P. Nagarajan
Ramachandra Gudde
Ramesh Srinivasan *Editors*

Essentials of Laboratory Animal Science: Principles and Practices

Essentials of Laboratory Animal Science: Principles and Practices

P. Nagarajan • Ramachandra Gudde • Ramesh Srinivasan
Editors

Essentials of Laboratory Animal Science: Principles and Practices

Editors
P. Nagarajan
Experimental Animal Facility
National Institute of Immunology
New Delhi, Delhi, India

Ramachandra Gudde
Central Animal Facility
Indian Institute of Science
Bangalore, Karnataka, India

Ramesh Srinivasan
Veterinary Pharmacology and Toxicology,
Madras Veterinary College
Tamil Nadu Veterinary and Animal
Sciences University
Chennai, Tamil Nadu, India

ISBN 978-981-16-0986-2 ISBN 978-981-16-0987-9 (eBook)
https://doi.org/10.1007/978-981-16-0987-9

© The Editor(s) (if applicable) and The Author(s), under exclusive licence to Springer Nature Singapore Pte Ltd. 2021
This work is subject to copyright. All rights are solely and exclusively licensed by the Publisher, whether the whole or part of the material is concerned, specifically the rights of translation, reprinting, reuse of illustrations, recitation, broadcasting, reproduction on microfilms or in any other physical way, and transmission or information storage and retrieval, electronic adaptation, computer software, or by similar or dissimilar methodology now known or hereafter developed.
The use of general descriptive names, registered names, trademarks, service marks, etc. in this publication does not imply, even in the absence of a specific statement, that such names are exempt from the relevant protective laws and regulations and therefore free for general use.
The publisher, the authors, and the editors are safe to assume that the advice and information in this book are believed to be true and accurate at the date of publication. Neither the publisher nor the authors or the editors give a warranty, expressed or implied, with respect to the material contained herein or for any errors or omissions that may have been made. The publisher remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

This Springer imprint is published by the registered company Springer Nature Singapore Pte Ltd.
The registered company address is: 152 Beach Road, #21-01/04 Gateway East, Singapore 189721, Singapore

Preface

Laboratory animals have a long history of use since human started the practice of medicine. The early days of laboratory animal use saw a high growth phase. During this period, science progressed a lot with many vaccines and drugs. However, ethics and welfare were not given much importance. The awareness on animal ethics and a need to control laboratory animal research was felt in the middle of the twentieth century. Since then, there was some decline in the use of animals. Activism and public interest in the field of animal usage ensured restrictions in animal usage and welfare measures.

While the importance of welfare was stressed, it was realized that animals play a crucial role in the development of science and continuous research. Countries across the globe started bringing in regulations regarding use of animals. Governments stepped in to regulate animal usage. Now a balancing act is required by the stakeholders, *viz.* the veterinarians, the scientists, management, and the regulators. On one side, many interesting developments in animal research including use of newer species such as zebra fish, gene knockouts, and humanized animal models are revolutionizing in the field of *in vivo* research. On the other hand, emphasis on 3Rs (Replacement, Reduction and Refinement) and the implementation of severity classification and humane endpoints have made the field of *in vivo* studies thought-provoking and meaningful.

The scenario in India is not vastly different from other countries as witnessed all around. India brought into force the CPCSEA guidelines in the early 1990s and has been amending it for the better as and when required. Research in India has got a huge boost with potential for contract research. Many laboratory animal facilities are prepared to get certified, to be ready for a harmonized accredited unit, and to attract international research houses towards India.

The above factors have made sure that animal research in India is growing by leaps and bounds, both in quantity and quality. It is a felt need that proper reference materials are not readily available in the area of animal research. There have been many institutes of repute that offer training in laboratory animal husbandry and research techniques. These institutes also come up with training manuals that provide information on specific areas of handling, anesthesia and specific protocols. However, a comprehensive textbook in the area of laboratory animal sciences that caters to scientists, managers, and veterinarians has been elusive till date.

Laboratory animal science has seen a vast change in the country, and there is a substantial increase in the activity related to laboratory animal research. Regularly conferences are conducted to bring the latest technology to the forefront. There are encouraging business tie-ups with international companies for the supply of quality animals and high-end equipment. In the last few years, many scientists have been trained by international accredited training resources. All these have led to a good accumulation of related knowledge. We realized that there were highly experienced professionals contributing a lot in laboratory animal sciences within the country. All that was required was to bring them to a common platform. We are glad that many practicing professionals have come forward voluntarily to contribute the chapters.

We, the editors, felt the need for a comprehensive book that encompasses all the important components. The idea of bringing out a book was the brainchild of Dr. P. Nagarajan (one of the editors and an author) who firmly believed that adequate knowledge, theoretical and working, was available within the country that has to be harnessed. The initiation for the book project started in late 2019. The idea snowballed and became serious in mid-2020. Though the global pandemic and the subsequent lockdowns put some brakes in the progress, we were able to accomplish the task in due time.

The book has been designed, keeping in mind, to cover all aspects of the required knowledge domain. It has been appropriately divided into sections, such as Principles, Techniques, and Ethics and regulations. We are confident that a student of laboratory animal science would be able to obtain maximum extract that a practicing scientist needs after reading the book. Keeping pace with the developments in the area, we have taken care to include special chapters such as GLP, in vitro fertilization, and humanized mouse that discuss the current state-of-the-art techniques to throw light on the newer areas of the science.

The task of editing a book can be quite challenging, starting with separation of chapters and sections, identification of authors, and working with deadlines and many acts of commissions and omissions. It is also interesting since we have gathered so much knowledge and have been able to establish rapport with leading scientists. Looking back, we feel greatly satisfied with the outcome. We fervently hope that the book will be eagerly taken up and will accomplish its purpose of spreading the knowledge of laboratory animal science, instilling the ethical values, and training the scientist and student in the newer techniques.

We wish to thank the publishers Ms. Springer India Nature Pvt. Ltd. for venturing to help us out in this task and doing all they can to ensure that the publication is done in time. We wish to thank all the authors, who were willing to share their knowledge, cooperated with us through the rigors of corrections and recorrections, with the unified motive of bringing out a useful book.

New Delhi, Delhi, India P. Nagarajan
Bangalore, Karnataka, India Ramachandra Gudde
Chennai, Tamil Nadu, India Ramesh Srinivasan

Acknowledgments

The editors and authors would like to acknowledge the following experts who have helped in the review of various chapters. The corrections and suggestions of the reviewers have helped us to improve the quality and presentation of chapters. Without their support, this book would not have become a reality.

- Prof. Klas Abelson, University of Copenhagen, Denmark
- Prof. Vera Baumans, Division of Laboratory Animal Science, Utrecht University, Netherlands
- Prof. Patri Vergara, Universitat Autonòma de Barcelona, Spain
- Dr. Christian Lawrence, Aquatic Resources Program, Boston Children's Hospital, USA
- Dr. Lakshmanan Annamalai, Director of Anatomic Pathology at Audentes Therapeutics, USA
- Dr. Rajesh K Anand, Technical advisor, Samitek Instruments, India
- Dr. Ramanamurthy B, National Centre for Cell Science, NCCS, India
- Dr. Aurelie Jory, National Centre for Biological Sciences, India
- Dr. Pralhad Wangikar, PRADO, Preclinical Research and Development Organization, (Place?)
- Dr. M.A. Akbarsha General Secretary, Society for Alternatives to Animal Experiments, India
- Dr. S.C. Parija, Orissa University of Agriculture & Technology, Bhubaneshwar

Contents

Part I General Principles and Regulations

1 Laboratory Animals in India: Past, Present, and Future 3
Nappan Veettil Giridharan

2 Laws, Regulations, Policies and Guidelines Governing the Care and Use of Laboratory Animals 23
Suresh Kumar, Manoj Kumar Rajput, and Sandeepa Bhan Tickoo

3 Ethics, Animal Welfare and Regulation: The Indian Perspective . . . 39
Vijay Pal Singh and Shikha Yadav

4 Planning and Designing of Laboratory Animal Facilities 53
Krushnarvind B. Patel, Vikas Galav, and S. G. Ramachandra

5 Quality Control of Laboratory Animals 85
Tarani Kanta Barman

6 Basic Biostatistics for Laboratory Animal Science 107
Suresh Kumar and Pardeep Kumar Yadav

7 Alternatives to Animal Experiments in Research and Regulatory Testing 137
Shikha Yadav and Vijay Pal Singh

Part II Biology and Husbandry of Laboratory Animals

8 The Anatomy and Physiology of Laboratory Mouse 159
Sarita Jena and Saurabh Chawla

9 The Anatomy and Physiology of Laboratory Rat 187
Saurabh Chawla and Sarita Jena

10 The Anatomy, Physiology, and Husbandry of Laboratory Rabbit . . . 211
Sarita Jena and Saurabh Chawla

11 The Laboratory Guinea Pig 239
Saurabh Chawla, Sarita Jena, and Sunita Nayak

12 The Laboratory Hamster 253
Padmakar Tambare and Yogeshkumar Murkunde

13 The Housing and Husbandry of Zebrafish (*Danio rerio*) in a Laboratory Environment 277
Kalidas Kohale

14 Nonhuman Primates in Biomedical Research 313
Surender Singh, B. G. Roy, S. G. Ramachandra, and P. Nagarajan

15 Mouse Genetics and Breeding 343
Satheesh Kumar Panneer, Shailendra Kumar Arindkar, and Perumal Nagarajan

16 Laboratory Animal Nutrition 373
Suresh Kumar, Manoj Kumar Rajput, and Pradeep Kumar Yadav

17 Bacterial and Parasitological Diseases in Laboratory Animals 405
Jerald Mahesh Kumar and Yadav Govind

18 Microbiology and Pathology of Viral Diseases in Laboratory Animals 447
M. J. Mahesh Kumar and Sachin Singh

19 Laboratory Animal Behaviour and Its Applications in Biomedical Research 479
V. S. Harikrishnan

Part III Laboratory Animal Techniques and Practices

20 Nonsurgical Techniques in Laboratory Animals 499
S. Ramesh and B. Vasanthi

21 Clinical Examination of Laboratory Rodents and Rabbits 521
Krishnamurthy Venkataraman and B. Sangeetha Raajkamal

22 Anaesthesia, Analgesia and Euthanasia of Laboratory Rodents and Rabbits: A Practical Approach 541
V. S. Harikrishnan

23 Biosafety Consideration in Laboratory Animal Facilities and Working on Infectious Agents 563
Aravindh Babu R. Parthiban, Azhahianambi Palavesam, Karthik Kumaragurubaran, and Dhinakar Raj Gopal

24 Occupational Health Hazards and Disaster Management in Laboratory Animal Facilities 585
Neeraj Khatri, Priyanka, Ashish Kumar Dubey, Ram Bharti, and Bhupinder Singh Chopra

25 Preclinical Imaging for Laboratory Rodents 607
Pradip Chaudhari

Part IV Specialty Areas of Laboratory Animal Science

26 Management of Specific Pathogen-Free (SPF) Mice and Rats 633
G. H. Mohan, R. V. Shwetha Reddy, and C. Yogesh

27 Good Laboratory Practice (GLP) Requirements for Preclinical Animal Studies 655
K. Srinivasan, K. Tikoo, and G. B. Jena

28 Assisted Reproductive Technologies (ARTs) in the Laboratory Mouse 679
Rahul Thorat

29 Generating Transgenic Animal Models: Recent Technological Advancements 709
Neerja Wadhwa, Nirmalya Ganguli, and Subeer S. Majumdar

30 Mouse Models of the Humanized Immune System 725
Kshama Jain, Prakriti Sinha, K. Varsha Mohan, and Pramod Kumar Upadhyay

31 Necropsy Procedures for Laboratory Animals 743
Yogeshkumar V. Murkunde

32 Surgical Models of Laboratory Animals 783
R. K. Shakthi Devan

About the Editors

Perumal Nagarajan is working as Head, Scientist at Laboratory Animal Facility, National Institute of Immunology, New Delhi, India. Being a veterinarian, he has been working on laboratory animals for more than 20 years. He has vast knowledge and expertise in care, management, breeding, and medicine of laboratory animals including genetically modified animals, immunodeficient animal model, humanized animal models, and non-human primates that are being used in research. Before joining NII, he worked at Central Drug Research Institute (CDRI) and National University of Singapore (NUS). He had undergone diverse trainings in laboratory animals including imaging modalities and microsurgery abroad. His research interest is on animal models for various diseases and in particular metabolic diseases. He has published more than 35 research articles in peer-reviewed international journals and authored book chapters. He is also serving as a reviewer for various international journals, and he is a member of several national and international scientific societies related to laboratory animal science.

S. G. Ramachandra graduated from Bangalore Veterinary College in 1987 and subsequently completed post-graduation and PhD in Veterinary Pharmacology and Toxicology from Bangalore Veterinary College. He has been working in the field of Laboratory Animal Science for the last 29 years and is currently working as Chief Research Scientist and In-charge of the Central Animal Facility at the Indian Institute of Science, Bangalore. He has undergone several advanced trainings on laboratory animal medicine and surgery in the USA, Germany, Taiwan, and Japan. He is associated with various regulatory authorities such as CPCSEA (Ministry of Animal Husbandry and Dairying, Govt. of India) and NGCMA (DST, Govt. of India) as laboratory animal expert. He has organized several national and international conferences/workshops on laboratory animal science. He has established the Laboratory Animal Scientists Association (LASA) and served as Founder President for 9 years (2004–2013). He has also served as Vice President of Asian Federation of Laboratory Animal Science Associations (AFLAS), Japan. Established “Journal of Laboratory Animal Science” and serving as Editor-in-Chief. He has been nominated as “Fellow” by the National Academy of Veterinary Science, New Delhi, and bestowed with “Nakula” award by the Karnataka Veterinary Association for his significant contribution in the area of Laboratory Animal Science. He has provided

technical guidance in establishment of more than 15 laboratory animal facilities in the country. He is also actively involved in research and developed rapid diagnostics kits for the detection of six different pathogens of laboratory animals. He has published 90 research papers in peer-reviewed national and international journals. He has delivered 90 "invited" lectures at national and international conferences including Germany, Thailand, Korea, China, Sri Lanka, South Africa, Taiwan, and the UK.

Ramesh S is currently serving as Professor and Head, Department of Veterinary Pharmacology and Toxicology, Madras Veterinary College, Tamil Nadu Veterinary and Animal Sciences University at Chennai, India. He has a teaching experience of 26 years. He teaches the subjects of pharmacology and toxicology to veterinary graduate, post-graduate, and doctoral students. He has so far guided 10 dissertation works. His research interest areas include pharmacokinetics of antibacterial drugs, veterinary drug residues, and antimicrobial chemotherapy. He has published about 30 research papers in peer-reviewed journals. He has authored one chapter in a book and brought out practical manuals for students and trainees. He has developed e-learning contents in veterinary pharmacology. He has acquired knowledge and expertise in laboratory animal sciences such as biology, housing, handling, ethics, design, pharmacology, and toxicology. Since 2013, he has organized the Certificate Course in Laboratory Animal Sciences at Chennai, India, the only course outside Europe to be accredited by FELASA. He has also developed training modules in laboratory animals catering to research scholars and laboratory animal caretakers. He serves in the editorial of Journal of LASA India and is a member of many scientific societies.

Part I

General Principles and Regulations

1 Laboratory Animals in India: Past, Present, and Future

Nappan Veettil Giridharan

Abstract

The science on laboratory animals encompassing fields of biosciences, medicine, and veterinary disciplines is just over 100 years old. It's the science of captive domesticated animals for research—its breeding, maintenance, experimentation, and welfare. The science flourished with the advent of laboratory rodents, especially mice and rats over the years, and these along with other smaller and large animals contributed to the growth of biomedical sciences unraveling biology – basics as well as applied aspects. This introductory chapter sums up its small beginning in the USA and Europe and its spread across Asia, including India. India has over 1750 registered establishment of private and public animal facilities currently catering to biomedical research in the country. Starting from its modest beginning, catering mainly to the pharma industry in Mumbai and Ahmedabad, to the establishment of state-of-the-art animal facilities at the university level, India indeed has traversed a long path. The history, its present status in India, and what needs to be done to get this field on par with those in developed countries are discussed here.

Keywords

Laboratory animals · Mice · Rats · NIN · CDRI · CRI · DBT · IISER · CDRI · National Centers · CPCSEA · Animal transport · Manpower · Alternatives to animals

N. V. Giridharan (✉)
National Institute of Nutrition, Jama-I-Osmania, Hyderabad, India

© The Author(s), under exclusive license to Springer Nature Singapore Pte Ltd. 2021

P. Nagarajan et al. (eds.), *Essentials of Laboratory Animal Science: Principles and Practices*, https://doi.org/10.1007/978-981-16-0987-9_1

1.1 Introduction

'A laboratory animal is a man-made biological tool, nurtured in a controlled environment, maintained on a standard diet, free from pathogens, and of defined genetic background.'

Man always depended on animals for his survival, be it for food, for competition, or for companionship. His quest to know about the surroundings he lived in made him experiment on animals for acquiring knowledge about himself and the world around him. The great Greek physician Gallo (129–200 AD) used deer to demonstrate that the arteries carried blood and not air, as it was believed then. We have come a long way since then and have added an array of specially bred animals under controlled conditions, now clubbed under a broad name, "laboratory animals," the backbone of any biomedical research. These essentially include rodents, lagomorphs, carnivores, primates, farm animals, and even a variety of reptiles and birds and lower forms, which were brought from the wild, bred, and maintained under defined environmental conditions to adapt to our specific experimental needs.

Many people have intense feelings about the use of animals in biomedical research. Heated and emotional debates are common, but if we ponder, can we do away with animal experiments? Tables 1.1 and 1.2 effectively sum up the giant

Table 1.1 Major advances in basic research that depended on animal experiments

1600s	Discovery of blood circulation
	Discovery of the function of the lungs
1700s	Measurement of blood pressure
1800s	Vaccination to stimulate immunity
	Understanding of infectious diseases
1900s	Discovery of antibodies
	Understanding of hormone systems
1920s	Discovery of vitamins
1930s	Discovery of the mechanism of nerve impulses
	Discovery of tumor viruses
1940s	Understanding of embryonic development
1950s	Understanding of the control of muscle activity
	Understanding of energy metabolism
	Understanding the mechanism of hearing
1960s	Discovery of monoclonal antibodies
	Understanding the biochemical functions of the liver
1970s	Understanding of transplantation antigens
	Understanding the way, the brain functions
	Discovery of prostaglandins
1980s	Development of transgenic animals
	Understanding the basis of memory
1990s	Understanding autoimmune disorders
	In vitro fertilization, cloning, gene manipulation
2000s	Regeneration, stem cell

Table 1.2 Major medical advances that depended on animal research

1920s	Insulin for diabetes
1930s	Modern anesthetics for surgery, diphtheria vaccine
1940s	Broad-spectrum antibiotics for infections, whooping cough vaccine
	Heart-lung machine for open-heart surgery
1950s	Kidney transplants
	Cardiac pacemakers and replacement heart valves, polio vaccine
	Drugs for high blood pressure
	Hip replacement surgery
1960s	Corneal transplants
	Rubella vaccine
	Coronary bypass operations
	Heart transplants
	Drugs to treat mental illness
1970s	Drugs to treat ulcers
	Improved sutures and other surgical techniques
	Drugs to treat asthma
	Drugs to treat leukemia
1980s	Immunosuppressant drugs for organ transplants
	CAT scanning for improved diagnosis
	Life-support systems for premature babies
	Drugs to treat viral disease
1990s	Laparoscopic surgical techniques
	Breast cancer links
	Gene therapy for cystic fibrosis
2000s	Stem cell therapy

strides we have made in the field of biology and medicine, which would not have been possible without research on animals [1]. As one can see, the results from animal research have a direct bearing on the well-being of people as well as on animals themselves. Broadly, laboratory animals are used in basic biology and medical research, the development of diagnostic tests and new treatment for diseases, preparation of animal products, and testing of chemicals and drugs for biosafety, for understanding genetic disorders, and for biology and medical education. Laboratory animal science encompasses fields of bioscience, medicine, veterinary, and other related disciplines. Its basic aim is to know about the characteristics of animals used in research to understand the appropriate way to handle them for experimental procedures. It is an advanced area of specialization and needs a basic understanding of biology and medicine coupled with skills of management of handling and experimentation.

1.2 Development of Science Across the World

Rodents, especially mice and rats form the bulk of laboratory animals, and almost 90% of basic applied research revolves around them. These along with guinea pigs and rabbits were quite popular as pet animals and for their meat and fur (especially rabbits) as early as the seventeenth century. Under captive breeding, many of these animals lost their native color (gray, brown, or black) and became albino (white) and became less ferocious and easy to handle. This enhanced their value as pets and also paved the way to be used in biomedical research. William Harvey (1678) was the first scientist to use albino mice to study reproduction and blood circulation. Priestley and Lavoisier popularized them in the eighteenth century by using them for anatomy and physiology studies, and even Mendel used them for genetic studies in the early nineteenth century before switching on to peas, because of the objection from the Church [2]. But the whole credit for establishing mouse as a defined laboratory animal goes to Little in the late nineteenth century who was studying the coat color of these animals using albino mice from Switzerland (hence Swiss mouse) [3]. This led to the C57 inbred stock of mice, which along with its derivatives form 85% of mice used in research. Jackson Laboratory, USA, the first commercial company that started in 1907, is credited with the establishment of over 300–400 inbred strains of mice used in various research laboratories across the world [4].

A parallel development was also happening in the usage of rats. Wild brown rats rampant in Europe and a threat to human life as pests and carriers of diseases (esp. plague) necessitated an industry of rat catchers. Many used it as a livelihood, and the adventurous ones among them went ahead and bred them in captivity for using them for their meat and more commonly for rat-baiting, a gambling sport quite popular then. Specific mud pits were built and wild rats were released into them. Terrier dogs were let loose and bets were made on the time taken by the animals to finish the rats in the pit [5]. Over time, breeding of these rats for such purpose produced rats with less ferocity and colors varying from hooded (frontal part all black and the rest white) to white (red albino). As early as 1828, such rats were used for a fasting experiment. Over the next 30 years, rats were used for several experiments especially nutrition and reproduction [6]. Eventually, a "laboratory rat" became the first to be domesticated for purely scientific work, with the establishment of the Wistar Institute in Philadelphia, USA, in 1906[5]. The outbred Wistar rat strain was established by Donaldson, Greenman, and King and from this came all the major rat strains used in biomedical research—Sprague-Dawley, Holtzman, Fischer 344 (all albinos), and Long-Evans (hooded). Copenhagen rats (black) called the gutter rats were rampant in the gutters of Denmark, were developed independently, and are used sparingly for specific infectious studies. Japanese scientists led by Dr. Kuramoto, who analyzed 118 rat inbred strains from all over the world, established that the hooded rats from Japan that were accidentally brought to Europe and the USA were the ancestors of all the inbred rats used in research today [7]. Keeping rats as domesticated pets was quite popular in Japan in the eighteenth century, especially the hooded rats, which were often referred to as Japanese rats.

Guinea pigs, rabbits, hamsters, primates, farm animals, fishes, and reptiles and a variety of lower organisms were added to the ever-evolving laboratory animal list. With the advancement in genetics, especially in molecular biology, mutants (natural, induced), transgenics, and knockout animals specific for a defined work have emerged, and the current biomedical scientists have a bounty of arsenals to pick up to do research of his or her choice, a far cry from earlier days [8,9]. The USA and Europe formed the cradle of laboratory animal sciences and its later development in developing strains for specific work in biomedical research. Developing countries like India that gained independence in 1947 took time to keep pace with these developments, but there were flashes of use of lab animals here while the British was in power. Let us examine them and see how this branch of science grew subsequently to present status in India.

1.3 Development in India

The earliest users of laboratory animals in India were from pharma and vaccine industries, some biomedical research institutes, medical and veterinary colleges, and university departments. Laboratory mice, rats, guinea pigs, rabbits, and primates were available here as early as 1910. Haffkine Institute, Parel, Mumbai, started in 1828 by Dr. Haffkine, can be called the first user of lab animals, who were into the production of plague and rabies vaccine and anti-snake venoms. Swiss mice, guinea pigs, and New Zealand rabbits were being maintained as early as the 1950s. Mumbai was the hub of the pharmaceutical industry, and CIBA-Geigy and Hoechst research centers (both closed now) were pioneers to establish modern animal facilities, matching the standards of those days, and the latter was even maintaining Beagle dogs, pretty early in India. In the government sector, the Central Research Institute, Kasauli (1905); Regional Research Laboratory (now called IICT), Hyderabad; Central Food and Technological Research Institute (CFTRI), Mysore; Indian Veterinary Institute (IVRI), Bareilly; Central Drug Research Institute (CDRI), Lucknow; Chittaranjan Cancer Research Institute; Bose Institute and Institute of Chemical Biology, Calcutta; VP Chest Institute in Delhi; Indian Institute of Science (IISc), Bangalore; St. John’s Medical College, Bangalore; Drug Research Centre, Baroda; Hindustan Antibiotics Ltd, Pimpri, Pune; All India Institute of Medical Sciences, New Delhi; JIPMER in Pondicherry; Cancer Research Institute (CRI) in Parel, Mumbai (now ACTREC); Pasteur Institute of India, Coonoor; National Institute for Research in Tuberculosis; Adyar Cancer Institute, Chennai; National Institute of Nutrition (NIN), Hyderabad; and National Institute of Virology, Pune, were some of the early users of laboratory animals. Among the universities, Madras University; Annamalai University, Chennai; University of Mysore; Banaras Hindu University, Varanasi; BITS, Pilani; University of Delhi; University of Punjab; Osmania University, Telangana; Andhra University, Vishakhapatnam; University of Rajasthan; and University of Kerala were some of the earliest users of laboratory animals.

Of these institutes mentioned above, the role of CRI, Mumbai; NIN, Hyderabad; CDRI, Lucknow needs to be mentioned specifically for bringing laboratory animal science to the center stage in the country. In 1955–1956, an all Indian survey on the condition of laboratory animals and the animal house facilities in India was conducted under the sponsorship of UNESCO, and the person responsible for getting the UNESCO support was the then director of CRI, Prof V R Khanolkar, a well-known pathologist. The animal house in charge of CRI visited more than 130 animal facilities of various biomedical research and teaching institutes and pharmaceutical concerns and prepared an exhaustive report on the prevailing conditions of laboratory animals in the country. The report showed the appalling conditions in which animals were maintained in the various animal facilities of the country, and UNESCO felt the need for a separate body to look into these matters, and thus Laboratory Animal Science Information Service (LAIS) was born in 1957, under its patronage [1]. During the next year, LAIS collected information on the species and strains of lab animals used in the country and published them as bulletins for the use of research scientists in the country. The unit was taken over by the Indian Council of Medical Research (ICMR) in 1959 and maintained as a separate unit in CRI. It was mainly a service unit publishing biannual bulletins every year giving information on the laboratory animals bred and maintained and the purpose for which they are used, but LAIS felt the need for training personnel working in the animal facilities to enable them to handle animals properly for maintenance as well as for experiments. So, a course was started in 1968 using the animal facilities of CRI and Haffkine Institute, with a duration of 3 months. After that every year the course was conducted, and 10–16 candidates from different animal facilities were trained till 1975. Next year, the unit was shifted to NIN, Hyderabad, and this course was reviewed and modified to a training program of 6-week duration. Apart from India, the training course also attracted participants from neighboring countries like Sri Lanka, Malaysia, Nepal, Bangladesh, Indonesia, and Thailand, all sponsored by WHO.

In Mumbai, LAIS had no access to animal facilities (it was using the facilities of CRI) of its own, so for expanding its activities, the unit was shifted to NIN, Hyderabad, where the staff of LAIS took over the breeding of small laboratory animals, housed in the facility and became part of it. But it maintained its independent character and was named as Laboratory Animal Information Service Centre (LAISC) with a separate budget of its own. The center grew in stature with the acquisition of NIN animal facilities and added a new dimension to the training program by chalking out a senior supervisory training course in 1978, meant for graduates and postgraduates of biology, veterinary, and medicine working in the animal facilities with a duration of 3 months. The center also started supplying lab animals within the state and also outside on a small scale. It started publishing LAIS center News twice a year in April and November, giving information on various activities of the center and carrying articles pertaining to care breeding and experimentation of animals, and this was sent to 400 institutes on gratis.

As the country moved toward the era of modern biology in terms of molecular biology and biotechnology, the demand for good quality animals of defined genetic

and health status became a necessity, and the Department of Biotechnology (DBT) stepped in and a new facility named "National Infrastructure Facilities for Laboratory Animals" was built in NIN premises attached to the existing LAIS building. The new facility with a built-in area of 24,000 square feet had separate administration, washing area, breeding rooms, and a hostel cum conference hall for conducting training programs. Breeding nuclei of BALB/c, C57 and nude mice, SD rats, and NZ rabbits were brought from the USA and UK and maintained here, taking care of all environmental conditions required for an animal facility as per international standards. Separate isolator rooms were built, and breeding nuclei of mice and rats were maintained in microbiologically defined, specific pathogen-free status in isolators. The NIFLA was later merged with LAISC, and it was renamed as the National Centre of Lab Animal Sciences (NCLAS) and became a full-fledged center of ICMR with a separate budget of its own. The center started supplying good quality lab animals—mice, rats, hamsters, guinea pigs, and rabbits—to over 180 institutes in the country [1].

NIN animal facilities were preparing its lab animal diets for feeding mice, rats, rabbits, guinea pigs, and monkeys under its care based on international nutritional lab animal dietary guidelines using locally available dietary ingredients. Once NCLAS became a reality, pelleting machines were purchased, and the diets were pelleted for the first time with defined pellet size for rodents, rabbits, and monkeys. The center started supplying animal diets in large quantities with the acquisition of additional machinery, and diet supply became a major activity of the center. With its thorough grounding in nutrition, it also started supplying custom-made nutrient deficiency and energy-dense experimental diets as per the specific needs of the scientist. With support from ICMR and DBT, the center also set up microbiology, clinical biochemistry, histopathology, and genetics laboratories. With the establishment of these laboratories, the lab animals were regularly monitored, for their microbial status and genetic purity. This ensured that good quality animals could always be made available for research workers within the institute and outside.

To improve the care and maintenance of rhesus monkeys in the center, a new primate facility that can accommodate 40 monkeys with adequate playing and exercise area was built in 2004 with the help of ICMR, and several experiments involving testing of drugs and vaccines were conducted here. The center also got into the limelight with the identification, characterization, and establishment of two mutant obese strains of rats—WNIN/Ob and WNIN/GR-Ob, the former with obesity and euglycemia and the latter with impaired glucose tolerance. These were the first inbred mutant obese rats' strains from Wistar stock in the world with the highest weight (1.4kg) recorded so far (Figure 1). It is a unique rat model quite different from the established models developed abroad, as it has all the symptoms of metabolic syndrome in one animal. Its gene defect is not due to leptin or leptin receptor genes, but at a locus close to the latter, on chromosome 5[10]. It is assumed that its defect could be due to an error in the leptin signaling mechanism, and work is in progress to pinpoint the gene in question. It can boast of a lineage starting from 1920, when Wistar rats were first introduced in India, at Pasteur Institute, Coonoor, where the seed for the current NIN was planted in the form of an inquiry unit studying

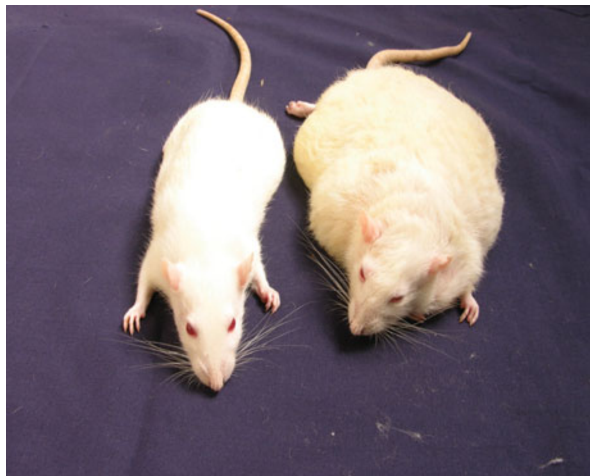


Fig. 1 WNIN/Ob rat. Adult lean (left) and obese (right) of the same age

deficiency diseases in the country. The unit moved to Hyderabad in 1958, and in 1969, it was named as National Institute of Nutrition (NIN) under the aegis of ICMR, New Delhi. The Wistar strain had come from the Wistar Institute, Philadelphia, and was inbred all these years and was christened as WNIN in 1986 [11], and it is from this, the mutant strain emerged in 1996. Active research is pursued using this animal model, and over 100 research papers are published in reputed journals of India and abroad [11].

The latest development of this national center is its elevation to a full-fledged institute on its own merit in 2018 when NIN celebrated its centenary year. It is now called as the National Animal Resource Facility for Biomedical Research (NARFBR). This new venture is under construction in Genome Valley, Hyderabad, and has grandiose plans for microbial containment facilities (BSL-3) for drug and vaccine testing and transgenic and knockout animal production and maintenance and has large animal facilities for canines and primates and also for low vertebrates like zebrafish. It will also strive to develop, test, and validate alternatives for the replacement of animals for drugs, vaccines, and biotech products.

The CDRI, Lucknow, which had a conventional house earlier also became a national center for laboratory animals with emphasis on primates and alternatives to animal experimentation, under the tutelage of DBT and CSIR. The center runs its training programs for animal house personnel and also prepares its own laboratory animal diets. CRI, Parel, Mumbai, moved to Navi Mumbai in 2002 to become the basic research wing of the Advanced Center for Treatment, Research and Education in Cancer (ACTREC) and built a new animal facility to suit the new agenda of the institute for developing animal models for cancer studies. While it stayed put in Parel, it was breeding and maintaining several inbred strains of mice exclusively for cancer studies and had the distinction of developing the first inbred mouse strain in the country, the ICRC strain which could develop both skin and blood cancers. The first transgenic mouse was also made here which hosts a gene for a growth modulator

and is developed as a model for squamous carcinoma. The new animal facilities have several mouse strains and have regular animal house training at undergraduate and graduate levels.

Apart from these pioneer institutes which catered to the growth of laboratory animal science in the country, there were parallel development in the 1970s to 1990s, and some important research institutes came up with their specific research agenda which could boast of better animal facilities than that built earlier, and they could also upgrade them with generous funds from government research funds as years went by. Many of them have in-house training program for their staff, and some of the seniors in these facilities could avail of higher training in the subject in the USA, Europe, and Japan under the patronage of their respective institutes. Some important institutes in this category are the Tata Institute of Fundamental Research (TIFR), Mumbai; Center for Cellular and Molecular Biology (CCMB), Hyderabad; University of Hyderabad; Nizam's Institute of Medical Sciences, Hyderabad; Indian Immunologicals Ltd, Hyderabad; National Institute of Mental Health and Neurosciences (NIMHANS), Bangalore: National Centre for Cell Science (NCCS), Pune; National Institute of Immunology (NII), New Delhi; Post Graduate Institute of Medical Education & Research, PGIMER, Chandigarh; Sree Chitra Institute of Medical Sciences and Technology (SCTIMST), Thiruvananthapuram; Rajeev Gandhi Institute of Biotechnology, Thiruvananthapuram; National Institute for Research in Reproductive Health Mumbai; National JALMA Institute for Leprosy, Agra; International Centre for Genetic Engineering and Biotechnology (ICGEB), New Delhi; National Institute of Pharmaceutical Education and Research (NIPER), Mohali; Disease-Free Animal House; Haryana Agricultural University, Hisar, National Dairy Research Institute, Karnal, Haryana; National Institute of Occupational Health (NIOH), Ahmedabad; National Brain Research Centre, Manesar, Haryana; Indian Institute of Toxicology Research, Lucknow; National Institute of Cholera and Enteric Diseases, Kolkata; National Institute of Biologicals, Delhi; Institute of Microbial Technology, Chandigarh; Serum Institute, Pune, National Chemical Laboratory, Pune; Cochin University of Science and Technology (CUSAT), Kochi; and Jawaharlal Nehru Centre for Advanced Scientific Research (JNCASR), Bangalore.

TIFR, which started in 1945, has five centers all over India, and among which the National Center for Biological Sciences (NCBS), Bangalore, built in 1992 is worth mentioning as it has got the state-of-the-art facility for transgenics and knockouts and embryo preservation units under Animal Bio-Resource Center (2002). This, along with the Institute for stem cell Science and Regenerative Medicine and Center for Cellular and Molecular Platforms (Bangalore, 2009), is referred as the Bangalore Life Science Cluster or Bangalore Bio-Cluster. CCMB which started in 1976 was the first Institute of Molecular Biology in the country and has a modern facility with several rodent strains, which are genetically and microbiologically monitored periodically to keep them pure and healthy. Additionally, it has an embryo freezing unit and a transgenic lab. The CCMB has a fly house exclusively dedicated to *Drosophila* research. The Centre for DNA Fingerprinting and Diagnostics (CDFD) which started in 1996 was an offshoot of CCMB, which initially operated in a rented building, and

had no access to its own animal facilities. But in 2018, it moved over to a permanent research facility in Uppal, Hyderabad, with a state-of-the-art animal facility. NCCS and NII maintain various mouse strains and are specialized in cell lines to carry out cancer and other biomedical research areas. NII also initiated a program of wild rat breeding to develop some Indian inbred strains from them. SIMMT, Thiruvananthapuram, is involved in developing medical devices like heart valves and others and has both small and large animal facilities (for sheep as well as pigs). RCBG in Thiruvananthapuram is the first molecular biology research lab in Kerala (2001) which started with the help of Japan and works on viral diseases and has a fairly modern animal facility to cater to their needs. NIRRH, Mumbai, started in 1996 and later upgraded its animal facilities to modernity. It has rodent strains and keeps bonnet monkeys in open scrolls and is the only facility in the country having captive breeding of marmosets. A separate primate facility under its management for rhesus, bonnet, and langur monkeys are being built in the outskirts of Mumbai and once completed will be the biggest primate facility in the country. JALMA Institute of Leprosy under the aegis of ICMR was established in 1996 dedicated to mycobacterial infections like leprosy and even imported armadillos to maintain leprae culture. The armadillos did not survive the harsh weather conditions of India, so now the cultures are maintained on mouse footpads. They have a biocontainment facility to maintain their animals. ICGEB is an institute started by funding from the United Nations in 1983 and became an autonomous research center in 1994 and deals with system biology and has facilities for transgenic work. IICB in Kolkata was started in 1935, and was named Indian Institute of Experimental Medicine in 1956 under CSIR, and later in 1989 got the present name. Indian Institute of Toxicology Research and Industrial Toxicology Research Center under CSIR, are specialized in testing drugs and chemicals and have upgraded their facilities to meet the current standards. The Sikkim Manipal Institute of Medical Sciences, Manipal, and Amrita Institute of Medical Sciences (AIMS), Kochi, have modern animal facilities. The former concentrates on stem cell research, while the latter works on developing nanomedicines and their delivery systems. AIMS has a well-established nude mice colony and has a special robotic animal surgery facility for small and large animals. Additionally, it has a small animal MRI unit, which can perform an MRI scan on small animals.

The pharma industry of India, the bulk users of lab animals, played a major role in establishing laboratory animal science in the country and right from the 1960s established several drugs, vaccines, and biological manufacturing and testing research laboratories, which invariably needed state-of-the-art animal facilities to carry out their agenda. The Indian government took the lead first in establishing drug and pharmaceutical public limited companies (IDPL) in Hyderabad, Gurgaon, and Rishikesh in 1961. Out of the three, the IDPL in Hyderabad became a sick unit, and efforts are now being made to revive the company. The government started pharma institutes like NIPER, at Hyderabad (in the IDPL premises) and Mohali, and both have modern animal facilities, which are meant for pharmacy students to carry out drug research. Vikram Sarabhai Chemicals Ltd. in Vadodara funded by the Atomic Energy Commission was established in 1987 and was concentrating on

nutraceuticals. They were using conventional animal facilities for a long time but moved on to modern biology by establishing the Dr. Vikram Sarabhai Institute of Cell and Molecular Biology at the Vadodara University campus in 2012, catering to transgenic and knockouts.

There are several private industries at the forefront of production and testing of modern drugs and vaccines and biologicals in India, and most of these are concentrated in Ahmedabad, Vadodara, Mumbai, Hyderabad, Chennai, and Bangalore. Some of the earlier players in this list are Cipla Limited, Wockhardt, Unichem, Bharat serums and Vaccines Limited, and Lupin Limited, all in Mumbai, Alembic in Chennai, and Biological E Limited in Hyderabad. They were having conventional animal facilities earlier, but most of them have updated their facilities subsequently to meet international standards. Pharma companies built later (after the 1980s) came up with better animal facilities, and some of them went on to improve them further, to meet international standards required for their recognition at that level. Some of the well-known companies are Aurobindo Pharma, Reddy Research Laboratories, Bharat Biotech, and Hetero Drugs Ltd, all at Hyderabad; Ranbaxy which later became Sun–Pharma, Torrent Pharma, and Zydus Cadila, all at Ahmedabad; Lab Animal Research Sciences, under Reliance Life Sciences, Glenmark Pharma, Piramal Group, and PAN-Biotech, all at Mumbai; Syngene International Ltd, Bangalore; and Panacea Biotec, New Delhi. Companies like Torrent Pharma, LARS, Reliance Life Sciences are fully engaged in commercial preclinical testing for drugs, vaccines, and biologicals. Biocon Pvt Ltd, Bangalore, is the seventh leading biopharma company in the world, and they are into the manufacturing and testing of generic pharma ingredients, novel biologicals, biosimilar insulin, and antibodies. In 1904, it established Syngene International Ltd, as a custom research company and expanded it further in 2009 in collaboration with Bristol-Myers Squibb Pharmaceuticals Limited, USA. In 2012, it joined hands with Abbott for the development of nutraceuticals.

The country has about 40 GLP accredited animal facilities, which include several of the research facilities described above both at private and public levels. They are essentially commercial preclinical testing and evaluation labs. Some important such facilities which do only preclinical testing are the International Institute of Biotechnology and Toxicology (IIBAT) (specialized in genotoxicity); Orchid Chemicals and Pharmaceuticals, Chennai; Aurigene Discovery Technologies Limited; RCC Laboratories India Private Limited; Vimta Labs Ltd; Vivo Bio Tech, Hyderabad; Laila Nutraceuticals, Vijayawada; Jai Research Foundation, Gujarat; Eurofins; Himalaya Drug Company; Natural Remedies, Bangalore; Immex Ltd; Indofil Industries, Mumbai; Syngenta Biosciences, Goa; Shriram Institute of Industrial Research, New Delhi; etc.

The dawn of the 2000s witnessed bold initiations from the government to raise biomedical research in the country to new levels on par with developed countries. One is at the basic life sciences education level and the other for providing high biosafety-level facilities to address infectious diseases through microorganisms especially viruses. The establishment of the Institute of Science Education and Research (IISER) is a new attempt to improve the standard of collegiate education

in basic sciences with cutting-edge research tools at undergraduate levels. IISERs are established across the country in Kolkata, Pune, Mohali, Bhopal, Thiruvananthapuram, Tirupati, and Berhampur through funding from the Ministry of Human Resource Development. The IISERs built in Pune, Bhopal, and Kolkata, which are in view, now have animal facilities that meet international standards. The center at Pune is earmarked for generating in-house transgenic and knockout animals and acts as a repository for such animals. It has currently over 15,000 research animals of all varieties, mouse to zebra fish to *Drosophila* all maintained under biosafety containment. The institute is into in-house breeding to meet its requirement, but they also supply animals outside. They also offer services to transgenic creation, embryo/sperm cryopreservation, and genotyping and also offer surgical experimental animal models. With infectious diseases on the rise in recent times, the government is in the mood of creating BSL-3 ABSL-3 facilities in the country. Last year, such a facility was inaugurated at IISC Bangalore campus at the newly renovated Center for Infectious Diseases and Research (CIDR). The facility is accessible to the research community working in high pathogenic infectious diseases such as TB, HIV, Japanese encephalitis, and other BSL-3 pathogens and can experimentally infect animals via the aerosol route to develop novel diagnostics, drugs, and vaccines against such diseases. A similar facility is already available in IVRI, Izatnagar, against animal infection, and on the same line, another setup is coming in Tamil Nadu Veterinary and Animal Sciences University (TANUVAS), Chennai. NCCS, Pune campus, will soon have a BSL-3/ABSL-3 facility, which will become part of the Pune Bio-Cluster. As mentioned earlier, the NABFR coming up in Genome Valley, Hyderabad, will also be set up on the same lines.

1.4 Fall Out on the Use of Laboratory Animals

The history of laboratory animals will not be complete without the mention of parallel developments that happened with the rise in the use of animals worldwide. These include opposition to animal use, yet, more opportunities for employment in the manufacturing sector, as well as human resource development, formations of associations and organizations for discussing new development in the area, and awareness to enforce the ethical treatment of animals.

1.4.1 Opposition to Animal Experiments

Opposition to use animals for research happened all the time, despite giant strides made in biomedical sciences using laboratory animals. The earliest movement was recorded in 1826 with the formation of the Royal Society for the Prevention of Cruelty against Animals (RSPCA) in the UK. This subsequently became SPCA (Society for the Prevention of Cruelty against Animals) with branches all over Europe and the USA. New players joined the movement later, like People for the Ethical Treatment of Animals (PETA) and Humane Society International (HSI),

based in the USA. Initially, their protests were peaceful like writing articles in media and giving talk shows. But toward 2000, the movement became violent wherein animals were forcibly released while under experiment, and in California and Florida, the research facilities were even torched. The ripples started in the West found its way into India too, where animal activists resorted to forcible closure of some of animal facilities in the country and even released monkeys and dogs which were on drug and vaccine testing [1].

Such a rigid attitude by animal activists confused the public, who were caught between activists and scientists, the latter claiming it for the alleviation of humans and even animals from diseases. Looking at the alarming scenes that were developing globally, international bodies like the World Health Organization (WHO), Universities Federation of Animal Welfare (UFAW), Animal Welfare Institute (AWI), Council for International Organizations of Medical Sciences (CIOMS), and International Council of Laboratory Animal Science ((ICLAS) stepped into the scene and had a hard look at the prevailing conditions of laboratory animal upkeep and maintenance under normal and experimental conditions. They came out with guidelines for use of animals in biomedical research, which included general principles of space, and special provision and care for laboratory animals, under breeding, maintenance, and experimentation. Textbooks and pamphlets were published for public and animal users emphasizing the need for care and empathy for animals under captivity.

From the government side, strict legislations were enacted in Europe and the USA, and other developing countries, making violation of animal rights punishable even to the extent of the closure of animal facilities. The animal rights included a wide spectrum of guidelines including housing, record keeping, specific humane procedures for handling and oral feeding, injections, withdrawal of body fluids, pain perception, surgery, anesthesia and euthanasia, and insistence for trained personnel to look after and manipulate animals for experiments. Though there are individual differences in the specific interpretation of laws between various countries, certain features remained common among them. A centralized body at the national level and an ethical committee at the local research institute are mandatory to all these laws.

Though India had a head start in the area of laboratory science as early as the 1960s along with other countries, our progress has been tardy due to lack of funds and government apathy. Among the scientific organizations, only the Indian Council of Medical Research (ICMR) recognized the importance of laboratory animals and created a unit called LAIS as mentioned earlier. The survey conducted by this unit showed lacunae in several aspects of animal upkeep and maintenance, in terms of housing, record keeping, and lack of trained personnel in the field. But conditions remained the same for a long time, till the animal rights movement which started in the West also made its impact here in the early part of this decade. Several government institutions faced the wrath of these animal activists, and some of them were even closed for poor housing and husbandry conditions. This forced the government to have a relook at the existing laws of the country, which was enacted in the 1960s (and modified in 1982) called the Prevention of Cruelty of Animals Act. Under Chapter 4 of the same act, though there was a provision for

control of experimental animals, the laws lacked teeth and the offenders got away paying paltry sums. To combat the strong protests from animal activists, and to improve the general conditions of the animal facilities, a Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) which covered breeding too was set up in 1991 with a member secretary at the helm along with other experts in the field. It was made mandatory that all animal facilities in the country be registered, and a local institutional committee is set up to scrutinize and approve the research projects using animals. The CPCSEA was reconstituted thrice, 1996, 2012, and again in 2019. The committee works under the chairmanship of an additional secretory under the Ministry of Environment and Forests with 17 experts drawn from diversified biological fields, including animal welfare members and persons of prominence having public interest. With such strict enforcement of legislation, animal facilities across the country received more funds from government and private organizations, resulting in an overall improvement of animal facilities in the past one decade, unlike the earlier times. We can now boast of state-of-the-art animal facilities in the country in private and public sectors, as mentioned earlier.

1.4.2 Formation of Agencies and Associations

The International Committee on Laboratory Animals (ICLA) was conceived as early as 1956, by the initiation of UNESCO, CIOMS, and the International Union of Biological Sciences (IUBS) to promote a high standard of laboratory animal quality, care, and health. In 1979, it was renamed as ICLAS (International Council of Laboratory Animal Science) which has representatives from every country where animals are used for biomedical research. The ICLAS constantly provides information on laboratory animals and has a scholarship program for personnel to update their knowledge and skill in laboratory animal science. AFLAS (Asian Federation of Laboratory Animal Science) is another international body that caters to the interest of Asian countries. This was conceived in 2003, which initially had only six members and now have representations from every Asian country where animals are used, including India.

Besides these two international organizations, there are associations and agencies in every country that promote the interest of laboratory animals and the personnel working with them. Some of the well-known agencies which started in the early 1960s include the American Association for Laboratory Animal Science (AALAS), The Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC), Animal Welfare Institute (AWI), Institute for Laboratory Animal Resources (ILAR),—all US-based; Canadian Council on Animal Care (CCAC); Canadian Association of Laboratory Animal Science (CALAS) based in Canada; Federation of European Laboratory Animal Science Associations (FELASA), in the UK representing Europe without Scandinavian countries; Scandinavian Laboratory Animal Science Association (SCANLAS) based in Finland; Central Institute for Experimental Animals (CIEA) based in Japan; and China Laboratory Animal Science and Teaching Center, China.

India had its first Lab Animal Science Association in 1990 in CDRI, Lucknow, called Laboratory Animal Science Association of India (LASAI) which had members from research institutes of India, both scientists and technicians. A new association exclusively for laboratory animal scientists called Laboratory Animal Scientist's Association (India) based in Hyderabad came into being recently. Both these associations periodically conduct meetings and have conferences and symposia annually covering the latest developments in the field and also discuss issues concerned with personnel working in the area.

1.5 The Way Ahead

It has been an impressive journey so far, from the Haffkine days to the period of IISER now. There are currently over 1750 registered (with CPCSEA) animal facilities in the country, out of which 40 are GLP accredited and 20 of them are AALAS and FELASA certified. Animal welfare laws have become more stringent, and the current infrastructure and the quality of animals maintained there reflect this. But isn't it time to sit back and reflect on what has been achieved so far and see whether any lacunae still exists and think of ways and means to improve them? Let's see where we do stand with respect to these aspects.

1.5.1 Human Resource Development

With several good animal facilities in the country and with greater awareness from administrators, skilled-trained animal caretakers (trained in-house or from centers which provide them) are no longer a problem in most of the animal facilities. But there is a distinct lack of trained manpower at the senior level (in charges/scientists/facility managers) that needs to have persons with an overall knowledge of the field. The problem is more confounded with the addition of new concepts in biosciences like molecular biology, biotechnology, and stem cell biology. Laboratory animal science as a subject is not covered in any of the veterinary, medical, pharmaceutical, or biological courses. Some of the veterinary courses have allocated some meager hours for such an important field, and the syllabi included are very superficial. It does not find a mention in the medical courses. Though in pharmacy and biological courses practical classes of anatomy and physiology of animals are included, there is not much coverage on the care, breeding, and experimental and management aspects. It should be made compulsory that every person working in either at the managerial level or experimentation should have a certificate encompassing all aspects of laboratory animal science. It can be of short duration for research workers who are going to use the lab animals for their work, confining to the practical aspects of handling and experimental procedures. But those who manage the facilities or those who take up research work in this area need to have adequate training, say a 6-month diploma course or through a postgraduate course of 2 years. This can be 1 year for the veterinary and medical and biology postgraduates and can be

conducted by centers like NARFBR, CDRI, or by central universities or even at the newly created IISER. In fact, years back, a syllabus for such a course was prepared by NARFBR (NCLAS then), and the scientific advisory committee approved the course in principle. But no progress was made on this further due to retirement of senior persons and the lack of enthusiasm to take the matter further by those who followed them.

1.5.2 Upkeep of Quality Animals

Though there is a vast improvement in the infrastructure provided to housing animals, by generous allotment of funds by the government and private sectors, upkeep of animals in terms of genetics and health is found wanting in many of the facilities. Identification and maintaining the pedigree of animals in terms of their genetic status (especially inbred, mutants, transgenic, and knockouts) is a must, so not to lose its original trait and to avoid accidental mixing of strains in the facility. Though the health status of animals in terms of microbial profile and histopathology images wherever needed can be maintained at an individual level by the facility, there needs to be a centralized approach concerning monitoring the genetic status of animals using the latest technique of DNA fingerprinting. Those who have the laboratory facility to support this can do this in once in 3 years and keep the records for scrutiny if necessary. Others can submit the samples to a specified government or private laboratory assigned by the government (say CPSCEA) for a fee. This will ensure that all the institutes, private or government, are maintaining animals of uniform genetic status, especially if they are involved in breeding for their own use or others. This needs to be done only for inbred strains, mutants, and transgenic animals and need not be done for random-bred animals and also for animals beyond mice and rats. One need not say how pedigree records are essential in such a scenario, and every animal in the breeding nuclei needs to be numbered and the record maintained. If one look at the history of strain development, let it be inbred, mutant, or gene manipulated, one can see how several animal strains evolved with such a painstaking approach of selective breeding. Weights at birth, weaning, and adulthood have to be done randomly in each generation of the colony, and a constant vigil needs to be kept on the physical and physiological status of animals under one's care, lest one doesn't miss any random mutation that could occur in the animal population.

1.5.3 Ensuring the Availability of Animals

Though we have animal facilities in thousands, the number of breeders for well-defined quality of animals is not adequate for the research needs of a vast country like ours. This is more glaring when one requires mutants and gene-manipulated animals, and so more such centers need be created by upgrading the existing facilities, as done in IISc, CCMB, and TIFR, or equipping the remaining IISER

centers to the level provided to Pune and Bhopal. Such facilities can provide custom-based gene-manipulated animals for a reasonable price, or research workers can come and work and create the animals of their choice. Till such time, one has to depend upon the import of animals from abroad, and there is an urgent need to make the rules more user-friendly to avoid the unnecessary bureaucratic hurdles that delay the work and kill the enthusiasm to take up research. Another area of concern is the shortage of large animals like dogs, pigs, and monkeys for experimental work. Unlike Western countries, there are very few animal facilities in the country even now, where controlled captive breeding of these large animals is undertaken. With a long duration of gestation and weaning and limitation in litter size especially in the case of monkeys, enough animals cannot be raised by these facilities to meet the experimental needs of the country. So there is an urgent need to establish two or more national centers of excellence to breed these animals in captivity and supply them to experimental workers. The centers can provide experimental space in their centers or extend their expertise to maintain them in the user's facility (if they have experimental space for large animals) during the period of experimentation. Though we have rhesus, bonnet, and langur monkeys, minipigs and beagle dogs need to be imported. It's high time we develop our own stock from wild or breed them here by importing a sufficient number of breeding pairs.

Talking about the availability of animals, one should ensure that they travel well to their destination in good condition, by road, train, or air. Transport cages should be of the prescribed guidelines, and adequate food and water (through succulent fruits) provided throughout the journey. At no time, the animals should be exposed to harsh weather outside, and at transit points (be an airport or railway station) in major cities, the government should ensure air-conditioned rooms for animals while embarking or disembarking.

1.5.4 Encouraging "Make in India" Animal Equipment Suppliers

One which adds to the cost of maintenance of animal facilities is the animal house equipment needed for maintaining animals, from cages and racks to biomedical equipment for animal experiments for physiology and pharmacological work. We have come a long way from the wood and metal cages of yesteryears to isolators and individually ventilated cages of plastic. Biomedical instruments to measure body temperature, exercise and activity cages, lung and cardiac function, systems to deliver materials to the brain directly, and whole-body CT scans and animal MRI is are now available. We have adequate manufactures of conventional plastic cages currently, but for isolators and individual ventilator cages of international standards, and physiology equipment, we still have to depend on to other countries companies. Attempts should be made to encourage Indian entrepreneurship for these items under Make in India program.

