inactivity and obesity also alter weight-bearing abilities of joints and constitute OA risk factors for younger individuals (Gabay 2008).

Degradation of cartilage extracellular matrix (ECM) in OA occurs by proinflammatory cytokines-instigated matrix metalloproteinases (MMPs) and aggrecanases which is a shared feature of rheumatoid arthritis (RA) and OA, the most common arthritic diseases. In contrast with repairable bone fractures, adult human cartilage has limited capacity to regenerate due to its avascularity, hypocellularity, chondrocyte senescence and hyporesponsiveness to chondrogenic/anabolic growth factors such as transforming growth factor beta (TGF-β) superfamily members. When all the physical (exercise, weight loss), pharmacological (analgesics, hyaluronic acid, NSAIDs, COX2 inhibitors, corticosteroids treatment) and surgical approaches (such as lavage and debridement) fail to manage symptoms of OA (pain) and cartilage has been damaged beyond repair, joint (knee, hip) replacement surgery (arthroplasty) is performed, which also lasts for about 10–12 years and, then, fails. Thus, it is important to prevent cartilage injuries and repair cartilage at a much earlier stage.

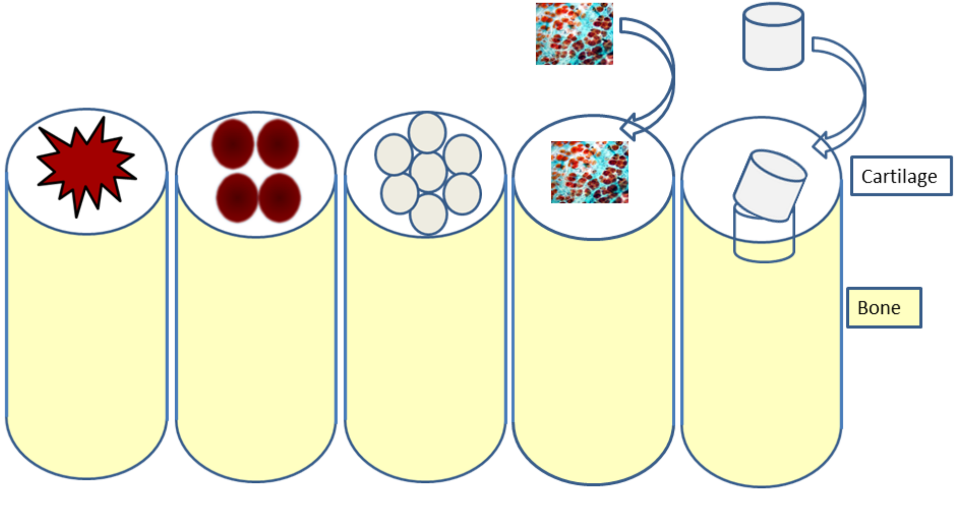

Figure 14.1. Schematic representations of surgical techniques for repairing cartilage. Cartilage focal injury can be repaired by: removing loose pieces of cartilage (debridement) and drilling holes until bone marrow and formation of blood clot which leads to repair (microfracturing); by removing circular pieces from non-weight bearing areas and their implantation as a mosaic (mosaiplasty); biopsy of cartilage, growth and multiplication of chondrocytes and injection of chondrocytes at the lesion site under a periosteal flap (Autologous Chondrocyte Implantation); removal of osteochondral graft from cadaver or patient and insertion of the graft of the size matching with lesion with a pin (osteochondral allograft transplantation). Normal cartilage (white) and bone (light brown) are also indicated (inspired from and based on: https://cartilage.org/patient/about-cartilage/cartilage-repair/.

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Surgical focal cartilage repair approaches involve debridement (removal of loose non-functional pieces of cartilage), bone marrow stimulation by microfracturing (microdrilling until subchondral bone and bone marrow, from where new cartilage develops), mosaicplasty (an autograft of healthy circular osteochondral tissues from non-weight bearing regions of the knee into drilled tunnels in a mosaic pattern within sections of defective cartilage), autologous chondrocyte implantation (ACI-for younger patients) and allograft or xenograft (for lesions greater than 2 cm) transplantation (Figure 14.1) (Clouet 2009; Katz 2010). However, bone marrow stimulation leads to substandard fibrocartilage, autografts do not integrate well as shown in a sheep model (Gelse 2015) and allografts may bring infections to the host and loss of cell viability. The problems of tissue regeneration technology include a limited availability of donor cells for transplantation, the formation of fibrocartilage of poor biomechanical quality, chondrocyte necrosis, chondrocyte dedifferentiation and poor integration with the native cartilage (Umlauf 2010).

Thus, there is an urgent need to develop and evaluate effective and durable new treatments for cartilage trauma and joint diseases-associated lesions by using a variety of experimental repair models. Here, we discuss various components of cartilage regeneration and recent developments in the field using specific examples.

CHONDROGENESIS

Cartilage originates from the mesoderm of the implanted embryo and constitutes one of the earlier steps of bone development. Fibroblast Growth Factor 2 (FGF2) primes mesenchymal stem cells (MSCs) by condensation for chondrogenesis causing them to express and deposit collagens type I, III and IV. This is followed by stimulation of chondrocyte progenitor cells with TGF-β to start differentiation into prechondrocytes, early chondroblasts, columnar chondroblasts, pre-hypertrophic chondrocytes (all expressing type II, IX and XI collagens, aggrecan), hypertrophic chondrocytes (expressing type X collagen) and calcified terminal hypertrophic chondrocytes (Goldring 2012). Overall, the chondrocyte differentiation steps are promoted by, amongst others, the SOX9 and RUNX2/3 master genes and antagonized by TWIST1, SOX5/6, and MEF2C/D through their inhibition of the 2 master factors (Liu 2017) which may provide interesting therapeutic targets for the treatment of OA. During OA, the aforementioned hypertrophic differentiation and calcification undergone by articular chondrocytes coincides with the expression of the hypertrophic chondrocyte markers, collagen type X and matrix metalloproteinase 13 (MMP-13) putatively regulated by growth arrest and DNA-damage-inducible protein beta (GADD45β). The changes in the expression profile of these differentiating cells in addition to the ensuing ECM remodelling leads to their eventual ossification (Goldring 2006).

In contrast with growth plate chondrogenesis where chondrocytes at the end of bones progressively undergo osteochondral ossification, for articular chondrocyte differentiation, MSCs lead to prechondrocytes, early chondroblasts and articular chondrocytes; the latter normally never undergo hypertrophic differentiation, thereby constituting the permanent articular cartilage. However, during OA, articular chondrocyte may undergo abnormal hypertrophic differentiation (Pitsillides 2011). The biological and biochemical agents which promote articular cartilage formation and prevent terminal differentiation may be of

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therapeutic value for permanent cartilage repair and regeneration. For instance, Triiodothyronine may prevent terminal hypertrophic differentiation.

Nonetheless, the current inability to stunt the maturation of grafted cells before reaching a fibrocartilaginous state (an intermediate of hypertrophic differentiation characterized by the co-expression of type I and II collagens and loss of tissue elasticity) constitutes one of the greatest hurdles of cartilage regenerative therapy. This challenge stems from the irreversible changes to the ECM upon exposure to chronic physical stress which, in part, drives the aberrant expression of genes responsible for the pathogenesis of joint-related degeneration.

IN VITRO AND *EX VIVO* MODELS OF CARTILAGE REPAIR

Various types of cultures such as 2-D monolayer-cultured cells, suspension, micromass as well as pellet cultures of articular chondrocytes representing different cell compactions with variable densities of chondrocytes have been used as *in vitro* models to test cartilage regenerative molecules and growth factors. For this purpose, rat, rabbit, bovine, equine, porcine, dog and human chondrocytes are used. Upon subculturing, monolayer chondrocyte cultures progressively lose their cartilage phenotype (Sox9, aggrecan and collagen 2A1 expression) as demonstrated by a loss of cartilage-specific Alcian Blue and Safranin-O (glycosaminoglycan)/fast green staining. Attempts are continuously being made to maintain the chondrocyte phenotype by growing them at high density and in 3-D cultures in agarose and alginate beads. Treatment with recombinant acid ceramidase (rAC; which maintains bioactive lipids such as ceramide) resulted in improved phenotype in rat, horse and human chondrocytes. Furthermore, the addition of rAC to feline, equine and rat bone marrow resulted in a 2-fold enrichment of MSCs and their chondrogenic differentiation (Simonaro 2013). Such approaches may improve cartilage repair technology. Because of its large size and easy accessibility, the bovine femur is a popular cartilage source and a repair model. In a study, a trephine drill head was used to explant an osteochondrol tissue from femoral condyle and used to punch defects as an *ex vivo* model maintaining the native cartilage matrix environment (de Vries-van Melle 2012). Pellet cultures were found superior to monolayer and suspension culture in repairing defects in such explants (Schmutzer 2017). Porcine femoral condyles have been used to generate osteochondral test tissue cylinders to evaluate adhesive properties of fibrin (Dehne 2012). Chondrogenesis-stimulating growth factors such as TGF-β1 and BMP-2 also enhanced cartilage repair in such models.

ANIMAL MODELS TO STUDY OSTEOARTHRITIS AND CARTILAGE REPAIR

Mature axolotl salamander diarthrodial joints are phenotypically similar to the developing synovial joints in mammals and have the remarkable and exceptional ability to regenerate its amputated limbs due to blastema, limb bud-like structures (Mitogawa 2015). Thus, they have been projected as vertebrate models for studying joint development and repair (Cosden-Decker 2012).

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Small animal models used for inducing OA-related cartilage loss are mice, rats, guinea pigs and rabbits (Little 2012). The surgical destabilization of medial meniscus (DMM) or anterior cruciate ligament (ACL) transaction leads to OA which can be used to study disease progression and therapies for preventing cartilage degradation (Culley 2015). The injection of type II collagen antibodies in mouse paws induces cartilage loss without causing inflammation which can be used to study cartilage protective therapies (Croxford 2013). The commonly used mouse strain for genetic modification, C57Bl/6 has been developed as articular cartilage repair model by full-length injuries to patellar groove of 3-weeks (young), 4-weeks (juvenile) and 8-weeks old (adult) mice with a 27G needle followed by studying repair. Young and juvenile animals showed superior repair after 8 weeks (Matsuoka 2015). Transgenic mouse strains overexpressing human MMP-13 in joints lead to cartilage loss and can be used as repair therapy models. There are numerous other enzymatically- and chemically-induced and genetically modified models causing OA-like cartilage loss or protecting against cartilage degradation.

Among large animals, goat, sheep, minipigs such as Göttingen minipig (Christensen 2016) and horses are well suited to induce chondral defects (Madry 2015). ACL and DMM models of cartilage loss can also be developed in large animals such as dog. The number of osteochondral defect research animals can be reduced if both joints are used, as observed in a rabbit model (Orth 2013). Further, bone marrow stimulation in rabbit trochlear groove appears to induce more chondrogenesis compared to medial femoral condyle (Chen 2013).

Due to the close genetic proximity of primates, the *Cynomolgus* monkey model may be highly pertinent to conduct preclinical cartilage repair research and ultimately translate these findings to clinic. We achieved high-quality repair in microfracture and collagenase-induced cartilage injury models with chondrogenic bone marrow-derived mesenchymal stem cells (BMSCs) delivered by acellular dermal matrix scaffold (Jiang 2014, Ma 2013). Earlier, osteochondral defects were treated for 6−12 weeks by microfracture-based bone marrow stimulation in *Cynomolgus* monkeys (Gill 2005). However, the long-term properties of the repaired cartilage (fibrocartilage formation and eventual degeneration) are not known. Use of nonhuman primates and dogs for cartilage research may be troubling due to ethical unacceptability by the society.

GROWTH FACTORS THAT PROMOTE ARTICULAR CARTILAGE FORMATION

The same growth factors responsible for embryonic cartilage development may also play a role in adult cartilage repair. TGF-β signalling is required for limb regeneration in Axolotls (Lévesque 2007) and for the formation of joints, as TGF-β type II receptor gene knockout mice lack interphalangeal joints (Spagnoli 2007). Human OA cartilage responds poorly to TGF-β due to IL-1β-mediated decreased receptor II and due to up-regulation of inhibitory Smad7 (Verdier 2005). It is a major growth factor for repairing joint injuries and for preventing cartilage degradation. Cartilage regenerative factors include TGF-β1/BMP, FGF and Wnt family members. TGF-β1, TGF-β3 plus dynamic compression could promote cartilage formation with compressive properties closer to those of native cartilage. FGF-18 promotes cartilage growth and prevents degradation (Huey 2012). Recombinant human FGF-

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18 treatment in the sheep chondral defect microfracture model displayed increased cartilage-specific type II collagen staining and improved cartilage repair scores (Howard 2015). Treatment of monkey bone marrow-derived MSCs with BMPs, BMP-2, BMP-4, BMP-7, BMP-9, growth differentiation factor 5 (GDF-5), TGF-β and IGF-1 in different combinations may extend the differentiated chondrocyte phenotype, upregulate chondrocyte gene expression and result in better-quality and longer-lasting cartilage. BMPs induce chondrogenesis and cartilage repair (Reddi 2003). BMP-7 and GDF-5 expression levels are reduced in murine arthritic cartilage, indicating impaired repair during the pathogenesis (Bobacz 2008). BMP-7 was also shown to protect cartilage in a rabbit OA model (Badlani 2008). TGF-β1 and BMP-7 induced chondrogenesis in human embryonic stem cells, but these articular chondrocytes did not express lubricin or proteoglycan-4 with lubricative properties (Nakagawa 2009). Overexpression of BMP-2, BMP-4 or TGF-β1 genes also induced chondrogenesis in human MSCs, but, subsequently, led to hypertrophic differentiation (Steinert 2009; Pagnotto 2007). BMP-4 can induce redifferentiation of rabbit chondrocytes *in vitro* and *in vivo* (Lin 2007). Low-level of combined BMP-2, TGF-β1 and IGF-1 expression resulted in larger cartilage aggregates (Steinert 2009a). IGF-1 and BMP-7 synergized to enhance human OA chondrocyte survival and increased bovine spine disc (nucleus pulposus) regeneration (Kim 2010). Adenoviral-based or plasmid-based IGF-1-expressing chondrocytes (gene therapy) improved early cartilage healing in horses and rabbits (Goodrich 2007; Ortved 2015; Madry 2013) (Table 14.1).

Table 14.1. Some of the different approaches of cartilage repair; please see text for details and references

CARTILAGE REPAIR APPROACHES (partial list)	
Types of cartilage repair	Procedures/factors/molecules
Surgical	Debridement and microfracturing, mosaicplasty, autologous chondrocyte implantation, osteochondral allograft transplantation
Cell Therapy	Chondrocytes (autologous-, allogeneic- and xenogeneic) Chondrogenic stem cells (embryonic, adult, reprogrammed or induced pluripotent cells), bone marrow-derived stem cells, umbilical cord-derived stem cells, human Wharton's jelly MSCs, peripheral blood mononuclear cells
Pharmacological small molecules (chondrogenic)	Kartogenin, Triiodothyronine, TGF-β+Rac1 inhibitor, TD-198946, RNA interference, microRNAs
Chondrogenic growth factors	Fibroblast growth factor, Fibroblast growth factor-18, Growth differentiation factor, Bone morphogenetic protein, Transforming growth factor beta, Insulin-like growth factor
Scaffolds for cartilage repair	Extracellular matrix, Platelet-rich plasma, Chitosan, Autologous platelet-enriched fibrin, Hyaluronic acid
Inhibitors of cartilage degradation	Doxycycline, FGFR1 inhibitor (G141), Trichostatin A, Tissue inhibitors of metalloproteinases or TIMPs, Synthetic matrix metalloproteinase (MMP) and aggrecanase (ADAMTS) inhibitors, RNA interference, microRNAs

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CELLS USED TO REGENERATE AND REPAIR CARTILAGE

Neither MSCs, nor chondrocytes have been effective in generating proper quality of cartilage. MSCs can differentiate into chondrocytes, fibroblast-like chondrocytes and hypertrophic chondrocytes (expressing type X collagen and MMP-13) which produce a mixture of fibrocartilage and hypertrophic cartilage that eventually fails as a tissue due to its poor biomechanical quality. The microfracture surgery technique also results in fibrocartilage. Delivery of *in vitro* differentiated chondrocytes also does not maintain the proper chondrocyte phenotype, and hyaline cartilage markers such as type II collagen and SOX9 gradually decline. Thus, any therapeutic cells which besides generating hyaline cartilage also produce fibrocartilage and hypertrophic cartilage result in organ failure.

Autologous chondrocytes implantation requires harvesting cartilage and, then, implanting them in a different tissue of the same individual. To increase their number by expansion, if chondrocytes are cultured as monolayer, they dedifferentiate into fibroblasts and start expressing type I and III collagens; implantation of such cells result in fibrocartilage formation. Attempts to redifferentiate such cells into chondrocytes are being made by using various culture conditions and stimuli (Huey 2012). Bianchi et al. have grown high density passage 2 human chondrocytes on a membrane in 3-D system in serum-free redifferentiation medium with TGF-β3 which resulted in a continuous layer of articular cartilage-like tissue without a scaffold (Bianchi 2017). Allogeneic (from same animal species) and xenogeneic (from different animal species) chondrocyte transplantation result in immunological rejection and may also bring infections to the host. Nevertheless, conditions for acquisition, preservation and storage of osteochondral allografts have been established by using human and goat knee experimental models (Bugbee 2016). Platelets have been shown to promote chondrocyte proliferation *in vitro* and cartilage repair in rat OA model by increasing adenosine diphosphate (ADP) and BMP-7 (Zhou 2016). Human peripheral blood mononuclear cells (PBMC) and derived MSC-like adherent (1:20 and 1:2 ratios) cells under hypoxic conditions were used with a collagen-glycosaminoglycan (GAG)-scaffold to treat mountain sheep osteochondral defects and were found to regenerate cartilage, suggesting easily accessible PBMCs as a new source of cartilage repairing cells (Hopper 2015). The lifespan of older rabbit chondrocytes could be increased by transfecting with human telomerase reverse transcriptase (hTERT) and glucose-regulated protein 78 genes suggesting that older chondrocytes may also be useful for repair (Sato 2012).

USE OF MSCS TO REPAIR CARTILAGE

Embryonic, adult and induced pluripotent stem cells (iPSCs) are potentially attractive sources for articular cartilage regeneration (Sakata 2015). Sources of adult MSCs include synovial membranes, synovial fluid, bone marrow and adipose tissue among others (Mazor 2014). Bone marrow is a rich source of multipotential cells (Jones 2002; Sekiya 2002). Other sources include muscle and periosteum (De Bari 2001). Co-expression of TGF-β and cartilage-specific transcription factor, SOX9, by gene transfer in BMSCs resulted in chondrogenic markers expression for 21 days and reduced hypertrophic differentiation (Tao 2016). Intraarticular injection of mouse synovial MSCs from C57BL6 and MRL/MpJ "super-

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healer" strains lead to increased cartilage repair in a mouse joint injury model after 4 weeks (Mak 2016; reviewed in Rai 2014). Adipose tissue contains directly usable stromal vascular fraction (SVF) or their adherent derivatives for cartilage repair (De Francesco 2015). Among adipose-derived MSCs (ADSCs) and differentiated chondrocytes in a rat OA model, the latter were more effective in regenerating cartilage defect as measured by various cartilage markers (Latief 2016). However, the testing of different types of MSCs in rodent models gave inconsistent results; most studies showing a statistically significant positive score in arthritis improvement while other showing statistically significant deterioration in arthritis score or no impact (reviewed in Hynes 2016). Both autologous chondrocytes and allogenic MSCs introduced in rabbit osteochondral defect for 6 months had similar regenerative capacities (Tay 2012).

As a new source of adult MSCs, human Wharton's jelly MSCs (hWJMSCs) were used to repair a rabbit femur patellar groove full-length defect for 16 months that was not rejected and yielded properly structured hyaline cartilage (Liu 2017). Delivery of umbilical cord blood-derived mesenchymal stem cells (hUCB-MSCs) with a hyaluronic acid (HA) hydrogel composite resulted in trochlear groove osteochondroal defect (5 mm wide-10 mm deep) repair with superior hyaline cartilage in minipig models after 12 weeks (Ha 2015). hUCB-MSCs with 4% HA hydrogel gave best results in a rat model (Park 2015).

TGFβ3 and Rac1 inhibitor (NSC23766) treatments were able to commit ADSCs to chondro-lineage differentiation mostly into articular chondrocytes (Zhu 2014). Activation of TGF-β signaling pathway in human pluripotent stem cells-derived chondrogenic progenitors leads to articular chondrocytes which produce stable articular cartilage *in vitro* and *in vivo* . In contrast, treatment of the same cells with BMP-4 leads to hypertrophic chondrocytes which start endochondral ossification *in vivo* (Craft 2015). A small thienoindazole derivative compound, TD-198946 regulating Runx1 could induce chondrogenic differentiation and prevent hypertrophy in metatarsal organ culture in addition to preventing and repairing OA in surgically-induced mouse model (Yano 2013). Kartogenin is a powerful inducer of differentiation of MSCs into chondrocytes, is chondroprotective *in vitro* and in two OA animal models (Johnson 2012). Kartogenin conjugated with chitosan nanoparticles and microparticles were retained in rat joints longer and were more preventive of degeneration (Kang 2014). TGF-β treatment *in vitro* could induce condensation of human MSCs into mesenchymal cell bodies (recapitulating cartilage development) followed by chondrogenic differentiation into clinically-relevant and mechanically functional pieces of articular cartilage, with the ability to repair *in vitro* cartilage defect (Bhumiratana 2014). Thus, these promising studies suggest that it may be possible to conduct selective differentiation into only articular chondrocytes. It remains to be seen if these approaches can be translated to human cartilage repair in clinic.

Due to the scarcity of chondrogenic cells, other sources of such cells are being sought. Many adult cells such as keratinocytes, fibroblasts, synovial cells and blood cells can be reprogrammed by transfecting with factors such as Oct4, Sox2; K, Klf4, c-Myc, Nanog and Lin28 into iPSCs and then into chondrocytes which, in turn, can be potentially useful for cartilage regenerative medicine (Liu 2016). Human iPSCs could be 3D bioprinted into cartilage mimics with a nanofibrillated cellulose (NFC) composite bioink and co-printed with irradiated human chondrocytes (Nguyen 2017). However, safety issues such as host immune reactions, genetic aberrations and teratoma formation need to be addressed before their application in clinic (Lietman 2016) (Table 14.1).

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SCAFFOLDS TO DELIVER CELLS AND FACTORS FOR CARTILAGE REPAIR

Current tissue engineering strategies to repair cartilage involve autologous cells in resorbable delivery biomaterials or scaffolds; they provide mechanical stability, 3-D cell distribution and improved differentiation. Such systems deliver stem cells, growth factors and chondrogenic genes (Sittinger 2004). Scaffolds designed for cartilage repair should have the following properties: an ability to match growth and degradation rates, to remove scaffold degradation by-products and harsh chemicals, to maintain round chondrocyte morphology and phenotype and to match compressive properties of native cartilage in addition to having sufficient surface and tensile abilities (reviewed in Huey 2012). Scaffolds with nanomaterials, functional mechanocompatible scaffolds, multilayered scaffolds, and extracellular matrix scaffolds for articular cartilage surface regeneration have been developed (Sakata 2015; Benders 2013). The following approaches are used to introduce these biomaterials into host tissue: i) implantation of engineered constructs of MSC-seeded scaffolds, and (ii) direct delivery of an appropriate population of MSCs by intra-articular injection. Platelet-rich plasma (PRP)-augmented scaffolds prepared and administered with variable methods showed improved outcomes in some animal models or had negative effects or no impact relative to controls (reviewed in Sermer 2015). PRP containing BMSCs were able to significantly regenerate early-stage degenerated rabbit intervertebral disc (a cartilage-like tissue) (Wang 2016). RPR and autologous BMSCs (undifferentiated and differentiated into chondrocytes) promoted repair of articular cartilage in a collagenase-degenerated OA rabbit model (Hermeto 2016). Chitosan implantation (which coagulates in situ at body temperature) in microdrilled defects suppressed fibrocartilage scar tissue formation in rabbits promoting bone remodeling and blood vessel migration in cartilage lesion (Mathieu 2013). Chitostan-PRP hybrids displayed greater cell recruitment and regenerative capacity than PRP only (Chevrier 2017). Bone marrow stimulation with an ultrapurified alginate gel in rabbit patellar groove resulted in more hyaline-like cartilage compared to untreated defect and bone marrow-stimulated defect (Baba 2015). Autologous platelet-enriched fibrin (APEF) scaffold alone without BM-MSCs has generated thick cartilage tissue in a 15 mm chondral defect in horses (Goodrich 2016). In the same model, autologous progenitor cells and fibrin gave a better cartilage repair score than allogenic cells with fibrin (Frisbie 2015). A collagen sponge vehicle was used to conduct a matrix-assisted (autologous) chondrocyte implantation (MACI) in horses, which after 53 weeks resulted in a cartilage with compressive and frictional properties similar to native tissue, but with inferior shear abilities (Griffin 2015; Nixon 2015). A scaffold using pig peritoneum-derive acellular matrix conjugated with BM-MSC affinity peptide (E7) able to recruit MSCs was used to repair rabbit femoral cartilage microfracture defects resulting in a lack of transplant rejection and stable hyaline cartilage generation as analyzed by histological assessment, synovial fluid analysis, magnetic resonance imaging (MRI) and nanomechanical methods (Meng 2017). Furthermore, gellan-gellan-sulfate sponge-soaked with tenascin-C was proven to be an effective method to repair rabbit patellar groove osteochondral defect (Ikemura 2015). Despite many trials of scaffolds, no material matches the compressive, friction and tensile (value of 5–25 MPa) properties of natural cartilage which confer the tissue its ability to withstand various deformations and motions. Recently, soft hydrogel scaffolds have been reinforced by 3-D printed high-porosity microfibre networks. The stiffness and

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elasticity of these composite scaffolds was similar to that of articular cartilage enabling chondrocytes to maintain their innate morphology and phenotype (Visser 2015). Scaffold-free technologies have also been developed, which recapitulate native cartilage without scaffold (reviewed in Lee 2017) (Table 14.1).

INTEGRATION OF ENGINEERED CARTILAGE INTO NATIVE TISSUE

The proper integration of repaired cartilage in surrounding tissue is essential for biological fixation, load distribution and biomechanical signal transmission. Both vertical and lateral integration of the engineered or transplanted tissue is required for successful functioning of cartilage. The neocartilage may appear histologically integrated, but may not be functionally integrated. The non-integration is due to cell death at the edges of the tissue and the non-adhesive properties of cartilage ECM; these issues can be dealt with by treatment with anti-apoptotic agents and ECM degrading enzyme (reviewed in Huey 2012).

PROMOTING CARTILAGE REPAIR BY INHIBITING CARTILAGE DEGRADATION

Traumatic injuries and aging may lead to cartilage degeneration. Additionally, surgically grafted cartilage may also degenerate with time. Therefore prevention of such degradation may promote repair. Cartilage degradation can be induced in mice by destabilization of medial meniscus (DMM). Within this model, the degradation can be reduced by the deletion of fibroblast growth factor receptor 1 (FGFR1) and a novel non-ATP-competitive FGFR1 inhibitor, G141. The same was shown in chondrocytes and human cartilage explants (Xu 2016). A carbohydrate-based drug candidate, tri-butanoylated N-acetyl-D-galactosamine analog (3, 4, 6-O-Bu3GalNAc) promoted cartilage production *in vitro* in MSCs and reduced OA in rat OA model (Kim 2016). Cell therapy of intervertebral disc nucleus pulposus with BM-MSCs increased TGF-β, which inhibited IκB phosphorylation and NF-κB activation and suppressed disc degeneration (Yang 2015). PRP injection in rabbit discs also prevented degeneration (Gui 2015). Trichostatin A was shown to prevent the degradation of transplanted autologous osteochondral tissue in the rabbit model (Hou 2015). Doxycycline, an antibiotic shown to inhibit MMP-13-mediated cartilage degradation in pellet cultures and in rats, induced chondrogenesis in hBM-MSCs and promoted osteochondral defect repair in Sprague-Dawley rats (Lee 2013). Other agents capable of preventing cartilage degradation include, synthetic MMP/aggrecanase inhibitors, endogenous tissue inhibitors of metalloproteinases (TIMPs), cytokine antagonists (IL-1 receptor anatagonist, soluble IL1R, sTNFR), apoptosis inhibitors (Bcl-2). Such strategies can prolong the survival of engineered or repaired cartilage.

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ASSESSING THE QUALITY OF CARTILAGE REPAIR

Various methods can be used to evaluate the quality of the repaired cartilage. These include macroscopic evaluation, non-destructive structure evaluation by high field (9.4 Tesla) magnetic resonance imaging (MRI) (Goebel 2015) and micro-computed tomography (micro CT), biochemical, biomechanical and molecular biological, histological and immunohistochemical methods for which scoring systems such as those of International Cartilage Repair Society (ICRS), modified O'Driscoll Scores and Osteoarthritis Research Society International (OARSI) can be applied (Madry 2015; Orth 2012). MRI is also being adapted for smaller murine models of cartilage defect repair (Mak 2015).

CONCLUSION

The current problems of cartilage tissue repair/regeneration technology include a limited number of donor cells for surgical transplantation, the formation of fibrocartilage of poor biomechanical quality, chondrocyte necrosis, chondrocyte dedifferentiation into fibroblasts and poor integration of new cartilage with the native cartilage. The resulting tissue, thus, lacks the elasticity to efficiently protect joints. Currently, surgical research has produced various treatments such as bone marrow stimulation by microfracturing, mosaicplasty, autograft and allograft transplantation using small and large animal models of cartilage loss (DMM model) and repair. Similarly, biomedical research strategies involve stimulation of chondrogenesis particularly towards articular cartilage phenotype (by Triiodothyronine, recombinant acid ceramidase, different combinations of TGF-β1 and BMPs, FGF-18, GDF-5, IGF-1, TGFβ3 plus Rac1 inhibitor-NSC23766, TD-198946, Kartogenin), cell therapy with autogenic, allogenic and xenogeneic chondrocytes, platelets, PBMCs, embryonic and adult MSCs and reprogrammed iPSCs. For the efficient delivery of chondrogenic cells, genes and molecules into defect sites, various kinds of scaffold systems such as ECM-based, acellular matrix, platelet-rich plasma-enriched, alginate, chitosan, fibrin, etc. as well as nanomaterials and 3-D printing in various combinations are being used (Table 14.1). Proper integration of implanted or engineered cartilage can be achieved with limited enzymatic treatments at the edges. All these strategies may be effective in the short-term. However, the issue of further differentiation of the inoculated cells into a state favoring further degeneration still needs to be addressed. This may have to do with the innate differences between the microenvironment of the pathological and healthy joint which may affect the outcome of treatments. To this end, numerous inhibitors of cartilage degradation and chondroprotective agents may be useful. So what still remains to be determined is how one may stunt this process at the right stage of chondrogenesis (articular cartilage) without allowing for further differentiation into the later stages and their analogues (fibrocartilage). In the future, it will be possible to selectively silence antichondrogenic factors/genes by RNA intereference (RNAi) and promote cartilage repair due to injuries, disease and aging as recently reported (Lolli 2017). Furthermore, this new repertoire of treatment options also requires a matching set of non-invasive diagnostic and surveillance tools to monitor the progression of disease and the effectiveness of treatment as well as improve the success of prophylactic treatment from lifestyle changes to proper rehabilitation at trauma sites. With regards to this, resources must also be allocated to the

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development of new clinically-relevant biomarkers specific to OA (as opposed to generalized inflammatory markers). Although we haven't addressed this, great strides have been made in this regard with the avenue of various cartilage synthesis and degradation serum-, urine-synovial fluid-borne biomarkers, (such as aggrecan/collagen II neoepitopes). From both diagnostic and therapeutic viewpoints, the many challenges underlying cartilage repair do not outweigh the field's potential for growth. In the future, only a treatment inducing durable cartilage tissue repair that is not subject to degradation and which can closely mimic the native cartilage tissue has a chance of meeting FDA approval.

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Chapter 15

EXPERIMENTAL MODELS OF FRACTURE REPAIR

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ABSTRACT

Orthopaedic trauma causes major morbidity and mortality around the world. Bone fractures are caused by trauma, diabetes and osteoporosis, whereas their complications frequently cause long-term disability and, even, death in the elderly. Various *in vitro*, *in silico* and *in vivo* models have been developed to mimic the pathogenesis of various human diseases and biological processes as well as study potential treatment options. Animals such as mice, rat, rabbit, Yucatan mini-pig, goat, sheep, pig, dog and non-human primates (NHPs) are used to study methods of accelerating fracture healing by surgical, biomechanical, biological, engineering and cell therapy approaches. Many models are designed to mimic compromised human bone repair and microbial infections. The quality of fracture repair is analyzed by radiography, micro-computed tomography, histology, histochemistry, immunohistochemistry and molecular biology techniques. Biomechanical testing such as torsion and bending is performed to assess the strength of repaired bones. We discuss various approaches that promote healing of fractures using different models. The findings may be applicable to bone repair in human trauma-, osteoporosis-, and diabetes-associated fractures.

Keywords: bone, fracture, repair, models, *in vivo*

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ABBREVIATIONS

BMP2:	Bone morphogenic protein 2
BMSCs:	Bone marrow-derived mesenchymal stem cells
CCL2:	Chemokine (C-C motif) ligand 2
COX-2:	Cyclooxygenase-2
DBM:	Demineralized bone matrix or decalcified bone matrix
FGF:	Fibroblast growth factor
GDF:	Growth and differentiation factor
HA:	Hyaluronic acid or hydroxyapatite
HSCs:	Hematopoietic stem cells
IGF-1:	Insulin-like growth factor
IL-6:	Interleulin-6
iPSCs:	Induced pluripotent stem cells
k-wire:	Kirschner wire
LIPUS:	Low-intensity pulsed ultrasound
MCP-1:	Monocyte chemoattractant protein-1
Micro/μ-CT:	Microcomputed tomography
NHPs:	Non-human primates
NSAIDs:	Non-steroidal anti-inflammatory drugs
OI:	Osteogenesis imperfecta
PDGF:	Platelet-derived growth factor
PTH:	Parathyroid hormone
RA:	Rheumatoid arthritis
SA:	Staphylococcus aureus
SD:	Sprague Dawley
T2DM:	Type 2 Diabetes mellitus
TGF-β:	Transforming growth factor beta
VEGF:	Vascular endothelial growth actor

INTRODUCTION

Bone is made up of several cell types which are responsible for its remodeling and resorption. Bone cells originate from mesenchymal stem cells (MSCs) and hematopoietic stem cells (HSCs). MSCs lead to undifferentiated mesenchymal stem cells or preosteoblasts which differentiate into osteoblasts. Single-nucleated preosteoblasts are found in canals, endosteum, periosteum, and marrow and play an important role in fracture healing by differentiating into osteoblasts. Bone lining cells originate from osteoblasts and remove organic components of mineralized matrix in response to parathyroid hormone secretion whose action is offset by calcitonin. Osteocytes result from the final differentiation of osteoblasts surrounded by matrix and are found abundantly in mature bone to form a cell network. Osteoclasts are multinucleated (3–20, when active) lysosome-rich, mitochondria-harboring cells which result from the differentiation of hematopoietic stem cells. These cells

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are involved in bone demineralization and resorption during bone repair to subsequently recruit bone-forming osteoblasts (Alghazali 2015).

Various traumatic injuries (such as sports-related and work place-related injuries, combat casualties and motor vehicle accidents among others), bone tumor removal, infections, osteoporosis and diabetes cause bone fractures in humans. Fractures occur when the load forces such as bending, tension, compression, shear, and torsion exceeds the elastic limit of the bone. In return, these fractures may cause four major types of bone defects: calvarial (skull) defect**s**, long bone or segmental defect**s**, cortical defect**s** and cancellous bone defect**s**. Fracture repair is a complex process which involves an initial inflammatory response, revascularization, soft and hard callus formation, initial bony union and slow bone remodeling (reviewed in Claes 2012). While bones have enormous regenerative and healing capacity, some fractures are subject to delayed union or nonunion, in which case bones have failed to heal. Standard bone fracture regeneration involves biomechanical support by a cast and with devices such as braces, nails, screws and plates promoting revascularization (blood supply) at the trauma site. The outcome of fracture repair depends upon the severity of trauma, the quality of realignment, stabilization techniques and the onset of infection or chronic pathogenic inflammation (reviewed in Claes 2012).

Bone grafting is a very common surgical procedure to repair fractures performed in instances of delayed unions, nonunions, osteotomies, arthrodesis and multi-fractures in addition to replacing bone due to cancer and cyst removal. Critical-size defects constituting the minimum length of fractures that do not heal spontaneously within a defined time period, are commonly used to model nonunion. They may be used to study defective fracture healing caused by diabetes, sepsis, rheumatoid arthritis (RA), osteoporosis and several other conditions. This process may also be impaired with aging and after cancer radiation therapy (Giganti 2014; Inyang 2014). Additionally, a variety of cellular mechanisms underlie fracture repair. Central to this process is osteogenesis, the differentiation of donor endogenous osteoprogenitor cells into osteoblasts and osteocytes. Another crucial mechanism, osteoinduction, involves the stimulation of host MSCs primarily with transforming growth factor beta (TGF-β) superfamily members or recruitment of circulating osteoprogenitors into graft site to differentiate into osteoblasts. Moreover, osteoconduction is a property that enables the growth of new blood vessels and bone using the graft as a scaffold. This scaffold serves to fill the bone defect, an area lacking bone, with cells, growth factors and vessels, thereby enabling healing.

The artificial scaffold should preferably be porous, sterilisable, degradable and compatible with the structure of the target bone defect, the ultimate goal being its replacement with natural bone. The materials such as hydroxyapatite, demineralized bone matrix (DBM) and ceramics have osteoconductive properties; DBM is a source of TGF-β, insulin-like growth factor (IGF-1) and fibroblast growth factor (FGF) which lead to chondroblastic differentiation of mesenchymal cells followed by bone generation through osteochondral osteogenesis. Finally, osteointegration is the proper integration of graft into host bone tissue. The initial phase of fracture healing resembles that of inflammation, but the magnitude and duration of this phase is not known (reviewed in Giannoudis 2015). The current gold standard for fracture repair is by autogenous bone graft where bone tissue harvested from the same individual (in areas such as iliac crest, fibula and scapula in humans) is implanted into a different bone of the same person (autograft). Although it is a painful and invasive procedure, this prevents infections and immune reaction against exogenous elements, enabling optimal

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healing. However, the extraction of autograft from iliac crest may lead to blood loss, nerve injury, hernia, infection, arterial or urethral injuries, pelvic instability, fracture and pain. Similarly, allografts (from donor of the same species) and xenografts (graft from a donor of different species) may lead to viral infections and rejection by host immune system necessitating the use of *in vitro*, *in silico* and animal models to develop new approaches and materials (biomolecules, cell therapies and biomaterials) to repair fractures. Excellent reviews have been written for the appropriate use of rodent and other animal models for mimicking human bone fracture healing and regeneration (Garcia 2013; Peric 2015; Bigham-Sadegh 2015). Here we focus on the recent developments over the last 5 years in the field.

PHASES OF FRACTURE REPAIR AND BASIC MECHANISMS OF OSTEOGENESIS

Stages of fracture healing have been best studied in rat and essentially consist of four phases: hematoma formation and inflammation, soft callus formation, hard callus formation and bone remodeling (reviewed in Alghazali 2015) (Figure 15.1). After fracture and rupture of blood supply, the initial acute inflammatory phase is started in the surrounding soft tissue. Plasma and leukocytes exudate and bone ends die off. At the gap, fibrinogen is converted into fibrin, leading to fracture *hematoma formation* (sealed blood clot) which contains platelets, macrophages, osteoclasts, and various inflammatory cells, such as granulocytes, lymphocytes, and monocytes, proinflammatory and anti-inflammatory cytokines. The hematoma acts as scaffold to attract polymorphonuclear neutrophils (PMNs) which release macrophage-attracting chemokines such as CCL2 (chemokine (C-C motif) ligand 2) and IL-6 (interleulin-6). The dead cells and debris are cleared from hematoma. Afterwards, lymphocytes migrate to the site of injury and start an adaptive immune response. Inflammatory cytokines such as IL‑1, IL‑6, TNF-α, receptor activator of nuclear factor κB ligand (RANKL), macrophage colony-stimulating factor 1 (MCSF1) as well as bone morphogenetic proteins (BMP 2, BMP 4, BMP 5 and BMP 6) are released. Subsequently, angiogenic factors (first, angiopoetin‑1, and, then, vascular endothelial growth factor, VEGF) are released due to the hypoxic microenvironment of the fracture site. Endothelial cells migrate from periosteal vessels into the hematoma and initiate neovascularization; the new vessels providing osteoprogenitor cells. Fibroblasts synthesize collagen and the hematoma is transformed into a tissue mass rich in collagen, cells and capillaries. In rats, this phase lasts 7 days and promotes angiogenesis and differentiation of MSCs. Antiinflammatory drugs such as cyclooxygenase-2 (COX-2) inhibitors impair this phase.

The type of repair depends upon biomechanical conditions and the degree of movement at the site of fracture. Under low interfragmentary movements, direct primary bone repair takes place by normal bone remodeling. During this process, osteoclasts originating from hematopoietic stem cells resorb bone and create tunnels where blood vessels are formed which recruit progenitor cells (undifferentiated mesenchymal cells) to differentiate into bone-forming osteoblasts (which synthesize type 1 collagen, osteocalcin and osteopontin) ultimately connecting bone fragments. Under more movement of fracture site, indirect secondary bone healing process takes place resulting in soft callus and, then, into bony callus. This phase partially overlaps with the inflammatory phase. In 3-7 days, osteoblasts

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originating from periosteal precursor cells contribute to bone formation. The undifferentiated pluripotent cells (such as bone marrow) are recruited and could be differentiated with growth factors-promoted differentiation of mesenchymal cells into chondrogenesis or cartilage formation resulting in *soft callus*. In 7–10 days, callus formation is driven by chondrocytes and cartilaginous tissue formation which progresses to the fracture site reaching to maximum size by 14 days. Fibroblasts synthesize fibrous tissue which also contributes to fibrocartilage formation that holds fracture fragments together. During *hard callus* formation, after 10–14 days, proliferating chondrocytes become hypertrophic, deposit collagen, release calcium (mineralization) and undergo apoptosis in a manner similar to growth plate endochondral ossification. The mineralized cartilage tissue is invaded by blood vessels. Vasculature and increased oxygen promote osteoblast differentiation. The blood vessels enable the recruitment of MSCs and monocytes; the latter differentiate into osteoclast-like cells, which resorb the calcified cartilage and MSCs differentiate into osteoblasts which fill the lacunae with new woven bone and bridge the gap. The osteoblasts mature into osteocytes and establish new osteons called secondary osteons. Bony bridging is regarded as successful repair.

In the final stage, *bone remodeling* occurs by resorption of the periosteal callus by osteoclasts, thereby creating channels for osteoblast recruitment. The cells later mature into osteocytes that form secondary osteons resulting in the restoration of original bone shape, structure and strength. Irregularly woven bone is converted into lamellar bone. Vascularization and proinflammatory cytokines are reduced during this phase and takes 5–8 weeks in rats (reviewed by Claes 2012) (Figure 15.1). In other mature animals, all these phases may take from 3 (mice) to 24 weeks (NHPs) (reviewed in Peric 2015). The integrity of bone tissue is maintained by a continuous balance between synthesis by osteoblasts and resorption by osteoclasts in response to hormones and local growth factors such as bone morphogenetic protein (BMP), transforming growth factor beta (TGF-β), platelet-derived growth factor (PDGF), epidermal growth factor (EGF), fibroblast growth factor (FGF) and insulin-like growth factor (IGF). These factors, particularly BMPs are important drivers of bone fracture repair.

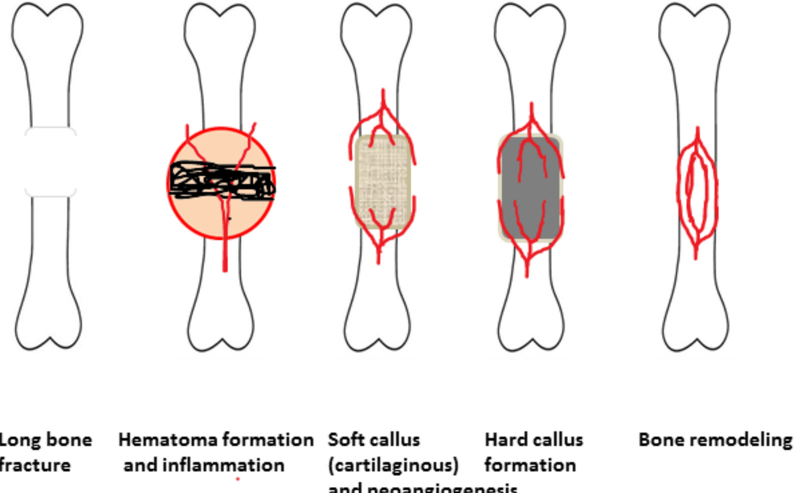

Figure 15.1. Depiction of four essential stages of bone fracture healing/repair. Please see text for details and references.

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BONE REPAIR MODELS

In vitro and *In Silico* Models

Development of *in vitro* and *in silico* models including computer simulation of fracture surgeries and repair could help patient recovery and facilitate compliance with the guiding principles of 3Rs (replacement, reduction, refinement) for ethical utilization of animals. An *in vitro* fracture (wound) healing assay was developed on the microgrooved polycaprolactone substrates to study the effect of microgroove widths and depths on the osteoblast-like cell (MG-63) migration and fracture healing (Zhang 2015). For bone repair and regeneration via drug delivery, a biomineralized (calcium phosphate), temperature-responsive and injectable hydrogel system containing hyaluronic acid (HA) and poloxamer was developed (Huh 2015). Vertebral bodies from fresh-frozen cadaveric thoracolumbar spines were used to study the ability of a novel biodegradable copolymer, poly (propylene fumarate-co-caprolactone) [P(PF-co-CL)] to repair vertebral lytic defects (Fang 2014). Since angiogenesis plays an important role in fracture repair, a computer-based multiscale model of osteogenesis and sprouting angiogenesis, incorporating lateral inhibition of endothelial cells (MOSAIC) has been developed and tested (Carlier 2012).

In vivo Models

Just as in any other branch of the health sciences, animal models should have clear endpoints and reflect real clinical situations such as: normal fracture repair (healing without delay), delayed union (repair after fracture or osteotomy in a prolonged fashion), established hypertrophic non-union (abundant callus made up of fibrocartilage that does not bridge the fracture), established atrophic non-union (fractures that do not show any signs of repair including lack of callus formation in a defined time frame; 16 weeks in small animals), fractures with a segmental defect and fractures at risk of delayed or non-union (such as non-healing, critical size defects, open and infected fractures from compromised patients) (Mills 2012). The goal of animal models is to study the efficacy and safety of the surgical procedures, drugs, bioactive molecules or cell therapy approaches. For certain clinical situations, appropriate animal models may not be generated due to major differences between animals and humans.

There are quite a few examples of this distinction. One of the most common differences involves bone healing being faster in small animals relative to large animals and humans. Additionally, in contrast to mammals, teleosts can regenerate the amputated bony fin rays. However, a better model was developed by introducing bone crush injury in adult caudal fin of zebrafish to closely mimic fracture bone repair in mammals (Sousa 2012). Less invasive ectopic bone models such as intraperitoneal, intramuscular and subcutaneous locations of animals (mostly in rodents) can be used to evaluate or screen the bone-generating capacity of implanted stem cells, growth factors and osteoinductive materials (scaffolds) within a period of 3 to 24 weeks. However, the ectopic models have limitations such as eventual reabsorption of newly formed bone and the lack of mechanical stimulus required for proper bone remodelling. Orthotopic models deal with bone directly or area around it.

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As animals have different life expectancy, and older animals may have impaired quality and repair capacity, the age of the animal must be taken into account when choosing a model. Similarly, hormonal cycles in female animals may affect bone repair and turnover, therefore choice of the appropriate gender is also required. For instance, older thyroparathyroidectomized and ovariectomized rats have delayed healing of femoral fractures and reduced bone mineral density and could serve as models of osteoporosis fractures in postmenopausal women.

Several animal models such as rats (36% of models used), mice (26%), rabbits (13%), dogs (9%), non-human primates (3%) and sheep, pigs and cats (2%) have been used to study fracture repair. They differ widely among themselves and relative to humans (particularly rodents) in terms of biochemistry, biomechanics and anatomy of normal bone and healing processes (reviewed in Mills 2012). Smaller animals (rodents, rabbit-used in 80% of studies) may cost less while pigs, sheep and dogs (20% of studies) may have higher maintenance (feeding, housing etc) costs. Dogs and non-human primates (NHPs), though closer to humans, are used as a last resort due to the ethical concerns of general public. Rat and rabbit models are generally used for calvarial defects and rabbits and dogs for segmental defects. Due to the short duration of bone maturation, rabbits are popular for bone healing studies such as testing of growth factors, biomaterials and stem cells. For creating calvarial defect in rabbits, an anteroposterior midline 4–5 cm skin incision is made over the cranial vault and the periosteum is elevated and retracted to expose the cranial bone to make a 15 mm defect using a trephine. For segmental defects, rabbit radius offers several advantages (Bigham-Sadegh 2015). Different type of bones and their defects may have different rates of regeneration.

RODENTS

Due to their small bone size, mice are not ideal for bone fracture repair studies, but are preferred due to low cost, ease of handling and the availability of biomarkers and genetically modified lines; nevertheless, segmental defects and fracture stabilization have been performed in this model (Clough 2015). Due to the similarity of human and rat receptors, rat osteoblasts, osteocytes and osteoclasts respond to drugs similarly to those of human bones. However, rodents lack the Haversian system and have permanently open growth plates. In mice models, bone healing was found to be delayed in open femur osteotomies relative to open fractures after stabilization with intramedullary screws (Klein 2015). For studying atrophic non-union fractures, a rat model was developed where osteotomy of the femur diaphysis, removal of periosteum and endosteum, isolation of the fracture site using a latex artefact (Penrose drain tube) and reduction of the fracture using an intramedullary pin was performed, removal of the latex and repair followed after 125 days (Roberto-Rodrigues 2015). To develop reliable nonreunion murine models, 10 months old Col1/ColII double transgenic mice were subjected to femur fractures of 0.6 mm (sub-critical) and 1.6 mm length (critical) and stabilized with plates and screws. Subcritical fractures completely healed in 5 weeks while critical fracture did not heal when analyzed by histology or micro-CT and, thus, could serve as non-union models to test therapeutic interventions (Chaubey 2013). A poly (2-hydroxyethyl methacrylate)-nanocrystalline hydroxyapatite (pHEMA-nHA)-vancomycin synthetic bone graft preabsorbed with 3μg rhBMP-2, promoted repair of critical-size (5 mm)

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infection-prone rat femoral segmental defects (Skelly 2014). A rodent closed fracture model was developed that involved creation of a medial parapatellar incision, dislocation of the patella, boring an 18 gauge hole through the center of the femur, delivery of an adjunct (if applicable), fixation of the k-wire in the greater trochanter of the femur, suturing of muscle and skin, and finally, creation of the mid-diaphyseal fracture with a three-point bending fracture device (Drissi 2015). To study mechanisms of intramembranous bone regeneration, fully stabilized, single-cortex defects in mouse femurs were developed (McGee-Lawrence 2015). To investigate the occurrence of non-unions of fractures, an integrative *in vivo* and *in silico* approach enabling possible treatment strategies was developed. In these models, the dynamics of blood vessel formation, oxygen supply, growth factor production and cell proliferation and the interactions thereof with the host environment were taken into account (Carlier 2014). A geriatric fracture model was generated by using 25-months old C57BL/6 mice (equivalent to 70–85 years old humans) through creation of transverse, traumatic tibial diaphyseal fractures. A 40-day analysis revealed that their molecular program was intact, but with a reduced callus expansion and bone volume (Lopas 2014).

SHEEP

Due to the similar weight, size, bone structure, and bone remodeling process, sheep could be analogous to humans. To develop a nonunion process in a noncritical segmental tibial defect in sheep, in Group 1 (experimental), a defect was created by surgically stripping the periosteum from the edges of a distal tibial osteotomy, keeping the edges 5 mm apart, and placing an incomplete O-shaped silicone ring in the gap. In group 2, control sheep, a simple fracture at the distal end of the tibia was created followed by fixation with interlocking nails in both groups. After 8 weeks, the control group depicted typical repair process while experimental group showed a fracture line with rounded edges and a scarce callus formation mostly consisting of fibrous tissue (Lozada-Gallegos 2013). Sheep model was also used to assess spinal fusion status after anterior cervical discectomy and fusion (ACDF) procedure which demonstrated that cyclic arginine-glycine-aspartic (cRGD) and mineralized collagen matrix were the superior promoters of spinal fusion compared to the collagen matrix alone and cRGD was as effective as rhBMP-2 to increase the incidence of fusion events (Scholz 2013).