1.5.5 Encouraging Basic Research in Laboratory Animal Sciences

Worldwide, working in laboratory animal science is considered as a service activity, and India too is no exception. The youngsters do not consider it as an exciting area to work. This should change as animal house personnel are an integral part of the success of any experimental outcome and his or her skills and knowledge assure that the experiment is executed well. For an innovative mind, the field does offer some challenges starting from housing, analyzing environmental variables in the outcome of an experiment, developing appropriate animal models to developing alternatives to animal experimentation, etc. All our animal facilities are built as per the guidelines of the West, and not based on what this country requires. Unlike Western countries, our problem is one of keeping heat and dust away, and so far, nobody here seems to have put their thoughts on this. A symposium was conducted at NIN at the time of the inauguration of the new facility addressing these issues, which was attended by architects, civil and electrical engineers, and energy experts, and several ideas for dissipation of heat and dust were discussed then. It's high time we revive those ideas again and build something, which suits our climatic conditions rather than aping American and European designs and models. Temperature, humidity, ventilation, light, sound, and diets are some of the variables that can alter the results of an animal experiment. Though Western countries have come out with some interesting observations, we haven't done anything to study their interaction in the outcome of an experiment. For example, it would be worthwhile to look at the effect of soft music on the breeding and behavior of animals and the possibility of obtaining uniform results while conducting an experiment. Bedding is an integral part of rodent animal breeding and maintenance, and one can study the effectiveness of using different materials available here, like paper pulp, coconut husks, or any local plant materials, etc. Animal enrichment by providing hiding places and providing toys and some inanimate objects is attempted in Western countries, but we have not given any thought to such innovations to elicit a better behavioral response from animals. Developing animal diets that suit the nutrient requirement of the species using local food ingredients for normal up keeping and for developing experimental diets to study lifestyle diseases is an interesting area.

Many of our animal facilities are maintaining inbred strains of mice and rats for a long time, and it's quite possible that one can come across some aberrations in terms of their anatomy and physiology. If one notices such abnormalities, they can be selectively bred and see whether it is useful for any specific biomedical inquiry. We have such models in front of our eyes, like the obese mutant rats and nude rats developed at NIN, bilateral blind rats developed at CCMB [13], microphthalmia mice developed at NCRC [14], etc. Developing minipigs (as done in the USA and Germany) and an Indian breed of dogs from our mongrel stock to replace the beagle dogs (imported from abroad at high costs) is an area one could explore. In recent times, minipigs have been sighted in the deep forests of Assam, and it will be worthwhile to pursue the lead and see that they can be developed in captivity. There is a fundamental question to the use of albino mice and rats for drug toxicology and safety evaluation tests as it is observed that the response or toxicity varies

when one uses colored mice or rats· One often wonders how results from such rodents could be interpolated to humans who are not albinos [15]. The albinism phenotype is due to tyrosinase deficiency [16] that affects the final conversion to melanin which gives the color coat to the animals. This defect in tyrosine metabolism affects several systems in the body [14] wherever melanin is involved, and strictly these animals cannot be considered as normal to carry out any studies that have applications in humans. It's to be noted that wild white rats are seen in the south and Rajasthan, and it would be a worthwhile effort to catch some of them and develop a normal Indian rat strain that could replace the existing albino strains in vogue. Alternatives to animal experimentation are taken very seriously in the West (the Netherlands, Hopkins University, and USA in the forefront), and this is one area one can innovate [17]. Computer modeling and use of subcellular fractions and slices of animal or human tissue in preliminary toxicity screening use of lower vertebrates (zebra fish) and invertebrates (*Drosophila*, nematodes, horseshoe crabs, hydra, sea urchins, and sponges) for basic and experimental studies come under this.

1.6 Conclusion

There are a sufficient number of animal facilities in the country, thanks to generous funding from the government and the private sector, and there is no need to construct more now or in the future. What we need now is to consolidate what we already have, by closing those that do not meet the required standards (which is the case with some of the state-level institutes; medical, veterinary, and pharmacy colleges; and even some universities), upgrade or update which show promises, and continue to sustain those built with high standards. Those that have lost out can be given access to the nearby big facilities, government or private, through a memorandum of understanding between the concerned parties or through legislation if needed. The animal rights movement in the country which witnessed some ugly skirmishes earlier with animal activists is more or less settled now with the establishment of a central CPCSEA with more powers and an institutional ethical committee at the local level. Though this is sufficient to ensure proper use of laboratory animals, it has only a regulatory role in implementing the guidelines that are laid. This is under an animal board that has a broad spectrum of animals (wild, domestic, pets) and birds under its purview to look after and is attached to the Ministry of Environment and Forest. We need another apex body under the Ministry of Human Resource Development, which exclusively looks after the needs of laboratory animals in the country. It can periodically evaluate the performance of various facilities, which use laboratory animals, and can give course correction in terms of reduction or expansion, whenever needed. This body will have the information on all the animals in each of the animal facility and its utility in terms of research and development. The so-called laboratory animal body will have representatives from ICMR, CSIR, ICAR, DBT, DST, Atomic Energy Commission, UGC, pharma, and drug industries. Quality research requires quality animals and administration should ensure that this remains so at all times.

References

1. Giridharan NV, Kumar V, Muthuswamy V (2000) Use of animals in Scientific Research. ICMR BOOKLET (PDF), Indian Council of Medical Research, New Delhi. www.researchgate.net
2. Vanderberg JG (2000) Use of house mice in biomedical research. ILAR Journal 41(3):133–135
3. Hedrich H (2004) The house mice as a laboratory model : a Perspective. Elsevier Science
4. Crow JF (2002) CC Little , Cancer and inbred mice. Genetics 161(4):1357–1361
5. Jax mice and Research services (2016). C River .com Charles River Laboratories
6. Hedrich HJ (2000) Part 1. The history and development of rat as a laboratory model. In: Krinke GK (ed) The Laboratory Rat. New York Academic Press, pp 3–8
7. Kuramoto T, Nakanishi S et al (2012) Origins of albino and hooded rats: implications from molecular genetic analysis across modern laboratory strains. PLoS one 7(8):e 43059
8. Giridharan NV (1988) Animal models of obesity and their usefulness in molecular approach to obesity. Indian Med Res 108:225–242
9. Panchal SK, Brown L (2011) Rodent models for metabolic syndrome research. J Biomed Biotehnol 2011:2011. 351982
10. Kalasikham RR, Batulla KK, Kirlampalli V, Friedman JM, Nappanveettil (2013) Obese locus in WNIN/Obese rat maps on Chromosome 5 upstream of leptin receptor. PLoS One 2:e7679
11. Giridharan NV, Lakshmi CN, Raghuramulu N (1997) Identification of impaired glucose tolerant animals from an inbred Wistar rat colony. Lab AnimSci 47:428–431
12. Giridharan NV (2018) Glucose and energy homeostasis: Lessons from animal studies. Indian J Med Res 148:659–669
13. Rao SH, Sesikeran B (1992) Congenital anophthalmia in CFY rats: a newly identified autosomal recessive mutation. Lab Anim Sci 424(6):623–625
14. Kohale K, Ingle A, Kelkar A, Parab P (2004) Dense cataract and Micropthalmia—New Spontaneous mutation in BALB/c Mice. Comparative Medicine 54(3):275–279
15. Creel D (1980) Inappropriate use of albino animals as models in research. Pharmacology Biochem and Behavior 12(6):969–977
16. Blaszcyk WM, Arning L, Hoffman KP, Eppelen JT (2005) A tyrosinase missense mutation causes albinism in Wistar Rat. Pigment cell Res 18:144–145
17. Lowa A, Jertic M, Georgia F, Hedrich S (2018) Alternatives animal testing in basic and pre-clinical research of atopic dermatitis. Exp Dermatol 27(5):476–483

2 Laws, Regulations, Policies and Guidelines Governing the Care and Use of Laboratory Animals

Suresh Kumar, Manoj Kumar Rajput, and Sandeepa Bhan Tickoo

Abstract

A mature and uniform interlinking legal system of regulation, policies and guidelines across countries is necessary for proper care and use of laboratory animals in experimentation. Initially, there was a vast disparity in animal welfare legislations and regulations across the globe. But now, most of the countries have incorporated the internationally accepted standards for animal care and welfare and are working towards accomplishing global harmonization. Many non-profit international organizations such as the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC International) perform a key role in establishing as well as promoting best standards for animal care throughout the world through voluntary assessment and accreditation programmes. This chapter highlights the important regulations, laws, policies and guidelines on care and use of experimental animals.

Keywords

Regulations · Animal welfare · 3Rs · Occupational hazards and laboratory animals

S. Kumar (✉) · M. K. Rajput
National Institute of Biologicals, Noida, UP, India
e-mail: suresh.kumar@nib.gov.in

S. B. Tickoo
Shriram Institute for Industrial Research, New Delhi, India

© The Author(s), under exclusive license to Springer Nature Singapore Pte Ltd. 2021
P. Nagarajan et al. (eds.), *Essentials of Laboratory Animal Science: Principles and Practices*, https://doi.org/10.1007/978-981-16-0987-9_2

2.1 Introduction

Laboratory animals are used in research and testing all over the world. The global research communities acknowledge the ethical concerns of modern societies in the use of animals in experiments. To ensure the humane care and use of laboratory animals, most of the countries have evolved their own regulatory framework. Almost every country has made exemplary progress in laws, regulations and guidelines that ultimately supported the regular improvements in the welfare of research animals as well as the quality of science [1]. However, there are still a few nations that do not have legal framework for animals in research and need to rise to meet the international harmonization [2]. This chapter seeks to discuss the laws, regulations and guidelines governing laboratory animal welfare across the globe. Apart from this, this chapter also attempts to cover the guidelines related to safety and occupational hazard, 3Rs and ethics for reliable and authentic knowledge in scientific writings related to laboratory animal research publications.

2.2 Laws, Regulations and Guidelines for Laboratory Animal Welfare

The use of laboratory animals in experimentations has fostered a diverse range of attitudes since the nineteenth century. The first law relating to animal protection and protection of experimental animals (Cruelty to Animals Act) was published in 1822 and 1876 by the United Kingdom [3, 4]. Thereafter, other countries have also made laws for the care and protection of animals used for experimental purposes. In the North American region, the United States enacted the Animal Welfare Act (AWA) and the Health Research Extension Act (1985). The AWA was revised in July 2020 and named as Animal Welfare Act and Regulations "Blue Book" (July 2020). In Canada, the Canadian Council on Animal Care (CCAC) plays a central role in setting and sustaining standards for the ethical use and care of animals in research [5]. In Latin America, Brazil has laws and regulations relating to research animal care and use. The rest of Latin American countries are yet to form any legal framework in this area [6].

In Europe, the European Directive (Directive 2010/63/EU) was amended in 2010 to harmonize the standards for animal care, animal housing, training, project reviews and authorization across all member countries [7]. Japan, China and Korea have enacted the regulations, laws and guidelines in 1983, 1973 and 1991, respectively, for the care and use of laboratory animals. The incorporation of the principles of the 3Rs is common to these countries [8]. Singapore, Thailand, Indonesia and Malaysia known as other Pacific Rim countries have varied levels of regulatory frameworks. Singapore has a strong research environment and has published the "Guidelines on the Care and Use of Animals for Scientific Purposes", in 2004 by the National Advisory Committee for Laboratory Animal Research (NACLAR 2004) encompassing the responsibility of animal care staff, scientist and institutions when researching animals based on concepts of the 3Rs.

In India, the Prevention of Cruelty to Animals Act was enacted in 1960. Subsequently, the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) was formed to ensure the humane care of animals before, during and after the experiment on animals based on five founding principles including 3Rs [9]. In Australia, the protective legislation for animals varies from state to state. However, all the states follow the Australian Code of Practices for the Care and Use of Animals for Scientific Purposes enacted in 1969 and last amended in 2013 including the principles of the 3Rs [10]. Most of the African and Middle Eastern countries still have not framed the legislation nor established regulatory oversight, policies or guidelines except for some exceptions [11]. The details of key acts, regulations and guidelines on laboratory animal welfare are provided in Table 2.1.

2.3 Guidelines on Safety and Occupational Hazards in Laboratory Animals

The occupational safety and health considerations of the personnel involved in the care and use of laboratory animals is the legal and ethical obligation of the facility provider/management of the organizations. To provide appropriate measures for safety and health of the personnel involved, a successful management plan is to be employed that identifies the hazards and eliminates or minimizes the risk of injury or illness to employees and staff members and damage to equipment, property and environment. The details of various guidelines related to safety and occupational hazards in lab animals are provided in Table 2.2.

2.4 Guidelines on Alternatives to Animals in Research and Testing

The concept of 3Rs (Replacement, Reduction and Refinement) given by William Russell and Rex Burch (1959) have revolutionized the thinking on modern laboratory animal science research and quality of animal studies. The principles and techniques of 3Rs are broadly adopted by various national and international organizations across the world. Several 3Rs centres and institutes are continuously working for developing as well as confirmation of alternative methods to replace animal experimentations. The various guidelines on alternatives to research and testing on animals are detailed in the chapter “Alternatives to Animals Experiments in Research and Testing” of this book.

Table 2.1 Details of key acts, regulations and guidelines on laboratory animal welfare

S. no.	Acts, regulations and guidelines	Country/region	Important points	References/useful links
1.	The Animal Welfare Act and regulations. Revised in 2020 and named as Animal Welfare Act and Regulations "Blue Book"	United States	Only federal law in the United States that provides the minimum standards and regulates the animal breeding and selling for use in biomedical research and as pets. The facilities should have an Institutional Animal Care and Use Committee (IACUC) to oversee their animal research	https://www.nal.usda.gov/awic/animalwelfare-act
2.	Public Health Policy on Humane Care and Use of Laboratory Animals (PHS Policy), based on the Health Research Extension Act, 1985	United States	Protects all vertebrate animals and applies to research facilities receiving US Public Health Service (PHS) funds. It also includes the US Government Principles, the *Guide*, and the AVMA Guidelines	https://olaw.nih.gov/policies-laws/hrea-1985.htm
3.	Directive 2010/63/EU (Revision of 1986 Directive 86/609/EEC)	European Union	Provides minimum standards for animal housing and care Implementation of 3Rs in laboratory animals	[7]
4.	Animals Scientific Procedures Act 1986 (ASPA) and amended in 2013	United Kingdom	The protected animals have been regulated in the scientific procedure or other experiments. This is to protect them from pain, distress and suffering. The Act requires a three-tiered licencing system: personal licence, project licence and certificate of designation	https://www.legislation.gov.uk/ukdsi/2012/9780111530313
5.	National Research Council (NRC) Guide for the Care and Use of Laboratory Animals (NRC 1997). Revised in 2011	Multinational	It provides the recommendation on use and care of animals in different programmes, institutional responsibilities, animal house management, veterinary care and physical plant. The animal use and care projects and programmes are accredited on these primary standards across the world	[12, 13]

6.	Guide for the Care and Use of Agricultural Animals in Research and Teaching (2010)	Multinational	It provides species-specific guidance regarding handling, transportation, environmental enrichment, etc. it is one of the standards adopted for the AAALAC accreditation process	[14]
7.	Prevention of Cruelty to Animals Act, 1960, and Breeding of and Experiments on Animals (Control and Supervision) Rules of 1998, 2001 and 2006	India	Committee for Control and Supervision of Experiments on Animals (CPCSEA) ensures that animals are not subjected to unnecessary pain or suffering before, during or after the performance of experiments on them	cpcsea.nic.in
8.	Drugs and Cosmetics Act 1940 and rules thereunder 1945 (amended in 2019)	India	It specifies the environmental conditions of the animal facility where the animals undergo tests based on requirements of the Prevention of Cruelty to Animals Act, 1960	http://vbch.dnh.nic.in/pdf/Rules%20and%20regulations%20of%20Drug%20and%20Cosmetics%20act.pdf
9.	Animals Protection Act 71 (1962) and Guidelines on Ethics for Medical Research: Use of Animals in Research and Training (South African Medical Research Council, 2004)	South Africa	No specific provisions to the use of animals in research in this act. It specifically refers to the 3Rs. The medical research has published Guidelines on Ethics for Medical Research: Use of Animals in Research and Training	https://www.animallaw.info/sites/default/files/AnimalsProtectionAct71–62.pdf
10.	Guidelines for the Ethical Review of Laboratory Animal Welfare (GB/T35892–2018)	China	It covers the ethical review and animal welfare management in the production, transportation and use of laboratory animals and quality management thereof	[15]
11.	Act on Humane Treatment and Management of Animals and various guidelines (1973), amended in 2012	Japan	It emphasizes on 3Rs for animal experimentation. There is self-regulation within each animal facility to encourage flexible animal research with administrative guidance and voluntary guidelines	[1]
12.	Animal Protection Act (APA) and the Laboratory Animal Act (LAA)	Korea	Both laws include the 3Rs and require for the establishment of Institutional Animal Care and Use Committees	[1]

(continued)

Table 2.1 (continued)

S. no.	Acts, regulations and guidelines	Country/ region	Important points	References/useful links
13.	Australian Code for the Care and Use of Animals for Scientific Purposes (8th edition, 2013)	Australia	It promotes humane care and use of animals in scientific studies. Also guides the animal ethics committees, investigators and institutions for animal carers	[16]
14.	Animal Welfare Act 1999 Part 6	New Zealand	Two guidelines of an act: Good Practice Guide for the use of animals in research, testing and teaching and guidelines for the welfare of livestock from which blood is harvested for commercial and research purposes	[17]
15.	NACLAR Guidelines (2004)	Singapore	The guidelines promote responsible and humane care and use of animals for scientific purposes and are based on the principles of the 3Rs	[9]
16.	Animals for Scientific Purposes Act 2015 and Ethical Principles and Guidelines for the Use of Animals for Scientific Purposes, NRC of Thailand (1999)	Thailand	Any procedure for scientific purpose on animals should be in synchronization with universal ethics and standard for animal welfare	[9]
17.	Law of the Republic of Indonesia No. 18, 2009 Law of the Republic of Indonesia No. 41, 2014 Government of Republic of Indonesia's Regulation No. 95, 2012	Indonesia	Nos. 18 and 41 are dedicated to the veterinary public health and animal welfare No. 95 describes principles for the ethical treatment of animals in research, handling, transportation, housing, husbandry and care, use, humane treatment, euthanasia, etc.	[9]
18.	Malaysian Animal Welfare Act, 2015	Malaysia	Legislates care and use of experimental animals. It is applicable to all fields of science in Malaysia	[9]
19.	AAALAC International	Multinational	It guides on voluntary accreditation of animal care and use programmes across the world	www.aaalac.org

20.	Canadian Council on Animal Care (CCAC)	Canada	It sets standards, guidelines and policies on animal use in science. It assesses the institutions and ensures the use and humane care. Also, formation of animal care committee	[18] https://www.ccac.ca/en/about-the-ccac/
21.	World Organisation for Animal Health (OIE) animal welfare standards, World Organisation for Animal Health	Multinational	Animals should only be used in conditions when no alternative method is available. The animal use should be ethically justified, and it should cause as little pain or distress as possible. The importance of 3Rs is emphasized	[17] https://www.oie.int/fileadmin/Home/eng/Health_standards/tahc/2018/en_chapitre_aw_research_education.htm
22.	Law 11,794/2008 Decree 6899/2009	Brazil	National Council for the Control of Animal Experimentation (CONCEA) requires institutions to establish an ethics committee on the use of animals for day-to-day enforcement of the law and regulations	[19]
23.	AVMA (American Veterinary Medical Association) Guidelines for the Euthanasia of Animals: 2020 Edition	United States	The guidelines mainly present the criteria for euthanasia, method and agent of euthanasia and also an emphasis on applying appropriate pre-euthanasia	https://www.avma.org/sites/default/files/2020-01/2020Euthanasia-Final-1-17-20.pdf
24.	ISO (International Organization for Standardization) 10,993–2	Multinational	It stipulates the minimum standards needed to assure the welfare of animals used in tests to evaluate the biocompatibility of materials used in medical devices. It also gives instructions for the refinement of the test method and replacement of animal tests by alternative scientifically valid methods	[20]
25.	OECD (Organisation for Economic Co-operation and Development) principles for GLP (Good Laboratory Practice) concerning the safety testing of any chemical substance	Multinational	Guidelines for the harmonization of safety chemical testing by reducing the number of animals or by the alternative testing system for the welfare of animal methods used in testing	http://www.oecd.org/env/ehs/testing/animalwelfare.htm

(continued)

Table 2.1 (continued)

S. no.	Acts, regulations and guidelines	Country/ region	Important points	References/useful links
26.	Federation of European Laboratory Animal Science Associations (FELASA)	Multinational	Publish the guidelines, recommendations and reports on all aspects of laboratory animal science (LAS) in Europe and beyond	http://www.felasa.eu/
27.	American Association for Laboratory Animal Science (AALAS)	Multinational	It is the association of professionals that promotes rational laboratory use and animal care for the betterment of the people and animals	https://www.aalas.org/

Table 2.2 The details of various guidelines related to safety and occupational hazards in lab animals and workers

S. no.	Area of risks	Guidelines	Important points	References/useful links
1.	Occupational Health & Safety (OH&S) risks	Occupational Health and Safety Assessment Series (OHSAS 18001)	Internationally recognized system for overseeing the organization's activities and processes to reduce or eliminate Occupational Health & Safety (OH&S) risks to employees	https://www.certificationeurope.com/certification/ohsas-18001occupational-health-and-safety-management/
		North Dakota State University (NDSU) Guidelines for Occupational Health & Safety (1997; latest rev. 2015)	To address the health and safety of researchers involved in the use of animals	https://www.ndsu.edu/fileadmin/policesafety/docs/OccupationalSafetyandEnvironmentalHealth.pdf
2.	Biosafety	Design and management of research facilities. In Laboratory Animal Medicine	Design to create an efficient barrier control programme	[21]
		Biosafety in Microbiological and Biomedical Laboratories	Biosafety level in the animal facility	[22]
		NRC (National Research Council). 2011a. Guide for the Care and Use of Laboratory Animals	Guide on animal care and operational practices to meet applicable regulations and standards	[23]
		Canadian Biosafety Standard (CBS)	Guidelines related to design construction and operation of facilities used for handling of pathogens or toxins or stored therein	http://canadianbiosafetystandards.collaboration.gc.ca/cbs-ncb/index-eng.php
		Laboratory Biosafety Manual	Categorized the pathogens in risk groups (RGs) from 1 to 4 by the World Health Organization (WHO)	https://www.who.int/csr/resources/publications/biosafety/WHO_CDS_CSR_LYO_2004_11/en/
3.	Hazard communication and signage	Globally harmonized chemical hazard warning symbols (Global Harmonization System (GHS))	Inform the workers about the appropriate use of chemicals in animal facilities	https://www.osha.gov/dsg/hazcom/ghsguideoct05.pdf

(continued)

Table 2.2 (continued)

S. no.	Area of risks	Guidelines	Important points	References/useful links
4.	Personal protective equipment (PPE)	NIOSH (National Institute for Occupational Safety and Health) respirator selection logic	Devices such as respirators used to protect workers from harmful dust, insufficient oxygen environments, fogs, smokes, mists, particulates, gases, vapours and sprays	http://www.cdc.gov/niosh/docs/2005-100/pdfs/2005-100.pdf
		National Institutes of Health (NIH), Animal Research Advisory Committee, Guidelines for personnel protection in animal facilities, 2016	Protective clothing requirements and zoonotic diseases in laboratory nonhuman primates	https://oacu.oir.nih.gov/sites/default/files/uploads/arac-guidelines/ppe.pdf
			Guidelines for staff working in nonhuman primate facilities. PPEs suggested on risk assessment	https://oacu.oir.nih.gov/sites/default/files/uploads/arac-guidelines/ppe.pdf
		Occupational Safety and Health	PPEs suggestion in the animal facility	[13]
		CROSSTEX MaskEnomics filtration guide	Guidelines for the selection and use of face masks for mucous protection	http://www.crosstex.com/home.asp
5.	Hearing protection	Occupational Safety and Health Administration (OSHA), the United States, Department of Labour	Control measures for occupational noise like engineering controls, administrative controls or PPEs	https://www.osha.gov/SLTC/noisehearingconservation/
6.	Medical evaluation	OSHA Respirator Medical Evaluation Questionnaire (Mandatory)	Initial evaluation of employees for allergies to animals related to prior employment	https://www.osha.gov/pls/oshaweb/owadisp.show_document?p_table=STANDARDS&p_id=9783
7.	Immunization of workers	Biomedical Waste Management Rule (BMWM) 2016	Recommended for tetanus and hepatitis B vaccine for the workers involved in biomedical waste	https://dhr.gov.in/sites/default/files/Biomedical_Waste_Management_Rules_2016.pdf
		Centers for Disease Control and Prevention (CDC)	Information on rabies virus serology for the workers having high occupational risk and exposure to rabies	http://www.cdc.gov/rabies/specific_groups/doctors/serology.html

		Reference serum revisited	Storing a worker's serum for possible future reference	[24]
8.	Routine periodic medical evaluations	Laboratory animal allergy (LAA)	Information on testing of the workers for evidence of an allergy to animal proteins	[25, 26]
9.	Animal-related occupational injuries and illnesses	Occupational Safety and Health (OSH)	Animal handling occupational illnesses and injuries. Measures to minimize the risk of disease and other occupational hazards	[27]
10.	Zoonosis	Centers for Disease Control and Prevention and published papers	Tuberculosis, rubella and herpes B virus	[28, 29, 30] http://www.cdc.gov/tb/publications/factsheets/statistics/TBTrends.htm https://www.cdc.gov/herpesbvirus/index.html

2.5 Education Training and Regulatory Guidelines on Laboratory Animals

Everyone involved in animal experimentation and care must be adequately trained before working with lab animals. The training programme should be like that it can accommodate a range of educational qualifications frequently encountered in research institutions. Before 1985, training programmes for veterinarians and animal technicians were available in Europe and the United States. Two laws on the care and use of animals were made by the US Congress [31]. According to these laws, all animal research funded by PHS (Public Health Service) should comply with the PHS Policy on Humane Care and Use of Laboratory Animals [32]. All persons having responsibilities for the use of animals must have adequate qualification and training including researchers and members of Institutional Animal Care and Use Committees (IACUCs).

Europe adopted the Directive 86/609/EEC in 1986 for the Protection of Animals Used for Experimental and Other Scientific Purposes (EU Directive) [33]. The Council of Europe (CoE) in the same year accepted the Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Procedures [34, 35]. Both these directives have provisions on the competence of persons involved in animal experimentation. Several other countries including Canada and New Zealand have developed guidelines on the competence of personnel. The training course should include subjects such as animal husbandry, gnotobiology, experimental techniques, anaesthesia and ethical features. The European Science Foundation (ESF), an organization from 30 European countries, stated that courses on laboratory animals should also include information on animal alternatives, welfare and ethics [36]. In countries like Denmark, Sweden, Finland, the Netherlands, France, Belgium and the United Kingdom, a training course has been made mandatory by law. In other countries like Germany, Spain, Portugal and Italy, training courses are not mandatory as per law, but they conduct the training.

According to the US Animal Welfare Act, 1996, institutions are responsible of providing training in areas like the proper use of anaesthetics, tranquilizers and analgesics, humane methods of animal maintenance and experimentation and the availability and use of methods that limit the use of animals or minimize animal distress [37, 38]. Despite the guidelines published on Care and Use of Laboratory Animals by the National Research Council of Education and Training [39], the training courses of many institutions have major differences. The International Council for Laboratory Animal Science (ICLAS) has considered harmonizing training courses. In this regard, a subcommittee of the Working Group on Harmonization examines general principles for training that have been formed. FELASA Accreditation is recognized as the premier accreditation and was introduced in 2003 and is recognized as the premier accreditation scheme in Europe for laboratory animal science (LAS) courses [40]. This scheme necessitates the development of quality training programmes on laboratory animals in Europe and internationally.

FELASA encourages professional competence of all personnel working with animals and the implementation of 3Rs. A Certificate Course on Laboratory Animal

Science (CCLAS) that started in India in 2013 promotes judicious and scientific handling and management of laboratory animals. A MoU was signed between TANUVAS (Tamil Nadu Veterinary and Animal Sciences University), CPCSEA (Committee for the Purpose of Control and Supervision of Experiments on Animals), NIAW (National Institute of Animal Welfare) and Laboratory Animal Scientists' Association (India) (LASA India) to start this course in India. This course aims to bridge the quality standards prescribed by the FELASA for personnel involved in animal experimentation in India. Based on the success of this course, FELASA has accredited this course for rodents and fish species. Outside Europe, this is the only course accredited and audited by experts from FELASA. The objective of this programme is to guide the judicious use of laboratory animals to achieve scientific results in animal experimentation.

2.6 Guidelines on Publications Related Work on Animals

Clear and accurate reporting of data is very important for the reproducibility of results. To address this issue, ARRIVE (Animal Research: Reporting In Vivo Experiments) guidelines were framed in 2010. These guidelines instruct the authors and journals to report minimum necessary information for in vivo experiments. But still, the impact of these guidelines in reporting has been limited. Many in vivo researchers are not aware of reporting the information as per ARRIVE guidelines. The revised ARRIVE provides information about 21 items. It covers best practices of conduct of animal studies for improving standards of the experimental process and publication. The ARRIVE guidelines apply to all studies involving live animals. These are also to be used by funding institutions and ethical review boards to ensure best practices and reporting in animal research.

The ARRIVE guidelines prescribe the "Essential 10" for minimum reporting requirements. The initial focus is to instruct authors, journal staff, editors and reviewers on "10 critical information". Once the "Essential 10" is regularly reported in publications, the information on other items may be added until reaching all 21 items. The ARRIVE Essential 10 are study design, sample size, inclusion and exclusion criteria, randomization, blinding, outcome measures, statistical methods, experimental animals, experimental procedures and results. As per revised ARRIVE guidelines, the recommended items to be described are abstract, background, objectives, ethical statement, housing and husbandry, animal care and monitoring, interpretation, generalization, protocol registration, data access and declaration of interests. It is the responsibility of authors to fulfil the requirements of guidelines and regulations related to the use of animals. They must have approval for the study from the relevant ethics committee and provide the institution details where the research was approved. The protocol licence numbers should also be indicated [41].

2.7 Conclusion

The harmonization in laboratory animal welfare and use programmes has been made largely due to the globalization of science. A group of few countries yet have not adopted the guidelines, regulations and policies directing the care and use of laboratory animals. Further, there are still many pressing issues with the long-standing legal regimes as established by countries like the European Union, the United States and Australia. Therefore, additional efforts have to be required to form international organizations to set up harmonized global laboratory animal laws, policies and regulations. This chapter can be a catalyst for further improvement and implementation of legislations, policies and guidelines in the sphere of laboratory animal use and care as aspired globally for quality science.

References

1. Ogden BE, Pang W, Agui T et al (2016) Laboratory Animal Laws, Regulations, Guidelines and Standards in China Mainland, Japan, and Korea. ILAR J 57:301–311
2. Vasbinder MA, Locke P (2016) Introduction: global laws, regulations, and standards for animals in research. ILAR J 57:261–265
3. Marie B (2012) The history of Animal Welfare Law and the future of animal rights. Electronic Thesis and Dissertation Repository 803, https://ir.lib.uwo.ca/etd/803
4. Lyons D (2011) Protecting animals versus the pursuit of knowledge: evolution of the British animal research policy process. Soc Anim 19:356–367
5. Griffin G, Locke P (2017) Overview of Canadian and US legal requirements for animals in research. ILAR 57:271–284
6. Rivera E, Hernandez R, Carissimi A et al (2017) Latin American laboratory animal legislation. ILAR 57:293–300
7. Olsson AS, da Silva SP, Townend D et al (2016) Protecting animals and enabling research in the European Union: an overview of development and implementation of the Directive 2010/63/EU. ILAR 57: 347–357
8. Kong Q, Qin C (2010) Analysis of current laboratory animal science policies and administration in China. ILAR e-Journal 51:e1–e10
9. Retnam L, Chatikavanij P, Kunjara P et al (2016) Laws, regulations, guidelines and standards for animal care and use for scientific purposes in the countries of Singapore, Thailand, Indonesia, Malaysia, and India. ILAR J 57:312–323
10. Marston H, Timoshanko A, Lidbury B (2017) Australian regulation of animal use in science and education: a critical appraisal. ILAR 57:324–332
11. Mohr B, Fakoya F, Hau J et al (2017) The governance of animal care and use for scientific purpose in Africa and the Middle East. ILAR 57:333–346
12. National Research Council of the National Academy of Sciences (2011) Guide for the care and use of laboratory animals. National Academy Press, Washington
13. Guillen J (2017) Laboratory animals: regulations and recommendations for the care and use of animals in research. Academic Press, Cambridge
14. Federation of Animal Science Societies (FASS) Guide (2010). https://www.adsa.org/Publications/FASS-2010-Ag-Guide
15. MacArthur Clark JA, Sun D (2020) Guidelines for the ethical review of laboratory animal welfare People's Republic of China National Standard GB/T 35892-2018. Animal Model Exp Med 3:103–113

16. Timoshanko AC, Marston H, Lidbury BA (2016) Australian regulation of animal use in science and education: a critical appraisal. ILAR J 57:324–332
17. Bayne K, Bayvel AC, Williams V (2013) Laboratory Animal Welfare: international issues. Academic Press, pp 55–76. https://doi.org/10.1016/B978-0-12-385103-1.00006-3
18. Griffin G, Locke P (2016) Comparison of the Canadian and US Laws, Regulations, Policies, and Systems of Oversight for Animals in Research. ILAR J 57:271–284
19. Filipecki ATP, Machado CJS, Valle S et al (2011) The Brazilian Legal Framework on the scientific use of animals. ILAR eJ 52:e8–e15
20. International Standard (ISO) 10993–2, 2nd ed, 2006
21. Lipman N, Leary S (2015) Design and management of research facilities. In: Fox JG, Anderson LC, Otto G, Pritchett-Corning KR, Whary MT (eds) Laboratory animal medicine, 3rd edn. Elsevier/Academic Press, Amsterdam, pp 1543–1597
22. Centers for Disease Control and Prevention and National Institutes of Health, Biosafety in Microbiological and Biomedical Laboratories (2009) 5th ed., U.S. Government Printing Office, Washington, DC
23. Management of Animal Care and Use Programs in Research, Education, and Testing (2018) 2nd edition. Weichbrod RH, Thompson GAH, Norton JN, editors. CRC Press/Taylor & Francis, Boca Raton, FL
24. Lehner NL, Huerkamp MJ, Dillehay DL (1994) Reference serum revisited. Contemp Topics Lab Anim Sci 33:61–63
25. Bush RK, Stave GM (2003) Laboratory animal allergy: an update. ILAR J 44:28–51
26. Gross NJ (1980) Allergy to laboratory animals: epidemiologic, chronological and physiologic aspects, and a trial of cromolyn in its management. J Allergy Clin Immunol 66:158–165
27. Schmitt JM, Wilson DE, Raber JM (2018) Occupational safety and health. In: Weichbrod RH, Thompson GAH, Norton JN (eds) Chapter 14, Management of Animal Care and use programs in research, education, and testing, 2nd edn. CRC Press/Taylor & Francis, Boca Raton, FL
28. Fox JG, Otto G, Colby LA (2015) Selected zoonoses. In: Fox JG, Anderson LC, Otto G, Pritchett-Corning KR, Whary MT (eds) Laboratory animal medicine, 3rd edn. Elsevier/Academic Press, Amsterdam, pp 1543–1597
29. Walther BA, Ewald PW (2004) Pathogen survival in the external environment and the evolution of virulence. Biol Rev 79:849–869
30. Cohen JI, Davenport DS, Stewart JA et al (2002) Recommendations for prevention of and therapy for exposure to B virus (Cercopithecine herpesvirus 1). Clin Infect Dis 35:1191–1203
31. Animal Welfare Act-AWA (1985) Food Security Act of 1985—Animal Welfare. Also called The Improved Standards for Laboratory Animals Act, Public Law 99–185. GPO Office of the Federal Register, Washington DC
32. Public Health Service-PHS (2002) Public health service policy on humane care and use of laboratory animals. US Department of Health and Human Services, Washington DC
33. Council Directive 86/609/EEC (1986) Council Directive 86/609/EEC of 24 November 1986 on the approximation of laws, regulations and administrative provisions of the Member States regarding the protection of animals used for experimental and other scientific purposes
34. Council of Europe-CoE (1993) Resolution on education and training of persons working with laboratory animals adopted by the Multilateral Consultation on 3 December 1993
35. ETS123 (1986) European convention for the protection of vertebrate animals used for experimental and other scientific purposes
36. European Science Foundation-ESF (2001) Policy Briefing 15:1–6
37. Animal Welfare Act-AWA (1996) Animal Welfare Act as Amended (7 USC, 2131, 2156)] The complete Animal Welfare Act including all amendments (1970, 1976, 1985, 1990) following the 1966 enactment. This version is current through 1996 and can be found in United States Code, Title 7, Sections 2131 to 2156

38. National Research Council-NRC (1991) Education and training in the care and use of laboratory animals. National Academy Press, Washington DC
39. Guillen J (2012) FELASA guidelines and recommendations. JAALAS 51:311–321
40. Federation of European Laboratory Animal Science Associations-FELASA (1995) FELASA recommendations on the education and training of persons working with laboratory animals: categories a and C. reports of the Federation of European Laboratory Animal Science Associations Working Group on education accepted by the FELASA Board of Management. Lab Anim 29:121–131
41. Percie du Sert N, Hurst V, Ahluwalia A (2020) The ARRIVE guidelines 2.0: updated guidelines for reporting animal research. PLoS Biol 18:e3000410

3 Ethics, Animal Welfare and Regulation: The Indian Perspective

Vijay Pal Singh and Shikha Yadav

Abstract

The understanding of the term "animal welfare" varies according to individuals, and discussions over this often turn into a battle of wits. However, if we are to improve our standards of animal welfare to a global scale, we must cohalesce those with competing agendas such as animal welfare pertains to much of human well-being and to the quality of science on which we pride ourselves constantly. It is important to develop an effective approach for bridging the gap between what are often perceived as competing agendas concerning human/society and animal welfare. To achieve this, we need to have an effective regulation requiring a high ethical standard. The existing gap between animal welfare and animal ethics, and also between regulation, act, and law, is narrow and blurred. It is important to note that scientific achievement from laboratory animal science and thus the progress of science and medicine will continue to be assessed through the yardstick of animal welfare and care.

Keywords

Animal welfare · Ethics · Law · Well-being · Animal protection · Laboratory animals · IAEC · CPCSEA · Five freedoms · Act · Rules

V. P. Singh (✉)
Council of Scientific and Industrial Research, Institute of Genomics and Integrative Biology, New Delhi, India
e-mail: vp.singh@igib.res.in

S. Yadav
National Institute of Biologicals, Ministry of Health and Family Welfare, Government of India, Noida, UP, India

© The Author(s), under exclusive license to Springer Nature Singapore Pte Ltd. 2021

P. Nagarajan et al. (eds.), *Essentials of Laboratory Animal Science: Principles and Practices*, https://doi.org/10.1007/978-981-16-0987-9_3

3.1 Introduction

Historically, the majority of our understanding of our bodies and our crucial systems started from understanding similar organisms in the animal kingdom. Even till now, testing of drugs and development of various therapies, vaccines, hormone therapy, various cosmetics, etc. are done by experimenting with animals. Animals are used widely for assessing the safety of almost all new chemicals, pesticides, consumer goods, medicines, medical devices, vaccines, and many other products. This is done not only for the fulfillment of requirements of regulatory authorities of the respective countries but also advancement of health of people, animals, and the environment.

In the last two centuries, animals have been used in biomedical research to understand human and animal anatomy, physiology, and diseases and for the discovery of medicines. Animal-based research has been essential so far to uncover the processes within the biological system as well as for preclinical testing. The biggest breakthrough made in pharmacology in the nineteenth and the twentieth century was shouldered by animal testing programs. Every Nobel winning research in physiology and medicine over the last century was backed by intensive data based on animal usage in the experiments. The scenario led to a huge number of research programs involving animals rather indiscriminately often not justifiable to the requirement of that experimental data or not.

The following are some of the areas where the animals are still being used:

- **Drug development**: For the development of new drugs, and repurposing of old drugs, for both humans and animals.
- **Toxicity (safety) testing**: To test the safety of new products, i.e., drugs, food additives, agrochemicals, etc.
- **Basic research**: It is curiosity-driven research for the purpose of advancing knowledge and discovering new models of human diseases.
- **Education**: To train new researchers and surgeons.

3.2 Early History

India encountered in the late nineteenth century and early twentieth century institutionalization of bacteriology, as it started with Imperial Bacteriology Laboratory situated at Poona (1890) (today's Pune); the Bacteriology Laboratory situated at Agra (1892); the Plague Research Laboratory at Bombay (today's Mumbai) (1896); the Pasteur Institute of India, Kasauli (1900), Coonoor (1907), Rangoon (1916), Shillong (1917), and Calcutta (today's Kolkata) (1924); and the Central Research Institute (CRI) at Kasauli (1905). The establishment of these institutes brought to the fore discussion of animal ethics in laboratory research as these newly formed institutes consumed animals in huge numbers. For instance, the production of a single (sample) vaccine at the Pasteur Institute needed 6000 animals per year. But it is also true that despite Victorian moralities and Hindu animal sympathy, no animal experiment legislation was ever enforced in British-ruled India, which developed

around the current Cow Safety Movement. Breezing through the historical logs, it shows that the animal experiments in India were initiated much earlier, i.e., in the 1860s than the nineteenth century. As the British tried to introduce different chemical medicines in India, animal experiments became a necessity. Around the same time, animal welfare activities were also initiated; in 1861, Colesworthy Grant (1813–1880), a British painter living in Calcutta at the time, the first Indian Society for the Prevention of Cruelty to Animals (SPCA) was founded. Grant was mainly troubled by the suffering of animals he encountered on the streets every day or of the ones in misery due to the drought in Calcutta.

As the issue of welfare of animals, especially experimental animals, got accelerated internationally, India too followed the lead. High-level scientific communities in India took efforts to deal with the issues of welfare and regulation on animal experiments by preparing various guidelines.

3.3 Rules and Regulation of Laboratory Animals

The international and national legislations aim for enhancement of animal welfare, standardization/harmonization of policy, and, ultimately, improvement of research output. In the West, it was initiated in1876 as Cruelty to Animals Act (UK); 1891, Law of Vivisection (Denmark); 1944, Animal Welfare Act (Sweden); 1966, 1988, 1998, and 2003, Animal Welfare Act (United States); and 1977, Animal Experimentation Act (the Netherlands). In the United Kingdom, in any "procedure," the Home Office must license to include vertebrates (and the common octopus) that may cause "pain, discomfort, distress, or lasting harm." Article 51A (g) of the Constitution of India reads that it is a fundamental "duty of every citizen of India to protect and improve the natural environment including forests, lakes, rivers, and wildlife and to have compassion for living creatures."

The first law to prevent cruelty to animals in India was enacted in 1890 (11 of 1890) by the British government. After 70 years, in 1960, the Prevention of Cruelty to Animals Act (PCA Act), 1960, was enacted, and amended in 1982, intending to prevent the infliction of unnecessary pain or suffering on animals. Under Chapter IV, Section 15, of the PCA Act 1960, a statutory body by the name CPCSEA (Committee for the Purpose of Control and Supervision of Experiments on Animals) was constituted to supervise and control experiments on animals. CPCSEA has come up with the Breeding and Experimentation of Animals (Control and Supervision) Rules 1998, later amended in 2001 and 2006, followed by a set of guidelines for the care, maintenance, and use of laboratory animals, namely, "CPCSEA Guidelines for Laboratory Animal Facility" in the year 2005 and "Guidelines on the Regulation of Scientific Experiments on Animals" in June 2007. The Council of Europe (2009) brought out the European Convention ETS 123 for the protection of animals used for experimental and other scientific purposes (revised in 2007) and European Directive 2010/63/EU (86/609/EEC) for the protection of animals used for experimental and other scientific purposes (revised in 2010).

The main acts and rules regarding laboratory animals in India are as follows:

Acts

- No. 59 of 1960—The Prevention of Cruelty to Animals Act, 1960.

Rules

- The Breeding of and Experiments on Animals (Control and Supervision) Rules, 1998.
- The Breeding of and Experiments on Animals (Control and Supervision) Amendment Rules, 2001.
- The Breeding of and Experiments on Animals (Control and Supervision) Amendment Rules, 2006.

Rules and regulatory systems are required for laboratory animals to avoid unnecessary pain and suffering to animals and to identify the balance of harm caused to animals in relation to benefit to the community from scientific animal experiments.

Some milestones in the Indian legislation concerning animals:

- 1960: Prevention of Cruelty to Animals (PCA) Act 1960, amended in 1982.
- 1964: Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA).
- Central Insecticides Board and Registration Committee under Insecticides Rules, 1971, for Pesticide Safety Testing.
- 1972: Wildlife Protection Act.
- 1992: Indian National Science Academy (INSA) "Guidelines for Care and Use of Animals in Scientific Research," revised in 2001.
- 1998: "Breeding of and Experiments on Animals (Control and Supervision) Rules, 1998," amended in 2001 and 2006.
- 2001: Indian Council of Medical Research (ICMR) "Guidelines for Use of Laboratory Animals in Medical Colleges."
- Drugs and Cosmetics Rules, 2005.
- Drugs and Cosmetics (Eighth Amendment) Rules, 2015, about phytopharmaceutical drugs.
- Drugs and Cosmetics Rules 1945, 158 (B) guidelines for issue of license concerning Ayurveda, Siddha, or Unani drugs. This concerns the need for safety and evidence of effectiveness needed for these drugs.
- Food Safety and Standards Act 2006, necessitating the food safety testing for approval for human consumption.

3.4 Welfare Issues in the Use of Laboratory Animal

3.4.1 What Is Welfare?

The welfare of an individual is its attempts to cope with its environment [1]. Both the level of inability to cope and the ease or difficulty of coping are included. Coping refers to having control of mental and bodily stability. The term welfare can be applied to live animals as well as humans and is an attribute of an individual.

Practically, animal welfare aims to reduce the negative experiences such as pain, suffering, and discomfort and to improve the environment of the animal, i.e., improved basic needs, enrichment, proper handling, etc. Welfare is generally a relative term concerning its need, health of an animal, distress, adaptation (coping), and naturalness, i.e., animal must live a reasonably natural life.

3.4.2 Why Welfare?

In the words of Mahatma Gandhi: "Ethically they had arrived at the conclusion that man's supremacy over the lower animals meant not that the former should prey upon the latter, but that the higher should protect the lower." The motivation for animal welfare is both moral and scientific. As we have a moral and legal obligation to treat animals with compassion, it is important to note that scientifically good and reproducible science will happen only if the welfare of an animal is well taken care of. Animal welfare is not a choice; it is a need for both animal and good science. It was first recognized in 1980 that animals and their response systems are subjected to challenges from their environment. Apart from that, it is really important to understand that only adaptation and living in harmony with nature is not enough to assess good welfare. Animals used in experimentation are subject to the conditions offered by responsible persons. Therefore, homeostatic balance is strongly determined by both genotypic and phenotypic constraints which subsequently influence the quality of experimental results thereby.

Scientific measures of animal welfare

- Physiological marker of pleasure.
- Behavioral signs of pleasure.
- Strongly preferred behaviors shown.
- Variety of normal behaviors shown or suppressed.
- Normal physiological and anatomical development which are possible.
- Behavioral aversion shown.
- Physiological coping.
- Immunosuppression.
- Disease occurrence.

(continued)

- Behavioral struggle to cope.
- Behavior pathology.
- Brain changes.
- Body damage occurrence.
- Diminished ability to grow or breed.
- Shortened life expectancy.

3.4.3 How to Measure Welfare

There are many ways to measure welfare. Animal welfare can be measured qualitatively by observing the following indicators:

- Longevity.
- Health.
- Behavior.
- Expressions.
- Physiology.
- Immunity.
- Reproduction.

Assessment of the following "five freedoms" can be a litmus test to measure welfare because addressing the five freedoms ensures that the animal lives a naturally healthy life [2].

1. **Freedom from hunger and thirst** by providing ready access to fresh water and diet to maintain full health and vigor.
2. **Freedom from discomfort** by providing an appropriate environment including shelter and a comfortable resting area.
3. **Freedom from pain, injury, or disease** by prevention or rapid diagnosis and treatment.
4. **Freedom to express (most) normal behavior** by providing sufficient space, proper facilities and company of the animal's own kind.
5. **Freedom from fear and distress** by ensuring conditions and treatment which avoid mental suffering.

3.4.4 Difference between Welfare and Well-being

Welfare is a broader concept that describes what is expected from the environment to respect the needs of animals. Well-being on the other hand is a subjective state,

which is related to welfare but does not relate directly. Sick animals also have good care and welfare, but well-being is (due to sickness) very poor.

3.5 Animal Welfare in India

The Animal Welfare Division of the Ministry of Animal Husbandry and Dairying and Fisheries is entrusted with the responsibility of implementing the provisions of the Prevention of Cruelty to Animals Act, 1960 (59 of 1960). Two statutory bodies, viz., Animal Welfare Board of India (AWBI), and Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), were set up under this Act. Apart from this, there is a subordinate body, namely, the National Institute of Animal Welfare (NIAW) (at Ballabhgarh, Haryana), for imparting training and education on diversified subjects in animal welfare including laboratory animal management, behavior, and ethics. Activities relating to animal welfare are looked after by the Animal Welfare Division of the Ministry of Animal Husbandry and Dairying and Fisheries with the mandate of preventing the infliction of unnecessary pain or suffering on animals.

3.5.1 Institutes in India and Abroad for Laboratory Animals

3.5.1.1 Government Institutes

NIAW: The National Institute of Animal Welfare (NIAW) was established to promote awareness and disseminate information about animal welfare among the public and to impart education in this discipline professionally with a structured framework given the intrinsic value of animals and their contribution not only to the country's economy but also in terms of social and environmental issues.

CPCSEA: Committee for the Purpose of Control and Supervision of Experiments on Animals.

The main functions of CPCSEA are:

1. Registration of establishments conducting animal experimentation or breeding of animals for this purpose.
2. Selection and assignment of nominees for the Institutional Animal Ethics Committees of the registered establishments.
3. Approval of animal house facilities based on reports of inspections conducted by CPCSEA.
4. Permission for conducting experiments involving the use of animals.
5. Recommendation for import of animals for use in experiments.
6. Action against establishments in case of violation of any legal norm/stipulation.
7. Conduct training program for the nominees of CPCSEA.
8. Conduct/support conferences/workshops on animal ethics.

3.5.2 Guiding Principles for Ethical Use of Animals for Experiments

CPCSEA at its tenth meeting dated December 20, 2004 (Agenda Item No. 10.6), adopted five guiding principles that emerged after brainstorming sessions (2004–2005).

Principle No. 1: Experiments involving operations may be carried out for the purpose of advancement by the new discovery of physiological knowledge; or knowledge which is expected to be useful for saving or for prolonging human life or alleviating suffering; or for significant gains in the well-being of the people of the country; or for combating any disease, whether of human beings, animals, or plants.

Principle No. 2: Animals lowest on the phylogenetic scale and with the least degree of sentience should be first considered for any experiment designed with the minimum number of animals to give statistically valid results at 95% degrees of confidence. Replacement alternatives should be given due care and sound justification provided if alternatives, when available, are not used.

Principle No. 3: Proper use of animals in experiments and avoidance/minimization of distress and pain inflicted on experimental animals should be an issue of priority for research personnel, and unless and the contrary is established, investigators should consider that procedures that cause pain or distress in human being will also cause pain or distress in animals. All scientific procedures adopted with animals that may cause more than momentary or slight pain/distress should be performed with appropriate sedation, analgesia, or anesthesia.

Principle No. 4: Persons using experimental animals have a moral responsibility for the welfare of animals during and after their use. Investigators are responsible for the aftercare/rehabilitation of animals after experimentation and may be permitted to euthanize animals only in the following situations:

(a) When the animal is paralyzed and is not able to perform its natural functions, it becomes incapable of independent locomotion, and/or it can no longer perceive the environment in an intelligible manner.
(b) If, during the experimental procedure, the animal has been left with severe recurring pain wherein the animal exhibits obvious signs of long-term extreme pain and distress.
(c) In situations where non-termination of the animal experimented upon would be life-threatening to human beings or other animals (for euthanasia: AVMA guidelines).

Costs of aftercare/rehabilitation of animals post-experimentation are to be part of the research cost and should be in a positive correlation with the level of the sentience of the animals.

Principle No. 5: The living conditions of animals should be appropriate for their species and contribute to health and comfort. Normally, the housing, feeding, and care of all animals used for biomedical purposes must be directed by a veterinarian or a scientist trained or experienced in proper care, handling, and use of the species

being maintained or studied. In any case, veterinary care shall be provided as indicated.

3.5.3 Associations

Apart from government institutions, some associations like LASA and LASAI are doing great work in the area of animal welfare.

(a) **Laboratory Animal Scientists' Association (India) (LASA):** LASA is a national-level association of people who work with laboratory animals. The association promotes animal welfare, ethics, and responsibility and the principles of 3Rs of Russell and Burch. LASA publishes a journal, *Journal of Laboratory Animal Science*, to promote the advancement of laboratory animal science.
(b) **Laboratory Animal Science Association of India (LASAI):** LASAI is also a national-level association to promote laboratory animal science. It works for laboratory animal welfare, welfare assessment, and ethics and also in the development of human resources.
(c) **Society for Alternatives to Animal Experiments-India (SAAE-I)**: SAAE-I is a national-level organization for 3Rs, with emphasis on alternative methods such as in vitro, in silico, and model organisms of lesser sentience than mammals where such are available. Where such alternative methods are not available, animal experiments may be conducted with the utmost animal welfare and ethical concerns.

3.6 Animal Ethics

Ethics is a systematic reflection of morality, and morality is an entity of norms and values. These entities and norms are different in different societies. But one common principle of morality is not to harm anyone without reason which is reflected in our legislation which enjoins us to be compassionate toward animals and avoid unnecessary pain and suffering. Deep ethical application for the animals is of prime importance for all the researchers working with laboratory animals. Such ethical issues must be considered at the individual level, the institutional level, and the national level. At an individual level, each researcher must follow all the ethical guidelines established at the institutional level. The head of the establishment, where animals are maintained for the experiment, must form an Institutional Animal Ethics Committee for experimentation to make sure that all experiments conducted on animals are rational, do not cause undue pain or suffering to animals, and are with a minimum number of animals and that no alternative methods can be used (the 3R principle).

Ethics Is Different from Law

Ethical norms are broader and more informal than law. An action may be legal but unethical or sometimes illegal but ethical.

3.6.1 Ethical Guidelines for Use of Animals

1. Animal experiments should be considered only after due analysis of their necessity for human or animal health and the advancement of knowledge.
2. Animals selected for experimentation must be of appropriate species, quality, and age. Only a minimum number of animals should be used to obtain scientifically and statistically valid results.
3. Animals should be treated with kindness, and investigators should take proper care by avoiding or minimizing discomfort, distress, or pain.
4. Investigators should assume that all procedures that induce pain in humans may cause pain in other vertebrate species until proven otherwise.
5. All procedures that cause more than momentary pain or distress should be performed under appropriate sedation, analgesia, or anesthesia following accepted veterinary practices. Surgical or other painful procedures should not be performed on unanesthetized animals.
6. At the end of, or whenever appropriate, during, an experiment, the animal that would otherwise suffer severe or chronic pain, distress, discomfort, or disablement that cannot be relieved or repaired should be euthanized with the minimum of pain, fear, and distress.
7. The best living conditions should be provided to laboratory animals. Normally, the care of animals should be under the supervision of a veterinarian or a person having adequate experience with laboratory animal care.
8. Investigators and personnel in-charge of animal facilities should see to it that personnel conducting experiments on animals possess appropriate qualifications or experience for conducting the required procedures. Training should be provided by an institution to those involved in animal experimentation.
9. Wherever possible, alternative in vitro system should be used to replace and reduce the number of animals used for experiments.

3.6.2 Responsibilities for the Implementation of Ethical Guideline Rest with the Institutional Animal Ethics Committee (IAEC)

1. Every experiment shall be performed by or under the supervision of a person duly qualified on that behalf, that is, degree holders in veterinary science, medicine, or laboratory animal science of a university or an institution recognized by the government for the purpose and under the responsibility of the person experimenting.

2. That experiments are performed with due care and concern, and as far as possible, experiments involving surgeries are performed under the influence of some anesthetics of sufficient power to prevent the animals from feeling pain.
3. That animals which, in the course of experiments under the influence of anesthetics, are so injured that their recovery would involve serious suffering are ordinarily medically allowed to death while still under influence of anesthetic or euthanized.
4. That experiments on animals are avoided wherever it is possible to do so. Experiments on larger animals are avoided when it is possible to achieve the same results by experiments on small laboratory animals such as guinea pigs, rabbits, mice, rats, etc.
5. That as far as possible, experiments are not performed merely to acquire the manual skill. That animals intended for the experiments are properly looked after before, during, and after the experiments. That suitable record is maintained concerning experiments performed on animals.
6. IAEC should focus mainly on ensuring ethical and methodical handling of animals during and after experiments, that animals do not suffer or undergo less suffering.
7. IAEC will review and approve all types of protocols for research involving small animal experimentation before the start of the study.
8. For the approval of experimentation on large animals, the case is required to be forwarded to CPCSEA in a prescribed manner with the recommendation of IAEC.
9. IAEC is required to monitor the research throughout the study and after completion of the study; IAEC shall obtain the periodic reports on research development and shall ensure a visit to the animal house facility and laboratory where the experiments are conducted. The committee has to ensure compliance with all regulatory requirements, applicable rules, guidelines, and laws.

3.6.3 Composition of IAEC

The Institutional Animal Ethics Committee shall include eight/nine members as follows:

A) Members from the establishment (05 members):
   1. One biological scientist.
   2. Two scientists from different biological disciplines.
   3. One veterinarian involved in the care of animals.
   4. One scientist in charge of the animal facility of the establishment concerned.

B) Members from outside, nominated by CPCSEA:
   1. Main nominee of CPCSEA.
   2. Link nominee of CPCSEA.
   3. A scientist from outside the institute.
   4. Socially aware nominee.

The chairperson of the committee and member secretary would be nominated by the establishment from among the above five members. However, if the establishment wants to propose its administrative head, who is from a nonscientific background, as chairperson, then six members of IAEC may be proposed. Having a veterinarian in IAEC is mandatory for judging the level of care and handling of laboratory animals in a given protocol.

3.6.4 Challenges of the IAEC

- Interpretation of the principle of reduction by ethics committees leads to underpowered studies (Type II error). Hence, reduction may be trumping optimal experiment design.
- Rigor should be central to the ethical review of experiments using animals. Available evidence suggests that this is either not being done at all or not being done well.
- Harm and benefit evaluation of what will be the benefit of the work? Who will benefit from the work? How will one assess benefit? When will the benefit be achieved?
- If the project fails the test, has the system failed itself?
- Caring "on-comouse" of the same level as a human patient in the clinical trial of a new medicine.
- Assigning of value or rights to being, depending on their affiliation to certain species.
- Laboratory animal education should be mandatory as per CPCSEA.

3.7 Conclusion and Future Perspectives

While we agree that complying with the guidelines, rules, and regulations may not make animal experiments "translational" to humans, due to unmanageable species differences, we should endeavor to ensure that animal experiments are planned in advance and prudently, taking into account welfare, ethical, and regulatory provisions, and with as much scientific rigor as possible. Otherwise, they are destined to be unworthy from the very beginning.

In the future, we should consider and implement relevant animal welfare, ethics, and law in practice:

1. Competence-based teaching in laboratory animal science should be done at regular intervals to raise researchers' awareness of regulatory, ethical, and welfare measures.
2. Rigorous two-tier peer review process adopted by funding agencies and research institutions before funding animal research.
3. Provision of mandatory certified training of IAEC reviewers.

References

1. Broom, D. (2014). Sentience and Animal Welfare. CABI, UK
2. Brambell, FWR. (1965). Report of the technical committee to enquire into the welfare of animals kept under intensive livestock husbandry systems. (Command Rep. No. 2836). London: Her Majesty's Stationary Office

Further Suggested Reading

Committee for the Update of the Guide for the Care and Use of Laboratory Animals, Institute for Laboratory Animal Research, NRC of National Academies. Guide for the Care and Use of Laboratory Animal, (2011) Eighth edition, National Academies Press, Washington DC

Compendium of CPCSEA (2018) Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Environment, Forest & Climate Change, Government of India, Animal Welfare Division. India

NC3Rs 3Rs in Toxicology and Regulatory Sciences. National Centre for the Replacement, Refinement & Reduction of Animals in Research (https://www.nc3rs.org.uk/3rs-toxicology-and-regulatory-sciences)

NC3Rs Gremace Scales. National Centre for the Replacement, Refinement & Reduction of Animals in Research (https://www.nc3rs.org.uk/mouse-grimace-scale)

Tandon PNK, Muralidhar K, Gupta YK (2014) Use of animals in scientific research, Indian National Science Academy, New Delhi

van Zutphen LF, Baumans V, Beynen AC (eds) (2001) Principle of laboratory animal science. Elsevier

Planning and Designing of Laboratory Animal Facilities

4

Krushnarvind B. Patel, Vikas Galav, and S. G. Ramachandra

Abstract

Planning and designing a laboratory animal research facility is an intense and innovative process. It must support the proposed research activities of the institute. The features required in the design and construction differ between institutes depending on the goals and objectives. A safe, consistent, and hygienic environment should be provided for animals and staff working for animals. The shape, size, and features of the animal rooms will depend on the intended use. Operationally, vivarium infrastructure space is separated into the animal housing area and support area. Generally, animal housing space allocated 30% and support space 70%, which varies depending on size and functionality of vivaria. Typically, the smaller facilities require a percentage of support area.

Managing an animal facility is very challenging and needs the harmonized effort of different staff working in the facility. Besides dedicated support areas, it is vital to pay attention to the micro- and macroenvironment while designing the animal facility. Designing and installation of the HVAC system are critical. This chapter describes the features and critical aspects of the modern animal facility.

K. B. Patel
Center for Comparative Medicine, Institute of Liver and Biliary Sciences (ILBS), New Delhi, India
e-mail: kbpatel@ilbs.in

V. Galav
Post-Graduate Institute of Veterinary Education and Research (PGIVER-RAJUVAS), Jaipur, Rajasthan, India

S. G. Ramachandra (✉)
Central Animal Facility, Indian Institute of Science, Bangalore, Karnataka, India
e-mail: sgr@iisc.ac.in

© The Author(s), under exclusive license to Springer Nature Singapore Pte Ltd. 2021

P. Nagarajan et al. (eds.), *Essentials of Laboratory Animal Science: Principles and Practices*, https://doi.org/10.1007/978-981-16-0987-9_4

Keywords

Vivarium · Housing · Architectural features · Functional areas · HVAC · Environmental control and monitoring

4.1 Introduction

It is well established that the reliability and reproducibility of research using animals are augmented by controlling research variables. One of the primary requirements for this is a well-planned, optimally designed, and adequately maintained animal facility. Variables such as physical, chemical, biological, psychosocial, and genetic factors (Fig. 4.1) can impact the outcome and quality of animal research. Controlling genetic variables is mostly nature dependent; however, controlling other variables is primarily reliant on the floor plans, engineering features, and overall operation of the animal facility. Different methodologies in planning, designing, and managing the

Fig. 4.1 Schematic illustration of variables that confound animal research outcome

environmental standards keep on advancing to achieve better performance levels and operational efficiency.

Environmental variables directly affect the animal's biology and result in variation in biological data, thus confounding the interpretation of experiments. In modern science, animal welfare and comfort are given paramount importance. Hence, science-driven environmental control should be put in place in the animal facility for the animal's well-being. The responsibility for the animals' well-being to provide its comfort and assist scientists solely lies with laboratory animal specialists.

Therefore, the floor plans, engineering features, and overall operation of the facility are vital for good animal welfare of laboratory animals, which in turn responsible for a good science. This chapter describes the planning process and salient design and engineering features for a laboratory animal facility to provide optimal standards in animal care and use.

4.2 Approach to Facility Planning

It is always advisable that before designing the animal facility, a clear consensus must be arrived on the goals, plans, and objectives of the proposed facility. Planning is identifying, defining, and detailing the facility needs with feasibility checks that are likely to be incorporated in the facility's design. Planning an animal facility requires a genuine understanding of the needs of all stakeholders, including researchers, veterinary team, managers, engineers, and administration.

To develop a good laboratory animal facility, a thorough knowledge of laboratory animal medicine and its husbandry and understanding architectural requirements are mandatory. There are various categories of facilities for housing laboratory animals such as conventional, barrier, and containment facilities. These are defined as follows:

In conventional housing, animals are reared in conditions devoid of specific barriers and containment.

In the barrier facility, housing animals are protected from exposure to undesirable microbes.

In containment housing, housing is designed to prevent leaking of hazards that are biological, chemical, or radiological in nature.

4.2.1 Stage I: Understanding the Needs

The first stage in planning involves identifying the needs of the research animal facility. Representatives of all stakeholders, including research groups, lab animal specialists, veterinarians, engineering personnel, occupational health and safety personnel, and research policy administrators should create a "Stakeholder Group" to discuss, review, and determine the specific needs of the animal facility. Such requirements are categorized in terms of the following:

1. Diversity and dimensions of research projects.
2. Animal species and quantity to be housed.
3. Range of animal types/models (transgenic, immunocompetent, immunocompromised animals, etc.)
4. Cage types or housing.
5. Laboratory or support space.
6. Cage-changing/cage-washing systems and schedules.
7. Decontamination and sanitation.
8. Feed, bedding, and water quality and their use and disposal.
9. Environment, vibration, and noise controls, including the heating, ventilation, and air-conditioning/air handling units (HVACs/AHUs) and backup power requirements.
10. Movement of personnel and material and functional relationship with various support areas.
11. Access to engineering maintenance.
12. Vermin control.
13. Animal transport/loading-unloading areas.
14. Animal quarantine requirements.
15. Biomedical waste collection, storage, and disposal requirements.
16. Biocontainment and overall security and safety needs.
17. Physical location of the animal facility (interconnected with research labs, independent or standout facility, etc.)
18. Overall space requirements to meet the above requirements.

4.2.2 Stage II: Reviewing and Feasibility Studies

In the second stage of planning, a detailed review of the setout needs or master plan proposed is done in consultation with facility managers, veterinarians, designing experts, and budgeting authorities. Requirements defined by the user groups in phase I should be reassessed in terms of future research policies and priorities of the institute, competing research needs, oversight in terms of animal quantity, quality requirements, space or energy requirements and the availability of budget, and fixed as well as running expenses for the animal facility. Estimating life cycle-based costs of the animal facility is essential for creating various facilities/equipment/systems in animal facilities than its initial costs.

Exhaustive feasibility studies are carried out at this stage to thoroughly reassess the scope and accommodate the necessary functionalities of the proposed animal facility plan. Consultants can assist in this exercise to identify and refine the operational challenges, project constraints, constructability, critical issues, logistics, and transition scenarios to establish a probable costing of the project and develop a preliminary schedule for implementing the plan.

4.2.3 Stage III: Designing Stage

Following the feasibility studies of the proposed plan, a final decision on the location for research animal facilities should be decided and, subsequently, the project's conceptual design.

The design team would prepare floor plan layouts, building elevations, contraction finishes, electrical and plumbing systems, and configuration of significant engineering systems to confirm the building's functionality and integrate it into the building's chosen site and context. The average life span of any animal facility building is approximately 15–20 years. However, since the pace of scientific research always supersedes it, most animal facilities may require modifications or expansions every 5–10 years. Therefore, a creative conceptual design for the project to optimally utilize available spaces, to be ready for future realignment, redesigning, or expansion, is required.

It is advisable to follow a "validating and commissioning system" in a phased manner for animal facilities, instead of only "commissioning" the complete project at the final stage. This concept should always be inbuilt from the design stage itself. Smart virtual 3D technologies could help ascertain the functionalities and adjacencies of animal holding and supporting areas while foreseeing the movement of personnel and materials between various sections of the facility. This assists in designing the facility fulfilling all future needs smartly.

The design team should present specific information until the last phase and suggest if any amendments or changes are required. This would help to finalize the design details and subsequently handed over to the construction team.

4.2.4 Stage IV: Construction and Commissioning

Once the project design is ready, it is submitted to the department concerned for obtaining permits, approvals, and budget clearance. All the drawings and specifications should be rechecked to ensure smooth and coordinated completion of the construction.

Looking at the complexity of the animal facility, creating functional designs, getting the construction started and completed in a coordinated manner, and ensuring that they function as designed are extremely important to any successful management of research animal facility.

4.3 Locations and Size

The location of the facility is very critical for establishing the facility. The site selection is critical in areas where natural calamities such as geophysical (earthquakes, volcanic activity), hydrological (avalanches, floods), and meteorological (cyclones, tornadoes) [1–3] calamities are frequent. Site selection for containment level animal facilities requires special consideration concerning the environment, bioterrorism, and biohazard safety. Local environmental factors and HVAC system design play a major role in designing.

The location should preferably be away from other research facility given the considerations of health, security, human comfort, etc. However, for proper planning for access to research staff, supplies must be done. Besides, the facility should be planned and designed accordingly to diminish contamination within animal rooms and redundant personnel exposure to animals and its waste products.

The proposed animal facility size can be calculated by considering institute's own or others experience in allocating vivarium space in comparison to wet laboratory space or number of in vivo research investigators/scientists [4–7].

4.4 Arrangement

Single-floor animal facility especially located at the ground floor is generally the most efficient operationally as it does not require vertical transportation of material and personnel [8, 9]. In a multistory facility, the configuration is different from a single-story facility with two dedicated elevators so that clean and soiled dirty materials are transported unconnectedly. The main disadvantage is that the multistory is less efficient to operate than a single-story building.

4.5 Circulation

Movement of material, people, and animals inside the facility as well as entry-exit from the facility is called circulation. In designing an animal facility, clean area (clean corridors, animal rooms, clean cage holding area, procedure room, etc.) and service area (cage washing area, necropsy area, and dirty area for carrying dirty cages) should not be intermixed. Various factors like staff, animals, consumable items, chemicals, equipment, and facility-generated waste [8, 9] contribute to the effect of circulation. Hence, careful consideration in designing elevators, corridors, entry, and exit patterns must be given, to avoid contamination.

In a single-corridor system, there is one-way traffic movement containing one central corridor with animal holding rooms on both sides resembling a U-shape (Fig. 4.2). The single corridor can be uni- or bidirectional. The disadvantage of a single corridor is there are higher chances of cross-contamination and mixing of contaminants. However, adopting proper procedural controls, engineering controls, and the use of personal protective equipment (PPE) could prevent intermixing and contamination. Usually, husbandry activities and transfer of clean materials (bedding, food, cages, etc.) should be performed in the morning, and the transport of dirty materials including cages and bedding materials should be done in the evening. In the dual-corridor system, separate corridors for the movement of clean and dirty operations will be built that prevent cross-contamination between animal rooms. Secondly, husbandry activities and procedure activities could be planned with flexibility for any time of the day. The disadvantage of the dual corridor is the high initial cost since 15–20% larger space is required as compared to a single-

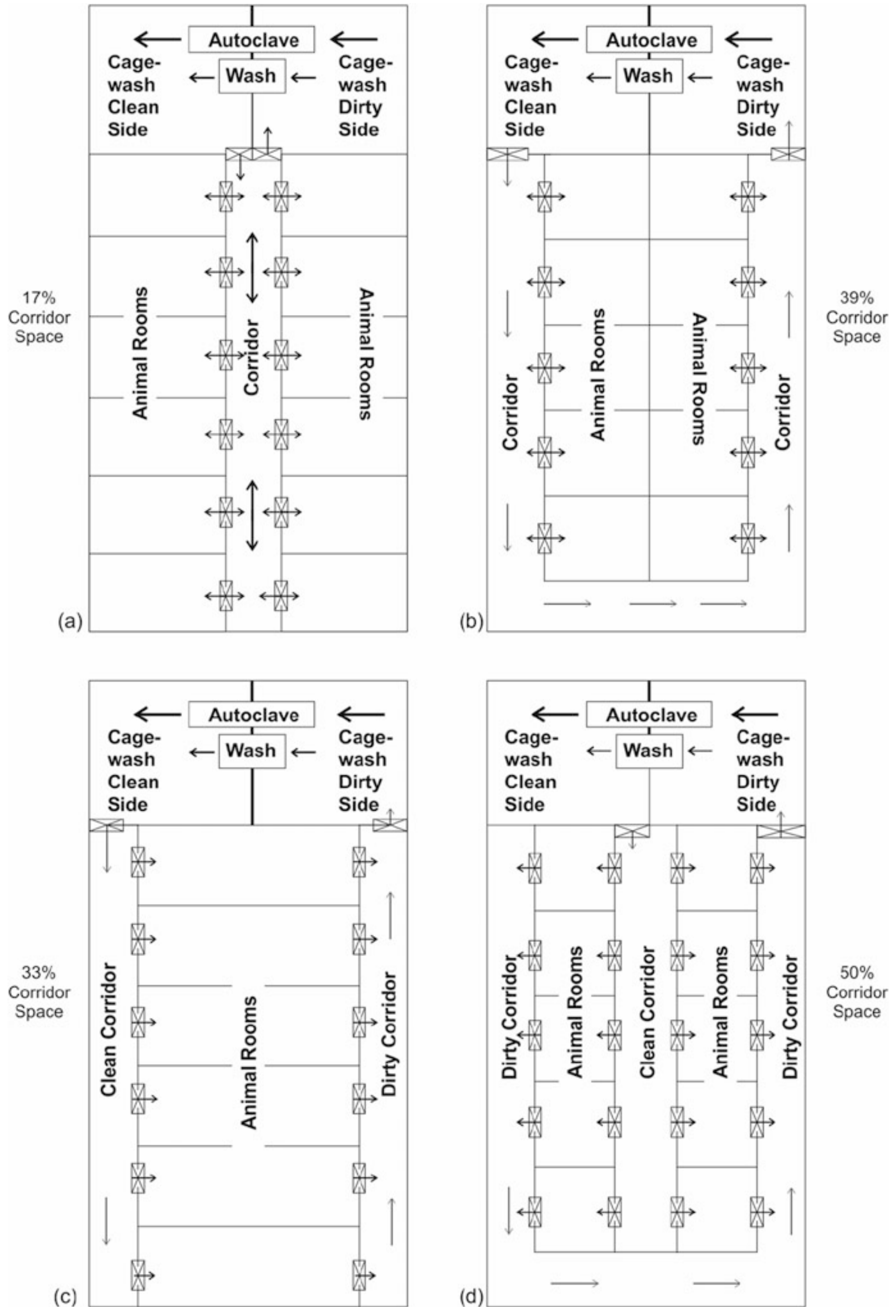


Fig. 4.2 Facility design (**a**) single-corridor with bidirectional flow; (**b**) single-corridor with unidirectional flow; (**c**) dual-corridor with large animal rooms; (**d**) dual-corridor with relatively small animal rooms

corridor system. It also increases labor costs under the recurring cost. In both systems, horizontal circulation patterns are usually followed.

4.6 Functional Area

Animal facility space can be divided into animal housing and support space as per the functional use of the space. The ratio of animal housing to support and use spaces differs among facilities, depending on requirements [8, 9]. However, the following areas are essential for animal holding and support areas for a laboratory animal facility:

Functional area:

I. Animal housing area.
   - Animal holding rooms.
   - Breeding rooms.
   - Animal quarantine rooms.

II. Support area.
   - Procedure room.
   - Surgery room.
   - Necropsy rooms.
   - Behavioral studies.
   - Laboratories for diagnostic procedures.
   - Imaging rooms.
   - Washing areas for cage and types of equipment.
   - Clean and dirty cage loading docks.
   - Autoclave/sterilization area.
   - Clean cage and equipment storage/sterile store.
   - Reception areas for personnel (changing rooms) and animals (loading bays).
   - Administrative space for animal care staff and others.
   - Shower/change room and break area/small meeting room.
   - Toilets and janitors.
   - Storage area for animal feed, bedding, chemicals/biologicals, and wastes.
   - Pharmacy – drug dispensing.
   - Waste disposal room.
   - Cold room.
   - HVAC/service space.
   - Security features control room.
   - Incineration/alkaline hydrolysis.

The above requirement of specific functional areas is determined by considering the size of animal care and use program, species housed (small/large), animals maintained in special conditions (i.e., gnotobiotic, specific pathogen-free, immuno-compromised, small/large, natural, or environmentally controlled housing), types of research programs, etc.

4.7 Structural Features

4.7.1 Interior Surfaces

The interior surfaces of an animal facility should support thorough cleaning and, hence, be resistant to detergents, moisture-proof, relatively smooth, and free of cracks, crevices, and pinholes. The interior surfaces should be easily and safely repairable while facility operations are going on. Any interior surface penetrations and junctions in the animal room, clean area, and procedure room should be sealed to facilitate balancing of relative air pressures and restrict vermin access.

4.7.2 Floors

The floors should be resistant to disinfectant wash, high-temperature water, and detergent cleaning and also support the continuous movement of cages and equipment inside the facility.

The floor should have a concrete base, preferably monolithic or having a minimal number of joints for effective sanitation and prevention of microbial accumulation. Vitrified tiles or seamless epoxy, polyurethane, and methyl methacrylates like resins with different thicknesses are some of the routinely used flooring materials. Vinyl sheet flooring with appropriately welded joints/seams can be considered as alternate flooring option. In areas where noise reduction is required, the preferred flooring option is hardened rubber base aggregates flooring. Properly installed sturdy ceramic tile flooring can be a choice of flooring for cage sanitation area, which offers advantage of minimal maintenance. The sturdy tiles joints should be grouted specially to make them nonporous and chemical- and moisture-resistant. Animal holding rooms and hallways have trolleys and racks movement which generates more noise on grouted tile flooring and makes it an unacceptable flooring option for these areas [8, 9]. Do not underestimate the importance of correct installation of flooring as the best flooring material selected may not meet the required expectation, if installed improperly.

4.7.3 Walls and Ceilings

In an animal facility, walls and ceilings are required to be cleaned and sanitized with detergents and disinfectants along with even a high-pressure water so they should be smooth finished, free from moisture impacts, nonabsorbent, and able to withstand cleaning with detergents, disinfectants, and high-pressure water [8–10]. The walls should be crack-free with sealed pipes and service sleeves to prevent vermin. Concrete blocks can be used as walls with fiberglass strengthened plastic panels. A metal frame can also be used with drywall. However, wooden framing is not recommended. The protective guardrails or wall curbs should withstand sanitizing

and should prevent the entry of vermin. It is essential to seal the ceiling-to-wall junction to restrict vermin access. A suspended ceiling is usually avoided.

4.7.4 Doors and Windows

Animal facilities require an easy movement of large size equipment of animal rooms, i.e., racks, cage change stations, trolleys, etc., so to facilitate this movement, single door and double doors should have minimum 120 and 180 cm opening, respectively [10]. Stainless steel or fiberglass-reinforced polyester door frames are a more economic and maintenance-free option in the long run. Usually, it is preferable to consider doors with automatic door closure mechanism along with protective hardware, i.e., shielded handles, door seals/sweeps, kick plates, jamb guards, etc. Doors needed to open into animal rooms for safety reasons; if doors require opening toward a corridor, there should be a recessed vestibule. Viewing panel/port is recommended on doors for observation and safety reasons, which needs to be covered to avoid disturbances in animal circadian rhythm. Especially, rat and mouse holding room's doors, viewing panel can be covered with red color glass as they have dichromatic vision and perceive red color as dark [11–13]. If heavy traffic is anticipated, automatic sliding or hinged doors or wall-mounted push plates are provided. Door seals should be provided for balancing ventilation and controlling the air movement.

4.7.5 Drainage

Floor drains with liquid drain traps kept should be installed, and the floor should have a proper slope. To control humidity, drainage should permit the quick water clearing and drying of surfaces to reduce the duration of increased relative humidity [14]. Generally, 4-inch diameter drain pipes can be adequate to serve the animal facility and larger drain pipes ($\geq$6 in.) for cage sanitation areas or other areas with high water usage. An additional flushing mechanism at the rim and/or trap of the drain or an in-line comminutor will help in solid waste disposal. At present, many lockable drains are used to prevent the backflow of sewer gases, insects, and other contaminants. Rodent rooms can be sanitized satisfactorily without hosing down, hence may not need drains. The installation of floor drains in all the animal rooms with covering/capping will cater to future housing of non-rodent species.

4.7.6 Corridors

Corridors should be 6–8 feet wide to facilitate personnel and equipment movement in animal facilities. Corners between floor and walls should be coved for easy and thorough cleaning. Wall protective systems such as crash or bump rails should be provided at an appropriate height by considering facility equipment. In high noise areas such as cage washing facilities, dog housing, nonhuman primate areas, etc.,

corridor should be provided with enclosed entryways with two doors or other mechanisms to contain the noise. Enclosed entryways with two doors also serve the purpose of airlocks to follow the containment principles at required places. Efforts should be made to provide accessibility of utilities (pipes of water, drain, HVAC ducts, valves, electric conduits, etc.) for service purpose via false ceiling trap doors or interstitial space from corridors to prevent the service-related entry into animal holding rooms. Installation of ancillary items like fire alarm, fire extinguishers, telephones, Internet boosters, etc. should be done at appropriate place and height with some protection (covering/bumper guards) so it can prevent damage by large equipment movement through hallways.

4.7.7 Exterior Windows

Windows in animal rooms should be avoided as they create a potential security risk, a problem with temperature control, and difficulty in strict control of photoperiod (critical for rodents' breeding colonies). However, windows are recommended for large animals' (nonhuman primates, dogs, etc.) housing areas as they serve the purpose of environmental enrichment.

4.7.8 Vermin Control

The animal facility should be free from pest infestation by planning and implementing sound vermin control programs, which can prevent, control, or eliminate pest's infestation. Written and properly implemented pest control programs will help to eliminate pests. During construction, it's imperative to properly seal all cracks, joints, power outlets, and wall ceiling edges. Animal housing areas should have a minimum interior build-in if needed, for effective cleaning and eliminating hiding places for vermin.

Holes, blind end spaces, gaps, and cracks in the facility should be adequately sealed and treated with amorphous silica dust to remove the harborage of pest. The ingress of pest and insects into the facility should be reduced by using high-pressure sodium lamps or dichrome yellow lamps at external entry ports. High-velocity air curtains, insectocutor, and other pest control mechanisms are helpful tools to reduce the ingress of flying insects and should be installed at appropriate places. Improper usage of pesticides in animal facility especially in animal holding rooms can cause toxic effects on animals, adversely affect the normal physiology and in turn negatively impact the in-vivo research outcome of animal research [15]. Hence, consideration should be given to use nontoxic types of vermin control, which disrupt the growth and reproduction of pest such as insect growth regulators [16, 17].

4.7.9 Noise and Vibration Control

Noise and vibrations are other potential variables for animals causing changes in breeding and behavior, as well as several other physiological effects involving cardiovascular and stress pathways [18]. Rodents and humans can tolerate noise up to 80 dB, but guinea pigs, primates, and cats cannot tolerate more than 60 dB [19]. The primary noise areas are cage washing and sanitation areas, housing areas of canine, and some nonhuman primate species rooms. The noise from these areas can be prevented using high-density masonry walls, double entry doors, sound-attenuating doors, soundproof walls, subitizable sound attenuating panels, etc. Whistling noise may be produced in an animal room when there are improper ventilation ducts and outlets and improperly sealed rooms and when air balancing is not proper. To reduce noise levels in a facility, commercially available sound attenuating panels can be considered for use in a noise-generating area of the facility [20]. Besides, noise level inside the animal rooms can be reduced by appropriate placement of emergency alertness systems (alarms and speakers) used for people. Rodent facilities should consider using emergency alarms which produce noise frequency of 400 and 500 kHz and do not generate an ultrasonic noise.

The noise and vibration effects on rodents are well documented by performing several studies and found that these tend to increase corticosterone levels and alter various biochemical parameters or reproductive efficiency. The source of vibration is mainly from mechanical equipment in the animal facility and other building components or remote sources. As per Section 5-2 of US guidelines (NIH 2008), vibration velocity must be less than 100 μm/s in an animal facility, down to 50 μm/s for animal behavior and holding rooms. Building structure requires special consideration when they are located adjacent to high vibration-generating infrastructures, i.e., busy highways, metro, railway, or public bus stations, etc. to minimize vibrations in an animal research facility. It is of paramount importance to know the vibration-generating places around and to keep engineering mechanisms (vibration suppression systems) to either separate or reduce the vibrations [21].

4.8 Engineering Features

4.8.1 Heating, Ventilation, and Air-Conditioning (HVAC)

The environment immediately around the animals, within the animal room, and in the overall animal facility is very crucial for animals as well as people, which can be maintained by HVAC system. Important components of this system are to supply clean air, maintain consistent temperature and relative humidity in species-specific ranges, maintain appropriate specified pressure gradients in different areas, exhaust out air contaminants including heat, and maintain specified fresh air changes. HVAC

system design requirements are specific for animal research facilities compared to other types of buildings, which are explained in "Laboratory Animal Rooms," section of The American Society of Heating, Refrigerating, and Air Conditioning Engineers (ASHRAE) Handbook of HVAC application [22]. HVAC system cost is the highest in different components of designing and building a modern animal facility.

4.8.2 Air Quality

The air quality delivered to the facility depends on external air quality and further treatment of this air by filtration. The supply air source and exhaust air source should not be mixed. The supply air can be filtered with different pore size filters starting from course prefilters to fine HEPA (high-efficiency particulate air) filters with 85–99.97% efficiency for different areas as per the need. For instance, HEPA-filtered air may be provided to maintain defined microbial status rodents and operation theatre, while other areas are provided with less efficient filtered air, i.e., 95%. It is to be noted that individually ventilated caging (IVC) system and cage change station are equipped with HEPA filters, which preclude the need of HEPA filtering complete air delivered to the animal rooms (except immune compromised animal rooms) and reduce the cost of HVAC installations substantially.

4.8.3 Ventilation

The various factors that determine ventilation systems in an animal facility are:

1. Type of species and numbers/density.
2. Recommended room temperature and relative humidity.
3. The load needed for each room for heating and cooling.
4. The heat generated by the animal inside the room.
5. Caging system (isolated static, filter top, or individual caging systems).
6. Use of biosafety cabinet, cage changing station, and fume hoods.
7. Sanitation and cleaning methods in animal rooms.
8. Particulate matter, airborne contaminants, and noise.
9. Institutional animal care standards/compliance to legislation.

Air handling units (AHUs) need to be independent for both supply and exhaust with 100% supply of air from outside/fresh air. The ventilation rate or fresh air changes per hour (ACH) should be in the range of 10–20 ACH, depending on the density of animals, heat load, particulate matter, and other contaminant gases like ammonia and CO_2 in the room. The rodent room equipped with individually ventilated caging system having 60–100 ACH with the provision of exhausting air from IVCs directly out of the room needs around 5–6 ACH [23]. Levels of important gaseous contaminants like ammonia (NH_3) and carbon dioxide (CO_2) inside animal

cages (microenvironment) and in holding rooms (macroenvironment) are critical to the health of both animal occupants and human workers. These levels are closely linked to ventilation rates. Ammonia acts as an irritant to mucosa, and at more than 25 ppm, it may cause physiological changes and research interference. CO_2 is a respiratory and cardiovascular stimulant and increased levels can cause suffocation. The American Conference of Governmental Industrial Hygienists, 2004 [24], can further be referred as it recommends safe exposure limit to gaseous contaminants and required ventilation rates for effective control.

HVAC systems are of two types: constant volume reheat (CVR) type and variable air volume (VAV) type. CVR systems used in animal facilities take 100% fresh air from outside and condition (filter, cool, heat, humidify) it and deliver it into the facility at constant flow rate. Different areas of animal facility have different heat loads and need different ventilation rates but almost have similar temperatures. VAV is the preferred HVAC system over CVR as it can give variable flow as required in different areas. While using VAV system, one needs to consider effectiveness of ventilation in terms of fresh air distribution in the room and removal of various air contaminants such as particles, allergens, microbes, etc.

4.8.4 Exhaust

There should be a balanced exhaust air system relative to the AHU supply. Exhaust air from animal rooms needs to be cleared outdoors without recirculation into any other room. To minimize the potential for cross-contamination, airflow direction should always be inward to the animal rooms every time.

There should be separate exhaust air systems for isolation rooms, research areas, fume hoods, biosafety cabinets, cage washers, central sterilization areas, toilets, janitors, and locker rooms.

Some of the equipment like fume hoods, certain types of biosafety cabinets, cage washers, autoclave, etc. need special consideration for exhaust ventilation. Biosafety cabinets and fume hoods need separate direct exhaust system. In built, cage and rack washers are directly connected with separate exhaust. Autoclaves and cage and rack washers release heat and moisture and must be provided with dedicated exhaust mechanism like a canopies just above the doors of these equipment. The cage wash area requires a dedicated exhaust system, which includes exhaust fans and sloping nonferrous ducts, to reduce high heat and moisture loads in this area. Ventilated rodent cage system exhaust should be directly connected to the building HVAC system as filtration mechanism of ventilated rack takes care of only microbial and particulate contaminants and excludes gaseous contaminants and heat load. Different ways to connect supply and exhaust air of ventilated racks to building ventilation have been reviewed [25–27]. Individually ventilated racks are supplied with HEPA-filtered air, and exhausted air is passed through HEPA filter by the provision of separate stand-alone ventilation units equipped with blowers, prefilter, and HEPA filters. In some configurations, ventilation units may be fixed on top of the ventilated racks or the wall shelves and connected with flexi ducts.

Equipment and their ventilation connection strategies are important to decide at the early phase of planning of an animal research facility. It affects the physical couplings of the rack and can impact overall Cubic Feet per Minute (CFM) requirement and air handling unit capacity for that area/room. Direct connection of ventilated racks with facility exhaust efficiently removes only gaseous contaminants but not the heat load. About 20–35% of the heat load of exhaust air is dispersed in the animal room by convection and radiation [28].

Overall, many factors influence HVAC capacity, types, performance, and energy-efficient design, which should be decided by HVAC experts after critically considering all the variables at the planning stage. Installation of HVAC system contributes substantially to fixed costs and later also in operating costs, so decisions on the suitability of HVAC system are important for a cost-effective and maintenance-friendly animal facility. HVAC installation is also crucial from the welfare point of view and is often subject to scrutiny by ethics committees for its capacity to aid animal welfare as per legislation.

4.8.5 Plumbing

The early decision during the facility design stage is critical for adequately concealing pipes to avoid pest entry in the facility. Creating additional plumbing for new sinks and drains becomes challenging. It is wiser to make provision at the initial stage, for any future plumbing requirements than to meet the roadblocks later.

4.8.6 Sinks

Sinks in animal rooms are useful for handwashing, dumping water post sanitation, and some miscellaneous use while working with animals. So, depending on water use in animal rooms, sinks are desirable in animal rooms, but it adds to additional costs to provide drainage too. For ABSL-3 animal rooms, provision of a sink is a must [29, 30]. Wall-mounted hands-free or automatically operated stainless steel sinks are generally preferred to avoid impedance in-floor sanitation and effective use of animal room space. Providing dedicated floor sanitation equipment for each animal room is a good sanitation practice. One sink per suite may be sufficient if there is no risk of transmission of infectious agent transmission. In modular animal facilities, sinks can be avoided inside animal rooms with stringent standard operating procedures to address cleanliness and sanitization of personnel moving between rooms.

4.8.7 Floor Drains

Generally, the drainage is not needed for the rooms in which animals are housed in cages with dry bedding material though there are advantages and disadvantages in

providing drainage in these types of animal rooms. There are many disadvantages such as installation cost, confounding pest control, the potential for sewage backing up and sewer gas escaping into the room, etc.

Animal rooms and washing areas intended for hose-down cleaning must need drains with an appropriate location and size. Depending on the need for dry waste disposal through drains, an extra rim and/or trap flush fitting mechanism is selected. Generally, considering the arrangements of pens/trough, i.e., on a single side or room, on a double side with an aisle toward the walls, or in the center, a minimum floor slope of 1.6 cm per meter toward the drain should be provided. Drain pipe diameter should be 4 or 6 inches depending on water and waste material required to pass through the drain pipes. Detailing of drain specifications for small or large animal facilities and any future requirements must be addressed at the planning stage.

4.8.8 Drinking Water

Drinking water may be provided through automatic watering or water bottles. Depending on the preference and on the quality of groundwater, reverse osmosis (RO) water, acidification, chlorination, or potable water may be provided. It is essential to decide on all these points at the planning stage because each option needs special plumbing and equipment.

Reverse osmosis is simple and relatively inexpensive and widely used to provide filtered uniform quality water to laboratory animals at different locations worldwide. With water bottle usage, chlorination is not the right choice as chlorine dissipates; hence, acidification could be a better choice.

4.8.9 Hoses

Hoses are required in sanitation areas and animal rooms. Generally, hose reels dropping from a ceiling or upper wall with the centralized supply of pressurized re-circulating warm water (~ 49 °C) for the full facility are highly recommended.

4.8.10 Safety

Safety eyewash and shower stations, fire emergency signage and controlling equipment, biohazard signages, CCTV cameras, and emergency public address systems should be placed throughout the facility as per the local regulations.

4.8.11 Steam and Gas Service

Gases (O_2, CO_2, N_2, etc.) like compressed air, vacuum steam, hot, and cold water utilities are required for animal facilities. Generally, clean steam (not a pharmaceutical grade) produced by using reverse osmosis water is required for autoclave, HVAC heaters, humidification, cage washers, etc. in an animal facility. Steam generators may use chemicals which can have potential to interfere with animal physiology and research outcome; hence, clean steam is recommended for animal facilities' use. The autoclave chamber also needs to be provided with clean steam, which is used for autoclaving animal holding room's requirements. Compressed air is also specifically required for the autoclave. Vacuum and other gases depending on the requirement of animal facilities should be provided through gas manifold for safety reasons. This requires proper planning at the animal facility design stage to accomplish detailed plumbing work.

4.8.12 Bulk Detergent Delivery

Detergents are caustic and a large quantity of detergent near the cage sanitation equipment poses a safety concern. Detergents, acid, and neutralizing agents are stored in bulk near the receiving dock with proper precautions and directly piped to cage sanitation areas. The publication of Dysko and colleagues [31] is a useful resource on this topic for further reference.

4.9 General Animal Housing Concepts

4.9.1 Animal Holding Rooms

There could be two types of animal holding rooms depending on the usage of amount of water for room sanitation or housing: (1) rooms require a large amount of water (e.g., water-living animals and large animals such as canine, porcine, and nonhuman primates) and (2) rooms require small quantity of water (e.g., rodents and rabbits). One can design all animal rooms to support either both categories or of a single (one/other) category.

Floors sloping to floor drains become essential for category 1 animal rooms, where large amounts of water are used, but category 2 animal rooms do not require floor sloping or even drains. The room designed to accommodate both the categories may not meet the complete requirement of either.

One room size does not fit all and hence, there is no ideal room size for the animal room. Factors such as species, types of cage and rack system, and their arrangements in the room are to be considered in deciding room size and shape. For effective utilization of space, it is advisable to decide on these factors at the design stage itself.

Rodent racks (single- or double-sided) are arranged in different orientations and styles to make effective utilization of space and ease of daily operation. Depending

on the types of racks and room size, racks can be arranged like library style with leaving aisle in the center, single-sided racks against the walls, and mix of both types by making a central row of double-sided rack in the room and all around toward the walls' single-sided racks. Fixed furniture and stationary equipment other than a sink should be avoided in animal rooms as they reduce the effectiveness, disrupt sanitation operations, and also provide harboring space for vermin. Sizes of animal rooms and doors and dimensions of racks, trolleys, and sterilizers are required to be looked into for better utilization of space and effective workflow.

4.9.2 Animal Cubicles

These are small animal rooms within a big animal housing space that can provide isolation based on species, microbial status, experimental projects, etc. and can accommodate one or two racks. They are composed of three solid sides and the fourth side with hinged or shutter-type glass doors. Especially when more than one animal cubicle is arranged facing one another in a single large animal housing space, there should be at least 1.5 m distance between the opposite side facing cubicles. Generally, the concept of animal cubicles is used for small animals but can be used for large animals too [9, 32]. Owing to the advent of microisolation caging system for rodents, the popularity of animal cubicles has reduced, though cubicles effectively prevent the spread of airborne infectious agents among cubicles, when filter top cages are used. Open top conventional cages do not effectively contain the spread of airborne infectious agents among cubicles in the same room. Curry (1998) and Foster (1993) [33, 34] have explained several engineering options for animal cubicles with their advantages and disadvantages. These days, even prefabricated cubicles are also commercially available with the provision of lighting and ventilation with/without HEPA filtration.

4.9.3 Conventional Animal Housing

The animal room, facility, or area of a facility designed and operated without barrier or containment principles is known as a conventional facility. The facility, though it maintains good hygiene and proper welfare measures, does not have any provision for containing the entry of contamination.

4.9.4 Barrier Animal Housing

Barrier housing refers to the entire or specific area within a facility and its management practices, which are made to arrest the entry of diseases-causing agents. The barrier facility concept has evolved owing to the need to maintain specific pathogen-free (SPF) animals for the research community [35]. Now, barriers have become important for the researcher and research facilities as well because of the need for

diseases-free animals for study and usage of immunodeficient and genetically engineered animals in research. The barrier essentially means “keeping out” infectious agents. Static and ventilated types of microisolation caging equipment and flexible film isolators are commonly used to create barriers and work reasonably well. They are labor-intensive compared to barrier area or whole barrier facility.

The basic difference between a room-level barrier and a whole “barrier” facility is that the cages and supplies are required to be cleaned and sterilized outside with wrapping and transported inside the room-level barrier. In the barrier area/facility, wrapping, unwrapping, and transportation back are not needed as the autoclave opens inside the barrier.

In a large rodent barrier facility, cage washing and sanitation facility may be provided inside. This eliminates the need for autoclave cages routinely by using a cage washing machine at a minimum of 82.2 °C unless there is a disease outbreak. The best option is to provide an autoclave of appropriate size along with a cage washing and sanitation area inside the barrier [4]. Irradiated feed and bedding with double-bag packaging may eliminate the need of autoclaving them in the barrier. The outer bag of irradiated material should be removed outside the barrier, and the external side of the inner bag must be sanitized before taking into the barrier.

Different microbiological control levels are designed and managed for barriers depending on the provision of control on supplies and personnel entry in the barrier facility. Double door autoclaves, dunk tanks, entry-exit air showers/air locks with interlocking mechanism, and the required numbers of double doors are the key components to make the highest level of barrier facility. These vestibules are used to take packed sterile supplies and animals into filter boxes by chemically sanitizing external surfaces of packages into the barrier. Vestibules are also used to take out soiled equipment and trash. A barrier designed dunk tank filled with liquid chemical sterilant can be used for carrying heat-sensitive sterile materials inside the barrier by packing in watertight packaging.

Generally, personnel enter into a barrier by wearing the sterile garments covering the head to toe and other required personal protective equipment’s via interlocking double door air locks. Many times air showers equipped with HEPA filters and/or wet showers are added in the personnel entry port design, which offers enhanced protection to the barrier. Refer to Hessler (2009) [36] for further details about maintaining the rodent barrier facility.

Different research programs may require a barrier with specialized areas for performing animal procedures, analysis/testing, creating transgenic/knockout, animal imaging, irradiation, etc. Genetically engineered rodent-producing laboratories located within the barrier need a quarantine area to follow the quarantine of all the recipient mothers and cross verify their health status upon weaning the young ones. Animal cubicles are recommended for this purpose.

4.9.5 Containment Animal Housing

“Containment” is done to isolate the hazardous agents (biological, chemical, or radiological) to which animals have been exposed by using animal housing systems, design, and operational processes. Containment helps in protecting the workers, other animals, and the outside environment from potentially hazardous agents. “Containment” can be achieved at the cage level, the room level, and the area level within an animal facility or at the level of the entire facility. Animals are frequently used for studies in which hazardous chemicals, biologic agents, and/or radionuclides are administered. Specific engineering features may be used for controlling different types of hazards and to maintain a safe environment [29, 37]. The hazardous agents should be contained as close to the source as possible in animal facilities, and there should be an appropriate laboratory with the necessary equipment and animal procedure space inside containment facilities [38].

4.10 Support Areas

4.10.1 Administrative, Training, and Personnel Health and Hygiene

To manage an animal facility, coordinated effort of different categories of staff and dedicated office space is required [39]. The administrative area can be provided nearby to the animal facility to address the animal biosecurity concern. Training for animal care and use staff is a critical component of animal care and use program, and this area should include space for training, conferencing, and library. However, the space for hands-on training in animal procedures should be in the animal facility. The office space and its type and its area can be decided based on the overall activity of the facility. Animal technicians and supervisors spend most of the time in the animal housing areas; hence, their offices may be located throughout animal housing areas. The sitting space of veterinary technicians may be arranged in the facility adjacent to laboratories or the surgery area.

A safe, efficient, and healthy working environment must be provided for personnel working in the facility [40]. Animal facility environment poses potential risks such as animal allergens, infectious agents, chemical hazards, and physical hazards. To reduce the potential risks for transferring hazardous agents between home and the animal facility, animal care technicians are required to wear dedicated facility clothes. Other personnel working in the facility need to wear protective outer garments over their street clothes before accessing animal housing areas or at the entry of the animal facility. Support facilities should include a toilet, shower, locker rooms, and resting room for eating and drinking. A laundry room for laundering uniforms and surgical linens may be considered though commercial laundry service also meets the requirement.

4.10.2 Quarantine

There is a potential health risk to existing animal colony from externally procured animals. To mitigate any such risk, newly procured animals are required to be isolated or contained in the quarantine area for a defined time or simply a room designated for the purpose. BSL-2 standards established by CDC-NIH [29] provides useful guidelines for rodent quarantine facilities and practices. Rehg and Toth [41], Otto and Tolwani [42], and Lipman and Homberger [43] have elaborated effective quarantine programs for animal facilities. The quarantine facility must ensure the acclimatization of externally procured animals and provide containment until their health status meets established criteria.

Primary cage-level containment for rodents is desirable and provided by a static or ventilated isolator caging system along with the use of a ventilated change station or Class II, Biosafety Cabinet (BSC) for animal manipulation and cage changing. Secondary containment may be provided by different methods such as a flexible-wall, vertical-flow, and/or a holding room or rooms isolated physically from the facility.

The quarantine areas should be maintained at negative air pressure with the provision of proper airlocks. Material and personnel entry-exit procedures are very critical for maintaining containment. These can be achieved by providing dedicated PPE for quarantine personnel and, dedicated steam sterilizer for a quarantine area. If a separate steam sterilizer is not possible, the material must be bagged and external surfaces are chemically decontaminated upon exiting the quarantine area for remote sterilization.

There are many options for quarantine such as microisolation cages, gnotobiotic isolators, single-use disposable cages, etc., and each has its own merits and demerits [44]. One can select one or a combination of these depending on the facility needs.

4.10.3 Feed and Bedding Storage

Provision for sufficient storage space for essential items required for animal husbandry and care is necessary because of the possible inconsistency in the supply/procurement chain. Bedding and feed storage areas should be separate. These storage areas should be vermin-proof and free from the risk of toxic and harmful substances. Feed store should be located on a clean side or with a path to carry into a clean area without exposing it to the soiled area. Generally, feed and beddings are delivered on pallets and necessitate wide paths and doors between the receiving port and the storage areas.

The maximum recommended storage temperature for natural ingredient feed is 21 °C (70 °F) and relative humidity below 50% [10]. Purified and chemically defined diets are required to be stored at 4 °C (39 °F) as their shelf life is shorter in comparison to natural ingredient diet [45]. Cold storage space is also required for fresh food items (i.e., fruits, and vegetables). Safety testing laboratories often require the administration of potentially hazardous test substances by mixing in feed for

long-term studies, and this demands a highly specialized feed preparation area. The design criteria, finishes, and functions of storage areas have been deliberated by Ruys (1991) [46].

4.10.4 Housekeeping and Supply Storage

The location of housekeeping and supply storage space should be considered critically with the provision of drainage. These storage areas should include storage rooms for sanitation supplies, including floor scrubbers, mop, etc.

4.10.5 Receiving and Shipping

Animal facilities procure consumable materials (e.g., feed, bedding, disinfectants, detergents, disposables, animals, etc.) in a large quantity and ship out bedding disposal, waste, trash, and animals. Due to large volume of supplies, it is essential to provide receiving and shipping dock or a separate area for receiving and shipping. Looking to the animals shipping and receiving requirement, there should be a provision of environmentally controlled room besides receiving dock for temporary housing of animals in transit.

To facilitate easy unloading and loading of materials, a dock should be provided with a lift for different height adjustments to accommodate various sizes of vehicles. Alternatively, automatic rollup doors equipped with flying insect air shields and a standard hinged door for personnel access may be provided.

4.10.6 Waste Storage, Removal, and Disposal

A large quantity of waste such as soiled bedding, general waste, and animal carcasses is generated in animal facilities and needs to be removed from the facility. In the animal facility, soiled bedding makes the bulk of the waste and is disposed of inside bedding disposal stations and shifted to a trash container located outside the facility.

Due to stringent guidelines, most pollution control boards do not permit on-site incinerators. Most of the hazardous waste and animal carcasses are collected and segregated into different color-coded containers/bags which are then handed over to approved disposal agencies. There may be a need to provide a temporary storage space for these packed wastes (except animal tissue and carcasses) until it is handed over to a commercial disposal agency. Refrigerated and/or freezer space preferably near the dock and separated from other cold storage is essential for the storage of carcasses and animal tissue waste [10].

4.11 Specialized Facilities

4.11.1 Animal Procedure Laboratories

Most survival animal procedures are conducted inside the animal facility. Taking the animals in and out of the facility for simple procedures may be detrimental to the animal. Hence, a significant percentage of the animal facility area is devoted to the animal procedure area.

Even though most routine procedures on rodents are performed in the animal change stations inside the animal room, still rodent facilities require a dedicated procedure space for performing more complicated procedures. Animal procedure space is necessary for a rodent facility, and this may include laboratories for surgery, diagnostic and experimental imaging, whole-body irradiation, animal procedures, etc. [26].

Depending on the nature of research activity and the concerns regarding cross-contamination potential in shared rooms, the facility may identify separate procedure room/rooms. These rooms should be equipped with examination tables and require utilities such as oxygen, carbon dioxide, nitrogen, air, vacuum, water, etc., anesthetic scavenging mechanism, biosafety cabinets, chemical hood, euthanasia chamber, freezers, and other amenities/equipment. The size of the procedure room should be based on proposed activities and necessary equipment. Shared procedure rooms for specific pathogen-free rodents are not advised because of the increased potential for cross-contamination. If animals are housed in microisolation/individually ventilated cages, animal procedures may be performed in biosafety cabinets or cage changing stations.

4.11.2 Surgery

Dedicated space is required for conducting major survival surgical procedures on non-rodent mammalian species [10]. The surgical room design should depend on the number of surgeries performed, complexities of procedures, and species on which surgery is performed. Basic components of surgery room include operating rooms, surgeon preparation, animal preparation, postsurgical recovery space, and storage for equipment and supplies. Ideally, a separate room should be provided for non-rodent surgery. Adjacent office space for veterinary technicians and veterinarians may be included while designing a full-fledged complex surgery room.

Rodent aseptic survival surgery needs are less stringent and hence a dedicated surgery suite/room may not be required. If the facility intends to do many rodent surgical procedures, then a surgical room near the rodent housing areas should be provided [41]. These surgeries may also be performed in a biosafety cabinet to provide a sterile environment. Backdraft workstations may be used as rodent surgery tables when volatile anesthetics are used [47, 48].

4.11.3 Diagnostic Laboratories and Necropsy

Diagnostic laboratory facilities are an essential component of any modern animal facility. The requirement may vary from a simple laboratory to a more comprehensive diagnostic laboratory. This laboratory should preferably be located adjacent to or a part of the administrative building. The necropsy room should contain an autopsy table and benchtop workstations. This should support gross dissections, perfusions, and trimming of tissues. Tables and workstations should be down with a backdraft to draw vapors and low-level aerosols away from the prosector with the provision of an integral sink to collect fluids and permit easy wash. Ideally, the necropsy area should be in a relatively isolated area adjacent to refrigerated space or with a refrigerator to store animal carcasses [36].

4.11.4 Imaging and Special Research Support Facilities

Diagnostic imaging equipment, including x-ray and ultrasound machines for large animals, is typically located in the surgery suite. Many facilities opt for imaging instruments such as MRI, CT scanners, PET and micro-PET scanners, ultrasound fluorescence and bioluminescence in vivo imagers, and confocal microscopes to support present-day research. Ideally, the imaging facility should be located inside the barrier to limit the biosecurity risk. If the imaging facility needs to be kept outside the barrier facility, then additional support spaces, such as procedure laboratories and housing space, should be provided. Alternatively, the imaging facility can be made directly accessible from both inside and outside the barrier. In addition to imaging equipment, a whole-body irradiator for small rodents may be either located inside the rodent barrier or accessed from both inside and outside the barrier. Location of non-radioisotopic imaging equipment and radioisotope equipment must be considered critically at the building planning stage [36].

4.11.5 Cage Servicing and Sanitation

One of the most important areas in the animal facility is the cage cleaning and sanitation areas followed by disinfection/sterilization area. The type of cage sanitation equipment and the amount of space required in the cage sanitation area depend on the species housed, cage types, cage rack capacity of the facility, and cage sanitation program [49].

A dedicated central area for sanitizing cages is desirable over the decentralized area to prevent the risk of colony exposure to adventitious agents. The separation of clean and soiled activity within the area is critical and achieved by pass-through cage sanitation equipment and a wall. Doors on pass-through equipment should ideally be interlocked to ensure that both doors cannot be opened simultaneously.

The clean area is divided into two sections with cage processing and storage. The clean storage area is separated from the hot, moisture-laden environment of the cage

wash area. There may be a bulk autoclave between the clean side of cage wash and the clean/sterilized cage storage area in some rodent barrier facilities.

Drains, minimally 4 inches in diameter (6 inches preferred), should be provided to avoid the accumulation of standing water. Trap or rim flushing drains should be considered as some amount of bedding material may reach and stuck into drains.

The area of floors, walls, and ceilings at the washing area should be constructed to withstand a hot, moisture-laden environment and exposure to a variety of chemicals and movement of heavy equipment. Floors should be monolithic and non-slippery and consist of seamless and chemical- and heat-resistant polymer composites, usually epoxy aggregate, or ceramic tile with epoxy grout [50].

Walls should be constructed of moisture-proof and impact-resistant cement concrete plastering or epoxy-coated cement masonry units. Solid ceilings constructed of epoxy-coated gypsum board and access panels for mechanical service with options of aluminum or stainless steel access panels, or a suspended lay-in ceiling made of rust-resistant aluminum or fiberglass grid or fiberglass panels, should be used. Personnel safety should be ensured by providing safety showers, eyewash stations, adequately insulated hot water and steam lines, sound attenuation, etc. At the entry, dynamic safety devices that prevent staff from becoming trapped inside must be fixed [10].

4.11.6 Communications

The telephone lines should be provided throughout the facility except for animal holding rooms. If required, wireless or fiber-optic LAN-based Internet connections are preferred in most rooms (including animal rooms). This is also needed for research and animal care data acquisition and transmission as the pen and paper data transfer would pose biosafety and biosecurity risk. Video cable lines may also be considered in selected rooms especially surgical training rooms or for CCTV purposes [26].

4.11.7 Security and Access Control

Animal research facilities must need a sound security and access control system for many reasons, such as protecting against destructive tactics by miscreants, protecting valuable animal research resources, reducing the potential risk of infection, maintaining research integrity, and preventing bioterrorism when working with hazardous agents. For providing better security and access controls, it is ideally preferred to employ a multilayered system utilizing both external and internal access controls [47]. Security personnel, physical barriers, and control devices can also be utilized to suffice for perimeter control measures. Security and access control must be provided at all external doors of the animal facility. For all those access points that are in very limited use, alarms are provided to alert that they have been opened.