PIGS AND NON-HUMAN PRIMATES

Anatomy, microstructure and remodeling of pig bones resemble those of humans. The skeleton, posture, structure, composition and remodeling pattern of non-human primate bones is also similar to humans, but their use is constrained due to limited availability, high cost and ethical reasons. Adult common marmoset monkeys (*Callithrix jacchus*) have been suggested as models due to anatomical characteristics similar to those of humans including absence of growth plates, the presence of a Haversian system and true remodeling of cancellous and cortical bone.

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Overall, rats have been proposed to be a first line of small models, followed by primates, pig and dog, while rabbit and marmoset could serve as intermediate models. European Medicines Agency (EMA) suggests that primates, sheep and pigs are the ideal animal models for testing new drugs prior to clinical trials in humans (Peric 2015).

COMPROMISED BONE REPAIR AND RELATED MODELS

As previously stated, there are several clinical situations where endogenous bone fracture repair is impaired. These may include Type 2 Diabetes mellitus (T2DM), rheumatoid arthritis (RA), lupus, hypothyroidism, malnutrition, alcoholism, smoking, infection of the fracture site, radiation use and non-steroidal anti-inflammatory drugs (NSAIDs) therapy (Table 15.1). In systemic chronic inflammatory diseases such as RA and type II diabetes, there is bone loss and osteoporosis, ultimately leading to fracture risk. This is mainly due to stimulation of osteoclastogenesis by proinflammatory cytokines resulting in an imbalance between bone formation and bone resorption. Acute inflammatory conditions such as sepsis and polytrauma also impair fracture healing in patients and animals due to a systemic increase of inflammation (Claes 2012). Generally, the ageing world population may explain the increasing incidence of cases of impaired bone regeneration.

Fracture healing is impaired in diabetes partly through plasminogen activator inhibitor-1 (PAI-1) whose deficiency partly restored healing by increasing alkaline phosphatase-positive cells (Mao 2014). Type 2 Diabetes (T2DM) models were generated by placing male C57BL/6J mice at 5 weeks of age on high-fat diet. Tibial fractures were introduced at 17 weeks. These mice had delayed fracture healing and weakened biomechanical properties with callus adiposity rather than osteoblast lineage (Brown 2014). In the same diabetic mouse model, skeletal repair was found to be impaired by increased levels of TNF-α-driven suppression of Indian Hedgehog (Ihh) expression which could be rescued by delivery of purified Ihh via slow release hydrogel to the fracture site (Tevlin 2017). In another T2DM model, subcritical femoral defects were created in diabetic fa/fa and nondiabetic +/+ Zucker Diabetic Fatty (ZDF) rats and internally stabilized. Vehicle or 75 μg/kg/d parathyroid hormone (PTH 1−84) was injected for 12 weeks. Skeletal effects were evaluated by μCT (micro computed tomography), biomechanical testing, histomorphometry and biochemical markers, and defect regeneration was analyzed by μCT. PTH was able to partially reverse adverse skeletal effects more effectively in normal rats compared to diabetic animals (Hamann 2014). Obese diabetic mice lacking leptin receptor (db/db) could also serve as model as they are deficient in postnatal regenerative osteogenesis in ectopic osteogenesis and fracture healing models (Roszer 2014). Compared to control (C57BL/6) mice, leptin-deficient obese ob/ob mice showed increased fracture callus volume and delayed callus maturation; such impaired healing could be reversed by local application of leptin (Khan 2013). Similarly, treatment of Sprague Dawley (SD) rats with recombinant leptin presented better femoral fracture healing and biomechanical properties than control rats (Liu 2017). Intramedullary delivery of Ultralente insulin, a form of insulin which can last up to 36 hours, in femoral fractures of non-diabetic Wistar rats contributed to early-stage fracture healing by increasing osteogenic markers expression, subperiosteal angiogenesis, and mineralized tissue formation (Paglia 2013).

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Table 15.1. Diverse approaches which promote bone fracture repair by different mechanisms. We also list certain conditions and factors which either cause fractures or impair their repair. Please see text for references

INDUCERS AND INHIBITORS OF BONE FRACTURE REPAIR	
Types of fracture repair	Procedure/factors/molecules
Surgical	Immobilization/stabilization and realignment by cast and braces, nails, screws, K-wire and plates Bone grafting (autografts, allografts and xenografts) for delayed unions, nonunions, osteotomies, arthrodesis and multi-fractures
Biological, Pharmacological	Osteogenesis-inducing agents Osteoinduction by transforming growth factor beta (TGF-β) superfamily including bone morphogenetic proteins (BMP-2), growth and differentiation factor-5, Insulin, Insulin-like growth factor (IGF-1), Fibroblast growth factor (FGF), Platelet-derived growth factor, Epidermal growth factor Osteoconduction by growth of new blood vessels (angiopoetin‑1 and vascular endothelial growth actor) on graft scaffolds Osteointegration Strontium ranelate, Statins (Simvastatin, Lovastatin), Parathyroid hormone-related peptide, Stromal-derived factor, sclerostin antibody Low-intensity pulsed ultrasound (LIPUS)
Cell therapy	Bone marrow-derived mesenchymal stem cells, Bone marrow-derived mononuclear cells, Osteogenic cells from adipose tissue, muscle, umbilical cord blood, peripheral blood, dental pulp Induced pluripotent stem cells Endothelial progenitor cells (through angiogenic factors) Fat tissue pericytes
Biomaterials/Scaffolds/ Bioengineering (for osteogenic cell, molecule, factor delivery)	Scaffolds: Bioactive inorganic materials: Tricalcium phosphate and Hydroxyapatite Biological polymers: Collagen, decalcified bone matrix (DBM), Collagen-glycosaminoglycan, fibrin (promotes wound healing, cell attachment and proliferation), Chitosan (a polysaccharide), Hyaluronic acid (HA), platelet-rich plasma (PRP) Synthetic polymers: Polyfumarates, polyurethane, poly (glycolic-co-lactic acid) (PLA), polyglycolic acid (PGA), copolymers of PLA and PGA (PLGA) and polycaprolactone Inorganic-organic composites such as collagen-hydroxyapatite
Inhibitors of fracture repair or compromised fracture repair	Diabetes, Osteoporosis, Sepsis, Rheumatoid arthritis, Lupus, Infections (causing osteomyelitis), Aging, Cancer radiation therapy, Hypothyroidism, Malnutrition, Alcoholism, Smoking, Use of non-steroidal anti-inflammatory drugs (NSAIDs) and Cyclooxygenase-2 inhibitors, TNF-α, Corticosteroids

Osteoporosis is a major factor causing fractures in elderly patients. Bone mineral density is a major predictor of osteoporotic fractures, which is controlled by genetic factors. Loss of

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EN1, a non-coding variant, leads to bone loss (Zeng 2015). As a model of osteoporosis, mice were ovariectomized at 9 weeks of age and subjected to drill-hole surgery in the right tibial diaphysis at 11 weeks (Matsumoto 2016). 12 week post op ovariectomized rats were subjected to unilateral transverse osteotomy on the proximal tibiae and effectiveness of semaphorin 3A (Sema3A) to enhance callus volume and density at 4 weeks post-fracture and ossification at 8 weeks was shown (Li 2015). In a mouse femoral osteotomy model, ovariectomy-induced osteopenia (reduced bone density) impaired the middle and late stages of bone healing (Pang 2015). Strontium ranelate (SR) was shown to be beneficial in osteoporotic bone healing in rats even when applied after fracture (Komrakova 2015). Simvastatin at 20 mg dose was shown to stimulate repair in osteoporotic female rat femur fractures stabilized with k-wire (Issa 2015). Leptin receptor null Lepr(-/-) mice have been used as a model of diabetic osteoporotic fractures to demonstrate parathyroid hormone-related peptide (PTHrP)-driven stimulation of fracture repair by increasing osteoblast gene expression and bone formation (Liu 2015). Concerns have been raised about many of the osteoporotic models as they do not exactly imitate human postmenopausal and senile osteoporosis or secondary osteoporosis (reviewed in Simpson 2015).

Osteogenesis imperfecta (OI) is a human genetic disorder characterized by multiple fractures due to a mutation in the collagen type I gene. *Oim* mice (B6C3fe-a/a-*oim*) constitute a model of severe OI and have a guanine deletion at nucleotide 3,983 in *COL1A2* resulting in the absence of normal heterotrimeric collagen $\alpha1(I)_2\alpha2(I)_1$, replaced by homotrimeric $\alpha1(I)_3$, which accumulates in the extracellular matrix. The homozygous *oim* animals have brittle bones, multiple fractures, and skeletal deformities (Chipman 1993). Additionally, Brtl/+ mice constitute a heterozygous model of OI containing a Gly349Cys substitution in one COL1A1 allele, and displaying a low ductility phenotype. At 8 weeks of age, Brtl/+ has an increased osteoclast number, which mimics the upregulated bone turnover of OI patients (Davis 2012). Primed human first trimester fetal blood mesenchymal stem cells (hfMSCs) treated with SDF1 (stromal-derived factor) upregulated CXCR4 and enhanced engraftment *in vivo* in both *oim* and wild-type bone and bone marrow (Jones 2012).

Use of knockout and genetically defective animals (mostly mice) may serve as models of compromised situations. Fracture healing was impaired in NOD/scid-IL2Rγcnull immunodeficient mice due to delayed endochondral ossification and decreased osteoclast activity suggesting the requirement of an intact immune system for bone healing (Rapp 2016). Dystrophin-utophin double knockout mice are potential models of Duchenne muscular dystrophy (DMD; where patients exhibit lack of dystrophin expression, osteopenia, fragility fractures, and scoliosis) and display a reduced bone healing in addition to abnormalities in skeletal muscle, cartilage and intervertebral discs (Isaac 2013).

Models of Bone Repair under Infections

In humans, bone allograft transplantation can transmit bacterial (such as *Clostridium* species and *Treponema pallidum*-causing syphilis) and viral diseases such as HIV, Hepatitis B and C. Bacterial infections may cause septic arthritis, delayed union and osteomyelitis. Therefore, it is necessary to study bone repair under infection situations in animal models. Some examples are presented here. Skeletally mature C57bl/6 mice were subjected to a

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transverse osteotomy of the femur, which was treated with titanium fracture fixation plates and screws to keep in position. This model could then be infected with *Staphylococcus aureus (SA)* to study the immune response of the fracture (Rochford 2016). A similar model using Balb/c mice was developed to investigate a low grade acute SA osteitis by femoral defects stabilized by titanium locking plate and, then, infecting with SA. After debridement at 7 and 14 days, mice were sacrificed at 28 days and displayed delayed healing (Windolf 2013). In mice, a novel clinically-relevant model was established utilizing a locking fracture fixation plate to enable debridement of a bioluminescent *Staphylococcus aureus*-infected segmental bone defect during a revision surgery to study the therapeutic effects of placing a vancomycin-laden spacer in the defect, which reduced infection and osteolysis (Inzana 2015). A rabbit humerus model was developed using plating and nails to study prevention of implant-related bone infection and osteomyelitis (Arens 2015). Retaining an implant in the presence of a *Staphylococcus aureus* infection without antibiotic leads to a weaker callus and impedes callus maturation relative to noninfected controls in a Sprague-Dawley rat model (Bilqili 2015). A novel broad-spectrum antibiotic CSA-90 (cationic steroid antibiotic-90) and rhBMP-2 were found to promote osteogenesis *in vitro* and in a rat open fracture model infected with *S. aureus* (Schindeler 2015). Many factors such as bacterial (*Staphylococcus epidermidis*) infections cause osteomyelitis and could prevent fracture repair and induce non-unions as demonstrated in a recently developed pre-clinical *in vivo* rat femur osteotomy model (Lovati 2016).

BIOLOGICAL AND BIOCHEMICAL AGENTS AFFECTING FRACTURE REPAIR RESPONSE

Biological molecules enhancing repair include growth factors (mainly BMPs), vascularization-promoting angiogenic factors (such as VEGF, FGF, PDGF, IGF), immunomodulatory cytokines, anti-cytokines, corticosteroids, parathyroid hormone, growth hormone, steroids, calcitonin and vitamin D. Different combinations of the factors may stimulate osteogenesis, osteoblast differentiation and angiogenesis. BMPs are already in clinical use, but have several side effects (Peric 2015). Tibial bone defects in male Wistar albino rats were used to demonstrate effectiveness of *Potentilla fulgens* extract to enhance trabecular bone formation as measured by osteonectin and osteopontin expression in osteoblasts and osteocytes (Koparal 2016). In a rat calvarial defect (5 mm) model, collagen sponge and fibrin glue were shown to promote new bone formation over 4–8 weeks period, the latter being better than the former (Santos 2015). Analysis by radiography and micro-CT revealed that platelet-rich plasma increases bone formation, bone area and torsional stiffness in animal long bone defect models (reviewed in Gianakos 2015). Blockade of a Wnt signalling pathway inhibitor, sclerostin, by injection with its associated antibody in a mouse tibial midshaft osteotomy model resulted in earlier healing of the fracture (Alzahrani 2016). Similar results were obtained by systemic sclerostin mAb (monoclonal antibody) administration in a rat femoral osteotomy model (Feng 2015). In rheumatoid arthritis-like collagen-induced arthritis (CIA), in male DBA/1 mice, sclerostin antibody prevented or reversed the decrease in axial and appendicular bone mass, but did not prevent or repair local bone erosion (Marenzana 2013). Lovastatin and tocotrienol were shown to upregulate the

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expression of genes related to tibial fracture healing in ovariectomized Sprague-Dawley female rats, an osteoporosis-like model (Ibrahim 2015). In metaphyseal small plug defects and diaphyseal segmental bone defects in rat femora, injectable lovastatin microparticles with biodegradable polyurethane (PUR) scaffolds resulted in increased new bone formation and bridging (Yoshii 2014). Vitamin C, an antioxidant and cofactor, improves bone health and could potentially prevent osteoporotic bone fractures (Aghajanian 2015). However, in a human clinical trial, no benefit for fracture repair was found (Ekrol 2014). Nutritional supplementation of mice subjected to closed tibial transverse fractures with basic milk protein displayed a larger and harder callus with increased chondrogenesis- and osteogenesis-associated gene expression and biomechanical properties (Yoneme 2015). In a murine tibial fracture or fragility fracture model, low-dose recombinant human TNF-α treatment within 24 h of injury increased fracture repair, which works through the recruitment of neutrophils and monocytes by increasing CCL2 (Chan 2015). In contrast, high levels of TNF-α in transgenic mice (hTNFtg mice) had a negative impact on fracture healing in that the mice displayed more soft calluses and decreased biomechanical stability while TNF-α inhibition by Infliximab reversed these effects without interfering with fracture healing (Timmen 2014). It was recently shown that, after fracture, a C-X-C motif-ligand-12 (CXCL12) (+)-BMP2 (+) perivascular cell population is recruited along the endosteum, followed by a timely increase of BMP2 leading to downregulation of CXCL12 that determines the fate of the CXCL12(+)-BMP2(+) to osteogenesis instead of their role to angiogenesis. These results may lead to novel pharmacological and cell therapy approaches for fracture repair (Myers 2015). Delivery of BMP-2 by nanofiber mesh-alginate (NMA-rhBMP-2) in rat femoral large defect improved bone formation and biomechanical function compared to the gold standard of autograft treatment (Krishnan 2015). Delivery of adenovirus-mediated BMP2-expressing allogenic cells encapsulated in poly (ethylene glycol) diacrylate (PEGDA) hydrogel in a 5 mm rat femoral defect lead to more rapid healing (in 3 weeks) compared to high dose rhBMP2 (2-3 months) (Sonnet 2013). Basic fibroblast-derived growth factor containing a polycystic kidney disease (PKD) domain and collagen-binding domain (CBD) and demineralized bone matrix (DBM) increased callus formation and bone mineral density in a mouse femur fracture model (Saito 2015). In critical-sized 15-mm rabbit, radius defects treated with a growth factor (BMP2, GDF-5; growth and differentiation factor-5 and swap mutant GDF-5V453/V456 or BB-1)-loaded collagen carrier resulted in strong angiogenicity and osteogenicity; BB1 being the most osteogenic, as shown by micro-CT and immunohistochemistry analysis (Kleinschmidt 2014). Treatment of transversely cut rat femur at the midsection with a nonselective endothelin 1 receptor blocker, bosentan (100 mg/Kg) resulted in better radiographic healing score and callus/new bones formation relative to control group (Aydin 2015). BMP2 and debridement promoted repair in rat femur non-union fractures (Schützenberger 2014). In the germline, neuropeptide Y knockout mice, closed tibial fracture repair was delayed due to decrease in bone callus volume and strength (Sousa 2013). Monocyte chemoattractant protein-1 (MCP-1) is expressed in osteoblasts of ulnar stress fracture model and may have an important role in the early phase repair and activated remodeling (Wu 2013). Delivery of VEGF in hydroxyapatite-containing gelatin scaffold yielded favorable results in critical size rabbit tibial defect repair (Ozturk 2013). AAV-DJ harbored COX-2 vector gene therapy led to a ~5-fold increase in infectivity in MSCs, which enhanced COX-2 expression and significantly promoted fracture union in mice at 21 days by inducing osteoblastic differentiation of MSCs (Lakhan 2015). Lentivirus-mediated Wnt10b

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gene overexpression in rat femur non-union fracture model enhanced BMP2 expression, bone mass density and bone mineral density in callus leading to complete healing by 8 weeks (Gao 2015).

UTILIZATION OF STEM CELLS AND CELL THERAPY IN FRACTURE REPAIR

Stem cells are undifferentiated cells which can proliferate and self-renew themselves, that originate from the ectoderm, mesoderm and endoderm of the implanted embryo. Multipotent cell can differentiate into different cell-types of a particular germ layer. For instance, bone marrow-derived multipotent stem cells (MSCs) can be differentiated under appropriate conditions into osteogenic, chondrogenic and adipogenic lineage and, therefore, can be used for cell therapy approaches to repair bone fractures. Besides the bone marrow, other sources of osteogenic multipotent cells include adipose tissue, muscle, umbilical cord blood, peripheral blood, dental pulp, etc., from humans, pigs, rodents, rabbits, dogs, cats, small ruminants, and horses which may differ from the bone marrow in terms of phenotype, morphology, proliferation and osteogenic potential. Induced pluripotent stem cells (iPSCs) are generated by reprograming adult cells such as fibroblasts to express embryonic stem cell characteristics (reviewed in Alghazali 2015). Other than being a source of osteogenic cells, transplanted MSCs contribute to repair by excreting various cytokines and growth factors. For cell therapy, autologous MSCs can be first differentiated into osteogenic cells, and then transplanted at the fracture site. Endothelial progenitor cells can also be used to release osteogenic and angiogenic factors leading to neovascularization and repair at the site of fracture. In all MSC-based repairs, the properties and fate of these cells should be monitored with appropriate markers. Cell therapy and tissue engineering approaches have been combined to include growth factors, scaffolds and osteogenic cells to enhance regeneration (Peric 2015). The role of MSCs in bone repair has been investigated in several animal models. To investigate the intrinsic progenitor cells in non-union fractures, Wistar rats were subjected to an atrophic nonunion at the tibial midshaft by stripping the periosteum and endosteum as well as creating a 1.0 mm noncritical gap. The fracture was subsequently stabilized. The ensuing results revealed that despite the systemic increase in bone marrow-derived mesenchymal stem cells (BMSCs), an impaired local response at non-union fractures was observed, but the cells could be differentiated under osteogenic and chondrogenic conditions, but not under adipogenic conditions (Tawonsawatruk 2014). In a mouse model of segmental bone defect, the combination of IGF1 and CXCR4 antagonist, AMD3100, was able to stimulate mobilization of BMSCs to the fracture site and increase bone growth and healing through Akt, ERK and Smad signaling pathways (Kumar 2012). As stated above, diabetes impairs bone formation and fracture healing. In a streptozotocin-induced diabetes type I mouse model, increased TNF-α reduced mesenchymal stem cell (MSC) number by decreasing proliferation and increasing apoptosis in the repair area, thus reducing regenerative potential (Ko 2015). Enriched $CD146^{+}NG2^{+}CD45^{-}$ cells from mouse fat tissue pericytes could be differentiated into osteoblasts *in vitro*, and could colonize cancellous bone scaffolds and contribute to regeneration of large bone defects *in vivo* (Konig 2016). Low-intensity pulsed ultrasound (LIPUS) and bone mesenchymal stem cells C3H10T1/2 promoted femoral bone-

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defect healing after 4 weeks in Sprague-Dawley rats by increasing proliferation *in vitro* and *in vivo* (He 2015). LIPUS also promoted osteogenesis, neovascularisation and repair in steroid (lipopolysaccharide and methylprednisolone)-induced femoral osteonecrosis rabbit model (Zhu 2015). In an ovariectomized rabbit (osteoporosis-like conditions), allogenic fetal BMSCs and decalcified bone matrix scaffolds were seeded in osteogenic media and transplanted into fracture defect which resulted in more bone tissue after 3 months compared to tissue engineering with BMSCs or scaffold alone (Wang 2015). Osteogenic cell sheets made from rat BMSCs containing osteoblasts and extracellular matrix and inserted in a critical fracture healing model of Fischer 344 inbred rats revealed enhanced bone regeneration and maximum bending load as determined by biomechanical testing relative to controls (Shimizu 2015). In ovariectomized osteoporotic mice, the implantation of mouse MSC-derived osteoblasts loaded in 3-D collagen I gel resulted in increased trabecular bone volume relative to matrix alone suggesting usefulness of stem cell therapy for bone (Taiani 2014). Endothelial progenitor cells (EPCs) from the bone marrow of syngeneic rats loaded in a gelfoam scaffold introduced in bone defect were able to heal the fracture after 10 weeks by increasing BMP2 and promoting angiogenesis (Li 2014). Intravenous injection of CD271-selected human bone marrow MSCs and labelled with infrared fluorochrome, cypate, resulted in homing of BMSCs at mouse femur fracture site (Dreger 2014). In a murine transverse femoral fracture model, injection of AMD3100, an antagonist of the chemokine receptor 4 (CXCR4) lead to mobilization of endogenous circulating mesenchymal stromal cells, hematopoietic stem cells and endothelial progenitor cells and a larger fracture callus and better regeneration (Toupadakis 2013). In a dog femoral neck fracture model, delivery of autologous uncultured bone marrow-derived mononuclear cells (BMMNCs) through fibrin glue and a modified cannulated screw resulted in effective fracture healing (Licheng 2013).

BIOMECHANICAL APPROACHES TO REPAIR FRACTURES

The purpose of fracture fixation is to align the fracture fragments to enable healing. Stabilization with an intramedullary nail may enhance indirect repair in most models. Low interfragmentary movement leads to direct conversion of mesenchymal cells into bone, moderate movement enables cartilage matrix conversion to callus/bone (endochondral ossification) and high movement inhibits bone repair. These goals can be achieved with external splinting (with plaster casts or braces) or internal splinting (intramedullary nails and plates). Such techniques affect the quality of revascularization, an essential step for repair (Claes 2012). For direct repair, plating may lead to repair, but may leave weakness due to the required screw holes. In an ovine osteotomy model, active locking plates were shown to stimulate circumferential callus and yield faster and stronger healing compared to standard plates (Bottlang 2016). Stabilization of CD1 mouse femur midshaft fractures with screws lead to faster bone repair compared to delayed non-stabilized fractures (Histing 2016). In ovariectomized mice subjected to drill-hole surgery in tibia, noise-like whole-body vibration and intermittent administration of parathyroid hormone enhanced osteoporotic bone healing (Matsumoto 2016). The fixation method of fracture could also impact healing. In a numerical predictive model and its testing in an ovine long bone transversal fracture model, an optimized, moderate axial stiffness together with certain shear stiffness was shown to enhance

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fracture healing (Steiner 2014). In a critical size rat femur defect model, initial low-stiffness fixation of the defect, followed by high-stiffness fixation during the healing process (termed reverse dynamization) with rhBMP-2 on collagen sponge accelerated healing (Glatt 2012). Similarly in a critical size (6 mm) defect in rat femora receiving rhBMP-2 in alginate hydrogel, compliant fixation plate samples adapted to more efficiently distribute loads in the defects (Boerckel 2012). There are now numerous computational analysis approaches available to predict and monitor fracture repair (reviewed in Anderson 2014).

USE OF BIOMATERIALS TO REPAIR BONE FRACTURES

Fracture repair may be impaired because of interruption in blood supply, inflammation and infections. Thus, various biomaterials and scaffolds are used to give a template for mechanical support for cells and tissues and guide them for regeneration, deliver repair-promoting drugs and thereby enhance bone repair. The ideal biomaterials should have osteoinductive, osteoconductive and osteointegrative properties so that the need for autologous grafts or allografts is eliminated due to abovementioned complications (infections, rejection). Over time, such materials should be resorbed and replaced with natural regenerated bone tissue. A bone tissue engineering system should have following properties: a) enable cell adhesion and growth; b) degradation products of its scaffold should not cause inflammation and toxicity; c) provide mechanical support and strength for cells; d) be porous to enable cells and molecules to circulate efficiently; e) its degradation products should be fully absorbable f) there should be balance between resorption and regeneration (Alghazali 2015). Biomaterials used for scaffolds may be classified as bioactive ceramics, bioactive glasses, biological or synthetic polymers and their composite materials. Bioactive inorganic materials include tricalcium phosphate and hydroxyapatite (HA) which mimic the mineral phase of bone and can help in osteointegration. Biological polymers include collagen, decalcified bone matrix (DBM), fibrin (promotes wound healing, cell attachment and proliferation), chitosan (a polysaccharide) and hydrogel containing hyaluronic acid (HA). Examples of synthetic polymers are polyfumarates, polyurethane, poly (glycolic-co-lactic acid) (PLA), polyglycolic acid (PGA), copolymers of PLA and PGA (PLGA) and polycaprolactone. Moreover, nano-hydroxyapatite/collagen constitutes an example of inorganic-organic composite that is similar to real bone. Synthetic extracellular matrix-like materials such as hydrogels contribute to repair by their immunomodulatory properties. The third-generation biomaterials are both bioactive and degradable as they contain both cells and molecules (antimicrobials, pharmaceuticals, growth promoters) along with a scaffold (Peric 2015; Alghazali 2015). Scaffolds may be classified as hydrogel, porous and fibrous (mimicking collagen architecture), polymer-bioceramic composite scaffolds, nature/biological-inspired scaffolds and hybrid scaffolds (reviewed in Alghazali 2015).

Here are some examples of the use of different scaffolds in animal models. Three rat models closely mimicking human fractures were developed. In the first model, for a healing control group, the right tibia were fractured and stabilized with a PDLLA (poly DL-lactide)-coated k-wire. In the second, hypertrophic rats were subjected to an open osteotomy and stabilized like the previous. Finally, rats of the atrophy group were also osteotomized and treated with Fumagillin locally via the coating system of the k-wire. Fracture repair was

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followed by radiography, micro-CT and osteogenic gene expression for 3, 7, 14, 21 and 42 days. At the end of 42-days, only the first model displayed fracture gap, but not the others (Minkwitz 2015). Human scaphoid fracture non-union was modeled in Yucatan mini-pigs by performing a 3 mm osteotomy of the radiocarpal bone followed by immediate fixation or filling with a dense collagen gel and delayed fixation (Behrends 2015). In one study, dogs were subjected to an osteotomy in the mandibular body, which was fixed with a device manufactured with biodegradable poly-L-DL-lactic acid (70:30). The dogs were euthanized at 2 and 18 weeks and bone repair at the osteotomy site was observed (Sverzut 2015). Implantation of Beta-tricalcium phosphate (β-TCP) bone substituted with 75% porosity in a subchondral bone plate of pigs was the most effective material in repairing cancellous bone after 3 months (Matsuo 2015). In rabbit radius osteotomy defect model with positive (autogenous bone graft) and negative (empty sham) controls, it was concluded that the collagen-glycosaminoglycan scaffold could be useful for a low load-bearing defect. The rhBMP-2 containing collagen-glycosaminoglycan and collagen-hydroxyapatite scaffolds may be suitable for established nonunion defects (Lyons 2014). The use of standardized animal models such as rat, rabbit and sheep to test new materials and for simulation of patient fractures has been suggested (Reifenrath 2014).

MEASURING THE STRENGTH OF REPAIRED BONE FRACTURE TISSUE

Techniques to verify the quality of repair tissue can be non-invasive or invasive. Non-invasive methods comprise of physical examination and activity testing the weight-bearing ability of repaired tissue, imaging technologies such as plain radiography, contrast radiography, magnetic resonance imaging, quantitative computed tomography (qCT), peripheral quantitative CT (pQCT), and micro CT (μCT) and plain and color Doppler ultrasonography. These techniques assess bone geometry, mass and structure. X-ray with or without pQCT is the method of choice for *in vivo* follow-up due to its good correlation with the mechanical testing of bone strength (Peric 2015). Micro-CT offers advantages of rapid 3-D analysis of trabecular morphology without destroying the sample and its usage for other analyses. The invasive approaches comprise of microscopic techniques (transmission electron microscopy, scanning electron microscopy, phase contrast microscopy, laser microscopy and immuno-florescent microscopy), biomechanical testing (using special apparatus and criteria such as stress, strain, load, deflection, force, displacement, ultimate strength, fracture stiffness), biochemistry and molecular biology methods (measuring various, serum and urine biomarkers, proteins, DNA, RNA markers). The strength of callus and bone can be measured *ex vivo* by 3-point or 4-point bending and correlated with radiological assessment. Several stains and histopathological techniques can be used to measure specific components of bone (for example: alkaline phosphatase for osteoblast, Tartrate Resistant Acid Phosphatase or TRAP for osteoclasts, Von Kossa and counterstaining with toluidine blue for mineralized bone and cartilage in callus, calcein, tetracycline and alizarin for labeling mineralizing bone surfaces among others). There are numerous scoring systems for each method of evaluation for bone healing (reviewed in Bigham-Sadegh 2015). By comparing the sensitivities of different methods it was discovered that whole-bone stiffness is of limited reliability to assess the healing quality particularly at the late stages of the healing of long bones (Chen 2015).

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The comparison of ultrasonometric (ultrasound propagation velocity or UV) and computed-tomographic imaging techniques to study bone healing using sheep tibiae revealed that both methods are similarly effective in monitoring the post-operative healing status after a regular and resection osteotomy (Barbieri 2012). Methods have been developed to measure mouse femoral bone fracture toughness during crack initiation and propagation (Carriero 2014).

CONCLUSION

Considering the increasing population of aged people in the world, cases of bone fractures and compromised bone repair are also likely to increase. As currently used repair approaches such as autografts, allografts, xenografts and tissue engineering have several caveats, efforts are being made to repair fractures by tissue engineering using growth factors, nanostructured scaffolds and biomaterials and stem cells. Hypertrophic cartilage, which has all the signals for bone tissue formation, vascularization, remodeling and functional bone marrow development, may accurately model bone tissue through endochondral ossification. Techniques such as 3-D printing may also have enhanced repair potential in the future.

Through multidisciplinary technologies and approaches, numerous animal models are available to create and repair diverse types of bone fractures. However, experimental animal models should reflect the human fracture scenarios as closely as possible. Therefore, such models should be carefully selected to test the aforementioned materials and surgical methods which could benefit human patients as the ultimate goal is to improve their quality of life.

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Chapter 16

EXPERIMENTAL SOLID ORGAN TRANSPLANTATION IN SMALL ANIMALS

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ABSTRACT

Clinical organ transplantation for patients with end-stage organ failure has become an acceptable choice of treatment. Experimental solid organ transplantation modes in small animals have offered a unique advantage for addressing mechanistic questions of transplant rejection. In particular, rodents (rats/mice) are frequently selected to achieve experimental conclusions that probably can form the basis of clinical strategies, because their anatomical, physiological and immunological characteristics are similar to those of humans. Therefore, currently most of research projects are still performed in rodents. Nowadays, multiple organ transplant models in small animals have been developed and extensively used in transplantation research, based on their technical feasibilities. Of note, patterns of allograft rejection or tolerance vary between donor and recipient strain combinations, and among different transplanted organs. This raises concerns about the selection of an ideal model when using small animals as solid organ transplantation models. In this chapter, we will briefly review several most commonly used vascularized solid organ transplantation models in rodents, and hope that it may provide a better understanding and useful choices for certain organ transplants in small animal models.

Keywords: experimental model, solid organ, transplantation, rodent, microsurgery

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ABBREVIATIONS

ALT	Alanine aminotransferase
APCs	Antigen presenting cells
AST	Aspartate aminotransferase
BUN	Blood urea nitrogen
CAN	Chronic allograft nephropathy
CAV	Cardiac allograft vasculopathy
CCA	Common carotid artery
Clcr	Creatinine clearance
ECG	Echocardiography
EJV	External jugular vein
EKG	Electrocardiography
IVC	Inferior vena cava
LA	Left atrium
LV	Left ventricle
MHC	Major histocompatibility complex
MRI	Magnetic resonance imaging
MST	Median survival time
PET	Positron emission tomography
RA	Right atrium
RV	Right ventricle
SCr	Serum creatinine
SMA	Superior mesenteric artery
SVC	Superior vena cava
UBM	Ultrasound biomicroscopy

INTRODUCTION

Life-saving solid organ transplantation is one of the most remarkable therapeutic successes in modern medicine. It has progressed initially from early stage of animal experiment to routine, reliable and effective clinical practice during the past 70 years. In the early days of experimental organ transplantation, big animal models were most commonly used, because anastomotic techniques were relatively easier with larger vassals. The most important development in this filed was from late 19th into early 20th century while French surgeon Alexis Carrel first used sutures to anastomose blood vessels in animals (Doyle 2004). Subsequently, his anastomotic techniques were adopted by other surgeons and became the basis for the standard vascular anastomotic techniques used today. In the early 1900s, Dr. Carrel performed kidney transplantation into the neck of the same dog, which allowed this animal to survive for several years and have offspring (Carrel 1905). A few years later, he also successfully performed kidney transplantation between different dogs. Due to his pioneering vascular suturing techniques, he received a Nobel Prize in 1912 in Physiology or Medicine.

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In parallel to advances of microsurgical techniques and instrumentation, experimental organ transplantations in small animals were also being conducted from the 1960s, and became preferred models. Compared with big animals, the disadvantage of small animal is their smaller size for performing microsurgery. Thus, a higher level of microsurgical technical skill is demanded to achieve microsurgery in small animals. However, small animals, such as rodent models offer certain advantages over big animal models owing to the relative ease and comparative low cost of breeding. Moreover, there has been an increased availability of genetically, both transgenic and knockout, well-characterized strains of rodents. Nowadays embryonic stem cells have been isolated and genetically manipulated in mice, rather than in other mammalian animals (Niwa 2001). For immunology research, the mouse immune system indeed represents the ideal platform, and provides sufficient homology for pathway determinations and mechanistic studies (Anderson 2013). In addition, there have been a large number of specific monoclonal antibodies and immunological reagents available for rodents. These facts make the rodent models invaluable in transplant research.

Nevertheless, there are several potential limitations in rodent models. Unlike other large animal models, mice do not constitutively express class II antigens on vascular endothelium (Pescovitz 1984; Choo 1997). Methods of tolerance induction in mice have been limited by inability to repeat with large animals, especially in nonhuman primates and humans (Sachs 2003; Kirk 2003). In addition, allograft survival in rodent models varies depending on specific transplanted organs and donor-recipient strain combinations. Zhang et al. (Zhang 1996) demonstrated that all mouse intestinal allografts were rapidly rejected, and heart allografts also developed acute rejection without spontaneous acceptance, whereas kidney grafts showed both spontaneous acceptance and rejection depending on donor-recipient strain combinations. In contrast, the majority of liver allografts were spontaneously accepted despite fully major histocompatibility complex (MHC)-mismatched strain combinations (Zhang 1996). The rejection pattern of the mouse uterus grafts is similar to that of heart transplants (El-Akouri 2006). In general, the allograft rejection in mice appears less severe, compared to that in rat models (Zhang 1996). Clearly, further studies are warranted to determine values and limitations of rodent transplantation models, and identify physiological barriers to tolerance induction.

To date, more than 95% of organ transplantation studies are performed in rodents in research projects (Wang 2012). With state-of-the-art microsurgical technique, solid organ transplants in rodents including heterotopic heart, kidney, liver, small bowel, lung and uterus can be performed with a high success rate. All of these organ transplantation models are useful tools in fundamental organ transplantation research. However, each organ graft has a unique rejection pattern, and therefore its application varies depending on the specific need of research. In this chapter we will focus on the heterotopic heart, kidney, liver, small bowel and lung transplantation models in rodents (mice/rats), as these methods have become the predominant models used owing to their technical feasibilities, and the most needs for preclinical research when assaying transplantation immunity and developing novel therapeutic approaches that prevent allograft rejection.

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HETEROTOPIC HEART TRANSPLANTATION IN MICE

The heterotopic heart transplantation in mice nowadays is one of the most frequently applied transplant models. It was described for the first time by Corry in 1973 (Corry 1973). The surgical procedure was comparable to the heart transplantation in rats as performed by Ono et al. in 1969 (Ono 1969). The technique involves end-to-side anastomoses of the donor ascending aorta and pulmonary artery to the recipient abdominal aorta and inferior vena cava (IVC), respectively. After grafting, the heart graft quickly starts contracting. This leads to a retrograde blood flow from the recipient abdominal aorta via the donor ascending aorta directly into the coronary arteries, thus irrigating the myocardium by capillary bed. Then, blood flow is drained from coronary circulation back into the coronary veins, coronary sinus, the right atrium (RA) and the right ventricle (RV), and finally reaches the recipient IVC via the main pulmonary trunk. Hence, the presented model is associated with a non-functional heart graft because it does not pump blood maintaining physiological blood pressure despite heart graft contractions.

Since the model was introduced in 1973, many modified methods have subsequently been reported to continuously improve and refine techniques (Gu 2007; Wang 2006; Wang 2003; Li 2004). Basically, two different sites, abdominal or cervical, can be experimentally used for the fully vascularized heterotopic heart transplantation in mice. A suture technique of the cervical heterotopic heart transplantation was first performed in 1991, with the anastomoses of the donor innominate artery and pulmonary artery to the recipient common carotid artery (CCA) and external jugular vein (EJV), respectively (Chen 1991). Because of the small size in diameter of cervical vessels, this alternative technique is technically more difficult to perform vascular anastomoses with the sutures, and in general, more frequently produces complications. Subsequently, cuff techniques, instead of sutures for the vascular anastomoses, were established by several authors (Matsuura 1991; Tomita 1997; Wang 2005; Feng 2005; Gu 2007). Indeed, in the cervical model, the cuff technique is preferred over the suture technique in terms of revascularization, due to its lower technical requirement and higher surgical success rate (Zhou 2010). However, regarding the abdominal model, vascular sutures cannot be substituted by cuff techniques, due to its exclusive fashion of end-to-side anastomosis in revascularization of the graft. Since the vascular suture method is much more frequently associated with anastomotic complications than the cuff technique, the abdominal model has even been considered to be replaced by the cervical model with cuff techniques (Wang 2007). However, the carotid artery is much smaller in diameter than the ascending aorta of the donor, so that anastomoses are not easier to perform (Doenst 2001) in the cervical site, despite the introduction of cuffs. Moreover, the transplanted heart graft positioned in the abdominal cavity is likely self-guided and less twisted (Doenst 2001). Therefore, most of researchers still prefer the abdominal model based on their experiences and abilities with this method to produce reliable results with limited experimental variables (Hasegawa 2007). Nevertheless, the cervical model as a second-choice site provides the possibility for the heart re-transplantation or the second graft transplant for the purpose of testing donor-type organ-specific tolerance, when the abdominal site has been occupied by a transplanted heart graft (Chen 1991).

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The function of the transplanted heart is evaluated by palpating the heartbeat of the graft in recipients. This sensitive diagnostic method is easy, effective and reliable for monitoring transplant rejection. Such advantages of the immediate vascularization and the ease of monitoring rejection noninvasively make the model excellent, and popular to study alloreactivity that results in acute rejection. In addition, several alternative means, with advantages and limitations, have been employed for graft rejection evaluations, such as electrocardiography (EKG) (Superina 1986; Mottram 1988), ultrasound biomicroscopy (UBM) (Zhou 2007), echocardiography (ECG) (Książek 2016; Scherrer-Crosbie 2008), and magnetic resonance imaging (MRI) (Figueiredo 2009). However, the most simple and reliable form of assessing rejection is still the direct abdominal palpation of the graft (Zhang 1996).

Donor MHC antigens elicit graft rejection by interacting directly with recipient T cells, or indirectly as donor MHC–derived peptides expressed on recipient MHC molecules (Felix 2000). Fully MHC-mismatched (both class I and class II MHC allelic differences) heart allografts are quickly rejected within 7–10 days (Qian 1996; Chong 2013; Schenk 2008; Kwun 2011; Corry 1973). The survival times of heart grafts from MHC class I or class II-deficient mice are significantly prolonged to 30–40 days in fully MHC-mismatched hosts (Qian 1996). Moreover, single class I or class II MHC-mismatched heart allografts or MHC-matched/multiple minor histocompatibility antigen-mismatched allografts can generally take a considerable amount of time to be rejected, and can survive for more than 60 days (Hattori 2012; Schenk 2005). This lack of acute rejection is probably because of $CD25^+$ regulatory T cell mediated-inhibition of alloreactive T cells, which play a dominant role in mediating acute graft rejection (Schenk 2005).

The orthotopic heart transplantation in mice is technically challenging, and to our knowledge, is currently unavailable (Figueiredo 2009). Instead, the heterotopic heart transplant model in mice has been extensively used to address basic immunologic questions of rejection and tolerance. To date, this technique is considered to be the best model in transplantation study. However, the retrogradely perfused, beating but nonfunctional graft, with a total mechanical unloading of left ventricle (LV), is the shortcomings of the model, which leads to mural thrombi form in the LV and progressive atrophy of the transplanted graft (Baldwin 2014). To address this issue, Figueiredo et al. (Figueiredo 2009) established the partial LV loading model in mice by heterotopic combined heart/lung transplantation. However, whether the volume-loaded heterotopic heart transplantation is superior to the classical myocardial unloading model in rodents remains controversial (Didié 2013; Tang-Quan 2010; Liu 2015).

Heterotopic Heart Transplantation in Rats

The heterotopic abdominal heart transplantation model in rats, anastomosing donor ascending aorta and pulmonary artery to the recipient abdominal aorta and IVC, respectively, by an end-to-end fashion, was first described in 1964 by Abbott et al. (Abbott 1964). This technique opened a new avenue in terms of establishing fully vascularized organ transplantation models in small animals. However, the end-to-end anastomosis procedure completely shunted the blood flow of the recipient lower extremities to the heart graft, leading to severe complications of paraparesis and paraplegia, consequently restricting the

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recipient survival. Subsequently, Ono and Lindsey (Ono 1969) modified the model with end-to-side anastomoses for both the donor ascending aorta to the recipient abdominal aorta and the donor pulmonary artery to the recipient IVC, allowing an adequate blood flow to and from the lower extremities of the recipient. As a result, the refined technique achieved a 90% graft and recipient survival. Since then, the classical Ono and Lindsey model of the rat heterotopic heart transplantation has been widely used in research. Over the last 4 decades, there have been numerous modifications reported for simplifying the technique and improving reliability of the model. Nowadays, the rat heart transplantation models used for locating a heart graft involve cervical, femoral, or abdominal sites in recipients. Each site has inherent advantages and disadvantages. The model should be selected cautiously, depending on the specific needs of research. The abdominal site model using the end-to-side anastomosis of relatively larger vessels likely is performed easily, with a higher success rate. Thus, the abdominal site model is still the most preferred option for the rat heterotopic heart transplantation in many centers, because the required microsurgical suturing technique is technically less challenging. As compared to abdominal site model, the principal drawback of the cervical and femoral site models is that vessel diameters are smaller (Fensterer 2014), making anastomoses more challenging, which may lead to an increased failure rate. However, Ma et al. (Ma 2011) carried out a study to compare two models of cervical and abdominal heart transplantation in rats, and concluded that two models are comparable in terms of both achievement of success rate and the histologic features of acute allogeneic rejection. Moreover, due to the feasibility of the end-to-end fashion of the arterial and venous anastomoses, the cervical site model provides the possibility for using cuff techniques for the rat heart transplantation (Gu 2007; Xiu 2001; Matsuura 1991). The cuff technique is favored, because it reduces the graft ischemic time and enhances the operative success rate of the small vessel anastomosis procedure when performed by surgeons without a high level of microsurgical skills (Zhou 2010). Overall, the femoral site model is technically difficult because of vessels at the femoral site are very thin and the operative space is narrow, consequently lengthening the procedure time and increasing the ischemic time of grafts (Gordon 2007a; Gordon 2007b). However, the femoral site model enables easier handling of grafts, and allows more precise evaluation of the rejection endpoint through using the ECG (Gordon 2007a). On the other hand, cervical and femoral models may provide more possibilities for the re-transplanting of the second or third heart grafts, in the transplantation studies.

The graft function in terms of heartbeat is monitored by direct abdominal palpation that provides immediate, accurate evaluations with non-invasiveness. In addition, ECG (Voiglio 1995), EKG (Wada 1999) and fluorescence-based surveillance (Morgan 1999) have been reported for monitoring transplant rejection with limited predictive accuracy or practicability.

Fully MHC-mismatched rat heart allografts, such as Dark Agouti (DA; $RT1^{av1}$) donor to Wistar Furth (WF; $RT1^{u}$) recipient (Syrjälä 2014), DA donor to Lewis (LEW; $RT1^{l}$) recipient (Ma 2011), Brown-Norway (BN) donor to LEW recipient (Wada 1999), LEW.1W ($RT1.A^{u}$ B^{u} D^{u}) donor to LEW.1A ($RT1.A^{a}$ B^{a} D^{a}) recipient (Pêche 2006), RA ($RT1^{p}$) donor to PVG ($RT1^{c}$) recipient (Koshiba 2003), and LEW or ACI ($RT1^{a}$) donor to Buffalo ($RT1^{b}$) recipient (Goss 1993) are rejected within 10 days. Partially MHC-mismatched rat heart allografts of LEW donor to F-344 recipient with certain immunosuppressive drugs showing a chronic heart graft rejection, have been used to study cardiac allograft vasculopathy (CAV) (Hölschermann 2000; Suzuki 1999; Fujino 2012).

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In the classical Ono and Lindsey model of the heterotopic abdominal heart transplantation in rats, blood from the recipient abdominal aorta via the donor ascending aorta enters the coronary circulation. Due to the closed aortic valve, the LV is unloaded in this model. Thus, this technique is a less adequate model for the hemodynamic or function study, because its total mechanical unloading LV of the transplanted heart leads to substantial cardiac atrophy and functional deterioration. To circumvent these limitations, several alternative surgical techniques in order to partially load the LV have been proposed (Asfour 1999; Klein 1991). Asfour et al. (Asfour 1999) developed a volume-loaded heterotopic heart transplantation model via the anastomosis of donor pulmonary artery to left atrium (LA). In addition, the donor ascending aorta and superior vena cava (SVC) is end-to-side anastomosed to the recipient abdominal aorta and IVC, respectively. In this model, some of the coronary circulation blood flow return to the LV via the additional anastomoses, thus allowing for a partial loading of the LV. Comparing the partial volume-loaded and classical volume-unloaded models, Didié et al. (Didié 2013) concluded that the partial volume-loaded model shows superior morphological as well as functional preservation. However, there are controversies surrounding this topic. The extra surgical procedures required in the partial volume-loaded model result in longer ischemic time of the heart graft. And moreover, the mixture of deoxygenated blood returning to arterial coronary circulation may cause the myocardial chronic ischemic injury and dysfunction (Tang-Quan 2010). In this regard, it was argued that the classical non-loaded heterotopic heart transplantation in rats remains a more practical model relevant to preclinical transplant studies (Tang-Quan 2010).

KIDNEY TRANSPLANTATION IN MICE

The first kidney transplantation in mice was performed by Skoskiewicz et al. in 1973 (Skoskiewicz 1973). The technique they reported was similar to the model in rats described by Fisher et al. in 1960s (Lee 1967). Because of the small size of vessels (less than 0.4 mm in the diameter of the mouse renal artery), the technique is extremely difficult in vascular anastomoses with much higher incidences of surgical complication, an almost 30−50% mortality rate, so that its wide use was limited over next twenty years. Since 1990s, several modified techniques have been reported to overcome the small size of the vessels in using the donor aorta (Kalina 1993) rather than the base of renal artery to form the arterial anastomosis. Such a refinement significantly improved the technique, making it more practical and reliable for studying immunological mechanisms of transplant rejection. Nevertheless, to date, the kidney transplantation in mice is still highly challenging, and can be performed only in a few transplant research centers in the world.

Since the model established, there have been many modified techniques reported (Kalina 1993; Zhang 1995; Han 1999), thus greatly contributing to the development of the model. Similar to clinical renal transplantation, basally, a functioning kidney transplant requires establishment of vascular flow through arterial and venous anastomoses between donor and recipient vessels, and the connection of the donor ureter into recipient bladder. To date, the most commonly used experimental kidney transplantation method in mice is a heterotopic rather than orthotopic model, which creates an arterial anastomosis by the end-to-side anastomosis of the donor suprarenal (Kalina 1993) or infrarenal (Zhang 1995) aorta to the recipient abdominal aorta such that the donor aorta acts as a conduit to the renal artery. The

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donor renal vein can be connected to the recipient IVC by an end-to-side anastomosis, or connected to the recipient renal vein by a cuff technique (Chen 2013). The urinary tract reconstruction can be performed by anastomosis between the donor and recipient bladder patch (Kalina 1993; Zhang 1995; Chen 2013) or direct insertion of the donor ureter into the recipient bladder (Han 1999). Although many researchers currently still prefer the *bladder patch*-to-*bladder anastomosis technique* for restoration of urinary tract continuity (Kalina 1993), it has been suggested that direct implantation of the donor ureter into the recipient bladder is a quicker and easier procedure, which is associated with reduced surgical complications (Han 1999).

Unlike the model of the heterotopic heart transplantation, the kidney transplantation can be as an ideal life-supporting model, when both native nephrectomies are performed in the recipient, since its survival is dependent on the viability of the graft. To create a truly transplant dependent model, the second native nephrectomy can be immediately performed after grafting. Alternatively, some researchers preferred removing the second native kidney on the 4th−7th postoperative day to avoid an initial period of acute renal failure caused by surgical procedures (Skoskiewicz 1973; Chen 2013; Russell 1978; Benson 1985; Pratt 2002). Nevertheless, in many cases, for example, for short-term immunological and histological assessments, the recipient native kidney has been left *in situ*, so that the recipient mouse is not dependent on the transplanted graft for survival (Wang 2004; Inoue 1991; Wyburn 2006). Such an advantage of the mouse undergoing a limited surgical procedure reduces the surgical mortality, and prevents animals from the adverse effects of the gradual renal failure.

The function of the kidney graft in the nephrectomized recipient is assessed by the quantification of serum creatinine (SCr) and blood urea nitrogen (BUN), as well as creatinine clearance (Clcr) (Wang 2010), similar to clinical renal transplantation. Using the I-Stat portable clinical analyzer (Heska Corp., Fort Collins, CO), the SCr can be sequentially monitored with less than 0.2 ml whole blood sample from the retro-orbital sinus or lateral tail veins without sacrificing recipients (Bickerstaff 2008).

The kidney transplantation in mice has provided a potential model for studying acute and chronic graft rejection that is clinically more relevant. Several different combinations of inbred mouse strains have been used in kidney transplantation studies with varying progression and pattern of kidney allograft rejection. Acute allograft rejection can be observed in some strains of fully MHC-mismatched combinations, including C57BL/6 recipients of BALB/c or B10.BR allografts (Qi 1999; Brown 2007). Generally, kidney allografts with either a single class I or Class II MHC-mismatched strain combinations can survive for a long period without immunosuppression (Skoskiewicz 1973; Russell 1978). However, even with the fully MHC-mismatched combination such as C57BL/6 recipients of DBA/2 allografts, the majority of kidney grafts are accepted (Brown 2007), whereas heart grafts are usually rejected in this strain combination. Underlying mechanisms remain to be further elucidated. Of note, survival results vary among different research groups. For example, BALB/C allografts in C57/BL6 recipients have been reported with a survival time as low as 8 days (Qi 1999), versus that over 100 days in the other study (Lutz 2007). Mechanisms contributing to the discrepancy in allograft survival time observed in different groups remain unclear. Some authors were concerned that surgical factors may be involved (Tse 2013). Overall, the kidney transplantation model in mice is technically more demanding to perform reliable surgical procedures, and it is difficult to monitor allograft rejection as well, compared with the heterotopic heart transplantation model, while limiting its wide use.