In small lab animal facilities with few rooms and a limited number of people, lock and key systems may marginally manage security. For large animal facilities with complex research programs, access needs for many people and different levels of containments may require microprocessor-controlled security systems as even numeric coded lock systems become unmanageable and ineffective. These systems are designed to control the access by means of electronic key or proximity cards with aligned readers, and it also provides important data trail like time of entry-exit, location, and personal identification of each entry-exit. The primary goal of providing internal security and access control system is to protect both the integrity of research and animal health. Looking to high sensitivity of containment areas and possibility of key cards being shared with unauthorized persons, highest security controls including biometric reading devices like palm, fingerprint, or retinal scanners are preferred. Additional electronic and video surveillance systems provide for enhanced security. High-quality cameras with wide-angle optics installed at critical locations are very useful security arrangement to record activities that can be reviewed later, if necessary. It is facilitating, if the security systems are managed by animal facility personnel because for managemental reasons, frequent, short-notice changes in access may be required.

4.12 Environmental Control and Monitoring

4.12.1 Lighting

Light intensity and photoperiods are important environmental parameters for animals as they affect physiologic and behavioral processes of animals, most notably reproductive function [51–53]. The standard fluorescent lighting is preferred in animal rooms that are having recessed or surface-mounted ceiling fixtures along with watertight, gasketed luminaires. Separate room-wise automatic timer-controlled lights are preferred in animal facilities. These controls could be integrated with mechanical or electrical or BMS control. Out of these three types, the mechanical type of control is more likely to malfunction. A 12-h light/12-h dark (L/D) cycle inside animal rooms is commonly used for most lab animal species. However, breeding rodents and zebra fish prefer longer photoperiods (14:10) [54]. It is important to keep constant monitoring and checks on photoperiod as continuous illumination may go undetected. Continuous lighting with no dark periods is found associated with cessation of estrus cycling, chronic vaginal cornification, and development of excess ovarian follicles [55]. Exposure to even minimal lighting during the dark phase can alter the circadian rhythm and may introduce variability in experiments [56].

Illumination of 325 lux intensity, at approximately 1 meter above the floor, is considered adequate for providing routine animal care [57]. The effect of duration and intensity of light is recognized to be associated with retinal degeneration in albino rodents [58]. To perform any procedure, personnel require a higher intensity of light, and hence, two levels of lighting can be provided in rodent holding or

procedure rooms. This control can be accomplished by controlling required lamps with an automatic timer, which can provide uniform 325 lux illumination, and remaining lamps are controlled using automatic sensor-based or manual time-out feature which can automatically turn off lamps after a prescribed interval. Depending on need, even a third set of control can be installed for red lamps in rodent holding rooms to perform the task in the dark cycle. Animals with pigmented eyes are resistant to retinal damage at typical indoor lighting levels. Animals having pigmented eyes, like dogs and nonhuman primates, are housed by providing light intensity of 800–1100 lux with no damage to the retina.

4.12.2 Air-Conditioning and Air Balancing

Air balancing refers to controlling the movement of air from one area to another area by maintaining appropriate relative air pressure differential throughout the facility. Regardless of any fluctuations occurring in the room or duct pressure, the HVAC system is recommended to have independent terminal supply and exhaust devices so as to maintain a constant supply or exhaust air volumes.

To maintain differential air pressures, volumetric flow tracking and direct pressure control are often used. In the volumetric flow tracking system, building management system (BMS) adjusts to control the terminal devices for maintaining a set differential in supply and exhaust air volumes. In the direct pressure control systems, air pressure sensors provide data to the BMS, which subsequently adjusts the terminal dampers to maintain the desired pressure.

To maintain pressure differential, two-terminal devices are available, one of which measures airflow with an in-line sensor, and this sends signals to other control device which maintains a constant volume of air. The control device is either of a motorized butterfly damper type or pneumatically inflatable/deflatable vanes.

These devices control the supply and also the exhaust airflow rates to create a differential pressure between adjacent animal areas (i.e., corridor and rooms/serving area.). Consistent pressure differential creates directional air movement, which reduces the likelihood of any backflow of particulates, or fomites, or infectious agents, or volatile chemicals used within adjoining areas avoiding any cross-aerosol contamination.

It is important to recognize that the pressure differential will be altered whenever the door to the room is opened. In most clean areas, the static pressure differential is maintained at between 0.02 inches and 0.08 inches of water column (WC), while the quarantine and hazardous use areas are maintained at higher static pressures. Excessively higher static pressure differential (±0.25 inches WC) is avoided as it makes the door opening and closing extremely difficult.

The relative air pressures can be aligned based on established management practices, i.e., conventional, containment, or barrier. In conventional single-corridor facility, the corridor is typically balanced positively to the animal rooms, except for rooms that are designated as “barrier” or “clean” rooms, which are balanced positively to the corridor. Thus, it is highly preferred to provide adequate system

flexibility in conventional single-corridor facility, which can automatically alter the animal room pressure differential without rebalancing the whole facility air balance.

There are two reasons for keeping relative pressure differentials. A positive room air pressure keeps contaminants out. A relatively negative room pressure helps to contain a disease outbreak until detected. The corridors are kept relatively positive to rooms so that it makes the corridor free of animal allergens to reduce occupational hazards [59, 60].

Generally, dual-corridor facilities are maintained with positive relative air pressures in a clean corridor than in animal rooms, and the soiled corridor is at negative relative air pressure relative to animal rooms.

4.12.3 Temperature and Relative Humidity (RH) Control

The temperature inside the animal holding room is the single most critical parameter because its variation from specified ranges can lead to health disturbances and significant variation in experimental outcome. To set and maintain the required room temperature for a species housed in any particular room, it is preferred to have separate temperature control arrangements for separate rooms. Generally, the HVAC should have capabilities to maintain the temperature between 18 and 29 °C (65 and 85 °F). This range could be narrower if animal facilities are designed for rodents only. Ideally, the HVAC system should be able to maintain temperature ±1 °C (±2 °F) around any set point required for a specific animal holding room [61]. Rabbits require lower room temperatures as low as 18 °C (65 °F), and rarely temperature above 26.6 °C (80 °F) is needed for some relatively exotic species. Animal room temperature and animal cage temperature vary depending on the use of a static or ventilated caging system. In cages, the temperature will be higher by a few degrees than in the room, but it is biologically insignificant. Animal rooms for hairless rodent strains, such as nude mice and rats, are required to be maintained at 25.6–27.8 °C (78–82 °F) [62].

Relative humidity (RH) in animal rooms is recommended to be maintained between 30% and 70% [10, 44]. To maintain this range of RH in rooms, a well-designed HVAC system that supplies 12.8 °C (55 °F) air nearly saturated with water vapor is ideal. In cold-temperate regions, RH in external air varies dramatically. To maintain RH in a specified range, the moisture can either be added or removed from the supply air via mechanical means. It is very challenging to maintain RH consistently. Humidification is achieved by either injecting steam or atomizing water into the supply air stream [50], and dehumidification is achieved by lowering the temperature of the supply air as it passes over a cooling coil in the air handler and subsequently reheated to the required room temperature.

4.12.4 Redundancy

Environmental parameters in specific ranges are required to be maintained 24 × 7 throughout the year in animal facilities for scientific as well as animal welfare reasons. For effective control of the animal house's environment, the HVAC systems are designed with redundant critical components. Among many approaches for supplying redundancy, the parallel redundant systems or $N + 1$ systems (e.g., the number of air handlers required to meet 100% of the requirement plus one additional air handler) is the preferred one. Other options to provide redundancy for chillers and boilers may include connecting the animal facility HVAC with other low priority area and inbuilt provisions for quick re-connection of chilled water or steam sources. However, a separate chiller and boiler to provide redundancy is an ideal option.

4.12.5 HVAC Control and Environmental Monitoring

Standalone or integrated BMS (building management system) types of HVAC control and environmental monitoring systems are available to control temperature and relative humidity. Additional parameters can be included, such as supply and return air volumes, pressure gradients, day and night light cycle, microisolation racks, supply-exhaust motors, power supply, HVAC components, etc. The sensors for the BMS are in the common exhaust duct of a room or group of rooms to monitor temperature, RH, pressure gradients, and supply and return airflows. Besides, the fully automated system should be designed with integrated alarm and alerts to the designated persons via signal beeps, phone alerts, or email.

4.13 Conclusion

Planning and designing of any lab animal facility is closely interlinked with research goals, institutional needs, budgetary provisions, and availability of resources with the institute. Understanding of user, institutional and regulatory requirements, planning and designing of facility to fulfill goals, accommodating possible futuristic expansions, feasibility check at all stages, coordination in phases of construction, and above all the phase-wise validation and commissioning of facility are the most important aspects that cannot be ignored.

For reaching to a successful design, a planned collaborative team approach is required that accommodates inputs from all the stakeholders, to come up with an optimally designed facility which once constructed practically supports all the desired user/institutional activities.

A clear understanding of the technical aspects of physical requirements like location, size, circulation, and functional areas (quarantine, holding, washing, sterilization, biomedical waste, etc.); civil parameters related to flooring, walls, corridors, doors, windows, etc.; and most importantly environment vis-à-vis biosafety controls must be looked into and clearly defined while designing stage itself. Technical

details for all such requirements must be well defined and should be at hand when designs are being discussed with architectural and construction teams to avoid any future failures.

A well-managed, stage-wise discussion among stakeholders, with clarity on all micro and macro parameters, usually provides a 360-degree view of all aspects of the desired lab animal facility. This certainly helps to make sure that things are done right the first time and any later roadblocks can be avoided.

References

1. Schub T (2002) The year of the flood: tropical storm Allison's impact on the Texas Medical Center. Lab Anim (NY) 31:34–39
2. Takeshita M, Kanayama T, Suma M, Hiohara T et al (1997) Earthquake damage to a laboratory animal facility. Vet Med Sci 59:63–65
3. Vogelweid C, Hill J, Shea R, Johnson DB et al (2005) Earthquakes and building design: a primer for the laboratory animal professional. Lab Anim (NY) 34(7):35–42
4. Lipman NS (2009) Rodent facilities and caging systems. In: Hessler JR, Lehner NDM (eds) Planning and designing research animal facilities. Elsevier Inc., San Diego, CA, pp 265–288
5. Lipman (2007) Design and management of research facilities for mice. In: The mouse in biomedical research, 2nd edn. Elsevier, London, pp 271–319
6. Cole MN, Hessler JR (1991) Space standards. In: Ruys T (ed) Handbook of facilities planning, vol 2. Van Nostrand Reinhold, New York, pp 67–75. "Laboratory animal facilities"
7. Tyson KW, Corey MA (1999) Facility design: forecasting space requirements. Lab Anim 28:28–31
8. Hessler J, Leary S (2002) Design and management of animal facilities. In: Fox J, Anderson L (eds) Laboratory animal medicine, 2nd edn. Academic Press, New York, pp 909–953
9. Hessler JR (1991) Animal cubicles. In: Ruys T (ed) Handbook of facilities planning, vol 2. Van Nostrand Reinhold, New York, pp 135–154. "Laboratory Animal Facilities"
10. ILAR (Institute of Laboratory Animal Resources) (2011) Guide for the care and use of laboratory animals, 8th edn. Institute of Laboratory Animal Resources, Commission on Life Sciences, National Research Council, National Academies Press, Washington, DC
11. Jacobs GH, Fenwick JA, Williams GA (2001) Cone-based vision of rats for ultraviolet and visible lights. J Exp Biol 204:2439–2446
12. Lyubarsky AL, Falsini B, Pennesi ME, Valentini P, Pugh ENJ (1999) UV- and midwave sensitive UV- and midwave sensitive cone-driven retinal responses of the mouse: a possible phenotype for coexpression of cone photopigments. J Neurosci 19:442–455
13. Sun H, Macke JP, Nathans J (1997) Mechanisms of spectral tuning in the mouse green cone pigment. Proc Natl Acad Sci USA 94:8860–8865
14. Gorton RL, Besch EL (1974) Air temperature and humidity response to cleaning water loads in laboratory animal storage facilities. ASHRAE Trans 80:37–52
15. Gunasekara AS, Rubin AL, Goh KS, Spurlock FC, Tjeerdema RS (2008) Environmental fate and toxicology of carbaryl. Rev Environ Contam Toxicol 196:95–121
16. Garg RC, Donahue WA (1989) Pharmacologic profile of methoprene, an insect growth regulator, in cattle, dogs, and cats. JAVMA 194:410–412
17. Donahue WA, VanGundy DN, Satterfield WC, Coghlan LJ (1989) Solving a tough problem. Pest Control 57:46–50
18. Turner JG, Parrish JL, Hughes LF, Toth LA, Caspary DM (2005) Hearing in laboratory animals: strain differences and nonauditory effects of noise. Comp Med 55(1):12–23
19. Peterson EA (1980) Noise and laboratory animal. In E. C. Melby (ed.), Defining the laboratory animal and its environment. Lab Anim Sci 30(2):422–439

20. Carlton DL (2002) Affordable noise control in a laboratory animal facility. Lab Anim 31:47–48
21. ASHRAE (2007) Chapter 47: Sound and vibration control. In 2007 ASHRAE Handbook: HVAC Applications. Atlanta
22. ASHRAE (American Society of Heating, Refrigerating and Air Conditioning Engineers) (2007) Environmental control for animals and plants. In: 2007 ASHRAE Handbook: HVAC applications, chap. 22. ASHRAE, Atlanta
23. Geertsema RS, Lindsell CE (2015) Effect of room ventilation rates in rodent rooms with direct-exhaust IVC systems. J Am Assoc Lab Anim Sci 54(5):521–526
24. American Conference of Governmental Industrial Hygienists (2004) Threshold limit values for chemical substances and physical agents and biological indices. ACGIH, Cincinnati, OH
25. Hessler JR, Frasier DP (2009) Heating, ventilation and air conditioning (HVAC): special considerations. In: Hessler JR, Lehner NDM (eds) Planning and designing research animal facilities. Elsevier Inc., San Diego, CA, pp 461–478
26. Hessler JR, Höglund H (2002) Laboratory animal facilities and equipment for conventional, barrier, and containment housing systems. In: Hau J, Van Hoosier G (eds) Handbook of laboratory animal science, vol. 1, selection and handling of animals in biomedical research, 2nd edn. CRC Press, London, pp 127–172
27. Lipman NS (1993) Strategies for architectural integration of ventilated caging systems. Contemp Top Lab Anim Sci 32:7–10
28. Reynolds SD (2009) Using computational fluid dynamics (CFD) in laboratory animal facilities. In: Hessler JR, Lehner NDM (eds) Planning and designing research animal facilities. Elsevier Inc., San Diego, CA, pp 479–488
29. CDC-NIH (Center for Disease Control-National Institutes of Health) (2009) Biosafety in microbiological and biomedical laboratories, 5th edn. U.S. Department of Health and Human Services, Public Health Service, Centers for Disease Control and Prevention, and National Institutes of Health, U.S. Government Printing Office, Washington, DC
30. Hessler JR, Broderson R, King C (1999) Rodent quarantine: facility design and equipment for small animal containment facilities. Lab Anim 28:34–40
31. Dysko RC, Huerkamp MJ, Yrjanainen KE, Smart S, Curran R, Maute CJ, Thompson WD (2009) Plumbing: special considerations. In: Planning and designing research animal facilities. Academic Press, pp 425–453
32. Hessler JR (1993) Animal cubicles: questions, answers, options, opinions. Lab Anim 22:21–32
33. White WJ (1996) Special containment devices for research animals. In: Richmond JY (ed) Proceedings of the Fourth National Symposium on Biosafety: Working Safely with Research Animals. Centers for Disease Control, Atlanta, GA, pp 109–112
34. Curry G, Hughes HC, Loseby D, Reynolds S et al (1998) Advances in cubicle design using computational fluid dynamics as a design tool. Lab Anim 32:117–127
35. Foster HL, Foster SJ, Pfau ES (1963) The large scale production of caesarean originated, barrier-sustained mice. Lab Anim Care 13:23–25
36. Hessler JR (2009) Barrier housing for rodents. In: Hessler JR, Lehner NDM (eds) Planning and designing research animal facilities. Elsevier Inc., San Diego, CA, pp 135–146
37. Adelberg E, Austrian R, Bachrach HL (1989) Biosafety in the laboratory. National Academies Press, Washington DC
38. Lehner NDM, Crane JT, Mottet MP, Fitzgerald ME (2009) Biohazards: safety practices, operations and containment facilities. In: Hessler JR, Lehner NDM (eds) Planning and designing research animal facilities. Elsevier Inc., San Diego, CA, pp 347–364
39. Faith RE, Corey MA, Nelan R (2009) Animal care and administration space. In: Hessler JR, Lehner NDM (eds) Planning and designing research animal facilities. Elsevier Inc., San Diego, CA, pp 187–202
40. NRC (National Research Council), National Academy of Sciences (1997) Occupational health and safety in the care and use of research animals. National Academy Press, Washington, D.C.
41. Rehg J, Toth L (1988) Rodent quarantine programs: purpose, principles, and practice. Lab Anim Sci 30:323–329

42. Otto G, Tolwani R (2002) Use of microisolator caging in a risk-based mouse import and quarantine program: a retrospective study. Contemp Top Lab Anim Sci 41:20–27
43. Lipman NS, Homberger F (2003) Rodent quality assurance testing: use of sentinel animal systems. Lab Anim 32:32–39
44. Gullino P, Ediger R, Giovanella B, Merchant B, Outzen HCJ, Reed ND et al (1976) Guide for the care and use of the nude (thymus-deficient) mouse in biomedical research. ILAR News 19:3–20
45. Fullerton FR, Greenman DL, Kendall DC (1982) Effects of storage conditions on nutritional qualities of semipurified (AIJN–76) and natural ingredient (NIH–07) diets. J Nutr 12:567–573
46. Ruys T (1991) Handbook of facility planning: vol. 2, laboratory animal facilities. Van Nostrand Reinhold, New York
47. Cunliffe-Beamer TL (1993) Applying principles of aseptic surgery to rodents. AWIC Newsletter 4:2–6
48. Brown MJ (1994) Aseptic surgery for rodents. In: Niemi SM, Venable JS, Guttman HN (eds) Rodents and rabbits: current research issues. Scientist Center for Animal Welfare, Bethesda, MD, pp 67–72
49. Leary SL, Majoros JA, Tomson JS (1998) Making cage wash facility design a priority. Lab Anim 27:28–31
50. Grumman DL (1986) Air-handling systems ready reference manual. McGraw-Hill, New York, p 170
51. Lipman NS, Leary SL (2015) Chapter 36—Design and management of research facilities. In: Fox JG, Anderson LC, Otto GM, Pritchett-Corning KR, Whary MT (eds) American College of Laboratory Animal Medicine, Laboratory Animal Medicine, 3rd edn. Academic Press, pp 1543–1597
52. Drickamer L (1982) Acceleration and delay of sexual maturation in female mice via chemosignals: circadian rhythm effects. Biol Reprod 27:596–601
53. Drickamer L (1984) Seasonal variations in acceleration and delay of sexual maturation in female mice by urinary chemosignals. J Reprod Fertil 72:55–58
54. Drickamer L (1990) Environmental factors and age of puberty in female house mice. Dev Psychobiol 23:63–73
55. Mulder J (1971) Animal behavior and electromagnetic energy waves. Lab Anim Sci 21:389–393
56. Hoffman JC (1973) The influence of photoperiods on reproductive functions in female mammals. In: Greep RO, Astwood EB (eds) Handbook of physiology, endocrinology II, part I, section 7. American Physiological Society, Washington, DC, pp 57–77
57. Dauchy R, Sauer L, Blask D, Vaughn GM (1997) Light contamination during the dark phase in "photoperiodically controlled" animal rooms: effect on tumor growth and metabolism in rats. Lab Anim Sci 47:511–518
58. Bellhorn R (1980) Lighting in the animal environment. Lab Anim Sci 30:440–450
59. Weihe W (1976) Influence of light on animals. In: McSheehy T (ed) Control of the animal house environment, vol 7. Laboratory Animal Ltd, London, pp 63–76
60. Huerkamp MJ, Gladle MA, Mottet MP, Forde K (2009) Ergonomic considerations and allergen management. In: Hessler JR, Lehner NDM (eds) Planning and designing research animal facilities. Elsevier Inc., San Diego, CA, pp 115–128
61. Canadian Council on Animal Care (CCAC) (2003) Guidelines on laboratory animal facilities—characteristics, design, and development. CCAC, Ottawa
62. Reeb-Whitaker CK, Harrison DJ (1999) Practical management strategies for laboratory animal allergy. Lab Anim 28:25–30

Quality Control of Laboratory Animals

5

Tarani Kanta Barman

Abstract

The quality of laboratory animals is an essential component of reproducible biomedical research. The introduction of transgenic laboratory animals has transformed biomedical research but has complicated the management of colonies and the production of immunodeficient strains. Quality control in laboratory animals has emerged as a critical attribute in facility management to ensure high-quality, reproducible experimental data. The quality of laboratory animals is influenced by many factors. Among these factors, genetic purity and health status are the most important factors. Outbred animals are heterozygous, and their high genetic diversity resembles genetic polymorphism of humans, whereas inbred laboratory animals are characterized by homozygosity and represent a narrow genetic repertoire. Nucleic acid markers such as restriction fragment length polymorphisms, random amplified polymorphic DNA, microsatellite DNA, DNA fingerprints, and single nucleotide polymorphism are typically used as genetic markers of quality control in laboratory animals. Health monitoring is usually accomplished by a microbiological quality control program. Bacteria, viruses, and parasites cause considerable morbidity and mortality in laboratory animals and affect the quality of the research program. For microbiological monitoring, representative samples from a group of animals are tested for specific etiological agents to classify the health status of the colony. Health monitoring is critical in immunodeficient and disease-susceptible research animal models so that they remain free of opportunistic and pathogenic organisms. In this chapter, an overview of the various aspects of laboratory animal quality control is presented with special emphasis on health and genetic monitoring.

T. K. Barman (✉)
Department of Immunology and Microbial Disease, Albany Medical College, Albany, NY, USA
e-mail: barmant@amc.edu

© The Author(s), under exclusive license to Springer Nature Singapore Pte Ltd. 2021

P. Nagarajan et al. (eds.), *Essentials of Laboratory Animal Science: Principles and Practices*, https://doi.org/10.1007/978-981-16-0987-9_5

Keywords

Quality control · Health monitoring · Genetic monitoring · Specific pathogen-free

5.1 Introduction

Quality control is defined as the sum of all measures undertaken to ensure the identity and purity of a particular product [1]. Quality control, as widely used in manufacturing and service sectors, involves testing of products and services and checking their specifications that were set as per the prevailing standards. Good quality control helps organizations meet customer/consumer needs and regulatory expectations for their products, goods, and services. Quality control has also become an essential component of laboratory animal production and management systems.

The quality of laboratory animals is influenced by many factors such as genetics, microbial infection, parasitic infestation, nutritional status, and physical facilities where animals are raised. The environmental conditions such as air quality, temperature, humidity, light, and sounds inside the animal production and breeding facility can have a substantial impact on the quality of laboratory animals and research programs. Among these parameters, genetic and microbiological monitoring of colonies for its purity and health, respectively, are considered the most important. Genetic monitoring is a method of screening of genetic purity in inbred strains and maintenance of heterogeneity in outbred colonies for quality control of breeding and production of laboratory animals. Genetic monitoring is usually limited to the analysis of DNA. However, it may entail the analysis of RNA, cell surface markers, antigens, and protein. The genetic background of novel laboratory animals (transgenic animals) particularly gene(s) knockout mice and rats may markedly influence the experimental results and reproducibility.

Microbial infections including bacterial, viral, and parasitic infections influence the health of laboratory animals and may result in a variable and non-reproducible experimental data and potentially erroneous interpretations. The introduction of such infected animals from one facility to another may jeopardize the quality of research studies using these animals. Quarantine, isolation, and culling practices are generally followed to prevent microbiological contamination of colonies when introducing new animals in an animal facility from outside. Besides, different barrier systems are used to contain the microbial contamination of breeding colonies in laboratory animal facilities. For a successful quality control program in laboratory animals, a good understanding of the microbiological status of animal stocks is important.

In this chapter, our focus will be health monitoring, genetic monitoring, and environmental monitoring for quality control of laboratory animals. These are the backbone of quality control of the animal facility.

5.2 Quality Standards

The minimum standards of quality of laboratory animals are described in some key guideline documents. The *Guide for the Care and Use of Laboratory Animals (the Guide), (NRC 2011)* plays an important role globally. These guidelines may be institute-specific and/or country-specific. There are several important European guidelines available online such as FELASA (the Federation of European Laboratory Animal Science Associations) guidelines. Many other countries too have country-specific guidelines. Accreditation by the AAALAC International (Association for Assessment and Accreditation of Laboratory Animal Care International) is a peer-review process to make sure that the key standards are met. The organization and its standard operating procedures of animal care and use in housing, management, and procedural techniques including occupational health and safety of personnel ensure the appropriate use and well-being of animals.

The AAALAC International is governed by several principles:

- A qualified person should manage the laboratory animal facility.
- All personnel caring the animals should be trained and experienced in the care of laboratory animals.
- The physical facility and method of care should permit to maintain the animals in a state of welfare and comfort.

Good Laboratory Practice (GLP) is a lawful obligation for institutions undertaking nonclinical health and environmental studies to obtain a license and to be compliant. The International Organization for Standardization (ISO) is mainly a business standard to augment customer satisfaction, which provides a management tool to control and increase the effectiveness of the business activity. The purpose and management needs of an organization dictate or determine a facility's specific goals to introduce an appropriate quality system. Implementation of a quality system grants recognition for that standard and provides assurance to standards of animal care and use, improves the quality of animal experimental data, and contributes to the tenets of *three Rs* (replacement, reduction, and refinement). The Institute for Laboratory Animal Research (ILAR) was founded to create guidelines for the care and use of laboratory animals aimed at better humane care leading to better science. ILAR emphasizes on the improvement of health, welfare, and psychological well-being of the research animals through the principles of three Rs. Through its international program, it promotes these principles in developing countries. The International Council for Laboratory Animal Science (ICLAS) fosters international cooperation, humane use of research animals, and improving and maintaining the quality of laboratory animals through ethical principles.

5.3 Health Monitoring

Health monitoring (HM) is a vital component of a laboratory animal microbiological quality control program. It is of great importance for immunodeficient and disease-prone research animals so that they remain free of opportunistic pathogens. The main purpose of health monitoring is to detect latent infections in animals which not only affect the quality of experimental results but, if zoonotic, may also endanger the health of laboratory personnel. Poor microbial quality may impact the laboratory animal production system by increasing the morbidity and mortality in breeding stocks. The microbiological quality of laboratory animals can essentially influence the strength and reproducibility of experimental data and animal welfare. Hence, it is imperative for breeding and experimental facilities to establish a health monitoring program as an integrated part of any quality assurance system. Laboratory animals are classified into several groups based on microbiological status. They are conventional animals, specific pathogen-free (SPF) animals, and gnotobiotic animals.

Conventional animals are maintained under traditionally accepted animal husbandry practices. They do not fall under any microbiologically defined set of animals such as axenic, SPF, or pathogen-free animals. The conventional animals are composed of animals that are microbiologically undefined or minimally defined and harbor common rodent pathogens. They are still widely used in biomedical research particularly in teaching and research laboratory and in preliminary experiments. In Indian conditions, many animal facilities still raise animals under conventional husbandry practices. However, in many developed countries, most of the research animals fall between gnotobiotic and non-containment conventionally raised animals. Most of the commercial stocks are caesarean-derived, have microbiologically defined flora, and bred and maintained in barrier facility to prevent the entry of new microbes.

The **specific pathogen-free** (SPF) animals are used in research more commonly these days. The definition of SPF is still under debate, and there is a lack of consensus in the scientific community. Animals free of one or more specific pathogens such as *Mycoplasma*, *Salmonella*, and *Escherichia coli* may be defined as SPF. SPF is usually defined by each program. It does not have a standard inclusion-exclusion list across institutions. These animals are derived from cesarean section aseptically and then associated with defined microflora. These animals are then transferred from strict controlled isolators to controlled barrier facilities. The barrier facility is designed not to permit the entry of pathogenic microorganisms, where all materials entering the barrier facility are subjected to sterilization protocols that destroy all pathogens. In the barrier facility, all staff have to wear personal protective equipment and clothing during any activity.

The term **gnotobiotic** is originated from the Greek words *gnotos*, which means known, and biota which means microflora. The gnotobiotic animals have completely known microflora. Gnotobiotic animals may be axenic (germ-free) or associated with a restricted flora of nonpathogenic bacteria. However, axenic animals are not truly germ-free since they inherit murine leukemia viruses from their parents. Production of axenic rodents is extremely difficult because it requires rederivation

by cesarean section or embryo transfer and their maintenance in sterile isolators provided with sterilized water, feed, and bedding. Microbial monitoring is difficult and extremely rigorous because contamination with any microbes makes them non-axenic. The anaerobic and microaerophilic gastrointestinal flora are essential for normal physiology and disease resistance. Thus, axenic rodents are physiologically abnormal and susceptible to disease. Economically, they are very expensive and are not usually used in research. However, their usage may increase in the future with the expansion of microbiome research and its impact on human health.

Besides, several other conceptual terminologies are used for microbiological quality of laboratory animals such as murine pathogen-free (MPF), barrier reared (BR), optimal hygienic condition (OHC), health monitored (HM), and virus antibody-free (VAF) animals.

Health monitoring plays an important role in the categorization of these animals. There are several general factors in the design of a health monitoring program. The health monitoring program is adapted to individual and local needs, research objectives, prevailing specific agents, national monitoring systems, and local regulations. The health monitoring program of each animal facility should establish a sound documentation system and ensure that effective micro/parasitic aims have been fulfilled. The animal facility should have a dedicated self-contained microbiological unit with separate traffic for the movement of man, material, and animals. Handling of breeding and experimental facility should be considered separately. The breeding facility should have dedicated staff, and seldom new animals are to be introduced to protect microbial quality. But in the experimental facility, a large number of researchers work on different experiments in addition to the animal caretaker. The introduction of new animals and biological materials is common in an experimental facility.

The diagnosis of the presence of an organism does not essentially indicate that it must be eradicated. The selection of agents depends on multiple factors such as influence on animal health, scientific research, prevalence, ability to infect human host, species specificity, and host immune status. Some agents are species-specific or have a comparatively restricted host range, while other organisms may infect man and animals alike. **Biological materials** also possess the risk of zoonosis. Humanized immunodeficient mice are being used to study the human immune system. These animals accept human tissues and may amplify the incoming pathogens such as human immunodeficiency virus. Microorganisms can also be transmitted by biological materials such as embryonic stem cells having mouse hepatitis virus, contaminated serum, monoclonal antibodies, and viral stocks. Data on historical prevalence may be helpful to decide the organisms to be monitored. The prevalence of a particular agent in an area depends not only on host factors but also on **biocontainment** methods such as the practice of open cage, micro-isolation cage, frequencies of animal movement, and daily routine. **Opportunistic pathogens** can cause disease in immunocompromised animals. So, it is necessary to monitor such animals for devious commensals. Emerging agents that influence animal health and research pose challenges to health monitoring due to a lack of diagnostic tools.

Health monitoring itself directly does not affect the quality of animals but provides facts about the routine colony management. With the increasing national and international partnership and frequent exchange of research animals among researchers/investigators, the need for health monitoring increases to prevent the introduction of new pathogens into the facility. Reliable health report has become an important component of every animal house as every collaborating party will request for health report before exchanging any consignment. Detailed health monitoring before and during experimentation is the only way to demonstrate the presence or absence of unwanted pathogens and to explain experimental failure in each facility. Nowadays, scientific publications too require defining the health status of the animals used in research.

5.4 Potential Sources of Contamination

The source of infection could be anything including materials used inside the facility, equipment and appliances, laboratory staff, and the animals itself received from other facilities. If infected animals are introduced in the facility, it could be a potential source of contamination to the breeding stock. Personnel working in the animal facility may transmit some pathogens and help to transmit pathogens from infected animals to healthy animals if biosafety measures are not followed strictly. The common agents known to be transmitted from humans to immunodeficient rodents are *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Streptococcus agalactiae*. Feed and bedding materials can also carry many infectious agents. *Pseudomonas aeruginosa* is a common contaminant of water sources. Vermin such as insects, cockroaches, bugs, mosquitoes, and wild rats may also play as a potential source of infection. Hence, appropriate decontamination methods such as disinfection and sterilization should be in place in every animal facility. Sterilization of feeds (irradiation, autoclaving, etc.), water (use of RO water, filtration, etc.), and bedding materials (autoclaving) are common practices of providing **biosecurity measures**. For effective decontamination, quality checking of these methods is also important. For example, the efficiency of autoclave should be monitored by chemical and biological agents in addition to the recording of time, temperature, and pressures.

5.5 Interference of Microbes in Research

Bacteria, viruses, and parasites cause significant morbidity and mortality in laboratory animals and affect the experimental data and reproducibility. Hence, microbial qualities of laboratory animals are very important for the overall success of the animal breeding and housing facility. The lethality of many infections depends on the strain used. C57BL/6 mouse is resistant to mousepox, whereas BALB/c is susceptible [2]. High mortality in susceptible strains will have an adverse impact in research as well as a breeding program, while in resistant strains, the virus impacts

the immunological research by changing the phagocytic response of animals. Biological samples such as serum or, tumor taken from resistant strains may spread the virus to other animal facilities. Similarly, different strains of rats have different susceptibility to *Mycoplasma pulmonis* which leads to failures in research models of infection and inflammation. Many microbes do not cause disease in rodents but interfere with the experimental reproducibility. Contamination of rodents with Sendai virus may affect the results of vaccine testing by affecting the B and T lymphocytes. Mouse hepatitis virus may negatively impact the physiological and toxicological studies by elevating the liver enzymes. It also activates natural killer cells and interferons and influences immunological research. These are some of the indicators of potential infections that may seriously affect the quality of animal experiments. Detailed information on interference by microbes is available in other sources [2–5].

Besides, there are infectious diseases that may transmit from animal to human beings and may cause occupational hazards to veterinarians and animal care staff. Some of them are rabies, influenza, *Salmonella*, *Trichophyton* sp., and *Microsporum* sp. Ebola and hantaviruses can also be transmitted from animals to humans. Many biological products such as vaccines are produced in animals. Hence, it is important that laboratory animals should be free from zoonotic diseases.

5.6 Microbiological Quality by Health Monitoring

Prevention is better than cure. So, routine health monitoring is the mainstay for maintaining the microbiological quality of breeding stocks. There are several guidelines available for microbiological monitoring of laboratory animals. Periodic monitoring should be carried out for parasitic, bacteriological, virological, and mycological infections. Periodic screening of healthy stocks is the best practice to prevent infectious diseases. Health monitoring should be conducted under the guidance of experienced veterinary microbiologist and pathologist. The animal house caretaker, staff, and laboratory technician play a critical role in the health monitoring program. For successful health monitoring, there should be effective communication and active participation among animal caretakers, facility staff, managers, and veterinarians. Strict health monitoring and good colony management practices will result in microbiologically good quality animals.

The importance of health monitoring in laboratory animals is now recognized internationally. However, the system of health monitoring in terms of frequencies of testing, sampling methods, test procedures, and list of pathogens differs from country to country and even from institution to institution within the same country. Implementation of health monitoring program depends on funding, availability of staff, diagnostic laboratory facility, husbandry practices, building and infrastructure, the objective of the research projects, and the source of animals. The Federation of European Laboratory Animal Science Association (FELASA) guidelines prescribe many rules and regulations for health monitoring. For detailed guidelines, refer to www.felasa.eu.

5.6.1 List of Microbiological Exclusion for SPF

Most laboratory animals described as SPF must be free of pathogens on an exclusion list. The pathogens eliminated to define the SPF animals are called exclusion list. Commercial breeders provide with each consignment a comprehensive list of pathogens excluded to define the SPF status. Globally, these exclusion lists for rodents and other popular laboratory mammals have been considerably standardized due to the globalization of biomedical research and the endeavors of lab animal science organizations. Competition among international breeders also helped to evolve the exclusion list. An example of an exclusion list is shown in Box 1 and Box 2. Many diagnostic laboratories have developed a test for those agents. The exclusion list of SPF rodents is more extensive than those of rabbits and other laboratory animals and has included all known pathogenic bacteria, viruses, protozoa, fungi, and parasites. The list of infectious agents on the SPF exclusion list is institution- and vendor-specific and defined by research needs. The list includes all known mouse viruses such as mouse hepatitis virus, Sendai virus, and lymphocytic choriomeningitis virus, regardless of virulence because they are invasive and alter the metabolism of host cells. The pathogens that cause disease in immunocompetent host are categorized as **primary pathogens** such as *Mycoplasma pulmonis*, *Helicobacter hepaticus*, *Salmonella* sp., *Citrobacter freundii*, and *Clostridium piliforme*. Other opportunistic pathogens that cause disease in immunocompromised host such as *Staphylococcus aureus*, *Streptococcus pneumoniae*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, β-hemolytic streptococci, and *Pneumocystis* fungi are included in the list. Both ectoparasites and endoparasites are also included in the list. Complying with SPF standards can be difficult if the prevalence of infection is high and the barrier system and aseptic practices are inadequate to prevent the infections. However, stringent compliance reduces the risk of colonies being infected. This makes the exchange of animal models and collaboration easy.

Box 1: Example of Exclusion Lists of Microorganisms
Pathogenic bacteria/fungi

- *Pasteurella* spp.
- *Mycoplasma pulmonis.*
- *Bordetella* spp.
- *Corynebacterium kutscheri.*
- *Streptobacillus moniliformis.*
- *Salmonella* spp.
- *Helicobacter* spp.
- *Citrobacter rodentium.*
- *Filobacterium rodentium* (CAR bacillus).

(continued)

Box 1 (continued)
- *Clostridium piliforme.*
- Dermatophytes.

All parasites/protozoa

- Mites/lice/fleas (ecto).
- Tapeworms/pinworms (endo).
- Giardia.

Opportunistic microbes

- *Staphylococcus aureus.*
- *Streptococcus pneumoniae.*
- *Pseudomonas aeruginosa.*
- *Klebsiella pneumoniae.*
- *Corynebacterium bovis.*
- *Proteus* spp.
- *Helicobacter hepaticus.*
- Beta-hemolytic *Streptococcus* spp.
- *Yersinia* spp.
- *Pneumocystis murina.*
- Protozoa/trichomonads.

Box 2: Example of Exclusion Lists of Viruses
- Mouse hepatitis virus (MHV).
- Pneumonia virus of mouse (PVM).
- Reovirus 3.
- Sendia virus.
- Theiler virus (GD VII).
- Hantaan virus.
- Ectromelia virus.
- Lymphocytic choriomeningitis virus (LCMV).
- Mouse norovirus (MNV).
- Minute virus of mouse (MVM).
- Mouse parvovirus (MPV).
- Mouse adenovirus (MAV).
- Mouse rotavirus (MRV).
- Polyomavirus.

(continued)

Box 2 (continued)
- Mouse thymic virus (MTV).
- Mouse cytomegalovirus (MCMV).
- Lactic dehydrogenase virus (LDHV).
- K-virus.
- Murine chapparvovirus (MuCPV).

5.6.2 Sampling for Health Monitoring

Health monitoring in laboratory animals is a population event and not an individual event. Practically, it is not possible to subject all animals to health monitoring. Generally, a fraction of animal area selected for testing and whatever results will come would reflect the health of the entire colony. Therefore, sampling plays a vital role in health monitoring. In a given population, a sufficient number of animals should be monitored to get meaningful information. As per the recommendation of the Institute for Laboratory Animal Research (ILAR), for a conventional barrier facility with a population size of 100 or more animals, at least nine animals should be sampled randomly. This will give 95% confidence levels for correctly diagnosing infection with a 30% prevalence rate. In the same population for 99% confidence levels, 13 samples will be required to be taken on a random basis. The sensitivity and specificity methods used for diagnosing the infection also influence the number of samples. For example, molecular techniques such as PCR require a lesser number of samples than conventional culture methods. Serological methods are preferable to culture or PCR methods for diagnosing viral infections because antibodies persist for a long time and are easier to detect than culturing viruses. However, a second diagnostic method is required to confirm the issue of false-positive results. Often, different strategies are required to decide the number of samples in breeding and experimental colonies. Sentinel animals are used by many institutions.

5.6.3 Sentinel Animals

Random sampling is the best strategy in a large breeding population. However, this method may not be possible in experimental animals or some knockout mice colonies. Serological testing of some immunodeficient mice gives false-negative results where random testing may not be a good idea. In this type of setting, sentinel animals are used for health monitoring. Sentinel animals are generally taken from breeding stocks with known microbiological status and are housed with the animals considered for health monitoring. Though they are often taken from an outside source, it is always advisable to take those animals from the same parent stock. Sentinel animals should be housed at least for 1 month before testing and preferably for a longer period of 2–3 months for antibody surveillance. A longer period is

necessary for monitoring seroconversion against *Mycoplasma*, *Streptobacillus*, and *Pasteurella*. Sentinel animals are generally exposed to all potential infection by transferring soiled bedding from all cages during cage changing. Though this is a standard method, sometimes it may not be adequate to transmit certain pathogens. Sentinel animals can also be directly exposed to experimental animals by placing them in the same cage. However, the use of contact animals may not be possible in every condition. A combination of both methods can also be used for better results. Sentinel animals can also be used for animal populations housed in individually ventilated cages (IVC) and isolators. This requires maintaining sentinel animals on soiled bedding taken from an adequate number of cages under investigation. At least one sentinel cage containing two to five animals is used for 50–80 IVCs.

5.6.4 Plenum Testing or Rack Exhaust Testing

In a move to reduce the number of research animals and improve pathogen detection, many animal housing facilities are implementing a sentinel-free system. This includes testing samples from the animal environment such as plenum testing or exhaust air dust or environment PCR instead of animal samples. If the exhaust plenum test is unproductive, cage filter tops should be sampled for the PCR test as a substitute to soiled bedding sentinels. However, all methods have their limitations. If the prevalence of pathogens is low, then the indirect sentinel method is not very effective. For example, filter paper from the cage without mice is not effective for several rodent pathogens including murine norovirus, *Pasteurella pneumotropica*, and *Entamoeba muris*. Exhaust air of IVC was found to be better for the detection of murine norovirus than sentinel animals. Environmental sampling such as exhaust plenum testing or rack exhaust testing continues to evolve based on the design of IVC racks. Some IVC racks are designed in such a way that exhaust air is filtered before entering the plenum, thus making the testing of exhaust plenum ineffective. Usually, nucleic acid from pathogens is detected by PCR methods in environmental testing.

5.6.5 Frequency of Testing

The frequency of monitoring depends on the threat involved in the colonies, purpose of raising breeding colonies, and funding conditions of the facility. The FELASA guideline recommends testing of animal quarterly. Many commercial breeders test animals every 4–6 weeks. If health monitoring is for a particular agent, then the frequency of testing may be more or less. If the impact of the pathogen is serious on the colonies, the testing frequencies can be increased.

5.6.6 Methodology of Testing

The testing methods are categorized as bacteriological, mycological, virological, and parasitological. Experienced microbiologists are required in the testing of bacteria, viruses, and fungi, and parasitologists can take care of endo- and ectoparasites. Involvement of experienced pathologists is necessary in case mortality observed in the colonies.

For **bacteriological testing**, the culture method is considered the gold standard. However, the most common practices in the industry are PCR and serology. Though culture and isolation are the best methods, serology can be used in some cases where the culture method is difficult. Isolation of bacteria is generally done by inoculating clinical specimens in nonselective and selective media. Morphologically, unique colonies are then examined by Gram staining, biochemical test, and stereotyping. Traditional culture methods are also supplemented with PCR testing. *Clostridium piliforme*, *Mycoplasma* sp. *Corynebacterium* and *Bordetella bronchiseptica*, and *Pasteurella* are tested by serology. For **mycological testing**, Sabouraud's dextrose agar is used for the culture of common fungi. Fungal skin cultures should be incubated at 25–30 °C. For dimorphic fungi, plate should also be cultured at 37 °C. Staining techniques should be used in addition to biochemical testing and serology. For **virological testing**, culture and serology are used depending on conditions. Culture methods are used for isolation and identification of virus particles. The presently available serological test includes the hemagglutination (HA) test, hemagglutination inhibition (HAI) test, immunofluorescence assay (IFA), enzyme-linked immunosorbent assay (ELISA), and multiplex flurometric immunoassay (MFIA). The sensitivity and specificity of these tests differ, so these criteria should be kept in mind while selecting the test. For **parasitological testing**, the examination of external parasites should be carried out with naked eyes. Hair sampling is used for the examination of parasites and their eggs. Transparent tape is commonly used for the examination of parasites. Perianal tape test is used for the detection of pinworms. Fecal examination is a very popular method for the examination of internal parasites. Thick and thin blood smears are used for microscopic examination of blood parasites. Tissue sections are used occasionally to detect parasites in organs. The traditional diagnostic methodologies employed for the health monitoring of laboratory animals are briefly described below.

5.6.7 Direct Gross and Microscopic Examination

Gross and microscopic examination of animal tissues for pathology, specifically pathognomonic lesions consistent with infectious agents, continues to be the basic diagnostic methodology. Direct examination of tissues for pathology, microbes, and parasites may be performed in combination with additional techniques to arrive at a precise diagnosis.

Gross and histopathology evaluation of tissues and organs can be undertaken to study abnormalities during routine health monitoring. For routine histopathology,

selected tissue specimens with gross lesions are fixed in formalin, embedded in paraffin blocks, cut into sections, stained with hematoxylin and eosin, and examined under a microscope. Special stains can be used to identify certain pathogens (e.g., Gram's stain for bacteria, Warthin-Starry stain for spirochetes, Gomori silver stain and PAS stain for fungi) or specific pathologic processes (e.g., trichrome stain for collagen deposition, PAS stain for mucin/mucus accumulation, silver stain for neurodegeneration). For parasitological examination, low-power dissection microscopy is used to examine the hair and skin of laboratory animals for mange, mites, and lice and the digestive tract for helminths. Ova of mites can also be detected via perianal tape tests in heavily infested animals. Mucosal scrapings of the guts in the wet mount are used to diagnose enteric protozoa by phase-contrast microscopy. The limitation of the gross and microscopic examination is its low analytical sensitivity and high turnaround time.

5.6.8 Cultural Methods for Bacteria and Fungi

Several synthetic cell-free media are used for the identification of pathogenic bacteria and fungi. The basic biology of some important bacterial pathogens is summarized in Table 5.1. For bacteriological and fungal culture, animal tissue samples are collected aseptically, mostly from the nasopharyngeal cavity and large intestine. These sites are rich in numerous microbiomes that can grow on culture plates and make it difficult to identify colonies of interest. To overcome these challenges, specimens are cultivated on selective media that contain supplements, such as dyes or antibiotics, to inhibit the growth of unwanted bugs. For example, MacConkey's lactose agar (MLA) contains crystal violet and bile salts that selectively inhibit Gram-positive bacteria and support only the growth of Gram-negative bacteria. A combination of antibiotics is used to selectively control the growth of the contaminating microbiome during the isolation of *Helicobacter* spp. from intestinal specimens. However, *Bordetella bronchiseptica* isolated from the trachea contains few contaminant bacteria, making it easier to view pure colonies. If the number of bacteria is low in specimens, then enrichment media should be used. Enrichment media selenite broth is used to rescue *Salmonella* spp. from feces or the intestinal tract. On the MLA plate, lactose fermenters produce pink, whereas non-lactose fermenters yield colorless colonies. After the growth of pathogens on the agar plate, they are characterized further for morphology and colony size and stain with Gram staining. Motility can be assessed under a phase-contrast microscope. Additional biochemical, sugar fermentation and molecular tests are performed to further characterize the isolated bacteria. Serotyping may be performed to verify the identity of an isolate. The serotyping of *Salmonella* is done based on the presence of the somatic (O) and flagellar (H) antigens, whereas β-hemolytic streptococci are identified by specific cell wall carbohydrate (C) antigens, which are the basis of the Lancefield classification system. For fastidious and non-cultivable pathogens such as *M. pulmonis*, serological and/or PCR test may be used in place of culture method. Some bacteria such as *Clostridium piliforme* and *Filobacterium rodentium*

Table 5.1 List of important bacterial pathogens with colony characteristic, clinical samples, and cultural conditions

Organisms	Colony characteristics	Gram's staining	Selective/differential media	Clinical samples	References
Pasteurella pneumotropica	Small, smooth, white-gray, transparent, nonhemolytic colonies on blood agar	Gram-negative	Blood agar containing clindamycin, gentamicin, potassium tellurite, and amphotericin B (CGT medium)	Oropharynx, nasopharynx, and lesioned organs	[6, 7]
Mycoplasma pulmonis	Transparent, typical indented centers and black, granular, radiations, fried egg colony on agar	–	PPLO selective media	Oropharynx, lungs, trachea, uterus, ovary	[8, 9]
Bordetella spp.	Smooth, convex, pearly and glistening, grayish-white on charcoal blood agar with cefalexin	Gram-negative	Charcoal blood agar with cephalexin, modified cyclodextrin solid medium with cefdinir	Oropharynx, nasopharyngeal swab, trachea, and lungs	[10]
Corynebacterium kutscheri	Small yellowish or grayish-white, 0.1–0.3 mm in diameter. Some strains give hemolysis on blood agar	Gram-positive	Furazolidone, nalidixic acid, and colimycin supplemented heart infusion agar base (FNC agar) and Hoyle's medium base	Oropharynx, nasopharynx, cervical lymph node, feces	[11]
Streptobacillus moniliformis	Circular, convex, grayish, smooth, and glistening on blood agar. Puff-ball appearance on liquid media, L form shows fried egg appearance	Gram-negative	No selective media. Grows on trypticase soy agar with 20% blood or serum	Oropharynx, blood, abscess, affected tissues	[12, 13]
Salmonella spp.	On XLD typical colonies are opaque/yellow, pink, or red colonies with black centers	Gram-negative	Xylose lysine deoxycholate agar (XLD), XA medium, with D-arabinose, SS agar, bismuth sulfite agar, Hektoen enteric agar, brilliant green agar	Intestine or feces	[14]
Citrobacter rodentium	Usually small, circular, convex dark pink colonies on MacConkey agar. Rough or mucoid forms have also been reported. Colorless on EMB	Gram-negative	Violet red bile glucose agar, **MacConkey bromocresol purple broth,** BRILA MUG broth	Intestine or feces	[15, 16]

(CAR bacillus) do not grow on artificial media but can be cultured in a cell line or embryonated chicken eggs. Not all microbes can be cultured on cell-free media, so alternative methods such as serology and PCR may be developed to detect and monitor such pathogens as described below.

5.6.9 Serological Assays

Serology is the most widely used methodology to detect rodent viral infections, which are among the most widespread adventitious agents of rats and mice. Though virus isolation is considered a gold standard, it cannot be routinely performed in all laboratories due to constraints of time and budget and technical expertise. Antibodies generated in response to viruses are usually measurable by 14–28 days post-infection [17] and last for months and sometimes for the whole life of the rodent. Commonly used serologic techniques include blood hemagglutination (HA) test, hemagglutination inhibition (HAI) test, complement fixation, and virus neutralization test, immunofluorescence assay (IFA), immunoenzyme assay (IEA), multiplex fluorescent immunoassay (MFIA), and enzyme-linked immunosorbent assay (ELISA). Serology is used for monitoring infections by detection of specific antibodies produced in response to infection. Though the serological tests to detect serum antibodies are quite specific, sensitive, fast, and relatively cheap, they have several disadvantages. They are inadequate for infant and immunodeficient mice that cannot produce antibodies. The presence of antibodies is not indicative of active infection. Assays that detect IgG cannot detect early exposure to infection.

5.6.10 Molecular Diagnostics

Molecular techniques are used to detect and characterize all types of pathogens such as viruses, bacteria, fungi, and parasites. These techniques are highly specific, robust, quick, and cheap tools for cloning, sequencing, amplifying, comparing, and analyzing gene sequences to identify those that are shared by related pathogens or strains. The molecular techniques appear appealing over traditional serology because they can detect microbes reliably from any biological materials/samples. The common technique used in molecular methodology is PCR, which is highly sensitive and rapid and can detect infection early and from a small quantity of biological materials. It can even differentiate closely related infections. The likely limitation of this test is occurrence of false positives and false negatives which may be due to improper purification of nucleic acids.

The annealing of a known fragment of RNA or DNA to matching nucleic acid sequences is the principle of the molecular assay for infectious agents. The probes for hybridization assays are directly or indirectly labeled with a radioisotope, an enzyme that acts on a chromogenic substrate, or a fluorescent dye. Assays start with immobilization of the test nucleic acid in situ or by blotting onto nitrocellulose membranes. Free probes are removed by washing or digestion with an enzyme. Then

the signal emitted by the probe tag or the enzyme-substrate reactions is measured, and the intensity of hybridization is ascertained. The limitation of this assay is the potential detection of the infectious nucleic acid template by PCR in the absence of infection. The high sensitivity of the PCR assay makes it especially prone to false positive in case of even minute contamination of clinical samples. This can be overcome by estimating the copy number by qPCR which can help discount low-copy positive results not associated with an active infection. The common respiratory RNA viruses prevalent in the rodent population are mouse pneumonia virus (pneumovirus) and Sendai virus (paramyxovirus). Tissues collected from the respiratory system are preferred materials for diagnosis. Mouse rotavirus is another example of an RNA virus that is detected in infected young one from birth to 2 weeks of age with diarrhea. Fecal samples are ideal for the identification of virus that sheds in the feces up to 10 days after infection.

5.6.11 Health Monitoring Report

Laboratory health monitoring reports are detailed reports of the health status of colonies maintained by a vendor or animal facility. Usually, these reports are supplied by vendors for all purchases of animals. Every animal facility intending to take animals from another facility can request for a health report to reduce the probability of introducing any microbes. The animal facility should provide the health report of its animals to the researcher, and the report can be included in the publication as a specification of animals. FELASA has developed a common standard format for health reports used internationally for harmonization. For detailed information for preparation of a health monitoring (HM) report, the readers can consult the FELASA guideline [18].

5.7 Genetic Monitoring of Laboratory Animals

The genetic materials of the laboratory animals are one of the main factors that may influence experimental reproducibility. Broadly, animals are classified as outbred and inbred animals based on genetic makeup.

The outbred animals are highly heterozygous and resemble the genetic polymorphism of human. Therefore, they are used for drug screening and toxicological evaluation. Outbred stocks have high fertility, are easy to produce, require less husbandry cost, are resistant to diseases, and have a longer life span and are easily available. They are widely used in testing, teaching, and general experiments [19]. Outbred stocks are genetically undefined colonies. The objective of outbred stocks is to maintain the maximum level of heterozygosity to maintain original allelic forms and frequencies stable over generations. The foundation colony is unique in the production system. To keep gene and genotype frequencies stable in a closed population, no new breeders are to be introduced. The population size in terms of the number of breeding pairs should be at least 200 [20]. The reproduction

life should be 30 weeks and the mating scheme should be four-unit Robertson's mating system. Computer-based mating plan should be followed for best results where every individual will have an equal chance to contribute to the next generation. No selection criteria should be adopted to increase productivity. The genetic drift can be slowed down by keeping a high interval between generations. The risk of inbreeding can be minimized by using a subgroup mating scheme such as four-unit Robertson's mating scheme and circular group mating scheme in a block of three, four, and five. Future breeders are taken from the fourth litter and a maximum of three breeders per pair is ideal. The long-term goal of outbred foundation colonies is to maintain maximum polymorphisms, to produce colonies genetically similar to foundation stocks, to maintain stable allelic frequencies from generation to generation, and to avoid the introduction of new allelic form. The higher the number of foundation stocks with several independent origins, the higher the chances of genetic polymorphism.

Inbreeding in simple terms is defined as mating between blood-related animals. Genetically, inbreeding increases homozygosity and decreases heterozygosity. Inbreeding may result in decreased biological vigor due to inbreeding depression. Inbreeding depression is characterized by decreased fertility, reduced adaptability, stunted growth, and increased susceptibility to diseases. An inbred strain refers to strains of animals that have been mated by successive brother-sister mating for more than 20 generations. The resultant animals are genetically identical to each other, and hence inbred strains are extensively used for experimental purposes because they produce reproducible data in experimental replicates. As a result of continuous inbreeding, substrains may evolve. Generally, substrain refers to inbred strains that are distinct from other branches. Substrains evolve between 20 and 40 generations of brother-sister mating, and branches get separated from each other for breeding more than 100 generations. A line of inbred animals with definite genetic traits is called a strain, for example, C57BL/6J. F1hybrids are produced by mating two inbred strains.