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KIDNEY TRANSPLANTATION IN RATS

The first report of the rat kidney transplantation was presented at the American College of Surgeons Meeting in Chicago in 1961 by Bernard Fisher and Sun Lee (Shrestha 2014), and subsequently published in the middle of the 1960s (Fisher 1965; Lee 1967). Initially, the kidney graft was transplanted into the recipient in an orthotopic position. End-to-end anastomoses were performed with the donor renal artery and vein to the remains of the recipient renal artery and vain after nephrectomy. The urinary tract was also reconstituted by an end-to-end anastomosis between the donor and the recipient ureters. Since then, several modified microsurgical techniques with respect to improving surgical procedures have been reported over the last four decades. Each of them with certain advantages and disadvantages has contributed to the development of the model.

Currently, commonly used microvascular surgical techniques include (1) the end-to-end anastomosis of the donor renal artery and vein to the recipient renal artery and vein; (2) the end-to-side anastomosis of the donor renal artery and vein to the recipient abdominal aorta and IVC; and (3) the sleeve technique for the renal artery and the cuff procedure for the renal vein (Schumacher 2003; Lopez-Neblina 1994; Zhang 1991; Zhu 2009). In the end-to-side anastomosis fashion, the graft vessels should have a patch to increase the size of vessels, making anastomoses easier. In addition, Zhu et al. (Zhu 2009) described a cervical heterotopic kidney transplantation technique in rats, where the donor renal artery and vein were end-to-end anastomosed to the recipient left CCA using a sleeve anastomosis, and to the recipient EJV with a cuff technique, respectively. The donor bladder patch was exteriorized to form a cervical cutaneous stoma. Vascular conduits from the donor abdominal aorta and IVC in continuity to the transplanted organ vessels are often employed in other organ transplantation models, such as the small bowel transplantation in rats (Wasserberg 2004; Santiago 1992; Bakonyi Neto 1999; Wasserberg 2003). Based on this technique, Karatzas et al. (Karatzas 2007) used vascular conduits from donor abdominal aorta and IVC in continuity to renal vessels, to perform arterial and venous end-to-side anastomoses to the recipient abdominal aorta and IVC, respectively. This method is similar to the procedure of the kidney transplantation in mice. Although the extra procedures are required for harvesting the donor abdominal aorta and IVC, the technique likely makes vascular anastomoses easier. There are several procedures available to restore the continuity of the urinary tract, including the anastomosis of (1) the donor bladder patch to the recipient bladder, (2) the donor ureter to the recipient ureter with or without a ureteral stent, and (3) a direct insertion of the donor ureter into the recipient bladder.

Specific technical factors may affect outcomes of the graft function and survival. Spanjol et al. (Spanjol 2011) showed that the end-to-end vascular anastomosis has a better result with respect to the transplanted renal graft survival compared to the end-to-side fashion. Schumacher et al. (Schumacher 2003) argued that the end-to-end or end-to-side vascular anastomoses are preferable to the sleeve technique, because the latter in small arteries may cause the reduced blood flow, and subsequently result in a higher incidence of stenosis. Regarding urinary reconstruction, due to inadequate blood supply to the distal ureter and bladder of the donor, the anastomosis of the donor bladder to recipient bladder is associated with ischemia and necrosis at the anastomotic site, leading to urine leakage with fatal consequences. A ureteral stent is often adopted in the clinical kidney transplantation to reduce the incidence of urine leaks and ureteral stenosis (Wilson 2013; Gomes 2013). However, the

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placement of a stent within a ureter, which may be associated with the calculi formation and obstruction of the urinary tract, should be avoided (Martins 2003). Overall, direct implantation of the donor ureter into recipient bladder is an easier and more reliable procedure, which should be recommended for the urinary reconstruction in the model.

Basically, post-transplanted renal grafts are monitored by renal retention parameters, such as SCr and BUN. Xue et al. (Xue 2016) showed that the increasing levels of serum hepcidin and SCr were significantly correlated with impairment of renal function with the progression of chronic allograft nephropathy (CAN), suggesting that hepcidin may be a potential biomarker for monitoring the impaired renal functions after kidney transplantation. Moreover, Reuter et al. (Reuter 2009) proposed a non-invasive, entirely image-based method for assessment of renal allograft rejection in the rat model, based on high-resolution whole body small animal positron emission tomography (PET), following injection of ^{18}F-fluorodeoxyglucose. In addition, non-specific symptoms frequently presenting in acute or chronic rejection, such as proteinuria, oliguria, hypertension, graft tenderness and peripheral edema, should be observed.

Acute renal allograft rejection commonly occurs in fully MHC-mismatched rat strain combinations, such as WF to LEW (Li L 2016; Yang 2015), LEW-BN to LEW (Grabner 2013; Reuter 2009) and DA to LEW (Grau 2011) rat combinations. For example, without immunosuppression, DA grafts in LEW recipients lead to death of recipients from acute rejection around 7 days after kidney transplantation (Grau 2011).

Despite dramatic improvements in developing new immunosuppressant therapies, late graft loss after kidney transplantation remains an unsolved problem (Jevnikar 2008; Xue 2016). The CAN is the leading cause of late allograft loss. The kidney transplantation model in rats has provided an important tool for understanding the pathogenesis and molecular biology, and identifying potential treatment of the CAN (Shrestha 2014). The rat CAN model was first established by White et al. in 1969 (White 1969), using the F344 rat as the donor and the LEW rat as the recipient without immunosuppression to produce chronic allograft rejection. In such a rat CAN model, impairment of renal function and pathogenesis are similar to that occurred in clinical human transplantation. Therefore, this model is regarded as a standard model, and most commonly used to study the CAN (Shrestha 2014). The F344-to-LEW model can be employed to induce CAN because the rat strains differ partially at MHC (class I and class II) and various non-MHC loci, thereby allowing transplant without immunosuppression, which cannot be done in other fully MHC-mismatched rat strain combinations. However, with use of certain immunosuppressive agents, other combinations, such as DA-to-BN, DA-to-WF, DA-to-LEW, SD-to-WF, and LEW-to-BN have also been used to induce the CAN, depending on the aim of the individual study (Shrestha 2014).

LIVER TRANSPLANTATION IN MICE

Based on the technique of the orthotopic liver transplantation in rats (Lee 1973; Zimmermann 1978; Kamada 1979), the orthotopic liver transplantation model in mice was established by Qian et al. in 1991 (Qian 1991). In the model, the suprahepatic inferior vena cava of the donor and recipient was end-to-end anastomosed with a continuous suture method, and then cuff techniques were used for anastomoses of the portal vein and the infrahepatic vena cava, respectively. Biliary continuity was restored by tying the ducts over a

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tube stent. It was suggested that the anhepatic time should be limited to less than 20 min (Qian 1991). In this model, hepatic rearterialization of liver graft with reconstitution of the hepatic artery was not included. Therefore, it is not physiologically accurate, which was considered as a shortage of the model. However, due to the technical simplicity, it remains popular today at many research centers (John 2007; Conzelmann 2003; Nakagawa 2012; Que 2004; Li 2008; Birsner 2004). Later, Tian et al. (Tian 2002) developed an arterialized liver transplantation model in mice, where an arterial segment consisting of the hepatic, celiac and aorta from the graft was end-to-side anastomosed to the recipient abdominal aorta. The data demonstrated that arterialization of liver transplants in mice results in an improved function compared with grafts without hepatic artery (Tian 2002). Consistent with this study, Shen et al. (Shen 2005) showed that arterialized mouse liver transplants have tremendous benefits compared with non-arterialized grafts regarding ischemia injury and recipient survival after a prolonged cold ischemia (24 h). These findings suggested that arterial reconstruction might be a necessary procedure for consistent animal survival and reduced liver injury, and thus provide a superior and physiologically relevant model for liver transplantation research. Based on the previous technologies, Tian et al. (Tian 2003) established arterialized partial (50% and 30%) mouse liver transplantation model. The surgical procedure is similar to the standard model, but partial hepatectomy is achieved by removal of relevant lobes on the donor liver. The result indicated that the partial liver transplantation between a graft volume of 30% and 45% is feasible in the mouse model, which is similar to clinical patients (Tian 2003).

After transplants, the liver graft function is monitored by determination of the serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels as markers of hepatocyte injury (Iu 1987), and alkaline phosphatase levels served as a marker of bile duct injury (Tian 2002). In addition, other biochemical determinations, such as bilirubin, total protein, and albumin are often included (Qian 1991).

In mice, liver allografts are accepted across MHC barriers without requirement for immunosuppressive therapy (Qian 1994; Jiang 2006). For example, liver grafts can be permanently accepted with a median survival time (MST) >100 days in the strain combination of the C57BL/10 (B10; H2b) to C3H (H2k) mouse. In the same strain combination, heart grafts are quickly rejected with an MST of 10 days (Qian 1994). The underlying mechanisms are still unknown. There have been many proposed mechanisms regarding the ability of the transplanted liver to be accepted by the recipient. Dahmen et al. (Dahmen 1994) found that cells isolated from the recipient spleen and liver graft-infiltrating cells exhibit a strong donor and third-party alloreactivity *in vitro*, though hyporesponsive *in vivo* . This phenomenon has been named as "split tolerance." Findings have suggested that restoration of apoptotic activity of liver graft-infiltrating cells and recipient spleen cells, especially $CD8^+$ T cells, is critical for the development of liver transplant survival and tolerance (Qian 1997; Li 20011a; Li 2001b). Li et al. (Li 2008) showed that $Foxp3^+CD25^+CD4^+$ regulatory T cells were increased within liver grafts and recipient spleens. Depletion of recipient $CD25^+CD4^+$ T cells increased proliferative response of $CD4^+$ T cells and cytotoxic T lymphocyte induction of $CD8^+$ T, and induced acute liver allograft rejection (Li 2008; Jiang 2006). Similarly, blockade of CTLA4, an effector molecule of $Foxp3^+CD25^+CD4^+$ regulatory T cells, protects allo-reactive T cells from apoptotic death, leading to liver graft rejection (Li 2005). These findings suggest that Treg-mediated immune suppression may play an important role in the induction of liver transplant tolerance.

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However, why this occurs only in liver grafts, but not in other organ grafts, remains to be elucidated.

LIVER TRANSPLANTATION IN RATS

The orthotopic liver transplantation in rats was pioneered by Lee et al. in 1973 (Lee 1973), in which all anastomoses were performed using hand-suture techniques. Due to relatively longer operative time, an extracorporeal portosystemic shunt was used in the model. Later, the technique was simplified by elimination of the hepatic artery anastomosis and the extracorporeal portosystemic shunt (Lee 1975). However, because of technical demand and long operative time, this model has not been widely used (Ishii 2013). Since the introduction of the cuff technique making a vital improvement of the model in 1979 by Kamada et al. (Kamada 1979), the method has remained the gold standard, and became one of most commonly used model worldwide. In this model, the suprahepatic inferior vena cava is end-to-end anastomosed with a running suture technique, and the portal vein, the infrahepatic vena cava and the bile duct are reconstructed using cuff method. This technique has significantly simplified microvascular anastomoses and shortened the operation and especially anhepatic time in the recipient, thus enabling higher success rates of the long-term survival. However, hepatic artery reconstruction of the graft is not included, although it is routinely performed in clinical transplantation. Engerman et al. (Engemann 1982) argued that rearterialization of the liver graft was necessary, and established an end-to-side anastomosis between the donor aortic segment and the recipient infrarenal aorta to reconstruct hepatic artery. Later, Howden et al. (Howden 1989) developed an end-to-end sleeve anastomosis between the donor celiac axis and the recipient right renal artery. However, in his technique, an extra surgical procedure of right nephrectomy is required, making the method less optimal. Hereafter, there have been numerous techniques reported to establish hepatic rearterialization, greatly contributing to the development of the rat liver transplantation (Gao 1993; Huang 2011; Hasuike 1988; Steffen 1989; Howden 1989; Li 2002; Lehmann 2005; Liu 2015; Tan 2005).

Hepatic artery reconstruction is considered as an important step in human and large animal liver transplantations (Uchiyama 2002). However, it remains controversial in the rat liver transplantation. Some studies showed that there were neither significant improvement in recipient survival, nor histologic differences in hepatocyte or bile duct architecture between arterialized and non-arterialized grafts, suggesting that arterial reconstruction might be an unnecessary procedure of the operation (Kamada 1992; Kamada 1983). Thus, arterialization does not have a major contribution to the outcome of the rat liver transplantation. Therefore, biliary complications are more likely to be technique-related rather than the result of ischemia associated with the non-arterialized method (Kamada 1992). On the contrary, Nosaka et al. (Nosaka 1999) reported that the non-arterialized model is associated with higher rates of biliary complications and liver defects. Moreover, several studies showed that the arterialized model of liver transplantation in rats has benefits over nonarterialized transplants in many respects, such as improvement of the recipient survival after prolonged cold ischemia (Gao 1993), antigen presentation (Engemann 1982), microvascular perfusion and graft function

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(Post 1992). Clearly, further studies are warranted for eliciting this issue, especially to exclude surgical technical factors that may markedly influence the outcome of the survival.

The bowel infarction, as a consequence of a long period of portal triad clamping, may cause recipient early deaths within the first three hours after reperfusion (Oldani 2012). Therefore, the ideal anhepatic phase should be limited within 20 minutes. The acceptable ischemia time was considered to be up to 24 hours (Oldani 2012).

In many species, the acceptance or mild rejection *of* liver allografts are often observed, whereas their skin, heart and kidney allografts undergo acute rejection. In particular, in rats without immunosuppressive treatment, liver allografts can be permanently accepted between certain strain combinations (Houssin 1979). For example, a fully MHC-mismatched combination of organ transplants between PVG donors and DA recipients, hearts or kidneys are rejected by <10 days, while livers survive >100 days (Ganbold 2012). Furthermore, PVG rats grafted with a DA liver have been shown to be systemically tolerant with the permanent acceptance of a simultaneous or subsequent graft of DA heart, kidney or skin (Kamada 1980; 1984; 1985). Overall, liver graft has a unique ability to be accepted, and can protect other simultaneously transplanted organs against rejection. This was of particular interest for the clinical setting. However, the underlying mechanism of the phenomenon of non-rejection that is only restricted to liver, but not other organs, is still unclear. In short, due to its technical feasibility, the rat liver transplantation has become a reliable and practical model for studying transplantation immunity, regarding mechanisms of graft rejection, tolerance, and preservation-induced injury in liver transplantation. Nevertheless, its technique is still challenging with a steep learning curve (Hori 2010).

SMALL BOWEL TRANSPLANTATION IN MICE

The small bowel transplantation in mice was first reported by Squiers et al. in 1992 (Squiers 1992). They described a heterotopic technique including end-to-side anastomoses of the donor superior mesenteric artery (SMA) with a Carrell patch to the recipient abdominal aorta, and the donor portal vein to the recipient IVC, respectively. After vascular reperfusion of the graft, ends of the grafted bowel are brought out as stomata and secured to the skin on the abdominal wall. Using this technique, Zhong et al. (Zhong 1993) performed a large number of 247 cases of the small bowel transplantation in mice, with a 13% and 87% success rate for their first 132 cases and last 30 cases, respectively. This report indicated that this procedure is technically difficult and the microsurgical expertise is required for achieving consistent results. Later, He et al. (He 1998) developed a paratopic model of the small bowel transplantation in mice. The primary difference from the previous heterotopic model is that the distal end of the donor small bowel is end-to-side anastomosed to the recipient jejunem, while the proximal end of the small bowel is still brought out as a stoma. More recently, Liu et al. (Liu 2005) described an orthotopic technique for the small bowel transplantation in mice. The vascular anastomosis is similar to the manner as described for the previous heterotopic method. Differently, the entire recipient small bowel is removed, leaving approximately 1.0 cm on each end of the recipient jejunum and ileum, which then are end-to-end anastomosed to the end of the donor jejunum and ileum, respectively.

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Because the small bowel is the most immunogenic organ among all the solid organs (Zhang 1996b), its transplantation has been still unsatisfactory compared to other organs in clinical and experimental transplantation studies. A possible explanation for the high incidence of its rejection over other organs, such as kidney and liver, is that the small bowel consists of a large number of lymphoid organs, such as lymph nodes and Peyer's patches, as well as massive antigen presenting cells (APCs). However, the unique pattern of the alloimmune response to a small bowel allograft remains to be elucidated. In the mouse model, small bowel grafts with MHC-mismatched strain combination are rapidly rejected without any cases of spontaneous acceptance (Zhang 1996b). Moreover, some immunosuppressive agents can effectively prevent rejection of other organs, such as kidney and heart, but not small bowel grafts (Zhang 1996a). Overall, the small bowel transplantation model in mice offers a useful method to study immunology and physiology of the gut transplantation, as well as to investigate immunosuppressive agents. However, this model is limited to use due to extremely technical challenges (Liu 2007).

Small Bowel Transplantation in Rats

The first heterotopic small bowel transplantation model in rats was described by Monchik et al. in 1971 (Monchik 1971). Later, Kort et al. (Kort 1973) developed the model of the orthotopic small bowel transplantation in rats. In both methods, the small bowel is transplanted into the recipient by end-to-side anastomoses of the donor aortic segment to the recipient infrarenal aorta, and the donor portal vein to the recipient IVC, respectively. In the orthotopic model, the recipient native small bowel is removed, and it is replaced by the transplanted small bowel graft in continuity. In contrast, in the heterotopic model, the recipient small bowel is kept intact, so that the small bowel graft is not functionally involved in the continuity of the recipient digestive tract. In general, two different methods have been used for heterotopically locating small bowel grafts (Zhong 1991). In the first one, both ends of the graft are placed as stomata (ileostoma and duodenostoma) in the abdominal wall of the recipient. In the second one, the distal end of the graft is end-to-side anastomosed to the terminal ileum of the recipient, while the proximal end of the graft is exteriorized and sutured to the abdominal wall as a stoma (Nakao 2002). In addition, instead of sutures, the cuff methods have been reported for vascular anastomoses in the model, where the recipient left renal vessels were dissected and the left native kidney was removed, and then the small bowel graft was connected to the recipient renal vessels by a cuff technique (Wallander 1988). Moreover, the rat small bowel can be transplanted in the cervical area with the connections of the donor SMA to the recipient CCA and the donor superior mesenteric vein or portal vein to the recipient EJV, respectively, using the suture (Sonnino 1990) or the artery sleeve and venous cuff (Zhu 2008) techniques.

The well-established rat small bowel transplantation model has been extensively used for investigating various reactions related to the small bowel transplantation. Compared with the orthotopic model, the heterotopic model involves relatively simple techniques, and therefore has a higher survival rate (Nakao 2002). Furthermore, the heterotopic model is available for endoscopic evaluation and biopsy through the stoma (Toyama 1995; Nakao 2001). In fact, the heterotopic model has been the commonly employed model for studying immunological

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reactions of the small bowel transplantation and evaluation of immunosuppressive strategies, although it does not represent a physiologic state of the small bowel. In contrast, the small bowel grafted in the orthotopic model is under physiologic conditions, making it more clinically relevant and suitable for studies of the graft function. Overall, both heterotopic and orthotopic models have advantages and shortcomings. The selection of an appropriate model is important for achieving a specific research goal.

ORTHOTROPIC LUNG TRANSPLANTATION IN MICE

Until 2007, the orthotropic lung transplantation in mice has been first developed by Okazaki et al (Okazaki 2007). Of note, unlike other solid organ transplantation model, which can be anastomosed directly by sutures, the bronchial and vascular anastomoses required for the lung transplantation in mice are all performed using cuff techniques. This technique involves pulling the donor pulmonary artery, pulmonary vein and bronchus through a Teflon Catheter Cuff (24G, 20G and 20G, respectively). Then, vessels and the bronchus are folded over the cuff in order to expose the endothelial surface of vessels or epithelial surface of the bronchus, which is secured with a 10-0 nylon tie. Subsequently, the lung is transplanted by inserting cuff-equipped donor pulmonary artery, vein and bronchus into the respective structures of the recipient, which is also secured with a 10-0 nylon tie.

Clinical lung transplantation is far worse than other solid organ transplantations for long-term survival outcomes (Trulock 2006), mainly due to bronchiolitis obliterans syndrome that is considered as a manifestation of chronic allograft rejection (Chan 2004). The underlying mechanisms are not clearly known. To this end, the heterotopic tracheal transplantation model in mice has been developed to study lung transplantation biology (Hertz 1993). Due to the technical simplicity of this model, it has been widely used for studying immunological mechanisms of lung transplantation leading to obliterative airway diseases (Maruyama 2005; Higuchi 2005). However, the heterotopic tracheal transplantation model in mice lacking vascularization and aeration makes it difficult to draw conclusions about changes that can be only observed in whole lung transplantation. Thus, orthotropic lung transplantation in mice offers a physiological model of lung transplantation for investigating non-immunologic and immunologic mechanisms that contribute to lung allograft injury, despite its technical challenge.

ORTHOTOPIC LUNG TRANSPLANTATION IN RATS

The orthotopic lung transplantation in rats was first reported by Asimacopoulos et al. in 1971 (Asimacopoulos 1971). It was performed with sutures to anastomose the pulmonary artery, the pulmonary vein and the bronchus between the donor and recipient. Because of technical difficulties of the model in the vascular and bronchial anastomoses, its wide use as a standard experimental model of the lung transplantation was limited for more than ten years. In 1989, Mizuta et al. (Mizuta 1989) introduced the cuff technique, instead of sutures for anastomoses of vessels, making the model technically simpler, and more attractive. Later, they also applied the cuff technique in the bronchial anastomosis (Mizuta 1991). Since then,

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this method has become the standard experimental model of the lung transplantation in rats, owing to its technical feasibility and reliability. Subsequently, based on Mizuta's model, several modifications have been reported to simplify the surgical procedure and enhance the quality of the model (Goto 2012; Reis 1995; Sánchez 2007; Santana Rodríguez 2004). Of note, Goto et al. (Goto 2012) introduced cuffs to all of vessels and bronchi in both donor and recipient sides, and then connected them by interposing pieces of the donor descending aortas. This technique provides the possibility of using donor aorta in other organ transplantations.

Lung transplantation has been optioned for patients with end-stage lung disease. However, its outcomes have been greatly limited by the primary graft dysfunction and the bronchiolitis obliterance syndrome (Christie 2010). Experimental animal models of orthotopic lung transplantation have provided a useful physiological model for investigating mechanisms leading to lung allograft injuries from acute or chronic rejection. Because of its low cost, availability of inbred strains, and technical feasibility and reliability, the rat lung transplantation has been considered to be one of the best models, offering a valid alternative for those performed in large animals. However, despite the introduction of cuff methods, its surgical technique is still complex, and therefore it requires a long-term learning process for surgeons to achieve reliable results (Santana Rodríguez 2004).

CONCLUSION

Small animal models of solid organ transplantation provide unique tools for gaining insights into mechanisms of organ transplant rejection, and for developing novel therapeutic approaches that prevent rejection and induce transplantation tolerance. Rodent models of transplantation have become the first choice for fundamental transplantation research, due to their technical feasibility, the availability of numerous well-established transgenic and knockout inbred strains, low costs and simple handling. Nevertheless, there are limitations when translating information obtained from these models to clinic, due to certain inherent differences between rodents and humans in the genetics, physiology and immunology. These limitations can be overcome by using large animals, such as nonhuman primates, which are more close to human being in many ways. Finally, small animal solid organ transplantation models will continue to be important and remain indispensable tools for basic transplantation research.

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Chapter 17

LARGE ANIMAL MODELS IN ORGAN TRANSPLANTATION

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ABSTRACT

Large animal models have been and continue to be an essential link in development of clinical protocols in treatment of patients with end stage organ disease through organ transplantation. Rodent models are often the first step in testing hypotheses or assessing new therapies. Rodent models are however often not representative for clinical scenario and the large animals are indispensable in evaluating safety and efficacy of new techniques, treatment protocols or drug candidates. Porcine models are extremely valuable in testing, validating and fine-tuning organ procurement, preservation, and implantation techniques and protocols prior to clinical implementation. Safety and efficacy of immunosuppressive agents and immunomodulation protocols such as costimulation blockade and immune tolerance will ubiquitously have to be tested in large animals such as non-human primates prior to clinical trials. Furthermore, innovative approaches such as xenotransplantation are first developed in porcine to primate models before human trials. This chapter provides an overview of the current state of large animal models in transplantation research. Additionally, porcine models for kidney, liver, heart and lung transplantation are discussed and detailed protocols are provided.

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Keywords: large animal, transplantation, liver, kidney, heart, lung, porcine, non-human primate, immunosuppression, donor, recipient

Abbreviations

ABG	arterial blood gas
CBD	common bile duct
DCD	donation after cardiac death
DIC	disseminated intravascular coagulation
ECMO	extracorporeal membrane oxygenation
Gal	galactose-a-1, 3 galactose
GDA	gastroduodenal artery
HA	hepatic artery
ICA	internal carotid artery
IJV	internal jugular vein
INR	international normalized ratio
IVC	inferior vena cava
MHC	major histocompatibility complex
NHP	non-human primates
PVR	pulmonary vascular resistance
SLA	swine leukocyte antigen
SVC	superior vena cava
TRAIL	tumor necrosis factor-alpha-related apoptosis-inducing ligand
vWF	Von Willebrand factor

Introduction

Large animal research plays a crucial role in moving a therapy into a more complex *in vivo* system and evaluating the feasibility of a therapy. As therapies are developed for clinical application, they require validation in models that are reasonably predictive of their performance in humans. Large animal models have been used to study the safety and the efficacy of proposed transplant protocols, prior to moving forward with clinical trials. Large animal models have proven to be indispensable to design practical therapeutic protocols and validating mechanistic pathways originating from *ex vivo* and rodent studies (Kirk 2003). In this chapter, we describe why large animal models are needed to advance transplant science in humans. Kidney, liver, and heart-lung models in pigs are further described in detail.

Why Are Large Animal Models Needed?

Immunosuppressive drugs and biologicals have a narrow therapeutic-toxic range and their efficacy and side effects in humans cannot necessarily be extrapolated from rodent studies. Large animal models have been instrumental in predicting efficacy and toxicity of

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new agents and in developing clinical protocols. Chronic immunosuppression is associated with significant morbidity and many short-term and long-term complications such as infections, diabetes and malignancies. There has been an ongoing quest for immunosuppression-free transplantation through induction of immunologic tolerance towards the graft, while maintaining an otherwise intact immune response. In small animals, tolerance has been successfully achieved. Unfortunately, those strategies have proven to be less successful in humans (Levin 1985; Buhler 2002; Millan 2002; Oura 2015). Small animal models might therefore not be adequate in representing the complex human immune network associated with a longer lifespan and much broader exposure to variety of pathogens.

Currently, the shortage of donor organs is the single foremost challenge in the care of patients with end stage organ disease awaiting transplantation. By the end of 2015, nationwide (USA) over 60,000 patients were waiting for a kidney transplant, 14,000 were on liver transplant waiting list and 400 patients on the heart list. During that year, 7,125 liver transplants were performed, while full third of patients died or were removed due to being too sick to undergo transplant. The wait list mortality rate has been increasing during the last several years and reached a new high at 12.1% in 2014. It is clear that shortage of organs is having a detrimental effect on chances of survival for patients in need of a lifesaving transplant. Many approaches have been studied to increase the donor pool. Use of marginal and extended criteria organs has slightly increased overall access to transplant but the general picture has not changed. If successful, use of animal organs for human transplantation could change the current dire situation. Experimental xenotransplantation has come a long way and large animal studies have been paving the road towards clinical trials.

The most commonly used large animals for transplantation studies are dogs, pigs and non-human primates (NHP). Each specific large animal model has its advantages but also its limitations.

Dogs were the animal model of choice in early transplant experiments. Initial successful studies using azathioprine and 6-mercaptopurine were done in dogs (Calne 1982). Over time, canine studies have significantly decreased as social status of dogs has evolved.

NHP models are mostly used in studies evaluating biologics and antibodies, as there is a high degree of interspecies cross-reactivity in targets of monoclonal antibodies. Furthermore, NHPs are the recipient of choice for xenotransplantation studies, using porcine organs (Cooper 2014).

Pigs have been used extensively as they have several benefits. Their use has been more socially accepted as they are used for human consumption. Additionally, pigs have been amenable to genetic modifications. This has opened a window of opportunity to develop organs and tissues that are protected against human immune system. Although this goal has not been reached, major progress has been made in preclinical studies using NHPs as recipient of genetically modified pig organs (Ekser 2012; Cooper 2014). The first genetically altered pig models were those expressing human complement regulatory proteins such as CD59 and CD55 (Fodor 1994; Cozzi 1995). Since then, several other targets have been explored including transgenic expression of human tumor necrosis factor-alpha-related apoptosis-inducing ligand (TRAIL) to control cellular rejection (Klose 2005), human Fas ligand to protect porcine cells from human CD8+ T cells and natural killer cells (Lee 2014), and many other targets. However, the main hurdle in using porcine organs in primates has been hyper-acute rejection caused by the presence of preformed antibodies against galactose-α-1, 3 galactose (Gal). Similar to ABO antibodies, this antibody – antigen interaction causes

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acute endothelial injury, activation of platelets and coagulation cascade and subsequent organ thrombosis and failure. Pigs with reduced or complete deletion of Gal antigen expression were available at the turn of the century (Costa 1999; Phelps 2003). As expected, organs from Gal knock-out pigs were less prone to hyper-acute rejection (Kuwaki 2005; Yamada 2005). With lack of hyper-acute rejection and proper immunosuppression controlling cellular and antibody-mediated rejection, the main hurdle to long term graft survival has been coagulation incompatibility between the host and the donor and development of thrombotic microangiopathy of the pig heart heterotopically transplanted in a baboon (Houser 2004). For unclear reasons, the problem of coagulation incompatibility is more pronounced for kidney transplantation. The host develops consumptive coagulopathy with thrombocytopenia, reduced fibrinogen levels and an increased international normalized ratio (INR) and fibrin degradation products (Ierino 1998; Buhler 2000; Cozzi 2004; Lin 2010). Efforts made to address the coagulation incompatibilities include recombinant expression of membrane-tethered anticoagulants antagonizing tissue factor (Chen 2004), inhibiting platelet activation by Von Willebrand factor (vWF) deletion (Cantu 2007), or protein C activation by transgenic expression of human thrombomodulin (Petersen 2009). In a recent study using Gal knock-out / human CD55 transgenic pig donors, bilaterally nephrectomized baboons survived for over 4 months. Researchers selected NHPs with low anti-porcine antibody levels. Hosts were induced with T-cell depletion and received maintenance treatment with co-stimulation blockade (Higginbotham 2015).

PIG MAJOR HISTOCOMPATIBILITY COMPLEX (MHC)

In swine, the major histocompatibility complex (MHC) is called the swine leukocyte antigen (SLA). It is located in chromosome 7, and most of its class I and II regions have been sequenced. It was first described in commercial breeds, and subsequently, MHC inbred miniature pig lines were established at the National Institutes of Health and the University of Oklahoma (Chardon 1999; Pedersen 2011). The NIH inbred miniature swine MHC (SLA) genotypes (Homozygous AA, CC, and DD herds) were selectively bred from the original breeding pair designated AB and CD. The B allele was lost in the initial breeding (Sachs 1976; Flye 1999). Availability of genetically defined large animal model benefits the studies of transplant immunobiology, and also provides source of homozygous antisera for chemical characterization of MHC products (Sachs 1976).

Pig Kidney Transplant Model

For many reasons outlined above, pigs are the most widely used animals in preclinical kidney transplant studies. Here, we describe a porcine auto-transplant model. Depending on the scope of the study, timing of the donor and recipient surgeries are critical. Several factors need to be taken into consideration and the logistics will be different for autologous transplant versus allo- or xeno-transplant, short versus long preservation times, etc. For the purpose of this work, a heterotopic autologous porcine kidney transplant model will be described. This

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protocol is partially based on the work of Kaths et al. (Kaths 2016). Preoperative and anesthesia management is described elsewhere in this chapter (Porcine liver transplant).

Donor Surgery

The animal is placed in supine position. Depending on the scope of the study, central or peripheral venous access has to be established. For central access, the internal jugular vein can be catheterized. Alternatively, an angiocatheter can be inserted into an ear vein for peripheral venous access. A combination of metronidazole 500 mg and Cefazolin 1 g is used for perioperative antibiotic prophylaxis.

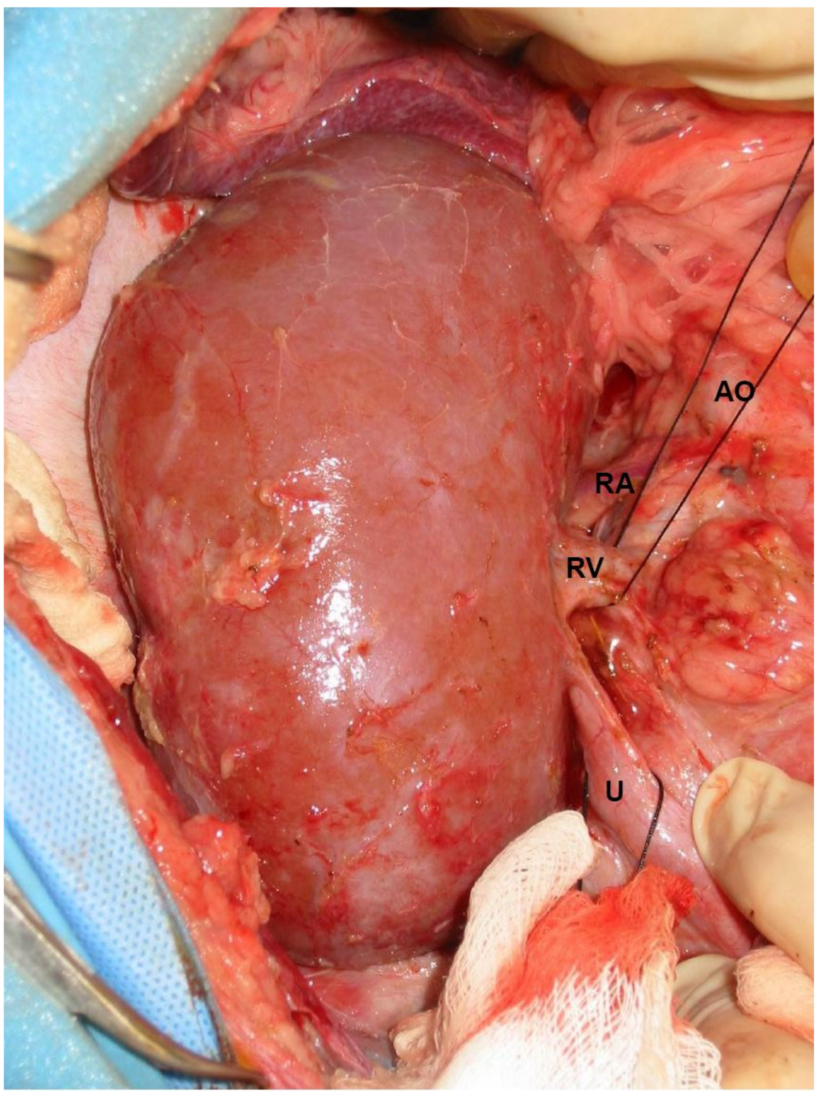

Figure 17.1. Right kidney in donor's abdomen. Renal artery, vein and ureter have been dissected free. Kidney has been completely mobilized. AO: aorta, RA: renal artery, RV: renal vein, U: ureter.

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Abdomen and pelvis are prepped and draped in a sterile fashion. The abdomen is entered through a midline laparotomy and a retractor is needed for adequate exposure. The right kidney is approached by moving the bowls to the animal's left. A sterile surgical towel is helpful to keep the bowl out of the surgical field. The right ureter is then identified and encircled with a vessel loop. The ureter is then dissected free from surrounding tissues using electro cautery. Care needs to be taken to leave sufficient tissue around the ureter in order not to devascularize it. Next, the renal vein and artery are dissected free, all the way to their origin at the level inferior vena cava (IVC) and the aorta (Figure 17.1). The renal artery is prone to spasm which if occurs will reduces the blood flow to the organ. The degree of spasm can be reduced by minimizing direct manipulation of the artery itself and by topical application of papaverine directly over the artery. The kidney itself is further dissected free and lifted form its bed.

Once the kidney including the renal vessels and ureter is completely mobilized, the distal ureter is ligated using (2/0) silk tie and transected proximal to the tie. Heparin is given intravenously (100 U/kg body weight) and 5 minutes are allowed for circulation. Using a vascular clamp, the renal artery is clamped at its origin close to the aorta. Next, the renal vein is clamped at the level of the IVC. The vessels are then transected using a surgical blade or a sharp pair of scissors leaving just enough tissue to safely oversew the stumps. The retrieved kidney is then immediately transferred to a sterile bag resting on an ice basin. Through an arterial cannula inserted in the renal artery, the kidney is flushed with cold preservation solution (e.g., Histidine-tryptophan-ketoglutarate) until clear and at least 500 ml. Depending on the protocol, the kidney can be preserved using static cold storage surrounded by preservation solution or pumped until transplanted. In the donor, the renal artery and vein stumps are over sewn using non-absorbable suture (6/0 Prolene). After adequate hemostasis, bowls are placed in their natural position and fascia is closed using a monofilament (number 1 PDS) running suture, followed by skin closure.

Recipient Surgery

When the focus of the study is on variables that will affect intrinsic graft features such as preservation and ischemia reperfusion, often an auto-transplant model is most appropriate. On the other hand, when investigating rejection, tolerance and other aspects of immune response, an allo- or xenotransplant model will be better suited. In either case, the recipient usually undergoes native nephrectomy at the time of transplant to allow for a precise post-transplant measurement of the graft function. Here, we will describe an auto-transplant case where contralateral nephrectomy is performed during the transplant operation, just prior to the graft implantation. In case of an allotransplant, bilateral nephrectomies will be required.

Preparation for the procedure, anesthesia and antibiotic prophylaxis is similar to the donor procedure as described above. Animal is placed in supine position and the abdomen and pelvis are prepped and draped in sterile fashion. Abdomen is reopened through the previous incision and retractor is assembled for adequate exposure. Right nephrectomy is then performed by dissecting free the renal vessels and the ureter, ligating and transecting them and removing the kidney similar to the graft nephrectomy described above. The bowl is moved to the left of the abdomen to expose the infrarenal aorta and IVC. The distal portions of both vessels are then cleared off the overlaying neuro-lymphatic tissue to accept the renal

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vessels. The lymphatic channels should be left intact as much as possible or ligated when transected. Care should also be taken not to avulse the posterior lumbar branches. Enough length is dissected free to accept the vascular clamps. After meticulous hemostasis, heparin (100 IU / kg body weight) is administered intravenously.

Venous anastomosis: Implantation is started by clamping the IVC with a side biting vascular clamp such as a Satinsky clamp. A longitudinal venotomy is performed using a surgical blade (Number 11) and extended with scissors to match the width of the renal vein. Four 6-0 prolene sutures are placed in the four quadrants of the venotomy. The kidney is wrapped in a surgical sponge submerged in sterile slush and brought into the field. The four quadrant stiches are placed in the renal vein. The upper corner suture is tied down and anastomosis is performed using running suture. Once one side of the vein is anastomosed, kidney is flipped to the other side and the other wall is anastomosed with the same suture to a few mm to the first corner. Through the small remaining opening, a Marks needle connected to a syringe containing heparinized saline is inserted and a few cc's is injected to distend the vein and safely place the last few stiches to complete the anastomosis. The two lateral stay sutures are very helpful as they keep the vein laterally open. They can be removed once the anastomosis is completed. Alternatively, one can suture the back wall from inside, then bring the needle out and suture the front wall from outside, without moving the kidney.

Arterial anastomosis: Aorta is clamped using similar side biting vascular clamp. An arteriotomy is made using a number 11 scalpel and enlarged using an aortic punch (3.5 or 4 mm) to match the size of the artery. Two 6-0 Prolene sutures are placed proximally and distally in both the aorta and renal artery. One is tied down and used to make the anastomosis in a running fashion. It is extremely important to include all the layers of the vessel wall with each stich, to avoid dissection after reperfusion. Gentle bulldog clamps are placed on both the renal artery and vein. Subsequently, the aortic and caval clamps are removed and any anastomotic bleeding is addressed prior to removing the bulldog clamps. It is important to maintain adequate blood pressure (above 100 mm Hg) to assure optimal perfusion of the graft. Additional IV fluid or vasopressors (norepinephrine drip) may be necessary to maintain arterial pressure. The wrapping around the kidney is removed. Topical Papaverine is applied to the renal artery to avoid potential spasm. The venous bulldog is removed first, followed by the arterial one. Irrigation of the abdominal cavity with warm sterile normal saline helps warming up the organ faster, and improves the blood flow (Figure 17.2).

Ureteral anastomosis: The graft's and recipient's ureters are trimmed to appropriate length enabling a comfortable anastomosis without tension or redundancy. Both ureters are then spatulated on opposite sides. This reduces the risk of anastomotic stricture. The ureter anastomosis is then performed using 6-0 PDS in a running fashion.

The bowel is then placed back in its original position. The fascia is closed using number 1 PDS in a running fashion. Skin is closed using non-absorbable suture (silk or nylon). Animal is kept warm, analgesia provided and blood pressure is closely monitored and until complete recovery. After extubation and recovery from general anesthesia, animal is returned to separate housing. Analgesia is administered routinely for the first few days and IV fluids as needed if oral intake is insufficient. Site of surgery is monitored closely for signs of infection. Surgical site infection can be treated with intravenous cefazolin and metronidazole.

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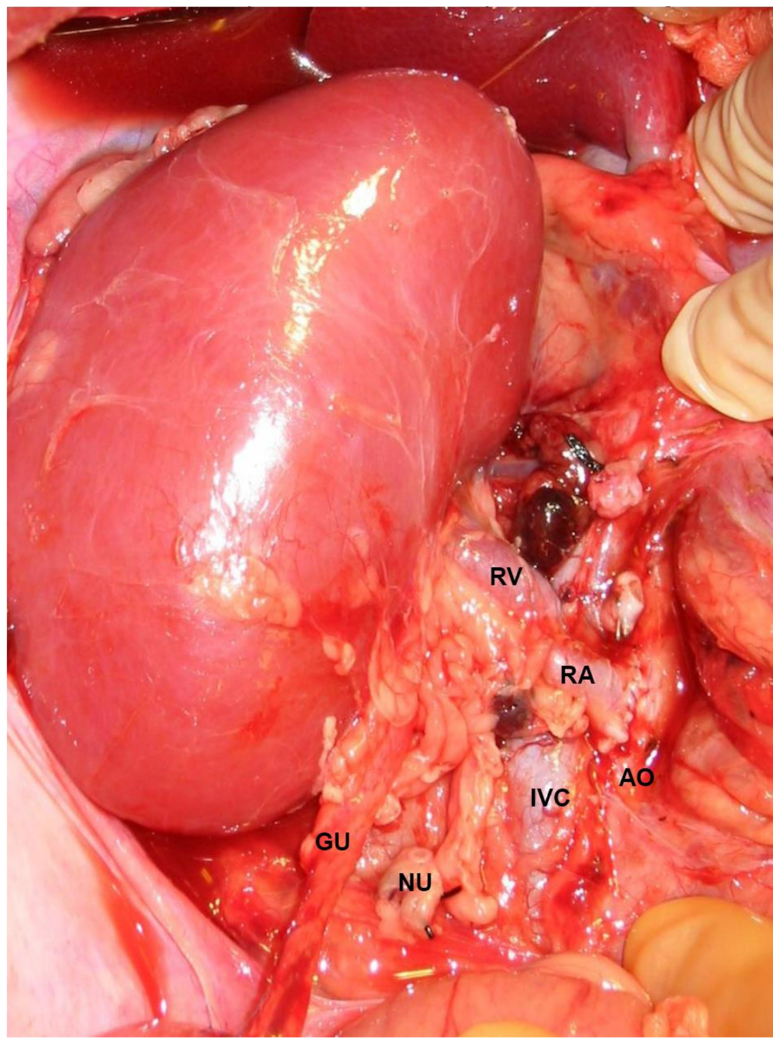

Figure 17.2. Kidney graft anastomosed to the aorta and the IVC after reperfusion. Note that ureter anastomosis has not been done yet. AO: aorta, RA: renal artery, RV: renal vein, GU: graft ureter.

Liver Transplantation in Pigs

The physiological and anatomical characteristics of the pig closely resemble those of humans in many ways, and pigs are cheaper and their use is more acceptable from the public's perspectives than non-human primates. In addition, in contrast with non-human primate species, inbred (genetically defined) lines of pigs are available, and different from mice, pigs constitutively express class II antigens on the vascular endothelium (Oike 2000), and the immunosuppressive drug metabolism is more similar to humans than rodents (Tata 2009). All of these characteristics facilitate the study of rejection and tolerance immunology. As a result, the pig has been extensively used in transplant immunobiology (Flye 1992). The early era of clinical liver transplantation and transplant research was based on dog experiments (Goodrich 1956; Moore 1959; Starlz 1960; Todo 1985) including the first

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successful liver transplant by Welch in 1955 (Goodrich 1956). Later, with more animal research restriction and animal protection activists' protest, experiments in dogs became less acceptable. Another reason is that unlike dogs, pigs more closely resemble the man in that they appear to have no hepatic vein sphincters and therefore do not manifest hepatic outflow block after liver transplantation, a frequent problem with dog liver transplants (Starlz 1960; Memsic 1986). Thus, the pig became the most used large animal model of liver transplantation and has been important for the development of clinical transplantation. The pig liver transplant model has been instrumental to train transplant surgeons; to test new surgical techniques; and to investigate organ preservation, organ rejection, and immunological tolerance (Steinig 1990; Gianello 1993; Oldhafer 1993; Hertl 1999; Hojo 2003). Arguably, the most attractive use of the porcine model however, is as a potential source of xenogenic cells and organs for human use (Ekser 2011; Elliott 2011). The production of transgenic pig lines that express human molecules which control host complement reactions against pig tissues, or lines which express a decreased amount of the Gal (α 1, 3) epitope, constitutes the first significant step towards the use of pig tissues in humans (Chardon 1999; Ekser 2011). This section reviews the techniques used for liver transplantation in pigs as well as pre- and post-operative management.

Pig Liver Anatomy

The pig liver can be divided into eight segments in a similar way to those described in the human liver by Couinaud, with the exception of segment I, or caudate lobe, which is adjacent to the visceral surface of the right lateral lobe and contains the cava within the parenchyma. Each segment is separated by the others by prominent fissures and has its own arterial supply, venous and biliary drainage. The left lateral lobe can be divided into segments II and III, and the right lateral lobe into segments VI, VII and I. The left medial lobe consists of segment IV, and the right medial lobe can be divided into segments V and VIII (Court 2003) (Figure 17.3).

The portal vein has two main branches, which divide at the hilum very close to the liver parenchyma. There are commonly one or even two communicating branches of the portal vein, which traverse the fissure between the right lateral and right median lobes of the liver. There are no communicating branches between the right and left median lobes, thus allowing complete division of right and left hemi-livers (Court 2003). The hepatic vein has four main branches coming directly from the IVC and draining the left lateral, left medial, right medial and right lateral lobes, respectively. Segment I drains directly into the IVC. In the pig, the retrohepatic IVC and the hepatic vein confluence are entirely intrahepatic (Court 2003). The main hepatic artery (HA) splits into a variable number of branches, but most commonly there are three branches visible anterior to the portal trunk. Usually the HA gives off one or two branches to the right lobe, a branch to the right medial lobe, a branch to the left lateral and left medial lobes.

The right and left hepatic biliary ducts generally drain their respective hemi-livers before joining into the common hepatic duct. The segmental distribution of the biliary ducts follows that of the portal vein as they lie together within the Glissonian sheaths. The left hepatic duct is generally of much larger caliber than the right, and drains both the left median and left lateral lobes. The right hemi-liver is drained by two separate hepatic ducts, similar to the arrangement in the human liver (Court 2003).

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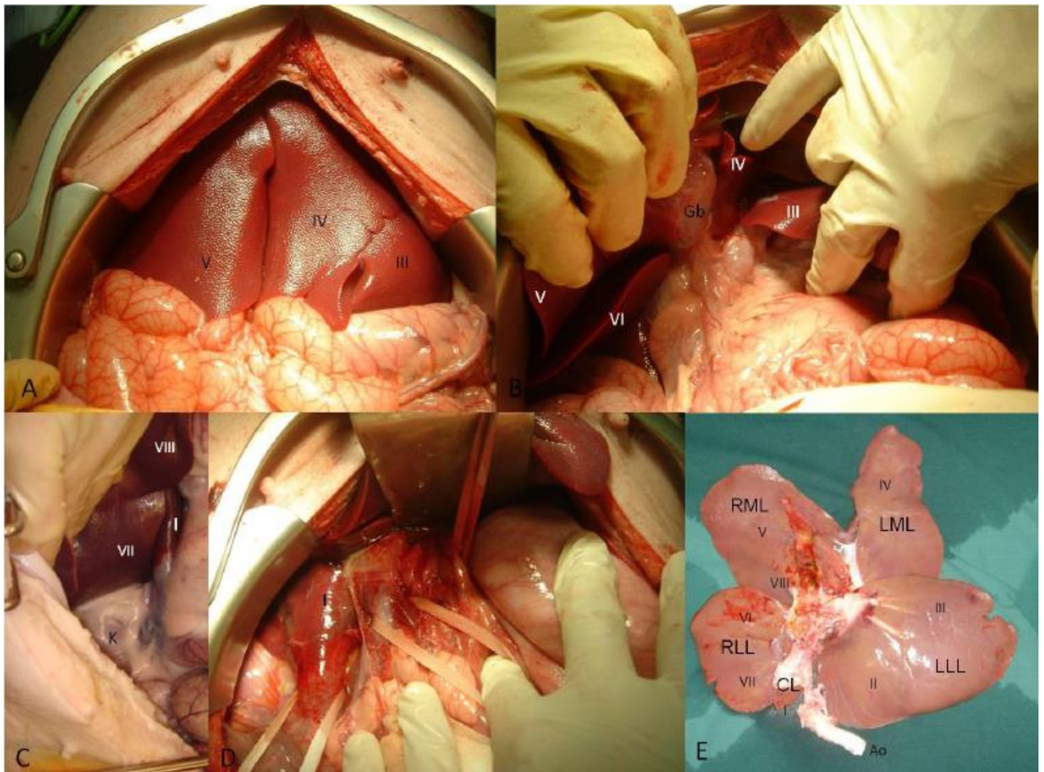

Figure 17.3. Anatomy of the pig liver. A) anterior view, B) visceral aspect without dissection, C) Infra-hepatic IVC, D) IVC, Portal vein and hepatic artery are isolated; E) Visceral aspect of the graft (gallbladder was removed). Segments of the liver. CL (segment I), RLL (segments VI and VII), RML (segments V and VIII), LML (segment IV), LLL (segments II and III). Gb: Gallbladder, K: kidney, RLL: right liver lobe, CL: caudate lobe, RML: right medial lobe, LML: left medial lobe, LLL: left lateral lobe. Ao: aorta.

PRE-OPERATIVE MANAGEMENT

Most investigators relied on miniature pigs, 25–35 kg, as both donors and recipients. The dissection of anatomical structures is more difficult in older and heavier animals due to intra-abdominal fat accumulation. To facilitate exposure and to avoid abdomen compartment syndrome donor weight should preferably be lower than recipient weight. The animals are transported to the laboratory at least 2 days prior to the surgery to minimize stress. The pigs are housed in temperature and light-controlled cages on a 12–12 h light-dark cycle and provided with food and water *ad libitum*. The animals are fasted at least 12h before the procedure. Unless it is relevant for the research objectives, animals do not need immunological matching, nor preoperative cross-matching. All surgical procedures are performed in a sterile fashion.