Co-isogenic strains are inbred strains that differ at one locus through mutation taking place in that strain. Due to genetic drift, co-isogenic strains may accumulate genetic differences over time which can be overcome by backcrossing with parent strains. A congenic inbred strain is defined as a new strain that is constructed by introducing a gene from donor strain into an inbred strain by repeated backcrosses. Segregating inbred strain maintains a particular allele or mutation in a heterozygous state. All the above three inbred strains are similar in genetic characteristics except for quite a few genes; these strains are used to study individual genes. However, they are generated by different procedures.

Recombinant inbred strain can serve as a great tool for genetic mapping and provide a brilliant technique for determining genetic correlations among complex characters. Recombinant inbred strains are produced by crossing two inbred strains for 20 or more generations of brother-sister mating. Recombinant inbred strains are highly homozygous just like any inbred strains. Recombinant congenic strains are produced by the crossing of two inbred strains, followed by backcrossing of the hybrids to one of the parents. By repeated backcrossing, the whole chromosome or

its part can be substituted resulting in consomic strains. This may also lead to lethality.

5.7.1 Genetic Quality Control

Genetic quality can be monitored in inbred strains directly or indirectly to detect changes in the genes. During the production of inbred strains, the genetic material may change continuously due to genetic drift, mutation, and genetic contamination. Genetic contamination is a common incident observed in the animal breeding facility, while genetic drift may occur due to random genotype change during breeding. A mutation may happen due to the deletion and insertion of nucleotide in the genome. Therefore, regular monitoring is essential for maintaining the genetic quality of the animals. The most common source of genetic contamination of inbred strain is the accidental mating of one inbred strain with animals of other origins. This can result in a huge exchange of alleles. Spontaneous mutations also cause genetic variation which is difficult to control. So, genetic quality control is important to ascertain the uniformity and authenticity of a strain. The polymorphic genetic markers are considered gold standards of genetic quality control of inbred laboratory mice and rats. On the contrary, outbred stocks can only be screened for genetic heterogeneity. The genetic monitoring of outbred stocks helps to select future breeders. Tips for quality control of inbred/mutant strains are given in Box 3.

Box 3: Tips for Quality Control of Inbred/Mutant Strains

- Follow brother × sister mating.
- Analyze SNP of breeders before start.
- Replace inbred lines periodically from a high-quality vendor.
- Use pedigreed or cryopreserved founders to refresh lines.
- Minimize drift by backcross to the parent strain every ten generations.
- Confirm mutation of alleles with phenotyping and genetic monitoring.
- Test newly created lines genetically.
- Validate both the background and mutations in newly purchased strain prior to use.
- Reduce human error by quality training.

The function of many genes is studied in genetically altered animal models. However, every gene functions in the framework of the genome as a whole. The phenotype of a gene mutation is controlled by a large number of modifier genes. For example, two congenic strains having the same mutation can display different phenotypes depending on their genetic background. Another example is substrain when genetically modified lines are raised in two different environments genetic drift may occur leading to a difference in substrain. This implies the importance of genetic

background in the analysis of mutation that affects phenotype leading to misinterpretation of experimental results [21–25].

Multiple common genetic monitoring strategies exist such as monitoring of biochemical, immunological, and cytogenetic markers. Protein isoforms such as isozymes can be used for biochemical monitoring. Glycoprotein markers present on B and T cells are also used as immunological markers in addition to immunoglobulin and complement factors. The karyotype of the mouse can be used as cytogenetic markers. Nucleic acid markers are usually used as genetic markers which include restriction fragment length polymorphisms (RFLP), random amplified polymorphic DNA (RAPD), microsatellite DNA, DNA fingerprints, and single nucleotide polymorphism (SNP). Genetic contamination can be determined by a minimum number of markers called critical subsets. If the animals were received from certified breeders and their genetic profile is known, then critical subset monitoring is acceptable. The current database that provides genetic profiles of inbred strain can be used to design a critical subset of markers. However, the genetic profile of many substrains remains unknown, so markers used should be verified before it is used.

5.8 Environmental and Miscellaneous Factors that Influence the Quality of Laboratory Animals

Biosecurity in an animal facility that consists of all procedures taken to eliminate, exclude, contain, and eradicate adventitious pathogens plays an important role. Animals are reared in barrier facilities provided with filtered air and sterile as well as disinfectant materials and equipment to exclude pathogens. **Barrier systems** help to exclude the opportunistic pathogens that can be a potential hazard in immunodeficient rodent housing. Filter-covered cages and individually ventilated cage systems effectively control the spread of infections. Pressure differential such as negative pressure differential is used for the containment of pathogens.

The physical facility and environment of laboratory animals also influence the quality of animals. Environmental factors such as temperature, relative humidity, airflow, air change, dust and ammonia odors, sound levels, and illumination also influence the quality of laboratory animals, and hence all recommended standards should be followed strictly. Animal caging systems, cages, beddings, environmental enrichment, stock density, the efficiency of cleaning, and disinfectant and sterilization systems are also important for quality control. If the quality of feed, minerals, vitamins, and water is not maintained strictly, it may produce poor quality animals and may affect the quality and reproducibility of experimental data. Routine monitoring of these factors is necessary for the continuous production of healthy animals.

5.9 Conclusion

Evaluation of genetic and microbiological status of colonies for their genetic purity and health status, respectively, is considered as the important component of the quality management system of the animal facility. The quality standards vary from country to country and from facility to facility. However, attempts at harmonization are in place that would provide an international standard. Health monitoring is vital for the assessment of the microbial status of laboratory animals and the performance of multiple testing with a smaller number of animals. Well-defined genetic makeup is important to avoid unexpected experimental results. Reliable breeding, good colony management practices, and routine genetic monitoring are essential modules of a high-quality genetic monitoring program. Records of specific substrains used should be maintained properly to protect the reproducibility of research outcomes. Of late, health and genetic monitoring reports have become even more crucial with the expansion of genetically modified research animals and worldwide exchanges of such laboratory animals. Several international breeders and pharmaceutical groups have cemented the way with the establishment of global standards. Harmonization of quality control of laboratory animals would help researchers as well as evaluators to strengthen the authenticity of research findings.

References

1. World Health Organization (2020) Quality Control, https://www.who.int/medicines/areas/quality_safety/quality_assurance/control/en/ Accessed 24 June 2020
2. Baker DG (1998) Natural pathogens of laboratory mice, rats, and rabbits and their effects on research. Clin Microbiol Rev 11:231–266
3. Bhatt PN, Jacoby RO, Morse HC, New AE (1986) Viral and mycoplasma infections of laboratory rodents: effects on biomedical research. Academic Press, Orlando, FL
4. Lussier G (1988) Potential detrimental effects of rodent viral infections on long-term experiments. Vet Res Commun 12:199–217
5. Nicklas W, Hornberger FR, IIlgen-Wilcke B, Jacobi K, Kraft V, Kunstyr I, Mahler M, Meyer H, Gabi Pohlmeyer-Esch G (1999) Implications of infectious agents on results of animal experiments. Lab Anim 33(suppl 1):39–87
6. Knight DP, Paine JE, Speller DCE (1983) A selective medium for *Pasteurella multocida* and its use with animal and human specimens. J Clin Pathol 36:591–594
7. Frebourg NB, Berthelot G, Hocq R, Chibani A, Lemeland JF (2002) Septicemia due to *Pasteurella pneumotropica*: 16S rRNA sequencing for diagnosis confirmation. J Clin Microbiol 40:687–689
8. Lentsch RH, Wagner JE, Owens DR (1979) Comparison of techniques for primary isolation of respiratory mycoplasma pulmonis from rats. Infect Immun 26:590–593
9. Deeb BJ, Kenny GE (1967) Characterization of mycoplasma pulmonis variants isolated from rabbits I. identification and properties of isolates. J Bacteriol 93:1416–1424
10. Ohtsuka M, Kikuchi K, Shundo K, Okada K, Higashide M, Sunakawa K, Hiramatsu K (2009) Improved selective isolation of *Bordetella pertussis* by use of modified Cyclodextrin solid medium. J Clin Microbiol 47:4164–4167
11. Amao H, Saito M, Takahashi KW, Nakagawa M (1990) Selective medium for Corynebacterium kutscheri and localization of the organism in mice and rats. Jikken Dobutsu (Japanese) 39:519–529

12. Rygg M, Bruun CF (1992) Rat bite fever (*Streptobacillus moniliformis*) with septicemia in a child. Scand J Infect Dis 24:535–540
13. Elliott SP (2007) Rat bite fever and Streptobacillus moniliformis. Clin Microbiol Rev 20:13–22
14. Park SH, Ryu S, Kang DH (2012) Development of an improved selective and differential medium for isolation of salmonella spp. J Clin Microbiol 50:3222–3226
15. Rogers L, Power K, Gaora PO, Fanning S (2016) *Escherichia coli* and other Enterobacteriaceae: occurrence and detection. Encyclopedia of Food and Health:545–551
16. Luperchio SA, Newman JV, Dangler CA, Schrenzel MD, Brenner DJ, Steigerwalt AG, Schauer DB (2000) Citrobacter rodentium, the causative agent of transmissible murine colonic hyperplasia, exhibits clonality: synonymy of C. rodentium and mouse-pathogenic Escherichia coli. J Clin Microbiol 38:4343–4350
17. Lipman NS, Hemberger FR (2003) Rodent quality assurance testing: use of sentinel animal systems. Lab Anim 32:36–43
18. Mähler M. (Convenor), Berard M, Feinstein R et al. (2014). FELASA recommendations for the health monitoring of mouse, rat, hamster, Guinea pig and rabbit colonies in breeding and experimental units. Lab Anim 48:178-192
19. Festing MFW (1991) Genetic quality control of laboratory animals used in aging studies. Neurobiol Aging 12:673–677
20. Hedricj HJ (2012) The laboratory mouse, 2nd edn. Elsevier Academic Press, San Diego, CA
21. Mekada K, Abe K, Murakami A, Nakamura S, Nakata H, Moriwaki K et al (2009) Genetic differences among C57BL/6 substrains. Exp Anim 58:141–149
22. Geurts N, Martens E, Verhenne S, Lays N, Thijs G, Magez S et al (2011) Insufficiently defined genetic background confounds phenotypes in transgenic studies as exemplified by malaria infection in Tlr9 knockout mice. Rénia L, editor. PLoS One 6:e27131
23. Fahey JR, Katoh H, Malcolm R, Perez AV (2013) The case for genetic monitoring of mice and rats used in biomedical research. Mamm Genome 24:89–94
24. Fontaine DA, Davis DB (2016) Attention to background strain is essential for metabolic research: C57BL/6 and the international knockout mouse consortium. Diabetes 65:25–33
25. Dobrowolski P, Fischer M, Naumann R (2018) Novel insights into the genetic background of genetically modified mice. Transgenic Res 27:265–275

Further Readings

Petkov PM, Ding Y, Cassell MA, Zhang W, Wagner G, Sargent EE, Asquith S, Crew V, Johnson KA, Robinson P, Scott VE, Wiles MV (2004) An efficient SNP system for mouse genome scanning and elucidating strain relationships. Genome Res 14:1806–1811

Reardon S (2016) A mouse’s house may ruin experiments. Nature 530:264

National Research Council (2011) The guide for the care and use of laboratory animals. The National Academies Press (US), Washington, DC ISBN-13: 978-0-309-15400-0. ISBN-10: 0-309-15400-6

Nicklas W, Homberger FR, Illgen Wilcke B et al (1999) Implications of infectious agents on results of animal experiments. Lab Anim 33(Suppl 1):S39–S87

Livingston RB, Riley LK (2003) Diagnostic testing of mouse and rat colonies for infectious agents. Lab Anim 32:44–51

Hansen AK (1999) Handbook of laboratory animal bacteriology. CRC Press, Boca Raton, FL

Baker DG (2007) Flynn’s parasites of laboratory animals, 2nd edn. Blackwell Publishing, Ames IA

Groen A (1977) Identification and genetic monitoring of mouse inbred strain using biochemical polymorphism. Lab Anim 11:209–214

Festing MFW (1977) Inbred strains in biomedical research. MacMillan, London

Jackson Laboratories (2020) The Jackson Laboratory http://www.jax.org Accessed 28 June 2020

6 Basic Biostatistics for Laboratory Animal Science

Suresh Kumar and Pardeep Kumar Yadav

Abstract

Understanding biostatistics is crucial for the estimation of sample size, determination of the power of the study, and assessing the statistical significance of the research outcome. In modern days, the estimation of the optimal sample size is a challenging and difficult task for the researchers. Although the inputs for sample size estimation for studies are predominantly available, the justification of sample numbers is still an enigma for many researchers. This chapter covers all the basic aspects of biostatistics and methods of sample size estimation in a manner that will infuse confidence in researchers and motivate them for effective calculation of sample size and to derive competent findings from their research data.

Keywords

Biostatistics · Study design · Statistical methods · Power analysis · The sample size

6.1 Introduction

Biostatistics is a study of statistical application in biological science. The proper use of statistical tools plays a crucial role in robust experimental designing, optimal sample size estimation, and valid conclusion from the research outcome. Many researchers have inherent apprehensions about the use of biostatistics in their

S. Kumar (✉)
National Institute of Biologicals, Noida, UP, India
e-mail: suresh.kumar@nib.gov.in

P. K. Yadav
All India Institute of Medical Science, New Delhi, India

© The Author(s), under exclusive license to Springer Nature Singapore Pte Ltd. 2021

P. Nagarajan et al. (eds.), *Essentials of Laboratory Animal Science: Principles and Practices*, https://doi.org/10.1007/978-981-16-0987-9_6

research. Of late, the justification of the appropriate sample size for animal experimentation has been a bone of contention encountered by the researchers from various regulatory bodies, funding agencies, and ethical review committees. In light of these facts, we provide an overview of biostatistics including biological variables, sample collection, study design, and statistical methods and present methods of sample size estimation for animal experiments in a descriptive manner that ultimately helps the researchers in the selection of optimal animal sample size. However, it is imperative to mention that this chapter is not complete to unlock all the myriad aspects of this vast subject, and the reader is advised to consult reference books for more details.

6.2 Types of Biological Variables

Statistical analyses highly rely on the type of biological variables that are convoluted in the study. Therefore, a researcher must be familiar with these variables that are usually the chief elements in biomedical research. The types of biological variables used in statistics with their definition and examples have been provided in Table 6.1**.**

6.3 Types of Data

Data is the information collected to answer a research question. The accurate data collection is considered the most critical step to keep up the integrity of research [5]. Qualitative data are usually non numerical values that can only be observed and approximated. An example is the greater or lesser complexity of behavior in the rat. On the contrary, quantitative data mainly deal with counts and numbers that can be articulated in numerical terms, for example, which of behavior is more frequent in rats [6, 7]. Primary data refers to the original information that is collected to answer the research question, while secondary data is the information that has already been collected by other investigators [8].

6.4 Methods of Data Collection

The method of data collection is highly dependent on the nature of the study conducted by the researcher. The primary data are always unique to researchers and can be collected by using both qualitative and quantitative methods. Secondary data are usually collected from the primary sources. Overview of the data type, method of collecting data, and research method applicability has been provided in Table 6.2**.**

Table 6.1 Overview of important biological variables used in statistics with their definition and examples [1–4]

S. no.	Variable types	Definition	Example
1.	Categorical or nominal	Variables that are classified but cannot be arranged in any particular order (unordered). If only two categories exist called dichotomous (or binary) variable	Genotype, stress type, animal excitability state (fine/nervous/excited/uncontrollable) Binary examples are Strauss tail (yes/no), paw withdrawn from hotplate (yes/no)
2.	Ordinal	Categorical variable having two or more categories, with clear ordering	Disease state (mild/moderate/severe) Animal excitability score, sample appearance pain score
3.	Ranked	It is an ordinal variable in which each data point can be put in order (first, second, third, etc.)	Stages of cancer
4.	Interval	Comparable to an ordinal variable, but that the intervals between the values of the interval variable are similarly spaced	Age, height, weight, area of the lesions, and blood glucose levels
5.	Ratio	It has a meaningful zero, otherwise similar to interval variables	The value of “0” cm means a complete absence of length
6.	Independent	A variable that is being worked upon in an arrangement to watch the impact on a dependent variable. It is additionally called experimental/predictor and/result variable	In a study to see the correlation between the lack of exercise and weight gain in the mouse model. Here, the weight gain is the dependent variable (response variable) and lack of exercise is the independent variable (explanatory variable) Any bewildering variable (uncontrolled variable) like the amount of feed consumption that would also affect on weight gain is a confounding variable in this experiment
7.	Dependent	A variable that is dependent on an independent variable(s)	
8.	Confounding	An extra variable that is likely to have a hidden effect on the dependent variable. However, their effect cannot be distinguished from the independent variable	
9.	Lurking	A “hidden” variable that may influence the response variable, but it is not among the explanatory and response variables. Most importantly, it is not recognized by the researcher as playing a role in the study	Suppose a researcher is conducting a study on the effect of exercise on a person’s blood pressure. The other factors like whether a person smokes or stress levels that may also affect the blood pressure are examples of lurking variables
10.	Control	The experimental factors that have to be kept constant	The housing environmental conditions in laboratory animals like temperature, relative humidity, ventilation, noise, and illumination have to be controlled

(continued)

Table 6.1 (continued)

S. no.	Variable types	Definition	Example
			to avoid any extraneous effects in animal experimentation
11.	Continuous	A variable with an infinite number of values and can assume any value (interval value)	Body mass, height, organ weight, time-to-event, blood or brain concentration, body temperature, and latency in the water maze
12.	Discrete	A variable with only specific numeric value and recorded as a whole number (non-interval value)	Number of needle punctures, litter size, number of correct responses, clinical score, number of rearing, arthritis score (scale 0, 1, 2, 3, 4), and number of episodes of respiratory arrest
13.	Random	It is a set of possible values from a random experiment	The behavior observed in the morning may be different from the behavior in the afternoon due to circadian rhythms

Table 6.2 Overview of the data type, method of collecting data, and research method applicability [6]

S. no.	Research method	Data type	Method of collecting data	Research method applicability
1.	Experiment	Primary	Quantitative	To examine cause-and-effect relationships
2.	Survey	Primary	Quantitative	To comprehend the general characteristics of a population
3.	Interview/ focus group	Primary	Qualitative	For gaining a better understanding of a topic
4.	Observation	Primary	Qualitative/ quantitative	To understand how something occurs in its natural setting
5.	Literature review	Secondary	Qualitative/ quantitative	To place research in an existing body of work, or to evaluate trends within a research topic
6.	Case study	Primary/ secondary	Qualitative/ quantitative	To gain an in-depth knowledge of a specific context, or in circumstances when resources not available for the large study

6.5 Types of Study Designs

The credibility of good research outcomes hinges on the study design. This is a basic research tool used for the selection of appropriate methods, frequency of data collection required, type of statistical tests to be applied, and finally arriving at the

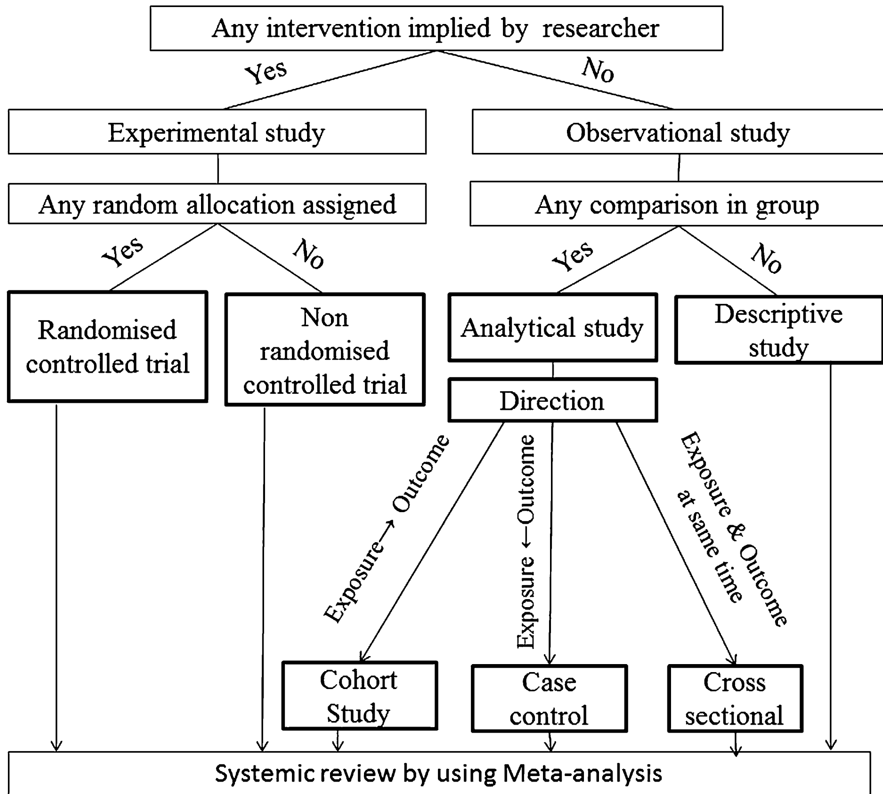


Fig. 6.1 Introduction to the study design

correct interpretation of data. Study designs can be broadly classified as observational studies (as shown in Fig. 6.1) wherein the researcher collects the information by observing the population or individuals or exposure without making any intervention or experiment, while experimental studies require a short intervention by the investigators to test the research hypothesis [9, 10].

6.6 Observational Designs

6.6.1 Exploratory Studies

Exploratory studies are performed when the prior knowledge about the phenomenon or research problem is very limited with no previous research has been conducted so far. These are usually conducted at a small scale for a limited duration to see the trend and pattern of response in an unknown field which ultimately helps in

generating the new hypothesis. However, these studies do not provide conclusive evidence. Thus, the test of significance is not required for these studies [11].

6.6.2 Descriptive Studies

Used to describe the data and characteristics of a population or phenomenon without establishing the causal relationship. Despite factual, accurate, and systematic data description, these studies cannot explain what causes the situation. It simply identifies what it is but does not answer like why it is that way, or how it came. However, the studies can lead to the formulation of a hypothesis which later can be verified by an analytical design. Examples are case study and case series [12, 13].

6.6.3 Analytical Studies

Used to establish the relationship between exposure and outcome by testing the hypothesis. These are further classified as cross-sectional, case-control, and cohort studies (Fig. 6.2).

In the cross-sectional study, it examines the disease (or other health-related states) and exposure states simultaneously that helps in developing a hypothesis. Case-control studies are used to investigate the relationship of exposure to the outcome

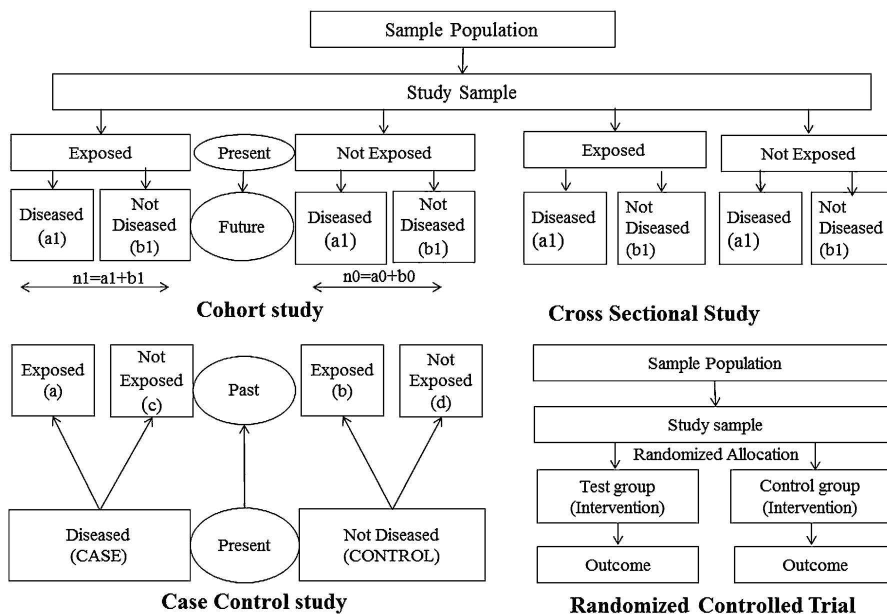


Fig. 6.2 Basic design of analytical and experimental studies

[9, 14]. Most often, it reflects the outcome followed by back trace to investigate exposures.

The odds ratio (OR; cross-product ratio or approximate relative risk, relative odds) is the ratio between the odds of disease in exposed and nonexposed animals.

OR = $(a/c)/\ (b/d)$ as illustrated in Fig. 6.2
OR = 1 means that exposure is not associated with the disease.
OR $<$ 1 means that the exposure may be protective against the disease.
OR $>$ 1 means that the exposure may be a risk factor for the disease.

The cohort study (longitudinal study) can be forward-looking (prospective) or backward-looking (retrospective) and is largely employed to explore the causes of disease and to establish links between risk factors and health outcomes. It usually defines the strength of the association between exposure and outcome [9, 14].

The relative risk (RR) or risk ratio is calculated by the ratio of the probability of an outcome in an exposed group to an unexposed group as illustrated in Fig. 6.2.

RR = $(a1/n1)/\ (a0/n0)$.
If RR = 1, the outcome is unaltered by the exposure.
If RR $<$ 1, the risk of the outcome is decreased by the exposure.
If RR $>$ 1, exposure increases the risk of the outcome.

6.6.4 Experimental Studies

Randomized controlled trials are prospective experimental studies that measure the cause-effect relation that exists between the intervention and the outcome (shown in Fig. 6.2). Random allocation helps in reducing the selection bias as well as allocation bias and mostly used in clinical trials [9, 14, 15].

6.6.5 A Systematic Review

A systemic review is a type of detailed literature review for gathering, appraising, and synthesizing evidence to address the predetermined research question systemically. It is considered good academic practice and usually based on the scientific principle of replication. This can be performed on any study design and often used a statistical procedure of merging mathematical data from numerous separate studies known as the meta-analysis [9, 16, 17].

6.7 Statistical Methods

Statistical methods are a set of mathematical formulas, models, and techniques that are used in the statistical analysis of raw research data. It has two broad categories, mainly descriptive and inferential.

6.7.1 Descriptive Statistics

Summarize and organize the data in a more meaningful way so that the relationship between variables in a sample or population can be easily understood [18]. The most recognized categories of descriptive statistics are the measure of central tendency and measures of variability or spread. Graphs, tables, and general discussions are mainly used in these two measures for the better understanding of analyzed data [19].

6.7.2 Central Tendencies

Refer to the center of a data set and mainly focus on the average or middle values of data sets. Examples are mean, median, and mode. Mean (often symbolized μ) is the arithmetic average, which is calculated by taking the sum of all measurements and then dividing by the number of measurements. It is highly susceptible to extreme values such as outliers. Median is the middle values of a data set arranged from the lowest to the highest or highest to lowest. It is not affected by extreme values. The mode is the most repeated number in the set of data. It is used when the values are widely varying. It is seldom used in medical studies [20].

6.7.3 Statistical Dispersion

Describes the dispersion of data within the set and focuses on the extent to which the distribution is spread toward the extremes, i.e., stretched or squeezed. Examples are variance, standard deviation range, kurtosis, and skewness. Variance is the average of the squared differences from the mean and indicates how close individual observation clusters about the mean value.

The standard deviation (often symbolized σ) measures the data set dispersion relative to its mean. Its value closure to the mean is indicative of a low standard deviation, which is concluded as the values in a statistical data set are close to the mean, while a high standard deviation signifies that the values are spread out over a wider range. It is calculated by variance (often symbolized σ^2), which is the average square deviation around the mean. The range is the simplest measure of statistical dispersion which measures from the smallest measurement to the largest one [21, 22].

Table 6.3 Summary of important characteristics of nominal, binomial, and Poisson distribution types [23]

S. no.	Characteristics	Normal distribution or Laplace-Gauss	Binomial distribution	Poisson distributions
1.	Symmetry	Symmetrical distribution of data on both sides of mean, i.e., bell-shaped curve	Asymmetrical distribution of data about it means, i.e., negatively or positively skewed curve	Asymmetrical distribution of data about it means, i.e., negatively or positively skewed curve
2.	Variability type	Continuous random variable distribution	Distribution of discrete random variables from a finite sample	Distribution of discrete random variables from an infinite sample
3.	Outcome	Used to calculate the probability of occurrences less than, more than, and between given values	The event can have only one of two possible outcomes such as yes/no, positive/negative, etc.	Useful in situations where the events occur in a continuous manner
4.	Statistical test	Parametric test	Nonparametric test	Nonparametric test
5.	Example	The probability that the weight of adult males' mice will be less than 20 g	Numbers of mice urinate during the restraining of 36 mice of treatment groups	Numbers of mice transferred to new cages per day in the animal facility

The skewness refers to the measure of symmetry, while kurtosis tells about the data being heavy-tailed or light-tailed in comparison to a normal distribution.

The correlation coefficient (often symbolized r) is the relationship between two variables which is used to measure the degree of the linear relationship between two continuous variables. The values of the correlation coefficient are always between −1 and +1. A positive value reflects the positive relationship (if one variable increase as the other increases), while a negative value indicates a negative relationship (if one variable increases, the other decreases). The complete absence of correlation is represented by 0 (if the two variables are independent of each other). The strength of the correlation is dependent on the slope of the regression line. The regression line is the straight line passing through the data that minimizes the sum of the squared differences between the original data and the fitted points. If the value is close to 1 (or − 1), the regression line has a steep slope and the correlation is high.

The exclusive pattern of data distribution is crucial for complete inferential statistics due to which the experimental observations show trends of inclination toward normal distribution. Statistical distribution detail has been depicted in Table 6.3.

6.7.4 Inferential Statistics

Inferential statistics are used to analyze the data from a sample to make predictions about the larger collection of the population. Hypothesis testing, standard error of the mean, and the confidence interval are mainly utilized to conclude the data in inferential statistics [18].

6.7.5 Hypothesis Testing

It is the essence of evidence-based research. Generally, two types of hypotheses are used for statistical analysis. The null hypothesis (often symbolized H_0) states that there is no existence of difference or relationship in the variables tested between the groups compared, and if indeed the difference is there, it is considered due to chance or error. The alternative hypothesis (often symbolized H_1) asserts that the difference or relationship does exist between the groups or variables. The final decision lies with the researcher to either accept a null hypothesis or reject a null hypothesis in favor of an alternative hypothesis [24].

6.7.6 Level of Significance

The probability of achieving the study results by chance if the null hypothesis is true termed as *p*-value. While the '*p*' value at which an event or result is as enough to be viewed as significant is known as the significance level. In most of the clinical research, '*p*' value <0.05 or 5% is considered significant. However, researchers may use the alternative standard like 'p' < 0.01 or 1% [25].

6.7.7 One-Tailed and Two-Tailed Test

In the testing of the null hypothesis if the alternative hypothesis shows a genuine difference between the groups (X and Y) in a specific direction (e.g., $X > Y$) is considered as a one-tailed test. However, if the alternative hypothesis is not exhibited in any particular direction (e.g., $X > Y$ or $X < Y$), then it is known as a two-tailed test. It is advisable to use a two-tailed test, when there is uncertainty, or perform animal studies [26, 27].

6.7.8 Types of Errors

In statistical hypothesis, there are two types of errors. Type I error (false-positive error, often symbolized "α") implies the probability of rejecting a true hypothesis like rejecting the null hypothesis, when it is true and accepting the alternative hypothesis. An example is the acceptance of an inactive compound as an active

compound. It is also known as the level of significance, while $1 - \alpha$ expresses the level of confidence. For example, $\alpha = 0.05$ means that the confidence level is 95% or 0.95.

Conversely, type II error (false-negative error, often symbolized β) is stated as the probability of accepting a false hypothesis like the accepting of a null hypothesis when it is not true and rejecting the alternative hypothesis. Importantly, type II error is more serious because once the compound is considered as inactive, there is a possibility that nobody will try it again. Thus, an active compound will be lost. Such errors should be ruled out or minimized by taking a larger sample and by employing a dose sufficient to make a difference in the trial [26, 28].

6.7.9 Outliers

During the analysis of data, sometimes, we found that one data point is extremely distant from the others (extremely high or low value). To find the outlier, we look at the data points that are further from the mean than three times the standard deviation and compute $\mu + 3\sigma$. Therefore, any values greater than $\mu + 3\sigma$ (approximate 99.7% of values are within this range) are considered outliers. While returning to the mean, compute $\mu - 3\sigma$, and any values less than $\mu - 3\sigma$ are also treated outliers. This could be because of the possibility of a mistake. The outliers are ought to be removed, to maintain a distance from invalid outcomes [27].

6.7.10 Confidence Interval or Fiducial Limits

Proposes the two extremes of a measurement within which 95% of observations would lie [27]. Suppose there are approximately 4 million of laboratory mice births every year in animal facilities situated across India and your animal facility has 4000 mice born per year. Using all the mice births at your animal facility as your sample (sample size = 4000), you intend to estimate the mean birth weight of all mice born in the animal facility in India. The sample mean (mean birth weight of mice pups) from your animal facility very likely will not be identical to the population mean (mean birth weight of mice pups from all animal faculties in India). The range of values that you expect to include the actual mean of the true population is referred to as the confidence interval. The values at either extreme of this range are called confidence limits. The probability of including the population mean within the confidence interval is the level of confidence; typically, 95% confidence intervals are used in research. A higher level of confidence (99%) will widen the range of the confidence interval.

6.7.11 Standard Error of Mean

(Often symbolized σM) measures how far the sample mean of the data is likely to be from the true population mean. This is calculated as SEM = $\sigma/\sqrt{N}$, where N is the sample size [27].

6.8 How to Choose the Right Statistical Test

The selection of statistical tests mainly depends on the characteristics and type of analysis of data required to address the research problem. The parametric tests are mainly used for normal distribution, while nonparametric tests are applied in skewed or non-Gaussian distribution. The detail of the general guidelines for choosing a statistical analysis is depicted in Table 6.4**.**

Post hoc tests are the modification of the "*t*" test and apply for interrelated multiple comparisons. Analysis of variance (ANOVA) only detects if a significant difference between the various groups exists or not, while a postdoc test is better in pinpointing the exact difference between the multiple groups of comparison. These tests are mainly applied for further exploration of data after a significant effect has already been established.

Dunnett's post hoc test is used when comparing one column (control group) with all other columns (experimental groups). Bonferroni, Turkey's, or Newman's tests are applied when comparing all pairs of columns including one column (control group) with all other columns (experimental groups) or between the other columns (experiment groups). These are general guidelines and should not be interpreted as rigid principles [28]. The comparison of different multiple comparison analysis statistics is provided in Table 6.5**.**

6.9 Sample Size Calculation

Justification of a sufficient sample size is highly conceptually debated by the investigator with regulatory bodies, funding agencies, and ethical review committees and even with journal editors for the publication of their work. A study with fewer subjects may miss a genuine impact in the investigation or produce an inconclusive result that could be considered unethical. Similarly, a study with too large samples will cause the wastage of animals or could expose the study subjects to unnecessary related risks. The estimation of sample size has been highlighted sufficiently for the clinical studies and trials; however, scanty reporting is available for animal studies in the published literature [30].

Table 6.4 Statistical test summary for different data types [19]

Aim	Type of data set			
	The continuous measurement from a normal distribution	Continuous measurement, rank, or score from the non-normal distribution	Binomial having two possible outcomes	Survival time
Type of test	Parametric test	Nonparametric test	Nonparametric test	Nonparametric test
Example of the data sample	Blood pressure readings from multiple patients	Giving ranks to several treatment efficiencies by one expert	Patient list recovering or not after a treatment	Time lapsed to the outcome of a disease
One group description	Mean, SD	Median, interquartile range	Proportions	Kaplan-Meier survival curve
One group compared to a hypothetical situation	*t*-test (if sample size >30, apply Z test)	Wilcoxon signed-rank test	Chi-square (χ^2) or binomial test	
Two paired[a] groups comparison	Paired *t*-test	Sign test or Wilcoxon test	McNemar's test	Conditional proportional hazards regression
Two unpaired[b] groups comparison	Unpaired *t*-test	Mann-Whitney test	Fisher's test (chi-square for large samples)	Log-rank test or mantel-Haenszel test
Three or more matched[c] group comparison	Repeated measures ANOVA	Friedman test	Cochran's Q test	Conditional proportional hazards Regression
Three or more unmatched[d] group comparison	One-way ANOVA	Kruskal-Wallis test	$\chi 2$ test	Cox proportional hazard
Association quantification between two paired samples	Pearson correlation	Spearman correlation	Contingency coefficients	
Value prediction from several measured or binomial variables	Multiple linear regression Or multiple nonlinear regression		Multiple logistic regression	Cox proportional Hazard regression

[a]Paired refers to two measurements in the same subject before and after a treatment (the relationship between the samples)
[b]Unpaired means the comparison is made between two measurements in two different groups (no relationship between the samples)
[c]Matched data samples are an extension of paired data samples when there are more than two samples
[d]Unmatched data samples are an extension of unpaired data samples when there are more than two samples

Table 6.5 The comparison of different multiple comparison analysis statistics [29]

Type of test	Criteria	Advantages	Disadvantages
t-tests on all pairs	All pairwise contrasts both simple and complex	• Simple to run on a computer or hand calculate • Widely available • Powerful • May be used with unequal sized groups	• Alpha inflation • Multiple type I errors • Unreliable results due to overestimation of differences among pairs
Dunnett	The contrast of the control group with each experimental group or combination of experimental groups	• Powerful • Good for finding small differences between experimental and control groups • Specifically tests the experimental groups directly against the control group and thus those differences are more clearly specified	• Not widely available • Does not test differences among experimental groups • Not ideal for exploratory statistical studies
Bonferroni	Tests selected contrasts, both simple and complex	• Preserves alpha • Can test differences among experimental groups as well as between experimental and control groups • Available in many statistical packages	• Groups must be equal in size • All contrast must be defined by the researcher • Not used in exploratory studies
Tukey	All possible simple contrasts	• Useful in confirmatory research when combinations of groups are not meaningful • Available in many statistical packages • Reduces risk of type I errors • May be used when group sizes are unequal	• Does not test complex contrasts • Subject to type II errors and not as powerful as other tests • Not ideal for exploratory studies • Not as available as Scheffe or Bonferroni
Newman-Keuls	All possible simple contrasts	• More powerful than the Tukey method • Available in some statistical packages • Reduces risk of type II errors • More likely to find small but significant differences	• Does not test complex contrasts • Requires equal group sizes • Subject to type 1 errors • Availability is variable
Scheffe	Tests of all possible contrasts, both simple and complex	• Good for both exploratory data analysis and for testing well-developed theories	• Alpha inflation higher than for other multiple comparison analysis statistics

(continued)

Table 6.5 (continued)

Type of test	Criteria	Advantages	Disadvantages
		• Can test pairs consisting of combinations of original study groups • Relatively powerful test • No need to define contrasts in advance • Available in many statistical packages • Reduced risk of type II errors	• Requires equal group sizes • Tests contrast not of interest • More subject to type 1 errors than other MCA statistics

6.9.1 Methods of Determining Sample Size

Given this limitation, we attempt to outline the most pragmatic techniques including common sense, the resource equation, and the power analysis method of computing the sample size. Among these, power analysis is considered the most scientific method. However, researchers often come across problems in applying this method. We also discuss alternative methods such as standardized effect size (SES) and KISS (keep it simple, stupid) for choosing the sample size.

6.9.2 Tradition or "Common Sense"

The majority of researchers select the sample sizes from the previous literature of similar type conducted successfully by the various researchers. Taking into account wide-ranging facts like the type of experiments, independent/dependent variables, experimental units, species, and strains makes the common-sense approach to be considered as a pragmatic approach [31]. Cox and Reid opine that "Except in rare instances, a decision on the size of the experiment is bound to be largely a matter of judgment and some of the more formal approaches in determining the size of the experiment have spurious precision." This approach is considered satisfactory with an experienced researcher who has already conducted several experiments similar to the one proposed. However, it could be very challenging for those starting a new research topic. It is to be noted that this approach further requires the "power analysis" approach for the final estimation of sample size [32].

6.9.3 Resource Equation Method

Based on previous experience substantially from industrial and agricultural research, this method is considered as an alternative to the power analysis approach for deciding the sample size in animal-based studies. This approach is most suitable for exploratory studies, wherein it is difficult to find the value of standard deviation

and the effect size [33, 34]. However, this method does not have the mathematical justification of power analysis.

The equation is:

$$E = (\text{the total number of experimental units}) - (\text{the number of treatment groups})$$

E should be between about 10 and 20, although these are not rigid limits.

Suppose if an investigator makes six groups to study the effect of the drug (one control group and five groups of different doses of the drug) with ten rats in each group. In this case, $E = (10 \times 6) - 6E = 60 - 6 = 54$, which is more than 20. Therefore, it will be considered that the sample size in this experiment is more than required. However, if the sample size is kept 4 per group, then E will be 18, which is the acceptable limit. Hence, it can be considered as an adequate sample size. Although this approach is easy, it is not recognized as scientific as the power analysis method.

This technique is appropriate when the result is a quantitative variable and analysis is done by the analysis of variance (ANOVA). Given this methodology as mentioned above, the acceptable range of degrees of freedom (DF) for the error term in ANOVA is between 10 and 20 [4, 9, 10]. Three common ANOVA designs applicable to animal studies are presented [35]. Summary of main formulas used for ANOVA designs in the calculation of sample size with examples is outlined in Table 6.6.

The concern about this technique is the presence of a slight "sweet spot" inside the limit of these two points. In the event of selecting animals below the lower limit, the possibility of type II error (false-negative result) increases considerably. If selecting the animals above the upper limit, the cost and use of animals will increase for only a modest gain. Further, in this strategy, if there are multiple medications, the number of test subjects per treatment can be decreased. Despite these shortcomings, this technique is of better value when compared to the common-sense method and simpler for new researchers who do not know statistics. However, many funding bodies and ethical review committees consider it as an "unrefined" technique and progressively requesting the utilization of power analysis to determine the sample size [33].

6.9.4 Power Analysis

This method was introduced by Jacob Cohen in the 1960s. Mounting evidence pointed out that the power analysis method is the most promising scientific tool used for the estimation of sample size by the researchers. This method is also used in clinical trials and clinical studies for the calculation of sample size. The power analysis is complex and based on several important variables that are largely decided by the researcher which may largely affect the precision and sensitivity of this method. Further, access to specialized software is normally required in this method,

Table 6.6 Summary of main formulas used for ANOVA designs in the calculation of sample size by resource equation methods with examples [35]

ANOVA design	Application	Example	Minimum n/group	Maximum n/group
One-way ANOVA	Group comparison	Tumor size comparison study (cm, change = posttreatment size – Pretreatment size) between three treatment groups	$10/k + 1$ $N = (10/3 + 1) = 4.3$ = rounded up to 5 animals/group	$20/k + 1$ $N = (20/3 + 1) = 7.7$ = rounded up to 8 animals/group
One within factor, repeated measures ANOVA	One group, repeated measurements	Study the effect of the drug on tumor sizes at four time points (pretreatment and posttreatment 1, 2, and 3) in a group of animals	$10/(r - 1) + 1$[a, b] $N = 10/ (4 - 1) + 1 = 4.3$ = rounded up to 5 animals	$20/ (r - 1) + 1$[a, b] $N = 20/ (4 - 1) + 1 = 7.7$ = rounded up to 8 animals
One-between, one within factor, repeated measures ANOVA	Group comparison, repeated measurements	Comparative study of three treatment groups with four repeated measurements (pretreatment and posttreatment 1, 2, and 3) of tumor sizes	$10/kr + 1$[b] $n = 10/ (3 \times 4) + 1 = 1.8$ = rounded up to 2 animals/group $N = n \times 3 = 2 \times 3 = 6$ animals If animal is required to be sacrificed at each Measurement, the total sample size is minimum (maximum) $N \times r = 6 \times 4 = 24$ animals	$20/kr + 1$[b] $n = 20/ (3 \times 4) + 1 = 2.6$ = rounded up to 3 animals/group $N = n \times 3 = 2 \times 3 = 6$ animals

k = number of groups, n = number of subjects per group, N = total number of subjects, r = number of repeated measurements
[a] $n = N$, because only one group is involved
[b] n must be multiplied by r whenever the experiment involves sacrificing the animals at each measurement

Table 6.7 Overview of the main factors that affect sample size calculation [38]

Parameters	Magnitude	Impact on the identification of the effect	Required sample size
P-value or alpha level	Small	Stringent criterion and difficult to achieve a significant difference	Large
	Large	Relaxed criterion. Significance is easier to attain	Small
Power	Small	Identification unlikely	Small
	Large	Identification more probable	Large
Effect size	Small	Difficult to identify	Large
	Large	Easy to identify	Small
Alternative hypothesis	One-tailed	Easy to identify (statistically significant difference in one direction only). Having a strong reason to believe for the effect to lie in one direction)	Small
	Two-tailed	More general criterion and used in animal research (statistically significant difference in any direction).	Large

which requires an additional level of understanding for the researcher. Moreover, the use of unfamiliar software and unfamiliar variables may also increase the chance for the wrong calculation to the researcher.

For applying this method, a researcher must be familiar with five key parameters including SD, effect size, power, significance level, and two-sided test. Accordingly, the sixth, i.e., the sample size, can be estimated with the help of exclusive software applications [36, 37]. The significance level (*p-value*) or sidedness of the test (one-tailed or tailed test) has already been discussed in the inferential statistics section. Overview of the main factors that affect the sample size calculation is outlined in Table 6.7.

6.9.5 Standard Deviation

Ideally, the information about the SD for a particular variable should be taken from similar types of previously published studies. However, if the information is not available, a pilot study may be conducted by taking the small numbers of untreated animals of the same characteristics. The pilot experiments require the maximum uniformity in animals. Further information about how we can keep the maximum uniformity is discussed under sample size [31, 39].

6.9.6 Effect Size

Effect size is the difference between the mean of two groups for quantitative data and the proportions of events in two groups. The investigator has to finalize how much minimum difference between the two groups can be considered as experimentally/ clinically significant before the start of the study. Generally, the bigger effect size is

easy to identify than smaller ones. Therefore, when planning an experiment, select the highest possible dose (or identical) that could reasonably be accepted but not reported to have any undesirable reactions.

Further, the most sensitive strains and species of animals can be chosen. For instance, Sprague-Dawley (SD) rats are not recommended for studies of the endocrine disruptor bisphenol, because of their lack of response to steroidal substances. Likewise, the variables that have a more pronounced responsive effect than others should be selected. While establishing the effective size, the value should be set at the lower end so that even the minimum difference can be reliably detected with that sample size [37, 40].

The effective size can be calculated based on substantive knowledge or previously published studies. It can also be determined by utilizing the conventions as suggested by Cohen for small, medium, and large effect sizes for many types of the test [41–44]. The details of the effect size threshold for various types of statistical tests are provided in Appendix 1. Moreover, the effective size (called Cohen's d) can also be calculated by using these formulae.

For two independent groups, the effect size (Cohen's *d*) is determined by subtracting the means and dividing the result by the pooled standard deviation [44].

For two-sample t-test = Difference in means of two groups (M2-M1/pooled standard deviation (SD_{pooled}).
For ANOVA = SD of group means difference/SD within the groups.

Cohen's *d* is considered as an appropriate effect size measure if two groups have similar standard deviations and are of the same size, while Glass's delta is an alternative measure if each group has a different standard deviation. Hedges' g is mainly used if there are different sample sizes and measures of effect size weighted according to the relative size of each sample.

Further, McGraw and Wong (1992) have recommended using Common Language Effect Size (CLES) (shown in column 5 of Appendix 3). CLES requires the understanding of the concept of probability, but no prior knowledge of statistics. They provided the case of heights of young adult males and females as an example, where the heights differ by an effect size of about 2 and make an interpretation of this difference to a CLES of 0.92 (i.e., 92 out of 100 blind dates among young adults, the male will be taller than the female) [45].

The calculations of effect size determination can also be performed with the help of effect size calculators and software.

6.9.7 Power

(Often symbolized as $1 - \beta$) is the probability of finding the true effect when one does exist or the probability of rejecting the null hypothesis when it is false. The power of 80–99% is usually specified, depending upon the research question. By

convention, it is mostly recommended at 80% (0.8). A high-power experiment has a greater chance to detect an effect [31].

6.9.8 The Sample Size

A suitable sample size for a proposed experiment is estimated using the power analysis. This approach is easy to understand and is less prone to error than the more conventional approach. An experiment is planned and designed to make it small as well as powerful enough to generate meaningful data. The animal's sample size can be reduced by taking some precautions during the designing of the animal experiments. Examples are the precise measurements, preferring continuous measurements over categorical measurements, selecting the paired data wherever possible, and performing one-tailed tests.

The sample size is largely determined by the effect of bias ratio or signal-to-noise ratio in animal studies. The term signal is mainly used for the magnitude of the difference between the means of the two groups (M1-M2) that has to be specified by the investigator, while the noise is a measure of the variation among the experimental subjects and expressed as the standard deviation. A smaller sample size reflects a high signal-to-noise ratio. Conversely, a larger sample size reveals a low signal-to-noise ratio.

6.9.9 Important Steps to Increase the Signal (Biological Difference(s))

- Scientifically and ethically improving the experimental design to maximize the difference between control and experimental groups.
- Using an inbred strain of mice which is expected to give the best response in the intended model.
- Increasing the dose of the test compound to obtain the optimum effect.
- Switching to species/strain of mice which is expected/reported to be more sensitive for the intended model.

6.9.10 Important Steps to Reduce the Noise (Standard Deviation)

By maintaining the maximum uniformity in animals or other experimental subjects as details provided below:

- Randomized block designs and within-subject are likely to give better control of variation than between-subject design [46].
- In cross-over study design, the variance between subjects is less due to its control in experimental groups.

- Homogenous model selection across different treatment groups, e.g., use of animals of the same sex, strain, and age.
- Assurance of high-quality genetic backgrounds in inbred strains and genetically engineered models (GEMs).
- Assurance of pathogen-free animals (strict quarantine and health monitoring).
- Control over microbiome-related effects.

6.9.11 Attrition

Numerous research protocols are related to a high probability of animals mortality. If the expected mortality is 10% attrition, then the sample size should be divided by 0.9 to calculate the real sample size. In the event when ten animals per group are required and the researcher is expecting 10% mortality, then the final sample size will be 11 animals per group (10/0.9 = 11.11). Similarly, in the case of 20% attrition, the sample size should be divided by 0.8. The equation for the amended example size = sample size/[1 − (% attrition/100)] [30, 47, 48].

6.9.12 Standardized Effect Sizes (SESs) (Cohen's d) and Sample Size

SESs are defined as the ES divided by the pooled SD (SD pooled) and mainly used in a meta-analysis which requires combining the results of several studies [49]. It can also be applied for the treatment response to a different variable because they are all expressed in the same units (SDs) especially in toxicity tests, where we generally see the effect of test chemicals on the measurement of hematology, clinical biochemistry, organ weights, etc. [50]. Further, SESs can also be used for calculation of sample size, if power, sidedness, and significance level by convention are fixed in the proposed experiment.

Based on human studies, Cohen also proposed that responses to a treatment resulting in standardized effect size (SESs, often specified as Cohen's effect size) of 0.2, 0.5, and 0.8 standard deviations (SDs) would represent small, moderate, and large treatment responses requiring sample sizes of 394, 64, or 26 subjects per group, respectively, to detect the effect by assuming 80% power, a 5% significance level, and a two-sided "*t*" test [31].

Further, Cohen suggested that SDs in laboratory animal-based studies are lower compared to human-based studies because of greater uniformity in age, weight, clinical or subclinical infection status, dietary habits, housing conditions, and use of inbred strains. Therefore, if we compare it with clinical trials, the higher SESs are observed in laboratory animals. Further, he also added that using a high dose of test substances and sensitive species and strains can help get a higher response in animal experimentation as compared to human clinical trials. On account of these findings, he suggested that SES of 1.1 "extra-large," 1.5 "gigantic," and 2.0 SDs "awesome" for laboratory animals would require sample sizes of 17, 8, and 5 subjects per group, respectively, with an 80% power, a 5% significance level, and a two-sided *t*-test [31].

The relationship between SES and sample sizes ranging from 4 to 34 subjects per group for 80% and 90% power, a 5% significance level, and one-sided or two-sided test has been provided in Appendix 2. For more extensive details, the reader is referred to the table provided by Ellis [49]. It is important to note that for any given sample size, there is a range of SESs and power levels. Taking an example with six animals per group, there will be a 90% chance of detecting an SES of about 2.1 SD, an 80% chance of detecting an SES of 1.8 SDs, a 70% chance of detecting an SES of 1.6 SDs, and a 60% chance of detecting an SES of 1.4 SD down to a 5% chance of detecting a nonexistent response (a type I error). In this process, sometimes the researcher will detect an effect that is smaller than the experiment was designed to be able to detect. Therefore, if a researcher wants to repeat an experiment, it is better to take a larger sample size than was used in the original experiment [31].

6.9.13 KISS Principle

The main goal of this method is to avoid redundancy and complexity and therefore being applicable in many domains of life [51]. For sample size estimation, it uses the combination of common-sense method and/or the resource equation method and then the conventional table or some simple arithmetic to estimate the ES that the experiment is likely to be able to detect for a given power.

In this approach, depending upon the proposed animal study details including the animal model details, experimental units, treatments, and outcome variables, at first, the mean and SD (ideally both low and high) of the variable of interest in control subjects are identified from the previous studies. Then the provisional sample size is selected either by using common-sense or resource equation and finding the SES from Cohen's convention table (Appendix 2) for the corresponding sample size. The SES is multiplied to calculate "predicted detectable ES" for the chosen levels of power. This can also be expressed in percentage. Finally, the decision is made if the predicted detectable ES is acceptable to the study, i.e., if it will detect a sufficiently small effect. In case it is not acceptable, a large sample size is selected and calculated again. For better understanding, we use an example below [31].

Example Does the red blood cell (RBC) count in mice get altered by a potential new drug?

Solution In the published study, the mean RBC count in C57BL/6 female mice is 9.19 with an SD of ±0.70 (n/ml). (It is made sure that it is the SD and not the SEM.) Based on previous studies, a provisional sample size of $n = 12$ mice/group is chosen. For a sample size of 12 with a 90% power and a two-sided test, SES = 1.39 (Cohen's d/SES value from Appendix 2). Therefore, the "predicted detectable ES" (SES*SD) is $1.39 \times 0.70 = 0.97$ (n/ml) or $(0.97/9.19) \times 100 = 11\%$ (expressed as %). A sample size of 12 mice per group is acceptable [31].

6.10 Sample Size Calculators and Biostatistics Software (Conducting a Power Analysis)

In recent times, many sample size calculators and biostatistics software are available that can help in the determination of sample size. Information about other freely available software and calculators for sample size calculation has been given in Appendix 4. To understand the concept of sample size calculation, here we have provided some examples by using the G power software in **Appendix 5.**

6.11 Conclusion

For good laboratory animal research, a proper understanding of biostatistics and its application in animal research studies is of paramount importance for a researcher. In this chapter, we have attempted to provide the basic concepts and principles in biostatistics including types of biological variables, data types, methods of data collection, various types of study designs, and statistical methods that help the researchers in designing animal experiments and analysis of data. The proper use of biostatistics will not only justify the sample size calculation but also provide a sound scientific and ethical judgment. The common-sense, resource equation, and power analysis methods are mainly used for computing the sample size. However, there is no single method of determining sample size that is considered entirely satisfactory.

Among these, power analysis is considered the most scientific method. However, sometimes researchers faced a sort of difficulty in applying this method owing to its complex nature. Most of the funding organizations and ethical review committees often demand the researcher to justify their sample sizes using a power analysis method only. In this context, the standardized effect size (SES) and KISS (keep it simple, stupid) approaches of choosing the sample size based on common-sense or resource equation methods combined with power analysis provide a simplified solution for the estimation of the sample size in laboratory animal experimentation. Further, the appropriate statistical knowledge analysis that is mentioned in this chapter will ensure proper reporting of the result as demanded by the various national or international guidelines on laboratory animal experimentation including Reporting of In Vivo Experiments (ARRIVE) guidelines. Many statistical software packages are readily available that will assist researchers to perform statistical analysis conveniently and efficiently.

Appendix

- Appendix 1: Threshold for interpreting effect size.
- Appendix 2: Cohen's d (SESs) for sample sizes of 4–34 subjects per group assuming 80% and 90% power, a 5% significance level, and a one-sided or two-sided test [31].

- Appendix 3: Common Language Effect Size (CLES) [30].
- Appendix 4: Summary of software or calculators*available for sample size calculation.
- Appendix 5: Examples by using the G power software.

Appendix 1 Threshold for Interpreting Effect Size [44]

Test	Relevant effect size	Effect size threshold		
		Small	Medium	Large
t-test for means	d	0.2	0.5	0.8
F-test for ANOVA	f	0.1	0.25	0.4
t-test for correlation	r	0.1	0.3	0.5
Chi-square	w	0.1	0.3	0.5
2 proportions	h	0.2	0.5	0.8

Rationale for these benchmarks can be found in Cohen (1988), and Rosenthal (1996) has later added the classification for "very large" descriptive category

Appendix 2 Cohen's d (SESs) for Sample Sizes of 4–34 Subjects per Group Assuming 80% and 90% Power, a 5% Significance Level, and a One-Sided or Two-Sided Test [31]

Sample size	80% one-sided	90% one-sided	80% two-sided	90% two-sided
4	2.00	2.35	2.38	2.77
5	1.72	2.03	2.02	2.35
6	1.54	1.82	1.60	2.08
7	1.41	1.66	1.63	1.89
8	1.31	1.54	1.51	1.74
9	1.23	1.44	1.41	1.63
10	1.16	1.36	1.32	1.53
11	1.10	1.29	1.26	1.45
12	1.05	1.23	1.20	1.39
13	1.00	1.18	1.15	1.33
14	0.97	1.14	1.10	1.27
15	0.93	1.10	1.06	1.23
16	0.90	1.06	1.02	1.18
17	0.87	1.03	0.99	1.15
18	0.85	1.00	0.96	1.11
19	0.82	0.97	0.93	1.08
20	0.80	0.94	0.91	1.05
21	0.78	0.92	0.89	1.03
22	0.76	0.90	0.86	1.00
24	0.73	0.86	0.83	0.96

(continued)

26	0.70	0.82	0.79	0.92
28	0.67	0.79	0.76	0.88
30	0.65	0.76	0.74	0.85
32	0.63	0.74	0.71	0.82
34	0.61	0.72	0.69	0.80

Appendix 3 Common Language Effect Size (CLES) [30]

Effect size interpretation (Robert Coe, 2002)	Percentage of control group below the average person in the experimental group	The rank of the person in a control group of 25 equivalent to the average person in the experimental group	The probability that you could guess which group a person was in from knowledge of their "score"	The probability that a person from the experimental group will be higher than the person from control, if both chosen at random (=CLES)
0.0	50%	13th	0.50	0.50
0.2	58%	11th	0.54	0.56
0.5	69%	8th	0.60	0.64
0.8	79%	6th	0.66	0.71
1.2	88%	3rd	0.73	0.80
1.4	92%	2nd	0.76	0.84
2.0	98%	1st	0.84	0.92

For the detail, see the link: https://www.psychometrica.de/effect_size.html

Appendix 4 Summary of Software or Calculators*Available for Sample Size Calculation

S. no.	Software		Platform	URL
1.	Stand-alone program	*G power	Windows and macOS	http://www.gpower.hhu.de
		PS	Windows	http://biostat.mc.vanderbilt.edu/wiki/Main/PowerSampleSize
		PASS	Windows	https://www.ncss.com/software/pass
		nQuery	Windows	https://www.statsols.com/nquery-samplemnsize-and-power-calculation-forsuccessful-clinical-trials
2.	Rb packages	Pwr	Windows, macOS, and Linux	https://cran.r-project.org/web/packages/pwr

(continued)

		Trial size	Windows, macOS, and Linux	https://cran.rproject.Org/web/packages/ Trial size
		PowerUpRc	Windows, macOS, and Linux	https://cran.rproject.Org/web/packages/PowerUpR
		Power SurvEpi	Windows, macOS, and Linux	https://CRAN.R-project.org/package=powerSurvEpi
3.	SAS	PROC POWER	Windows and Linux	https://support.sas.com/documentation/cdl/en/statug/63033/HTML/default/viewer.htm#power_toc.htm
4.	SPSS	SamplePower	Windows	https://www01.ibm.com/marketing/iwm/iwmdocs/tnd/data/web/en_US/trialprograms/U741655I36057W80.html
5.	Stata	Power	Windows, macOS, and Linux	https://www.stata.com/features/power-and-sample-size/
6.	Microsoft excel	Power Upc		http://www.causalevaluation.org/power-analysis.html
7.	Specialist simulation software	IcebergSim windows		http://icebergsim.software.informer.com/versions/
		FACTS	Windows	https://www.berryconsultants.com/software
		Clinical trial simulation	Windows and Linux	http://www.biopharmnet.com/innovation/trial_simulation/cts1.php

*Sample Size Calculations—Description of sample size calculations from the IACUC at Boston University Sample Size *Calculations EpiTools epidemiological calculators. http://epitools.ausvet.com.au/content.php?page = SampleSize. *Statistics calculators http://danielsoper.com/statcalc3/default.aspx

Appendix 5: Examples by Using the G Power Software

Comparing the Proportions from Two Independent Samples

Example: A scientist exploring a new treatment to reduce the development of tumors by 15% at 80% power with 5% significance in the control group of mice, where 30% of the mice developed tumors.

Solution

Firstly, the appropriate test is selected by using Table 6.4. In this case, Fisher's exact test will be applied to **compare two unpaired groups** in binomial distribution.

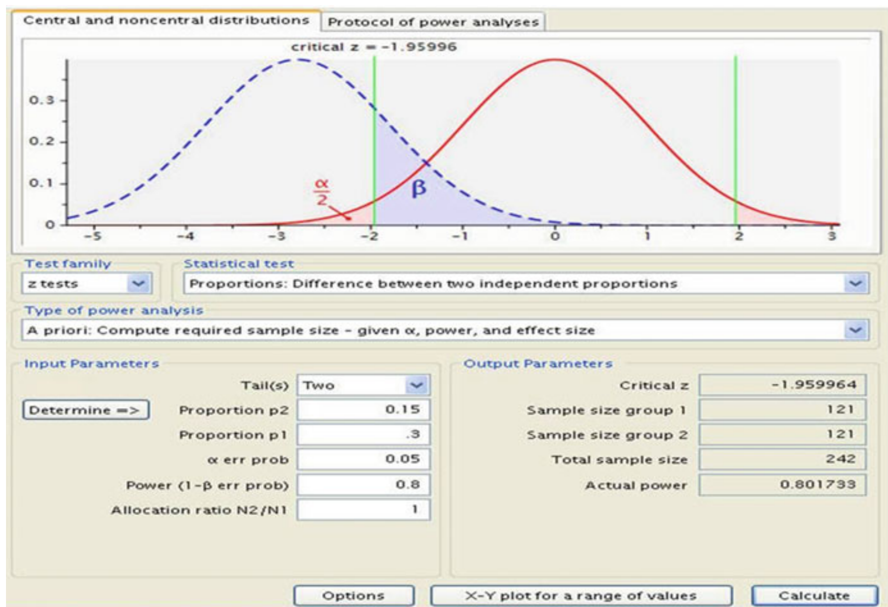
Follow the G power manual instruction: we follow a four-step approach.

1. Step 1: Go to the Test family. Select Fisher's exact test and choose "Exact."
2. Step 2: The Statistical Test: When looking for proportions and we have to compare two independent groups.
3. Step 3: Type of Power Analysis: When the significant threshold ($\alpha = 0.05$) is known, power (80%) is required. When results are available from the pilot study, the effect size can be calculated: go for an "A Priori" analysis = .
4. Step 4: It is tricky.

We need to Input Parameters. Well, it is the tricky one if we have no idea of the effect size, but in this case, it is possible to feed inputs. If we had conducted a pilot study, that can help to arrive at effect size through G*Power.

Parameters must be entered but the effect size is not known. Moreover, if pilot study results are entered, G*Power calculates the effect size.

If we are looking at a decrease of tumor size from 30% to 15%, two groups of about 121 mice would be needed at a significance of ($p < 0.05$) with 80% power. However, if it is aimed to have a decrease in tumor development from 40% to 10%, we would need two groups of about 36 mice for the same significance and 80% power.



Compare Means from Two Independent Samples

Example—In a study on the effect of an antihypertensive drug in rats, a difference of 30 mmHg (i.e., BP control – BP drug) is expected.

Previous work shows within-group standard deviation is 18 mmHg. The significance level is 0.05 (one-tailed) and the required power is 0.80. How many rats are needed per group if groups are of equal size?

Solution: First, we need to select the test from Table 6.4. Here, we compare two independent samples, continuous normal distribution. So, in these conditions, the unpaired *t*-test is applied.

Using G*Power, follow the four-step approach shown above as an example and put all these values: $d = 30/18$, $\beta = 0.80$, $\alpha = 0.05$, one-tailed test.

Result: Six rats per group.

(Use of G*Power is shown as an example of how to use software for our statistical needs. The authors understand that there is several other software that are user-friendly and can be used)

References

1. Bate ST, Clark RA (2014) The design and statistical analysis of animal experiments. Cambridge University Press, New York
2. Mayya SS, Monteiro AD, Ganapathy S (2017) Types of biological variables. J Thorac Dis 9 (6):1730–1733
3. Blank BE (2016) Elementary statistics. First president university press. http://math.wustl.edu/~brian/stats/2200-03.pdf

4. Banos JH (2018) Confounding variables. In: Kreutzer J, DeLuca J, Caplan B (eds) Encyclopedia of clinical neuropsychology. Springer, Cham
5. National Academy of Sciences (US), National Academy of Engineering (US) and Institute of Medicine (US) Committee on Ensuring the Utility and Integrity of Research Data in a Digital Age. Ensuring the Integrity, Accessibility, and Stewardship of Research Data in the Digital Age. Washington (DC): National Academies Press (US) (2009) Ensuring the Integrity of Research Data. Available from: https://www.ncbi.nlm.nih.gov/books/NBK215260/6
6. Kabir SMS (2016) Methods of data collection. An introductory approach for all disciplines, 1st edn. Book Zone Publication, Chittagong, pp 201–276
7. Casarrubea M, Aiello S, Di Giovanni G et al (2019) Combining quantitative and qualitative data in the study of feeding behavior in male Wistar rats. Front Psychol, 10. doi:https://doi.org/10.3389/fpsyg.2019.00881
8. Hox JJ, Boeije HR (2005) Data collection, primary vs. secondary. Encyclopedia of Social Measurement 1:593–599. https://doi.org/10.1016/b0-12-369398-5/00041-4
9. Saxena P, Prakash A, Acharya AS et al (2013) Selecting a study design for research. Indian J Med Spec 4(2):334–339
10. Martins SB, Zin AW, Zin W (2005) Study design. Anaesthesia, Pain, Intensive Care and Emergency Medicine—A.P.I.C.E. Springer-Verlag Mailand pp. 719–747
11. Singh K (2007) Quantitative social research methods. SAGE Publications India Pvt Ltd., New Delhi
12. Broder JM, Ethridge D (1995) Research methodology in applied economics. Am J Agric Econ 77(4):1069–1072
13. Fox W, Bayat MS (2007) A guide to managing research. Juta Publications, Kenwyn
14. Grimes DA, Schulz KF (2002) An overview of clinical research: the lay of the land. Lancet 359 (9300):57–61
15. Hariton E, Locascio JJ (2018) Randomised controlled trials - the gold standard for effectiveness research: study design: randomised controlled trials. BJOG 125(13):1716
16. Jahan N, Naveed S, Zeshan M et al (2016) How to conduct a systematic review: a narrative literature review. Cureus 8(11):e864. https://doi.org/10.7759/cureus.864
17. Burnes D, Sheppard C, Henderson CR et al (2019) Interventions to reduce ageism against older adults: a systematic review and meta-analysis. Am J Public Health 109(8):e1–e9
18. Satake EB (2015) Statistical methods and reasoning for the clinical sciences evidence-based practice, Ist edn. Plural Publishing Inc, San Diego, pp 1–19
19. Delorme A (2004) Statistical methods. J Neurosci 134:9–21
20. Manikandan S (2011) Measures of central tendency: median and mode. J Pharmacol Pharmacother 2(3):214–215
21. Morley A (2001) Statistical methods for anaesthesia and intensive care. Eur J Anaesthesiol 18 (10):701
22. Binu VS, Mayya SS, Dhar M (2014) Some basic aspects of statistical methods and sample size determination in health science research. AYU 35(2):119–123
23. Fay DS, Gerow K (2013) A biologist's guide to statistical thinking and analysis. Worm Book, ed. The C. elegans Research Community, WormBook, https://doi.org/10.1895/wormbook.1.159.1., http://www.wormbook.org
24. Mourougan S, Sethuraman K (2017) Hypothesis development and testing. IOSR J Bus Manage (IOSR-JBM) 19(5):34–40
25. Daniel WW (2002) Biostatistics. Hypothesis testing, 7th edn. John Wiley and Sons, Inc, New York, pp 204–294
26. Banerjee A, Chitnis UB, Jadhav SL et al (2009) Hypothesis testing type I and type II errors. Ind Psychiatry J 18(2):127–131
27. Dakhale G, Hiware S, Mahatme M et al (2012) Basic biostatistics for post-graduate students. Indian J Pharmacol 44(4):435–442
28. Motulsky HJ (1999) Amazing data with GraphPad prism. GraphPad Software Inc., San Diego, CA, pp 65–89. Available from: http://www.graphpad.com

29. McHugh ML (2011) Multiple comparison analysis testing in ANOVA. Biochem Med 21 (3):203–209
30. Charan J, Kantharia ND (2013) How to calculate sample size in animal studies? J Pharmacol Pharmacother 4(4):303–306
31. Festing MF (2018) On determining sample size in experiments involving laboratory animals. Lab Anim 52(4):341–350
32. Cox DR, Reid N (2000) The theory of the design of experiments. Chapman and Hall/CRC Press, New York Washington, DC
33. Festing MF, Altman DG (2002) Guidelines for the design and statistical analysis of experiments using laboratory animals. ILAR J 43(4):244–258
34. Mead R (1988) The design of experiments. Cambridge University Press, Cambridge
35. Arifin WN, Zahiruddin WM (2017) Sample size calculation in animal studies using resource equation approach. Malays J Med Sci 24(5):101–105
36. Cohen J (1988) Statistical power analysis for the behavioural sciences. Lawrence Erlbaum Associates, New York
37. Festing MF, Altman DG (2002) Guidelines for the design and statistical analysis of experiments using laboratory animals. ILAR J 43(4):244–258
38. Suresh K, Chandrashekara S (2012) Sample size estimation and power analysis for clinical research studies. J Hum Reprod Sci 5(1):7–13
39. Whitehead AL, Julious SA, Cooper CL et al (2016) Estimating the sample size for a pilot randomised trial to minimise the overall trial sample size for the external pilot and main trial for a continuous outcome variable. Stat Methods Med Res 25(3):1057–1073
40. Richter CA, Birnbaum LS, Farabollini F et al (2007) In vivo effects of bisphenol-a in laboratory rodent studies. Reprod Toxicol 24(2):199–224
41. Lenth RV (2001) Some practical guidelines for effective sample size determination. Am Stat 55 (3):187–193
42. Jones SR, Carley S, Harrison M (2003) An introduction to power and sample size estimation. Emerg Med J 20(5):453–458
43. Naduvilath TJ, John RK, Dandona L (2000) Sample size for ophthalmology studies. Indian J Ophthalmol 48(3):245–250
44. Babraham, Introduction to Power Calculation Version 2018.02 http://creativecommons.org/licenses/by-nc-sa/2.0/uk/legalcode
45. Coe R (2002) It's the Effect Size, Stupid. What effect size is and why it is important. http://www.leeds.ac.uk/educol/documents/00002182.htm . Accessed 12-14 Sept 2002
46. Festing MFW (2014) Randomized block experimental designs can increase the power and reproducibility of laboratory animal experiments. ILAR J 55(3):472–476
47. Fitts DA (2011) Ethics and animal numbers: informal analyses, uncertain sample sizes, inefficient replications, and type I errors. J Am Assoc Lab Anim Sci 50(4):445–453
48. Dell RB, Holleran S, Ramakrishnan R (2002) Sample size determination. ILAR J 43 (4):207–213
49. Ellis PD (2010) The essential guide to effect sizes. Cambridge University Press, Cambridge
50. Festing MFW (2014) Extending the statistical analysis and graphical presentation of toxicity test results using standardized effect sizes. Toxicol Pathol 42(8):1238–1249
51. Alwin DF, Beattie BA (2016) The KISS principle in survey design. Sociol Methodol 46 (1):121–152

Alternatives to Animal Experiments in Research and Regulatory Testing

7

Shikha Yadav and Vijay Pal Singh

Abstract

The term "alternatives" is used to describe any method resulting in the replacement of animals or reduction in the numbers used in that procedure or refinement of techniques that may minimize pain and suffering in the animals. Every year, we use millions of experimental animals of various species in biomedical research and in testing to assess the safety and effectiveness of drugs, cosmetics, and chemicals. The pain and suffering that these animals experience during these scientific experiments have always been an issue of serious debate. Several scientists, organizations, and institutes across the globe are working for developing and validating alternative methods, and progress has certainly been made in replacing methods using animals that have been in use for several years now with alternate methods. Some of these alternate methods have become a standard practice due to being more ethical, safe, cost-effective, quick, and accurate. However, it is certainly time now that the scientific communities consider using the available alternative methods like computer models, cell and tissue cultures, microorganisms, invertebrates, lower vertebrates, human tissues, and volunteers wherever possible and prioritize the replacement of animals over the refinement and reduction strategies. One of the most effective ways to advance this vision would be by increasing awareness in the scientific community about the available alternative methods and by sharing knowledge through education and training.

S. Yadav (✉)
National Institute of Biologicals, Ministry of Health and Family Welfare, Government of India, Noida, UP, India
e-mail: syadav@nib.gov.in

V. P. Singh
FSSAI, Ministry of Health and Family Welfare, Government of India, New Delhi, India
e-mail: vp.singh@igib.res.in

© The Author(s), under exclusive license to Springer Nature Singapore Pte Ltd. 2021

P. Nagarajan et al. (eds.), *Essentials of Laboratory Animal Science: Principles and Practices*, https://doi.org/10.1007/978-981-16-0987-9_7

The purpose of this chapter is to make the readers aware of the available alternative methods that they may use to replace, reduce, or refine the use of animals and to provide guidance on some of the available resources where they can look for these methods. We can certainly be hopeful that with the new, stricter regulations and smarter and scientifically validated alternatives being developed by scientists, the number of animals that are currently being used in research, testing, and education will continue to decline in the future.

Keywords

Alternative methods · In silico methods · Prediction of toxicity · Tissue cultures · Organ-on-a-chip models · Microorganisms · *S. cerevisiae* · *Salmonella* assay · *Drosophila melanogaster* · *Caenorhabditis elegans* · Zebrafish · Human volunteers

7.1 Introduction

The term “alternatives” is used to describe any method which results in the replacement of animals or reduction in the numbers used in that procedure or refinement of techniques that may minimize pain and suffering in the animals. This term was first introduced by David Henry Smyth in his book *Alternatives to Animal Experiments* in 1978, and it can be seen that Smyth’s term “alternatives” is synonymous with the term “three Rs,” i.e., replacement, reduction, and refinement which was given by W.M.S. Russell and R.L. Burch in their book *The Principles of Humane Experimental Technique* in 1959. Every year, we use millions of experimental animals of various species like mice, rats, hamsters, rabbits, guinea pigs, birds, dogs, nonhuman primates, etc., in biomedical research and in testing to assess the safety and effectiveness of drugs, cosmetics, and chemicals. The use of these laboratory animals in biomedical and behavioral research has helped to significantly increase our scientific knowledge and has contributed enormously to the betterment of human health by helping prevent, cure, and treat a large number of diseases. The laboratory animals also help us as tools in understanding the effects of several medical procedures and surgical experiments. However, the pain and suffering experienced by these animals during these experiments have always been an issue of serious debate. Animal rights activists and anti-vivisectionist groups find animal experimentation to be very cruel and unnecessary and call for the total abolition of all animal research regardless of its purpose or benefit to mankind. This approach too would certainly have some very severe consequences on the advancement of scientific research. And, it is equally important to mention here that scientists themselves do not want to use or cause unnecessary pain and suffering to the animals and that is why they readily accept all the regulatory and ethical controls over the use of animals in education, research, and testing. The scientific community is striving continuously to develop suitable alternatives. It includes “absolute replacement,” where animals are not required at all, or “relative replacement,” where a less sentient animal species is used. The purpose of this chapter is also to make the readers aware of the available alternative

methods that they may use to replace, reduce, or refine the use of animals and to provide guidance on some of the available resources where they can look for these methods. In the last few decades, a large number of promising non-animal methods have been developed such as complex cell cultures, computational models, organ-on-a-chip, etc., but they have not been very successful in replacing the animals completely. This is because they have not been able to mimic the diversity of different cell types and tissues present in a living organism; the complex interactions that occur between various cells, tissues, and organs in various locations in the body; and the influence of the tissue organization on the cellular environment. Although cell culture and tissue culture models may answer many questions about the molecular, cellular, tissue, and even organ functions, animal models are certainly required to investigate how the tissues and organ systems interact with each other.

7.2 3Rs: Centers, Institutes, and Databases

Although researchers are constantly working on ways to develop alternatives, it may not be possible to eliminate animal experiments in the near future. The actual shift from animal models to alternatives would depend on how well they reflect the complex human biology. But presently, it is certainly important for scientists to seriously consider using the available validated alternative methods and start prioritizing replacement over the refinement and reduction strategies. One of the most effective ways to advance this vision would be by increasing awareness among the scientific community about the available alternative methods and by sharing knowledge through education and training.

Several organizations and institutes across the globe are working dedicatedly for the effective implementation of the 3Rs, specifically for developing and validating alternative methods that can replace the use of animals in education, research, and regulatory testing. They also provide a lot of useful information about the 3Rs and available alternatives on their websites. Information on these institutes and their aims and missions are provided in Table 7.1, which may be helpful to the readers to obtain appropriate and updated information about the available alternatives as well as about the ones which are under the process of validation.

7.3 Alternative Methods: Historical Perspective

Continuous efforts are being made by the scientific community to replace animal tests since the 1960s. Earlier, to confirm pregnancy in a woman, her urine was injected into immature rabbits, and the human chorionic gonadotropin (hCG) in the sample of pregnant women would induce ovulation, which was detected only after the rabbit was killed. This test was replaced as gonadotropin could be detected directly using chemical tests to confirm pregnancy. Polio vaccine was used to be produced in primary monkey kidney cells for which a large number of monkeys were killed every year. By the 1970s, use of long-lived human or monkey cell

Table 7.1 Organizations working for alternatives to animal experiments

Organization	Main objectives and activities
Fund for the Replacement of Animals in Medical Experiments (FRAME) (established in London in 1969) https://frame.org.uk/	Promotes 3Rs and in particular "replacement" by validated reliable and reproducible alternative methods Works for eliminating the need to use laboratory animals in any kind of medical or scientific procedures
The Johns Hopkins Center for Alternatives to Animal Testing (CAAT) (established in the USA in 1981) https://caat.jhsph.edu/about/index.html	Promotes development of in vitro and other alternative techniques Facilitates acceptance and implementation of alternative methods Provides reliable information on alternatives to academia, government, industry, and the general public Educates and trains in the application of alternatives Website "Altweb" devoted to 3Rs and alternatives news and information Publishes open-access articles on alternatives to animal experimentation through *ALTEX* journal
European Centre for the Validation of Alternative Methods (ECVAM) (established in EU in 1991) https://ec.europa.eu/jrc/en/eurl/ecvam	Works for development, validation, and acceptance of methods to replace, reduce, or refine the use of animals in laboratories Developed publicly accessible Database on Alternative Methods to Animal Experimentation (DB-ALM) that provides summaries and protocols on alternative methods Developed Tracking System for Alternative Methods toward Regulatory Acceptance (TSAR) which tracks the progress of alternative methods for testing chemicals or biologicals like vaccines from time of submission to validation and final regulatory acceptance as a recognized test method. https://tsar.jrc.ec.europa.eu/test-methods?
The Interagency Coordinating Committee on the Validation Of Alternative Methods (ICCVAM) (established in the USA in 2000) https://ntp.niehs.nih.gov	Facilitates collaborations to promote development, regulatory acceptance, and use of alternative test methods Provides guidance to scientists developing alternative test methods Evaluates recommendations from expert peer reviews of alternative toxicological test methods and makes recommendations to federal agencies on their use The website provides details of alternative methods for chemical safety testing that have been accepted by the US and international regulatory authorities

(continued)

Table 7.1 (continued)

Organization	Main objectives and activities
Japanese Centre for the Validation of Alternative Methods (JaCVAM) (established in Japan in 2005) https://www.jacvam.jp/en/index.html	Promotes the 3Rs in animal experiments for the evaluation of chemical substance safety in Japan Collaborates internationally to establish guidelines for new alternative methods
Norwegian Reference Centre for Laboratory Animal Science and Alternatives (NORECOPA) (established in Norway in 2007) https://norecopa.no/alternatives/alternatives-to-animal-research-and-testing	National Consensus Platform of Norway for the advancement of "the 3Rs" in animal experiments Maintains databases like the following: Text Base that provides information on textbooks available in the field of laboratory animal science https://norecopa.no/textbase-database 3R Guide that contains 3R resources for those planning or conducting animal experiments https://norecopa.no/3r-guide-database NORINA database having information on more than 3000 audiovisual aids which can be used as alternatives in education and training https://norecopa.no/norina-database Inventory of 3Rs, Education and Training Courses and Resources that was produced by EU https://norecopa.no/european-commission-inventory-of-3rs-education-training-resources
Korean Centre for the Validation of Alternative Methods (KoCVAM) (established in Korea in 2009) http://www.nifds.go.kr/kocvamen/	Formulates and promotes policies concerning the development and approval of alternatives to animal testing Provides information regarding alternative test methods and educational programs for industry, academia, and research institutions Coordinates the process of validating and peer-reviewing alternative test methods and proposing validated test methods to the OECD for guidelines
Brazilian Centre for the Validation of Alternative Methods (BraCVAM) (established in Brazil in 2013)	Plays an important role in the development and validation of alternative methods, through the active participation of the National Network on Alternative Methods (ReNaMA) Prepares final recommendations on the validated test method and sends to the National Council for the Control of Animal Experimentation (CONCEA) which then is in charge of the regulatory adoption of all validated test methods in Brazil
Canadian Centre for the Validation of Alternative Methods (CaCVAM) (established in Canada in 2017) https://www.uwindsor.ca/ccaam/	Works with the Canadian regulators to expedite the development, validation, and regulatory acceptance of the new alternative methods
AltTox (http://alttox.org/)	A comprehensive resource for scientists and policymakers to promote the use of alternatives

(continued)

Table 7.1 (continued)

Organization	Main objectives and activities
	primarily for animal-based toxicity testing Its website AltTox.org is focused on providing information on the development, validation, and international acceptance of nonanimal toxicity test methods and by the national and regional regulatory authorities as replacements for many of the animal toxicity test methods which are currently required for regulatory submissions A section on toxicity testing, i.e., Methods, Approaches, Programs & Policies (MAPP), provides information on alternative methods for toxicity testing including the in vitro, ex vivo, in chemico, and in silico methods along with those in vivo methods which may still help in reducing or refining the animal use Provides details of agencies that have endorsed the scientific validity of these methods and the status of the regulatory acceptance at international (OECD) or at national or regional level (European Pharmacopeia, FDA, REACH, etc.) http://alttox.org/mapp/table-of-validated-and-accepted-alternative-methods/

cultures not only resulted in saving monkeys but also made the vaccine safer by eliminating the risk of contamination with animal viruses [1]. In the same year, the potency test of the yellow fever vaccine that was used to be performed on animals in lethal dose tests was replaced by the cell culture-based plaque reduction neutralization test [2]. Insulin that is used in diabetic patients was earlier extracted from the pancreas of cows and pigs, but obtained from bacterial cultures by using recombinant DNA technology. The mouse convulsion test that was used to test insulin required 600 mice per batch. The number of animals going into convulsions after the injection was used to measure the strength of insulin. However, as science progressed and the analytical techniques improved, animals were no longer used, and insulin was directly assayed by chromatographic techniques [3]. Earlier, all the new cosmetics were tested on animals such as rabbits to test their potential for skin irritation. However, in the past two decades, multilayered human epidermal cell culture models like EpiDerm™ by Mattec Corporation and EpiSkin™ by SkinEthic have been shown to provide accurate results for the irritation potential of any product that is to be used on human skin and have completely replaced the need for testing in animals. It is realized that in the near future, it might not be possible to replace the in vivo Draize eye test in rabbits with any one single in vitro eye irritation test which would predict the full range of irritation for all the classes of chemicals. However, careful combinations of several alternative test methods in a tiered testing strategy have been able to replace this in vivo test to some extent. The Bovine Corneal

Opacity and Permeability (BCOP) test method is an organotypic model that enables scientists to maintain the normal physiological and biochemical functions of the bovine cornea obtained from freshly slaughtered animals in vitro and has been adopted by OECD in July 2013 (TG 437) for identifying chemicals inducing serious eye damage and for chemicals not requiring classification for eye irritation or serious eye damage, thus resulting in a considerable reduction in the number of animals being used in Draize test. Regulations also make it mandatory to ensure that each batch of drug products that are intended for parenteral administration is free from pyrogens, and historically, the rabbit pyrogen test (RPT) was the required test. However, with the advancement in technologies, this animal-based test has gradually been replaced by alternative tests like bacterial endotoxin test (BET), monocyte activation test (MAT), and recombinant factor C (rFC) test in most of the cases, although the suitability of these alternatives has to be demonstrated in a product-specific validation to ensure quality control of parenteral drugs. Therefore, it can be seen that significant progress has been made in replacing animals completely or in reducing their use considerably, and these alternative methods that have been in use for several years now have become a standard practice and proved to be more ethical, cost-effective, quick, and accurate.

7.4 Available Alternative Methods and Recent Developments

7.4.1 Computer Models

Several in silico computer simulation software are available that help predict the various possible biological or toxic effects of a potential drug/chemical without the use of animals so that only the most promising molecules are then taken for in vivo experimentation, thus helping reduce the number of animals enormously. These software are available in the public domain and can be accessed either free of cost or on a fee-based service.

7.4.2 Prediction of Toxicity

Computer-aided drug design (CADD) software helps to screen the chemicals for potential biological activity in animals, by identifying the receptor binding site for the new potential drug candidate. Such software programs also help to tailor-make new drug molecules for specific binding sites, thus requiring testing in animals only in the final stage to confirm the results [4], thus helping in an enormous reduction in the total number of animals used in such studies.

Several software related to structure-activity relationship (SAR) such as Deductive Estimation of Risk-based on Existing Knowledge (DEREK), Toxtree, Ecostar, and OECD QSAR Toolbox have been developed that help predict multiple toxicity endpoints and species for new drug candidates. SAR programs are also available that help to predict the biological activity of potential drug molecules based on the

presence of certain chemical moieties found attached to the parent compounds. Quantitative structure-activity relationship (QSAR) mathematically describes the relationship between physicochemical properties and biological activity of the drug molecules. When compared to animal testing, this software is a faster and inexpensive way to predict several activities like carcinogenicity and mutagenicity of potential drug candidates [5]. The QSAR Toolbox developed by OECD [6] is a free software application used for filling gaps of (eco) toxicity data needed to assess the potential hazards of substances. It helps in providing reproducible and transparent chemical hazard assessment. Some of the other online toxicity prediction tools available are as follows:

(a) **Toxtree**—free stand-alone software that applies a decision tree approach to estimate toxic hazards.
(b) **PredSkin**—helps to predict the skin sensitivity potential of a chemical.
(c) **Endocrine Disruptome**—helps to predict the endocrine action of any molecule against 18 structures belonging to 14 nuclear receptors.
(d) **ProTox-II**—a web server which is used for the prediction of toxicity of chemicals.
(e) **eMolTox**—another webserver useful for toxicity prediction and drug safety analysis.

Besides, several in silico methods have been developed that are in use in the pharmaceutical industry to study and predict the physicochemical, pharmacokinetic, and pharmacodynamic parameters of drugs.

Pharmacodynamics/Target Prediction

(a) **iDrug-Target**—helps to predict drug-target interaction.
(b) **SEA-2D**—is used for similarity-based approach.
(c) **SwissTarget**—helps to predict drug-target based on 2D and 3D similarity approach.
(d) **PASS Online—**helps to predict over 4000 different kinds of biological activities like the mechanisms of action, pharmacological as well as toxic effects, interaction with metabolic enzymes and transporters, influence on gene expression, etc.

Pharmacokinetics (ADME)

(a) **SwissADME**—is used for computation of both physiochemical properties and pharmacokinetic parameters.
(b) **SOM Prediction**—helps to predict the potential metabolic sites of a molecule and its metabolic products.
(c) **admetSAR 2.0**—a free comprehensive tool for evaluating chemical ADMET properties,
(d) **pkCSM**—predicts small-molecule PK properties using graph-based signatures.

7.4.3 Adverse Drug Reactions

Owing to the huge global health burden and failure of drugs, predicting adverse drug reactions (ADRs) in the preclinical stages has become very important to reduce drug failures as well as the time and cost of development and also to provide efficient and safe therapeutic to the patients. In recent years, many computational methods for in silico ADR prediction [7] have been put forward, but it remains a challenge for the drug developers, as many deaths still occur every year due to ADRs that are not apparent until the particular drug has been marketed and used by the patients. Though computer models for identification of ADRs have limitations mainly owing to lack of complete human metabolomics data, they certainly can be a valuable tool in drug development as the early detection of ADRs would not only help to reduce costs but also help to considerably reduce the number of animals used in preclinical trials.

In the recent years, the use of these in silico methods is on the rise mainly due to the requirements for reducing animal testing according to the REACH (Registration, Evaluation, Authorization and Restriction of Chemicals) legislation of the European Union and the implementation of similar laws and regulations globally, rapid technological progress leading to the development of new reliable alternative methods, and also the economic incentives on using alternatives [6]. In recent years, there has been a rapid evolution in the implementation of these in silico methods into regulatory use, and these may gradually replace many of the classical in vivo tests.

7.5 Cell and Tissue Cultures

Scientists have been growing different types of animal and human cells in laboratories. The traditional two-dimensional (2D) cell cultures have been crucial in improving our understanding of cell biology and mechanisms of several diseases and also in the drug discovery and development process. They have successfully provided an alternative to the animal model in several types of studies like preclinical research of drugs, cancer research, studies on gene function, etc., thus contributing greatly to reducing the use of animals. These 2D cell cultures have the advantage of being simple and having a low maintenance cost. However, they have certain limitations also as the adherent cultures growing as monolayers on flat flasks do not mimic the natural structures and microenvironments of the cells and tissues or tumors, thus affecting the cell-cell and cell-extracellular environment interactions which in turn might affect several cellular functions. Moreover, the adherent cultures allow the researchers to study only one type of cell at a time. Therefore, scientists had been making efforts to develop better in vitro cell culture models that closely resemble the in vivo conditions.

Of late, three-dimensional (3D) cultures have been developed that provide a more realistic way to study diseases and test new therapies. The organ-on-chip models utilize a dynamic 3D environment very similar to the human body, providing good

opportunities for understanding the pathogenesis of several human diseases and also providing a better model to screen novel drug molecules. These models have been developed for all the major organs of the body including the heart, lung, and kidney to test the effect of any new drug candidate on these individual organs on a small, microfluidic scale simulating the biology as well as the physiology of the human organs. These miniature 3D cell culture-based "organ-on-chip" models have been designed to mimic all the processes of their regular-sized counterparts including electrophysiological responses, exchange of gases, and fluid filtration. This also allows the researchers to use a variety of imaging techniques to study the processes in the organs, which is a major advantage over the typical 2D and 3D cell cultures. Scientists can arrange single types of cells opposite to one another along a porous membrane, thus enabling the exchange of the cellular products as well [8]. The use of microfluidics technology offers the advantage of performing tissue culture in controlled environments and adjusted to optimize the pH, temperature, supply of nutrients, and disposal of waste [9]. Researchers have also integrated these organ-on-chip systems with various sensors and actuators so that the key parameters in the human body can be monitored and controlled more accurately [10]. These chips can contribute extensively to the discovery and development of new drugs as they can model the complex and dynamic processes of absorption, distribution, metabolism, and excretion (ADME) of drugs, which form an efficacy benchmark for any new drug molecule. These chips can be used to study various disease states, particularly those diseases that are specific to humans where the animal models are not able to provide answers such as those involving the brain or immune system. They are also useful for testing the efficacy of various drugs and vaccines to know how they may function when administered in vivo. In recent years, scientists have been attempting to develop innovative microfluidic designs so that these chips can closely simulate the actual organs. A group of scientists has developed a biomimetic microsystem that not only mimics the functional alveolar-capillary interface of the human lung but also reproduces the complex integrated organ-level responses, on the introduction of bacteria and inflammatory cytokines into the alveolar space [11]. In 2012, an organ-on-a-chip was developed to model pulmonary edema of humans which mimics the lung function in response to mechanical strain and cytokine IL-2 and also accurately predicted the activity of new drug candidates [12]. In 2015, a heart-on-chip system using microfluidics was developed to assess the effects of the cardiovascular drug which had the advantage of precisely regulating some conditions like flow rate, pulsatile flow, and shear stress in addition to having high-throughput capabilities [13]. A disease model of cardiomyopathy of BTHS based on heart-on-chip technology and iPSC-derived cardiomyocytes (CMs) from iPSCs of Barth syndrome (BTHS) patients was developed [14]. This model has been used successfully to test new treatment options involving pharmacology and genetic modification and also for identifying new potential therapeutic targets for BTHS [15, 16]. The two main factors that influence the time for which the drugs stay on the cornea are blinking and tear flow, and based on this, a corneal epithelium-on-a-chip model has been developed which mimics the cellular environment and the tear flow associated with the eye-blinking mechanism [17]. Drug transport and their permeability across

the rate-limiting barrier can also be analyzed. As the biology of the corneal epithelium on the chip is quite similar to that of humans, it can be used for understanding ocular pharmacokinetics and physiology and thus can also support the ophthalmic drug test method. A 3D tissue microfabrication construct has been developed comprising heart, lung, and liver cells in separate but interconnected chambers which has demonstrated the highly critical interorgan response to drug administration [18]. Bleomycin was shown to be cardiotoxic as determined by altered kinetics of heart cell beat followed by a complete cessation in this multi-organ system, while it was not cardiotoxic in the "heart-only" construct. This could be attributed to bleomycin-induced secretion of IL-1β in the lung tissue which is known to be cardiotoxic [19]. Thus, the multi-organ system paves way for analysis much closer to the "in vivo" techniques.

Models of human-on-a-chip are also being developed that would move closer to mimicking the whole human response and enable scientists to study the systemic effect of drugs on the human body. This includes individual vital organs on chips that are further connected by a microfluid circulation system in a microfabrication bioreactor. These models closely mimic the interactions between multiple tissues, realistic size ratios, and physiological fluid flow conditions of a human body [20]. They also enable the scientists to study the effects of various drugs on human cells that are organized in a similar anatomical way, which certainly offers a distinct advantage over the animal models, as these human cells in culture behave in almost the same way as they would behave in the body, thus reducing or replacing the need for animal testing.

Although these organ-on-a-chip and human-on-a-chip models have some limitations, the new emerging technologies have certainly improved their capability for translational research, high-throughput analysis, and precision medicine and thus can contribute enormously as alternative methods in the preclinical drug development studies and in estimating the toxicity of environmental contaminants.

7.6 Microorganisms

7.6.1 *Saccharomyces cerevisiae*

Saccharomyces cerevisiae (brewing yeast) is one of the most common eukaryotic microorganisms used for biological studies. Its cellular architecture is similar to multicellular eukaryotes, and the genome is very well characterized and studied. Besides, rapid growth, short generation time, ease of replica plating, and mutant isolation with a highly versatile DNA transformation system provide distinct advantages. The various membrane-bound organelles present in the yeast-like nucleus, peroxisome, mitochondria, and organelles of the secretory pathway are similar to the functions of mammalian cells [21]. *S. cerevisiae* has also been studied as a model to replace the traditional LD50 test invertebrates, and the LD50 tests in yeast model were found to correlate well with the customary LD50 test in mice, rats, and other laboratory animals for nearly 160 common drugs and other chemicals

[22]. It is also used as a model to study programmed cell death and cancer [23]. Its utility in studying the mechanisms of aging and longevity of multicellular organisms, however, has been limited [24]. Engineered yeast models have been developed to study the endogenous or heterologous proteins that lie at the root of complex human diseases and have proven to be powerful tools for understanding the molecular mechanisms of neurodegenerative diseases such as Parkinson's, Alzheimer's, and Huntington's disease [25, 26]. The yeast screening assays have also been greatly useful in the first-line high-throughput screening of potentially active compounds and help to greatly reduce the number of animals used in the discovery of new therapeutic agents.

7.6.2 *Salmonella* Species

The *Salmonella* assay also known as the Ames test has played an invaluable role in identifying rodent and human carcinogens [27, 28]. Over the years, this assay has undergone several modifications which have enabled the use of the very minute number of samples in semi-throughput modes [29] and testing of body fluids like urine [30], feces [31], cervical mucus [32], breast milk [33], and breast nipple aspirates [34]. Approximately 30,000 chemicals that are produced in an amount exceeding 1 ton/year are required to be tested by the *Salmonella* assay under the European Union's REACH legislation [35]. The flexibility of this assay has made it useful for almost every type of environmental and molecular epidemiology study, and the experience of the scientists with the *Salmonella* assay can be used for developing new approaches for predicting and understanding the toxicology of substances [36].

Several other prokaryotes, protists, and fungi can also be used as alternatives. *Bacillus subtilis* is a bacterial model that has been used for studying cellular differentiation. *Escherichia coli*, *Dictyostelium discoideum*, and *Schizosaccharomyces pombe* have been used as a model for molecular and genetic studies and *Neurospora crassa* as a model for genetics as well as for circadian rhythm and metabolic regulation studies [37].

7.7 Invertebrates

Invertebrates have been used as models in teaching and research since the eighteenth century. Due to the public and ethical concerns about the use of vertebrate animals in research, interest in establishing and using invertebrate models like nematodes, insects, crustaceans, mollusks, etc., has increased considerably in the last few decades. The most commonly used invertebrate models are *Drosophila melanogaster* and *Caenorhabditis elegans*.

7.7.1 *Drosophila melanogaster*

Drosophila melanogaster, commonly known as fruit fly, is one of the most widely used invertebrate models in research because of the various advantages that it offers over the other vertebrate models, especially mammals. It is easy to grow in laboratories and has an extremely low maintenance cost. It has a very short life cycle consisting of four stages, i.e., embryo, larva, pupa and adult, each of which is used to study various scientific concepts. The embryo is used as a model to study organogenesis and neuronal development, while the larva is useful for studying the physiological and developmental processes. In an adult fly, functions of various organs like the heart, lungs, gut, reproductive tract, and kidney are found to be similar to that in mammals [38]. *Drosophila* genome is 60% homologous to that of humans, its complete genome has been sequenced, and almost 75% of the genes that are found to be involved in human diseases have a functional homolog in this fly [39, 40]. The heart of the fly is an ideal model for studying cardiac development and cardiac diseases and also for understanding the underlying cellular and molecular mechanisms in morphogenesis because of the conservation of key genes and close similarity with the vertebrate cardiogenesis [41]. *Drosophila* is also used for studying the blood cell development of vertebrates because of having similarities with the mammalian mechanisms of hematopoiesis [42]. Researchers have developed diabetic, obese, genetically "lean," as well as hypoglycemic phenotypes of these flies which are excellent models to study the pathogenesis of important metabolic diseases like obesity and diabetes [43]. The insulin signaling in these flies is very similar to that in humans, making it a very good model to study the mechanisms by which insulin regulates metabolism [44].

Drosophila is a good model for studying human genetics due to several similarities in their development and behavior [45] and has also been used to study human diseases for comparing the resulting pathologic conditions by expressing the specific protein products found in human disease. It is also an excellent model to investigate neurodegenerative diseases like Parkinson's, Alzheimer's, and Huntington's diseases [46, 47]. The molecular mechanisms driving wound healing in *Drosophila* was found to resemble those involved in the tissue fusion events during animal development making it a good model for studying wound healing [48]. The excretory system of the fly has been very useful in understanding the development and differentiation of the renal system across species [49]. This fly has been used extensively to understand the molecular mechanisms regulating stem cell activity in all animals. This model was used to demonstrate apoptosis in response to damage or stress and the molecular signals that initiate tissue regeneration by activating the proliferation of the stem cells [50, 51]. *Drosophila* can also be used as a tool in the clinical drug discovery process as an initial, fast, and high-throughput screening alternative. An added advantage is that the genetic background of this fly can be manipulated very easily to mimic a diseased state which can then be used as a model to test the efficacy of potential drugs for that particular disease.

7.7.2 *Caenorhabditis elegans*

Caenorhabditis elegans is a eukaryotic, multicellular, transparent nematode with a length of 1 mm, which is a highly prolific breeder having a very short generation time. It has a 2–3-week-long life cycle in which embryogenesis takes place in 12 hours and an adult develops within 2.5 days. It can be easily grown in laboratories and is relatively inexpensive to maintain. *C. elegans* has almost the same number of genes as humans and has several similarities at genetic and molecular levels. It has been used as a model to understand the molecular mechanisms involved in many human diseases such as Parkinson's disease, Alzheimer's disease, cancer, diabetes, and some immune disorders [52–54]. It has also been used in the development and testing of new drug molecules for the treatment of these diseases [55]. *C. elegans* can be used as a model for forward and reverse genetics as their transgenic, mutant, and knockouts can be developed very easily and those expressing the green fluorescent protein (GFP) offer an added advantage of allowing the scientists to observe the cellular and metabolic processes in vivo [56, 57].

7.8 Lower Vertebrates

In cases where complete replacement of animals is not possible, efforts can be made to see if at least the higher animals can be "replaced" with lower vertebrates or invertebrates. The zebrafish is one such alternative that has become quite popular in recent years.

7.8.1 *Danio rerio* (Zebrafish)

Zebrafish is an important vertebrate model that offers several advantages over the other vertebrate models. They require a simple habitat and can be easily cost-effectively maintained in a laboratory. The short generation time of 3–5 months and large clutch size of around 250–300 help in providing sufficient animals for research. Another unique advantage of the zebrafish is that they have external fertilization which in turn facilitates observation of the development pattern as well as an experimental manipulation of the embryos. Moreover, the transparent embryos also allow the use of noninvasive imaging techniques for visualization of fluorescently labeled individual genes throughout the developmental process [58, 59]. Scientists have constructed 3D imaging in live zebrafish by using fluorescent probes, thus enabling high-throughput imaging with good reproducibility of results [60]. This model can also be used to study the pathogenesis of several human diseases [61]. The zebrafish embryos have been shown to exhibit similar responses as other mammals to drugs for cardiovascular diseases, cancer [62], and neurological drug discovery [63, 64]. The zebrafish possesses a molecular, structural, and functional similarity of the blood-brain barrier (BBB) with those of the higher vertebrates [65]. The small size and optical clarity of the embryos and larvae enable the use of

this model in high-throughput drug discovery programs [66]. Zebrafish is a powerful and validated vertebrate model for studying human neurodegenerative diseases like Alzheimer's disease [67] because of the close resemblance in the neuroanatomic and neurochemical pathways of zebrafish and the human brain. Emotional, physiological, and social-behavioral pattern similarities with that of higher animals have also been observed in zebrafish. Zebrafish age gradually like humans and also show similar aging-related changes across both cognitive and neurobiological spectra making it a good model to study the neurobiological mechanisms that underlie aging-related cognitive decline [68].

7.9 Human Tissues and Volunteers

Animals used in research have undoubtedly played a vital role in scientific and medical advances and have enhanced the lives of millions of people. With advances in technology and scientific processes, now it is certainly possible to use human tissues to replace some use of animals in research. All the tissues and organs donated by human donors or those collected at the time of surgeries or autopsy are useful for biomedical research and contribute to advancing the fundamental understanding of human biology, improve our knowledge of disease pathways and mechanisms, and help in developing better diagnostic techniques and in the discovery of better cures and treatments for all the diseases that occur in humans. By creating greater awareness about donating samples, it is possible to increase the availability of human tissues and organs for research, which not only allows the scientists to study diseases in tissues that are infected but also helps to reduce the number of animals used in scientific and medical testing.

Another way to prevent the use of animals is by safely using available human volunteers. Micro dosing is one method that utilizes the technological advances in analytical techniques and helps to assess the basic behavior of a new drug by injecting the novel molecule in human volunteers at doses which are much lesser than those that are expected to produce whole-body effects so that even the potentially harmful substances do not pose a threat [69]. It is also possible for the scientists to use sophisticated scanning technologies like CT, PET, and MRI to see abnormalities and track the progress of the treatment in the brains of the patients suffering from Alzheimer's disease, Parkinson's diseases, schizophrenia, epilepsy, and brain injury. Similarly, the recent advancements in brain imaging technologies have enabled the researchers to measure the human brain activity noninvasively and with high precision, thus creating hope of soon replacing the highly invasive and painful procedures that are presently done in monkeys [70].

7.10 Barriers to the Implementation of Alternatives and the Way Forward

The efforts made toward the use of alternatives have certainly helped in reducing the numbers of animals that are being used and in refining the experimental procedures to minimize their pain and suffering. Despite all these efforts, the actual success in eliminating the use of animals in research and regulatory testing has been minimal. Some of the factors that contribute to the delay in the acceptance and implementation of alternative are as below:

- The possibility of replacing animals with alternatives depends on the nature and requirements of each experiment. While isolated cell and tissue cultures are not able to mimic a complicated living system, it is not possible to use human volunteers in potentially harmful experiments. Therefore, it is very important to understand the scientific barriers to replacement in specific types of experiments so that scientists can focus on overcoming these barriers.
- The medical and veterinary colleges and some specific areas of biological research continue to depend on the use of animals.
- Often scientists have their preferred ways of performing experiments, and a lot of persuasion and training in alternative methods would be required to convince them to shift to the alternatives.
- Developing countries are not able to prioritize developing or implementing alternative methods due to a lack of funding and resources.
- Over the years, the increasing standard of ethical approval and control has certainly improved the implementation of 3Rs. However, the members of ethical committees need to be trained and sensitized about the available validated alternatives and then encouraged to perform very critical ethical review so that animal use could be avoided wherever possible.
- Developing alternative methods in product safety testing of medicines or chemicals is very challenging and time-consuming because of the existing laws and regulations in several countries. The period between the successful validation of the alternative methods and their regulatory acceptance and actual implementation is long. The processes of multinational agreements required for revision of the pharmacopoeial monographs and regulatory guidelines delays the process. The production of the new reference material for the new test systems in sufficient quantities is time-consuming and expensive.
- Although there are many successful alternative methods available in basic research, their use is mostly limited to the laboratories where they are developed. Publications with detailed methodologies, commercialization, and technology transfer of these methods are required to bring about their widespread use in the scientific community. Stricter enforcement of regulations to ensure the use of the alternatives where available would expedite the replacement of animals that are being used in research.

7.11 Conclusion

The scientific community certainly wants to replace all experiments that cause suffering in animals with humane alternatives. While several options are already available and can be used, some are not sophisticated enough to eradicate animal testing. However, it is certainly time now that the scientific community considers using the available validated alternative methods and prioritizes the replacement of animals, over the refinement and reduction strategies. With new, stricter regulations and smarter and scientifically validated alternatives being developed by scientists, we can certainly be hopeful that the number of animals used in research, testing, and education will continue to decline in the future.