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ANESTHESIA MANAGEMENT

Anesthesia is of crucial importance for achieving success in liver transplantation (de Lange 1984; Eisele 1986; Kaiser 2006). Animals receive 30 mg/kg ketamine intramuscularly. We use a heating pad and warm IV fluids to prevent hypothermia (body temperature is maintained at 39°C). An angio-catheter is inserted into an ear vein for IV access. For anesthesia induction, the pig receives 0.0035 mg/kg fentanyl and isoflurane 3% through face mask. After pre-oxygenation with 100% oxygen, it is intubated with an appropriate size cuffed cannula (7 mm endotracheal tube for a 30 kg animal) using an extra-long straight Miller laryngoscope. Intubation of the pig may be difficult because of long snout, copious oral secretions, tortuous larynx, and long epiglottis. Very often the epiglottis needs to be placed to the side just before advancing the tube. If intubation is not possible, ventilation can be achieved through a tracheostomy. After endotracheal intubation, maintenance of anesthesia is provided by a mixture of nitrous oxide (50%) and oxygen combined with isoflurane at 1−2%. The Tidal volume is 10 ml/kg and a rate of 15 breaths/min. Pancuronium 0.10 mg/kg is given to provide muscle relaxation and repeated at regular intervals as needed. Cefazolin 20 mg/kg IV is given before incision. An orogastric tube is placed to decompress the stomach and improve operative exposure. The left carotid artery is cannulated for continuous arterial pressure measurement and to collect arterial blood gases (ABGs), and the jugular vein is cannulated for central vein pressure measurement and for rapid infusion of fluids intra-op, and kept for daily blood checks. Urethral catheterization is done for continuous assessment of urine output. Arterial blood gases and blood glucose measurements are obtained during the case. If glucose is below than 70 mg/dl, dextrose 50% is administered. Low-dose dopamine infusion is started if necessary to keep mean arterial pressure above 70 mm/Hg (Eisele 1986). We keep IV fluids at about 20 ml/kg/h to provide around 10 ml/kg of urine per hour (Kaiser 2006). During the anhepatic phase, the level of anesthesia should be decreased to prevent hypotension; also during this phase, the animal receives blood, bolus of albumin or saline, and a dopamine drip to keep the blood pressure at normal levels. Calcium chloride and sodium bicarbonate are used after reperfusion. Sodium bicarbonate is given at 5 mEq/10 kg of body weight or more based on ABG results. Eisele showed that the average amount of sodium bicarbonate during a liver transplant and shortly after was 85 mEq (Eisele 1986). Frequently, after reperfusion, dextrose boluses are also required. Regarding blood transfusion, it is advised to use blood compatible products. Hemolytic transfusion reactions in previously untransfused pigs was believed to occur only rarely; however, there have been reports that the use of A–O incompatible transfusions in pigs undergoing liver transplants resulted in adverse reactions. Hunfeld showed that pigs that received A–O incompatible transfusions developed pulmonary hypertension and disseminated intravascular coagulation. Eight of 15 pigs died during or shortly after surgery due to hemorrhage and four pigs died due to severe bronchospasm (Hunfeld 1984). Sheil reported that two pigs that received A–O incompatible blood transfusions during liver transplants died due to disseminated intravascular coagulation (DIC), bleeding and progressive hypotension (Sheil 1972). Thus, it seems that it would be prudent either to use packed red blood cells, with a minimum volume of plasma, or to use A–O compatible blood for transfusion (Sheil 1972; Hunfeld 1984; Smith 2006).

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DONOR OPERATION

The donor operation should be timed so that the recipient is ready to receive the liver to minimize the period of organ ischemia, ideally using two surgical teams. The pig liver has a tolerance to ischemia similar or inferior to the human liver. Some animals start dying when cold ischemia is longer than 16 hours. Monbaliu showed that livers exposed to 15 minutes of warm ischemia function normally after transplantation, whereas all livers submitted to 60 minutes of warm ischemia display primary nonfunction and cause recipient death. Graft function and survival are occasionally observed after 30 and 45 minutes of warm ischemia (Monbaliu 2005). The abdomen is prepped and draped from above the xiphoid to the pelvis and a midline incision is made from the xiphoid to the pubis. In the lower abdomen, care is taken in the male pig by diverting the incision to the right to avoid the penis and the periurethral vascular plexus. A large Balfour retractor is placed and the abdominal wall retracted. First, the colon is mobilized, and the intestines covered with moist towels are hand retracted to expose the distal aorta, which is dissected and encircled. The left and right triangular ligaments are divided. The hepatic hilum is dissected and the hepatic artery, portal vein and common bile duct isolated. The common bile duct is isolated, ligated as low as possible, and divided. The gallbladder is incised and flushed with saline to remove sludge. The diaphragm pillars are divided, exposing the supra-celiac aorta for cross-clamping. Further dissection of the liver hilum can be done either before (warm dissection) or after (cold dissection) cold flushing. We prefer to do it after cold flushing to avoid vasospasm. Heparin is given (300 U/kg IV). After 5 min, the abdominal aorta is ligated distally and an 18-Fr cannula introduced and secured just above the aortic bifurcation. After cannulation, we collect 500 ml of blood into a bag containing citrate to use during or after the recipient operation. In addition to the aorta cannula, some groups place a cannula into the portal vein/splenic vein for in-situ flushing (Oike 2001). We prefer to flush the portal vein on the back-table. Next, the supraceliac aorta is cross-clamped, and cold flushing is initiated using 3L of Ringer lactate connected to a bladder irrigation tubing system. At the same time, either the suprahepatic or infrahepatic IVC is vented to allow venous drainage. At this point, the abdominal cavity is filled with slush. Next, attention is turned to the portal triad structures in the hepatoduodenal ligament, which are dissected as close to the duodenum as possible (Fondevila 2011). The gastroduodenal artery (GDA) is dissected and cut, the portal vein exposed and cut at the level of superior mesenteric vein and splenic vein. The proximal hepatic artery is skeletonized, the splenic artery and the left gastric artery are ligated and divided. The dissection is then carried to the origin of the celiac artery, and a segment of the aorta is removed with the graft. The suprahepatic IVC is cut with a cuff of diaphragm and the infra-hepatic IVC cut just above the renal veins. After hepatectomy, the portal vein, hepatic artery and bile duct are flushed with 1L of cold UW solution on the back table. On the back-table, excess of diaphragm is removed, the liver vessels are cleaned and branches are tied off; and the suprahepatic vena cava is trimmed to keep a short cuff (Heuer 2010) (Figure 17.4).

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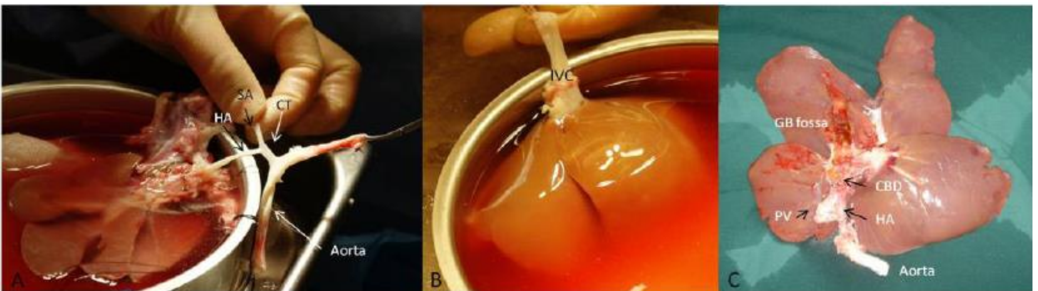

Figure 17.4. A–C. Back-table preparation on the liver graft. After donor hepatectomy the graft is placed on a basin with cold preservation solution. HA: hepatic artery, CT: celiac trunk, SA: splenic artery, PV: portal vein, CBD: common bile duct, GB: gallbladder.

LIVER TRANSPLANTATION TECHNIQUE

In the early reports of pig liver transplantation the recipient operative and perioperative mortality was very high, often approaching 50% (Calne 1968; Terblanche 1968; Chalstrey 1971; de Lange 1984). The learning curve is slow; de Lange showed that in the first series of 53 transplants the peri-operative mortality was 49%, and in a second series of 50 transplants the preoperative mortality was 20% (de Lange 1984). More recently, with advanced anesthesia and surgical techniques, the results are much improved. Fondevila found that with recipients of standard, whole-liver grafts, the 5-day survival rate was 100%; for the recipients of donation after cardiac death (DCD) grafts, the 5-day survival rate was 59%; and for the recipients of small-for-size grafts, the 5-day survival rate was 35% (Fondevila 2011). The time to perform the transplant procedure is in general less than 3 hours.

Various techniques of orthotopic porcine liver transplantation have previously been reported (Calne 1968; Terblanche 1968; Chalstrey 1971; Barron 1975; Woodle 1985; Filipponi 1989; Tanaka 1994; Flye 1999; Gruttadauria 2001; Oike 2001; Fondevila 2011). However, the main difference pertains to the use of veno-venous bypass. Because the pig retrohepatic IVC is entirely intrahepatic and unable to be separated from the surrounding hepatic parenchyma; liver transplant with retrohepatic caval preservation, the ''piggyback'' technique, is not able to be performed in the porcine model. Therefore, caval clamping during the anhepatic phase is unavoidable in pig liver transplantation (Fondevila 2011) and most groups use an external veno-venous bypass in order to minimize the adverse effects of portal and caval clamping (Calne 1968; Woodle 1985; Memsic 1986; Flye 1999). Another suggested approach is a side-to-side portocaval shunt with passive caval-jugular shunt (Gruttadauria 2001). However, some groups propose a simplified technique without the use of veno-venous bypass by showing that the severe hemodynamic alterations can be avoided, and the survival rates can be similar when the anhepatic phase is kept short (below 30 min) (Oike 2000; Ai 2007; Fondevila 2011). Both Oike and Fondevila showed that without bypass 87% and 100% of animals survived more than 1 week, respectively (Oike 2000; Fondevila 2011). Motsch also reported that there were no differences in operative mortality and the postoperative survival rate between the non-shunted and shunted groups despite a more pronounced but retrievable change in hemodynamics and acid-base equilibrium (Motsch 1987).

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Besides orthotopic whole liver transplantation, other models: auto-transplantation (Gruttadauria 2001), partial or split transplantation (Kamimura 1997; Hayashi 1998; Hayashi 1998; Hayashi 1998; Hojo 2003; Kelly 2004), auxiliary transplantation (Hagihara 1975; Ducerf 1995; Hayashi 1998; Fukueda 2006), small for size models (Yanaga 1995; Fu 2011; Hessheimer 2011), non-heart beating (DCD) models (Tabet 1997; Garcia-Valdecasas 1999) multi-visceral (Alessiani 1998; Yagi 1998; Ai 2007) have been described using the pig model.

Step-by-Step Orthotopic Liver Transplant Technique

After induction of general anesthesia, the animal is placed on its back on a heated electric blanket and secured to the operating table with restraining ropes. Then, the animal is intubated, and the cervical and abdominal areas are shaved and prepped. A paratracheal incision of about 10 cm is made on the right side of the neck, parallel to the trachea, and the internal jugular vein (IJV) and internal carotid artery (ICA) are dissected. A triple-lumen catheter is placed in the IJV for drug and fluid administration, with one port connected to a pressure transducer for venous pressure monitoring. A single-lumen catheter is placed in the ICA and connected to a pressure transducer for arterial pressure and heart rate monitoring. The large external jugular vein is found, isolated and prepared for later placement of the venous-venous shunt (Woodle 1985). At the end of the transplant procedure in the recipient, the neck incision is closed, and a Witzel tunnel is created to fix the venous catheter to the back of the neck. Permanent venous access is essential for frequent post-op and daily blood sampling.

The abdomen is opened via a midline incision and a large Balfour or Bookwalter retractor is placed (Figure 5). Then the liver is mobilized, first by dividing the left and right triangular ligaments. The terminal branches of the hepatic artery are ligated and divided as high as possible. The bile duct is doubly ligated and divided as high as possible (Figure 17.5). Once the liver is attached only to the IVC and portal vein, the veno-venous bypass can be initiated. The portal vein, infrahepatic IVC, and suprahepatic IVC are then clamped sequentially in that order. Next, the liver is excised leaving long segments of vena cava, portal vein, hepatic artery and bile duct with the recipient (Figure 17.6). The suprahepatic IVC is prepared by uniting the hepatic vein orifices, creating a common lumen on a large IVC cuff. The suprahepatic IVC anastomosis is performed end-to-end to the suprahepatic IVC of the graft with a running everting 4-0 polypropylene suture (Figure 17.7). After the suprahepatic IVC anastomosis, the liver is flushed with 1L of cold ringer lactate to wash out potassium rich solution from the liver while keeping it cold (If crystalloid flushing of the liver is not used, before opening the supra-hepatic IVC clamp the liver should be reperfused and 200 ml of blood released through the infrahepatic IVC or bypass tubing). The infra-hepatic IVC anastomosis is performed with a running 4-0 polypropylene suture in a similar way. Portal veno-venous bypass is discontinued (IVC to SVC bypass can be maintained). Then, recipient and donor portal veins are trimmed to avoid redundancy and kinking, and the anastomosis is performed with a running 6-0 polypropylene suture, leaving a growth factor of about 1 cm to prevent stenosis. The clamp on the suprahepatic IVC is released and we check for bleeding. After that, we release the infra-hepatic clamp, and check again for bleeding. Next, the portal vein clamp is released and the liver is reperfused (Heuer 2010; Fondevila 2011). After checking for hemostasis, we next dissect the hepatic artery, and a vascular clamp is placed

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proximal to the GDA. The donor common hepatic artery (with the GDA or splenic artery branch patch) or celiac artery is anastomosed to the recipient common hepatic artery (with a GDA branch patch) in an end-to-end fashion using polypropylene 7-0 running suture (Fondevila 2011). In the early reported series, the arterial reconstruction was performed by anastomosis of the long aorta segment attached to the graft to the infra-renal aorta (Terblanche 1968), or the aortic Carrel patch to the aorta (Calne 1968). After confirming stable hemodynamics, veno-venous bypass is interrupted. After arterial reperfusion, the gallbladder is removed (if not removed at the back-table). The distal common bile duct (CBD) of donor and recipient are trimmed to remove ischemic tissue and anastomosis is performed in an end-to-end fashion with PDS 6-0 suture in an interrupted fashion.

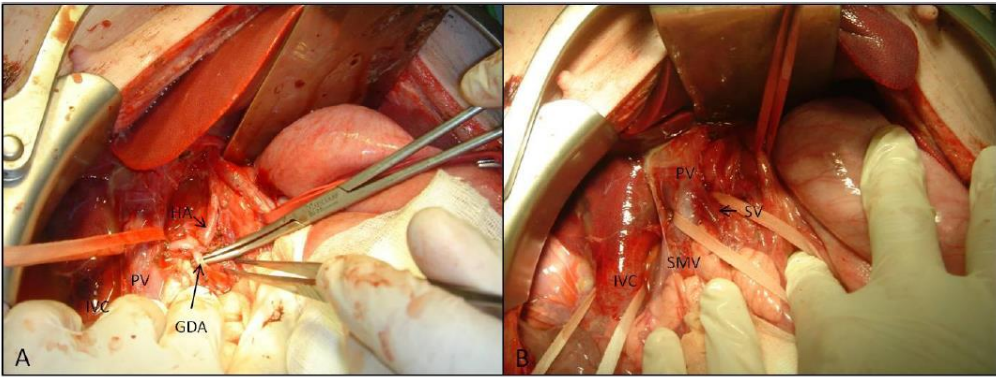

Figure 17.5. Liver pedicle after dissection of the hepatogastroduodenal ligament. HA: hepatic artery, PV: portal vein, GDA: gastroduodenal artery, SMV: superior mesenteric vein, SV: splenic vein.

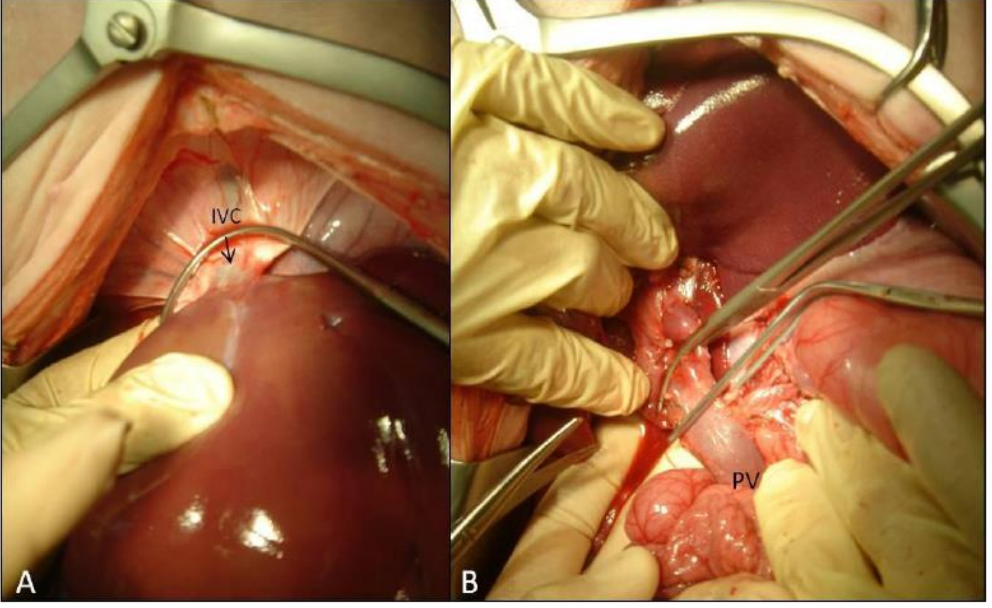

Figure 17.6. Recipient hepatectomy. A) A vascular clamp is placed on the supra-hepatic IVC taking a cuff of the diaphragm; B) The portal vein is clamped and cut as high as possible.

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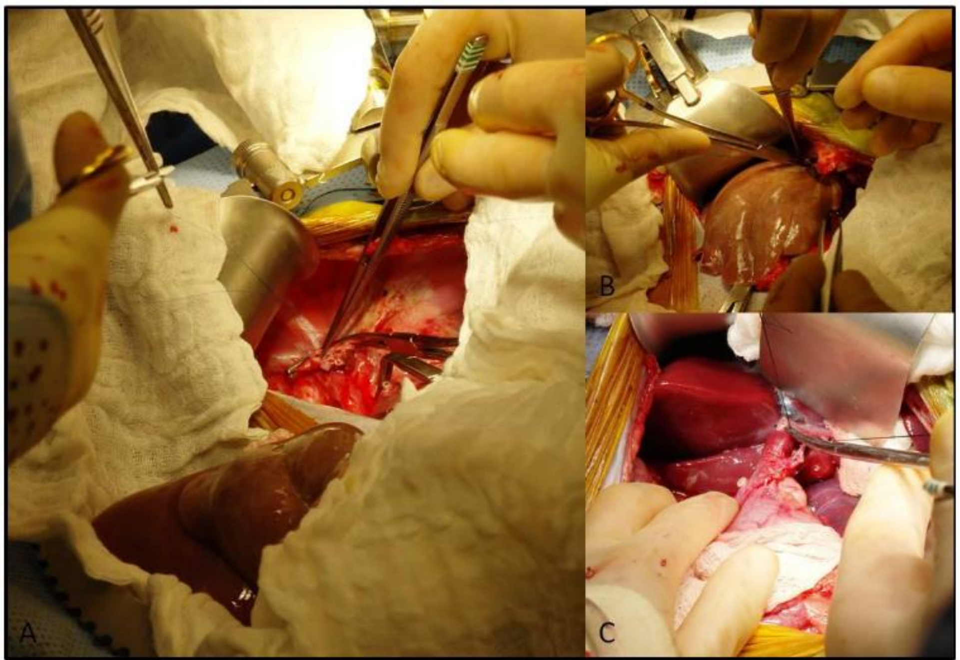

Figure 17.7. A) After recipient hepatectomy, the hepatic veins are opened to form a single IVC opening. The vascular clamp on the supra-hepatic IVC should have a cuff of diaphragm to increase the size of the venous cuff; B) The graft is placed in orthotopic position; C) Liver graft after portal reperfusion.

Veno-venous bypass is initiated usually just before completion of recipient hepatectomy. The bypass tube, primed with heparinized saline, is inserted through the external jugular vein (18−20 Fr cannula), and tightly secured. The infrahepatic vena cava is closed using a vascular clamp, and a bypass tube (24 Fr cannula) is inserted. The portal vein is ligated close to the liver at the level of its bifurcation. If we insert the bypass cannula directly into the portal vein instead of using the splenic vein, first the portal vein is clamped, a partial venotomy close to this ligature is performed, the tourniquet is loosened and the portal bypass tube (18−20 Fr cannula) is inserted for about 4 cm into the proximal portal vein and secured with a tourniquet. The portal vein venotomy is completed (above the cannula). At this point, the bypass circulation can be initiated (Heuer 2010). The porto-jugular bypass is interrupted just shortly before the portal vein anastomosis. When the shunt is removed, the splenic vein is ligated and the spleen can be either left in place (Flye 1999; Oike 2001) or removed (Stevens 1994). The external jugular vein is ligated cranially with a 3-0 silk ligature.

Memsic showed that pump-assisted veno-venous bypass is superior to passive bypass achieving an operative survival of 94%. He showed that pump-assisted veno-venous bypass had statistically significant improvements in portal decompression and hemodynamic stabilization. The intestines are less congested in the pump-assisted shunted animals compared to those with the passive shunt. The pump has the added advantage of a heat exchanger which, when used, can maintain the pig's body temperature (Memsic 1986). Motsch did not show differences in survival but confirmed better hemodynamics with bypass (Motsch 1987).

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Post-Operative Management

Postoperatively, animals return to warmed individual cages and are given free access to water and food. Venous blood samples are obtained frequently for acid-base balance and glucose measurements; and sodium bicarbonate or glucose is given if necessary. Animals are administered 250–500 mL of dextrose 5% + normal saline solution IV Q8 h, for the first 24–48 h. If the animal is not doing well, CVP measurement can guide fluid replacement. A cage that allows collection of urine also helps with fluid management. In addition, animals receive a Fentanyl patch on the skin for analgesia (alternatively, buprenorphin 0.1 mg IM Q8 h can be used for the first 48 h and thereafter as needed), antibiotics (Amoxicillin 15 mg/kg/day), proton-pump inhibitors (omeprazol 20 mg/daily) to prevent gastrointestinal bleeding, and immunosuppression according to protocols. Blood samples are obtained daily. The venous line is cleaned with antiseptics and flushed daily with heparinized saline to maintain patency. Aseptic technique to manipulate lines is extremely recommended in animals on long-term follow-up. If the white blood count increases, blood cultures should be drawn, and targeted antibiotic therapy should be continued until the end of the observation time. If follow-up is long or if animals become septic, IV lines need to be removed.

Spontaneous Tolerance, Rejection, and Immunosuppression

Calne and others showed that rejection of liver grafts in pigs were less frequent than in the dog (Calne 1967; Calne 1968; Terblanche 1968; Calne 1969; Dent 1971; Battersby 1974; Bockhorn 1981; Filipponi 1992). Calne found that without immunosuppression 23% of 55 outbred pigs lived more than 2 months (Calne 1967). Flye and colleagues showed that the median survival of technically successful liver allografts between pairs of outbred pigs (n=20) was 38 days (mean 145.7 ± 53.4 days), and between partially inbred swine matched at the SLA locus (n=17) was 79 days. Interestingly, mixed leukocyte reaction responsiveness did not correlate with the development of rejection. Five of 20 (25%) outbred pigs and 6 of 17 (35%) MHC-matched inbred miniature swine survived more than 100 days. On the other hand, all SLA-mismatched inbred recipients (n=26) died rapidly from massive liver rejection, with a median survival time of 9 days (Flye 1999).

There is no standard immunosuppression for pig liver transplantation. It depends on the goal of the experiment and planned follow-up period. In a MHC mismatch combination, it was shown that when cyclosporine at a dose of 20 mg/kg was given intramuscularly for a period of 21 days beginning on the day before the transplant, only one out of ten pigs had graft rejection while the others developed long-term acceptance (Bom-van Noorloos 1984). Oike showed that spontaneous tolerance to one haplotype-identical liver allograft [donor SLAdd class Id/d, class IId/d, and recipient SLAcd (class Ic/d, class IIc/d)] did not occur. All untreated animals (n=8) rejected their allograft and died within 28.1 ± 9.5 days. On the other hand, all tacrolimus treated animals developed a specific anti-donor unresponsiveness, and five (out of 14) survived long-term (112, 154, 406, 413, and 440 days), whereas several pigs died with a normal allograft function from either over-immunosuppression or other complications. Tacrolimus was given for 12 days through daily intramuscular injection at 0.1–0.4 mg/kg, in order to reach daily trough levels between 7 and 20 ng/ml (Oike 2000). Ducerf kept immunosuppression for the whole observation period. Initially, 500 mg of

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methylprednisolone IV is given. Then, cyclosporine (15 mg/kg) is given daily through gastrostomy tube for the first 10 days, and then mixed with food thereafter (Ducerf 1995). In his series, Fondevilla, gave immunosuppression for 4 days (tacrolimus 0.04 mg/kg IV Q24 h, methylprednisolone 125-100-75-50 mg IV Q24 h) even when the follow-up period was only 5 days (Fondevila 2011).

Heart and Lung Transplantation

Development of Heart Transplantation Animal Models

Heterotopic abdominal heart transplantation is the most important model to study immunosuppressive protocols or tolerance approaches in large animals. The heart is placed in a non-life supporting position, and even life-threatening complications like ventricular fibrillation of a rejecting heart do not automatically terminate the experiment. Other advantages over the orthotopic model in animal research are easy access for biopsies and technical simplicity in general. The technique is well established both in pig and NHP models. It was developed at the beginning of the 20th century by Carrel and Guthrie when they anastomosed the heart of a puppy to the neck vessels of an adult dog. In 1969, Ono and Lindsey described the first heterotopic heart transplant in the abdominal position which is the favored technique today (Ono 1969). There is extensive experience with heterotopic pig-to-baboon heart xenotransplantation at the Mayo clinic and at the National Institutes of Health. Using genetically modified pigs, heart graft survival in the xenotransplantation model is now greater than 1 year (Mohiuddin 2014; Mohiuddin 2015).

Combined Organ Transplants with Abdominal Heterotopic Heart Transplantation for Tolerance Induction

To study tolerance, heterotopic heart-kidney (Madariaga 2013; Madariaga 2015) and heart-lung transplants (Madariaga 2016) in major histocompatibility complex (MHC)-inbred miniature swine are used with 12 days of high-dose calcineurin inhibition. Heart-thymus and heart-kidney transplants in monkeys with a mixed-chimerism is yet another approach to study functional tolerance (Kawai 2011). We found that after kidney co-transplantation, stable long-term tolerance is induced without rejection of either organ after all immunosuppression is weaned. However, lung co-transplantation was unable to confer tolerance to the heart (Madariaga 2016).

Orthotopic Heart Transplantation in Pig-to-Baboon Xenotransplantation

The technique of orthotopic heart transplantation was developed by Lower and Shumway in Stanford (Lower 1960). In the 1960s they used dogs to develop the biatrial technique, where a donor cuff of right and left atrium is sewn to recipient cuffs of right and left atrium. These experiments led to the first human heart transplant performed by Barnard in 1967. Recently, this technique was changed to the bicaval technique where superior vena cava (SVC) and IVC are anastomosed separately, which seems to preserve the sinoatrial node and

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tricuspid valve function better. A meta-analysis of 41 papers comparing the two techniques found significant early advantages for the bicaval technique in terms of tricuspid valve regurgitation, sinus rhythm vs necessity of permanent pacemaker implantation, and even perioperative mortality. Long-term outcomes, however, were similar (Jacob 2009).

Today, only very few groups (University of Munich, Mayo Clinic) use orthotopic heart transplantation in a large animal model. Due to the necessity of cardiopulmonary bypass, full heparinization and the life-supporting position of the heart (in contrast to heterotopic abdominal heart transplant), this is a very complex procedure to perform in a preclinical animal setting. It is mostly used in pig-to-baboon xenotransplantation. The easier biatrial technique is used in Munich because we believe it suits better to the preclinical setting where bypass and operation times should be kept as short as possible as there is no ICU available postoperatively, and the baboon needs to be extubated shortly after the procedure.

Heterotopic Intrathoracic Heart Transplantation in Pig-to-Baboon Xenotransplantation

In this model, the original heart of the recipient is left in place and is supported by the new donor heart. Both hearts beat together as “one.” Heterotopic thoracic cardiac transplantation was originally developed by Barnard in Cape Town in the 1970s because preservation techniques and immunosuppression were imperfect (cyclosporine A was not yet available), and there was always a high risk of primary graft failure (Barnard 1977). The native recipient organ in place served as a hemodynamic backup in case the new heart would fail. Barnard also tried this technique as a life-saving procedure in cardiogenic shock after cardiopulmonary bypass (Barnard 1977). Today, the indication for heterotopic heart transplant is heart failure with irreversibly elevated pulmonary vascular resistance (PVR > 6 Wood units). The only alternative approach in such a clinical situation would be combined heart lung transplantation which is not ideal due to the shortage of lung donors. Recently, it has been shown that the implantation of a left ventricular assist device can reduce PVR into ranges where the patient then becomes a candidate for a normal orthotopic heart transplantation. This development is probably the end of *clinical* heterotopic HTx.

The concept of having a back-up heart, however, was shown to be very helpful in *preclinical* pig-to-baboon xenotransplantation. Bruno Reichart´s group in Munich used this technique and performed 21 heterotopic intrathoracic transplantations to test different immunosuppressive regimens and tried to achieve long-term survival (Abicht 2015). They concluded that this technique could be helpful when xenogeneic heart transplantation first comes to the clinic because the back-up function of the recipient´s native heart could replace extracorporeal membrane oxygenation (ECMO) in case of primary graft failure (Abicht 2015).

Development of Lung Transplantation Models

The first successful large animal model of lung transplantation was developed by a Russian physiologist with transplantation of individual canine pulmonary lobes in 1947. This was shortly followed by several groups successfully performing single canine lung transplant

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in the 1950s with the technical addition of using the bronchial artery to provide systemic blood supply to the airway and using a left atrial cuff for the pulmonary vein anastomosis instead of anastomosing individual pulmonary veins (Hardin 1954). Further development of the large animal lung transplantation model was limited until the 1960s when the use of postoperative immunosuppression (azathioprine and high-dose prednisone) increased postoperative survival as demonstrated in the experience of Hardy et al. with over 400 canine lung transplants (Hardy 1964; Alican 1971).

Following this extensive experimental experience, the first human lung transplantation was attempted by Hardy in 1963 (Hardy 1963); however, the patient died on postoperative day 18 from renal failure. For the next decade, several groups attempted lung or lobar transplants around the world with limited success (Cooper 2016). The main obstacle was dehiscence of the bronchial anastomosis. Further research in the canine model determined that use of high-dose prednisone prevented healing at the bronchial anastomosis (Lima 1981) and subsequently the initial immunosuppression regimen was changed to azathioprine and cyclosporine-A, with later introduction of prednisone. In addition, omentopexy was performed at the bronchial anastomosis to encourage collateralization and avoid anastomotic stricture (Dubois 1984).

The first successful human heart-lung transplantation program was started in 1981 with Shumway at Stanford (Reitz 1982), followed by a successful program of human lung transplantation by the Toronto Lung Transplant Group in 1983 (1986). Rapid advancements in survival outcomes ensued.

Currently, the main limitation to lung transplantation is the problem of chronic rejection. To further study chronic rejection, a large animal model of chronic lung rejection was developed using MHC-inbred miniature swine. Using MHC-inbred miniature swine allows transplants to be performed across reproducible MHC barriers (Sachs 1976). In this model, pigs received 12-day course of high-dose calcineurin inhibitor (Allan 2002) or cyclosporine, prednisone, and azathioprine (al-Dossari 1994) and were then followed by cellular immune assays and biopsies. All grafts survived for at least 5 months and developed manifestations of chronic rejections such as bronchiolitis obliterans, interstitial fibrosis and occlusive vasculopathy (al-Dossari 1994; Allan 2002).

PREOPERATIVE CARE AND ANESTHESIA

In the following section, we mainly present the protocols that are in use at the Center for Transplantation Sciences at MGH.

Animals are without food but with free access to water for 12 hours prior to the scheduled operation. Approximately 1 hour prior to the operation, the animal is sedated using ketamine analogue Telazol (1.4 mg/kg IM or IV, single dose, onset of action 15 minutes, duration of effect 90 minutes); glycopyrrolate (0.01 mg/kg IM or IV, single dose, onset of action 15 minutes, duration of effect 60 minutes); and xylazine (2 mg/kg IM or IV, single dose, onset of action 15 minutes, duration of effect 60 minutes). Within 1 hour of incision the animal is given cefazolin (1 g IV or IM, single dose).

Inhalational anesthesia is used (isoflurane). Pancuronium is administered (0.1 mg/kg) if paralysis is required (useful for lung transplantation).

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For postoperative pain control in pigs, animals are given buprenorphine (0.03 mg IM or IV, single dose, onset of action 1−2 h after administration, duration of action 8−12 h) and a fentanyl patch (1−4 mcg/kg/h TD, applied on the day of surgery, onset of action 3−4 h after application, duration of action 72 h continuous while patch is in place).

For postoperative pain control in NHP, animals are given buprenorphine (0.01 mg/kg IM or IV every 10–14 h) on a scheduled basis for the first three postoperative days and as needed thereafter. NHP recipients also receive ketorolac (3 mg daily IM) as an anti-platelet agent for the first five postoperative days.

CENTRAL LINE INSERTION

Central lines are useful for administration of drugs and fluid as well as for frequent blood draws to monitor drug levels and laboratory values. Central lines are generally kept in place for no longer than 60 consecutive days due to risk of systemic infection. Central line care involves sterile technique in accessing the lines and keeping the central line entry site clean. Central lines are flushed at least twice daily with heparinized saline (100 units/mL) to maintain patency. Jackets are placed on the animals to prevent damage or inadvertent line dislodgement.

The preferred central line accesses in pigs are internal and external jugular veins in the neck. The animal is placed supine and a 5 cm incision is made about one fingerbreadth lateral to the trachea. After exposing the internal jugular and the external jugular veins, 0-silk is used to ligate the vein in the cephalad direction. Gentle retraction is placed in the caudal direction and a small venotomy is made. The central line is tunneled through the skin with an entry point as far dorsal as possible to avoid trauma to the line when the animal is awake. The central line is then inserted into the venotomy and inserted so that its tip would rest in the SVC. The line is tested and then secured. The incision is closed in layers.

HEART TRANSPLANTATION: DONOR HEART HARVEST

After induction of general anesthesia, the animal is prepped and draped in the usual sterile fashion. The chest is opened via median sternotomy. The superior vena cava, inferior vena cava, pulmonary artery, and aorta are isolated with 0-silk sutures. Heparin 200 units/kg is administered. A purse string suture is placed in the ascending aorta with 5-0 Prolene and the aorta is cannulated with an 18 gauge angiocatheter or needle vent. The azygos vein is tied off and divided. In pigs, it is also critical to tie off and divide the hemiazygos vein to avoid bleeding in the recipient. The SVC and IVC are tied off and the aorta is cross-clamped. The heart is vented through the IVC and the pulmonary veins (when an isolated heart is transplanted) or left atrial appendage (when the lung is also harvested). Cardioplegia (e.g., 1L of Plegisol) is administered through the aortic cannula to arrest the heart. For topical cooling, iced saline is placed in the chest. The heart is transected and excised beginning at the IVC and SVC, pulmonary veins, then aorta and pulmonary artery. When the lungs are also used, the heart-lung block is excised *in toto* and heart and lungs are divided at the back table (Figures 17.8−10).

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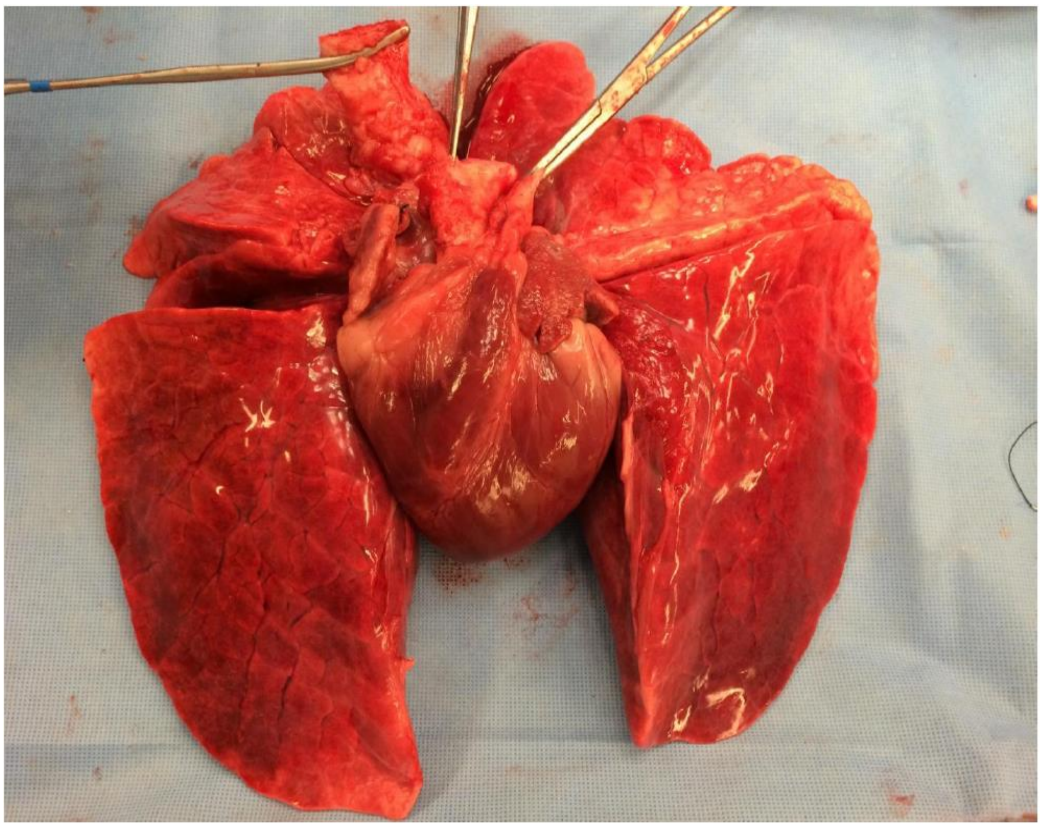

Figure 17.8. Heart and lung en bloc. Heart and lung are removed from the donor animal en bloc. Left clamp occluding the trachea. Middle forceps showing the aorta. Right clamp on the pulmonary artery.

In heterotopic heart and orthotopic lung transplantation, a cuff of left atrium is created for the lung, and the left atrium of the heart is closed with a 4-0 Prolene suture in a running fashion (Figure 17.11).

During back table dissection for isolated heterotopic abdominal heart transplant all pulmonary veins as well as the SVC and the IVC are tied off with 0-silk. We also create a 3 mm atrial septal defect in pigs to avoid thrombosis in the atria after heterotopic heart transplant.

For orthotopic heart transplant, a left atrial cuff is created by cutting open the pulmonary veins and a right atrial cuff is created by cutting open the IVC in the direction of the right atrial appendage (the SVC is tied off).

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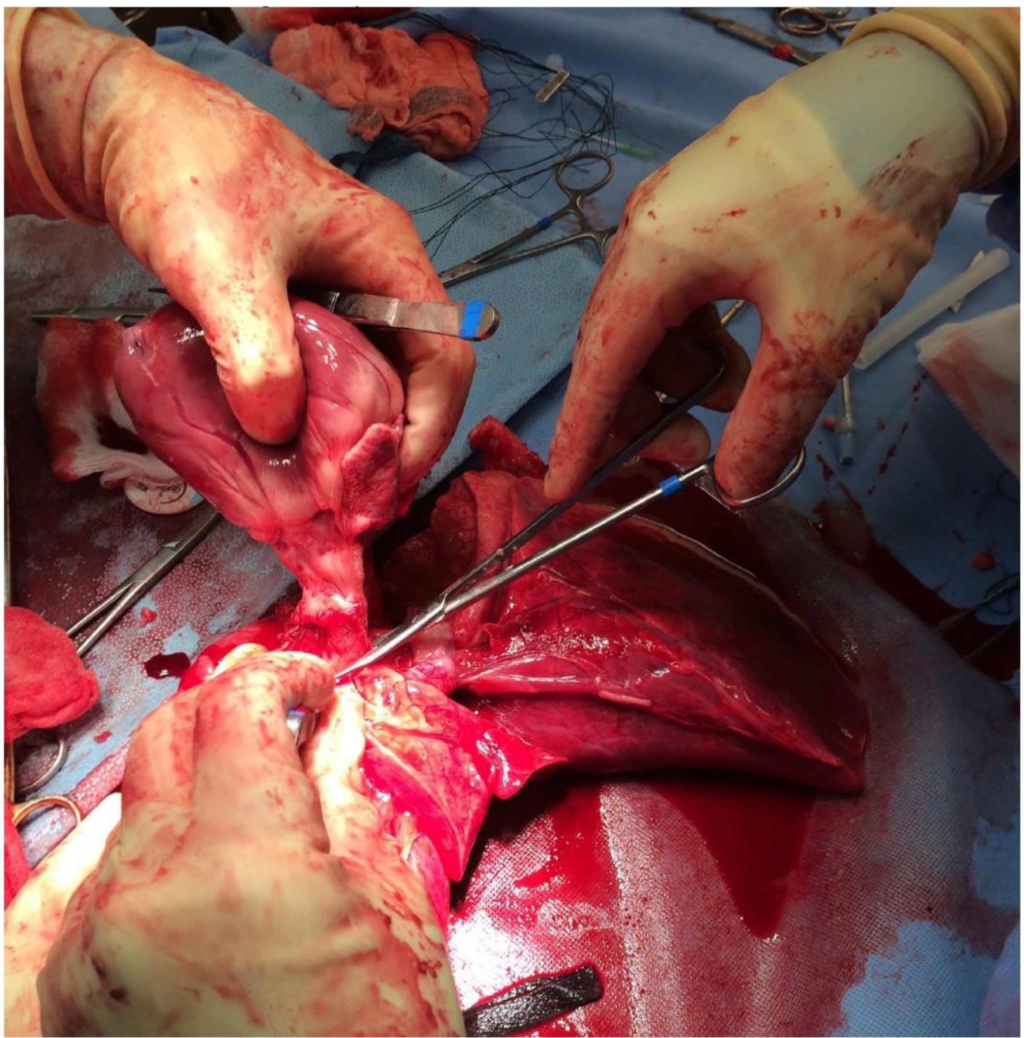

Figure 17.9. Separation of the heart and lung. The heart and lung are separated at the left atrium, leaving a left atrial cuff around the pulmonary veins.

Heterotopic Abdominal Heart Transplantation

After a midline laparotomy the viscera are retracted to expose the aorta and inferior vena cava which are dissected free. The heart graft is transplanted into the right side of the anim al (Figure 17.12). Heparin is administered (200 units/kg IV). After partial clamping using a

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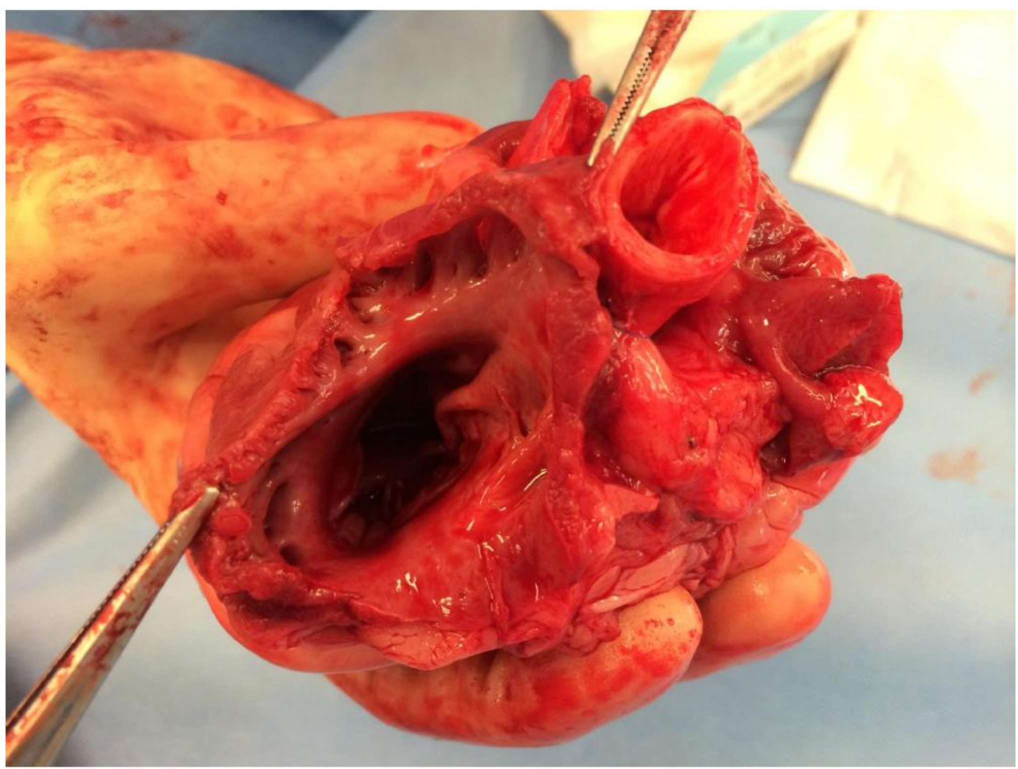

Figure 17.10. Heart alone with left atrium opened.

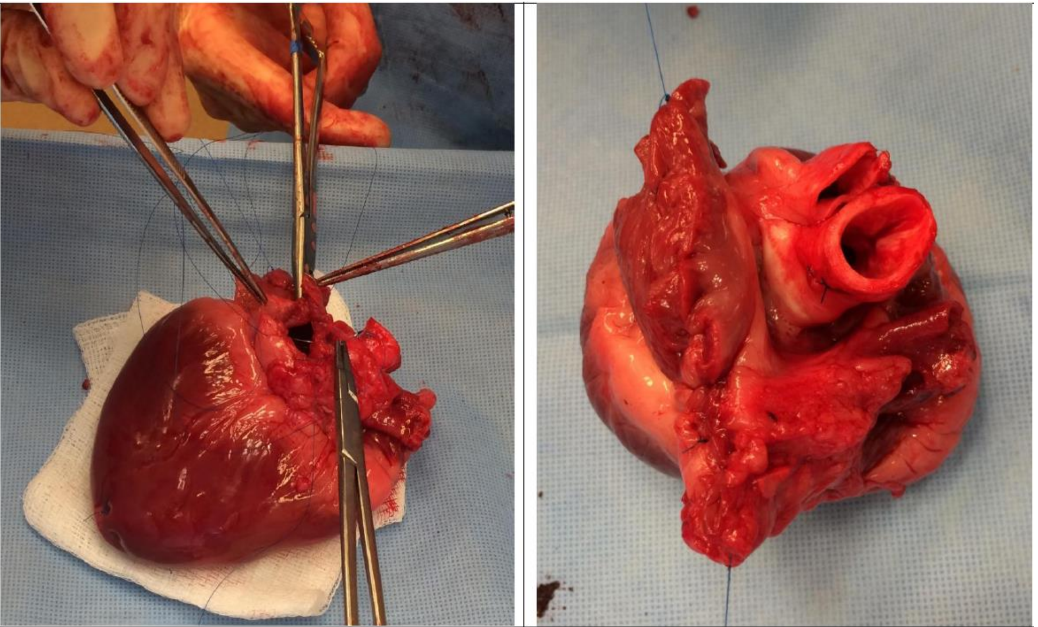

Figure 17.11. Closing the left atrium. The left atrium is closed with running 4-0 prolene suture. When the heart is harvested alone for solitary heart transplant, the pulmonary veins are divided and ligated.

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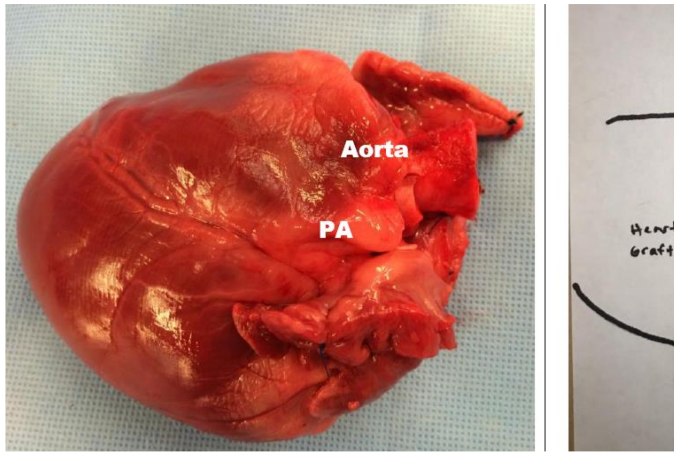
Satinsky clamp, the IVC is opened longitudinally with a blade and Potts scissors. An end-to-side anastomosis between the donor pulmonary artery and the recipient IVC is performed with 6-0 Prolene in a running fashion. After that, the abdominal aorta is partially clamped and opened longitudinally and the donor aorta is anastomosed to the recipient aorta using 6-0 Prolene in a running fashion. Before tying the knots and removing the clamps, de-airing with saline is performed. If the graft initially fibrillates, it is defibrillated to attain normal sinus rhythm. The animal´s abdomen is then closed in three layers: peritoneum with running 3-0 Vicryl, fascia with running 2-0 PDS, and subcutaneous with 3-0 Vicryl. The skin is stapled (in pigs) or sutured with monocryl (in NHP).



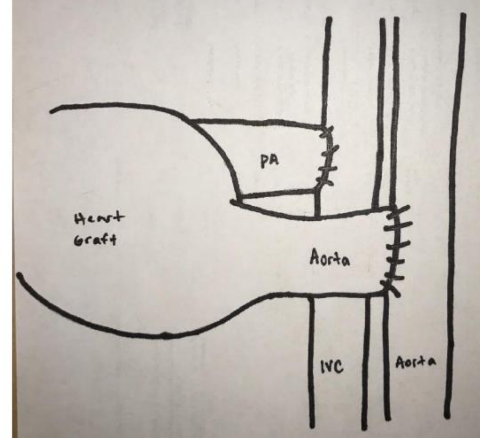


Figure 17.12. Prepared heart graft. Heart donor organ ready for heterotopic abdominal transplantation.

Orthotopic Heart Transplantation (Pig-to-Baboon, Munich Protocol)

After a median sternotomy, the pericardium is opened. Heparin is administered (400 units/kg IV), the aorta and both venae cavae are cannulated and total cardiopulmonary bypass is established. The animal is cooled to 30°C. Recipient cardiectomy is performed leaving cuffs of left and right atrium in place. Implantation of the donor heart is begun with the left atrial anastomosis (5-0 Prolene in a running fashion), followed by the right atrial anastomosis (5-0), the pulmonary artery (6-0) and the aorta (5-0). A needle vent is placed in the ascending aorta and the apex is punctured for de-airing before reperfusion. The heart is reperfused for 60 minutes, and the animal is rewarmed. Immunosuppression starts before reperfusion. Pacemaker wires are placed and cardiopulmonary bypass is weaned after 60 minutes of reperfusion. Protamine is given. Hemostasis is secured and a Blake drain is placed in the pericardium. The sternum is closed with wires, and the wound is closed in layers. Drains are removed and a jacket is put on before the baboon is wakened, extubated and put in the cage.

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Heterotopic Intrathoracic Heart Transplantation (Pig-to-Baboon, Munich Protocol)

The right-sided pulmonary veins are ligated, and the openings of the left pulmonary veins are transected to form the left atrial cuff. The right atrial cuff is created as usual. Management of cardiopulmonary bypass is identical to orthotopic heart transplant. After cross-clamping the aorta, Bretschneider cardioplegia is administered through the aortic root. The donor heart is put into the right side of the chest. Both atria are anastomosed with 5-0 Prolene in a running fashion (diamond-shaped for large opening). The aorta and pulmonary artery are anastomosed end-to-side, for the latter, an interposition with a vascular graft is necessary (for a more detailed description and pictures see Abicht et al. (Abicht 2015).

HEART BIOPSY AFTER HETEROTOPIC HEART TRANSPLANT

After induction of general anesthesia, the animal is placed in the left lateral decubitus position. An 8 cm incision is made in the right flank overlying the visibly beating heart graft. After the subcutaneous tissue and muscle are divided, the retroperitoneum is entered and the heart graft is exposed. A 4-0 prolene suture is used to make a pursestring stitch at the apex of the left ventricle, taking care to avoid any coronary arteries. A Tru-cut core biopsy needle is deployed through the apex into the septum and then withdrawn. After sufficient tissue is obtained, the pursestring stitch is tied and an additional mattress stitch using 4-0 prolene can be placed to achieve hemostasis. The incision is then closed in layers.

Lung Transplantation

Donor Lung Harvest

After induction of general anesthesia, the chest is entered through a median sternotomy. The SVC and IVC are encircled. The hemi-azygous and azygous veins are ligated. The space between the aorta and pulmonary artery is dissected and the aorta surrounded with umbilical tape to assist with later cross clamping. Heparin 200 units/kg IV is administered. A 4-0 prolene purse string sutures is placed in the pulmonary artery. An arteriotomy is made and the pulmonary artery is cannulated with cystoscopy tubing. The SVC and IVC are ligated. The aorta is cross-clamped, and cold preservative solution (e.g., Perfadex, XVIVO Perfusion; Englewood, CO) containing Alprostadil (prostaglandin E1, 500 ug/L) is perfused into both lungs through the pulmonary artery cannula. Alprostadil can also be administered directly into the pulmonary artery. The heart is vented by incising the left atrial appendage. Iced saline is placed in the chest and then the lungs and heart are removed *en bloc* for back table dissection.

During back table dissection, the left lung is separated from the heart by isolating the pulmonary artery, the left main stem bronchus and creating a left atrial cuff for the pulmonary vein (Figures 17.13 and 17.14).

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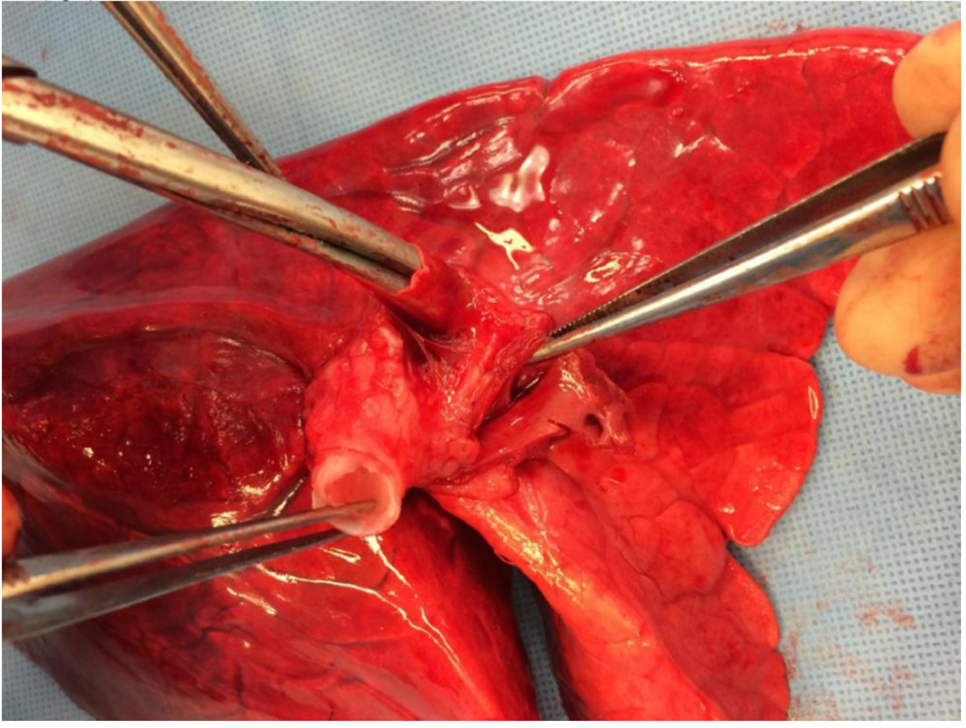

Figure 17.13. Lung preparation. L main bronchus (left), left pulmonary artery (middle) and left atrial cuff (right).

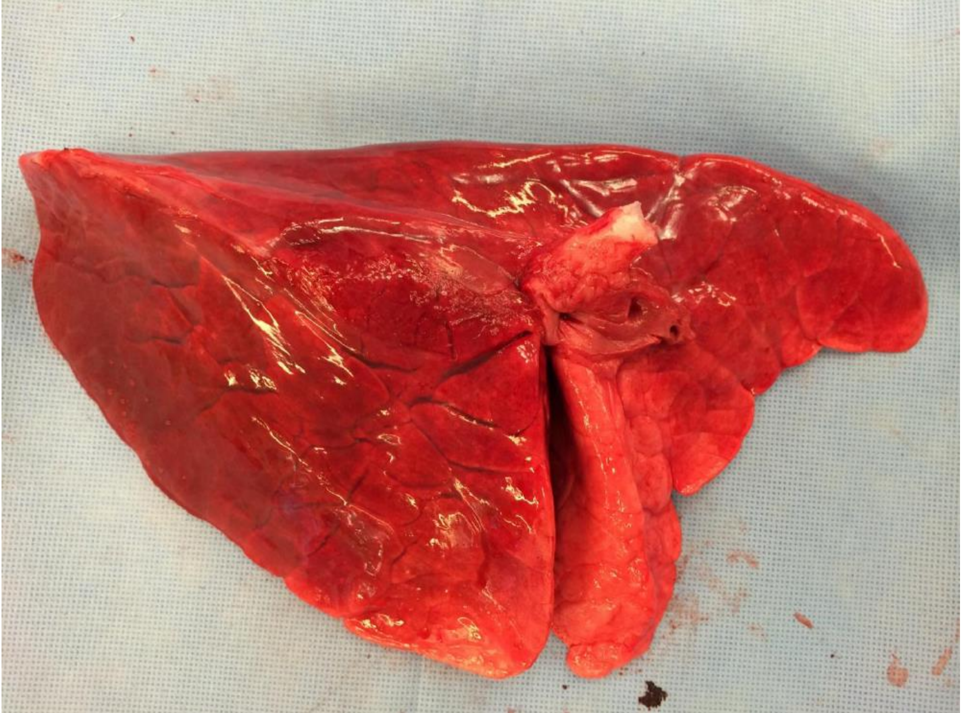

Figure 17.14. Prepared lung graft. Lung donor organ ready for transplantation.

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Recipient Lung Transplantation

The animal is placed in the right lateral decubitus position to expose the left chest. A posterolateral incision is made just below the left scapula. In the pig, rib 4 or 5 is exposed by resecting a small portion of the cartilage of the scapula. The rib is then cut with bone cutters and removed to optimize exposure. A rib spreader is inserted.

Upon gaining entry to the chest, the inferior pulmonary ligament is sharply incised. The left hilum was dissected. The superior pulmonary vein is usually small and can be ligated with suture. The inferior pulmonary vein is isolated. The left pulmonary artery is isolated. The left main bronchus is isolated. After the donor organ is ready, the recipient is systemically heparinized with 200 units/kg of heparin the pulmonary vein and the pulmonary artery are clamped with Satinsky clamps and then cut distally. The bronchus is cut and the left recipient lung is removed from the field.

We generally do not use bronchial blockers. Instead we have found it simpler to sew during periods of apnea and occlude the bronchus periodically to allow for ventilation.

The bronchial anastomosis is performed with 8 to 10 interrupted 4-0 vicryl stitches. The sutures are placed in an organized fashion. The donor bronchus is then telescoped down into the recipient bronchus and then the sutures are tied.

The recipient pulmonary vein to donor left atrial cuff anastomosis is then performed with running 5-0 prolene suture. The pulmonary artery anastomosis is also performed with running 5-0 prolene suture.

If there is significant size discrepancy between the donor and the recipient, a donor left upper lobectomy can be performed before implanting the lung.

After hemostasis is achieved, a Blake drain connected to bulb suction is tunneled through the skin and placed inside the chest. The thoracostomy drain is removed on postoperative day 2. The thoracotomy is closed with figure-of-eight #2 vicryl sutures to approximate the ribs and the muscle and subcutaneous tissues are closed in layers. The skin is stapled and interrupted #2 nylon retention sutures are placed. Sterile dressing with antibiotic ointment is then applied. A protective jacket is placed over the incision that also covers the central lines in the neck.

Lung Biopsy

After induction of general anesthesia, the animal is placed in the right lateral decubitus position. A 10 cm curvilinear incision is then made about 2 cm below the previous thoracotomy incision. The incision is carried down to a rib. The rib is isolated and shingled. After the pleural space is entered, the lung is examined. Biopsy can be performed sharply or with a stapler to wedge a piece of lung. If the biopsy is performed sharply, hemostasis can be achieved by using direct pressure and surgicell. The incision is then closed in layers. If the lung is densely adherent to the chest wall, a pleural catheter is not needed to evacuate air. If the lung is free of adhesions, a small Blake drain can be placed to evacuate air and removed on postoperative day 2.

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COMBINED HEART AND LUNG TRANSPLANTATION

Our group at Massachusetts General Hospital used a swine model of combined heterotopic heart and orthotopic left lung transplant to see whether a lung can confer tolerance to a heart the way a kidney does (Madariaga 2016). These experiments were carried out without cardiopulmonary bypass, which enabled a survival rate of almost 100% in a preclinical setting.

The recipients first underwent left orthotopic lung transplantation before heterotopic abdominal heart transplantation as described above. Heart grafts were placed on ice while lung transplantation was performed.

POSTOPERATIVE CARE AND MONITORING AFTER HETEROTOPIC HEART TRANSPLANT AND LUNG TRANSPLANT IN SWINE

Immunosuppression with calcineurin inhibitors (Cyclosporine A or FK506) is started in the OR and continued for 12 days. Trough levels are measured daily and doses adapted accordingly.

Heart function is monitored by daily palpation and electrocardiogram (ECG) using the AliveCor Veterinary Heart Monitor (AliveCor, Inc., San Francisco, CA). Routine biopsies after heterotopic abdominal HTx are performed via flank incisions at predetermined time intervals (POD 20−30, 50−60, 90−100) or whenever a decrease in palpation or QRS-wave amplitude occurs. Cardiac allograft rejection (heart survival time) was defined by either loss of a ventricular impulse on palpation, and/or QRS-wave amplitude of less than 0.3 mV, and/or the lack of ventricular contraction on echocardiography (Avitall 1988).

Lung function is monitored by clinical examination of breath sounds and weekly chest radiographs. Routine biopsies are performed on all transplant recipients via mini-thoracotomies at predetermined time intervals (POD 20−30, 50−60, 90−100) or whenever there was clinical suspicion for rejection.

CONCLUSION

Swine and primates have been extensively used in biomedical research, including transplant immunobiology (Swindle 1988; Swindle 1998; Critser 2009). Large animal transplant models have served a valuable role to train transplant surgeons; to test new surgical techniques; and to study the immunobiology of transplantation. Transplantation experiments in the large animals are complex, costly, and require adequate facilities and team-work. The learning curve is very steep, and the surgeon needs to be aware of technical modifications, and best model for the particular experiment. In addition, optimal anesthesia and post-operative care are essential to obtain good results. In the future, genetically modified pigs may provide a source of cells or organ grafts for clinical transplantation (Critser 2009; Sachs 2009; Elliott 2011; Gock 2011).

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Chapter 18

EXPERIMENTAL COMPOSITE TISSUES AND SOFT TISSUES TRANSPLANTATION

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ABSTRACT

Composite tissue allotransplantation (CTA) is the transplantation of body structures which include tissues from both ectoderm and mesoderm. Different from life-saving solid organ transplantation (SOT), the purpose of CTA is commonly to maintain appearance and function integrity of the recipient. There are some body structures that have been transplanted successfully around the world. Among them, the numbers of hand and facial experimental research transplantations and clinical cases are much more than that of other organs/tissues. In this article, firstly, we review the history and development of CTA. As these transplanted structures contain tissues with different degrees of immunogenicity, therefore, the immunogenicity of the whole composite tissue is complex. The history of CTA is also accompanied with the development of immunosuppressive therapy. Thus, we introduce the immunosuppressive treatments used in the experimental and clinical studies which mainly include immunosuppressive drugs, polyclonal or monoclonal antibodies and chimerism. Through the application of immunosuppressive therapies, the primary goal of CTA is to ensure the successful graft survived, and the final goals are to induce donor-specific immune tolerance and to further reduce the complications of immunosuppressive drugs. Secondly, two of the most commonly used research models, hand and facial transplantation are introduced using both experimental and clinical research situations. Finally, a brief introduction to several other transplantations, such as abdominal wall, larynx and penis allotransplantation is provided. Based on all those studies, we believe that with the advances in technology and immunosuppressive therapy, it can be expected that CTA will be beneficial to more and more patients.

Keywords: composite tissue, allotransplantation, immunosuppression, review

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ABBREVIATIONS

αβ-TCR:	αβ–T-cell receptor
AD-MSCs:	adipose-derived mesenchymal stromal cells
ALG:	antilymphocyte globulin
ALS:	antilymphocyte serum
ATG:	antithymocyte globulin
AZA:	azathioprine
BM-MSCs:	bone marrow-derived mesenchymal stromal cells
BMT:	bone-marrow transplantation
CIPS:	calcineurininhibitor-induced pain syndrome
CNI:	calcineurin inhibitor
CS:	corticosteroid
CsA:	cyclosporine A
CTA:	composite tissue allotransplantation
CTLA4Ig:	cytotoxic T-lymphocyte antigen 4 immunoglobulin
GVHD:	graft-versus-host disease
IDO:	indoleamine 2,3-dioxygenase
IL:	interleukin
LBN:	Lewis-Brown Norway
LEW:	Lewis
LF:	LF 15-0195
MLR:	mixed lymphocyte reaction
MMF:	mycophenolate mofetil
MSCs:	mesenchymal stromal cells
mTOR:	mammalian target of rapamycin
NF-κB:	nuclear factor-kappa B
NFAT:	nuclear factor of activated T-cells
NFAT-P:	phosphorylated nuclear factor of activated T-cells
PBMC:	peripheral blood mononuclear cell
RAPA:	rapamycin
SOT:	solid organ transplantation
SRL:	sirolimus
TAC:	tacrolimus
TGF- β:	transforming growth factor-β
WF:	Wistar Furth

INTRODUCTION

Composite tissue allotransplantation (CTA) is the transplantation of body structures which include the tissues originated from both ectoderm and mesoderm, such as skin, subcutaneous tissue, muscle, tendon, nerve, vessel, cartilage, bone and bone marrow. Around the world, several different body structures have been transplanted, mainly including hand, face, larynx, abdominal wall, tendon, knee, tongue and penis. The reasons for the damage or

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deficiency of these tissues are plenty, such as malignancy, electrical injuries, war damages, fire burns and animal bites. Compared to lifesaving solid organ transplantation (SOT), the purpose of CTA is more likely to maintain appearance and functional integrity.

HISTORY

The history of CTA is extremely long. The hypothetical proposition of replacing the incomplete or injured tissues can be traced back to a very long time ago. It begins with a legend back to the year 348AD, when a church sacristan got cancer which led to gaseous gangrene on one of his legs, and a healthy leg from a recently dead Ethiopian Moor was grafted to the sacristan by twin brothers, Cosmas and Damien (Kahan 1983). This seems to be the earliest record of CTA, although the final results of this story became a mystery. After that, not until the 16th century, a man's nose was sliced off in swordplay, and Gaspare Tagliacozzi, who is supposed to be the Father of Plastic Surgery, used a flap from the arm donated by a slave to repair it. Surprisingly, the nose lived for 3 years before a sudden rejection (Kahan 1983). In modern times, CTA was first proposed by Kleinert and Peacock with a digital flexor mechanism transplantation in 1957 (Peacock 1960).

With the development of transplantation, it was recognized gradually that not only microsurgery but also immunity plays an important role in CTA. In World War II, some British seamen and pilots were suffering from painful burns in U-boat attacks. When doctors carried out allogeneic tissue transplantation to the injured soldiers, the complications resulting from the immune rejection could not be ignored. Tom Gibson and Peter Medawar began the experimental research on tissue immunology; they were first to describe the rejection process, and for this contribution to the research, Peter Medawar received the Nobel Prize in 1960 (Tobin 2007).

Accordingly, with the in-depth studies, the role of immune system played in CTA cannot be ignored. The history of CTA is also accompanied with the development of immunosuppression therapy.

IMMUNOSUPPRESSION IN CTA

CTA is different from SOT as the transplanted tissues contain skin, subcutaneous tissue, muscle, tendon, fascia, nerve, blood vessel and so on. Their immunogenicity is discrepant. A research study–performed by Murray investigated the antigenic strength of different tissues and organs, and it showed that the skin was the most antigenic tissue (Murray 1971). Subsequent research showed that followed by the skin, the muscle presented as medium antigenic, bone and cartilage were further alleviative and least antigenic (Buttemeyer 1996). Therefore, the degree of immunogenicity of the composite tissues seems to be a complex question. Is it higher than any one of the tissues, or equal to the highest antigenicity tissue, or lower than the highest antigenicity tissue? The research carried out by Lee may provide some answers. During the research, they tested and compared the antigenicity of each individual tissue (skin, subcutaneous tissue, muscle, bone, and blood vessel) of a limb through a vascularized limb transplantation in rat and further compared the antigenicity with the whole

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limb transplantation. The result showed that the antigenicity of the whole limb is less than some of the individual tissue, and the severity of cellular and humoral immune responses was also different for different tissues (Lee 1991). This study shows the complexity of the immune response in CTA.

Because the immunosuppressive effect induced by the same immunosuppressive therapy was discrepant due to the different immunogenicity of different transplanted tissues, the researchers proposed split tolerance. This phenomenon was discovered earlier (Lustgraaf 1996; Heslop 1962). Generally, as the skin immune system has strong immunogenicity, skin is usually rejected first (Bos 2009). The immune system of skin is also complex; it contains immune cells of both innate and adaptive immunity such as macrophages, endothelial cells, dendritic cells, T cells and B cells (Bos 2009), and these immune cells could further secrete cytokines (Pidwell 2007). It has been reported that in a continuous experimental study, a musculoskeletal allograft was carried out in a minor histocompatibility mismatch swine modeland, the graft achieved a long-term survival with a short-course use of cyclosporine A (CsA) as the immunosuppressive drug (Lee 1998). However, in the skin transplantation from the same donor later, half of the recipients rejected it (Bourget 2001). Further studies showed that the dermis survived, but the epidermis was rejected (Mathes 2003). Another study had shown rejection of the same donor's skin in a bone marrow chimerical model but no rejection of successive heart transplantation (Fuchimoto 2001).

Similar to other organ transplantations, without the development of immunosuppression drugs and therapy, it is hard for CTA to achieve long-term survival. Therefore, it is necessary to introduce the immunosuppressive therapy in induction and maintenance of immunosuppression. It mainly consists of one or more agents from the following categories: immunosuppressive drugs, polyclonal or monoclonal antibodies and chimerism.

IMMUNOSUPPRESSIVE DRUGS

There are four main types of immunosuppressive drugs, corticosteroids (CS), calcineurin inhibitors (CNI) (CsA or tacrolimus (TAC)), antimetabolites [azathioprine (AZA) or mycophenolate mofetil (MMF)], and mammalian target of rapamycin (mTOR) inhibitor [sirolimus (SRL)] (Söderlund 2015). They can be used in both induction and maintenance therapy after transplantation.

CS has the anti-inflammation and immunosuppression effects with complex mechanisms. They can enter the cell directly through cell membrane and regulate the transcription of DNA mainly through inhibiting activator protein-1 and nuclear factor-kappa B (NF-κB) to affect the number and function of leukocytes in both innate and adaptive immune system (Auphan 1995). They play an important role in immunosuppression after CTA. However, the side effects of CS are obvious, for example, moon face, buffalo hump, hypertension, gastric ulcer, poor wound healing, water-sodium retention, osteoporosis and chronic adrenal suppression after long-term use of CS.

CNI is widely used for immunosuppression after CTA and the effects of preventing the proliferation and differentiation of T cells are obvious. Representative of CNI, CsA and TAC can enter the cell through diffusion or by combining with immunophilines. They then inhibit the calcineurin to dephosphorylate the phosphorylated nuclear factor of activated T-cells

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(NFAT-P). The dephosphorylated nuclear factor of activated T-cells (NFAT) could enter nucleus to promote the transcription of some cytokines such as interleukin (IL)-2, which could promote the occurrence of rejection. Therefore, the main mechanism of CNIs is to inhibit the secretion of cytokines including IL-2 (Reem 1992). A study had shown that the levels of CsA in skin in the first week after transplantation played important roles in the graft survival; when it reached the therapeutic levels in the first postoperative week, the allograft could have a greater possibility to achieve rejection-free status (Swearingen 2008). TAC also plays an important role in inhibiting rejection. In a Wistar Furth to Lweis (LEW) rats' limb allograft transplantation model, a combination therapy with CsA, ALS and TAC was used and as a result, the allografts survived well. More importantly, the levels of TAC in skin were 100-fold higher than that of other tissues such as blood and muscle. This study revealed that TAC had the function of immunosuppression and especially could inhibit the function of immune cells in skin (Solari 2009). The main side effect of CNIs is nephrotoxicity. It could present as acute and chronic disease with continuity in pathology from dose-related and reversible to irreversible symptoms with the vasoconstriction of renal afferent arterioles, leading to the occurrence of tubular atrophy and interstitial fibrosis (Pallet 2011). Other side effects include drug to drug interactions, neurotoxicity, hypertension, dyslipidemia, cholestasis, cholelithiasisand calcineurin-inhibitor induced pain syndrome (CIPS) (Söderlund 2015; Lindenfeld 2004; Grotz 2001).

As the representatives of antimetabolite drugs, the function of both AZA and MMF is to inhibit the replication of DNA and to affect the proliferation of both T and B cells through different mechanisms. When AZA enters the plasma, it converts to 6-mercaptopurine rapidly, then to thio-inosinemonophosphate, and further to a purine analog, which is the active metabolite of AZA (Elion 1993). MMF is the non-competitive inhibitor of inosine monophosphate dehydrogenase, which is important for the guanine nucleotides synthesis, and further for the replication of DNA (Ensley 1993). The side effect of AZA is mainly myelosuppression, and the side effects of MMF are leukopenia, nausea, vomiting and diarrhea.

SRL, also known as rapamycin (RAPA), which is a macrolide antifungal antibiotic secreted by *Streptomyces hygroscopicus* and found from the Easter Island in 1975 (Vezina 1975). It was first used as an immunosuppressive drug after transplantation in 1989 (Calne 1989). It targets and inhibits the enzyme mTOR, and further influence the proliferation of both T and B cells (Brown 1994; Waldner 2016). The side effects of SRL are drug to drug interaction, dyslipidemia, pancytopenia, delayed wound healing, oral ulcers and thrombocytopenia (Söderlund 2015; Lindenfeld 2004). However, there is a study which showed that the continuous use of SRL as maintenance therapy did not affect the follow-up operation and bone healing (Cavadas 2011).

ANTIBODIES

Antibody products are commonly used to induce immune tolerance. The main antibodies used in CTA are antilymphocyte serum (ALS)/antilymphocyte globulin (ALG)/antithymocute globulin (ATG), IL-2 receptor antagonists (basiliximab or daclizumab) and monoclonal antibodies targeting the co-stimulator of T cells.

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ALS/ALG/ATG contain non-selective polyclonal antibodies, which can interact with the antigens of T and B cells and cause the complement-mediated lysis, eventually leading to the reduction of T and B cells. Their side effects are mainly thrombocytopenia, leukopenia, cytokine release syndrome and serum sickness (Siemionow 2002).

In the process of immune rejection, IL-2 secreted by T cells can promote the activation, proliferation and differentiation of T cells (Söderlund 2015). Therefore, inhibition of IL-2 can delay the occurrence of rejection. There are some monoclonal antibodies such as basiliximab and daclizumab which work through inhibiting the IL-2 receptor. Additionally, no serious side effects of these antibodies have been reported.

As we know, T cells play an important role in immune rejection; therefore, by inhibiting the effect of T cells, we could partly reduce immune rejection after transplantation. Some antibodies could target the co-stimulator on the surface of T cells and further affect the downstream signaling pathway. The CD40-CD154 and CD28-CD80/CD86 co-stimulatory pathways could be blocked by different monotherapies (Bartlett 2002; Siemionow 2010; Tung 2008). Anti-CD3, anti-CD52 monoclonal antibody and cytotoxic T-lymphocyte antigen 4 immunoglobulin (CTLA4 Ig) could also block the function of T cells (Strome 2001; Wekerle 2000).

CHIMERISM

The final goal of immunosuppression is to induce donor-specific and immunosuppressive drugs-free immune tolerance. In order to achieve this goal, chimerism is widely used in CTA. Chimerism was first described by Owen in bovine biovular twins where each of them contains the blood cells of the other one without rejection (Owen 1945). This is thought to be a natural hematopoietic stem cell chimerism. In 1955, skin homograft transplantation achieved success by chimerism (Main 1955). Then, it was utilized successfully in allogenic skin transplantation in 1984 (Ildstad 1984).

There are two types of chimerism: microchimerism and macrochimerism. Microchimerism is transplantation of passenger leukocytes such as immature dendritic cells to the lymphoid organs of recipient. The recipient does not require pretreatment in this method. Macrochimerismis transplantation of bone marrow cells from donors; these stem cells could migrate to the bone marrow, thymus and other lymphoid organs of recipient and lead to the decrease of recipient T cells. But, this requires pretreatment in recipients; usually it is done through bone marrow irradiation to clear recipient hematopoietic cells in order to facilitate the donor hematopoietic system in the recipient. This method can induce donor-specific immune tolerance and prolong graft survival.

Chimerism is effective to induce and maintain long-term tolerance to CTA, however, some side effects of chimerism are lethal such as graft-versus-host disease (GVHD). It was first described by Vos in 1956 in the mice receiving total body irradiation, which upon receiving allogeneic bone marrow died 20 days after irradiation. The histological analysis showed that the mice died from multiple organ failure, and they called it secondary syndrome (Vos 1956).The reason for GVHD is that T cells from donor attack the tissues of recipient such as skin, intestinal mucosa, bile ducts and lymph nodes (Vriesendorp 2016). Therefore, it is important to achieve a balance between long-term survival and occurrence of GVHD. A

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study had been carried out with limb transplantation in MHC-mismatched rats and the results showed that the 20%−50% chimerism level is ideal which resulted in CTA survival of 100% and occurrence of GVHD reduced to 0% (Lin 2012).

Another cell-type used in inducing immune suppression is mesenchymal stromal cells (MSCs). MSCs are adult stromal cells with potential of self-renewal and multilineage differentiation. MSCs can be separated and obtained from bone marrow, adipose tissue and cord blood. In 2006, the international association for cell therapy had confirmed the identification criteria of MSCs such as plastic-adherence, expression of CD105, CD73 and CD90, and ability to differentiate into adipocytes, osteoblasts and chondroblasts (Dominici 2006). As the development of stem cell treatment in the induction of immunotolerance progressed and the MSCs appeared as perfect therapeutic candidates, their effect was also studied in CTA. MSCs could induce immune tolerance through cell-to-cell contact and cytokines secretion (Fryer 2015). Donor-derived MSCs could induce chimerism in the recipient resulting in no- or gentle GVHD. The side effects of MSCs are invasive recovery and delivery methods and related complications and their limited proliferation capacity (Meng 2007).

EXPERIMENTAL AND CLINICAL STUDY OF CTA

The successful clinical application of research is closely related to the results from animal experiments. Animal models, thus, play an important role in CTA research. Different models of functional studies and transplantation immunization studies have been explored. Also, for this purpose, several types of animals have been used. They range from the rodents (mouse and rat) to the dog (Lapchinsky 1973; Zhao 2016; Goldwyn 1966) and swine (Hettiaratchy 2004; Kuo 2009), and further to the nonhuman primate models (Hovius 1992; Stark 1987). In this part, we will address some animal models and related clinical advances. As limb and facial transplantation are more commonly done, and due to their better knowledge in both experimental research and clinical practice, we will focus on them in the following section.

LIMB COMPOSITE TISSUE ALLOTRANSPLANTATION

Limb transplantation is the most used animal CTA model. The limb mainly consists of tissues like skin, subcutaneous tissue, muscle, tendon, blood vessel, fat, nerves, cartilage and bone. Limb transplantation model is divided into functional and non-functional, or orthotopic and heterotopic models according to the different research priorities, such as immunosuppression or function.

The research of experimental limb transplantation was started in 1907 when Carrel had described leg transplantation between dogs (Carrel 1908). Although the issue of immune rejection was not raised at the time, the issues of postoperative nerve regeneration and limb function recovery had been considered. This was supposed to be the first animal model of limb transplantation in modern medicine. In 1936, both orthotopic and heterotopic limb transplantation was performed between young albino rat by Schwind, and with the connection of muscles and nerves, the transplanted limbs achieved muscular control and lived longer than

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3 months (Schwind 1936). 26 years later, Schwind found that limb transplantation between non-inbred genetic background rat aged no more than two weeks, could achieve immune tolerance induced by immature immune system (Schwind 1962).

With the discovery and usage of immunosuppressive drugs, the study of CTA came to a new stage. By the time, some immunosuppressive drugs were used in limb transplantation, representative drugs like CSs (Lance 1971), CsA, AZA and 6-mercaptopurine (Goldwyn 1966) could prolong survival time of grafts. In 1984, a study with the application of CsA in the orthotopic limb transplantation between BUF (H-1^b, Ag-B6) and LEW(H-1^l, Ag-B1) rats was performed; the limb allograft survived with a continuous dose of CsA (10 mg/kg/day), however, about one week after CsA was stopped, the allograft was rejected (Kim 1984). Right after that in 1985, Black reported that they achieved allograft tolerance in limb transplantation between Lewis-Brown Norway hybrid (LBN) (Rt-1^{l+n}) and LEW rats through using CsA 8 mg/kg/day for 20 days for induction treatment and 8 mg/kg/day twice weekly for maintenance treatment (Black 1985). At the same time, the inhibitory effects of different single immunosuppressive drugs on immunological rejection in limb transplantation were compared in another study. The effect of CsA (25 mg/kg), TAC (2 mg/kg) and MMF (30 mg/kg) on immunosuppression of limb transplantation in ACI to LEW rats were compared. The drugs were all used daily for 14 days as induction therapy, and then twice a week as maintenance therapy. The result showed that only TAC could maintain immunosuppression without signs of rejection, however, the rats died of bacterial pneumonia with mean survival time of 296.11 ± 29.78 days (Jones 2001). The first trial of limb osteomyocutaneous flap transplantation in large animals was done in pigs in 1998. With the combination use of CsA, MMF and prednisone, the survival time was prolonged. More important point is that the immunosuppressive therapy could be used in large animal CTA, bringing it close to the next step in its application to the human (Ustuner 1998).

With the extensive application of immunosuppressive drugs, the side effects are gradually emerging, such as lifelong use of anti-rejection drugs, infection, malignancy and organ toxicity. At the same time, researchers are trying to find other ways to induce lifelong and donor-specific tolerance. In addition to the use of immunosuppressive drugs, other treatments have also been explored. Poole described immunological effects of renal transplantation on limb transplantation. The researchers injected AS rat anti-August antiserum to AS rat at first, then the kidney of (AS × August) F1 rat was transplanted to AS rat, 156 days later, a limb was transplanted from (AS × August) F1 rat to AS rat. At last, the result showed that the two transplanted limbs survived for 92 and 207 days respectively, which means that long-term renal transplantation plays a role in the maintenance of immune tolerance in the later limb transplantation (Poole 1976). In 2003, Ozer demonstrated that combined use of CsA and ALS for 21 days could achieve donor-specific tolerance in semi-allogeneic rats' limb transplantation checked by donor and third-party skins; the donor skins were accepted and the third-party skins were rejected. In the peripheral blood, there were 35% to 42% donor-specific chimerism. This study in fact explored inducing donor-specific transplantation by chimerism (Ozer 2003). The same result was found in fully MHC-mismatched rats' limb transplantation later (Ozer 2003).

The study carried out by Foster showed that mixed chimerism induced by total-body irradiation and T-cell-depleted bone-marrow transplantation (BMT) in combination with CD28 blockade by CTLA4-Ig, could also induce donor-specific tolerance in rat limb transplantation models from ACI (RT1A^a) to Wistar Furth (WF) rats (Foster 2003). Another

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study was designed to observe the effect of combination of αβ–T-cell receptor (TCR) monoclonal antibody and CsA (αβ-TCR-CsA) treatment on reducing immune rejection. The results showed that by using αβ-TCR-CsA therapy for 7 days, could induce donor-specific tolerance as determined by skin transplantation *in vivo* and mixed lymphocyte reaction (MLR) *ex vivo* (Siemionow 2003). After that, they also demonstrated that thymus played an important role in this treatment for the induction of donor-specific tolerance (Siemionow 2006). In addition, subsequent study found that $CD4^+CD25^+Foxp3^+$Treg cells were important in the immune tolerance maintaining in CTA (Bozulic 2011). We had also reported an immunosuppressive protocol in mice vascularized limb transplant model. The combined use of anti-CD45RB monoclonal antibody (mAb) (3 mg/kg/day), LF 15-0195 (LF)(2 mg/kg/day) and RAPA(2 mg/kg/day) for 14 days right after the transplantation could achieve a long-term survival in mice limb allograft transplantation (Wang 2013). At the same time, in a recent study, Jindal used a human IL-2 fusion protein to react with IL-2 to further block its function. In combination use of ALS and CsA, in a rat limb CTA model, the allograft achieved long-term survival by suppressing effector T cells and by increasing the proliferation of Tregs (Jindal 2015). In order to study the influence on the expression of cell surface molecules during the process of rejection, pathological examination was performed on the skin from hand or forearm of patients with rejection. At different stages of rejection, some markers were expressed at higher levels, such as CD3, CD68, Foxp3, indoleamine 2,3-dioxygenase (IDO), intercellular adhesion molecule-1 (ICAM-1), E-selectin and P-selectin. When some of these markers were inhibited, the allografts could survive longer, and upon blocking E-selectin and P-selectin in the skin, the survival time of limb allograft in rat was significantly longer (Hautz 2010).

By infusing donor DCs to the recipient could induce microchimerism as introduced above. Dendritic cells (DCs), as an important antigen presenting cell in innate immune system, play an irreplaceable role in mediating immune rejection. But the immature DCs could induce immune tolerance to self-antigen. Thus, the immature DCs can be inducted into immune tolerance to the allogeneic graft antigens. In a rat limb transplantation model, DCs from recipient bone marrow were pretreated with alloantigen lysate and then applied the treatment combination of CsA and ALS. The results showed that they together prolonged the survival time of allograft and increased the percentage of $CD4^+CD25^+$ T cells (Kuo 2009).

Along with the discovery and research of MSCs in other SOT researches, they have also been used in the study of CTA. A combination therapy with ALS, RAPA, MSCs and BMT after body irradiation was carried out in a rat limb allotransplantation model. The combination use of MSCs was helpful for the induction of a stable chimerism without GVHD happening compared with other BMT treatment group alone (Pan 2010). Similar experiment was carried out in a swine limb transplantation model with the combined therapy of MSCs, BMT and CsA, and result showed that the therapy could prolong the survival time of allografts, and MSCs could also prevent the occurrence of GVHD led by BMT (Kuo 2009). The mechanism may be related to increasing $CD4^+CD25^+Foxp3^+$Treg cells both in peripheral blood circulation and skin of transplanted tissues, decreasing $CD3^+$T cell population and increasing the anti-inflammatory cytokines such as transforming growth factor-β (TGF- β) and IL-10 (Kuo 2011). In another study, the author compared the function of adipose-derived mesenchymal stromal cells (AD-MSCs) and bone marrow-derived mesenchymal stromal cells (BM-MSCs) in rat limb transplantation. The result showed that both AD-MSCs and BM-MSCs together with tacrolimus for 21 days could achieve long-term immune tolerance in

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vascularized composite allotransplantation. However, the chimerism induced by both of them was temporary for only 4 weeks (Plock 2015).

For the human hand transplantation, the first attempt was performed in 1964 by Ecuador when a bilateral amputee accepted a single hand transplantation and the immunosuppressive drugs given were AZA and steroids; unfortunately, after 2 weeks' treatment, the hand was rejected (Bozulic 2011). In 1998, a really successful hand transplantation was performed in France, where the induction therapies for immunosuppressive treatment were ALG, TAC, MMF and steroidsand maintenance therapies were TAC, MMF and prednisone (Dubernard 1999). Since then, with the development of immunosuppressive therapy, hand transplantation has been gradually well-developed around the world.

Facial and Scalp Composite Tissue Allotransplantation

Face contains the tissues of skin, muscle, bone, cartilage, nerve and vessel. In general, the purpose of facial transplantation is to repair the lesion and improve the appearance. However, skin is the largest part of face which has strong allogenicity. On the other hand, nerve regeneration is also an important part of facial transplantation, which is the basis of facial sensation and motor recovery. Neuroendocrine facial tissues contain fibroblasts, macrophages, mast cells, vessels and other immunogenic components which play an import role in local immune response.

The research of facial transplantation was carried out later than that of limb transplantation. However, with the guidance of the experience from the SOT and limb transplantation, it is developing faster. The rat model of facial transplantation is the commonly used model. Ulusal demonstrated that in a rat facial and scalp transplantation model between LBN rats ($RT1^{l/n}$) and LEW rats ($RT1^{l}$), the mono-treatment therapy of CsA for both induction (16 mg/kg/day) and maintenance (2 mg/kg/day) therapy induced the immunosuppression for more than 170 days (Ulusal 2003). At the same time, another rat hemifacial allograft transplant model was used to explore the immunotolerance and function recovery after transplantation with the same drug therapy, and the result showed that both donor-specific chimerism and functional tolerance were achieved (Demir 2004). Subsequently, an osteomusculocutaneous hemiface/calvaria flap model from LBN rats to LEW rats was performed, with CsA monotherapy for both induction and maintenance immunosuppressive therapy; the graft survived well and the average survival time was 154 days (Yazici 2006). However, most studies are hemiface transplantation, and compared to those, the whole face and scalp transplantation may have a more stringent demand for the vessels. In another face transplantation study, the authors confirmed that without vascular anastomosis, the large skin graft cannot survive. Furthermore, vascularized facial transplantation with CsA monotherapy could achieve better survival of allograft (Siemionow 2003).

Rat is a rodent and has a large evolutionary difference from human. Therefore, the orthotopichemiface/calvaria transplantation was studied in the rabbits transplanted from Dutch to Japanese White strains. The immunosuppressive drugs were CsA and prednisone, and composite tissues survived well without rejection. This was a trial of hemiface/calvaria transplantation in larger animals (Nie 2008). Similarly, another study was performed on the miniature swine to make a hemiface transplantation model, the transplant tissues included ear

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cartilage, nerve, parotid gland, lymphoid tissue, muscle and skin, using immunosuppressive treatment of CsA for only 4 weeks. Results showed that the allograft was rejected at 38−49 days, longer than that of untreated group. The skin and parotid gland could be used to monitor the rejection (Kuo 2009).

For the human facial transplantation, the first successful case was performed in France in 2005 when a 38 years old woman had a central and lower face transplantation from a brain-dead woman with distal nose, both lips, chin and cheeks. The immunosuppressive therapy was the combination of thymoglobulin, TAC, MMF, prednisone and infusions of donor bone marrow cells. The grafts survived well at least for 4 months after transplantation (Devauchelle 2005). Subsequently, 37 facial transplantations have been carried out in eight countries by the end of 2015, with 17 full face and 20 partial face transplantation. Five of them died because of different reasons such as sepsis, cancer, lymphoma, respiratory failure or suicide. Most of them had suffered acute rejection but the incidence of chronic rejection was low (Sosin 2016).

OTHER COMPOSITE TISSUE ALLOTRANSPLANTATION

In addition to the above two types of CTA, attempts have also been made to perform other CTAs. As the immunosuppressive therapies are usually similar with hand or facial transplantation, the following is a brief introduction to other CTAs.

Abdominal Wall Composite Tissue Allotransplantation

Abdominal wall allotransplantation is also conducted together with or after the intestinal, liver, or multivisceral transplantation, for the closure of the abdomen. Because the sizes of transplanted organs between donor and recipient are different, intestines get edema after reperfusion. It is important to maintain tension-free closure. It is also helpful to closely observe the occurrence of rejection (Gerlach 2016). Some different techniques are used in such cases. The types of transplanted abdominal wall usually are of full or partial thickness. According to the need for vascular reconstruction, it can be divided into vascularized or nonvascularized transplantation. Some animal models have been used for the research of abdominal wall transplantation (Ramirez 2014; Quigley 2013).

For the human, since the first abdominal wall transplantation was performed in USA in 2003 (Levi 2003), there were about 61 cases reported until the end of 2015. The most widely used method of transplantation is the full-thickness vascularized abdominal wall allotransplantation, and the major complications are delayed closure, wound infection, rejection and sepsis (Giele 2016).

Laryngeal Composite Tissue Allotransplantation

Laryngeal transplantation is suitable for the treatment of larynx irreversible trauma or low-grade malignant tumor with total laryngectomy. The goal of laryngeal transplantation is to make larynx functional, such as breathing, swallowing and voice. The animal models used

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for laryngeal transplantation mainly include rats and pigs. For the human, the first one was performed by Cleveland Clinic in 1998 where the transplanted tissue included the entire larynx, thyroid and parathyroid. The immunosuppressive drugs included anti-CD3 antibodies, CsA, MMF and methylprednisolone; the CsA was changed to TAC when a mild rejection happened 15 months after the transplantation. Subsequently, laryngeal function was recovered well (Strome 2001). After that, Duque reported 13 cases of laryngeal transplantation with 2-year graft survival rate of 90% (Duque 2007).

Penile Composite Tissue Allotransplantation

Penis contains tissues with different antigenic properties; they are mainly skin, muscle, fascia, sponge, nerve, neurofilm and blood vessels. In addition to the prevention of rejection, for the penile allotransplantation, it is more important to maintain the function of urination and erection. At the same time, it also involves complex ethical problems (Zhang 2010; Calne 2016). The experimental studies on penile transplantation are relatively few. Koga carried out early experimental penile transplantation in fully allogenic rats with TAC as the immunosuppressive drug, and achieved success in short-term survival (Koga 2003). Then a vascularized penile transplantation model was presented by Sonmez; with the treatment of CsA, the allografts survived longer than 200 days (Sonmez 2009). In another *ex vivo* experiment, the penile tissue from penile prosthesis surgery was co-cultured with allogenic or autologous peripheral blood mononuclear cells (PBMCs). The activation of PBMCs prompts rejection. This model could be used to evaluate the effect of different immunosuppressive therapies on the inhibition of rejection (Sopko 2016). At the same time, a penile transplantation model was established in Beagle dogs to research the blood supply recovery and tissue architecture changes (Zhao 2016).

The world's first penile transplantation was performed in China; however, the transplanted penis was removed 14 days after the operation because of the psychological problems but without the occurrence of rejection (Hu 2006). Subsequently not until 2015, a surgical team in Tygerberg hospital performed a successful penis transplantation (Bateman 2015).

CONCLUSION

In summary, compared with life-saving SOT, the purpose of CTA is to improve the life quality of the patients. With the development of medical technology and improvements in immunosuppressive treatments, both experimental and clinical CTAs are constantly developing. However, it still needs constant effort to achieve a balance between long-term allograft survival and low immunosuppressive side effects. At the same time, the ethical problems should also be considered. We believe that with the continuous technological development and maturity of CTA, it will benefit more patients in the future.

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Chapter 19

ISCHEMIA-REPERFUSION INJURY ANIMAL MODELS

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ABSTRACT

Ischaemia-reperfusion injury (IRI) is the principal pathological mechanism causing numerous widespread diseases. The complications induced by IRI including cardiovascular disease and cerebrovascular disease are a major reason of death in developed countries. IRI of native and transplant organs is an important cause of acute organ injury and a vital determinant of long-term organ dysfunction. The animal models of organ IRI are vital for understanding the underlying processes of IRI and development of efficacious treatment. Ischemic preconditioning (IPC) is a situation where preconditioning an organ with a sub-lethal/mild ischemic injury of short period can induce the organ's transient adaptive resistance to a lethal ischemic injury. Remote ischemic preconditioning (RIPC) is the phenomenon where a short sub-lethal ischemia and reperfusion in remote organs can induce ischemic tolerance in target organ. Ischemic postconditioning (IPoC) is a protective approach in which repeated brief IR is performed in the early phase of reperfusion after ischemia. The IRI animal models and different ischemic conditioning strategies in preclinical and available clinical application are reviewed in this chapter.

Keywords: ischemia-reperfusion injury, animal models, ischemic preconditioning, remote ischemic preconditioning, ischemic postconditioning

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ABBREVIATIONS

IRI	Ischemia-reperfusion injury
IPC	Ischemic preconditioning
RIPC	Remote ischemic preconditioning
IPoC	Ischemic postconditioning
IR	Ischemia-reperfusion
AKI	Acute kidney injury
ARF	Acute renal failure
BUN	Blood urea nitrogen
ROS	Reactive oxygen species
MI	Myocardial infarction
HF	Heart failure
LCA	Left coronary artery
LAD	Left anterior descending coronary artery
LV	Left ventricular
ECG	Electrocardiography
PTCA	Percutaneous transluminal coronary angiography
MPTP	Mitochondrial permeability transition pore
CABG	Coronary artery bypass grafting
STEMI	ST-elevation myocardial infarction
PCI	Percutaneous coronary intervention
ALI	Acute lung injury
ARDS	Acute respiratory distress syndrome
SMA	Superior mesenteric artery
PSS	Portasystemic shunt
AMI	Acute mesenteric ischemia
SCI	Spinal cord injury
RVM	Rice-Vannucci model
WMI	White matter injury
PVL	Periventricular leukomalacia
ICH	Intracerebral hemorrhage
IVH	Intraventricular hemorrhage
UCO	Umbilical cord occlusion
DTI	Diffusion tensor imaging
HI	Hypoxic-ischemic
HIE	Hypoxic-Ischemic Encephalopathy
CBF	Cerebral blood flow
BBB	Blood-brain barriers
MCA	Middle cerebral artery
tMCAO	temporary MCA occlusion
CP	Cerebral palsy
MHL	McGivney hemorrhoidal ligator band
CTT	Controlled tension tourniquet
ORB	Orthodontic rubber bands

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IOP	Intraocular pressure
FGR	Fetal growth restriction
CR	Controlled reperfusion

INTRODUCTION

Ischemia-reperfusion (IR) is a well-known pathological situation that is characterized by an early occlusion of blood supply to an organ or area followed by later vascular restoration and associated reoxygenation of downstream tissue (Eltzschig 2011). The restoration of blood supply to the affected organ/tissue produces remarkable organ/tissue injury which is known as IRI. IRI can develop as a consequence of trauma, hypertension, shock, sepsis, organ transplantation, or bypass surgery leading to end-organ failure such as acute renal tubular necrosis, bowel infarct, and liver failure. Furthermore, IRI can happen under different complications of vascular diseases such as myocardial infarction and stroke (Eltzschig 2011; Duehrkop 2014). IRI causes morbidity and mortality in extensive pathologies, including circulatory arrest, trauma, ischemic stroke, myocardial infarction, acute kidney injury, sickle cell disease and sleep apnea. Moreover, IRI is a great challenge during cardiothoracic, vascular and general surgery and organ transplantation. An imbalance in demand within the ischemic organ and metabolic supply induces extensive tissue hypoxia and microvascular dysfunction. Later reperfusion further increases the activation of innate and adaptive immune responses and cell death programs.

IRI is unavoidable in clinical transplantation. IRI-related impairment can be reduced by keeping the organ in a cold solution. But it is not possible to totally prevent such injury. Furthermore, IRI can cause delayed graft function which may affect the organ function and survival in short-term and long-term (Ponticelli 2015).

Acute IRI causes cell injury and death in lots of clinical conditions and in different tissues and organs including the brain, lung, heart, liver, spine cord, kidneys, stomach, intestine, pancreas, uterus and ovaries.

Ischemia conditioning is one strategy to improve IRI. IPC is a situation where preconditioning an organ with a sub-lethal/mild ischemic injury of short period can induce the organ's adaptive transient resistance to a lethal ischemic injury. IPC was first reported in dog heart study which showed that induction of mild ischemia followed by a period of reperfusion made the heart more resistant to a subsequent, ordinarily lethal ischemic insult (Murry 1986). Then the protective effect of IPC was verified in different animal models and in humans (Kloner 1998). IPC has been confirmed to be a potent method to induce tolerance to ischemic insults in most organs investigated and has been replicated in many laboratories around the world. IPC can decrease reperfusion injury and its systemic consequences. The main disadvantage of IPC is mechanical trauma to major vessels and direct stress to the target organ. So its clinical application is restricted.

RIPC is the phenomenon where a short sub-lethal ischemia and reperfusion in remote organs can induce ischemic tolerance in target organ. RIPC is a new approach where ischemia followed by reperfusion of one organ is thought to preserve remote organs (Tapuria 2008). RIPC was first described in 1993 and ischemia in the kidney followed by reperfusion decreased myocardial infarct size after ischemia (McClanahan 1993). Brief IR of the limb,

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gut, mesenteric, or kidney reduced myocardial infarct size in animal models (McClanahan 1993; Gho 1996; Birnbaum 1997). In humans, skeletal preconditioning has been used for myocardial protection with the beneficial effect being attributed to regulation of endothelial protection. The protective mechanisms of RIPC may involve adjustment of neurogenic pathways, elaboration of endogenous mediators, deduction of inflammatory cytokine production including tumor necrosis factor-α, and debilitation of IR-caused endothelial dysfunction (Meldrum 1998; Kharbanda 2002; Tapuria 2008). The mechanisms of RIPC's protective effect have yet to be explained. Investigations suggested that humoral and neural signalling pathways might be involved. More detailed information is available in some excellent reviews on the subject (Hausenloy 2013; Przyklenk 2013; Sluijter 2014).

Ischemic postconditioning (IPoC) is a protective approach in which repeated brief IR are performed in the early phase of reperfusion after ischemia. It was first reported in the dog heart (Zhao 2003). The protective effect of IPoC on IRI has been confirmed in different organs and animal species. IPoC is efficacious when it is performed in a remote organ or the target organ (Kerendi 2005; Zhao 2010). IPoC decreased myocardial infarct size by about 40% in animal models and in humans. IPoC has shown its clinical potential by decreasing IRI in different organs in several animal models. The strategies to reduce IRI such as IPC, RIPC and IPoC are safe, simple to perform and reproducible in human clinical trials.

RENAL IRI ANIMAL MODELS

Acute kidney injury (AKI) is a major kidney disease related to high mortality in patients. Clinically, ischemia is an important cause of AKI and can be induced by renal vascular occlusion, kidney transplantation or decreased cardiac output. IRI-induced acute renal failure (ARF) imitates the hemodynamic alterations-caused changes in renal function.

Different renal IRI animal models have been established and evaluated. Two types of warm renal IRI models are commonly applied: 1) bilateral renal IRI (Altmann 2012; Celie 2012; Zager 2012); and 2) unilateral renal IRI (Matsuda 2011; Tremblay 2002; Kitada 2002, Lee 2010, Yang 2016). The unilateral renal IRI model includes unilateral IRI without contralateral nephrectomy (Alikhan 2011; Godwin 2010) and unilateral IRI with contralateral nephrectomy (Matsuda 2011; Tremblay 2002; Kitada 2002, Lee 2010, Yang 2016). The bilateral renal IRI model is commonly used because it is considered more relevant to human pathological conditions where blood supply is normally affected in both kidneys (Altmann 2012; Lan 2012; Zager 2012). In the bilateral renal IRI model, decapsulation was carried out before renal ischemia (Megyesi 2002; Li 2009) which was shown to have renoprotective effects (Stone 1977). But, decapsulation was not performed in the majority of published studies of bilateral renal IRI. Renal IRI models were well established in large animals (Crane 2013; Lee 2006; Espinosa 1996). Rat IRI models then became the most popular animal model. The investigation with mouse renal IRI model has been extensively applied using genetically modified mouse since it was first developed in 1990 (Shoskes 1990). The mouse model of warm renal IR has crucial similarities with human IRI. The genetically modified mouse strains are very useful in investigating the molecular and cellular biology of IRI. The main disadvantage of renal IRI is the absence of cold ischemia. Usually, longer ischemia time is required to induce the same IRI effect on an organ for larger animals. Moreover, females seem to be more resistant to ischemia than males (Kennedy 2008).

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Porcine Renal IRI Model

The left kidney was exposed through midline abdominal incision after the anesthesia was induced and maintained (Crane 2013). The left renal artery was occluded by vessel loops after intravenous heparin (50 units/kg) and mannitol (350 mg/kg) were given. Pigs were subjected to hypothermic (5°C) or normothermic (33°C) ischemia. Sterile ice slush was filled in the abdomen cavity to produce hypothermic ischemia and ice slush was removed before reperfusion.