References

1. Bookchin D, Schumacher J (2005) The virus and the vaccine: contaminated vaccine, deadly cancers, and government neglect. St Martin's Press, New York
2. World Health Organization (WHO) (2007) guidelines for plague reduction neutralisation testing of human antibodies to dengue viruses. WHO/IVB/o707
3. Underhill LA, Dabbah R, Grady LT, Rhodes CT (1994) Alternatives to animal testing in the US P-NF : present and future. Drug Dev Ind Pharm 20:165–216
4. Vedani (1991) Computer-aided drug design: an alternative to animal testing in the pharmacological screening. ALTEX 8(1991):39
5. Matthews EJ, Contrera JF (1998) A new highly specific method for predicting the carcinogenic potential of pharmaceuticals in rodents using enhanced MCASE QSAR-ES software. Regul Toxicol Pharmacol 28:242–264
6. Raunio H (2011) In silico toxicology—non-testing methods. Front Pharmacol 2. Article 33:1–8
7. Kuang Q, Wang MQ, Li R, Dong YC, Li Y, Li M (2014) A systematic investigation of computation models for predicting adverse drug reactions (ADRs). PLoS One 9(9):e105889
8. Zhu J (2018) Application of organ-on-chip in drug discovery. J Biosci Med 2020(8):119–134
9. Bhatia SN, Ingber DE (2014) Microfluidic organs-on-chips. Nat Biotechnol 32:760–772. https://doi.org/10.1038/nbt.2989
10. Yu F, Hunziker W, Choudhury D (2019) Engineering microfluidic organoid-on-a-chip platforms. Micromachines 10:165. https://doi.org/10.3390/mi10030165
11. Huh D, Matthews BD, Mammoto A, Montoya-Zavala M, Hsin HY, Ingber DE (2010) Reconstituting organ-level lung functions on a chip. Science 328:1662–1668
12. Huh D, Leslie DC, Matthews BD, Fraser JP, Jurek S, Hamilton GA et al (2012) A human disease model of drug toxicity-induced pulmonary edema in a lung-on-a-chip microdevice. Science Translational Medicine 4:159ra147
13. Skommer J, Wlodkowic D (2015) Successes and future outlook for microfluidics- based cardiovascular drug discovery. Expert Opin Drug Discovery 10:231–244. https://doi.org/10.1517/17460441.2015.1001736
14. Wang G, Mccain ML, Yang L, He A, Pu WT (2014) Modeling the mitochondrial cardiomyopathy of Barth syndrome with iPSC and heart-on-chip technologies. Nat Med 20:616–623
15. Martins AM, Vunjak-Novakovic G, Reis RL (2014) The current status of iPS cells in cardiac research and their potential for tissue engineering and regenerative medicine. Stem Cell Rev Rep 10:177–190
16. Ribas J, Sadeghi H, Manbachi A, Leijten J, Khademhosseini A (2016) Cardiovascular organ-on-a-Chip platforms for drug discovery and development. Appl Vitro Toxicol 2:82–96

17. Bennet D, Estlack Z, Reid T, Kim J (2018) A microengineered human corneal epithelium-on-a-chip for eye drops mass transport evaluation. Lab Chip 18:1539–1551. https://doi.org/10.1039/C8LC00158H
18. Skardal A, Murphy SV, Devarasetty M, Mead I, Kang HW, Seol YJ, Atala A et al (2017) Multi-Tissue Interactions in an Integrated Three-Tissue Organ-on a- Chip Platform. Scientific Reports 7:8837
19. Didagelos M, Boutis A, Diamantopoulos N, Sotiriadou M, Fotiou C (2013) Bleomycin cardiotoxicity during chemotherapy for an ovarian germ cell tumor. Hippokratia 17(2):187–188
20. Esch MB, King TL, Shuler ML (2011) The role of body-on-a-chip devices in drug and toxicity studies. Annu Rev Biomed Eng 13:55–72
21. Mell JC, Burgess SM (2002) Yeast as a model genetic organism encyclopedia of life sciences. Mcmillan Publishers Ltd.
22. Koch HP, Hofeneder M, Bohne B (1993) The yeast test: an alternative method for the testing of acute toxicity of drug substances and environmental chemicals. Methods Find Exp Clin Pharmacol 15(3):141–152
23. Madeo F, Engelhardt S, Herker E, Lehmann N, Maldener C, Proksch A, Frohlich KU (2002) Apoptosis in yeast: a new model system with applications in cell biology and medicine. Curr Genet 41:208–216
24. Bilinski T, Bylak A, Zadrag-Tecza R (2017) The budding yeast Saccharomyces cerevisiae as a model organism: possible implications for gerontological studies. Biogerontology 18 (4):631–640
25. Pereira C, Bessa C, Soares J, Leão M, Saraiva L (2012) Contribution of yeast models to neurodegeneration research. J Biomed Biotechnol 2012:941232
26. Outeiro TF, Lindquist S (2003) Yeast cells provide insight into alpha-Synuclein biology and pathobiology. Science 302(5651):1772–1775
27. Kirkland D, Aardema M, Henderson L, Muller L (2005) Evaluation of the ability of a battery of three in vitro genotoxicity tests to discriminate rodent carcinogens and non-carcinogens I. sensitivity, specificity and relative predictivity. Mutat Res 584:1–256
28. Morita T, Asano N, Awogi T, Sasaki YF, Sato S, Shimada H et al (1997) Evaluation of the rodent micronucleus assay in the screening of IARC carcinogens (Groups 1, 2A and 2B): the summary report of the 6th collaborative study by CSGMT/JEMS MMS. Mutat Res 389:3–122
29. Kamber M, Flückiger-Isler S, Engelhardt G, Jaeckh R, Zeiger E (2009) Comparison of the Ames II and traditional Ames test responses with respect to mutagenicity, strain specificities, need for metabolism and correlation with rodent carcinogenicity. Mutagenesis 24:359–366
30. Cerná M, Pastorková A (2002) Bacterial urinary mutagenicity test for monitoring of exposure to genotoxic compounds: a review. Cent Eur J Public Health 10:124–129
31. De Kok TM, Van Maanen JM (2000) Evaluation of fecal mutagenicity and colorectal cancer risk. Mutat Res 463:53–101
32. Holly EA, Cress RD, Ahn DK, Aston DA, Kristiansen JJ, Wu R et al (1993) Detection of mutagens in cervical mucus in smokers and nonsmokers. Cancer Epidemiol Biomark Prev 2:223–228
33. Phillips DH, Martin FL, Williams JA, Wheat LM, Nolan L, Cole KJ et al (2002) Mutagens in human breast lipid and milk: the search for environmental agents that initiate breast cancer. Environ Mol Mutagen 39:143–149
34. Klein P, Glaser E, Grogan L, Keane M, Lipkowitz S, Soballe P et al (2001) Biomarker assays in nipple aspirate fluid. Breast J 7:378–387
35. Poth A, Jaeger M (2002) Alternative testing—the intelligent way to REACH compliance. AATEX 14:799–803
36. Claxton LD, Umbuzeiro GA, DeMarini DM (2010) The salmonella mutagenicity assay: the stethoscope of genetic toxicology for the 21st century. Environ Health Perspect 118 (11):1515–1522
37. Doke SK, Dhawale SC (2015) Alternatives to animal testing: a review. Saudi Pharm J 23 (3):223–229

38. Rothenfluh A, Heberlein U (2002) Drugs, flies, and videotape: the effects of ethanol and cocaine on drosophila locomotion. Curr Opin Neurobiol 12:639–645
39. Reiter LT, Potocki L, Chien S, Gribskov M, Bier E (2001) A systematic analysis of human disease-associated gene sequences in Drosophila melanogaster. Genome Res 11:1114–1125
40. Wilson-Sanders SE (2011) Invertebrate models for biomedical research, testing, and education. ILAR J 52:126–152
41. Medioni C, Senatore S, Salmand PA, Lalevee N, Perrin L, Semeriva M (2009) The fabulous destiny of the drosophila heart. Curr Opin Genet Dev 19:518–525
42. Crozatier M, Meister M (2007) Drosophila haematopoiesis. Cell Microbiol 9:1117–1126
43. Bharucha KN (2008) The epicurean Fly: using Drosophila Melanogaster to study metabolism. Pediatr Res 65(2):132–137
44. Teleman AA (2009) Molecular mechanisms of metabolic regulation by insulin in drosophila. Biochem J 425:13–26
45. Beckingham KM, Armstrong JD, Texada MJ, Munjaal R, Baker DA (2005) Drosophila melanogaster: the model organism of choice for the complex biology of multi-cellular organisms. Gravit Space Biol Bull 18:17–29
46. Bonini NM, Fortini ME (2003) Human neurodegenerative disease modeling using drosophila. Ann Rev Neurosci 26:627–656
47. Iijima K, Iijima-Ando K (2008) Drosophila models of Alzheimer's amyloidosis: the challenge of dissecting the complex mechanisms of toxicity of amyloid-beta 42. J Alzheimers Dis 15:523–540
48. Agnès F, andNoselli S (1999) Dorsal closure in drosophila. A genetic model for wound healing? C R Acad Sci III 322(1):5–13
49. Denholm B, Helen Skaer H (2009) Bringing together components of the fly renal system. Curr Opin Genet Dev 19(5–3):526–532
50. Ryoo HD, Gorenc T, Steller H (2004) Apoptotic cells can induce compensatory cell proliferation through the JNK and the wingless signaling pathways. Dev Cell 7:491–501
51. Jennings BH (2011) Drosophila—a versatile model in biology & medicine. Mater Today 14 (5):190–195
52. Link CD, Johnson CJ, Fonte V, Paupard M, Hall DH, Styren S, Mathis CA, Klunk WE (2001) Visualization of fibrillar amyloid deposits in living, transgenic Caenorhabditis elegans animals using the sensitive amyloid dye, X-34. Neurobiol Aging 22:217–226
53. Nass R, Merchant KM, Ryan T (2008) Caenorhabditis elegans in Parkinson's disease drug discovery: addressing an unmet medical need. Mol Intervention 8:284–293
54. Pujol N, Cypowyj S, Ziegler K, Millet A, Astrain A, Goncharov A, Jin Y, Chisholm AD, Ewbank JJ (2008) Distinct innate immune responses to infection and wounding in the C. elegans epidermis. Curr Biol 18:481–489
55. Artal-Sanz M, de Jong L, Tavernarakis N (2006) Caenorhabditis elegans: a versatile platform for drug discovery. Biotechnol J 1(12):1405–1418
56. Wicks SR, Yeh RT, Gish WR, Waterston RH, Plasterk RH (2001) Rapid gene mapping in Caenorhabditis elegans using a high density polymorphism map. Nat Genet 28:160–164
57. Jorgensen EM, Mango SE (2002) The art and design of genetic screens: Caenorhabditis elegans. Nat Rev Genet 3:356–369
58. Cooper MS, D'Amico LA, Henry CA (1999) Confocal microscopic analysis of morphogenetic movements. Methods Cell Biol 59:179–204
59. Luengo-Oroz MA, Ledesma-Carbayo MJ, Peyrieras N, Santos A (2011) Image analysis for understanding embryo development: a bridge from microscopy to biological insights. Curr Opin Genet Dev 21:630–637
60. Hammers MD, Taormina MJ, Cerda MM, Montoya LA, Seidenkranz DT, Parthasarathy R, Pluth MD (2015) A bright fluorescent probe for H2S enables analyte-responsive, 3D imaging in live zebrafish using light sheet fluorescence microscopy. J Am Chem Soc 137:10216–10223
61. Lieschke GJ, Currie PD (2007) Animal models of human disease: zebrafish swim into view. Nat Rev Genet 8:353–367

62. Hill AJ, Teraoka H, Heideman W, Peterson RE (2005) Zebrafish as a model vertebrate for investigating chemical toxicity. Toxicol Sci 86(1):6–19
63. Scalzo FM, Levin ED (2004) The use of zebrafish (Danio rerio) as a model system in neurobehavioral toxicology. Neurotoxicol Teratol 26:707–708
64. Guo S (2009) Using zebrafish to assess the impact of drugs on neural development and function. Expert Opin Drug Discov 4:715–726
65. Fleming A, Diekmann H, Goldsmith P (2013) Functional characterisation of the maturation of the blood-brain barrier in larval zebrafish. PLoS One 8:e77548
66. Lessman CA (2011) The developing zebrafish (Danio rerio): a vertebrate model for high-throughput screening of chemical libraries. Birth Defects Res C Embryo Today Rev 93:268–280
67. Saleem S, Kannan RR (2018) Zebrafish: an emerging real-time model system to study Alzheimer's disease and neurospecific drug discovery. Cell Death Discovery 4:45
68. Adams MM, Kafaligonul H (2018) Zebrafish—a model organism for studying the neurobiological mechanisms underlying cognitive brain aging and use of potential interventions. Front Cell Dev Biol 6:135
69. Lappin G (2015) The expanding utility of microdosing. Clin Pharmacol Drug Dev 4 (6):401–406
70. Bailey J, Taylor K (2016) Non-human primates in neuroscience research: the case against its scientific necessity. ATLA 44:43–69

Part II

Biology and Husbandry of Laboratory Animals

The Anatomy and Physiology of Laboratory Mouse

8

Sarita Jena and Saurabh Chawla

Abstract

Among the different types of vertebrate and invertebrate animals used in biomedical research, the laboratory mouse is the widely used vertebrate animal model. It remains the choice of model for many research hypotheses due to its small size, shorter life span, easy maintenance, short generation time and manipulable genetics. The mouse is used in understanding basic research, testing, teaching, genetics, physiology, psychology and many more areas of biomedical research. An important advancement in this regard is the whole-genome sequencing of both human and mouse; and both share synteny of chromosomes. This chapter reviews the existing and new unique features of the anatomy and physiology of mouse and their suitability to different experimental designs.

Keywords

Mouse · Animal model · Genome · Anatomy · Physiology · Husbandry

Abbreviations

AQCS	Animal Quarantine and Certification Services
FSH	Follicle-stimulating hormone
GG	Grueneberg ganglion

S. Jena (✉)
Institute of Life Sciences, Bhubaneswar, India
e-mail: saritajena@ilbs.res.in

S. Chawla
National Institute of Science Education and Research, Bhubaneswar, India
e-mail: saurabhchawla@niser.ac.in

© The Author(s), under exclusive license to Springer Nature Singapore Pte Ltd. 2021
P. Nagarajan et al. (eds.), *Essentials of Laboratory Animal Science: Principles and Practices*, https://doi.org/10.1007/978-981-16-0987-9_8

HVAC	Heating, ventilation, and air conditioning
ICC	Interstitial cells of Cajal
LA	Left auricle
LH	Luteinising hormone
LV	Left ventricle
MOE	Main olfactory epithelium
MUP	Mouse urinary protein
NALT	Nasal-associated lymphoid tissue
pRGCs	Photosensitive retinal ganglion cells
RA	Right auricle
RV	Right ventricle
SG	Salivary gland
SOO	Septal olfactory organ
VNO	Vomeronasal organ

8.1 Introduction

The house mouse is a commensal to man due to its adaptability to various environmental conditions. Its counterpart, the laboratory mouse, originated by mouse fanciers for their coat colour and that gradually extended to their use for scientific purposes. Though the laboratory mouse has been considered as the most extensively used and preferred animal model for biomedical research since the past four decades, it was used since the seventeenth century by William Harvey for reproduction and blood circulation studies and by Robert Hooke for the study of biological consequences of increased air pressure [1]. The advantage of short gestation, large litter size, short life span, easy and inexpensive maintenance features and suitability for various biomedical basic and applied research made it a way for the development of existing laboratory mouse and its overwhelming usage. An important reason in this regard is the whole-genome sequencing of both mouse and human. The whole-genome sequencing of mouse serves as an important informational tool for biomedical research on the basic understanding of the functions of the human genome. Despite differences in anatomy and physiology, genes from mouse and human are 99% identical [2], and it was reported that 30,000–40,000 human genes have murine counterpart [3]. Secondly, the ease of genetic engineering in the mouse yields unlimited possibilities for exploring the effect of alteration of gene expression and physiological characterisation of most of the regulatory and functional protein inside the human and animal body [4]. There are currently thousands of outbred and inbred strains available for research. The common uses of mice in scientific research include infectious diseases, oncology, immunology, drug discovery, vaccine safety analysis, etc.

The taxonomy of the mouse is as follows:

Kingdom – Animalia.
Phylum – Chordata.
Class – Mammalia.
Order – Rodentia.
Family – Muridae.
Subfamily – Murinae.
Genus – *Mus.*
Species – *musculus.*

8.2 Anatomy and Physiology

There are anatomical and physiological similarities and differences compared to humans in the digestive system, respiratory system, cardiovascular system, urogenital system, haematological system and nervous system. This similar or dissimilar features compared to a human have been used for studying many physiologic processes.

8.2.1 External Features and Musculoskeletal System

Adult mouse measures approximately 12–15 cm long from the nostril to the tip of the tail, and the length of the tail is almost half of the whole length of the body. The mice have soft, dense fur-covered throughout the body except for the tail. The mouse has rounded erect ears, protruding eyes with a pointed snout and cleft upper lip. They have short legs and a long, thin hairless tail.

Like other species, the skeletal muscle of the mouse consists of striated muscle fibres which are having multiple nuclei at the periphery of the cell, and the cytoplasm has contractile myofibrils [5]. The skeleton of the mouse has two divisions: the axial skeleton having a skull, ribs, sternum and vertebral column, and the appendicular skeleton having the fore and hind limbs and pectoral and pelvic girdle. The dorsal surface of the skull of the mouse has two distinct landmark points: the bregma and lambda which are the intersecting points of the sagittal suture with the coronal and lambdoid sutures, respectively. These are the landmark points or reference points for stereotactic surgery or for intraneuronal implantation or injection. The mouse skeletal system consists of both tubular and flat bones. Like other mammals, they have the shaft or diaphysis and the bone ends or the metaphysis as the architectural structure. Unlike other species, mice lack the distinct Haversian system [5] or osteons in the cortical bones (the compact bone). The mouse vertebral column has 59–61 vertebrae: 7 cervical, 13 thoracic, 6 lumbar, 4 sacral and 29–31 coccygeal [6]. In the intervertebral space of coccygeal vertebrae, 25 pairs of sesamoid bones support the tail [6]. There is metameric arrangement of bicipital muscles in the tail of the mouse [7].

8.2.2 Digestive System

The digestive system starts from the mouth and extends up to the anus. Apart from the functional parts of the digestive system, the salivary glands, pancreas and liver play important role in the process of digestion.

The dental formula of the mouse is 2(I 1/1,C 0/0,P 0/0,M 3/3). Mouse lacks deciduous teeth and has monophyodont dentition [8, 9], where the single set of teeth last for the entire life span. The incisor is open rooted and hypsodont [10], which grows continuously and high crowned throughout life, but the molars have fixed teeth. The odontoblasts produce secondary dentine, protecting the root pulp to exposure. There is a space between incisors and cheek teeth which is called diastema. There are three pairs of salivary glands (SG) present in the mice which include parotid, submaxillary, and sublingual. The parotid SG is located behind the ear and extended up to the shoulder region along the ventrolateral side of the neck. The sublingual SG is situated under the base of the tongue. The mandibular SG lies on the ventral side of the parotid SG [11]. The parotid saliva drains into the parotid duct, whereas the mandibular and sublingual glands drain into the mouth at small papillae near the incisors by Wharton's duct. The parotid salivary gland has a serous salivary secretion that contains amylase and lipase that aid in the process of carbohydrate and fat digestion, respectively. The sublingual secretion is entirely mucous type, while the submaxillary is a mixed type. The tongue of the mouse is flexible with a movable tip and extends from the rear end of the floor of the mouth. It has many taste buds (circumvallate and fungiform papillae) over the dorsal surface.

8.2.2.1 Oesophagus

The oesophagus connects the pharynx to the stomach and lies dorsally to the trachea. It has a 3–5-layer thick keratinised longitudinal and circular striated muscle layer [12, 13] and lacks the submucosal layer. Before swallowing or gulping of food, two sphincters get constricted; the epiglottis closes the windpipe to prevent aspiration of food into the trachea, and lower oesophageal sphincters constrict to restrict gastric reflux. During the process of swallowing, the pharynx constricts and food enters to the oesophageal opening. Then the peristalsis in the striated muscle layer of the oesophagus propels the ingesta along the length of the oesophagus [13]. The relaxation of the lower sphincter of the oesophagus allows the ingesta to enter the stomach.

8.2.2.2 Stomach

The stomach has three parts including cardiac (non-glandular), fundus (glandular) and pyloric regions (Figs. 8.1 and 8.2). The cardiac part is glandular and lined with keratinised epithelium like that of the oesophagus. Its function is limited to the storage of food before the process of digestion starts. The fundus is the actual functional part of the stomach. The fundus has three types of cells: the chief cells that produce the pre-pepsin (precursor of pepsin), non-chief cells (mucin secreting) and parietal cells that produce the precursor of hydrochloric acid. In the process of digestion, the ingested food mixes with both hydrochloric acid and pre-pepsin enzyme in the stomach. The pre-pepsin gets activated into pepsin in lower pH in

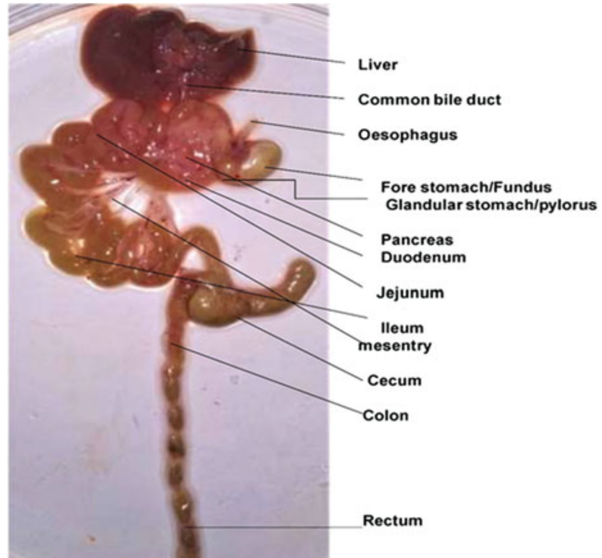


Fig. 8.1 Gastrointestinal tract of mouse

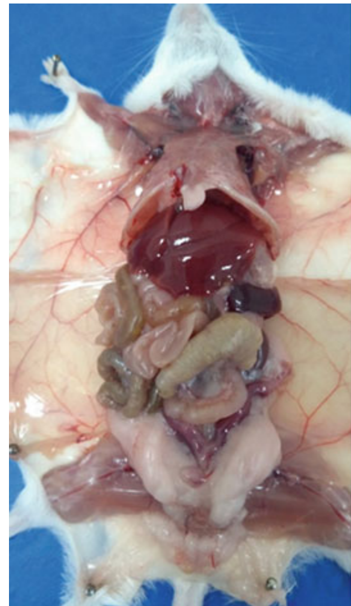


Fig. 8.2 Dissected abdominal picture of a mouse

the presence of hydrochloric acid. The contraction and relaxation of stomach muscle result in the mixing of the acid and enzyme and grinding of larger particles into smaller one, forming the "chyme" [11]. There is a valve called the pyloric valve which is present between the pyloric portion of stomach and the small intestine. The pyloric valve controls the movement of food by allowing only smaller particles to

pass to the duodenum. Two important glands are associated with the digestive system: the pancreas and the liver.

8.2.2.3 Pancreas

The pancreas of a mouse is not a well-defined solid structure like a human; rather, it is a diffused soft tissue that lies adjacent to the proximal small intestine [14]. The exocrine function plays an important role in the process of digestion. The exocrine part has two groups of cells: the acinar cells which secrete digestive enzymes and centroacinar cells or duct cells which secrete large volume of aqueous bicarbonate fluid that helps to buffer the effect of stomach acid. The acinar cells are arranged in grape-like clusters connected to ducts and that produce amylase (carbohydrate digestion), lipase (fat digestion) and precursors of protein-digesting enzymes like pepsin, trypsin, chymotrypsin and elastase which get activated by the presence of other enzymes in the duodenum.

8.2.2.4 Liver

The liver of a mouse is a four-lobed organ. The four lobes of the liver are the median lobe, left lobe, right lobe and caudate lobe which are joined dorsally [15] (Fig. 8.3). The gall bladder is situated at the base of the bifurcation of the median lobe. The liver plays the role of a multitasking organ performing various functions. Some of the important functions include synthesis and secretion of bile, metabolism and detoxification of drugs and toxic substances, storage of fat and glycogen, phagocytosis of foreign bodies and haematopoiesis in mouse [16]. The liver has the property of regeneration and the ability to alter its size as per functional demand. The liver of the mouse does not need stem cells for regeneration; rather, the hepatocytes can proliferate and differentiate as per demand [17]. Along with sources external to it, the liver plays an important role in maintaining stable blood cholesterol levels by converting it into cholic acid, the bile acid. Mouse produces 160 mg/kg/day of cholesterol, but in human the production is 10 mg/kg/day [18]. The bile fluid is a mixture of phospholipids, bile acids, cholesterol and bile pigment, i.e. bilirubin (a metabolic product of haemoglobin). The bile is collected in the bile canaliculi which opens into bile ducts, and the bile from bile ducts drains and gets stored in the gall bladder.

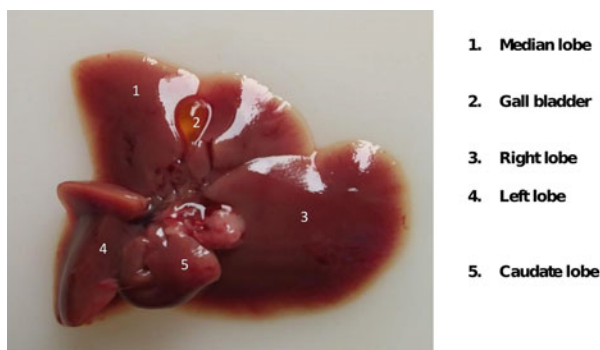


Fig. 8.3 Lobes of the liver of mouse

Inside the intestine, the fat content of the chyme stimulates the secretion of cholecystokinin which in turn stimulates the contraction of the gall bladder to release bile into the duodenum. The bile duct opens into the duodenum through a large papilla of smooth muscles called the sphincter of Oddi. It gets relaxed by the hormone cholecystokinin and allows bile to flow to the duodenum when the ingesta enters the duodenum. The bile acids are amphiphilic, having both hydrophilic and hydrophobic nature, therefore playing a vital role in the absorption of fat and fat-soluble vitamins. Again after absorption of fat, the majority of the bile acids are absorbed back to the intestine, and the portal veins pave the way to get recycled in the liver [16].

8.2.2.5 Intestine

The intestine is divided into the small intestine and large intestine (Fig. 8.1). In an adult mouse, the length of the small intestine is more than the large intestine, and it measures approximately 35 cm (from the stomach to caecum) consisting of the duodenum, jejunum and ileum. The large intestine is smaller in length and measures approximately 14 cm [16], and it consists of the caecum, colon and rectum.

8.2.2.6 Small Intestine

Many fingerlike projections which are called villi are present in the epithelial layer of the small intestine. There are many crypts present in between the villi. The high density of crypts and villi increases the surface area of the intestinal lumen, thus increasing the site of absorption of nutrients. The length and density of villi gradually decrease from the small to the large intestine. The crypt stem cells produce four types of cells by continuous proliferation and differentiation. The four types of cells are enterocytes, goblet cells, entero-endocrine cells and Paneth cells [19]. Except for the Paneth cells, other cell types get pushed up towards the tip of the villus for executing different functions, but the Paneth cells stay in the crypts. The enterocytes line the inner surface of the intestine, and they are the important cells responsible for the absorption of nutrients. The intracellular junction between the enterocytes form the tight junction which is responsible for the absorption of water and electrolytes. Goblet cells are the mucous-producing cells which form the mucous layer that protects and aids in enterocyte functioning. Entero-endocrine cells produce cholecystokinin and secretin. The Paneth cells secrete tumour necrosis factor, lysozyme and antibacterial cryptin and act as a barrier for translocation of bacteria. Paneth cells play an important role in homeostasis of the small intestinal epithelium, and loss or damage to these cells may result in reduced clearance of external bacterial invasion. In mice, the Paneth cells start developing at about 7–10 of postnatal age, whereas in humans, it occurs in utero at 22–24 weeks of gestation [20]. The duodenum receives the exocrine secretion of the pancreas and Brunner's gland and the bile from the liver which mixes into the chyme. The enzymes present in the secretions digest the proteins, fats and carbohydrates present in the chyme to amino acids, fatty acids and monosaccharides respectively. Principally, amylase digests the complex carbohydrates into the simpler form (glucose, fructose, galactose). The protein digestion starts in the stomach itself due to the effect of pepsin

followed by the effect of pancreatic enzymes. The activity of lactase that is present at birth gradually gets diminished by the age of weaning. In pups of the mouse, the immunoglobulin of the colostrum gets absorbed by the process of pinocytosis. The digested simpler form of protein and carbohydrates is transported by the use of ATP and sodium ion. Monosaccharides, amino acids and electrolytes are absorbed rapidly from the chyme in the duodenum followed by slow absorption of water-soluble vitamins, calcium, magnesium, iron and other essential minerals. Lipase and bile acids act on fat to digest it to short-chain fatty acids and monoglycerides which form emulsion due to the amphiphilic nature of bile acid and remain suspended in small droplet form in the chyme. The mixture of monoglycerides and fatty acids enters into the enterocyte cytoplasm through the brush border or the microvilli. Inside the cytoplasm, they again recombined to triglycerides which in turn combined to lipoproteins to form the chylomicrons. The chylomicrons get released from the enterocytes by the process of exocytosis, into the lacteals (lymph vessels present in the villi of the ileum), and enter the lymph ducts, which then enters the blood vessels. In this process of fat absorption, the fat-soluble vitamins A, D, E and K also get absorbed along with fat. The majority of the water absorption takes place in the small intestine and a few in the colon. The water absorption is executed by simple diffusion through the tight junction and due to osmotic gradient caused by the transport of electrolytes across the cell membrane.

The muscularis mucosa, a layer of thin smooth muscle below the mucosal layer, plays a role in the propulsion of gut content to move forwards by rhythmical longitudinal and horizontal contraction and relaxation of the muscles from the duodenum to the jejunum and ileum. The interstitial cells of Cajal (ICC) are present in the proximal part of the colon which play an important role in the process of impairment of the peristaltic movement. The ICC acts as a pacemaker that generates slow electrical waves that spread to the smooth muscle layer of the intestine, and they integrate the inhibitory and excitatory neurotransmission to balance the peristalsis [21]. The ileum is distinguished by the presence of lymph nodules or Payer's patches in the submucosa.

8.2.2.7 Large Intestine

The undigested food along with the desquamated epithelial cells and enzyme residues pass from the ileum to the colon through the caecum. The caecum is a large blind appendage-like structure present at the junction of the ileum and colon. The size of the caecum in the mouse is approximately one-third of the total length of the large intestine and functions as a fermentation vat. Here, the intestinal bacteria will act on the undigested materials. Finally, the remnants pass to the rectum through the colon. Here, the undigested remnants are stored, coated with mucus for lubrication and easy defaecation through the anus.

In addition to the process of digestion, the gastrointestinal tract also plays as a part of the immune system by functioning as both physical and immunologic barriers to external pathogenic invaders to protect the beneficial normal intestinal bacterial flora.

8.2.3 Respiratory System

The respiratory system of the mouse can be broadly divided into two parts, i.e. the upper respiratory tract, the conducting zone which forms the continuous passage through which the air can move in or out of lungs, and the lower respiratory tract, the respiratory zone where the gas exchange occurs. The upper respiratory tract consists of the nostrils, nasal cavities, pharynx, larynx, trachea, bronchi and bronchioles. The lower respiratory tract consists of terminal bronchioles, alveolar ducts and alveoli.

8.2.3.1 Nasal Cavity

The nasal cavity of the mouse extends from the external nares to the pharynx. It has two symmetrical longitudinal compartments separated by a cartilaginous nasal septum. The nasal cavity has three pairs of turbinate bones or conchae which are scroll-like, highly vascular structures [16]. The turbinate bones provide a warm and moist surface for the inspired air. Inside the nasal cavity of the mouse, there is an important structure present which is called the vomeronasal organ that is responsible for chemoreception of pheromone-mediated behaviours such as reproductive behaviour, suckling behaviour in neonates, acquisition of food by its smell, inter-individual social communication and territorial dominance behaviour [22].

8.2.3.2 Pharynx

The pharynx is a vertically elongated structure present posterior to the nasal and oral cavity. It serves as the intersecting point between the food pipe and windpipe and acts as a common passage for air and food. It has three parts including the nasopharynx, oropharynx and laryngopharynx. From the middle ear, the Eustachian tube opens into the lateral walls of the nasopharynx. The Eustachian tubes provide equalisation of air pressure in the middle ear with the outside environmental pressure [16]. At the entrance of the nasopharynx, there exist focal aggregates of nasal-associated lymphoid tissue (NALT). The NALT plays an important role in regional immune response like that of human tonsil [23]. The oropharynx connects the pharynx to the oral cavity and the laryngopharynx to the larynx.

8.2.3.3 Larynx

The tube-like structure that extends from the pharynx up to the trachea is called the larynx which is suspended from the base of the skull by the hyoid bone and fixed in its place by attachment to the trachea. The location of the larynx is ventral to the nasopharynx and posterior to the oropharynx. It acts as a gateway to the airway. The principal function of the larynx of a mouse is to keep the airway in the open position to allow inspiration and expiration and closing off the airway during swallowing by reflex action by the epiglottis whenever the mouse takes food or fluid. The larynx is composed of three unpaired (thyroid, cricoid and epiglottis) and three paired (arytenoid, cuneiform and corniculate) cartilages. These cartilages provide support and strength to the larynx. The sound production in the mouse is by the vibration of vocal cords which are attached to the arytenoid cartilages [16].

8.2.3.4 Trachea

The trachea is the last part of the upper respiratory tract extending from the base of the larynx to the point where it bifurcates into bronchi. It is a tubular organ of 5–10 mm long lined by the respiratory mucosa and supported by 15–18 C-shaped hyaline cartilages [24] with an internal diameter of 1.5 mm [23], placed parallel with each other and fibro-elastic tissue. The C-shaped cartilages have open-end dorsally, and a sheet of smooth muscle holds the tracheal rings by connecting to the end of tracheal rings. The epithelial lining consists of three types of cells, i.e. ciliated cells, non-ciliated secretory cells (goblet cells and Clara cells) and basal cells. The non-ciliated Clara cells are the major type of cells (50–60%). Mostly in other species, the Clara cells are present in terminal bronchioles. Clara cells produce mucus that covers the respiratory mucosa. Tracheal glands are present in the submucosa of the cranial part of the trachea [23].

8.2.3.5 Lungs

The lower respiratory tract, which is the site of the actual gas exchange, consists of the lungs. Lungs function with the help of terminal bronchioles, alveolar ducts and alveoli. The trachea bifurcates into two primary bronchi above the heart. The bronchi enter into the right and left lobes of the lungs and follow a monopodial branching system (parent branch divides into two symmetrical daughter segments), whereas in humans, there is a dichotomous branching system (daughter segments extend from an angle from the parent branch) [25]. The mouse does not have a respiratory bronchiole; rather, the smallest airway unit is the terminal bronchiole that is bifurcated into the alveolar ducts [26]. The alveolar ducts, alveolar sacs and alveoli combined to comprise the gas exchange units. The mouse has five lung lobes: only one lobe on the left side and four lobes, namely, superior, middle, inferior and postcaval lobe, on the right side (Fig. 8.4) [16]. The lung parenchyma consists of some specialised cell types within the conducting airway and the alveolar region. The airway epithelium consists of primarily two types of cells: non-ciliated dome-

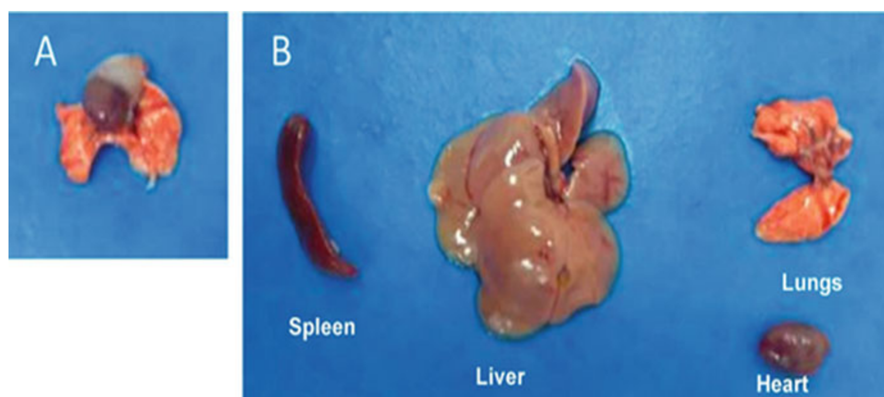


Fig. 8.4 (**a**). Lungs with heart, (**b**) Comparative representation of vital internal organs of mouse

Table 8.1 Comparative respiratory physiological parameters in mouse and human

Parameter	Mouse	Human
Respiratory rate (min^{-1})	106–163	12
Tidal volume (ml)	0.007–0.01	500
Minute ventilation (ml/min)	1.06–1.108	6000
Total lung capacity (ml)	0.9–1.44	6000
Residual volume(ml)	0.11–0.14	1500
Lung compliance (ml/cmH_2O)	0.053–0.13	200
Airway resistance ($cmH_2O.ml^{-1}$ s^{-1})	1.5	0.0016
$PaCO_2$ (mmHg)	34–35	40
PaO_2 (mmHg)	78–84	80–100
pH	7.37	7.4

Source: Adopted from Rao and Verkman (2000) [30]

shaped secretory bronchiolar Clara cells and ciliated cells. The Clara cells are the important cells for the repopulation of epithelial cells in the distal conducting airway and act as progenitor cells for bronchiolar epithelium. In mice, these cells have significant anti-inflammatory and immune regulatory functions as well as helping with clearance and reduction of surface tension in airways [16]. The Clara cells have the cytochrome P450 monooxygenase enzyme system which plays an important role in the metabolism and detoxification of inhaled xenobiotics. The alveolar epithelium is composed of both type I (~95%) and type II (~5%) cells. Type I cells are unable to divide and have very little or no capacity for repair if damaged. Type II cells can get repaired if damaged [27], and they are also responsible for the synthesis of surfactant and associated proteins [28, 29]. The lung has two important blood supplies: the bronchial circulation and the pulmonary circulation. For the functional needs to most of the parts of the lungs, the bronchial circulation provides the blood supply. The bronchial circulation is a branch of systemic circulation that arises from the aorta. The pulmonary circulation arises from the right ventricles and carries deoxygenated blood to the lungs to carry out the exchange of carbon dioxide with oxygen.

The breathing patterns of all the mice strains are not the same; some strains show rapid breathing with small tidal volume and others show slow breathing with larger tidal volume [16]. In mice, the diaphragm which is a thin dome-shaped muscular sheet that separates the abdominal and thoracic cavity plays an important role in the process of respiration. The diaphragm creates pressure gradients between the alveoli and atmosphere, by its contraction and relaxation to facilitate the process of respiration. The comparative respiratory physiology and lung anatomy of mouse and human are summarised in Table 8.1 and Table 8.2, respectively.

8.2.4 Cardiovascular and Haematological System

8.2.4.1 Cardiovascular System

The mouse heart is ellipsoidal or rugby ball-shaped and weighs about 100–200 mg and beats 500–800 times/min in comparison to the heart of a human which weighs

Table 8.2 Comparative anatomy of the lung in mouse and human

Anatomical structure	Mouse	Human
Number of lobes in the lungs	4 in the right side, 1 in the left side	3 in the right side, 2 in the left side
Lung parenchyma (%)	18	12
Alveoli (mean linear intercept)	80μm	210μm
Blood-gas barrier thickness	0.32μm	0.62μm
Trachea	Incomplete, C-shaped	Complete ring-shaped
Number of airway generation	13–17 generations	17–21 generations
Airway branching pattern	Monopodial	Dichotomous

Source: Adopted from Irvin and Bates (2003) [31] and Oakes et al. (2004) [32]

approximately 250–300 g that beats 60–70 times/min [33]. The heart is the chief organ responsible for pumping the blood to perfuse every part of the body. It is the hollow muscular structure located in the thoracic cavity in between the left and right lung lobes at both sides and covered externally by the pericardium. The heart is divided into the right and left part by a muscular septum. It is composed of four chambers, namely, right atrium (RA), right ventricle (RV), left atrium (LA) and left ventricle (LV). The right side of the heart is responsible for pulmonary circulation; the RA receives the deoxygenated blood from the body, and the RV pumps that blood to the lungs through the pulmonary trunk for the gas exchange to occur. The left side of the heart is responsible for systemic circulation; the LA receives oxygen-rich blood from the lungs, and the LV pumps the blood to the whole body through the aorta. The blood flow from the heart is controlled by four valves formed by the connective tissue and endocardium covering [13] that ensure the blood flows in one direction only, i.e. from the atrium to ventricles and from ventricles to their appropriate arteries. The right atrioventricular valve connects the RA to RV and it is a tricuspid valve (three distinct leaflets).The left atrioventricular valve is known as the bicuspid valve (two distinct leaflets) or mitral valve which connects LA to LV. These two valves control the one-way flow of blood from atria to ventricles and prevent the backflow during ventricular systolic contraction. The closing of valves creates the sound of the heartbeat. The other two valves are semilunar (three half-moon-shaped valve cusps), i.e. the pulmonary valve that lies at the originating point of the pulmonary artery trunk from the right ventricle and the aortic valve that is present at the starting point of the aorta from the left ventricle. These valves also allow one-way flow of blood from ventricles to the pulmonary trunk or the aorta and prevent the reverse flow when the ventricles relax following the pumping of blood. The heart is comprised of three layers. The layers from the inner to the outer side are the endocardium, myocardium and epicardium. Outer to the epicardium, there is a thin and delicate layer of the mesothelium. The inner layer or the intima of major blood vessels that supply or carry blood from the heart is lined with a layer of endothelium that also lines the endocardium of the heart in continuation of blood vessels [16]. The important structure in the cardiovascular system that initiates the electrical impulse to stimulate contraction or pumping mechanism of the heart is

Table 8.3 Blood pressure and heart rate by radio telemetry during various activities of mouse

Activity	Heart rate (bpm)	Systolic pressure (mmHg)	Diastolic pressure (mmHg)
At rest (sleep)	350–450	102–112	70–80
At rest (awake)	450–500	110–117	75–85
After light activity	600–650	126–138	94–109
At the time of weighing	700–750	140–155	105–120
At the time of hand restraining	750–800	140–155	105–120
When placed at different cage	750–800	140–155	105–120

Source: Hoyt et al. (2007) [16] and Kramer et al. (2000) [37]

called the sinoatrial node. In mouse, it is situated in the wall of anterior vena cava just above its emergence from the right atrium [34]. The bundle branches of His are morphologically asymmetrical, and the Purkinje cells are not so apparent in the ventricular walls of the heart of mouse [35]. Just distal to the aortic valve, the coronary artery branches from the aorta, lies embedded in the epicardium and supplies blood to the heart muscles. Observation of the coronary artery system is much difficult in the mouse as the arteries are deeply embedded in the myocardium. The striking differences in the mouse heart and vessels appear to be in the arrangement of the venous system of the heart. Cardiac veins are the prominently visible vessel on the epicardial surface of the left ventricle, far exceeding the visibility of the coronary arteries. The left cardiac vein, the largest coronary vein, originates from the ventral surface of the left ventricle near the apex of the heart and proceeds dorsally on the heart surface and drains into left anterior vena cava [36].

The blood pressure and heart rate fluctuate throughout the day depending upon activity level, stress and environmental attributes [16]. The heart rate and blood pressure during various activities of the mouse are presented in Table 8.3.

8.2.4.2 Haematological System

The bone marrow and the splenic red pulp produce the precursor cells of blood cells throughout the life of a mouse. There are three major cellular components of blood: erythrocytes (red blood cells), leucocytes (white blood cells) and thrombocytes (platelets).

- **Erythrocyte:** The mouse erythrocytes have a short life span of 30–40 days [38]. The erythrocytes are small compared to humans and other rodents. It is spherical, biconcave and anucleated. The Howell-Jolly bodies (remnant of nuclear DNA) can also be seen in the circulating erythrocyte of mouse [39].
- **Leucocyte:** The leucocytes found in mouse blood are lymphocytes, neutrophils, monocytes, eosinophils and basophils. Lymphocytes are the predominant circulating leucocyte in mice and basophils are rarely found in circulation. The leucocyte count depends upon site, method and time of collection. Therefore, they have limited contributions to disease diagnosis. Other lymphocytes are 7–12μm in diameter having round or oval dark-blue nuclei with pale blue scant cytoplasm.

Table 8.4 Haematological parameters of mouse

Blood parameters	Values
Red blood cell count	7–11 × 10^{12}/l
Haemoglobin	130–180 g/l
Haematocrit	0.40–0.50
Mean cell volume	40–55 fl
Mean cell haemoglobin	13–17 pg
Mean cell haemoglobin concentration	260–320 g/l
Red blood cell distribution width	11–15%
Platelet count	1000–2000 × 10^9/l
Lymphocyte	2–8 × 10^9/l
Neutrophil	0.5–3.0 × 10^9/l
Monocyte	0.05–0.10 × 10^9/l
Eosinophil	0.05–0.10 × 10^9/l
Basophil	Rarely observed

Source: Adopted from Everds (2004) [40]

Lymphocytes account for 60–90% of circulating leucocytes. Neutrophils account for approximately 5–20% of the total circulating leucocyte population. Neutrophils are 10–25μm in diameter and have a circular doughnut-shaped nucleus [40]. Ring-form or circular nucleus is a characteristic feature of murine neutrophils, eosinophils and monocytes.

- **Platelet:** The platelet count in mouse is relatively high (9–16 × 10^5/μl) [39]. The half-life of mouse platelets is approximately 5 days. Their size is approximately 1–3μm in diameter and 4–7 fl in volume [40].

The haematological parameters of the mouse are summarised in Table 8.4. Haematological parameters may vary depending on the status of mice like fasting status, hydration status, time of collection, the technique of collection, experimental manipulation, anaesthesia and sex [40]. More detailed information regarding the haematological values for a different strain of mouse can be found in https://phenome.jax.org [41].

8.2.5 Urogenital System

8.2.5.1 Urinary System

The important function of the urinary system of the mouse is the filtration of blood, maintenance of acid-base balance and water conservation. These functions are carried out by glomerular filtration of blood to remove the waste materials like uric acid, urea, creatine and creatinine; reabsorption of glucose, water, ions and amino acids; and tubular secretion. The kidney is also involved in the production of renin, which helps in the regulation of blood pressure and calcium absorption.

The kidney is composed of the outer cortex and inner medulla. The cortex has nephrons and tubules. The mouse kidney has approximately 14,000 nephrons in comparison to 1 million in humans [42]. The mouse kidney weighs more per gram body weight than any other mammal [16]. The renal corpuscle is the first part of

Table 8.5 Urinary parameters in mouse

Parameters	Value
Urine output	1.5–2 ml/day
pH	5.0–6.6
Specific gravity	1.022–1.048
Osmolality	1919–2650 mOsm/kgH_2O
Creatinine	372–462μg/day
Glucose	0.5–3.2 mg/day
Protein	0.5–2.7 mg/day
Urea	130 mmol/day/kg body weight
Creatinine clearance	302–346μl/min
Inulin clearance	283–351μl/min
Sodium	0.15–0.29 mmol/day
Potassium	0.34–0.37 mmol/day
Chloride	0.28–0.34 mmol/day

Source: Adopted from Loung (2017) [45]

nephron which surrounds the glomerulus and acts as the collection point for the glomerular filtrate that further passes to the tubules. The tubule has three parts: the proximal tubule, medullary loop (loop of Henle) and distal tubule which drains into the collecting ducts. The kidney's vascular system has two capillary systems: glomerular capillaries and peritubular capillaries. The glomerular capillaries remain under high pressure which helps in the filtration of waste, and after that the reabsorption of a large volume of fluid occurs through peritubular capillaries. The urine concentration and reabsorption of water start at the level of the loop of Henle. Further reabsorption occurs at distal tubules and collecting ducts due to the action of antidiuretic hormone secreted from the posterior pituitary in response to the increased osmotic pressure of blood.

Mouse urine is highly concentrated and excreted as a drop at a time. The total urinary output per day is less than 1.5–2 ml [43]. It contains a large amount of mouse urinary protein (MUP). MUP in the mouse is produced by the liver and circulated in the blood and excreted through the kidney. It acts as a pheromone transporter [44]. A large amount of protein excretion is normal in mice and male mice excrete more protein than females. In the urinary protein, tryptophan is always absent, whereas taurine is always present. The mouse excretes much more allantoin than uric acid, like rats. Unlike other mammals, creatine is a normal component of urine in mice [16]. The urine parameters of a mouse are presented in Table 8.5. However, these values vary with strains [45].

8.2.6 Reproductive System

The reproductive function of the mouse depends upon the external environment like light intensity, noise, temperature, humidity, nutrition and stress. The hypothalamic-pituitary axis is influenced by the above parameters directly or indirectly and, accordingly, regulates the release of hormone for ovarian and testicular function.

The follicular-stimulating hormone (FSH) is a gonadotropin hormone secreted by the anterior pituitary and regulates gametogenesis and reproductive processes in both male and female mice. The luteinising hormone (LH) and prolactin are also secreted from the anterior pituitary. LH is involved in the secretion of oestrogen and progesterone in female mice and androgen in male mice, whereas prolactin helps in lactation after parturition of pups. These gonadal hormones are critical in the maintenance of the reproductive tract and in exhibiting specific reproductive behaviour for successful mating. The female mouse gets sexual maturation at around 4 weeks of age and males at around 6 weeks of age [24]. The female mouse is polyestrous and comes to cycle at 4–5 days' interval. There are four phases in the oestrus cycle which include proestrus, oestrus, metestrus and diestrus. In proestrus, there is epithelial growth in the genital tract and marked by abundant epithelial cells in the vaginal smear. This is followed by oestrus where ovulation occurs with the presence of a mixed group of epithelial as well as cornified cells. The metestrus stage is characterised by the presence of massive cornified cells and few leucocytes followed by diestrus where the major cell types are leucocyte and mucous. Externally the opened vaginal orifice and protruded or swelled, the pink and moist vulva is the sign of proestrus and oestrus. In mouse, there occurs postpartum oestrus at about 14–24 hours after parturition, but the mating is not always fertile in this oestrus. The occurrence of oestrus and ovulation depends upon gonadal hormones (oestrogen, progesterone) and gonadotropin hormone (LH, FSH), respectively, and the cyclicity depends upon the diurnal rhythm of photoperiod. The female and male urogenital systems are illustrated in Fig. 8.5 and Fig. 8.6, respectively.

The pheromone also affects oestrus cycle. In mouse, the oestrus is suppressed when placed in the large group due to pseudopregnancy or prolonged diestrus (Lee-Boot effect), and this can be nullified by the introduction of pheromone from male mice (Whitten effect). Then the female mice may come to the oestrus 3 days after pairing with male mice. The introduction of strange male mice may prevent

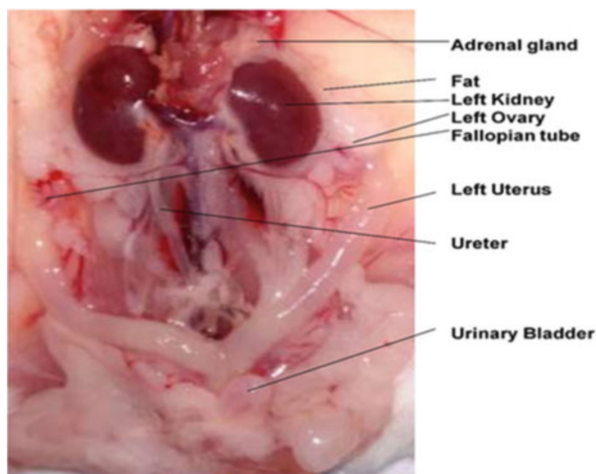


Fig. 8.5 Female urogenital system of mouse

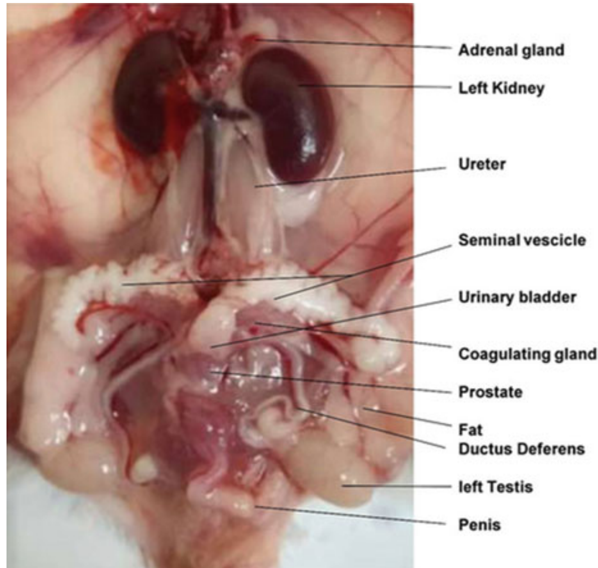


Fig. 8.6 Male urogenital system

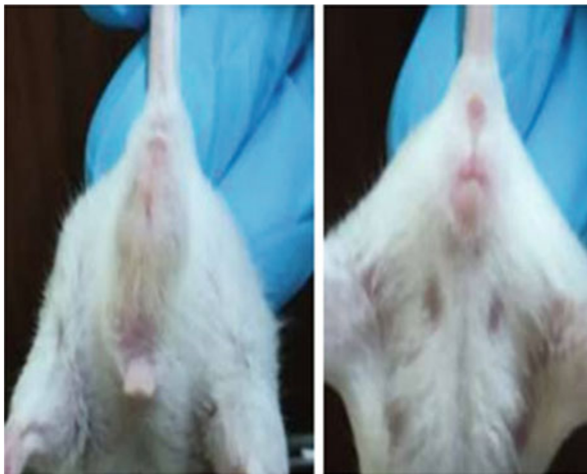


Fig. 8.7 Anogenital distance in male mouse (left) is more and less in female mouse (right)

implantation, and there may be a chance of pseudopregnancy in recently bred mice (Bruce effect). The mouse has five pairs of the mammary gland, three thoracic and two abdominal, which are very prominent in females. A prominent criterion that differentiated male from female is the presence of prominent genital papilla and comparatively greater anogenital distance in males (Fig. 8.7). The male reproductive system of mice consists of the paired testes, extra-testicular ducts, accessory sex

glands, urethra and penis. The extra-testicular ducts include the vas deferens, epididymis and efferent ducts. The mouse has accessory sex glands, namely, seminal vesicles, lobed prostate gland, bulbourethral gland, ampullary gland and preputial glands. The ampullary glands and preputial glands are unique to the mouse which are not found in humans [46]. Therefore, these glands should not be misinterpreted as abnormal findings.

8.2.7 Nervous System

The mouse has a smooth brain where the cerebral hemispheres lack the brain folding: the gyri and sulci. The cerebrum of the brain is divided into two symmetrical cerebral hemispheres by the longitudinal cerebral fissure. There are two prominent olfactory lobes anterior to the cerebrum, which contribute to a strong sense of smell in mice. Posterodorsal to the cerebral hemisphere lies the pineal body which regulates the circadian rhythm. The cerebellum is situated posterior to the cerebrum, which consists of two lateral lobular flocculus, central vermis and a pair of paraflocculus. Ventral to the cerebellum, there exists the medulla oblongata which is the most posterior part of the brain from which the spinal cord originates [47] (Fig. 8.8). The dorsal column of the spinal cord has to ascend sensory nerve fibres, whereas the ventral column of the spinal cord has descending motor nerve fibres. There is a segmental organisation of the spinal cord, and from each segment, a pair of spinal nerve roots originate. In mouse, there exist 34 pairs of spinal nerves (8 cervical, 13 thoracic, 6–7 lumbar, 4 sacral and 2–3 coccygeal) [48]. Each pair of the spinal nerve is attached to the spinal cord by rootlets and leaves the vertebral column through the intervertebral foramen. Some of the spinal nerves form the plexus, which gives rise to peripheral nerves [48]. The cervical plexus is formed by the first to fourth cervical spinal nerve. In mouse, the forelimbs receive the nerve supply through the brachial plexus that is formed by the fourth to eighth cervical and first and second thoracic spinal nerves. The lumbosacral plexus which supplies to the hind limbs is formed by the third to sixth lumbar spinal nerves [13]. The phrenic nerve is formed by branches of the fourth cervical spinal nerve and smaller branches of the third and fifth cervical nerve. The sciatic nerve is formed by the third and fourth lumbar spinal nerves [48].

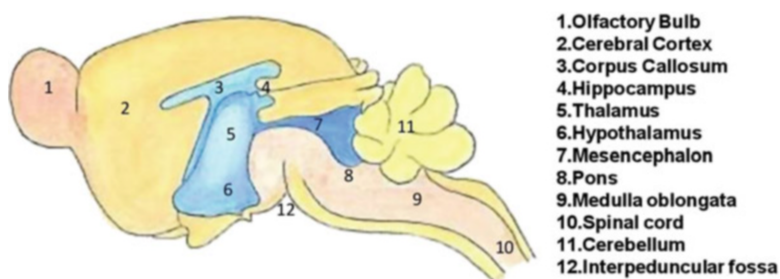


Fig. 8.8 Schematic diagram of brain of mouse

The brain consists of the functional cells, the neurons responsible for the conduct of electrical impulses and the supporting cells, the macroglia and microglial cells. The function of supporting cells is to maintain the homeostasis, myelin formation and protection and support to neurons. The brain weight and body weight of neonatal mice increase proportionately up to the 14th day of age. After that, the body weight increases but not the brain weight suggesting brain maturation [49]. The process of learning and memory starts in these 14 days. The myelination starts at around 7 days of age and completes by 57 days of age. The hearing response of pups starts at around 12 days old, and by 17 days of age, they start to respond like adults [50]. More detailed information regarding the brain of the mouse can be found in the Allen Brain Atlas (http://www.brain-map.org and http://www.nervenet.org/main/database.html).

8.2.8 Special Senses

8.2.8.1 Eye and Vision

The laboratory mouse shares similar anatomy and physiology of vision as that of its wild counterpart [51]. It has the same structural anatomy of the eye as that of other mammals. The mouse is a nocturnal animal, and it does not solely depend upon the visual system; rather, it relies upon the olfactory, tactile and auditory system for interaction with other animals, locating food and detecting predators. The mouse has a very poor vision which is found to be comparable to 20/2000 human vision [52, 53]. However, the visual system serves many complex non-visual functions such as detection of an object, light intensity, recognition of movement and differentiation of pattern.

Like other mammals, in the case of the mouse, the image is formed by the refraction of light on the retina through the cornea and lens. The mouse has a smaller axial length of 3.4 mm [53] and a comparatively large lens (Fig. 8.9). The retinal vasculature of the mouse is holangiotic type (fully vascularised). The retina is a highly stratified structure and approximately 200μm thick. It has two parts: the outer retina having the photoreceptor cell bodies (approximately 100μm thick) and the

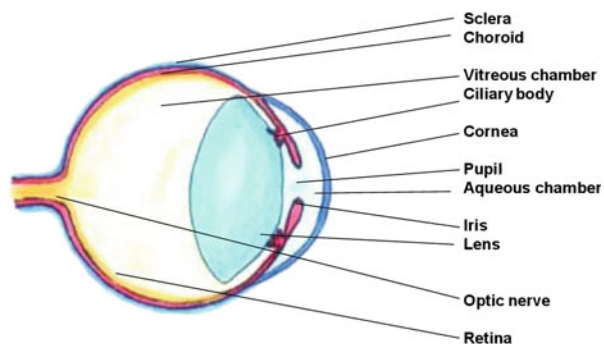


Fig. 8.9 Schematic diagram of eye of mouse

inner retina comprising of bipolar cells, amacrine cells and retinal ganglion cells. The photoreceptors receive the signals which are carried by bipolar cells to the amacrine and ganglion cells. The optic nerve is formed by the axons of the ganglion cells [54].

The photoreceptor layer contains both rods and cones which are responsible for low light (scotopic) and bright light (photopic) vision, respectively. The mouse is nocturnal, and the retina is mainly covered with rods, with approximately 6.4 million rods (~97%) and 200,000 cones (~3%) [53]. The mouse lacks a fovea or area centralis (the region with the highest cone density) in the retina [55]. The mouse has three types of visual pigments: a rod opsin, ultraviolet-sensitive cone opsin and middle wavelength-sensitive cone opsin. All three types of pigments are active with a peak sensitivity of $\leq$500 nm [54]. Due to the presence of ultraviolet-sensitive pigments, the mice have more sensitivity to ultraviolet light, whereas they are less sensitive to high wavelength light (e.g. red light) but can detect such light stimuli [54]. The detection of light stimuli not only helps the mouse for vision and image formation pathway but also helps in various non-visual functions such as circadian rhythm and regulation of sleep, mood and cognition. In the photoreceptor layer, a third type of photoreceptor exists apart from rods and cones called melanopsin-expressing photosensitive retinal ganglion cells (pRGCs) [54]. The melanopsin is responsible for non-visual functions in mice in the absence or degeneration of cones and rods too. These pRGCs are projected to the site for the circadian oscillation, the hypothalamic suprachiasmatic nuclei [56].