Canine Renal IRI Model

The kidney and renal hilum were exposed by laparotomy. The renal artery and vein were blocked by Satinsky clamps to induce normothermic renal ischemia (Crane 2013). The left kidney was exposed from the peripheral tissue and fat through median laparatomy. The left renal artery and vein were occluded by an atraumatic vascular clamp. The clamp was removed after 30 min of normothermic ischemia and then the right nephrectomy was done. Blood urea nitrogen (BUN) levels were significantly increased 14 days after reperfusion compared to before IRI. Blood BUN levels were significantly increased on 4 days after 90 minutes of warm unilateral renal ischemia with contralateral nephrectomy in dogs (Sekhon 2003). ARF was developed within 3 h by clamping the left renal artery for 1 h followed by reperfusion in uninephrectomized dogs (Yatsu 1998).

Bilateral Renal IRI Mice Model

The anesthesia is induced and maintained by isoflurane. The mouse is put on the homeothermic blanket of a homeothermic monitor system and covered by sterile surgical drape after the skin preparation (Wei 2012). The left kidney was exposed through left flank incision. The left kidney was gently pushed out of body cavity with cotton swabs to expose the renal pedicle. The pedicle was clamped by a microaneurysm. Sympathetic nerve damage may happen during the isolation of the renal artery in mice. Complete ischemia is confirmed by color change of the kidney from red to dark purple in a few seconds. Then, the left kidney was returned to the abdomenal cavity. Then the right kidney was exposed through the right flank incision and the renal pedicle was clamped using the same method. 22 and 35 minutes of ischaemia has been used for bilateral mouse renal IRI in most published studies. After the ischemia, the micro-aneurysm clip was removed for each kidney to start the reperfusion (Wei 2012).

Unilateral Renal IRI Mice Model

The left kidney and renal vessels were exposed via a dorsal incision after the anesthesia was induced and maintained by isoflurane. The left renal artery was clamped with a small vascular clamp for 50 minutes and released. Occlusion was confirmed visually by a change in

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kidney color, and reperfusion was confirmed by appearance of the original color. Then, the right kidney was nephrectomized via a dorsal incision. During surgery, body temperature was kept by putting the mice on a 38°C heating pad. Fifty minutes of ischemia and contralateral nephrectomy caused severe tubular damage that resulted in renal dysfunction in mice. Mortality occurred between 24 and 72 hours after IRI. The survival rate 7 days after IRI was 25% (Matsuda 2011). The left kidney and renal vessels were exposed by the abdominal incision after the anesthesia was induced and maintained by isoflurane (Yang 2016). The left renal artery was clamped with a small vascular clamp for 30 minutes. The right kidney was nephrectomized after the left kidney ischemia was finished.

Unilateral Renal IRI Rat Model

The right kidney was removed under light flurane anesthesia through a mid-abdominal incision. The left kidney was subjected to transient ischemia by occlusion of the left renal artery and vein using a micro-clip (Tremblay 2002). The surgical wound was temporarily closed, and the rats were placed on a heating pad kept at a temperature of 37°C for either 45 or 60 minutes of occlusion. The clip was then removed and the wound closed with a 3–0 suture. A 25% survival rate was observed 8 days after 60-minute renal ischemia.

Morphological studies have demonstrated that ischemia caused irreversible damages to the distal segments of the proximal tubules whereas more proximal segments suffer reversible injury after a short period (30 min) of normothermic ischemia (Shanley 1986).

ISCHEMIC CONDITIONING APPLICATION IN RENAL IRI ANIMAL MODEL

Contrast-induced AKI is a common and life-threatening complication after cardiac catheterization and no successful prophylactic treatment is available. IRI is certain to happen during transplantation.

RIPC by brief hind limb ischaemia had protective effect on renal IRI in rats (Wever 2011). Unilateral or bilateral RIPC were performed in rats. After 24 hours of reperfusion, Kidney function was ameliorated in both the bilateral RIPC group and unilateral group. Bilateral RIPC had better effect compared to unilateral RIPC (Wever 2011). RIPC by brief small intestinal ischemia reduced renal IRI in rats (Song 2007). RIPC was performed by three cycles of 8-minute ischemia and 5-minute reperfusion of the small intestine. Left renal ischemia was caused by a 45-minute renal artery blockage and reperfusion for 2 or 24 hours with right nephrectomy. Renal ischemic impairment was significantly improved. RIPC by 10 min of brief hepatic ischemia was shown to have protective effect on 45 min renal ischemia in rats (Atesx 2002). Repeated RIPC did not provide stronger protection against renal IRI compared to single dose RIPC in rats (Menting 2017).

IPoC was first reported to prevent mice ischemic ARF in 2007 (Szwarc 2007). IPoC ameliorated renal function after IRI in dog (Jiang 2010; Jiang 2012) and rats (Chen H 2008; Serviddio 2008; Eldaif 2010; Yun 2009; Zhang 2011; Miklos 2012). Renal injury parameters were remarkably reduced in IPoC compared with IRI only. IPoC prevented reactive oxygen

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species (ROS) production and prompted antioxidant activity which prevented cellular injury (Yun 2009). Moreover, mitochondria were more resistant to injury by IPoC, causing less apoptosis by up-regulation of antiapoptotic proteins as well as reduction of proapoptotic gene expression (Zhang 2011).

RIPC induced by three 5-min cycles of brief repetitive IR by clamping the exposed external iliac artery improved the early recovery of renal function in patients after kidney transplantation (Wu 2014). RIPC is a potential method to reduce transplantation-related IRI. RIPC induced by 4 cycles of 5 min lower limb ischemia and 5 min reperfusion using an arterial tourniquet cuff did not ameliorate renal function after live donor kidney transplantation in a double blind randomized clinical trial (Nicholson 2015).

The clinical protection effect of RIPC was demonstrated in a large-scale, multi-centre, multinational phase III renal transplantation with 406 live donor-recipient pairs (MacAllister 2015). Both recipients and donors received simultaneously the RIPC induced by four cycles of 5-min upper arm ischemia. Early RIPC was performed immediately before surgery and late RIPC was carried out 24 h before surgery. Early RIPC seems to provide renoprotection that potentially extend allograft lifespan by 2–3 years.

A multicentre, randomized controlled clinical trial is undergoing to evaluate whether RPIC of the leg of the patient ameliorates short and long-term graft function after kidney transplantation (Krogstrup 2015). RIPC reduced the renal damage in 42 patients with elective abdominal aortic aneurysm repair (Ali 2007).

CARDIAC IRI ANIMAL MODELS

The incidence of myocardial infarction is about one million people per year in the United States. Cardiac IRI is the typical pathophysiological characteristic for patients with the partial or complete arrest of cardiac circulation (Turer 2010). The first cardiac IRI model was developed in dogs (Jennings 1960). The histological results suggested that 30–60 minutes of IRI in dogs were comparable to the degree of necrosis usually observed after 24 hours of permanent coronary occlusion in human.

Myocardial ischemia is a usual clinical symptom characterized by low oxygen, low pH values, and high extracellular potassium concentration, which may trigger arrhythmias, myocardial infarction (MI), cardiac dysfunction and sudden death (Jennings 2013; Heusch 2014). Later reperfusion may repair the impaired myocardial structure and reduce heart function caused by ischemia. The effect of reperfusion is determined by the length and severity of prior ischemia (Lee 2012). Nonetheless, cardiac reperfusion could further initiate a complex inflammatory response, which may eventually cause cardiac IRI (Fauconnier 2011).

Different experimental models, including MI and cardiac IRI were developed depending on the duration and extent of coronary blood flow impairment and cardiac ischemic damage.

MI Animal Models

MI can be induced by different techniques of coronary arteries occlusions. These approaches were often applied to completely or partially block coronary artery branches in larger animals which were appropriate to generate MI and heart failure (HF) (Roth 1987;

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Harada 1994). Left anterolateral thoracotomy was carried out for occlude implantation after anesthesia was induced and maintained. A branch of the left coronary artery (LCA) was separated and the hydraulic occluder was put around the vessel. It was then inflated to cause incomplete constriction or full obstruction. An ameroid constrictor was implanted in a similar fashion but obstruction was reached by a dissimilar mechanism. The ring around the artery shrinks slowly due to the hygroscopic property of the casein plastic material. MI-induced HF model was produced by the hydraulic occluder or the ameroid constrictor in big animals (Harada 1994; Roth 1987).

HF model was first developed by coronary artery ligation in dogs (Hood 1967). Proximal left anterior descending coronary artery (LAD) was ligated to induce MI after orotracheal intubation and left thoracotomy was performed. The mortality of this model was more than 50% because of malignant ventricular tachycardias in the acute phase. Moreover, the average infarction size was 21% of the left ventricle and slight haemodynamic abnormalities were detected (Hood 1967). Additionally, the model is laborious and costly. But, this technique used in the pig induced a mortality of only 20%, mainly because of ventricular fibrillation and acute ventricular tachycardias (Iwanaga 2004).

Rat left ventricular (LV) MI model was first developed in 1979 (Pfeffer 1979). In short, orotracheal intubation and thoracotomy was performed after the anesthesia was induced and maintained. LCA was ligated in the proximal segment after the heart was exposed. Rats with small (4–30%) MI had no detectable damage in either baseline hemodynamics or peak indices of pumping and pressure-generating ability when compared to the sham-operated and noninfarcted rats. Rats with moderate (31–46%) MI had normal baseline hemodynamics but decreased peak flow indices and developed pressure. Congestive HF was developed when infarction was more than 46% at 21 days with elevated filling pressures, reduced cardiac output, and a minimal capacity to respond to pre- and after-load stress.

The degree of impairment of LV function is directly associated to the extent of myocardial loss (Pfeffer 1979). The mortality is strain-dependent. The mortality of Sprague-Dawley rats was 36% and Lewis inbred rats was 16% (Liu 1997).

The mouse is often used to characterise MI induced by coronary artery ligation. The surgical procedure to induce MI in the mouse is similar to the rat model (Gao 2000). But, a microscope is needed to precisely expose and ligate the relatively small LCA of the mouse. Mortality of MI mice is about 37–50% (Gehrmann 2001; Kuhlmann 2006). Most mice died within 1 h after ligation because of ventricular fibrillation and severe acute HF.

Myocardial IRI Model

Cardiac dysfunction induced by myocardial IRI is an important issue to be solved after percutaneous transluminal coronary angiography (PTCA) and thrombolysis are widely performed for acute MI therapy in humans. Further, cardiac dysfunction induced by myocardial IRI usually happens in cardiac surgery because reperfusion is inevitable during surgery with cardioplegia techniques and extracorporal circulation. Myocardial IRI animal models are well established. The duration of coronary artery occlusion affects the MI.

Regional myocardial IRI animal model is the classic model. The coronary artery is ligated with thread for small animals and a strip of moistened umbilical tape for big animals.

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However, a small plastic tube is put between the ligated vessel and the node in order to easily remove the occlusion.

Ischemia can be confirmed by the rapid regional whiteness of the myocardium andelectrocardiography (ECG) modifications. Reperfusion is confirmed by the occurrence of hyperaemia in the previously white region. Myocardial IRI animal model causes a higher infiltration of inflammatory cells, impaired fibrotic remodelling and increased neovascularisation in the area of infarction compared to the permanent occlusion model (Vandervelde 2006).

Myocardial IRI model was first developed in large animals. But rodent myocardial IRI models were often performed over the last decades. Myocardial IRI model in the closed-chest mouse was established to reduce inflammatory effects caused by surgery (Nossuli 2000). A thin suture was put under the LAD and the ends of the suture were threaded through a 0.5-mm piece of PE-10 tubing causing a loose snare around the LAD after thoracotomy was performed. The ends of the suture were externalized through each side of the chest wall, the chest was secured and ultimately the suture ends were hidden under the skin. The final IRI experiment was carried out a few days later after inflammatory cytokines levels returned to baseline. The LAD was obstructed by extracting the free suture ends in opposite directions and reperfusion was started by releasing them.

Ischemic Conditioning Application in Cardiac IRI Animal Model

Cardioprotection evoked by IPC is one of the most potent treatments for reducing infarct size after acute cardiac IRI. Mitochondria may play important roles in the protective effect of IPC. Signaling ROS are released by mitochondria in response to IPC and then suppress the mitochondrial permeability transition pore (MPTP) to reduce cardiac reperfusion injury and limit infarct size (Ferdinandy 2014). One considerable limitation of IPC is to perform the therapeutic intervention before myocardial ischemia. However, the beginning of myocardial ischemia is unforeseeable in MI patients.

IPoC by several cycles of short period ischemia has defeated this limitation (Zhao 2003).

The protection effect of IPoC has been verified in different species including man although the underlying mechanisms and signal transduction have not been yet understood (Skyschally 2009). Many of the signaling pathways underlying IPoC, but not all, are shared with IPC (Heusch 2006). The considerable disadvantage of IPC and IPoC as treatment for reducing cardiacl IRI is that both methods need to be performed directly to the heart. So the clinical application is limited.

RIPC was first described in 1993 and ischemia in the kidney followed by reperfusion decreased myocardial infarct size after ischemia (McClanahan 1993). RIPC by muscle stimulation with reduction of femoral arterial blood flow decreased myocardial infarct size in rabbit after 30 minutes of coronary artery occlusion and 4 hours of reperfusion (Birnbaum 1997). RIPC by 10 minutes limb ischemia significantly reduced reperfusion arrhythmias after 30 minutes left descending coronary artery occlusion in rats (Oxman 1997). RIPC by four 5-minute cycles of lower limb ischemia decreased myocardial infarct size after 40 minutes of balloon occlusion of the left anterior descending artery in pigs (Kharbanda 2002).

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RIPC by later non-invasive hind limb ischemia significantly decreased myocardial injury, infarct size, and incidence of ventricular arrhythmias after 30 minutes of left anterior descending coronary artery occlusion and 120 min of reperfusion in rats (Li 2010). RIPC by 15 minutes occlusion of the anterior mesenteric artery protected myocardium against infarction induced by 60 minutes coronary artery occlusion in rats (Gho 1996).

RICP by 10 minutes left renal artery occlusion in rabbit reduced myocardial infarct sizes induced by 30 minutes occlusion of left coronary artery and 2h reperfusion (Pell 1998).

RIPC induced by six cycles of 5 min limb occlusion and 15 min reperfusion considerably decreased heart IRI in rat caused by acute left anterior descending artery occlusion (60 min ligation and 180 min reperfusion). Furthermore, RIPC in bilateral upper and lower limbs showed a better effect in decreasing infarct size than RIPC in bilateral upper-limb and in bilateral lower-limb (Wu 2016).

RIPC performed by three 5-minute cycles of ischemia of the contralateral limb prevented endothelial IRI caused by 20 minutes of upper limb ischemia (inflation of a blood pressure cuff to 200 mm Hg) and reperfusion in humans (Kharbanda 2002). RIPC by two time periods of 3 min of ischaemia and 2 min of reperfusion around the right upper extremity with a tourniquet protected myocardium in patients undergoing coronary artery surgery by increasing anaerobic glycolysis (Günaydin 2000).

RIPC can provide cardioprotection at different time points of acute cardiac IRI. RIPC can be performed before myocardial ischemia (Przyklenk 1993); after the beginning of myocardial ischemia but before reperfusion (Schmidt 2007); at the beginning of myocardial reperfusion (Andreka 2007). RIPC was produced by 15 min obstruction of femoral arteries and provided a similar degree of cardioprotection when performed 25 min before MI, 10 or 25 min after the begining of MI and 10 min after the begining of reperfusion in in rat myocardial IRI (30 min of left coronary artery occlusion and 120 min of reperfusion) (Basalay 2012). Repeated daily RIPC for 28 days after MI reduces adverse LV remodeling and improves survival in a rat cardiac IRI (Wei 2011).

RIPC decreased myocardial damage in 42 patients with elective abdominal aortic aneurysm repair (Ali 2007). RIPC significantly decreased total serum troponin-T release at 6, 12, 24, and 48 h in 57 patients after elective coronary artery bypass grafting (CABG) and decreased 43% of troponin T area under the curve (Hausenloy 2007). Meta-analysis suggested that RIPC had an overall beneficial effect on cardiac damage biomarkers in 891 patients with CABG from 13 trials (Yetgin 2012).

RIPC produced perioperative cardiac protection and ameliorated the prognosis in 329 patients with elective CABG surgery (Thielmann 2013). A meta-analysis of 8 randomized controlled trials suggested that RIPC did not ameliorate the all-cause mortality at 30 days in patients with CABG surgery (Zhang 2014).

RPIC decreased MI size of ST-elevation MI (STEMI) patients with urgent revascularization by percutaneous coronary intervention (PCI) in five randomized trials (Rentoukas 2010). RIPC performed during ambulance transport increased median salvage index at 30 days in 333 patients with acute MI (Botker 2010). IPoC performed in the first minutes of reperfusion, decreased infarct size by 20% in 100 patients with primary PCI for anterior STEMI (Crimi 2013). RIPC decreased 30% concentration of biomarker released in STEMI patients. IPoC did not provide additional benefit (Prunier 2014). The protective effect of RIPC was verified in 197 STEMI patients by reducing 27% of MI size (White 2015). However, several large, multicentre, randomized, controlled clinical trials on cardioprotection

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have emphasized the challenges of translating ischemic conditioning cardioprotection strategies into patient benefit (Hausenloy 2016).

LUNG IRI ANIMAL MODELS

Lung IRI happens after lung transplantation, pulmonary thromboembolectomy, or cardiopulmonary bypass. Acute lung injury (ALI) causes the most serious symptom, the acute respiratory distress syndrome (ARDS) which is a clinical syndrome characterized by acute hypoxemic respiratory failure, bilateral pulmonary infiltrates consistent with edema, and normal cardiac filling pressures (Ware 2000).

Lung ischemia up to several hours is certain to happen during lung transplantation. Reperfusion of the transplanted lung can induce nonspecific pulmonary edema, alveolar damage and hypoxemia within 72 h after lung transplantation. 15% of the patients with lung transplantation encounter graft complications owing to lung IRI (de Perrot 2003).

IRI is a versatile pathological process that complicates the perioperative treatment of patients with lung transplantation. The most severe type can cause primary graft failure and is an important reason of morbidity and mortality after lung transplantation. Lung IRI generally shows quick damage in lung function after transplantation accompanied by fast progress of pulmonary edema, augmented pulmonary vascular resistance, and reduced airway compliance (Lee 2010). Lung IRI causes systemic effects in heart and liver and is distinguished by neutrophil sequestration and increased ROS in the circulation (Esme 2006; McMillen 1993).

Animal models can establish a bridge between clinical study and the preclinical investigation. Hypotheses produced in clinical investigations can be examined directly in animal models, and the results from *in vitro* investigations can be determined in animal models to evaluate their relevance in entire living systems. It is impossible to verify clinical hypotheses produced in clinical investigations and confirm the value of *in vitro* findings without animal models.

ALI animal models should replicate the mechanisms and consequences of ALI in humans, including the physiological adaptation and pathological alteration (Matute-Bello 2008). Single animal model cannot replicate all the features of human ALI/ARDS. Most of the animal models are associated with restricted features of human ALI/ARDS (Matute-Bello 2008).

ANIMAL MODELS OF LUNG IRI

Lung IRI models mainly include ventilated ischemia and anoxic ischemia. The most extensively applied models of lung ischemia are occlusion of the pulmonary artery, which keeps the venous return and bronchial circulation *intact,* and blockage of the hilum, which induces full ischemia and anoxia. The factors affecting the lung IRI models include animal species, desuflation vs. insuflation of the lung, the type of ischemia (hilum, pulmonary artery, venous return), experimental preparation (*in vivo* vs. isolated perfused lungs) and the length of both ischemia and reperfusion (Matute-Bello 2008). The lungs are perfused by two different vascular systems, the bronchial circulation and the pulmonary circulation. Ischemia

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can be induced by blocking the pulmonary artery, which maintains the bronchial circulation, or by occluding the hilum, which ceases all circulation.

Lung IRI induced by 2 h of ischemia and 2 h of reperfusion of the left lower lobe in cats triggered lung damage typified by the presence of neutrophils, macrophages, and RBCs in alveoli and alveolar edema (Neely 1995). Blockage of the pulmonary artery only causes less damage than obstruction of both the bronchial circulation and the pulmonary artery (Hamvas 1994; Pearse 1994). Reverse pulmonary venous circulation from the left atrium can happen if the hilum is not occluded, causing milder damage than full closure of the hilum (Hamvas 1992; Obermiller 1991). The degree of ischemia is also influenced by whether oxygenated air continues to ventilate the lungs while the circulation is occluded.

Ischemia without reperfusion in rabbits may or may not cause damage which is determined by the degree of inflation of the ischemic bed (Bishop 1994). When the reperfusion time remains persistent, such longer ischemic time induces worse lung damage in rats (Waddell 1996) and rabbits (Sakuma 1999). When the ischemic time is unchanging, the severity of damage increases with longer reperfusion time in cats (Neely 1995) and in rats (Khimenko 1996). So, ischemia and reperfusion times both influence extent of the following damage.

IRI can be performed either *in vivo* or in isolated lung preparations, and the experimental method does not seem to affect the development of injury. *In vivo* models enable the investigation of consequential alterations in the contralateral lung. Lung IRI results differ among species. Rabbits have been most extensively utilized in lung IRI models. In the dog, pulmonary postcapillary resistance was significantly increased after lung reperfusion (Jackson 1992). In rats, the permeability of the postalveolar venules was increased to cause lung edema (Khimenko 1999). Lung IRI-induced lung damage is distinguished by raised pulmonary vascular permeability and edema, hemorrhage and PMN infiltration. The degree of damage is worse when the lungs keep deflated during the ischemic period and when the pulmonary and bronchial circulations are blocked simultaneously.

Nonpulmonary IRI

Nonpulmonary IRI was induced by occlusion of blood supply except the bronchial circulation and the pulmonary circulation. The nonpulmonary IRI models were reported mostly in mice and rats. Models varied according to the ischemic anatomic area, the ischemic time, and the reperfusion time. The severity of lung damage is often associated to the volume of ischemic tissue than to the time of ischemia. Nonpulmonary IRI is usually reached by separating and blocking a specific artery for certain time and then starting perfusion. The superior mesenteric artery is the frequently utilized vascular bed in large animals (Fullerton 1996; Koike 1992; Koike 1995). Hindlimb ischemia was more commonly used in mice (Engles 1997; Welborn 1996). For example, 45 min of superior mesenteric artery occlusion induced moderate ^{125}I-albumin lung leak at 6 hr of reperfusion which was reversed at 18 hr in rats (Koike 1992).

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Ischemic Conditioning Application in Lung IRI Animal Model

IPC induced by 10 min IR remarkably decreased the injured alveoli to 23 +/- 13% which was caused by 2 h ischemia and 2 h reperfusion which was similar to 2 h ischemia and 1 h reperfusion in cat (Neely 1995). IPC induced by one cycle of 5 min of IR prevented the vascular and biochemical alterations in response to lung IRI caused by 2 h of normothermic ischemia in rat (Yildiz 2007). RIPC induced by three cycles of 5 min bilateral hind-limb IR significantly reduced pulmonary edema and respiratory failure in pig acute lung IRI (2 h ischemia/2.5 h reperfusion) (Harkin 2002). RIPC by three cycles of 5 min left common femoral artery IR protected against acute lung IRI and pulmonary hypertension in pig acute IRI (1.5 h ischemia/5 h reperfusion) (Waldow 2005). Intestinal RIPC improved the lung IRI by decreasing myeloperoxidase, malondialdehyde, TNF-α, and interleukin-1 levels in the serum and lung tissue and by increasing the NO level in the serum and lung tissue in rats (Wang 2015). Intestinal RIPC improved the function of anti-oxygen free radical, suppressed the release of pro-inflammatory cytokines and reduced apoptosis in lung IRI in rats.

IPoC by three successive cycles of 30-s reperfusion per 30-s occlusion before restoring full perfusion reduced pulmonary neutrophil accumulation/activation and lung IRI (40 min of left-lung ischemia and 120 min of reperfusion) and decreased systemic inflammatory responses (Xu 2011). IPoC induced by three cycles of 30 sec reperfusion and 30 sec reocclusion at the onset of reperfusion improved acute lung IRI which was comparable to IPC in rat (Liu 2009). IPC was induced by 10 min Superior mesenteric artery (SMA) occlusion and 10 min reperfusion before prolonged occlusion. Delayed IPoC did not have the protective effect.

Three cycles of lower limb RIPC before allograft reperfusion were performed in the recipients. RIPC did not significantly improve partial pressure of arterial oxygen/fraction of inspired oxygen ratio in a randomized controlled trial of 60 patients with bilateral sequential lung transplantation (Lin 2014). RIPC-treated group had less primary graft dysfunction and acute rejection. Anyway, the results in this small study warrant a large multicenter trial of RIPC in lung transplantation. RIPC provided pulmonary protection in 62 patients after elective open infrarenal abdominal aortic aneurysm repair (Li 2013).

Liver IRI Animal Models

Liver IRI is a main reason of liver impairment during liver transplantation and liver resection (Fondevila 2003; de Rougemont 2010). During liver resection, in-flow vascular occlusion for reducing intraoperative blood loss may induce warm liver IRI. In orthotopic liver transplantation, the liver undergoes cold-rewarming IRI when the donor liver is reperfused in the recipient (de Rougemont 2010).

Animals from pigs to mice can be used to investigate the complex biological processes of hepatic IRI and evaluate the protective effect of treatment agents. The animal studies can be used as a reference for human study with some limitations. The limitations include anatomical differences of the livers of various species and subspecies, the differences in hypothermia and ischemia tolerance and the experimental models differences. So, it is extremely vital to select

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the appropriate animal species and experimental model and to standardize the protocol according to the study.

The advantages of mice and rats include minimal logistical, financial or ethical problems, and availability for genetic alterations studies. Nevertheless, the disadvantage is that the results obtained from small animals are of restricted relevance to human beings because of their variable size and anatomy of the liver and their faster metabolism (Abdo 2003). The age and sex of the animals need to be considered. Remarkable variations in hepatic microcirculation were observed between young and older rats (Yahanda 1990).

14–16 weeks old rats are the most appropriate for liver IRI study. The younger rats may have technical problems and the older rats are more susceptible to fat accumulation and respiratory infections. Gender also influences experimental results. Hormone levels in female animals are dependent on the estrous cycle which definitely changes the ischemia tolerance of the liver. The male rats were less sensitive to IRI than female rats after normothermic liver ischemia (Gasbarrini 2001).

Partial Liver IRI

Partial hepatic IR I was developed by occlusion of the hepatic artery, the portal vein and the bile duct of the left and median lobes for 60–120 min and 24 hr reperfusion in rats (Yamauchi 1982). This 70% partial ischemia model has been generally used for hepatic IRI study (Massip-Salcedo 2008, Casillas-Ramirez 2008). Furthermore, a 30% partial liver ischemia model has been established to block the blood supply to the right lobe of the liver by occlusion of the hepatic artery and portal vein (Peralta 2000).

Partial hepatectomy under IR is usually performed to control bleeding during parenchymal dissection in clinical practice. Hepatic regeneration and IRI animal model is utilized to imitate the clinical situation of selective or hemihepatic vascular occlusion for liver resections. The portal triad supplying the median lobe is blocked by microvascular clamp after resection of left hepatic lobe in animal model. The congestion of the bowel can be avoided during the ischemic period by maintaining the portal flow through the right and caudate lobes. The right lobe and caudate lobes are removed at the end of ischemia. Reperfusion of the median lobe is achieved after the clamp is removed. This model does not need any portal decompression and also achieves some major standard such as good duplicability, reversibility, and easy operation (Selzner 1999, Ramalho 2009).

Total Hepatic IRI with Portocaval Decompression

Total hepatic IRI with portal decompression closely imitates the clinical situation of warm ischemia after the Pringle maneuver for liver resection and liver transplantation. The portacaval shunts for portal hypertension was first developed in rats (Blakemore 1945). Then a simple technique for portocaval shunt in the rat was reported (Bernstein 1959). Portosystemic shunt transferred the portal blood to the iliac vein after functional hepatectomy. The splenocaval shunt was developed in rat (Spiegel 1995). A portofemorojugular bypass was used in pigs (Uhlmann 2003).

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Total Hepatic IRI with Spleen Transposition

Total hepatic ischemia with spleen transposition was first established in rats for the surgical treatment of portal hypertension (Bengmark 1970). The spleen was relocated to a subcutaneous pouch. Acceptable portosystemic anastomoses was performed 2–3 weeks later. The spleen transposition produced reversal of blood flow in the splenic vein and triggered angiogenesis. An easy and simple experimental rat model of total hepatectomy, hepatic ischemia and extrahepatic portal obstruction was established in rat (Omokawa 1991). The spleen with its scarified capsule was firstly relocated to a subcutaneous pouch to perform portasystemic anastomosis. Total hepatectomy was easily carried out in a lobe-by-lobe fashion 2 weeks later. Anhepatic rats with a glucose infusion survived for 10 hours and all died of acute hepatic failure. Sixty minutes of hepatic ischemia was done in rats with a spleen transposition for a portasystemic shunt (PSS). The long time interval (3 weeks) for the establishment of satisfactory portosystemic collaterals is the disadvantage of this model. Total hepatic IRI with PSS using a 1-mm inner diameter tube was strongly comparable to partial hepatic ischemia in the pathophysiological profile during hepatic ischemia in rats. Rats without PSS badly endured 30 min of total hepatic ischemia. Rats with PSS or partial hepatic ischemia survived 60 min of total hepatic ischemia (Suzuki 1998).

Total hepatic ischemia in the mouse was first established by partial hepatectomy with clamping of the remnant liver (Yadav 1998). A partial (30%) hepatectomy was carried out with resection of the caudate, right lateral, and quadrate lobes and papillary process. The pedicles of the median and left lateral lobe at the level of the hilum were blocked by vascular microclamps to reach total ischemia. Spontaneous portocaval shunts through caudate branches and collateral vessels prevented mesenteric congestion. All animals survived 60 min of ischemia. However all animals died after 90 min of ischemia.

Different factors need to be considered before the selection of an experimental model of hepatic IRI. The severity of hepatic IRI is decided by the ischemia time (González-Flecha 1993). It is better to utilize an ischemia time with high survival for investigating the hepatic IRI mechanisms or the protective mechanisms of an agent. The ischemia time with low survival is more suitable for the relevance study of a drug in hepatic IRI. 60 min of warm ischemia caused reversible cell injury. Liver oxygen consumption recovered to control levels after reperfusion. 120–180 min warm ischemia induced irreversible cell damage after reperfusion. 90 min of ischemia was the cellular endpoint for hepatocytes (González-Flecha 1993).

Ischemic Conditioning Application in Liver IRI Animal Model

IPC was induced by 10 minutes ischemia and 10 minutes reperfusion prior to 60 minutes of ischemia in rat and mice model of hepatic IRI. IPC reduced liver IRI, as shown by lower hepatic inflammatory cytokines, lower serum aminotransferase levels, and less severe IR-related histopathologic alterations (Liu 2014). IPC protected against hepatic IRI, at least in part, by heme oxygenase-1-mediated autophagy. The proof from systematic review

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demonstrated that IPC conferred a protective impact in experimental steatotic livers with IRI which improved the survival and reduced histological injury (Chu 2015).

RIPC as a liver protective approach was first reported in rat left lobe of liver IRI (45 min ischemia/240 min reperfusion) (Lai 2006). RIPC by four 10-min cycles of IR to hind limb significantly reduced plasma alanine aminotransferase which was related to the induction of hemeoxygenase-1 (Lai 2006). Different RIPC protocols were used to verify the protective effects of limb RIPC against acute liver IRI in the rat, rabbit and mouse (Tapuria 2009; Kanoria 2006; Abu-Amara 2012). RIPC by four cycles of 4 min hind limb IR significantly increased sinusoidal flow, sinusoidal perfusion, red blood cell velocity, and reduced neutrophil adhesion and cell death in rat partial hepatic ischemia IRI (45 min ischemia/3 h reperfusion) (Tapuria 2009). Hemeoxygenase-1 had an important role in regulating hepatic microcirculation and endothelial function.

Liver IRI (25 min ischemia/120 min reperfusion) in rabbits increased serum aminotransferases and decreased mean arterial blood pressure, hepatic blood flow and peripheral oxygen saturation (Kanoria 2006). RIPC by three cycles of 10 min hind limb IR significantly improved these parameters and considerably increased hepatic venous nitrate/nitrite. RIPC by six cycles of 4 min hind limb IR significantly increased hepatic microcirculatory blood flow and reduced plasma transaminases in mice partial liver IRI (40 min ischemia/120 min reperfusion) (Abu-Amara 2012). The hepatic soluble guanylyl cyclase-cyclic GMP pathway was needed for mediating the protective effects of hind limb RIPC on hepatic microcirculatory blood flow in mice liver IRI.

In rat liver IRI model (30 min ischemia and 30 min reperfusion), 172 genes were found as the most highly affected, and these genes demonstrated similar patterns regarding the up- and down-regulated expression levels among IPC, IPoC, and IPC + IPoC groups (Knudsen 2012). IPC, IPoC, and IPC + IPoC demonstrated significant impacts on the expression levels of a large number of genes during early reperfusion. IPC, IPoC, and IPC + IPoC seem to effectuate their protective effects by adjusting the same genes and genetic networks (Knudsen 2012). These recognized networks are involved in keeping cellular homeostasis.

PANCREASE IRI ANIMAL MODELS

Pancreas transplantation is the most effective long-term treatment for insulin-dependent diabetes mellitus. However, long-term pancreas survivals are remarkably lower compared to other whole organ allografts. Increased sensitivity to IRI may, in part, be responsible for this clinical difference by direct damage to the allograft. IRI is the original stimulus for organ injury after transplantation. Tissues are susceptible to cold and warm ischemic injury before transplantation. Also damaged cells then are potent stimuli for the innate immune system which may induce the activation and maturation of alloreactive T and B cells and destroy the allograft damage (Brennan 2010). IRI may finally trigger acute rejection of the transplanted organ.

A mouse pancreatic IRI model was developed for the first time to distinguish the histologic and biochemical impairment related to IRI and to estimate the immunologic markers of ischemic injury to the pancreas in 2013 (Lunsford 2013). Total pancreatic ischemia induced by occlusion of the superior pancreaticoduodenal artery, inferior

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pancreaticoduodenal artery, and lieno-pancreatic artery for 10 minutes caused high mortality. Distal pancreatic ischemia induced by occlusion of the lieno-pancreatic artery and the lienal artery for 30 minutes caused time-limited and reversible pancreatic inflammatory process. Perceptible ischemic changes reversed after reperfusion. Serum amylase and lactate dehydrogenase reached the peak at 6 hour and returned to normal range by 120 hour after IRI. Biochemical evidence of inflammation was detected. Noticeable inflammation was observed by histologic scoring. Time-limited pancreatic inflammation and damage was detected by histologic and biochemical indices in this new mouse distal pancreatic IRI model.

Complete pancreas IRI rat model was first developed by occlusion of the four blood-supplying arteries (left gastric artery, gastroduodenal artery, splenic artery, and caudal pancreaticoduodenal artery) to the pancreas in 1995 (Hoffmann 1995). The complete pancreas IR caused pancreatic microvascular failure. The severity of changes was determined by duration of ischemia and duration of reperfusion. The morphological and biochemical changes showed that IR induced an inflammatory reaction similar to that of acute pancreatitis.

The whole rat pancreas ischemia was induced by occlusion of the left gastric artery, gastroduodenal artery, splenic artery and caudal pancreaticoduodenal artery with microvascular clip for 1 or 2 hours (Neeff 2012). The pancreas is highly susceptible to ischemic damage. IR of the pancreas caused acute pancreatitis in rats (Dobschuetz 2008; Lo 2009). IR induced an inflammatory reaction similar to acute pancreatitis. The rat pancreas IRI was induced by occlusion of the splenic and the gastroduodenal artery for 2 hours followed by reperfusion for 6 hours (Lo 2009). The superior and inferior pancreaticoduodenal arteries with vessels of the splenic artery were occluded using microvascular clamps for 30 minutes in rabbits. The reperfusion period was 30 minutes (Apostolidis 2012).

Ischemic Conditioning Application in Pancreas IRI Animal Model

Combined pancreas–kidney transplantation has been successfully implemented for patients with type 1 diabetes and end-stage renal failure (Wynn 2004). This operation can be complicated by graft pancreatitis, a contributing factor for which is acute IRI, resulting in graft loss (Büsing 1993). RIPC by 15 min of infrarenal aortic ischemia and reperfusion could protect the pancreas against acute IRI as evidenced by improved circulation, reduced inflammatory tissue response and histological damage (Oehmann 2007). RIPC could be one approach to ameliorate organ procurement in pancreas transplantation.

RIPC-mediated protection against acute pancreatic IRI has been confirmed by a subsequent study (Nikeghbalian 2009). RIPC of liver was performed by first clamping of the hepatic pedicle for 10 minutes in rats. Following this, IR of the pancreas was induced by first clamping the inferior splenic artery for 30 minutes, followed by reperfusion for one hour. RIPC of the liver decreased the severity of IR-induced pancreatitis (Nikeghbalian 2009). These effects were partly associated with the decrease of pro-inflammatory interleukin-1 beta. However, the protective effect of RIPC was not observed in rats (Warzecha 2008). RIPC was carried out by occlusion of femoral or renal artery twice for 5 min with 5 min interval. RIPC had no effect on pancreatic exocrine secretion and did not decrease the severity of IR-induced pancreatitis in rats (Warzecha 2008).

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GASTRIC IRI ANIMAL MODELS

Stomach is one of the most vulnerable organ systems to ischemia. Gastric IRI is a major and usual clinical situation which could cause mucosal injury. Gastric IRI was induced by different clinical situations which included hemorrhagic shock, peptic ulcer bleeding, ischemia gastrointestinal disease and vascular rupture or surgery (Guo 2014). Gastric IRI is a quite common condition causing mucosal damage and may influence mucosal repair by regulating the gene expression of growth factors. Thus, accurate comprehension of the molecular mechanism of IRI may help to explore new mucosal protective agents.

The gastric IRI rat model was developed. The celiac artery was exposed through abdominal midline incision and clamped for 80 min (Mogami 2012) or 30 min (Kyoi 2004) in rat. Reperfusion was initiated for 12 h or 48 h after 80 min ischemia (Mogami 2012). The gastric IRI mice model was also established. Gastric IRI was induced in mice by 30 min celiac artery clamping and 60 min reperfusion. Macroscopically visible injuries were constantly found in the stomach 60 min after the inception of reperfusion following ischemia for 30 min (Nakamori 2010).

ISCHEMIC CONDITIONING APPLICATION IN GASTRIC IRI ANIMAL MODEL

IPC was produced by two 5 min episodes of celiac artery occlusion and 10 min reperfusion. IPC increased plasma corticosterone and reduced the gastric damages caused by prolonged gastric IR (30 min occlusion of celiac artery and 3 h of reperfusion) in rats (Bobryshev 2009). RIPC by two cycles of 5 min myocardial or hepatic IR was first reported to protect gastric mucosa against severe gastric IRI (3h ischemia/3h reperfusion) as efficiently as gastric preconditioning (Brzozowski 2004A; Brzozowski 2004B) in rat.

Gastric IRI was induced by putting an elastic rubber band on the proximal part of the bilateral lower limb for ligature for 3 h and reperfusion for different period in rats (Wang 2011, Wang 2014). IPoC by three cycles of 30-s reperfusion and 30-s femoral aortic reocclusion were performed before reperfusion. IPoC decreased post-ischemic oxidative stress (Wang 2011). RIPC was carried out by three cycles of 30 s of reperfusion and 30 s of reocclusion of the femoral aortic immediately after gastric IR and before reperfusion for up to 24 h. RIPC provided effective functional protection and prevented gastric IRI by anti-inflammatory and antioxidant actions (Wang 2014).

INTESTINE IRI ANIMAL MODELS

The survival rate of patients with ischemic intestinal damage is usually less than 50% (Gregory 2011; Yasuhara 2005). Intestinal ischemia is related to different clinical conditions including hemorrhagic shock (Corcos 2013; Fishman 2014), trauma (Corcos 2013), cardiopulmonary disease (Sastry 2014), acute mesenteric ischemia (Yasuhara 2005), neonatal necrotizing enterocolitis (Nankervis 2008), volvulus (Schwartz 2013) and intestinal transplant rejection (Mallick 2004). Different intestine IRI animal models are decribed below.

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Complete Vascular Occlusion

Intestinal IRI model is usually produced by temporary vascular occlusion of the SMA with atraumatic vascular clamps or permanent vascular ligation of SMA. The SMA is exposed through a midline incision and blocked for a period of time (Ikeda 1998). Heparin is administered intravenously to prevent thrombus formation within the SMA and the circulation is reestablished after the atraumatic vascular clamps are released. SMA occlusion only was not reliable in inducing consistent and reproducible injury. SMA occlusion combined with ligation of collateral arcades caused a higher degree of injury and a greater but consistent level of mortality in the animals (Megison 1990). SMA blockage caused blood flow decrease of 63% in the proximal colon, 71% in the jejunum and ileum, 61% in the distal duodenum and 35% in the proximal duodenum. Blood flow decrease was not found in the mid and distal colon (Premen 1987). So the effect of SMA blockage is mainly dependent on the intestinal segment. The intestine segments may have different sensitivity to ischemic injury. The colon was more resistant to ischemia injury compared to intestine in rats (Leung 1992). The advantages of SMA ligation animal model are representation of occlusive causes of ischemia and easy to establish surgical model. The main disadvantages of SMA ligation animal model include 1) intestine segments resist differently to ischemic injury (Robinson 1981; Leung 1992; Chen 2013) and 2) undependable to produce constant damage and high mortality rate (Megison 1990).

SMA Embolization

A complete irreversible ischemia was reported in a porcine model by SMA embolization. The SMA was approached either by endovascular catheterization (Acosta 2007; Rosow 2005) or via percutaneously (Klein 1996). These models are especially helpful for imaging investigations to improve diagnosis in patients suspected of acute mesenteric ischemia (AMI). AMI is a complex of diseases and includes occlusive AMI or nonocclusive AMI. Occlusive AMI can be separated to acute or chronic and whether veins or arteries are influenced. Nonocclusive AMI damages all types of mesenteric ischemia with patent mesenteric arteries. It is usually related to patients over 50 years of age with aortic insufficiency, congestive heart failure myocardial infarction, renal disease, hepatic disease or undergoing cardiac surgery (Trompeter 2002). Nonocclusive AMI composes 20−30% of AMI with a mortality rate of 50%.

The advantages of SMA embolization animal model include representation of occlusive causes of ischemia, non-manipulation of intestinal tract (Acosta 2007, Block 2011) and perfection for diagnostic imaging studies. The disadvantages of SMA embolization animal model include only arterial supply blocked, poor model of chronic ischemia, need for advanced surgical technique, requirement of large animal and irreversible ischemia, prohibiting study of reperfusion.

Low-Flow Ischemia

Most of the original low-flow ischemia investigations were carried out in cats (Granger 1986; Kubes 1992; Nilsson 1994; Acosta 2007). This model well represents the type of

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intestinal injury induced by hemorrhagic shock or other events that greatly decrease–blood supply to an intestinal segment. In the low-flow ischemia model, blood flow is decreased to 20% of baseline levels (25–35 mmHg). A segment of ileum is typically separated by a midline incision. An arterial route is produced between the superior mesenteric and femoral arteries and pressure cannulas are utilized for vascular pressure measurement. Reduced blood pressure within the SMA (25–30 mmHg) is reached by application of an adjustable vascular clamp. Most damage related to this model has been attributed to reperfusion as a result of elevation in xanthine oxidase and neutrophil activation. It may not sufficiently represent clinical scenarios, in which injury induced by ischemia may be so severe that reperfusion injury is of limited significance.

The advantages of low-flow ischemia animal model include representation of nonocclusive causes of ischemia and ameliorated model of reperfusion injury. The disadvantages of low-flow ischemia animal model include increased difficulty of surgery and minimal ischemic injury.

Segmental Mesenteric Vascular Occlusion

Intestine ischemia model can be induced by segmental mesenteric vascular occlusion in which the blood flow within a discrete loop of intestine is interrupted by clamping the local mesenteric vascular supply and cross-clamping the bowel. In one animal, multiple loops can be undertaken to different lengths of ischemia with or without reperfusion, thus quantifying the degree of injury within each loop. It is not difficult to establish this model in pigs and in rodents (Grootjans 2013a; Grootjans 2013b). The advantage of this model is to provide multiple treatment groups and controls within a single animal. But, this model of strangulating obstruction depends on full occlusion of the arterial and venous blood supply. In clinical situation, the venous circulation is usually damaged before the arterial blood supply due to differences in vessel wall thickness and compliance (Park 1990). The animal species utilized may contribute remarkably to the outcome. Variations of the mucosal vascular supply in the small intestine exist among species. The pig and human villus microvascular architecture are extremely comparable (Bellamy 1973, Casley-Smith 1984). The mesenteric vascular differences may affect the physiological function of the intestinal mucosa among species and likely their sensitivity to injury. Intestinal permeability studies have shown remarkable differences between rodents and humans and considerable correlation between pigs and humans (Delahunty 1987; Bijlsma 1995).

The advantages of segmental mesenteric vascular occlusion animal model include representation of occlusive causes of ischemia and establishment of multiple loops of different duration of ischemia in one animal. The disadvantage of segmental mesenteric vascular occlusion animal model includes ligation of venous and arterial supply. Appropriate experimental design and data interpretation can be achieved according to the advantages and disadvantages of each model.

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ISCHEMIC CONDITIONING APPLICATION IN INTESTINE IRI ANIMAL MODEL

RIPC of the rabbit heart was the first report to have the protective effect on jejunal intestinal contractile function in a model of sustained (1 h) simulated ischemia in isolated, spontaneously contracting rabbit jejunum (Dickson 2002). RIPC by 15-min infrarenal aortic occlusion and reflow could protect against intestinal IRI in rats receiving small bowel transplantation as effective as IPC (Saeki 2011).

Surgery for the abdominal aorta disease induced intense and unexpected hemodynamic modifications caused by aortic clamping and unclamping (Eide 2005). The IRI of the splanchnic organs is the major complication of abdominal aorta occlusion (Cornet 2009). The intestine is very susceptible to IRI which is important in the induction of systemic inflammation response syndrome and multiple organ dysfunction syndrome (de Arruda 2006; Zanoni 2009). It is the important reason of morbidity in patients with major aortic repair (Back 2005). Supraceliac aortic occlusion reduced intestinal mucosal length by decreasing villous height and increased serum lactic dehydrogenase and lactate levels in rats. Both IPC and RIPC alleviated these histopathological and laboratory changes in rats (Erling Junior 2013). RIPC provided intestine protection in 62 patients after elective open infrarenal abdominal aortic aneurysm repair (Li 2013).

SPINE CORD IRI ANIMAL MODELS

The neurological function after spinal cord IRI could not be ameliorated, and the irreversible and delayed death of spinal neurons happened (Weir 2002). Spinal cord IRI is a usual problem after thoracic or thoracoabdominal aortic surgery (Kuniyoshi 2003). 400,000 spinal cord injury (SCI) patients in the United States and 14,000 new cases every year were reported (Sekhon 2001). Spinal cord IRI patients have a poor prognosis, usually resulting in severe paralysis or mortality (Sinha 2010).

Experimental work can improve the understanding of spinal cord ischemia and develop new methods to protect spinal cord during extensive aortic surgery. The consequence of spinal cord injury during and after aortic surgery is determined by the duration and degree of ischemia, failure to reestablish blood flow to the spinal cord after repair and biochemically-mediated reperfusion injury (Svensson 1997). Spinal cord IRI animal models have been established to explore the mechanisms involved in the rat (Taira 1996), rabbit (Naslund 1992), pig (Qayumi 1997) and mouse (Lang-Lazdunski 2000).

Rat Spinal Cord IRI Model

An aortic cross-clamping technique was utilized to produce the rat spinal cord IRI model (Lang-Lazdunski 2000). A longitudinal incision was performed along the left vertex of the sternum to the third rib. The aortic arch was isolated from the left common carotid and subclavian arteries. Attention was paid to avoid the left internal mammary artery, the left superior vena cava and the left recurrent laryngeal nerve. Heparin (100 UI/kg) was given

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intravenously 10 min before aortic clamping. The left subclavian artery and aortic arch were blocked simultaneously by micro-artery clamps. The artery clamps were removed after 14 min for reperfusion.

Mice Spinal Cord IRI Model

The application of genetically modified mice promotes understanding of pathological mechanisms in many diseases and improves therapy development. It is now possible to evaluate the effects of spinal cord IRI on specific genes and molecular signaling pathways.

The first spinal cord mice IRI model was established in 2000 (Lang-Lazdunski 2000). The anesthesia was induced and maintained by 2% isoflurane. A cervicothoracic procedure was utilized to isolate the aortic arch. The aortic arch was clamped between the left subclavian artery and the left common carotid artery. An additional clamp was put on the left subclavian artery (Lang-Lazdunski 2000). The clamps were removed 5 minutes after ischemia. This model was used infrequently because the surgical technique was difficult and mortality after surgery was high. Survival is limited to less than 1 week and paralysis happens often immediately. In order to control consistently different outcomes of spinal cord IRI model, a simple and reproducible mouse model of spinal cord IRI was developed by transient cross-clamp of the descending aorta through a lateral thoracotomy (Awad 2010).

The beginning and extent of paralysis is decided by aortic cross-clamp time and the intraoperative core temperature that was kept during the period of ischemia. By modifying these parameters, mice can be consistently stratified into two groups: 1) immediate and severe paralysis; and 2) delayed paralysis which mimicks the phenomenon of humans undergoing similar surgery (Awad 2010). This new model will enable the screening of neuroprotective therapeutics and will help disclose basic mechanisms of post IR pathology and functional impairment induced by aortic cross-clamp (Awad 2010).

Transient Cross-Clamp at the Descending Aorta (Awad 2010)

Intubated mice were put on their right side and the left forelimb was placed and fixed laterally beneath the mandible. The rib cage is exposed by a transverse incision performed below the left forelimb and shoulder. Muscle between the 2nd and 3rd rib was cut to fully expose the inferior vena cava, descending aorta, thymus and left atrium. The descending aorta was separated and blocked by a small aneurysm clip across the vessel. Aortic cross-clamp was kept for 3–11 minutes.

A simplified mouse spinal cord IRI model was reported recently (Wang 2010). Mice were anesthetized with isoflurane and endotracheally intubated. The middle segment of the thoracic aorta was blocked by cross-clamping for 0, 8, 10 or 12 min via left lateral thoracotomy. 90% blood flow reduction was detected in the lumbar spinal cord. Neuronal cell death and neurological deficit were related to the ischemia time. 80% of mice with 10 min aortic clamping survived for 28 days and had significant neurological deficit.

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ISCHEMIC CONDITIONING APPLICATION IN SPINE CORD IRI ANIMAL MODEL

The incidence of temporary or permanent spinal cord injury still fluctuates between 3% and 20% after extensive thoracoabdominal aneurysm repair (Murana 2015). Paraparesis and paraplegia still happen after the surgery. The beneficial effect of RIPC was shown in rabbit spinal cord IRI (Sapmaz 2015). RIPC and IPoC remarkably ameliorated the neurological outcomes in rabbit spinal cord IRI (Sapmaz 2015). RIPC and IPoC significantly reduced percentage of apoptosis and intracellular edema. Hind limb RIPC preserved spinal cord function after left subclavian artery and segmental artery occlusion in a porcine model, as indicated by the motor-evoked potential amplitudes (Haapanen 2016).

CEREBRAL IRI ANIMAL MODELS

Cerebral IRI causes wide-ranging damage and is associated with morbidity and mortality. Stroke and cardiac arrest are the main reason for cerebral IRI in adults. Cerebral IRI is induced by complications during labor and delivery which may cause neonatal hypoxic-ischemic encephalopathy in infants.

Stroke is the third most important cause of disability and death in USA (Lloyd-Jones 2010). Strokes are most frequently ischemic in origin, induced by vascular blockage in the cerebral circulation. Stroke causes a focal brain injury.

Another important cause of Cerebral IRI is cardiac arrest followed by resuscitation. Cardiac arrest induces full ischemia in the whole brain. 70,000 patients are resuscitated from cardiac arrest each year and 10–35% of resuscitated patients survive to hospital discharge with the severe brain damage induced by cerebral IRI (Bloom 2007; Nichol 2008).

Animal Models of Localized Hypoxic-Ischemic (HI) Injury and Stroke

The complex cascade of events after global and focal HI brain injury shares certain similarities to the development of injury. The difference is depending on brain regions affected. It is not easy to translate the animal results to clinical practice. Neonatal stroke is a complicated pathophysiologic process that is inadequately understood and difficult to study.

Rice-Vannucci model (RVM) is the most frequently used model to investigate HI injury in the newborn. Unilateral carotid ligation was performed in the postnatal day 7 rat with exposure to 8% hypoxia for approximately 1.5 hours results in a reproducible area of hemispheric ischemic injury (Rice 1981). The rat brain at the postnatal day 7 is histologically similar to a 32- to 34-week gestation human fetus or newborn infant (Vannucci 1997). This method was applied to explore neonatal asphyxia in rat pups, piglets, dogs and kittens (Roohey 1997). A model of neonatal stroke was established by 4 hours middle cerebral artery occlusion and 24 h reperfusion without craniectomy in rat pups (Ashwal 1995). The advantage of this model is relatively noninvasive, focal brain hypoxia and temporary occlusion.

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The most frequent types of ischemia-related injuries in preterm human babies (23–32 weeks of gestation) include periventricular white matter injury (WMI), periventricular leukomalacia (PVL), intracerebral hemorrhage (ICH) and intraventricular hemorrhage (IVH) (Volpe 2009). Ischemic injury at-term brain is different from those in preterm newborns. The injury at-term brain is displayed focally in gray matter regions (Kirton 2011).

Perinatal arterial ischemic stroke happens between 20 weeks of gestation to 28 days postnatal. The incidence is 1 in 2300 live infant births. Perinatal arterial ischemic stroke causes remarkable morbidity and severe lasting cognitive and neurological adeficits including neurodevelopmental disabilities, epilepsy, cerebral palsy, impaired vision and language function (Nelson 2007).

Animal Models for Ischemia-Related Preterm and Term Injury

Different models of hypoxia-ischemia, hypoxia, IVH and focal stroke have been established in small and large animals to imitate ischemic injuries observed in the human infant.

Large Animal Models

A birth asphyxia model was established in non-human primates by 15–18 min of umbilical cord occlusion (UCO) prior to birth (Traudt 2013). UCO caused death or moderate-severe cerebral palsy in 43% of animals. UCO animals showed poor weight gain, poor cerebellar growth, behavioral impairment and abnormal brain diffusion tensor imaging (DTI) (Traudt 2013). But the cost limited the use of non-human primate model.

The most frequently applied large mammal species to produce HI in the premature brain are pigs, sheep and rabbits. These mammal species have a white/grey matter ratio similar to the human brain.

Cerebral ischemia models in fetal sheep were first established in the near term fetus by bilateral transient occlusion of the carotid arteries (Williams 1992). The intrauterine pathophysiology and the contribution of other organs on the brain could be investigated in fetal sheep UCO model. WMI and deep grey matter injury was more observed in the preterm fetuses of these models (Mallard 1994). However, fetal models are complicated by maternal/placenta metabolism, which is not present in the human situation of hypoxic-ischemic encephalopathy (HIE). Newborn pigs with different combinations of hypoxia and ischemia demonstrated alterations in metabolism and cerebral blood flow (CBF) comparable to that detected in human infants with HIE (Laptook 1988). The HI models induced by temporary common carotid arteries occlusion/hypoxemia in newborn pigs (Thoresen 1995) and 30 min of cerebral ischemia in fetal sheep (Gunn 1997) have been involved in development of therapeutic hypothermia for term infants with HIE.

A majority of cerebral palsy cases are induced by prenatal HI. Rabbits have many advantages compared to other animal species; mainly, their motor development is in the perinatal period, similar to humans. Intrauterine ischemia is caused at 22 days gestation in rabbits as equivalent to the preterm, and at 29 days gestation to simulate at-term injury (Derrick 2007). HI in preterm rabbits causes impairment predominantly in subcortical areas,

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such as thalamus and basal ganglia (Derrick 2007). This model has shown a correlation between abnormalities on neuropathology and cerebral palsy-like hypertonic motor deficits and MRI in the newborn pups.

Rodent Models

Much rodent brain development happens after birth as in human. Different regions of the rodent brain mature at a dissimilar pace. It is not easy to follow a single postnatal day as a complete presentation of brain development in human. Comparative studies between rodents and humans have shown that the rodent brain at postnatal day 1 – day 5 is equivalent to 23–32 weeks of gestation in the humans which is appropriate for investigation of preterm injury. The rodent brain at postnatal day 7 – day10 is equivalent to 36–40 weeks of gestation in humans which is acceptable in brain injury investigation at term (Semple 2013). WMI models established included unilateral common carotid artery ligation and hypoxia in postnatal day 1 – day 3 rat, (Sheldon 1996; Back 2002), prolonged low grade gestational hypoxia (Baud 2004) and ibotenate injection in postnatal day 5 rats (Gressens 1996). HI model was established in postnatal day 7 rats (Rice 1981) and postnatal day 7–9 mice (Ek 2015) which was able to mimic HIE in the at-term human infant and acquire information about the pathophysiology of the disease.

HI increased blood-brain barriers (BBB) permeability to small and large molecules within hours after the HI (Ek 2015). Brain pathology was closely associated with decreases in regional CBF during the hypoxia. HI caused inflammation which contributed to the development of injury in the newborn brain (Doverhag 2010).

Genetic background (Sheldon 1998) and sex may impact mechanisms of neuronal cell death and damage severity (Johnston 2007). Various models have been developed to investigate the underlying mechanisms of perinatal arterial stroke, including models of irreversible middle cerebral artery (MCA) ligation (Renolleau 1998) and temporary MCA occlusion (tMCAO) in postnatal day 7–10 rats (Derugin 1998; Mu 2003) and postnatal day 9 mice (Woo 2012). Different severity of damage can be induced by the varied length of tMCAO (Woo 2012). The presence of recirculation in the tMCAO model (Fernandez 2012) imitates reperfusion, which often happens in arterial stroke in term infants.

Large animal cerebral ischemia models would be very appropriate in translational research because the white/grey matter ratio is comparable to the human brain and the brains are gyrencephalic. In neonatal models, newborn pigs are most suitable because their general brain and organ maturation at term are alike to that of humans. Global models combined with global hypoxia and hypotension have been established which cause permanent brain injury, organ failure, post-hypoxic seizures and abnormal neurology, alike to humans on survival. But, maintenance cost and long-term neurorehabilitation have greatly restricted the application of larger animals.

Rodents are not gyrencephalic species and their physiology, CBF regulation and white/gray matter ratios are extremely dissimilar from those in humans. These differences between rodents and human limit the translatability of investigations in rodents.

Cerebral palsy (CP) is a complicated multifactorial disease which influences about 2.5–3/1000 live term births and 22/1000 prematurely born babies. CP is associated with damage to the developing brain experienced before, during, or after birth. Spastic CP, the most

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frequent type, is maily connected to damage of the cerebral cortex and subcortical white matter and the deep gray matter. HI during the last third of pregnancy and around birth age is the main etiological factors of spastic CP.

The rodent HI model is the most appropriate, cost-effective, and extensively utilized animal model and has provided considerable knowledge on CP pathophysiology. But, investigation in large animals with greater similarity to humans was provoked due to the shortcomings of the rodent HI model. In the fetal instrumented sheep model, preterm fetus (95 days of gestation) was similar to 24–28 gestational weeks in humans which showed cerebral hemodynamics comparable to that in the human fetus, both in normal conditions (Papile 1985; Helou 1994) and in response to HI (Mallard 1992; Bennet 1998). Furthermore, the gyrencephalic structure of the sheep brain cerebrum and the important stages of neurodevelopment are equivalent to those in humans (Cook 1987). Long-term monitoring of the fetus and pertinent clinical interventions was established in large sheep according to that model (Gunn 1997).

The main disadvantage of the instrumented fetal sheep model is inability to investigate motor deficiencies, especially dexterity movements, related to HI injury (Back 2012). A non-human primate model established in baboons at 125 days of gestation, comparable to 26 weeks of gestation in human, has demonstrated equivalences with human preterm infants in the pattern of white matter damage (Griffith 2012). A HI model in a newborn piglet has shown neuropathological and electro-physiological disturbances comparable to those in the asphyxiated term human infant (Thoresen 1996). The model can imitate similar alterations in brain white matter to a human newborn (Kyng 2015). But, high mortality and differences in protocols cause instability in the extent of brain damage. The firm conclusions are difficult to make.

Ischemia-related damage to prenatal or early postnatal brain affects many critical neurodevelopmental processes that experience maturation alterations during these time frames, causing different structural-functional abnormalities later in life. Application of several IRI models from small and big animals allows the improved understanding of brain pathology and development of novel therapies for the immature brain because no single animal model can entirely summarize the complexity of the human condition.

Neurological injury after global brain ischaemia is still problematic. However, sudden death models bring undesirable factors for investigating the brain due to multiple organ injury. A new minimally invasive large animal model of isolated global brain ischaemia was developed by carotid and vertebral arteries occlusion for 30 min of normothermic ischemia in pigs (Allen 2012). Cardiac or pulmonary function was not affected. Multiple post-reperfusion seizures, post-brain oedema and extensive cerebral infarctions were found in this model.

ISCHEMIC CONDITIONING APPLICATION IN CEREBRAL IRI ANIMAL MODEL

IPC is really the best known strategy to prevent or reverse neurodegeneration (Lehotský 2009). IPC has confirmed so far to be one of the most successful infarct-limiting strategies available. IPC is an innovative strategy for cerebral ischemia therapy and protective effect has been confirmed in large and small animal models of focal and global cerebral ischemia (Koch

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2013). IPC in brain includes an early and a late period, during which different neuroprotective responses are evoked in specific time windows (time interval between sublethal injury and lethal injury). There is a rapid period for which the cumulative protective effect of released factors is maximal if the window between sublethal injury and lethal injury is about 1 h in duration (Perez-Pinzon 1996; Perez-Pinzon 1997). Delayed preconditioning evokes maximum protection if the window is expanded to several days after the preconditioning injury (Kato 1992a; Kato 1992b) and has been demonstrated to supply stronger and longer lasting neuroprotection than the early period.

RIPC is the preferred method which overcomes some of the limitations of IPC (Dezfulian 2013). However, it still must be provided before the onset of ischemia. Thus, the application of IPC and RIPC for acute stoke therapy is mostly limited owing to the unpredictability of acute stroke onset. RIPC by transient limb ischemia significantly reduced brain infarct size in a rat transient middle cerebral artery occlusion model of acute stroke (Hahn 2011). RIPC produced by tightening the upper two-thirds of both hind limbs for 15 or 30 min was first shown to have neuroprotection in a rat model of asphyxial cardiac arrest (Dave 2006). Rapid RIPC with three cycles of 15 min unilateral limb occlusion just before stroke decreased infarct size in rats. Stroke was induced by a permanent occlusion of the left distal middle cerebral artery and a 30 min occlusion of the bilateral common carotid arteries (Ren 2008). Delayed preconditioning with three cycles of 15 min limb occlusion 2 days before stroke also decreased infarct size. 4 cycles of 5 minutes of ischemia of the hind limb protected the brain injury induced by 60 minutes hypothermic circulatory arrest in piglets (Jensen 2011).

Neuroprotection has been reported in both rapid (0 –1 h after RIPC) and delayed (24 h) temporal windows as well as treatment after the ischemic event (Pignataro 2013; Sun 2012). RIPC by 20 min of femoral artery occlusion reduced 50% infarct size caused by 100 min of middle cerebral artery occlusion which was performed 10 minutes after RIPC (Pignataro 2013). Delayed RIPC induced by five cycles of 5 min limb occlusion 24 hrs before global ischemia did not prevent hippocampal CA1 neuronal death at 7 days after transient global cerebral ischemia. Global ischemia was induced by bilateral common carotid artery occlusion and hypotension (35 and 40 mmHg) (Saxena 2009).

IPoC decreased 50% infarct size *in vivo* and about 30% *in vitro* (Pignataro 2008). IPoC induced by 10 min ischemia and 10 mins reperfusion was demonstrated to be the most effective both *in vivo* and *in vitro*. IPoC has equivalent neuroprotection as IPC. IPoC considerably prevented global cerebral IR-caused behavioral deficits by activation of phosphoinositide 3-kinase-linked pathway (Rehni 2007). Global cerebral IR was induced by 10 min bilateral carotid artery occlusion and 24 h reperfusion. IPoC was produced by three cycles of 10 s carotid artery occlusion and 10 s reperfusion. IPoC remarkably decreased infarct volume at 48 hours and ameliorated functional outcomes at 4 weeks after neonatal HI brain injury in rat pups by opioid receptor/Akt pathway (Zhou 2011). HI was produced in postnatal Day 10 rat pups by unilateral carotid ligation plus 2 hours of hypoxia. IPoC was produced by 4 cycles of 10 minutes of IR on both hind limbs just after HI.

LIMB IRI ANIMAL MODELS

Lower extremity ischemia may happen because of traumatic or embolic/thrombotic vascular obstruction, or alternately owing to tourniquet utilization during surgical procedure.

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Reperfusion of the ischemic extremity is critical for tissue survival. But reperfusion may initiate complex inflammatory cascades which can provoke ischemic tissue damage and cause local and systemic inflammatory reaction. Locally, lower extremity IRI induces rigidity, muscle edema formation, loss of muscle viability, apoptosis and necrosis. Furthermore, lower limb IRI can cause multiple organ dysfunction (Yassin 2002).

Acute limb IRI is widespread clinical problem and is widely investigated in the pig (Harkin 2002), canine (Belkin 1990), rabbit (Summers 1995), rat (Sternbergh 1994) and mouse (Kyriakides 2001) models. Limb IRI is induced by a tourniquet or an elastic rubber band on the proximal part of the limb, clamping the femoral artery or iliac artery, or the infrarenal aorta (Bonheur 2004; Dick 2008; Lundberg 2003). But, ischemia induction methods, ischemia time and reperfusion time are different. The ischemia time varies from 2 h to 4 h in acute model rats. The reperfusion time differs from 1 h to 24 h (Dick 2008; Hosseinzadeh 2009; Chen 2009).

Unilateral Hind Limb IRI

The femoral artery and vein were exposed by a groin incision after the anesthesia was induced and maintained. A tourniquet (standardized weight of 450 g) was put below the femoral vessels to occlude collateral circulation (Dick 2008). The femoral artery was obstructed for 3 h using two microvascular clamps. The rat hind limbs were not exsanguinated, but venous return was allowed during the whole period of ischemia in order to avoid venous congestion and other injury through microcirculatory impairment, which would not present the clinical condition. Reperfusion was started after 3 h ischemia.

An acute rat unilateral hind limb IRI model with 2 h of reperfusion is adequate to evaluate basic parameters of IR and it may be acceptable for a first screening of drug candidates. 24 h of reperfusion will be needed for in-depth analysis of mechanisms of actions, muscle viability and distant organ damage (Duehrkop 2014).

Tourniquet-caused hind limb IRI mice model was induced by the McGivney hemorrhoidal ligator band (MHL) (Kyriakides 2001). Tourniquet models are commonly utilized for murine investigation of IRI models. Occlusion of several branches of the femoral and iliac arteries is needed to reach dependable limb ischemia in angiogenesis mice model. The rich collateral blood supply surrounding the murine pelvic girdle supplies considerable collateral flow often from iliac and tail branches.

The disadvantage of MHL model is to cause nonspecific neuromuscular injury because of the crushing force of the rubber band on the underlying tissue. The controlled tension tourniquet (CTT) enabled utilization of minimal amounts of reproducible circumferential tension to induce full hind limb ischemia. Reperfusion could also be easily reached by releasing the tightness on the winch (Bonheur 2004, Conrad 2005). Orthodontic rubber bands (ORB) induced similar tissue ischemia compared with MHL in murine models of limb IRI with less neuromuscular dysfunction. ORB may be the preferred model for selected studies of limb IRI (Crawford 2007).

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Retinal IRI Animal Model

Retinal IRI is a general clinical situation and is related to the neurons loss, the retina degeneration, retinal function loss, and finally vision loss (Cho 2009; Kim 2013). Retinal IRI induces the pathologic processes of some retinal diseases such as retinopathy of prematurity, acute glaucoma, diabetic retinopathy and central retinal artery occlusion (Andreeva 2014; Sun 2010). Retina is more susceptible to ischemic damage since it has very high oxygen consumption. Retina as an extension of central nervous system is a perfect model system to investigate the pathophysiology of IRI and evaluate various treatment agents in animal models (London 2013).

Rat Retinal IRI Animal Model

The rat pressure-induced retinal IRI model was generated in 1991 (Büchi 1991). Anesthesia was induced and maintained with isoflurane/O2. A 30-gauge needle was cannulated to the anterior chamber of left eye and was connected to a reservoir with 0.9% NaCl. The intraocular pressure was raised to 120 mm Hg for 30, 45, or 90 min. Retinal ischemia was verified by retinal edema and stasis in retinal arteries (Joachim 2012). The needle was removed at the end of ischemia and intraocular pressure (IOP) was returned to normal pressure. Reperfusion was verified with observation of episcleral veins.

Mice Retinal IRI Animal Model (Kim 2013; Silverman 2016)

Mice were kept on a heating pad to avoid hypothermia during the anesthesia. The left eye was dilated with 2.5% Phenylephrine HCl after the anesthesia was induced and maintained. A 30-gauge needle attached to sterile saline or PBS reservoir was inserted into the anterior chamber of left eye. The reservoir was elevated to produce an IOP of 120 mmHg for 1 h to cause retinal ischemia. Retinal ischemia was verified by blanching of the retina using an ophthalmoscope. The right eye was used as controls. The needle was removed after 60 minutes and reperfusion for the retina was started.

Pig Retinal IRI Animal Model (Gesslein 2010)

Endotracheal tubes were orally intubated for animals after the anesthesia was induced and maintained. Mechanical ventilation was maintained in the volume-controlled mode and regulated to acquire normocapnia. The animals were ventilated with a mixture of oxygen (70%) and dinitrous oxide (30%). A 30-gauge needle was cannulated into the posterior chamber of one eye. The IOP was raised to 80 mmHg by continuous infusion of a balanced salt solution for ophthalmic irrigation. The IOP was confirmed by a Tono-Pen®XL tonometer. The other eye was used as a control, and the same surgical procedure was carried out except for increase of the IOP. The cannulation needles were removed 1h after ischemia. Reperfusion of the retinal vasculature was started for 5 or 12 h during anesthesia. Ischemia was verified by indirect ophthalmoscopic examination and fundus imaging.

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ISCHEMIC CONDITIONING APPLICATION IN RETINAL IRI ANIMAL MODEL

RIPC by tightening a tourniquet around the upper thigh and releasing for three cycles improved retinal IRI induced by right middle cerebral artery occlusion and pterygopalatine artery occlusion for 60 min through the upregulation of antioxidative stress proteins (Zhang 2014). Retinal ganglion cells damaged by optic nerve transection caused extensive cell death. IPoC induced by several mild IR to the hind limb at 10 min or 6 h after optic nerve cut improved ganglion cell survival at 7 days post-injury. 10 min IPoC still had protection at 14 days post-injury (Liu 2013).

IPoC induced by seven cycles of 1 min/1 min IR reduced glial fibrillary acidic protein levels of Müller cells at 3 and 7 days post-ischemia and provided protection against rat rental IRI (Fernandez 2009). IPoC can effectively prevent injury after retinal ischemia when it was performed early (within 1 h) in the post-ischemic period (Fernandez 2009; Dreixler 2010). The rental protective effect of IPoC depends upon the ischemia time. IPoC has no synergistic effect with IPC. IPoC works, in part, by preventing apoptotic injury to the inner retina. IPoC has considerable promise for clinical translation to eye diseases that cause blindness by ischemia (Dreixler 2010). Indeed, delayed IPoC (24 h after prolonged ischemia) significantly improved the rental ischemia in rats (Dreixler 2011).

UTERINE IRI MODEL

Uterine transplantation is a treatment option for patients with unmanageable uterine factor infertility. Uterine transplantation has been well performed in mice (Racho El-Akouri 2003), sheep (Ramirez 2011) and rats (Wranning 2011). However, uterine IRI affected the success of uterine transplantation. Intraoperative treatment IRI decreased the incidence of acute rejection and ameliorated the long-term graft outcome (Land 2005). Tacrolimus reduced oxidative damage and protected tissue damage in the rat uterine IRI caused by distal abdominal aortic occlusion (Sahin 2014).

Uterine IRI was induced in rats by occlusion of ovarian arteries and the lower abdominal aorta for 30 min in the rat (Sahin Ersoy 2017). Unilateral uterine IRI was produced by occlusion of the uterine and ovarian arteries of the right uterine horn for 30 minutes in rats on day 17 of gestation which was effective for inducing fetal growth restriction (FGR) (Thaete 2006). This rat model was helpful because it imitated the imbalance of supply/demand for blood perfusion in gestational tissue. On the 15th day of gestation, unilateral mice uterine IR model was established by occlusion of the uterine and ovarian arteries to the right horn of the uterus for 30 minutes (Thaete 2013). Fetal and placental weights were decreased after IR which suggested that IR caused FGR as it did in rats. This mouse model is very important because it allows the investigation of IR-related developmental pathology at the molecular level in genetically modified mice.

The uterine IRI in cynomolgus monkey was induced by occlusion of the bilateral uterine and ovarian vessels for 0.5, 1, 2, 4, 8 h and then 3 h reperfusion. No abnormality was detected by both light and electron microscopy after 2 h ischemia and 3 h reperfusion (Adachi 2016). No noticeable change was found after 4 h ischemia. However, rough endoplasmic reticulum

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swelling and dilated nuclear pores were detected after 3 h reperfusion. The uterus of the cynomolgus monkey could tolerate 4 h warm ischemia.

OVARY IRI ANIMAL MODEL

IRI is a noteworthy problem following reperfusion treatment for ovarian torsion. Ovarian torsion is a rare but significant gynecologic surgical emergency that can affect women of all ages (Houry 2001). It is originally related with decreased venous ovarian blood flow, and ischemia and infarction can happen due to restriction of arterial blood flow (Wilkinson 2012; Sasaki 2014).

Rat ovary IRI model is often used which is induced by 360° ovary torsion and detorsion (Caglayan 2014; Celik 2014; Sak 2013; Yaman 2016). In the rat ovarian IRI model, the adnexa including ovarian tissues and the tube were rotated 360c clockwise and then secured to the abdominal wall (Sak 2013; Yaman 2016). Period of ischemia was 2−3 h and bilateral adnexal detorsion (reperfusion) was 2−3h.

Ischemia was induced by the lower part of the right ovary occlusion with vascular clips in rats (Ingec 2011; Kumbasar 2016). 1 h controlled reperfusion (CR) (on-off method: controlled reperfusion by opening and closing the clips (on/off) in 10-second intervals, for 5 times for a total of 100 seconds) was performed. CR improved ovarian IRI. CR decreased sterility and ovarian oxidative stress induced by IRI in a rat model of ovarian IRI with unilateral oophorectomy (Aksoy 2015).

Ovarian autotransplantation is a more utilized approach for preserving fertility in women with cytotoxic treatment for immunosuppressive disorders or cancer, which eventually provoke early ovary failure (Donnez 2005; Mhatre 2005). It was found in 34% of women receiving chemotherapy and 92% of patients having radiotherapy or chemotherapy for bone marrow transplantation (Meirow 1999). IRI of the graft ovary is one of the important factors affecting the graft revascularization and success of ovarian transplantation (Liu 2002).

ISCHEMIC CONDITIONING APPLICATION IN OVARY IRI ANIMAL MODEL

RIPC by 15 min iliac artery occlusion was found the best timing to reach functional support and preserve more viable ovarian follicles in rats with bilateral oophorectomy and ovarian transplantation (Damous 2008). RIPC group showed greater amounts of viable ovarian follicles and raised vascularization and vasodilatation than the control group. RIPC by the common iliac artery occlusion for a 15 min ischemia and 15 min reperfusion before ovarian transplantation significantly increased the estradiol levels in autologous ovarian transplants. Better graft morphology and more mature follicles were observed (Damous 2009).

CONCLUSION

IRI animal models from small animals to big animals have been established in different organs. IRI animal models can establish a bridge between clinical study and the preclinical *in*

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vitro investigations. Hypotheses produced in clinical investigations can be examined directly in animal models, and the results from *in vitro* investigations can be determined in animal models to evaluate their relevance in entire living systems. The animal models of of organ IRI are very important for understanding the underlying processes of IRI and development of efficacious treatment. Animal investigations suggest that IPC, RIPC and IPoC contribute to protection against IRI in different organs.

RIPC definitely has a reasonable prospect for ischemic protection of the heart and other organs (Heusch 2013). RIPC may protect against acute IRI in various noncardiac organs and tissues including the lungs, liver, kidneys, ovaries, pancreas, stomach and intestine in animal models (Candillo 2013). The main restriction in clinical translation of IPC is to block the blood supply of the target organ or tissue. Translation of IPC, RIPC and IPoC strategies from successful preclinical investigations to the clinical study has been relatively discouraging (Ferdinandy 2014). Ischemic conditioning cardioprotection strategies verified in animal heart IRI model have been evaluated in patients with acute MI or CABG surgery. Some large, multicentre, randomized, controlled clinical trials on cardioprotection have emphasized the challenges of translating ischemic conditioning cardioprotection strategies into patient benefit (Hausenloy 2016). Ischemic conditioning showed protective effects on kidney, lung and intestine in some single centers with small number of patients. Multicentre, randomized controlled clinical trials will be needed to evaluate whether ischemic conditioning has protective effects on IRI of different organs.

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Chapter 20

ENCAPSULATED ISLET TRANSPLANTATION: FROM ALLOGENEIC TO XENOGENEIC TRANSPLANTATION

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ABSTRACT

Islet transplantation is considered an ultimate modality for patients with diabetes mellitus. After adoption of the Edmonton protocol in 2000, clinical islet transplantation has increased the duration of insulin independence up to 5 years post-transplant. However, two major challenges limit the broad clinical application of islet transplantation: a shortage of organ donors and the need for immunosuppression. Islet encapsulation, a method of encasing islets in a semipermeable matrix to provide a physical immune barrier, is one of the strategies for overcoming these limitations. Islet encapsulation blocks immune-regulating cells with high molecular weights, while still allowing oxygen, insulin, and nutrients with low molecular weights to pass through the

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barrier. Currently, a number of encapsulation methods are being investigated in preclinical and clinical trials, including macroencapsulation, microencapsulation, conformal coating, and nanoencapsulation. Because of the shortage of pancreas donors, a xenogeneic pancreas may be considered as an alternate source for islet transplantation. In this chapter, we review the basic concepts of islet encapsulation and islet transplantation from allogeneic and xenogeneic sources.

Keywords: diabetes mellitus, islet transplantation, encapsulation, allogeneic, xenogeneic

ABBREVIATIONS

FACS	fluorescence-activated cell sorting
IEQ	islet equivalent
IXA	international xenotransplantation association
NHP	non-human primate
PEG	polyethylene glycol
SNU	Seoul national university
SPF	specific-pathogen-free

INTRODUCTION

Islet transplantation is considered to be both a treatment and a cure for diabetes mellitus (Figure 20.1). The first attempted islet transplantation was reported by Watson-Williams and Harshant in 1893, which predated the discovery of insulin (Srinivasan 2007). Watson-Williams and Harshant transplanted the minced pancreas of sheep to patients with diabetic ketoacidosis; however, their attempt failed after transient improvement of glycosuria for 24 h. In 2000, a landmark study on islet transplantation, the Edmonton protocol, revolutionized the field of islet transplantation (Shapiro 2000). Since then, many promising results in human islet transplantation have been reported with the use of immunosuppressive agents, including low doses of tacrolimus, sirolimus, and daclizumab (Shapiro 2006). In the late 2000s, the results of clinical islet transplantation showed marked improvement, with patients showing normal C-peptide levels, reduced HbA1c, and decreased islet reinfusion rates (Barton 2012). According to the Collaborative Islet Transplant Registry, the islet graft survival rate at 3 years post-transplant was 44% during the period 2007–2010, which was significantly improved compared with the rate of 27% in 1999–2002 (Barton 2012). This success may be partly attributed to changes in immunosuppression strategies.

Despite these improvements in islet transplantation, two major barriers-a shortage of human pancreas donors and the need for immunosuppression-still limit the widespread clinical application of islet transplantation (Frank 2004). The current protocol for islet transplantation requires at least two donor pancreases for successful islet transplantation, which increases the shortage of organ donors. Moreover, the use of immunosuppressants for preventing transplant rejection is toxic to islets as well as transplant recipients. In this regard, islet encapsulation could be an immune isolation tool that addresses these challenges (Figure 20.2).

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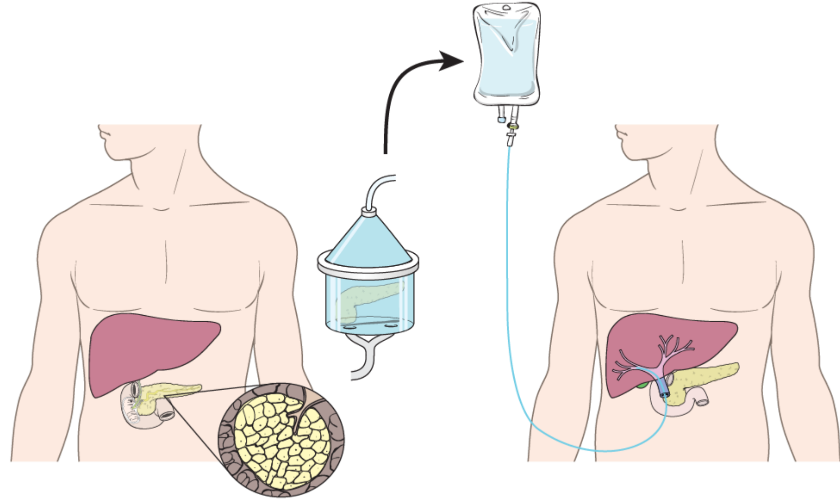

Figure 20.1. Conventional islet transplantation.

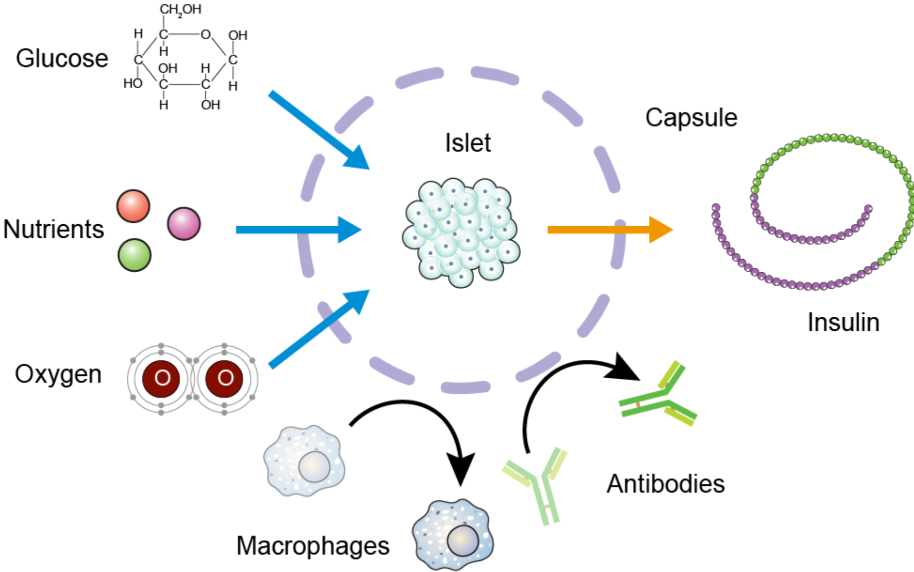

Figure 20.2. The principles of encapsulation technology.

By using an encapsulation technique, immune system components with high molecular weights, such as immune cells (7 μm) and antibodies (~150–900 kDa), can be blocked. Meanwhile, substances with low molecular weights, such as oxygen, insulin (~6 kDa), nutrients, and hormones, can pass through the barrier (Dufrane 2012). Therefore, islet encapsulation could prevent immune rejection, although protection from chemokines and cytokines with lower molecular weights is inadequate. Xenogeneic islets are one of the avenues to address the shortage of human pancreas donors. In this chapter, we describe experimental islet transplantation, including islet encapsulation and graft islet sources.

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STRUCTURAL APPROACHES FOR ISLET ENCAPSULATION

Structurally, islet encapsulation can be divided into four groups (Figure 20.3): 1) macroencapsulation; 2) microencapsulation; 3) conformal coatings; and 4) nanoencapsulation or layer-by-layer coating. The latter two types are cell surface modifications.

Macroencapsulation

In macroencapsulation, a large mass of islets is encapsulated in one or several large capsules. There are two main types of macrocapsules: intravascular and extravascular.

Intravascular Macrocapsules

In an intravascular macrocapsule, the islets are located in a hollow semi-permeable membrane containing many artificial capillaries. Using vascular anastomoses with an intravascular shunt, the device is directly connected to the host's systemic circulation; thus, it allows blood flow into the hollow fibers (Monaco 1991). The major advantage of this device is its proximity to the blood stream of the recipient. Islets are located near the fibers and can directly receive oxygen and nutrients from the blood stream, therefore improving graft survival and glucose-stimulated insulin secretion. In addition, the islets are protected from immune cells by the fiber membrane.

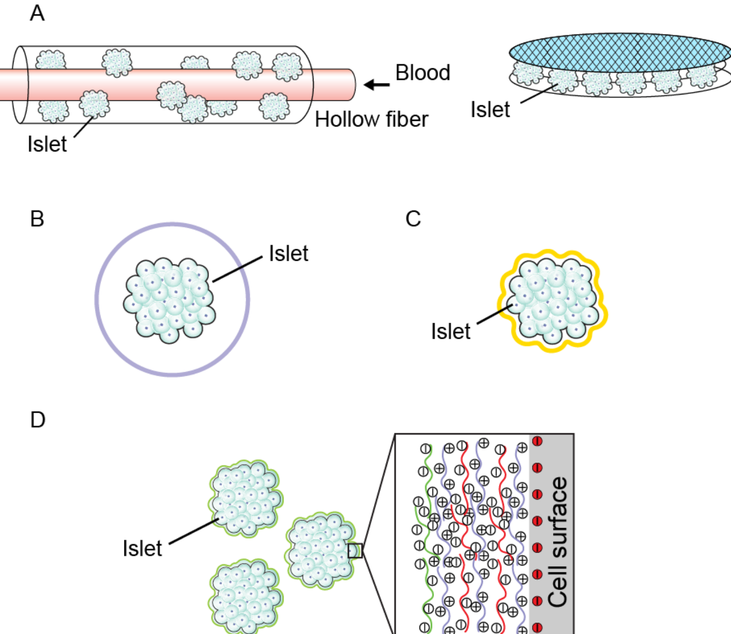

Figure 20.3. Structural approaches to islet encapsulation: macroencapsulation (A), microencapsulation (B), conformal coating (C), and nanoencapsulation (D).

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Researchers have demonstrated long-term islet function with these devices in a canine xenograft model without immunosuppression (Sullivan 1992). However, despite successful animal studies, embolization and thrombus formation within the lumen or at the anastomotic sites have been severe problems that required intense anticoagulation therapy (Vaithilingam 2011). The risks associated with vascular prosthetic procedures, such as bleeding, also make intravascular devices unsuitable for clinical applications. For example, the failure of carotid artery device connections resulted in the sudden death of a canine during long-term follow-up (Scharp 2014). For these reasons, the US Food and Drug Administration has not approved clinical trials of this hybrid artificial pancreas.

Extravascular Macrocapsules

Extravascular macrocapsules do not require intravascular shunts because they are based on the principle of simple diffusion chambers. A major advantage of these devices is the ease of retrieval or reloading. Other advantages include minimal surgical risk, no risk of thrombosis, the ability to transplant into different sites, and the ability to keep graft cells within the device (Schulz 2012). Macrocapsules also have greater mechanical strength than microcapsules. However, a major disadvantage is the relatively low ratio of surface area to graft islet volume. This interferes with oxygen diffusion and nutrient transport, leading to compromised islet viability and function. A low surface-to-volume ratio also slows the exchange of glucose and insulin (Pareta 2012). Packing density is another critical issue for clinical application. The islet density in the device has to be less than 5%–10% to maintain an adequate supply of oxygen and nutrients. Therefore, macrocapsules may be somewhat impractical: the relatively large devices need to be implanted to provide sufficient islet masses and cannot be placed at conventional transplantation sites (Van Suylichem 1992).

The macrocapsular devices have been tested in tubular and planar chambers (Figure 25.3A). The tubular devices are typically made of copolymers (including polyacrylonitrile and polyvinylchloride) and have a smooth or fenestrated surface (Colton 1995). Both the cylindrical shape and smooth surface contribute to greatly improved biocompatibility, thus preventing fibrosis. These devices showed successful results in a rodent model (Lacy 1991). In clinical setting, excellent graft survival at 2 weeks post-transplantation was reported in patients with type 1 and type 2 diabetes (Scharp 1994). However, there are two major disadvantages of tubular chambers: a susceptibility to rupture and the need for a large number of islets because of their low islet seeding density (Colton1995).

The planar diffusion chamber is made up of two flat sheets attached to a ring support, giving it a flat configuration. These chambers are more stable than tubular chambers and can supply oxygen to the whole graft. However, initial studies using this device failed because of pericapsular fibrosis (Brauker 1996; Scharp 1984). Several bilayer planar devices have been developed to improve vascularization and consequently provide effective immune isolation (Brauker 1995; Geller 1997). In the 1990s, Baxter Healthcare (Deerfield, IL, USA) developed a planar device with two composite membranes and a loading port. This macrocapsule, which was later named the TheraCyte® Implant System, was 4 cm long and teabag shaped, with a bilayered polytetrafluoroethylene membrane. Xenotransplantation studies using this device allowed neonatal pig islets to survive in diabetic mice for up to 16 weeks and in nondiabetic cynomolgus monkeys for up to 8 weeks (Elliott 2005). Preimplantation of TheraCyte devices

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has improved vascularization and reduced the islet doses required to normalize blood glucose levels in diabetic rodents (Sörenby 2008). Moreover, human fetal pancreatic tissue within TheraCyte devices differentiated into insulin-producing cells in a rodent model, although they were not fully mature (Lee 2009). Currently, several organizations (e.g., Betalogics of Janssen Pharmaceuticals, ViaCyte) and other academic investigators are conducting further investigations and testing various modifications to create a clinically applicable device (Scharp 2014).

An islet sheet is another type of planar flat-sheet device, which was developed in the late 1990s. In a pancreatectomized beagle model, euglycemia was maintained for 84 days after the implantation of islet sheets containing 75,000 islet equivalents (IEQs) of islets into the omentum (Storrs 2001). Later, human islets transplanted into the subcutaneous space of rats using islet sheets demonstrated islet survival after explantation (Lamb 2011). More recently, Krishnan et al. proposed a model using islet sheets and dorsal window chambers to monitor the microvascular environment around biomaterial implants (Krishnan 2014). They observed significant changes in blood flow, hemoglobin oxygen saturation, and vascular density within the first 2 weeks after transplantation of islet sheet devices.

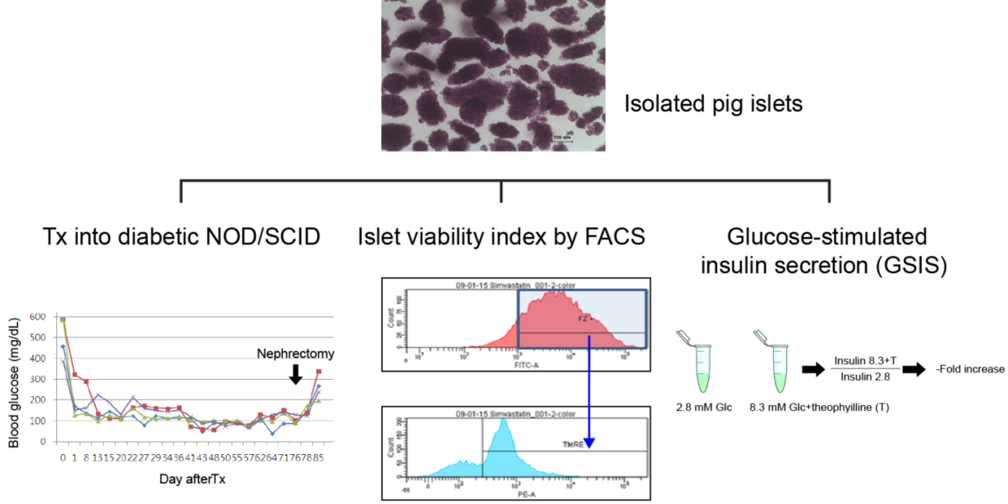

Figure 20.4. Quality control of the isolated pig islets. Portions of the isolated pig islets were tested for potency using three independent assays: NOD/SCID bioassay (*), FACS index (+), and glucose-stimulated insulin secretion (#).
For the NOD/SCID bioassay, four animals were rendered diabetic by injecting streptozotocin (200 mg/kg). Blood glucose levels were followed after pig islet transplantation (marginal mass: 2,500 IEQ) into subcapsules of the kidney.
For the FACS index, pig islets were dissociated into single cells and were stained with fluozin-3 and TMRE for measuring β-cells and mitochondrial potential, respectively. Then, the stained cells were analyzed by FACS. The FACS index was calculated by the fluozin-3-positive (β-cells) × TMRE-positive cell fraction.
For the glucose-stimulated insulin secretion, a small portion of the final preparation of the pig islets (100 IEQ, in triplicate) before transplantation was sequentially incubated in Kreb's HEPES buffer containing 2.8 mM glucose and then 8.3 mM plus theophylline for 1 hr, respectively. The secreted insulin was measured by enzyme-linked immunosorbent assay.

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An oxygen-refueled macrochamber device (called βAir) was developed by Beta-O2 Technologies Ltd. This device has two main compartments: an immune-protected compartment and an oxygen supply compartment. In this device, islets are immobilized in alginate, shielded from the immune system by three layers (a thin hydrophilized Teflon membrane, an alginate layer, and an alginate matrix with high guluronic acid), and supplied with oxygen by daily refueling through the subcutaneous port (Ludwig 2010). Animal studies using this device have shown positive results in rodents (Ludwig 2012) and pigs (Neufeld 2013).

Another sheet-type macroencapsular device was introduced in 2004 by Qi et al. (Qi 2004). This device used polyvinyl alcohol (PVA) hydrogel and was prepared by using a freezing and thawing technique. Xenogeneic transplantation using this hydrogel sheet demonstrated improved glycemic status and renal dysfunction in rodents (Sakata 2006). Furthermore, cryopreservation of islets using the PVA macroencapsulation method was reported to be effective up to 30 days (Qi 2010). Rodent models also showed insulin-positive islets at 24 weeks after allogeneic transplantation (Qi 2012). This device has not yet been part of a clinical trial, but it has unique characteristics and potential utility for the cryopreservation of graft islets.

A monolayer cellular device has a collagen matrix covered with gelled alginate on both sides (Dufrane 2010). In this system, the interaction of islets with the biological membrane is enhanced and the islet concentration per unit surface area is increased as the islets are placed in a monolayer on the collagen matrix (Dufrane 2012). In primates, subcutaneous implantation of this monolayer cellular device resulted in significant improvements in hyperglycemia (HbA1c $< 7\%$) for 6 months without immunosuppression (Dufrane 2010).

All of the previously mentioned studies indicate that extravascular macrocapsular devices are attractive candidates for clinical applications. However, low graft survival rates have hampered their clinical use.

Microencapsulation

Microcapsules are smaller than macrocapsules. They contain one or a few islets in a spherical semipermeable microcapsule (Figure 20.3B). Generally, the size of microcapsules is less than 1,000 μm in diameter (Jain 1999; O'Sullivan 2011; Scharp 2014). Microcapsules have many advantages over macrocapsules (Table 20.1). They are not only very simple to construct but are also mechanically stable. The simplicity of the manufacturing process allows for easy changes to key parameters, such as pore size, thickness, and permeability. The higher surface-to-volume ratio of their spherical configuration results in better diffusion rates (Van Schilfgaarde 1999). Finally, the transplantation of microcapsules is a simple procedure that does not require major surgery. Therefore, microencapsulated islets are currently the preferred system for approaching a bioartificial pancreas (De Vos 2002; Kizilel 2005; O'Sullivan 2011; Scharp 2014).

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Table 20.1. Comparison between macroencapsulation and microencapsulation

	Macrocapsule	Microcapsule
Encapsulation material	Alginate Polytetrafluoroethylene Acrylic copolymer Agarose Polysulfone Amphiphilic conetwork membrane	Alginate Poly-L-lysin Poly-L-ornithine Agarose Sodium cellulose sulfate
Site for transplantation	Peritoneum Subcutaneous adipose tissue	Peritoneal cavity Renal capsule Subcutaneous adipose tissue
Advantages	Use of allogeneic and xenogeneic islets without immunosuppression Easy implantation Easy retrieval Mechanically stable	Use of allogeneic and xenogeneic islets without immunosuppression Better oxygen and nutrient transport Easy implantation
Disadvantages	Higher dose of islets Risk of capsular rupture Inadequate graft oxygenation	Difficult to retrieve Capsule clumping in peritoneum Mechanically and chemically fragile

However, some disadvantages of the microencapsulation technique need to be addressed. The difficulty of retrieving implanted islets from the recipient is a major challenge. Early pericapsular fibrosis causes a foreign body reaction and negatively affects graft islet function (Gotfredsen 1990). Almost 10% of the capsules have shown fibroblast and macrophage overgrowth (Fritschy 1994). Insufficient biocompatibility of the capsules also can affect the function of encapsulated islets (Gotfredsen 1990; Tuch 2009).

In general, microcapsules are produced from polymers that form hydrogels under certain conditions. They are derived from either natural or synthetic routes. To improve biocompatibility, researchers have investigated various materials, including alginate, chitosan, methacrylic acid, methyl methacrylate, polyethylene glycol, 2-hydroxyethyl methacrylate, polycations and anions, agarose, collagen, silica, and dexamethasone (De Vos 2010; O'Sullivan 2011). In islet transplantation, alginate has been the most widely used hydrogel; it can be produced under physiological conditions for physiological pH, isotonic solutions, and room or body temperature (Ménard 2010; Sandler 1997). Alginate-based microcapsules do not interfere with islet function (Jain 1999; Opara 2010) and provide a microenvironment that promotes the functional survival of islets, presumably due to the mechanical support of the islets and the conservation of their structures. Because alginate molecules are negatively charged like the cellular surface, the binding of immune cells to alginate microcapsules is limited. Therefore, alginate is considered to be highly biocompatible. The long-term survival of islets and well-preserved graft function after transplantation have been demonstrated in both animals and humans (Jain 1999; Vaithilingam 2011). The first transplantation with alginate microcapsules was performed by Lim and Sun in 1980. Since then, many studies in rodent, pig, and canine models of diabetes have reported successful transplantation with

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microcapsules. However, in nonhuman primates (NHPs), only two groups have shown successful results (Elliott 2005; Sun 1996).

Alginate molecules are anionic copolymers of β-D-mannuronic (M) and α-L-guluronic acids (G), which readily form a gel in the presence of divalent cations such as Ca2+, Ba2+, or strontium (De Vos 2002; Kizilel 2005; O'Sullivan 2011; Opara 2010; Scharp 2014). The commercially available alginates originate from seaweed. Adequate purification of these natural compounds is necessary to maximize biocompatibility and islet viability while also minimizing immunogenicity. Alginates are polysaccharides composed of M and G linked together; the composition and ratio of G: M varies depending on the source. When dissolved, alginate forms a highly viscous solution. An air-driven droplet generator creates small droplets, namely microcapsules. The droplets are stabilized by immersion in a solution of divalent cations such as Ca2+ or Ba2+, which form strong cross-links with G and M monomers (De Vos 2009). Various factors (e.g., G and M composition, concentration, viscosity, gel homogeneity, capsule size) determine the characteristics of the capsules, including their strength, flexibility, durability, and permselectivity. Because divalent cations bind more strongly to G components than to M or MG components, microcapsules with a high content of G alginate tend to be more rigid and stable than those with a high content of M alginate (Mørch 2006). Microcapsules with high M alginate content tend to increase swelling, resulting in inadequately encapsulated islets. However, a study suggested that a high content of M alginate is more favorable than a high content of G alginate in terms of encapsulated islet survival (King 2003). Traditionally, calcium is one of the most commonly used cross-linking cations. The introduction of barium in microcapsules decreased the capsule size and reduced permeability to immunoglobulin G. Compared with calcium and barium, strontium shows immediate binding affinity when a high content of G alginates is used and an equivalent effect when a high content of M alginate is used.

One study suggested that the type of polyamine (poly-L-lysine, poly-D-lysine, or poly-L-ornithine) rather than the type of alginate (high-G or high-M) matters in immune response (Ponce 2006). A three-layer coating of alginate-polyamine-alginate creates a permeability barrier, which provides immune isolation for the microcapsules. The thickness of this barrier varies with incubation time and concentration (Jain 1999). Poly-L-lysine was the first permselective biomaterial used to create this barrier and is the most widely used (Lim 1980). Poly-L-lysine showed the least reactivity to the host compared with poly-D-lysine or poly-L-ornithine (Ponce 2006). However, a poly-L-ornithine coating was shown to mechanically support the microcapsules and markedly reduce immune response (Darrabie 2005; Tam 2011). More recently, a method incorporating CXCL12 chemokines in alginate microcapsules was introduced in an attempt to modulate the immune environment surrounding the islet graft (Chen 2015). Microencapsulation of islets using this method demonstrated a long-term (>300 days) immunoprotective effect in allogeneic and xenogeneic transplantation and a selective increase in intragraft regulatory T cells.

Conformal Coating

Traditional methods for microencapsulation produce beads of a consistent size. However, because islets vary in shape and size, these traditional methods can produce much larger sized capsules than the islet itself. The empty space inside the capsule results in core hypoxia and

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delayed insulin secretion in response to glucose (Yang 2015). This may, in part, contribute to the death and dysfunction of transplanted islets. Conformal coating is one of the technologies to overcome this problem. In conformal coating, the coating conforms to each islet's individual shape through a polymer bath (Figure 20.3C). This gives each islet a tight coating with very little additional space, which allows the islet to keep its own shape (Benchley 2013). Compared to conventional microcapsules, this technique greatly reduces the size and total volume of the islets for transplantation. Therefore, intraportal transplantation of conformally coated islets is possible.

The most common material used for the conformal coating of islets is a hydrogel polymer of polyethylene glycol (PEG). When exposed to photoinitiators such as ultraviolet or visible light, PEG can form a cross-linking covalently and polymerize (Shen 2005). Because the photoinitiator is bound to the surface of the islet, the polymerization can propagate outward at a controlled distance. Conformal coating produces thicker cross-linked hydrogels (50 to 70 μm) than nanotechnology, which is discussed later (Scharp 2014).

In 1997, Neocrin Inc. demonstrated successful xenogeneic transplantation of conformally coated porcine islets into diabetic rodents without immunosuppression (Hill 1997). However, the company failed to conduct further studies in larger animal models, presumably because of an aggressive reaction to the PEG coatings and xenograft tissue. Novocell then modified PEG triacrylate to improve the binding ability to photoinitiators and reactivity in large animals (Scharp 2014). An animal model with diabetic baboons demonstrated survival of allograft islets with minimal immune reactions up to 20 months (Scharp 2014). However, a clinical trial of two recipients with type 1 diabetes of 25–30 years failed to achieve insulin independence after transplantation. The longer duration of insulin use in humans was suggested as a possible reason for the different results between baboons and humans, with a reduced release of insulin and poor survival of the grafted islets (Scharp 2014). More recently, a computationally optimized method was introduced for conformal coatings (Tomei 2014). With this method, the islets can be completely encapsulated with a thin continuous layer of hydrogel. In diabetic mice, allogeneic transplantation of conformally coated islets resulted in euglycemia for more than 100 days with no foreign body reaction and normal revascularization.

Nanoencapsulation or Layer-by-Layer Coating

Nanoencapsulation is a more recently developed technique (Tendulkar 2013). Nanomedicine-the medical application of nanotechnology-is associated with tiny measurements at the level of 1–100 nanometers (nm). Nanoencapsulation encapsulates islets by alternating positively and negatively charged polymers on the surface of the cell aggregates (Figure 20.3D); it is also referred to as layer-by-layer coating when limited to the nanoencapsulation of islets for the treatment of diabetes (Wilson 2008). This approach was developed because of the reality of standard cell aggregate hydrogel coatings (that may leave openings in the coating) and, the diffusional concerns of hydrogel microcapsules (that may reduce the responsiveness of encapsulated islets to glucose changes). With nanoencapsulation, capsule thickness can be significantly minimized, which improves glucose-dependent insulin release as well as the diffusion of nutrients and waste products. In

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addition, a variety of agents also can be integrated to improve the survival and function of encapsulated islets.

Krol et al. coated islet surfaces with polyelectrolytes of poly (allylamine hydrochloride) and polystyrene sulfonate layers (Krol 2006). Wilson et al. demonstrated nanoencapsulated islet function both *in vitro* and *in vivo* using a PEG coating combined with biotin-PEG peptide conjugates with streptavidin (Wilson 2008). Furthermore, nanoencapsulation using biotin-PEG-GLP-1 was found to promote islet viability and insulin secretory capability in response to glucose (Kizilel 2010). More recently, nanoencapsulated islets coated with polylysine and polyglutamic acid effectively reversed hyperglycemia after allogeneic transplantation in diabetic mice for a month (Zhi 2012). Also in 2012, Dong et al. demonstrated that a nanoencapsulation technique induced normoglycemia for more than 3 months, even in major histocompatibility complex mismatched diabetic mice. They also demonstrated the longer survival of pegylated islets with nanoparticles containing leukemia inhibitory factor versus pegylated islets with empty nanoparticles or uncoated islets (Dong 2012).

Source of Graft Islets

Allogeneic Islets

Allogeneic islets are the most widely used source for islet transplantation. As in other organ transplantation, a major barrier in allogeneic islet transplantation is host immunity. Both the innate (within the first 2–3 days) and adaptive immune systems respond to destroy the graft after islet transplantation into the recipient (Gibly 2011). The autoimmune process and alloimmunity have been suggested to influence islet survival after allotransplantation in patients with type 1 diabetes (Harlan 2009; Hilbrands 2009). Islet encapsulation has been shown to protect allografts in experimental models. In addition, encouraging results have been obtained both in preclinical and initial human allograft studies, as discussed previously (Calafiore 2006; De Vos 2010; O'Sullivan 2011; Vaithilingam 2011). Encapsulation may protect islets from autoimmunity (Kobayashi 2006; Vaithilingam 2011) and allograft rejection in immunized hosts (Kumagai Braesch 2013). Although there has been much research on how to prevent an immune response in allogeneic islet transplantation, the availability of organ donors is still a major hurdle for human islet transplantation. The demand for donors far exceeds the supply.

Xenogeneic Islets

As in allogeneic transplantation, host immunity is a critical issue in xenogeneic transplantation. However, xenogeneic transplantation has an advantage by overcoming the shortage of organ donors. Many studies have investigated its clinical applications.

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Guidelines for Clinical Islet Xenotransplantation

In 1994, Groth et al. first transplanted fetal pig islet-like cell clusters into 10 diabetic kidney-transplant patients. Porcine C-peptide was detected in the urine of four patients for ~200–400 days after transplantation (Groth 1994). In Mexico, Valés-Gonzaléz et al. co-transplanted pig islets and Sertoli cells placed in a stainless steel chamber under the skin in pediatric patients with diabetes (Valdes-Gonzalez 2005). In China, Wang et al. transplanted neonatal pig islets into the arterial veins of the liver in 25 patients with type 1 diabetes using clinically available immunosuppression (Wang 2011). However, these early clinical trials showed an absence of clinical benefits to the patients who received porcine islet transplantation, as well as insufficient regulatory oversight: appropriate peer review of the protocol, efficacy, and safety of preclinical studies in NHPs should be guaranteed before clinical trials.

In 2008, key members of the International Xenotransplantation Association (IXA) and World Health Organization gathered in Changsha, China to develop principles and guidance for clinical islet xenotransplantation, referred to as the *Changsha Communiqué* ("First WHO Global Consultation on Regulatory Requirements for Xenotransplantation Clinical Trials: Changsha, China, 19–21 November 2008. The Changsha Communique," 2009). Since then, IXA has published consensus statements on clinical trial protocols for porcine islet products in type 1 diabetes (Hering 2009). This document describes several key issues, such as the ethical requirements and progress of an internationally regulatory framework, source pigs, pig islet product manufacturing and release testing, preclinical efficacy and complication data required to justify a clinical trial, strategies to prevent the transmission of porcine endogenous retroviruses, patient selection for pilot clinical trials of islet xenotransplantation, and informed consent in xenotransplantation clinical trials. In 2016, IXA updated this consensus statement (Hering 2016).

Clinical trials of porcine islet xenotransplantation should be conducted under appropriate regulatory oversight based on the guidance provided in the IXA consensus statement, which protects the patients from potential risks of infection and advises the investigators on obtaining public consensus on the safety of xenotransplantation. This point needs to be emphasized because islet xenotransplantation may be the key to the clinical realization of xenotransplantation. In this sense, NHP preclinical studies showing the efficacy and safety of the protocol should be a prerequisite. Matsumoto et al. reported that a clinical trial of encapsulated porcine islets in eight patients under regulation of the authority in Argentina could lead to a significant improvement of HbA1c and reduction of hypoglycemic unawareness events with an improved transplant estimated function (Matsumoto 2016).

Management of the Pigs and Islet Isolation

Our group acquired a specific-pathogen-free (SPF) miniature pig strain from the University of Chicago in 2004. Since then, we have been breeding and maintaining a closed herd in an SPF barrier facility. The characteristics of this Seoul National University (SNU) miniature pig, including microbiological monitoring, islet isolation, and the predictive

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parameters for high islet yield, have been reported elsewhere (Jin 2010; Jin 2011; Kim 2009). In microbiological monitoring, 41 viral pathogens, 35 bacterial pathogens, 2 fungal pathogens, and 25 parasites were screened for and confirmed to be negative, indicating that this closed herd has achieved designated-pathogen-free status. In addition, all SNU miniature pigs have been tested for the presence of porcine endogenous retrovirus (PERV) by reverse transcription-polymerase chain reaction. The results showed that PERV types A, B, and C were present in the genome. However, no reverse transcription activity of PERV (as assessed by *in vitro* reverse transcriptase assay) was observed in more than 50 monkeys that underwent pig islet transplantation. This observation is supported by the gene sequence data of PERV in SNU miniature pigs, which showed that most PERV genes were integrated into the chromosome as defective forms (data not shown).

The SNU miniature pigs also produced a higher yield of islets compared with other strains of pigs (~9,600 IEQs per gram of pancreas), the highest value that has been reported worldwide to date (Kim 2007). The characteristics of the pig contributed to this extremely high islet yield, including strain specificity, age, gender, pregnancy experience in female, as did the techniques of islet isolation and maintenance, including an efficient pancreas procurement procedure with no warm ischemia time, less than 20 min of cold ischemia time, and a good degree of pancreas distension by collagenase infusion. The selection of a high-yield pig using the parameters for successful islet isolation prediction contributed to a consistently high yield of islets (Kim 2009). Implementation of all of these procedures allowed our group to achieve consistent glycemic control in long-term (> 6 months) in diabetic NHPs using pig islet transplantation (Shin 2015; Shin 2016).

Quality Control of Isolated Pig islets

To achieve consistent glycemic control after the transplantation of porcine islets in NHPs, quality control of isolated islets is important. Table 20.2 lists the assays that can be used to determine the quality of isolated islets. In our pig-to-NHP islet xenotransplantation experiments (Shin 2015; Shin 2016), we performed three independent assays: 1) an islet cell viability test using a β-cell-specific fluorescent dye (Fluozin-3), a mitochondrial activity indicator (tetramethylrhodamine, ethyl ester [TMRE]), and a fluorescence-activated cell sorting (FACS) machine; 2) glucose-stimulated insulin secretion (GSIS); and 3) a nondiabetic obese severe combined immunodeficiency (NOD/SCID) mouse bioassay. Four streptozotocin-induced diabetic mice were transplanted with 2,500 IEQ pig islets under kidney subcapsules. Their blood glucose levels were monitored 2–3 times per week for at least 2 months (Figure 25.4). Our study showed that the isolated pig islets were >90% pure by dithizone staining, with >80% healthy β-cells by FACS and >60% diabetes correction capacity by the NOD/SCID bioassay (Shin 2015). Although the increase of insulin upon the glucose stimulation of pig islets overall exceeded one-fold, the results from the GSIS assay were highly variable and do not reflect the potency of the isolated pig islets, unlike those from other species (data not shown).

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Table 20.2. Assays to determine the quality of isolated pig islets

Test	Method	Advantages and disadvantages
Viability	β-cell viability by FACS using β-cell-specific fluorescent dye	Relatively easy to perform, but cell dissociation procedure may yield an artifact
Glucose-stimulated insulin secretion (GSIS)	Insulin-secreting capacity upon a glucose stimulus	Relatively easy to perform, but the results are highly variable and not correlated to transplantation outcome
Oxygen consumption rate (OCR)/DNA	Oxygen consumption rate by normal cellular metabolism; fractional cell viability	Relatively easy to perform, and the results are relatively well correlated to transplantation outcome
Mouse bioassay	Diabetes reversal capacity *in vivo* ; golden standard	Most difficult and requires a long time, but the results are well correlated to transplantation outcome
ADP/ATP	ADP/ATP ratio by normal cellular metabolism	Relatively easy to perform, but the results are variable

TRANSPLANTATION SITES

Determining the optimal site for encapsulated islet transplantation requires intense research. Several factors should be taken into consideration for future studies, including biocompatibility, revascularization, mechanical resistance, and retrieval of graft islets.

Generally, macrocapsules have been transplanted into the subcutaneous space. The major advantage of this location is its easy accessibility, which provides potential for biopsy access and retrieval. However, the poor vascular supply of the subcutaneous space may compromise islet function and engraftment. Because the macrocapsules are not permeable to vascular ingrowth, they may need a mechanism to enhance oxygen supply. Pretreatment with fibroblast growth factors in macroencapsulated islets demonstrated the beneficial effects of neovascularization and normoglycemia up to 3 months in rodents (Wang 2002). Implantation in prevascularized subcutaneous sites also showed long-term survival of graft islets without immunosuppression in rodents (Luan 2014). Several other sites have been suggested for islet transplantation, such as the pancreas, skeletal muscles, gastrointestinal tract, bone marrow, lymph nodes, spleen, and immune-privileged sites (thymus, testis, and anterior chamber of eyes) (Yang 2015). However, these sites have not been evaluated in encapsulated islet transplantation thus far.

The intraperitoneal space has been the most commonly investigated site for the transplantation of microencapsulated islets. The technical ease of accessibility and its capacity to accommodate a relatively large volume of transplanted material are major advantages of the intraperitoneal space. Although the intraperitoneal space has been used in most studies of encapsulated islets, the optimal site for encapsulated islet transplantation has not yet been

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elucidated. Difficulty in vascular network and retrieval of transplanted capsules are disadvantages of the peritoneal cavity. The clumping of capsules in biped humans and NHPs by gravity should also be solved for the clinical application of transplantation. It is generally accepted that vascularized sites would be better locations than the intraperitoneal space in terms of oxygen supply. Therefore, researchers have been investigating other transplant sites, such as the kidney capsule, subcutaneous space, and omentum.

Capsules implanted subcutaneously or under the kidney capsules showed increased viability and insulin secretion rates, as well as lower immune responses, compared with those implanted intraperitoneally (Dufrane 2006). However, these sites have a limitation of restricted volume. The implantation of encapsulated islets into the omentum via the omental pouch also showed successful results in rodent models of diabetes (Pareta 2014). The omental pouch has several advantages, such as unlimited space for islet transplantation, relatively easier retrieval of graft islets, improved proximity to vessels, and delivery of insulin to the portal circulation. Taken together, these observations support the previous suggestion that research should be focused on finding or creating a transplantation site to allow close contact between the vasculature and encapsulated islets rather than the unmodified peritoneal cavity (De Vos 1999).

INSULIN RELEASE FROM ENCAPSULATED ISLETS

Because macrocapsules have generally thicker walls and larger diameters than microcapsules, an impaired diffusion of nutrients and oxygen can threaten the viability of the islets and slow the kinetic release of insulin. As discussed previously, to guarantee an adequate supply for the cells, the islet density in the macrocapsules should be kept quite low (< 5−10%). Consequently, large devices are required to provide sufficient masses of islets (Lanza 2011). A capsule's components can also affect glucose-stimulated insulin secretion. For example, hydroxyl methylation of polysulphone permitted for efficient insulin release from the macroencapsulated islets (Lembert 2001), whereas blending polysulphone with polyvinyl pyrrolidone or sodium dodecyl sulfate did not show glucose-induced insulin release (Orive 2003). Although several *in vivo* and *in vitro* studies have shown maintenance of long-term normoglycemia and insulin release after retrieval of macroencapsulated islets (Kessler 1997; King 2001; Lembert 2001), the relatively large surface-to-volume ratio may slow the exchange rate of glucose and insulin, leading to inadequate regulation of glucose levels.

In this regard, microencapsulation may provide some advantages. However, the kinetics of insulin release from microencapsulated islets has been reported to be delayed in previous studies, which seems to be related to microcapsule volume (De Vos 2002; De Vos 2010; O'Sullivan 2011; Orive 2003). *In vitro*, insulin release usually peaks 10–15 minutes later in microencapsulated islets compared to naked islets. A similar delay in insulin release from microencapsulated islets was observed when glucose concentration decreased (Wang 1997). *In vivo*, intraperitoneal transplantation of microencapsulated islets cured diabetic mice in the long term, but with a delay of C-peptide release from the graft islets.

An intraperitoneal location may affect the kinetics of insulin release from microencapsulated islets. There are two time-dependent steps: the elevation of glucose levels in intraperitoneal spaces (which stimulates the graft islets to release insulin) and entrance of

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the secreted insulin from capsulated islets into systemic circulation. Intraperitoneal glucose concentration in rats has been shown to follow blood glucose levels with a delay of 5 minutes and a reduction of up to 80% of blood glucose levels (Velho 1989). Consequently, the signal for insulin release is delayed and reduced in intraperitoneal transplantation. There is also a delay before insulin in the peritoneal space is absorbed into the venous system (De Vos 1996). Previous studies suggest that absorption of insulin administered intraperitoneally is dependent on volume, concentration, and time (Schade 1981). The kinetics of insulin release may be further affected by other variables, such as the clumping of capsules, pericapsular cellular attachment, or modification of the peritoneal cavity. This may be further complicated after meals or during exercise, when there is a need to halt insulin secretion to prevent hypoglycemia (Omer 2004).

Islet cell aggregates can provide additional benefits in terms of insulin secretion and beta cell survival after microencapsulation, probably because of improved oxygenation (Zhi 2013). Islet cell aggregates are usually obtained by dispersing islets into single cells and allowing them to reaggregate in culture. By using this approach with rat islets, glucose-stimulated insulin release was improved *in vitro* compared with whole islets (Zhi 2013). In nanoencapsulation, the kinetics of insulin release may be improved due to the absence of dead space around the islets. However, studies regarding insulin secretion kinetics of nanoencapsulated islets are still scarce. Coating materials may also affect the kinetics of insulin secretion. The nanocoating of human islets with poly-(diallyldimethylammonium chloride) showed blunted insulin release (Krol 2006), whereas conformal nanothin PEG coatings of islets via layer-by-layer self-assembly of poly (L-lysine)-g-poly(ethyleneglycol)(biotin) and streptavidin demonstrated better insulin secretory function (Wilson 2008). More studies are needed to identify the factors regulating the kinetics of insulin release from nanoencapsulated islets.

TRACKING MICROENCAPSULATED ISLET TRANSPLANTS

Currently, the only way to evaluate the functional status of implanted microcapsules is invasive recovery surgery. There is a growing interest in improving the ability to monitor implanted cell-loaded devices. As an attempt to resolve this need, Barnett et al. developed radiopaque alginate-based microcapsules using either barium sulfate or bismuth sulfate, which can be monitored by X-ray (Barnett 2006). Another group successfully tracked microcapsules in real time with a bioluminescence approach (Tiernan 2014). Further research is needed to determine whether these emerging techniques could be applied to the clinical transplantation of microencapsulated islets.

CONCLUSION

The efficacy of encapsulated islet transplantation has been demonstrated in diabetic animal models. Although several techniques for islet encapsulation have been extensively investigated, the problems of biocompatibility and long-term survival of graft islets still need to be solved. In addition, other limitations exist, such as the detrimental effects of cytokines,

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delayed insulin secretion from encapsulated islets, choice of transplantation sites, and need to track microcapsules. Furthermore, even though technical success has been achieved in animal models, the shortage of organ donors has to be addressed prior to clinical application. Xenotransplantation with pig islets is a potential alternative source of islets, as discussed in this chapter. Induced pluripotent stem cells (Alipio 2010) and human embryonic stem cells (Motté 2014) also have been suggested as alternative sources of islets. The creation of guidelines and consensus statements on encapsulated islet transplantation from various sources may also affect the field of encapsulated islet transplantation. In the future, further extensive research is needed to address the unresolved factors in islet transplantation.

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Chapter 21

HEPATOCYTE TRANSPLANTATION

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ABSTRACT

Hepatocyte transplantation has been investigated as an alternative therapeutic modality to orthotopic liver transplantation in patients with liver disease. Since the implementation of a reliable isolation method for hepatocytes, several experimental animal models of induced or inherited liver failure have been developed to test their potential. So far, the transplantation of hepatocytes has been shown to be effective in restoring biochemical function in animal models of liver-based metabolic disease, to improve the survival of animals with experimentally-induced acute and chronic liver failure and to engraft and proliferate when the host conditions allow for growth advantage of the transplanted hepatocytes. Lessons learned from the experimental animal models have been translated into clinical attempts in patients with liver diseases. While some efficacy has been demonstrated, most patients eventually need a whole liver transplant due to the lack of long-term engraftment and functionality of hepatocytes. Therefore, before this procedure becomes a routine, we must overcome these obstacles for which further research will be needed.

Here we discuss the advances and hurdles of hepatocyte transplantation and describe some animal models used to study this promising alternative therapy.

Keywords: liver, hepatocyte, transplantation

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ABBREVIATIONS

HTx	Hepatocyte transplantation
OLT	Orthotopic liver transplantation
ALF	Acute Liver Failure
AILF	Acetaminophen Induced Liver Failure
ACLF	Acute on Chronic Liver Failure
NASH	nonalcoholic steatohepatitis
LFT	Liver Failure Tests
DSAs	Donor Specific Antibodies
CN1	Crigler-Najjar type 1 disease
UGT1	hepatic UDP-glucuronosyl transferase
HT1	Human Hereditary tyrosinemia type 1
FAH	furamylacetoacetate hydrolase
HCC	hepatocellular carcinoma
apo	apolipoprotein
LDL	low density lipoprotein
OTC	Ornithine transcarbamylase deficiency
BDL	Surgical bile duct ligation
LSEC	Liver sinusoidal endothelial cells
PMN	Polymorphonuclear cells
KC	Kupffer cells
DAMPs	Danger-associated molecular patterns
CCL4	Carbon tetrachloride
EDTA	Ethylenediaminetetraacetic acid
HBSS	Hank's Balanced Salt Solution
NAC	N -acetylcysteine
GMP	Good Manufacturing Practices

INTRODUCTION

Hepatocyte transplantation is an experimental procedure that has been widely used not only to improve the quality of life of patients awaiting for an OLT (Orthotopic Liver Transplantation), but also as a treatment for patients with end stage or genetic metabolic liver diseases by providing the metabolic and synthetic support that they need.

The first description that lead to the surge of hepatocyte transplantation dates back to 1898, where Ribbert et al. (Ribbert 1898) attempted to place small fragments of liver into the lymph nodes of rabbits and guinea pigs. He observed the animals for 4−5 weeks and found that hepatocytes and ducts were nearly normal for 1−2 weeks. However, after 2−3 weeks there was a gradual atrophy and bile ducts were necrotic or proliferated to form adenoma-like nodules.

A year later (1899), Lubarsch et al. intravenously injected hepatocytes in the pulmonary veins of rabbits, but they were degenerated in 3−4 weeks (Lubarsch 1899). While no evidence of function was observed in any of these attempts, it paved the way for other investigators to

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explore alternate sites to heterotopically graft minute amounts of liver tissue, such as subcutaneous tissue (Herxheimer 1926), peritoneal cavity, anterior chamber of the eye, kidney, small intestine, and stomach among others (Grisham 1964). Unfortunately, survival of hepatic tissue depended upon the development of collateral circulation, which did not occur and engrafted tissue either underwent total necrosis, or resulted in complete loss of hepatocytes.

Back in the days, there was no specific isolation technique to get hepatocytes; it was not until the mid-1960s, when Howard et al. (Howard 1967) used the combination of a mechanical and enzymatic digestion technique to isolate rat hepatocytes. Later, this technique was modified in 1969 by Berry and Friend, who described for the first time the successful preparation of isolated rat liver parenchymal cells using a collagenase perfusion method (Berry 1969). This method has been refined and optimized not only to achieve better yield (Seglen 1976) but to use the isolated hepatocytes clinically (Gramignoli 2015).

Since then, laboratory animal studies and some clinical trials have demonstrated that hepatocyte transplantation (HTx) can be used for the treatment of liver failure and inborn errors of liver metabolism (Dhawan 2015; Jorns 2016; Coppin 2017). This procedure has several advantages over OLT: it involves a simple technique, where implantation of hepatocytes into the recipient is usually done by infusion into the portal vein, or the splenic artery (Strom 1997; Fox 1998), although some other places have been also tested. The infusion can be performed on a scheduled basis, hepatocytes can be cryopreserved to be used in the future, and more importantly, one donor can be used for multiple patients (Mitry 2004).

From the immunological perspective, if hepatocytes are lost, they do so in a less severe manner than whole liver. These characteristics make hepatocyte transplantation an attractive alternative therapy for the treatment of patients waiting for a liver by improving their quality of life while the organ becomes available or by providing the necessary metabolic and synthetic support while the native liver regenerates.

This book chapter will describe the advances and hurdles of hepatocyte transplantation as an experimental technique in animal models and in clinical settings.

MICROSCOPIC ANATOMY OF THE LIVER

The liver is the largest organ in the body representing approximately 2.5% of the total body weight of an adult. In a resting state, 25% of the total cardiac output gets into the liver through the hepatic portal vein and the hepatic artery. The liver has regulatory, secreting, synthetic, metabolic and excretory functions. The principal cell performing all these functions is the “Hepatocyte” which accounts for roughly 80% of the liver mass (Rhoades 2009).

Hepatocytes are arranged in plates separated by capillary networks called sinusoids. The polygonal shape of the cells allows them for one side (basal membrane side) to be in contact with the sinusoids from where they receive nutrients and oxygen, and on the other side (apical side) with their neighboring hepatocytes, with whom they form bile canaliculi (Gissen 2015) (Figure 21.1).

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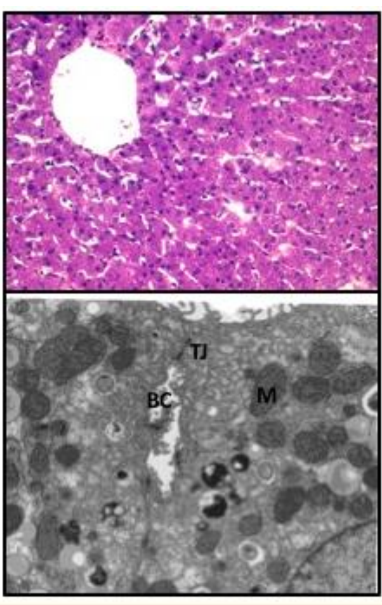

Figure 21.1. Microscopic anatomy of the liver. H&E (upper panel) and Transmission Electron Microscope picture of polarized hepatocytes (TJ: Tight Junction; BC: bile canaliculi; M: mitochondria).

Unfortunately, following isolation, hepatocytes lose their polarity which is extremely important when cells are transplanted, since for cells to function, they need to follow a similar arrangement in the organ they allocate. Defects in hepatocyte polarization lead to major pathophysiological consequences (Gissen 2015) including graft failure.

HEPATOCYTE ISOLATION

Hepatocyte transplantation encompasses 3 steps: The isolation of hepatocytes, the preparation of cell suspensions and its implantation in the recipient. Diverse methods have been recently described for the isolation of different sources of hepatocytes; while all of them are founded on the same concept, they varied based on the technique and the type of enzymes used. Regardless of the species, when hepatocyte isolation is performed from a whole organ, the general technique involves liver perfusion through the existing vasculature; and by injecting the solutions directly into the parenchyma when isolation is from small liver biopsies.

Human Hepatocyte Isolation

In the case of human hepatocytes, the main issue facing is the lack of adequate sources to get viable cells. Hepatocytes are generally obtained from discarded organs due to steatosis greater than 40−50% (Strom 1997), prolonged ischemia time (Muraca 2002), traumatic graft injury, capsular tear (Bilir 2000; Alexandrova 2005), blood group incompatibility (Fox 1998) as well as donor livers after the portion used for transplantation has been removed (Mitry

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2002; Mitry 2004). Other proposed sources are hepatic tissue from liver resections due to benign disease or after resection of a colorectal tumor (Gunasegaram 2008).

The isolation is performed using a two-step Ethylenediaminetetraacetic acid (EDTA) or EGTA/collagenase enzymatic perfusion technique. First, cannulation of the hepatic artery and the portal vein from discarded hepatic tissue is performed (Figure 21.2). The enzymatic digestion consists of two stages: a first stage of non-recirculating perfusion of the hepatic tissue with a HEPES-buffer free of calcium and containing EDTA to wash out the blood and weaken intercellular junctions; the second stage is a recirculating perfusion step where the same previous solution is used with the addition of $CaCl_2$ and collagenase to achieve complete digestion of the hepatic tissue. Both stages are performed at 37°C. The tissue is then mechanically disaggregated, filtered and washed to obtain a hepatocyte suspension (Figure 21.2), which will then be used for transplantation. While experimentally hepatocytes have been placed in culture for a few days allowing time for hepatocytes to recover from the harsh enzymatic treatment, clinically, this is not routinely performed.

Figure 21.2. Procedure for the isolation of human hepatocytes comprising: Liver cannulation and perfusion with digestive enzymes, mechanical dispersion of the liver, to obtain single cell suspension.

While most of the protocols are based on the use of Collagenase Type 1: 0.05% (Alexandrova 2005; Hughes 2006; Dhawan 2015), there have been some protocols using different collagenase and slightly different concentrations (Cho 2004). One of the most recent modifications to the protocol was the use of Liberase (a blend of collagenase I and collagenase II and dispase or thermolysin) and addition of the antioxidant *N* -acetylcysteine (NAC) (Bartlett 2014) with an overall 70% success rate for isolations from normal and diseased livers.

The yield and the viability of hepatocyte following the isolation will vary depending on the conditions of the tissue. Lower yields and viabilities have been observed when hepatocytes are isolated from fibrotic or steatotic livers, or with an increased gamma-glutamyltranspeptidase or aspartate aminotransferase activity and bilirubin content. Cold ischemia times and weight of the perfused livers also influence yield. Interestingly, yield is significantly increased by chemotherapy treatment (Lee 2014). Therefore, the number of hepatocytes obtained ranges from $2.0 \times 10^{(6)}$–$1.8 \times 10^{(10)}$ cells (Mitry 2003; Lee 2014).

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Mouse Hepatocyte Isolation

Hepatocytes are isolated by the same enzymatic digestion technique developed by Berry and Friend (Berry 1969) as an in situ two-step collagenase perfusion technique and as modified by Seglen (Seglen 1976).

Independently of the mouse or rat strained used, the following procedures are usually performed:

In general, all solutions should be prepared just before isolation and pre-warmed at 37−40°C. The isolation involves an *in vivo* perfusion, where a cannula is inserted in either the portal vein (Berthiaume 1999; Li 2010) or into the vena cava through the right atrium of the animal while the animal's heart is still beating (Severgnini 2012). The liver is then perfused with a solution that is usually Hank's Balanced Salt Solution (HBSS) containing EDTA to wash out blood, followed by a collagenase perfusion with concentrations that vary from as low as 0.03% to as high as 0.2% (Chung 2010; Sewing 2016). Several collagenases have been used, including collagenases type I, type II, Type III and Type IV. In some protocols, the addition of Elastase has been tested (Oliva 2008). After the perfusion is terminated, the surface of the liver sac is cut to release the hepatocytes, which are then collected and passed through a cells strainer, followed by a round of centrifugations and washes that will yield single isolated hepatocytes.

Several protocols use the additional step of cell purification through gradient centrifugation with Ficoll or Percoll. To avoid the manipulation of the cells which unfortunately results in a reduced cell number and viability, investigators have used a protocol that reduces the need for the gradient centrifugation purification step and results in high yields of viable and functional cells (Severgnini 2012).

Porcine Hepatocyte Isolation

The isolation for porcine hepatocytes resembles pretty much the human procedure (Figure 21.2). The differences lie on the concentration and type of collagenase used, and the addition of other enzymes to the perfusate to achieve better yield and viability.

The collagenase that is more commonly used in this species is type I and IV, and the concentrations range from 0.02% to 0.8% (Gerlach 1996). The addition of Neutral Protease and Deoxyribonuclease I have been also tested (Meng 2010). And so, does the use of Dispase and a four-step retrograde perfusion combining both dispase and collagenase (Maruyama 2003).

HEPATOCYTE TRANSPLANTATION

Since more than 40 years ago, the potential of Hepatocyte transplantation (HTX) has been investigated in different animal models of induced acute liver failure (Chen 2005; Tanaka 2006; Totsugawa 2007; Yamamoto 2010; Navarro-Alvarez 2010), chronic liver failure (Nagata 2003; Nakamura 2014); (Kobayashi 2000) and hereditary liver disorders (Matas 1976; Yoshida 1996).

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While a significant improvement of liver function and survival has been observed experimentally, unfortunately the outcome of hepatocyte transplantation in patients with liver failure has been disappointing, except when used as bridge to liver transplantation.

One cannot directly extrapolate the techniques and approaches used in these models to the clinical scenario, and individual case by case factors need to be taken into consideration. Among those, some important factors that need special attention when transplanting hepatocytes clinically are: the route of administration and the number of infusions and cells administered on each infusion, as the efficiency of the transplanted cells depends on those conditions. The administration of too many cells imposes a risk to the donor, by increasing the portal pressure (Dhawan 2015).

The average adult liver contains 2.8×10^{11} hepatocytes (Fitzpatrick 2009; Dhawan 2015). Therefore, it has been anticipated that in total, approximately 5−10% of the recipient's estimated liver cell mass, should be infused (Fox 1998) (Jorns 2012) (Muraca 2002). The final cell dose is usually adjusted based on the patient's body weight and clinical situation, but as a rule, 1−2% of liver mass may be the upper limit of hepatocytes that can be safely infused in each infusion (Horslen 2003; Dhawan 2015). To deliver the total dose required, multiple infusions will need to be done, separated by hours, or even days, with a careful monitoring of portal pressure which should not increase by more than 12 mm Hg to avoid significant portal hypertension.

Route of Hepatocyte Infusion

The infusion of hepatocytes experimentally and clinically is currently done by a slow infusion through the portal vein, the splenic vein or in the intraperitoneal cavity. The portal vein route has been generally used when attempting to transplant hepatocytes with the goal of correcting inborn errors of metabolism, which is usually done by catheterization via an ultrasound-guided approach of the portal or inferior mesenteric vein or by laparoscopic surgery (Fox 1998) (Figure 21.3). Using this route generates the advantage of having hepatocytes in their natural microenvironment right away, where they can respond to local growth factors and cytokines. Hepatocytes rapidly integrate in the liver parenchyma and start replacing missing liver functions; they can undergo proliferation and repopulation of the host liver, causing minimal stress when compared with whole liver transplantation (Figure 21.4).

On the other hand, in patients where the parenchyma is not intact, such as in the case of cirrhosis, the splenic arterial route has been mostly used, which is accessed through a catheter inserted in the femoral artery for implantation in the spleen. This organ is considered to be very attractive and convenient; its location makes it easy to access, its structure can accommodate a relatively large number of cells, and since it is a non-vital organ, it could be easily removed without fatal consequences if graft rejection ensues. Nevertheless, it is recommended that after each cell infusion a repeated Doppler ultrasound is performed to rule out the formation of thrombosis.

This route has been tested in patients suffering from ALF (acute liver failure) (Bilir 2000) or ACLF (acute on chronic liver failure) (Wang 2014). Direct intrasplenic injection has been experimentally proved to produce an engraftment far superior to that obtained using splenic artery infusion (Nagata 2003a; Nakamura 2014; Nagata 2003b; Ito 2007).

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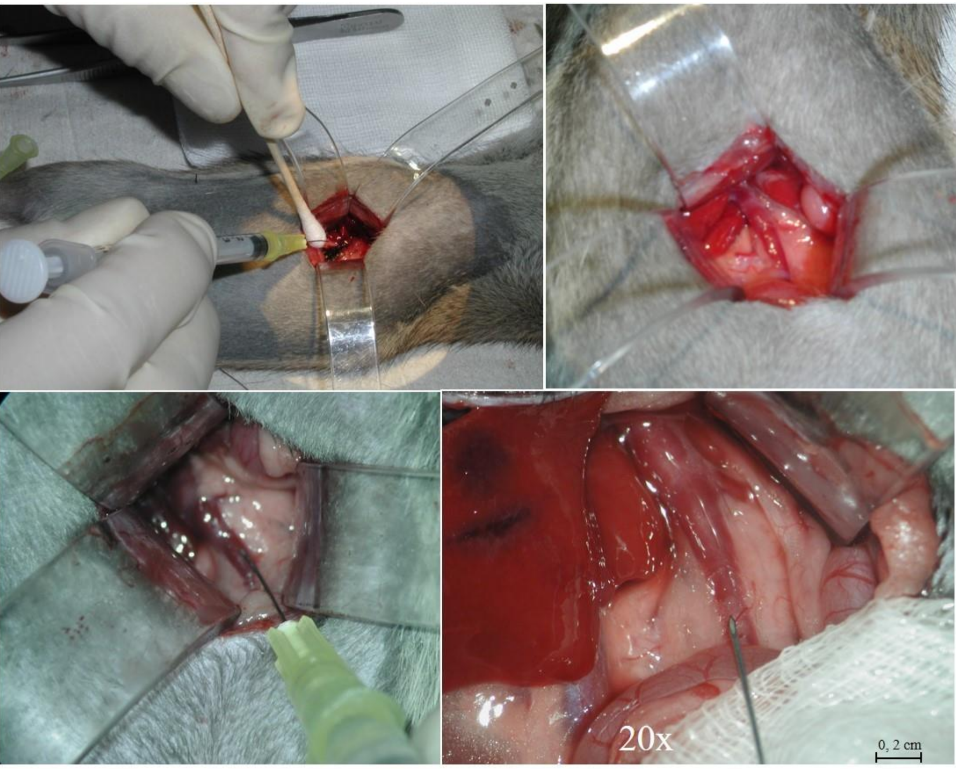

Figure 21.3. Injection of hepatocytes into the portal vein.

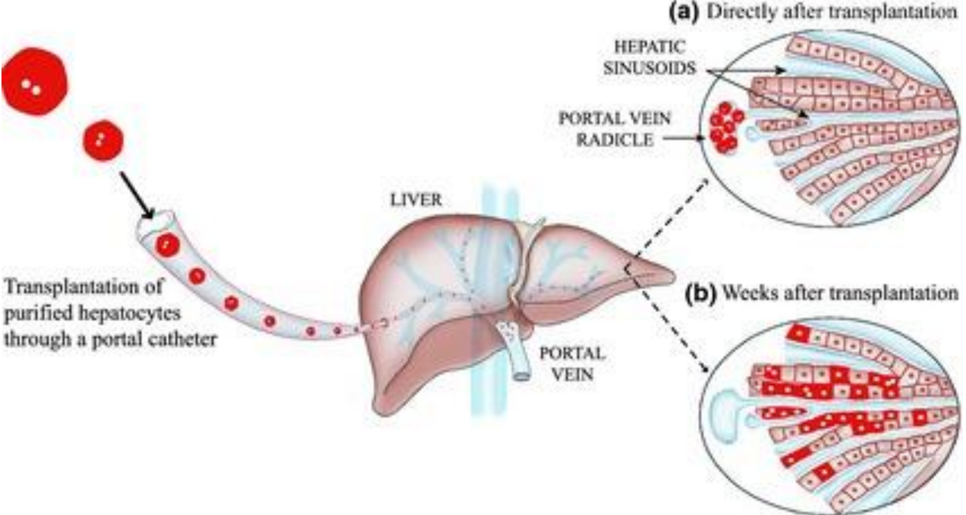

Figure 21.4. Integration of hepatocytes following injection.

An additional site that has been proposed especially for patients with acute liver failure, is the intraperitoneal cavity (Habibullah 1994). Usually these patients have severe coagulopathy and liver inflammation, which makes access to the portal system very

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challenging due to the higher risk of possible systemic embolization or portal vein thrombosis that can result from hepatocyte transplantation. Therefore, the peritoneal cavity is an alternative site for hepatocyte transplantation (Dhawan 2015). In order to avoid the host immune response, hepatocytes have been encapsulated in alginate microspheres. This allows short-term support, while providing time for the native liver to regenerate (Jitraruch 2014; Jitraruch 2017; Sgroi 2011). An optimized protocol to produce (Good Manufacturing Practices) GMP grade alginate-encapsulated human hepatocytes has been established and could be soon used in clinical transplantation (Jitraruch 2014).

INDICATIONS

Metabolic Liver Disorders

These disorders are characterized by inherited alterations in either hepatic enzymes or proteins with metabolic functions. In this situation, the liver parenchyma is usually intact, and the rest of the liver functions are normal.

The information regarding the potential of hepatocyte transplantation in these disorders comes mainly from animal models that have attempted to mimic human genetic liver diseases. These models have provided valuable insight into the physiological, biochemical and molecular mechanism underlying each disease.

1) Model for Human Crigler–Najjar syndrome type I: Crigler-Najjar type 1 disease (CN1) is a rare inherited metabolic disease characterized by complete absence of hepatic UDP-glucuronosyl transferase (UGT1), resulting in high levels of unconjugated bilirubin.
   The Gunn rat is a natural model for CN1 that allows us to study the mechanism of bilirubin conjugation and deposition in order to understand its toxicity. This rat lacks hepatic bilirubin UDP-glucuronosyl transferase activity (UGT1A1), thus resembling Crigler-Najjar syndrome Type I. In this rat model, transplantation of normal hepatocytes resulted in the production of bilirubin glucuronides in the bile with a concomitant decrease in their serum bilirubin levels (Rugstad 1970; Polgar 2017).
2) Model for Human Hereditary tyrosinemia type 1 (HT1): This is a recessive liver disease caused by deficiency of the enzyme furamylacetoacetate hydrolase (FAH), which is responsible for the last step in the tyrosine catabolism. Accumulation of fumarylacetoacetate, the substrate for FAH, and its precursor, maleylacetoacetate, are thought to be hepatotoxic. HT1 affects mainly the liver and the kidney where FAH is most expressed (St-Louis 1997). Patients with HT1 either develop acute fatal liver failure in infancy or can progress to cirrhosis and hepatocellular carcinoma (HCC) early in childhood (Gokay 2016). If not treated, patients die of acute and severe hepatorenal failure in the early infancy. There are several animal models that are useful to study this lethal disease; a mouse (Aponte 2001), a rat (Zhang 2016) and a porcine model (Hickey 2011), which are deficient in FAH. Fah-deficient mice and pigs are usually maintained using 2-(2-Nitro-4-trifluoromethylbenzoyl)-1, 3-cyclohexanedione (NTBC), which is an inhibitor of 4-hydroxyphenylpyruvate

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dioxygenase that blocks the accumulation of toxic metabolites such as fumarylacetoacetate and maleylacetoacetate. If NTBC is withdrawn, usually these animals develop progressive liver failure and renal tubular dysfunction. Hepatocyte transplantation into these species can rescue these animals demonstrating complete liver repopulation by the transplanted hepatocytes (Zhang 2016).

3) Model of Human familial hypercholesterolemia: This is an autosomal dominant disorder characterized by premature atherosclerosis, cardiovascular disease with markedly elevated plasma levels of LDL cholesterol and apolipoprotein (apo) B100 due to mutations in the low density lipoprotein (LDL) receptor gene. Several species have been used to develop experimental models for the study of this disease: In mice, the Ldlr-/- mice have high LDL cholesterol and apoB100 levels and developed extensive atherosclerosis on a chow diet (Powell-Braxton 1998). In rabbits, the Watanabe Heritable Hyperlipidemic rabbit also presents with elevated serum LDL cholesterol levels and early atherosclerosis. In these models, autologous hepatocyte transplantation of genetically corrected *ex vivo* with recombinant retroviruses demonstrated a significant and persistent decrease in total serum cholesterol (Chowdhury 1991).

Given the lower surgical risk and fewer consequences of graft loss when compared with LT, hepatocyte transplantation is a good alternative therapy for these patients, where replacement of the defective hepatocytes can be achieved through hepatocyte injection. In fact, hepatocyte transplantation has been performed in more than 30 children with inborn errors of metabolism including urea cycle defects, Crigler-Najjar Type I (Fox 1998), Type I Glycogenosis, tyrosinaemia type I, Ornithine transcarbamylase deficiency (OTC) (Ribes-Koninckx 2012), Factor VII deficiency, Refsum's disease, Familial Hypercholesterolemia (Grossman 1995) to name a few. An initial partial correction of the defect is expected by the transplantation of hepatocytes which significantly reflects in the improved clinical outcome of the disease. It is estimated that for children with metabolic disorders, engrafted cells representing 5% of the native liver would suffice to reconstitute an enzymatic defect. However, the outcome unfortunately doesn't last long, and it is thought to be due to a low initial engraftment and an insufficient proliferation of the transplanted cells, for which investigators have been looking at different approaches to improve engraftment and repopulation of hepatocytes such as host conditioning by radiation therapy (Soltys 2016).

Acute Liver Failure

Acute liver failure (ALF) is a clinical syndrome caused by liver damage by a large number of noxious agents which is unfortunately associated with a high mortality rate. There have been several animal experimental models of acute liver failure ranging from surgical models to drug models.

1. Surgical models of induced acute liver failure:
   a) The most widely used model is the 90% hepatectomy. This model leads to a 100% mortality in mice within 3−5 days (Navarro-Alvarez 2010). The animals die of liver failure with elevation of liver enzymes, hyperbilirubinemia, ascites

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and encephalopathy, thus recapitulating the clinical picture of acute liver failure observed in patients. The transplantation of isolated hepatocytes can improve the survival of the recipients in this model of ALF. Several sources of hepatocytes have been used such as syngeneic hepatocytes, xenogeneic hepatocytes (Tanaka 2006; Soto-Gutierrez 2007; Yamamoto 2010), stem cell-derived hepatocytes (Soto-Gutiérrez 2006) and immortalized human hepatocytes (Kobayashi 2000b). All of them consistently have demonstrated promising results.

2. Drug-induced liver failure: Several drugs have been used to induce acute liver failure:
   a) Acetaminophen: Acetaminophen hepatotoxicity is due to its toxic metabolite, *N*-acetyl-*p*-benzo- quinoneimine (NAPQI), which is produced by the cytochrome P450 system when alternative detoxification pathways are saturated. The main underlying mechanism of Acetaminophen Induced Liver Failure (AILF) is the massive necrosis of hepatocytes which leads to the release of danger-associated molecular patterns (DAMPs) and formation of the inflammasome complex in various cells such as Kupffer cells (KC). This activates innate immune cells creating a sterile inflammation which will further contribute to the necrosis of more hepatocytes (Jaeschke 2014). Several models of AILF have been created. The most studied animals are the mice. There have been several issues when developing this model, for instance, it is well known that gender makes a difference; female mice usually develop less necrosis in the liver than male mice, independent of their genetic background (Mohar 2014). However, the differences in their genetic background require dose adjustments for reproducible results, because they develop more or less injury (Masubuchi 2009). Acetaminophen is usually administered by a single intra-peritoneal dose in doses that typically ranged from 250 mg/kg to as high as 600 mg/kg (Navarro-Alvarez 2010; Mossanen 2015). The transplantation of hepatocytes in this model has significantly extended the survival of these animals (Tanaka 2006; Yamamoto 2010).
   b) D-Galactosamine: It is an amino sugar metabolized via the galactose pathways in the liver. It is used in experimental animals to induce acute or fulminant liver failure. D-Galactosamine leads to the depletion of uridine moieties within the liver, therefore altering the hepatocyte RNA metabolism, and resulting in massive hepatic necrosis (Bélanger 2005). Animals develop biochemical changes consistent with ALF, encephalopathy, hypoglycemia and coma, resulting in a high mortality if not treated. When used in large animals such as pigs, the dose has been typically 0.5 g/kg of D-Gal given via the jugular route 24 h prior to cell transplantation. Investigators have used this model to test the feasiblity of hepatocyte transplantation. In this model, Totsugawa et al. tested primary and immortalized human hepatocytes injected intraportaly at a dose of 1x10^9 representing the 5% mass of the pig liver. The transplantation of hepatocytes significantly improved the survival and the clinical parameteres of the animals (Totsugawa 2007).

3. Combined surgery and drug induced liver failure:
   a) Retrorsine + 70% hepatectomy: In this model retrorsine, a pyrrolizidine alkaloid that inhibits hepatocyte proliferation is used, followed by 70% hepatectomy which is usually performed 4 weeks later. Because retrorsine inhibits

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regeneration of the liver after resection, animals cannot recover from the loss of hepatocyte mass. The model produces the clinical picture of liver failure including high LFTs, hyperammonemia-induced hepatic encephalopathy, and hypoglycemia (Navarro-Alvarez 2010). This model is widely used to evaluate the repopulation of transplanted cells, since it allows a selective long-term survival and repopulation advantage to the engrafted donor hepatocytes (Liu 2012; Navarro-Alvarez 2014).

b) Carbon tetrachloride (CCL_4) + Ischemia: Although carbon tetrachloride is used mainly to induce chronic liver failure, there have been models that have used it for the creation of fulminant liver failure. In a large animal model using pigs, Yuasa et al. investigated different doses of CCL4 administered through the portal vein combined with 30 mins ischemia by occlusion of the portal vein. He demonstrated that the use of 7.5 ml, of CCL_4 plus 30 mins of ischemia, represented the most reliable and reproducible model of fulminant liver failure, recapitulating the biochemical, hemodynamic and pathological findings found in patients undergoing this dreadful complication (Yuasa 2008). These parameters make this a great model to study the effect of hepatocyte transplantation.

In all these models, the transplantation of hepatocytes has been shown to improve the survival and clinical parameters of the animals undergoing acute liver failure (Tanaka 2006; Totsugawa 2007; Yamamoto 2010; Navarro-Alvarez 2010) and to prevent the development of intracranial hypertension in preclinical studies using large animals with acute ischemic liver failure (Arkadopoulos 1998).

Hepatocyte transplantation performed on patients with liver failure aims at sustaining liver functions to serve as a bridge to OLT while improving the quality of life of the patient, or where possible, until liver regeneration is achieved (Strom 1999; Dhawan 2015; Gramignoli 2015). In this context, usually patients with severe ALF, grade III to IV encephalopathy and those who were not candidates for OLT are selected to undergo this experimental treatment. Significant clinical improvement has been observed, however, they are not stable enough in some cases and patients eventually died if they did not receive an organ on time. Following transplantation, there is usually 48- to 72-hour delay in functioning of the transplanted hepatocytes which might represent a recovery or engraftment period. The transplanted hepatocytes have been easily identified both on transjugular biopsy and on autopsy in the spleen and liver by light microscopy and fluorescent in situ hybridization (FISH) in some of the transplanted patients (Bilir 2000; Gramignoli 2015).

Chronic Liver Failure and Acute-on-Chronic Liver Failure (ACLF)

Defined as an acute deterioration of an existing chronic liver disease, which could be a known compensated disease or an asymptomatic undiagnosed chronic hepatitis, nonalcoholic steatohepatitis (NASH) or cirrhosis.

1) Drug induced: The most used animal model to study chronic liver failure is the CCL4- induced cirrhosis alone or combined with phenobarbital. It is widely studied in small animals such as mice (Navarro-Alvarez 2010) and rats (Kobayashi 2000b).

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The principle established is the same across species when using small animals and consists of giving a mixture of CCl4 and mineral oil diluted 1:9 by intraperitoneal injection twice a week for 6 weeks which can be extended up to 10 weeks depending on the progression of the disease. Protocols usually start at a dose of 0.20 ml, where the percentage of CCl4 in the mineral oil (v/v) is adjusted weekly based on changes in body weight. Animals in this protocol clearly recapitulate the clinical picture of patients with cirrhosis. They develop increased LFTs, hyperbilirubinemia, coagulation dysregulations, and clinically proven ascites. The transplantation of hepatocytes in this model has contributed to an improvement of biochemical parameters and survival.

2) Surgical: Surgical bile duct ligation (BDL) is the most common, quick and reproducible model of obstructive cholestatic liver injury in mice and rats. This model intends to reproduce the fibrosis and cirrhosis observed in the patient liver cirrhosis induced by hepatotoxins. Mice and rats in this model present with severe jaundice 21 to 28 days after the ligation (Kountouras 1984).

Despite these diverse models showing-significant benefit of hepatocyte transplantation in chronic diseases, clinically, OLT is the only cure for these patients. But, there have been some promising results indicating that HTx could be used as an alternative treatment (Pareja 2013; Wang 2014). Improvement of clinical parameters and liver function has been observed and in some cases of patients, has served as a bridge to liver transplantation (Pareja 2013; Gramignoli 2015).

IMMUNOSUPPRESION

Hepatocytes are subjected to a cell-mediated immune rejection; therefore an immunosuppresion protocol must be in place when performing hepatocyte transplantation. The first thing to consider is the ABO compatibility.

Following transplantation, the surviving allogeneic cells in humans have to overcome the adaptive immune system which might contribute to the gradual loss of graft function. Although the exact mechanisms by which hepatocytes are rejected are not very well understood; it is known that hepatocytes stimulate a cell-mediated immune response induced by both CD4 and CD8 T cells (Han 2009). Therefore, patients usually undergo an immunosuppression protocol consisting of the administration of a bolus of corticosteroids prior to hepatocyte infusion followed by double therapy with corticosteroids and calcineurin inhibitors such as cyclosporine or tacrolimus (Fox 1998; Dhawan 2010; Dhawan 2015; Gramignoli 2015; Khan 2017).

Although previously not considered, it has been recently demonstrated that hepatocyte transplantation is associated with the formation of *de novo* DSA antibodies, therefore it would be wise to reconsider the immunosuppression protocols currently used in order to take this factor into account (Jorns 2016). Further studies are required to better understand the immune mechanisms involved in graft loss after HT and to improve the current immunosuppressive protocols.

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Engraftment Improvement

Following transplantation, there is an inflammatory response mediated by innate immune cells, which accounts for the early clearance (80–90%) of the transplanted cells (Viswanathan 2014). Cells not only need to overcome this response, but they have to find their way to integrate into the hepatic parenchyma, which they achieve by disrupting the endothelial barrier. Therefore, to get the most beneficial effect from the transplanted cells, several attempts have been made towards decreasing that initial inflammatory response, and aid in the integration of hepatocytes in the liver.

Several drugs aiming at the same outcome of improving cell engraftment and liver repopulation have been tested in preclinical animal models. In an experimental setting, pretreatment of the recipient with Thalidomide has demonstrated a significant reduction in the hepatic recruitment of PMN and KC, with the concomitant decrease of the inflammatory responses mediated by cytokine and chemokines (Viswanathan 2016) whereas the use of etanercept (ETN) successfully neutralized TNF-α expressed by neutrophils (PMN) or Kupffer cells (KC) (Viswanathan 2014).

Thalidomide also demonstrated positive effects on endothelial injury and increased engraftment of transplanted cells (Viswanathan 2016). Additional attempts such as vasodilation of the hepatic sinusoids with ET-1 receptor blockers (Bahde 2014) or induction of injury to Liver Sinuoidal Endothelial Cells (LSEC) with cyclophosphamide or doxorubicin improved engraftment (Malhi 2002; Kim 2005).

Once cells are in the hepatic parenchyma, they would need to repopulate the liver. Therefore, scientists have worked towards ways to create growth advantage for the transplanted cells to enhance the engraftment and/or proliferation of donor cells. Among those, preconditioning of the liver before transplantation with either hepatic irradiation, portal vein embolization, or hepatectomy have been proposed. Some of which have already been tested clinically and have yielded superior liver repopulation (Soltys 2016).

Conclusion

Hepatocyte transplantation for the treatment of liver diseases has dramatically increased over the years. Although considerable progress has been made, especially for inborn errors of metabolism, its full potential has not proven successful in the treatment of acute and chronic liver failure which has been hampered by lack of long-term hepatocyte functionality. Established and newly developed models of liver failure have been so far an excellent working tool to investigate the effects of hepatocyte transplantation and will continue to improve our understanding of this alternative therapy in the years to come.

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