In albino mice, there are mutations in tyrosinase resulting in a lack of melanin in the coat and the retinal pigment epithelium. Hence, the colour of the eye is pink. The albino mice show compromised visual function due to a reduced number of rods and ganglion cell layers and are more prone to light-induced retinal damage [57]. Due to these paucities, usually, albino mouse is not chosen as a model for vision-related studies [54].

The mouse has three pairs of ocular glands, namely, Meibomian gland, Harderian gland and lacrimal gland [58].

- Meibomian glands are specialised sebaceous glands. It secretes in the inner margin of the eyelid and contributes lipid component to the tear film.
- The Harderian gland is a large horseshoe-shaped gland situated deep inside the orbit. The secretion of the gland has a pigment known as porphyrin which is pinkish-red or rust-red and fluoresces under ultraviolet light. The Harderian gland drains at the base of the nictitating membrane. Usually, the excess secretion gets wiped away during the act of grooming but that gets accumulated at the inner canthus (exhibiting bloody tear) or nostrils if the animal is under stress or grooms less.
- There are two pairs of lacrimal glands, the extra-orbital (also called exorbital) lies subcutaneously antero-ventral to the ear and the intra-orbital (also called infraorbital) lies at the outer canthus of the eyelids. In the mouse, the exorbital gland measures 3 mm in diameter, and the infraorbital gland is very small in size [58]. The common excretory ducts of both glands drain into the conjunctival sac dorsally.

8.2.8.2 Nose and Olfaction

The mouse depends upon the sense of smell or olfactory system for executing a wide range of activities which includes foraging, detecting food sources, recognising and bonding with social and mating partners and avoiding predators. These activities are associated with many behaviours of mice such as learning and memory, social interaction, fear and anxiety [59].

The olfactory system comprises of structures in the nasal passage, which function together for the olfaction. These structures are:

- Main olfactory epithelium (MOE).
- Vomeronasal organ (VNO).
- Septal olfactory organ (SOO).
- Grueneberg ganglion (GG).

The MOE covers approximately 47% of the total nasal cavity epithelium [58]. The MOE has also a high regenerative property. However, the specificity of odorant perception may not be restored always [60].

The VNO is a bilateral tubular structure situated ventrally at the base of the nasal septum. The lumens are lined by respiratory epithelium laterally and neuronal epithelium medially. It connects with the accessory olfactory bulb [58]. The main function of VNO is the perception of pheromones.

The SOO is a small island of olfactory cells, enclosed by the respiratory epithelium situated at the ventral base of the nasal septum.

The GG is a group of ganglion cells, surrounded by squamous epithelium, situated bilaterally in the dorsal meatus of the nasal vestibule [58]. Ganglion cells are present at birth with the dual function of perception of pheromone and temperature. The function of GG gradually decreases with age [58].

Olfaction is the sensory system by which the mouse acquires and processes chemical information from the external olfactory stimuli, which can be categorised broadly into two types:

- General odorants, from food or the environment that indicates the presence of food, or impending danger.
- Pheromones, which convey sexual or social cues, released from individuals of the same species.

The detection of smell is mediated through the MOE. The olfactory receptor neurons present in the MOE are bipolar and project their axons to the olfactory bulb. The olfactory receptor neurons with chemoreceptors send signals to odour- or pheromone-processing centres in the brain for the perception to act accordingly. Usually, general odorants activate receptors in a combinatorial fashion, whereas pheromones activate a narrow range of receptors that activate sexually dimorphic neural circuits in the brain [61].

It has been found that olfaction plays an important role in diet-induced obesity in the mouse. Loss of olfactory neurons in adult mice protects against diet-induced

obesity with increased thermogenesis in brown and inguinal fat depots. Also, loss of olfaction after induction of obesity can reduce fat mass and insulin resistance [62]. Loss of olfaction or inactivation of the main olfactory epithelium can induce anxiety-like behaviour in mice [63]. The olfaction has a major role in sexual behaviour too, as the sensory information reaches the brain and indirectly regulates hormone levels required for executing sexual and reproductive behaviour such as puberty, oestrus cycle, mating and pregnancy [64]. However, it has been seen that the female olfactory system is more efficient than males, and females tend to exhibit increased sensitivity and enhanced discrimination and identification abilities than males [65].

8.2.8.3 Ear and Auditory System

In mouse, the ear can be divided into three parts: the external, middle and inner ear. The external ear includes the pinna and external ear canal. The external ear canal and the middle ear are separated by the presence of the tympanic membrane (eardrum). The middle ear comprises of the tympanic cavity consisting of the tympanic bulla and the three ossicles or bony structures: the incus, malleus and stapes. The inner ear is comprised of the cochlea which is an important structure for auditory response and the vestibular apparatus responsible for the maintenance of balance, the non-auditory response. The vestibular apparatus consists of the semicircular canals, the utricle and the saccule. The internal acoustic meatus is the passage that extends from the posterior cranial fossa to the vestibular apparatus, through which nerve supply and blood supply to the internal ear occur. The Eustachian tube extends from the posterior wall of the tympanic cavity up to the nasopharynx. This tube is responsible for the maintenance of the equilibrium of pressure across the tympanic membrane.

The sound is transmitted as vibration to the ear ossicles, which further transmitted to the fluid-filled cochlea and organ of Corti, inside it. Inside this organ of Corti, there exists a highly specialised epithelium, the sensory hair cells that are mechanoreceptors and can trigger action potentials in response to sound or movement. From here, the nerve fibres carry the impulse to the brain.

Mouse hearing range varies from 1 to 100 kHz [66]. Upon exposure to noise beyond the hearing range, the mouse experience non-auditory effects such as a change in endocrine and cardiovascular function, sleep cycle disturbances, seizure and many behavioural changes [67].

8.2.8.4 Skin and Tactile System

The skin of the mouse consists of two parts, an outer epidermis of stratified squamous epithelium and an inner dermis continuous with adipose and loose connective tissue. The epidermis is relatively thin in haired areas than hairless parts such as the plantar surface of the paw, tail, snout, nipples and anogenital openings.

The dermis consists of collagenous and elastic connective tissue, blood vessels, nerves, fat cells and smooth muscle (arrector pili that lift hair follicles). The mouse does not have sweat glands and rete ridges (ridges of epidermal cells) [68].

Hair follicles are invaginations of the epidermis into the dermis. The body of the mouse is covered by a fine short hair coat called pelage hair or truncal hair. There is the presence of long hairs around the head called the vibrissae. The pelage hair comprises of four types of hairs [68]:

- Guard hairs—The long, straight, thick tactile hairs.
- Auchene hairs—Same length as guard hairs which have a gradual bend distally.
- Awl hairs—Short and thin hairs with a bend at the distal end.
- Zigzag hairs—Shorthairs giving a “Z” shape with two bends.

Apart from these hairs, many other types of hairs are also found in mice. The tail is covered with very short, thick fibres. Their ears have a variety of very short fine hairs and their eyes have vibrissae above their eyelids [68].

The sensory or tactile hairs are larger and longer than pelage hairs, and the follicles are large and contain abundant blood and nerve supplies. Tactile hairs are of two types: the vibrissae situated around the snout region and the guard hairs dispersed among the pelage hairs. The vibrissae are located in a grid-like layout on either side of the snout. They have the dense nerve terminals and sensory receptors, through which they transfer the touch signals. There exist two types of vibrissae: the micro vibrissae which are short and thin hairs surrounding the nose responsible for gaining the tactile information about object or texture and macro vibrissae which are longer present in the whisker pad and are responsible for transmitting spatial information [69]. The deflection of whisker opens the mechanogated ion channel in the nerve endings which in turn activate the action potential in the sensory neuron of the infraorbital branch of the trigeminal nerve. The trigeminal nerve projects to the thalamus barreloids. The axons from the barreloids project to the primary somatosensory cortex forming discrete barrel clusters in layer 4 of the cortex for tactile sensation. The layer 4-barrel map is arranged almost in the same layout or pattern as that of the arrangement of whiskers on the snout [70].

Another type of specialised mechanoreceptor system is found in the glabrous (hairless smooth skin) skin of the mouse. The mouse uses its forepaws to explore and to groom. Such exploratory behaviour involves the forepaw glabrous skin as the primary sensory surface [71]. It has been found that the forepaw skin is innervated with a very high density of mechanoreceptors, almost three times higher than that of the hind paw. Hence, they are highly sensitive to even slowly moving mechanical stimuli in comparison to the hind paw [71].

References

1. Guenet J, Bonhomme F (2004) Origin of the laboratory mouse and related subspecies. In: Hedrich H (ed) The laboratory mouse. Elsevier Academic Press, San Diego, pp 3–13
2. Waterston RH, Lindblad-Toh K et al (2002) Mouse genome sequencing consortium, initial sequencing and comparative analysis of the mouse genome. Nature 420(6915):520–562. https://doi.org/10.1038/nature01262

3. Grieder FB, Strandberg JD (2003) The contribution of laboratory animals to medical progress-past, present, and future. In: Hau J, Van Hoosier GL (eds) Handbook of laboratory animal science, 2nd edn. CRC Press, BocaRaton, FL, pp 1–11
4. Lorenz JN (2002) A practical guide to evaluating cardiovascular, renal, and pulmonary function in mice. Am J Physiol Regul Integr Comp Physiol 282(6):R1565–R1582. https://doi.org/10.1152/ajpregu.00759.2001
5. Krinke GJ (2004) Normative histology of organs. In: Hedrich H (ed) The laboratory mouse. Elsevier Academic Press, San Diego, pp 133–166
6. Shinohara H (1999) The mouse vertebrae: changes in the morphology of mouse vertebrae exhibit specific patterns over limited numbers of vertebral levels. Okajimas Folia Anat Jpn 76 (1):17–31. https://doi.org/10.2535/ofaj1936.76.1_17
7. Shinohara H (1999) The musculature of the mouse tail is characterized by metameric arrangements of bicipital muscles. Okajimas Folia Anat Jpn 76(4):157–169. https://doi.org/10.2535/ofaj1936.76.4_157
8. Kuijpers MH, van de Kooij AJ, Slootweg PJ (1996) Review article. The rat incisor in toxicologic pathology. Toxicol Pathol 24(3):346–360. https://doi.org/10.1177/019262339602400311
9. Goldberg M, Kellermann O, Dimitrova-Nakov S, Harichane Y, Baudry A (2014) Comparative studies between mice molars and incisors are required to draw an overview of enamel structural complexity. Front Physiol 5:359. https://doi.org/10.3389/fphys.2014.00359
10. Colby LA, Nowland MH, Kennedy LH (2020) Clinical laboratory animal medicine: an introduction, 5th edn. Wiley Blackwell, pp 74–123
11. Berdanier CD (2004) Gastrointestinal system and metabolism. In: Hedrich H (ed) The laboratory mouse. Elsevier Academic Press, San Diego, pp 245–260
12. Johnson LR (2003) Essential medical physiology, 3rd edn. Academic Press, San Diego
13. Krinke GJ (2004) Normative histology of organs. In: Hedrich H (ed) The laboratory mouse. Elsevier Academic Press, San Diego, pp 113–166
14. Dolenšek J, Rupnik MS, Stožer A (2015) Structural similarities and differences between the human and the mouse pancreas. Islets 7(1):e1024405. https://doi.org/10.1080/19382014.2015.1024405
15. Hollander CF, van Bezooijen CFA, Solleveld HA (1987) Anatomy, function and aging in the mouse liver. In: Chambers PL, Henschler D, Oesch F (eds) Mouse liver tumors. Archives of toxicology (supplement), vol 10. Springer, Berlin, Heidelberg
16. Hoyt RF, Hawkins JV, St Clair MB, Kennett MJ (2007) Mouse physiology. In: Fox JG, Barthold SW, Davisson MT, Newcomer CE, Quimby FW, Smith A (eds) The mouse in biomedical research, normative biology, husbandry, and models, vol 3, 2nd edn. Academic Press, San Diego, CA, pp 23–90
17. Tanimizu N, Ichinohe N, Ishii M et al (2016) Liver progenitors isolated from adult healthy mouse liver efficiently differentiate to functional hepatocytes in vitro and repopulate liver tissue. Stem Cells 34(12):2889–2901. https://doi.org/10.1002/stem.2457
18. Dietschy JM, Turley SD (2002) Control of cholesterol turnover in the mouse. J Biol Chem 277 (6):3801–3804. https://doi.org/10.1074/jbc.R100057200
19. Brittan M, Wright NA (2002) Gastrointestinal stem cells. J Pathol 197(4):492–509. https://doi.org/10.1002/path.1155
20. Lueschow SR, McElroy SJ (2020) The paneth cell: the curator and defender of the immature small intestine. Front Immunol 11:587. https://doi.org/10.3389/fimmu.2020.00587
21. Blair PJ, Rhee PL, Sanders KM, Ward SM (2014) The significance of interstitial cells in neurogastroenterology. J Neurogastroenterol Motil 20(3):294–317. https://doi.org/10.5056/jnm14060
22. Pérez-Gómez A, Stein B, Leinders-Zufall T, Chamero P (2014) Signaling mechanisms and behavioral function of the mouse basal vomeronasal neuroepithelium. Front Neuroanat 8:135. https://doi.org/10.3389/fnana.2014.00135
23. Navarro M, Ruberte J, Carretero A (2017) Respiratory apparatus. In: Morphological mouse phenotyping. anatomy, histology and imaging. Academic Press, pp 147–178

24. JacobyRO FJG, Davisson (2002) Biology and diseases of mice. In: Fox JG, Anderson LC, Lorw FM, Quimby FW (eds) Laboratory animal medicine. Elsevier Academic Press, San Diego, pp 35–120
25. Patra AL (1986) Comparative anatomy of mammalian respiratory tracts: the nasopharyngeal region and the tracheobronchial region. J Toxicol Environ Health 17(2–3):163–174. https://doi.org/10.1080/15287398609530813
26. Pan H, Deutsch GH, Wert SE et al (2019) Comprehensive anatomic ontologies for lung development: a comparison of alveolar formation and maturation within mouse and human lung. J Biomed Semant 10:18. https://doi.org/10.1186/s13326-019-0209-1
27. McElroy MC, Kasper M (2004) The use of alveolar epithelial type I cell-selective markers to investigate lung injury and repair. Eur Respir J 24(4):664–673. https://doi.org/10.1183/09031936.04.00096003
28. Fehrenbach H (2001) Alveolar epithelial type II cell: defender of the alveolus revisited. Respir Res 2(1):33–46. https://doi.org/10.1186/rr36
29. Crouch E, Wright JR (2001) Surfactant proteins a and d and pulmonary host defense. Annu Rev Physiol 63:521–554. https://doi.org/10.1146/annurev.physiol.63.1.521
30. Rao S, Verkman AS (2000) Analysis of organ physiology in transgenic mice. Am J Physiol Cell Physiol 279(1):C1–C18. https://doi.org/10.1152/ajpcell.2000.279.1.C1
31. Irvin CG, Bates JH (2003) Measuring the lung function in the mouse: the challenge of size. Respir Res 4:1. https://doi.org/10.1186/rr199
32. Oakes JM, Scadeng M, Breen EC, Marsden AL, Darquenne C (2012) Rat airway morphometry measured from in situ MRI-based geometric models. J Appl Physiol (1985) 112 (11):1921–1931. https://doi.org/10.1152/japplphysiol.00018.2012
33. Wessels A, Sedmera D (2003) Developmental anatomy of the heart: a tale of mice and man. Physiol Genomics 15(3):165–176. https://doi.org/10.1152/physiolgenomics.00033.2003
34. Tobias O (2001) Function and structure of the mouse sinus node: nothing you can see that isn't shown, cardiovascular research. 52(1):1–4. https://doi.org/10.1016/S0008-6363(01)00417-5
35. Miquerol L, Meysen S, Mangoni M et al (2004) Architectural and functional asymmetry of the his-Purkinje system of the murine heart. Cardiovasc Res 63(1):77–86. https://doi.org/10.1016/j.cardiores.2004.03.007
36. Michael LH, Taffet GE, Frangogiannis NG, Entman ML, Hartley CJ (2004) The cardiovascular system. In: Hedrich H (ed) The laboratory mouse. Elsevier Academic Press, San Diego, pp 207–224
37. Kramer K, van Acker SA, Voss HP, Grimbergen JA, van der Vijgh WJ, BastA (1993) Use of telemetry to record electrocardiogram and heart rate in freely moving mice. J Pharmacol Toxicol Methods 30(4):209–215. https://doi.org/10.1016/1056-8719(93)90019-b
38. Car BD, Eng VM (2001) Special considerations in the evaluation of the hematology and hemostasis of mutant mice. Vet Pathol 38(1):20–30. https://doi.org/10.1354/vp.38-1-20
39. O'Connell KE, Mikkola AM, Stepanek AM et al (2015) Practical murine hematopathology: a comparative review and implications for research. Comp Med 65(2):96–113
40. Everds N (2004) Hematology of the mouse. In: Hedrich H (ed) The laboratory mouse. Elsevier Academic Press, San Diego, pp 271–286
41. MPD Mouse Phenome database for mice by (J) strain, sex and age, with protocol detail https://phenome.jax.org/search/details/ssmeasures?searchterm=complete+blood+count+&ontavail=0
42. Schnermann J, Traynor T, Yang T et al (1998) Tubuloglomerular feedback: new concepts and developments. Kidney Int Suppl 67:S40–S45. https://doi.org/10.1046/j.1523-1755.1998.06708.x
43. Jung JY, Madsen KM, Han KH et al (2003) Expression of urea transporters in potassium-depleted mouse kidney. Am J Physiol Renal Physiol 285(6):F1210–F1224. https://doi.org/10.1152/ajprenal.00111.2003
44. Hurst JL (2009) Female recognition and assessment of males through scent. Behav Brain Res 200(2):295–303. https://doi.org/10.1016/j.bbr.2008.12.020

45. Loung RH (2017) The laboratory mouse. In: Kurtz DM, Travlos GS (eds) The clinical chemistry of laboratory animals, 3rd edn. CRC Press, pp 1–32
46. Knoblaugh SE, True L, Tretiakova M, Hukkanen RR (2018) Male reproductive system. In: Treuting PM, Dintzis SM, Montine KS (eds) Comparative anatomy and histology, a mouse, rat, and human atlas, 2nd edn. Academic Press, pp 335–363
47. Cook MJ (1983) Anatomy. In: Foster HL, Small JD, Fox JG (eds) The mouse in biomedical research, normative biology, husbandry, and models, vol 3. Academic Press, pp 102–120
48. Sengul G, Watson C (2012) Spinal cord. In: Watson C, Paxinos G, Puelles L (eds) The mouse nervous system. Academic Press, pp 424–458
49. Kobayashi T (1963) Brain-to-body ratios and time of maturation of the mouse brain. Am J Phys 204:343–346. https://doi.org/10.1152/ajplegacy.1963.204.2.343
50. Shnerson A, Willott JF (1979) Development of inferior colliculus response properties in C57BL/6J mouse pups. Exp Brain Res 37(2):373–385. https://doi.org/10.1007/BF00237720
51. Shupe JM, Kristan DM, Austad SN, Stenkamp DL (2006) The eye of the laboratory mouse remains anatomically adapted for natural conditions. Brain Behav Evol 67(1):39–52. https://doi.org/10.1159/000088857
52. Baker M (2013) Neuroscience: through the eyes of a mouse. Nature 502(7470):156–158. https://doi.org/10.1038/502156a
53. Huberman AD, Niell CM (2011) What can mice tell us about how vision works? Trends Neurosci 34(9):464–473. https://doi.org/10.1016/j.tins.2011.07.002
54. Peirson SN, Brown LA, Pothecary CA, Benson LA, Fisk AS (2018) Light and the laboratory mouse. J Neurosci Methods 300:26–36. https://doi.org/10.1016/j.jneumeth.2017.04.007
55. Warwick RA, Kaushansky N, Sarid N, Golan A, Rivlin-Etzion M (2018) Inhomogeneous encoding of the visual field in the mouse retina. Curr Biol 28(5):655–665.e3. https://doi.org/10.1016/j.cub.2018.01.016
56. Hattar S, Liao HW, Takao M, Berson DM, Yau KW (2002) Melanopsin-containing retinal ganglion cells: architecture, projections, and intrinsic photosensitivity. Science 295 (5557):1065–1070
57. De Vera Mudry MC, Kronenberg S, Komatsu S, Aguirre GD (2013) Blinded by the light: retinal phototoxicity in the context of safety studies. Toxicol Pathol 41(6):813–825. https://doi.org/10.1177/0192623312469308
58. Ramos MF, Baker J, Atzpodien EA et al (2018) Nonproliferative and proliferative lesions of the Ratand mouse special sense organs (ocular [eye and glands], olfactory and otic). J Toxicol Pathol 31(3 Suppl):97S–214S. https://doi.org/10.1293/tox.31.97S
59. Zou J, Wang W, Pan YW, Lu S, Xia Z (2015) Methods to measure olfactory behavior in mice. Curr Protoc Toxicol 63:11.18.1–11.18.21. https://doi.org/10.1002/0471140856.tx1118s63
60. Schwob JE (2002) Neural regeneration and the peripheral olfactory system. Anat Rec 269 (1):33–49. https://doi.org/10.1002/ar.10047
61. Touhara K, Vosshall LB (2009) Sensing odorants and pheromones with chemosensory receptors. Annu Rev Physiol 71:307–332. https://doi.org/10.1146/annurev.physiol.010908.163209
62. Riera CE, Tsaousidou E, Halloran J et al (2017) The sense of smell impacts metabolic health and obesity. Cell Metab 26(1):198–211.e5. https://doi.org/10.1016/j.cmet.2017.06.015
63. Glinka ME, Samuels BA, Diodato A, Teillon J, Feng Mei D, Shykind BM, Hen R, Fleischmann A (2012) Olfactory deficits cause anxiety-like behaviors in mice. J Neurosci 32(19):6718–6725. https://doi.org/10.1523/JNEUROSCI.4287-11.2012
64. Kelliher KR, Wersinger SR (2009) Olfactory regulation of the sexual behavior and reproductive physiology of the laboratory mouse: effects and neural mechanisms. ILAR J 50(1):28–42. https://doi.org/10.1093/ilar.50.1.28
65. Kass MD, Czarnecki LA, Moberly AH, McGann JP (2017) Differences in peripheral sensory input to the olfactory bulb between male and female mice. Sci Rep 7:45851. https://doi.org/10.1038/srep45851

66. Reynolds RP, Kinard WL, Degraff JJ, Leverage N, Norton JN (2010) Noise in a laboratory animal facility from the human and mouse perspectives. J Am Assoc Lab Anim Sci: JAALAS 49(5):592–597
67. Turner JG, Parrish JL, Hughes LF, Toth LA, Caspary DM (2005) Hearing in laboratory animals: strain differences and nonauditory effects of noise. Comp Med 55(1):12–23
68. Sundberg JP (2004) Skin and adnexa of the laboratory mouse. In: Hedrich H (ed) The laboratory mouse. Elsevier Academic Press, San Diego, pp 195–206
69. Adibi M (2019) Whisker-mediated touch system in rodents: from neuron to behavior. Front Syst Neurosci 13:40. https://doi.org/10.3389/fnsys.2019.00040
70. Petersen CC (2007) The functional organization of the barrel cortex. Neuron 56(2):339–355. https://doi.org/10.1016/j.neuron.2007.09.017
71. Walcher J, Ojeda-Alonso J, Haseleu J, Oosthuizen MK, Rowe AH, Bennett NC, Lewin GR (2018) Specialized mechanoreceptor systems in rodent glabrous skin. J Physiol 596 (20):4995–5016. https://doi.org/10.1113/JP276608

9 The Anatomy and Physiology of Laboratory Rat

Saurabh Chawla and Sarita Jena

Abstract

The laboratory rat is commonly used as an experimental model in biomedical research. Characteristics such as short life span, ease of breeding, short generation time, and requirement of smaller space have made laboratory rats as the most commonly used experimental animals along with mice in the field of basic and applied research. Animal studies fetch the best results by making the right choices of animal models. Despite decades of basic research done and data generated on rodent anatomy and physiology, researchers tend to overlook the proximity of these details to the human system while testing and selecting the right model and testing their hypothesis. This chapter reviews the important points of anatomy and physiology of laboratory rats in comparison to humans for a better understanding of the subject. The main focus has been on salient anatomical features of laboratory rat which is different and unique and may influence experimental design and outcome. Applied knowledge related to the husbandry of laboratory rats has also been covered.

Keywords

Rat · Anatomy · Physiology

S. Chawla (✉)
National Institute of Science Education and Research, Bhubaneswar, India
e-mail: saurabhchawla@niser.ac.in

S. Jena
Institute of Life Sciences, Bhubaneswar, India
e-mail: saritajena@ils.res.in

© The Author(s), under exclusive license to Springer Nature Singapore Pte Ltd. 2021
P. Nagarajan et al. (eds.), *Essentials of Laboratory Animal Science: Principles and Practices*, https://doi.org/10.1007/978-981-16-0987-9_9

9.1 Introduction

The laboratory rat is a descendent of brown Norway rat or *Rattus norvegicus*. Other common species of genus *Rattus* is known as black rats or *Rattus rattus*. The rat may be considered as one of the most successful species on earth due to its adaptability to a wide variety of climatic conditions with diverse eating habits aided by highly developed senses of smell, hearing, and touch. The ancestors of present-day laboratory rats seem to have originated in Central Asia and have become cosmopolitan in the last 200–300 years due to a rise in intercontinental trade and travel. Characteristics such as short life span, ease of breeding, short generation time, and small space requirement made laboratory rats along with mice as the most commonly used experimental animals in the field of basic and applied research. The laboratory rat has made a significant contribution in the area of biomedical research to date.

9.2 Scientific Classification/Taxonomy

Kingdom: Animalia
Subkingdom: Bilateria
Infrakingdom: Deuterostomia
Phylum: Chordata
Subphylum: Vertebrata
Infraphylum: Gnathostomata
Superclass: Tetrapoda
Class: Mammalia
Subclass: Theria
Infraclass: Eutheria
Order: Rodentia
Suborder: Myomorpha
Superfamily: Muroidea
Family: Muridae
Subfamily: Murinae
Genus: *Rattus*

9.3 General Characteristics

The laboratory rat generally has short hairs and a long, hairless, scaly tail. The ears are round and erect. The mouth has a pointed snout with a long whisker (vibrissae). Front and hind feet have five digits each with sharp claws (Fig. 9.1). They have moderately long legs and can stand upright on their two rear legs. The soles of hind feet have fleshy footpads. Self-grooming is an innate behavior and a most frequently performed activities in rodents [1]. Directional terms commonly used with rodents are given below and depicted in Fig. 9.2:

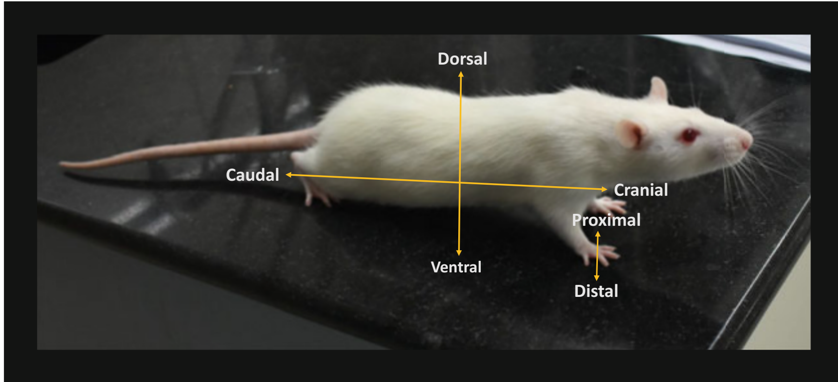


Fig. 9.1 Directional terms

Fig. 9.2 Rat feet depicting five digits and sharp claws

- Cranial: Toward the head.
- Caudal: Toward the tail.
- Dorsal: Toward the spine.
- Ventral: Toward the abdomen.
- Medial: Toward the midline of the body.
- Lateral: Toward the side of the body.

9.3.1 Size, Weight, and Life Span

The growth rate is a function of environment, nutrition, and strain. The size and body weight depend on strain or stock when raised under standard laboratory conditions.

For example, F344 and Wistar rats have a smaller body size than Sprague-Dawley rats [2]. Laboratory rats may grow big and may reach a weight of up to 800 g [3]. The body weight at birth is around 4.5–6 g. The weight of the neonate is around 5.9–6.4 g. Males generally grow faster when compared to females. Generally, an adult male weighs around 450–550 g, whereas an adult female weighs around 250–300 g [4]. Life span also varies depending on strain/stock or diet. Laboratory rats have an average life span of around 2–4 years.

9.3.2 General Appearance

The laboratory rat has a blunt snout with short ears. These are distinguishing features of rats as compared to mice which have a small head, pointed snout, and large ears (Fig. 9.3). Females usually have six pairs of mammary glands (Fig .9.4). Males and females can be distinguished based upon anogenital distance (right from birth) which is more in males as compared to females.

9.4 Special Senses

9.4.1 Eye

The average anterolateral diameter of the adult rat eye is around 6–7 mm. The rat's eyes are slightly exophthalmic (protruding), remain exposed, and dry out quickly during the long surgery. Albino rats lack melanin in their eyes. The iris as well as the pigment epithelial layer of the retina does not have melanin in comparison to non-albino rats. Thus, albino rats are prone to phototoxic retinopathy and retinal damage. The light passes through the cornea followed by the pupil. Then, light passes through the pupil. Like the human pupil, the rat's pupil size is highly variable. The diameter of the pupil responds very rapidly such that a contraction from 2 to

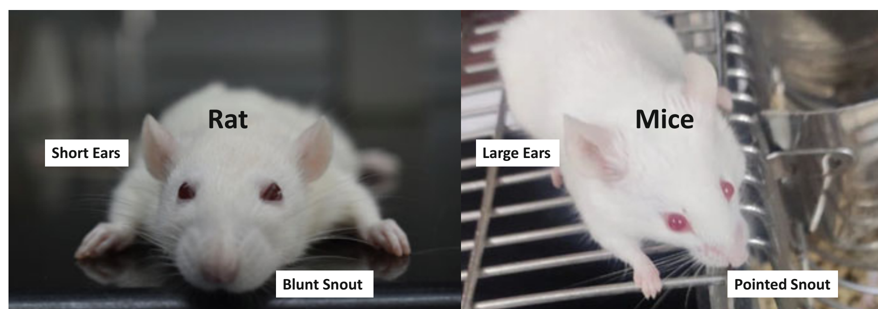


Fig. 9.3 Difference between rat and mice

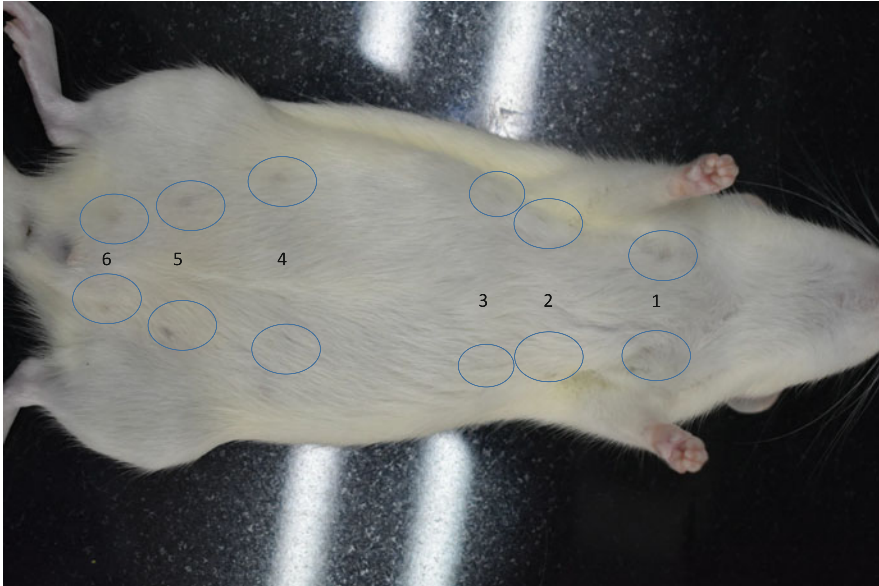


Fig. 9.4 Mammary gland in female rat—1: cervical, 2 and 3: thoracic, 4: abdominal, and 5 and 6: inguinal

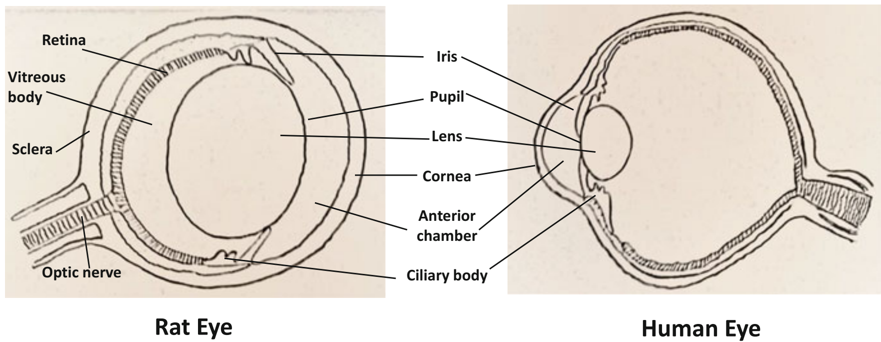


Fig. 9.5 Rat eye vs human eye

0.5 mm takes only half a second [5]. The size of the lens is relatively large in rodents, but it lacks flexibility as compared to humans (Fig .9.5). The retina of the rat's eye has rods and only two types of cones (green and blue). Therefore, the rats are color-blind to red. Rats have poor acuity or sharpness of vision [6]. Rats can also readily detect UV light due to the presence of UV-sensitive cones [7].

The Harderian glands are pigment-producing lacrimal glands located posterior to the eye globes. These glands are absent in humans. Red-colored secretory porphyrin-rich material that lubricates the eyes and eyelids is commonly and regularly

produced in rodents. Chromodacryorrhea is an excessive secretion of porphyrin pigment from the Harderian gland. This pigment drains through the nasolacrimal duct into the nose and may be seen as red crust deposited around the eyes and nose of the affected animal, sometimes also referred to as "bloody tears." Occasional small amounts of porphyrin are normal and wiped out by continuous grooming.

9.4.2 Ear

The primary function of the ear is to detect sound and maintain balance. The ear of a rat may be divided into the external, middle, and inner ear.

The external ear consists of the pinna and the external auditory canal which acts as a funnel to transmit the sound to the tympanic membrane which acts as the entrance to the middle ear. Further, the sound is transmitted as mechanical energy to the ear bones, namely, malleus, incus, and stapes. Thereby, the energy is transmitted as waves to fluid-filled cochlea further to the organ of Corti, which lies within the cochlea, generating action potentials in the nerve fibers from where the impulses are then carried to the brain.

Rats are very sensitive to sound and can even hear ultrasound as the range of the rat's hearing lies within 200 Hz to 80 or 90 kHz [8]. Rats are also known to emit ultrasonic vocalizations (USVs) and thus may be an important part of communication. Long USVs are emitted in adverse conditions, whereas short USVs are emitted during play or copulation [9].

9.4.3 Skin

The skin is the largest organ of the body based on the surface area. Apocrine sweat glands are absent in rodents. Eccrine sweat glands in mice are absent throughout the body except for the footpads. Rat footpads can be considered similar to that of human's palms and soles in having a thick epidermis and eccrine glands. Typical characteristics of the skin in contrast to humans are presented in the Table 9.1.

9.4.4 Rat Whiskers

Whiskers are modified hairs specialized as touch organs that grow in rows on either side of their nose. In rats and mice, the facial whiskers are swept back and forth rapidly 12 days after birth [10]. The whiskers differ from normal hairs as it is thicker and sealed by a capsule of blood known as blood sinus which is heavily innervated by sensory nerves [11]. Whiskers in rats are highly sensitive to touch and help them in exploring their surroundings. Thus, albino rats despite having a poor vision navigate their path using whisker touch. Whiskers not only help rats to navigate short distances but also help in in-depth perception and identification of objects.

Table 9.1 Comparison between the characteristic features of the skin of rat and humans

Characteristics	Rodents	Humans
Hair growth pattern	Mouse hair follicles remain in the resting phase (telogen) which means that hair growth is not continued until the next wave/cycle	Human hair on the scalp or beard remains in a growing phase (anagen) for months to years
Hair function	Thermoregulation	Does not play a significant role in thermoregulation
Fingerprints	No fingerprints on footpads (rete ridges absent)	Fingerprints are present as rete ridges are present
Sweat glands	Apocrine sweat glands are absent in rats. Mammary glands are modified apocrine glands present in rats. Eccrine sweat glands in mice are absent throughout the body except for the footpads	Present throughout the body
Biomechanical properties	The skin in rodents is thin and loose	In humans, the skin is thick, stiff at places, and adherent to underlying tissues
Hypodermal thickness	Hair cycle-dependent	Variable, depending on the region
Subcutaneous muscle layer	Present throughout as panniculus carnosus which aids in wound contraction	A similar layer present only in the neck region
Wound healing	Subcutaneous muscle "panniculus carnosus" facilitates wound contraction and apposition. Thus, healing is very fast	Healing primarily by reepithelialization

9.5 Digestive System

Rats have two prominent incisors (Fig. 9.6) and six molars. The dental formula of rats is $2 \times$ (1 1/1, C 0/0, P 0/0, M 3/3) $= 16$. The incisors are long front teeth having open roots, and thus, they continue to grow throughout the animal's life. Both mice and rats are monophyodont with a single set of teeth throughout their life span. Rats have three pairs of salivary glands in the head and neck region. Parotid glands are triangular and lie ventral to the ear extending along the lateral surface of the neck. The mandibular gland is quite prominent and oval and can be visualized in the ventral portion of the neck upon dissection. The sublingual gland lies adjacent to the mandibular gland. Salivary glands are connected to the mouth through a salivary duct.

Multilocular adipose tissue referred to as brown fat, also known as the hibernating gland, is located in the ventral and lateral portions of the neck and sometimes confused with salivary glands. The brown fat plays an important role in non-shivering heat production during cold exposure in rats. Rats are not known to hibernate; thus, the tern "hibernating gland" is a misnomer.

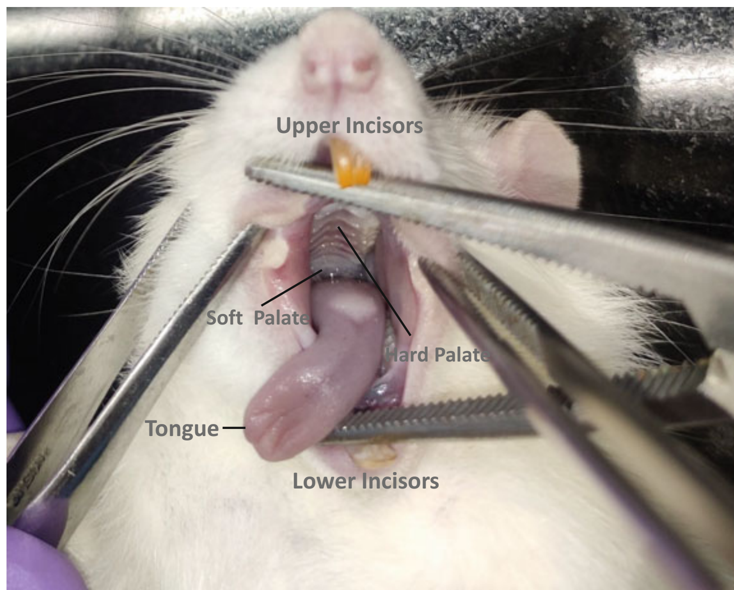


Fig. 9.6 Mouth and related parts

The tongue is long and attached to the rear of the oral cavity by a sheet of tissue known as the frenulum. The dorsal surface of the body of the tongue is rough due to the presence of numerous filiform papillae [12]. The tongue is made of skeletal muscle that is innervated by the hypoglossal nerves.

The hard palate and soft palate form the roof of the oral cavity (Fig. 9.6).

The esophagus is a distensible and muscular tube like other mammals that connect the pharynx and stomach. The esophagus transports food toward the stomach aided by the peristaltic movement. The epithelium of the esophagus is covered with a layer of keratin. A rat cannot vomit because of a powerful and effective gastroesophageal barrier which consists of a crural sling, the esophageal sphincter, and the intra-abdominal esophagus. Thus, rats have a powerful barrier between the stomach and the esophagus [13]. In humans, the pharynx is a common pathway for the respiratory and digestive system, whereas in rats, it has separate regions for both these systems.

The stomach is located close to the abdominal side of the diaphragm. The part continuous with the esophagus is the forestomach also known as pars proventricularis. The remaining part is the glandular stomach (corpus or pars glandularis). The forestomach in rodents occupies about three-fifths of the stomach area [14]. The part of the stomach which connects with the esophagus has lesser curvature, whereas the opposite end has a greater curvature. The forestomach is a thin-walled, nonglandular section, lined by keratinized stratified squamous epithelium and receives food from the esophagus through the gastroesophageal junction and serves as a holding chamber or initial reservoir for food. The forestomach and

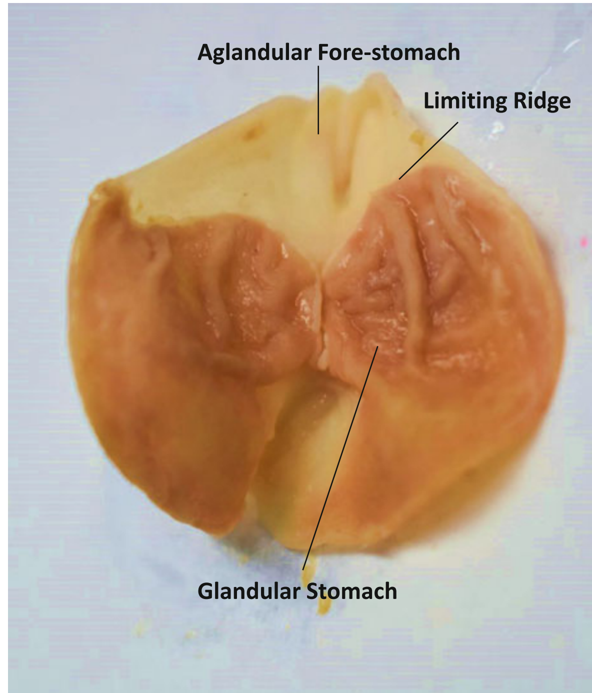


Fig. 9.7 The mucosal surface of the rat stomach

the glandular stomach are separated by a low fold of tissue called the limiting ridge (margo plicatus) (Fig. 9.7). The esophagus enters at the midpoint of the rat stomach at the lesser curvature near the limiting ridge. The glandular part is lined by the lamina propria and consists of simple tubular gastric glands containing mucus-secreting neck cells, pepsinogen-secreting chief cells, and HC1-secreting parietal cells. The terminal part of the stomach tract is connected to the duodenum at the pyloric ring. Protein digestion begins in the stomach by HCL and enzyme pepsin.

The small intestine is the major site for the digestion and absorption of nutrients. It begins from the pylorus part of the stomach to the terminal part of the ileum where it meets the caecum (Fig. 9.8). The small intestine has a smaller diameter than the large intestine though it is longer. The small intestine can be further divided into three parts: the duodenum, jejunum, and ileum. The duodenum is the first part of the small intestine (8 cm), followed by the jejunum in the middle (80 cm) and terminates at the ileum (3 cm).

The duodenum receives partially digested food in the form of acidic chyme from the stomach which stimulates the release of pancreatic enzymes via pancreatic duct into the duodenum. The duodenum receives bile produced by the liver through the common bile duct.

The pancreas in rats is diffuse, lobulated, pink in color, and located in the mesentery between the stomach, duodenum, and ascending and transverse colons in the rat. The pancreas may be divided into three parts, namely, the biliary,

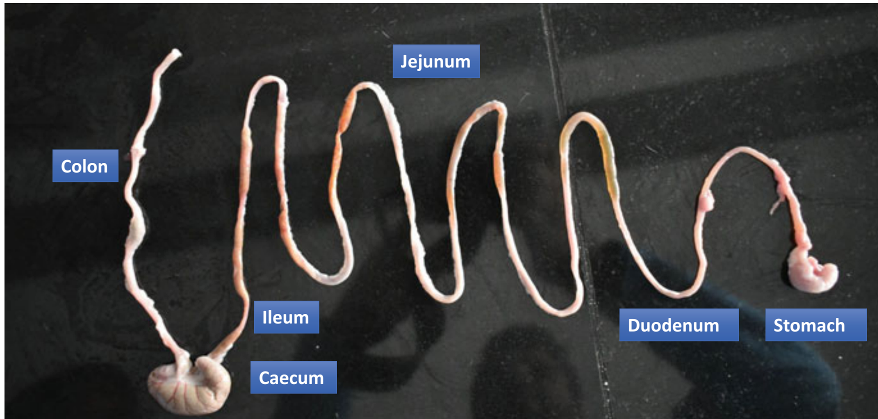


Fig. 9.8 Gastrointestinal tract

duodenal, and gastrosplenic portions. The coeliac and the superior mesenteric arteries supply the oxygenated blood to the pancreas, whereas the venous blood is drained by the portal vein. The pancreas plays a vital role in the digestive system which helps to break down carbohydrates, proteins, and lipids (fats). Islet cells are the endocrine cells of the pancreas that produce and secrete insulin as well as glucagon into the bloodstream which in turn regulates glycogen and glucose metabolism.

The main cell types present in the small intestine of the rat are the following:

(a) **Enterocytes**: Enterocytes form a barrier that offers only a very limited passage of material in both directions. Cell-cell adhesion is ensured by tight junctions, desmosomes, and adherence [15].
(b) **Brunner glands**: It secretes an alkaline fluid composed of mucin and thus protects the duodenal epithelium from the acid chyme of the stomach.
(c) **Paneth cells**: Paneth cells are found at the bottom positions of small intestinal crypts. Paneth plays an important role in the innate immune system and thus produces large amounts of α-defensins and other antimicrobial peptides, such as lysozymes and secretory phospholipase A2 [16].
(d) **Enteroendocrine cells:** Enteroendocrine cells are a highly specialized mucosal cell subpopulation, distributed throughout the small intestine [17]. They are present in crypts as well as villi. They produce a range of gut hormones that have key roles in the coordination of food digestion and absorption and insulin secretion and also have a role to play in appetite [18].

The small intestine terminates at the ileum. Lymph nodules called Peyer's patches are found abundant in the ileum.

The large intestine is divided into the cecum, colon, and rectum. Rats have large caecum which acts as a fermentation chamber containing a high concentration of

bacterial organisms which aids in the digestion of cellulose. The rat cecum is devoid of internal septa which is present in many other rodents. In rodents, the cecum is large and constitutes up to one-third of the large intestine. The size and length of the colon itself are smaller in rodents as compared to the human colon which is significantly bigger and longer. The rectum is extremely short in the rodents and is easily prolapsed under certain pathological conditions.

Mesentery in rats is formed by the double fold of peritoneum attached to the abdominal wall (Fig. 9.9). Mesentery consists of mesenteric arteries, veins, lymphatics, mesenteric fat, and mesenteric lymph node and is attached to the walls of the small and large intestine.

The liver of the rat is multi-lobulated and can be divided into four major lobes: (1) left lobe, (2) median/cystic lobe, (3) right lateral lobe, and (4) caudate (Fig. 9.10).

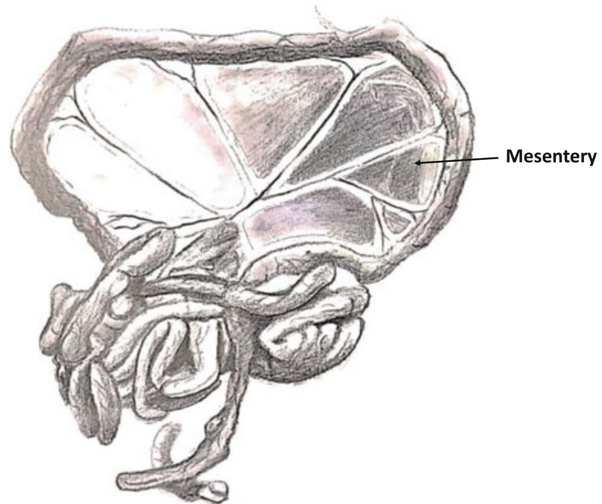


Fig. 9.9 Illustration of intestinal mesentery of rat

Fig. 9.10 Rat liver

The liver constitutes about 6% of the total body weight [19]. The liver plays a very important role in nutrient processing, energy homeostasis, and detoxification. Nutrients are absorbed from the gastrointestinal tract and transported to the liver via the portal vein. Histologically, hepatocytes in the liver are arranged as classic liver lobules and polygonal structures with portal tracts at the periphery and a central vein in the center. The gallbladder is absent in rat. Bile from each lobe of the liver flow through the common bile duct which enters the duodenum approximately 25 mm from the pyloric sphincter [20, 21].

9.6 Respiratory System

The primary functions of the respiratory system are gas exchange and olfaction. The respiratory system consists of the upper respiratory tract, the airway, and the lower respiratory tract. The upper respiratory tract starts from the nose and includes the parts outside the thoracic cavity. The lungs of the rat lie on each side of the heart in the chest cavity. The left lung of the rat is a single lobe, whereas the right lung is divided into four lobes (Figs. 9.11a and 9.12). The right lung consists of the right superior lobe, right inferior lobe, right middle lobe, and postcaval lobe. The left lung is smaller than the right lung (Fig. 9.13) [22]. The lung in the newborn rat is devoid of alveoli or alveolar ducts. The gas exchange in newborn rats occurs in smooth-walled channels and saccules, the prospective alveolar structures. Once the rat is

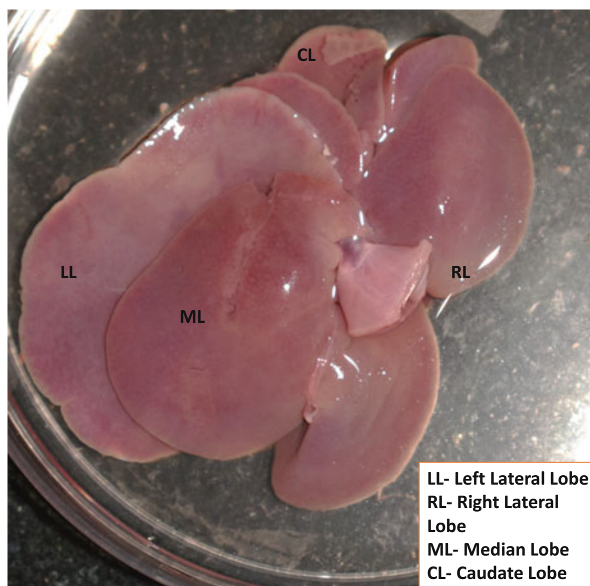


Fig. 9.11 Rat liver

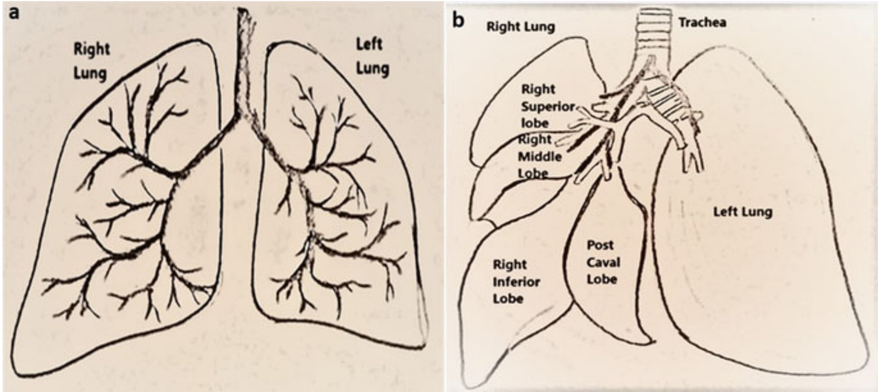


Fig. 9.12 Comparative anatomy of the rat (**a**) and human lungs (**b**)

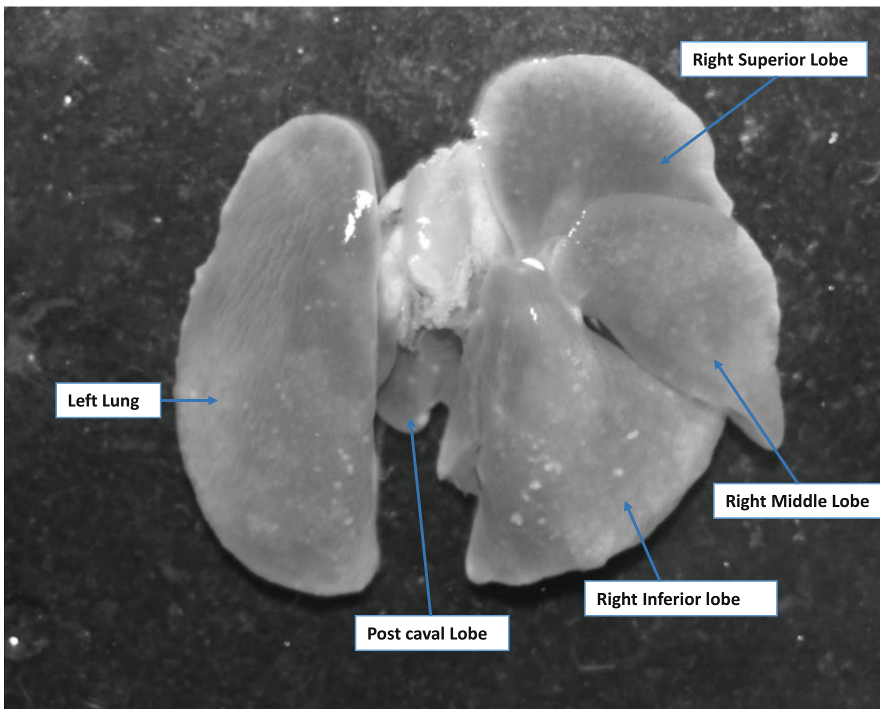


Fig. 9.13 Rat lung

4 days old, rapid restructuring of lung parenchyma occurs on day 4, and by day 7, the rat lung can be considered as morphologically more mature. Respiratory bronchioles that are not present at birth are visible by day 10. True alveolar septa are formed after day 13 after the expansion and thinning of the primary and secondary septa [23].

Table 9.2 Anatomical features of rat and human lung

Anatomical structure	Human	Rat
Number of lobes in the lungs	3 on the right side, 2 in the left side	4 on the right side, 1 in the left side
Lung parenchyma (%)	12	24
Alveoli (mean linear intercept (MLI))	210 µm	100 µm
Blood-gas barrier thickness	0.62 µm	0.38 µm
Trachea	Complete ring-shaped	Incomplete, C-shaped
Number of airway generation	17–21 generations	15–16
Airway branching pattern	Dichotomous	Both monopodial and dichotomous

Source: Adopted from Irvin and Bates (2003) [24] and Oakes et al. (2004) [25]

Air enters and is conditioned inside the nose, which is made of nasal bone and cartilage. The nasal passage leads to the nasopharynx.

The laboratory rodents such as rat exhibit a predominantly monopodial branching system. In contrast, dichotomous (axis divided into two branches) and trichotomous airway branching patterns are present in human and nonhuman primate lungs (Table 9.2).

9.7 Cardiovascular System

The heart of the rat (*Rattus norvegicus*) is located on the midline in the thorax surrounded by the lungs laterally. The apex (left ventricle) lies near the diaphragm. The left lung is smaller in rat and thus provides enough space at the left thoracic wall between the third and fifth ribs to facilitate cardiac puncture.

The wall of the heart chambers consists of three layers, namely, the internal endocardium, the middle myocardium, and the outermost known as the epicardium. The myocardium is the thickest of the three layers with thickness increasing toward the ventricles than in the atria.

The mammalian four-chambered heart has double circulation with an interior circuit within the thorax (Fig. 9.14). The pulmonary artery carries deoxygenated blood pumped via the left ventricle toward the lungs for oxygenation, whereas the pulmonary vein carries oxygenated blood toward the left atrium to the left ventricle from where the oxygenated blood is pumped throughout the body.

Rats in contrast to other large animals and humans have a left cranial vena cava and the common right cranial vena cava [26]. Humans have only the right (cranial) superior vein.

The right cranial vena cava empties the right atrium, and the left cranial vena cava is joined by the azygos vein and the caudal vena cava to enter the right atrium. In humans, the myocardium extends outside the heart no further than the extrapulmonary veins, whereas in rodents, the cardiac muscle extends beyond the atrium along pulmonary veins and into intrapulmonary veins, sometimes also known

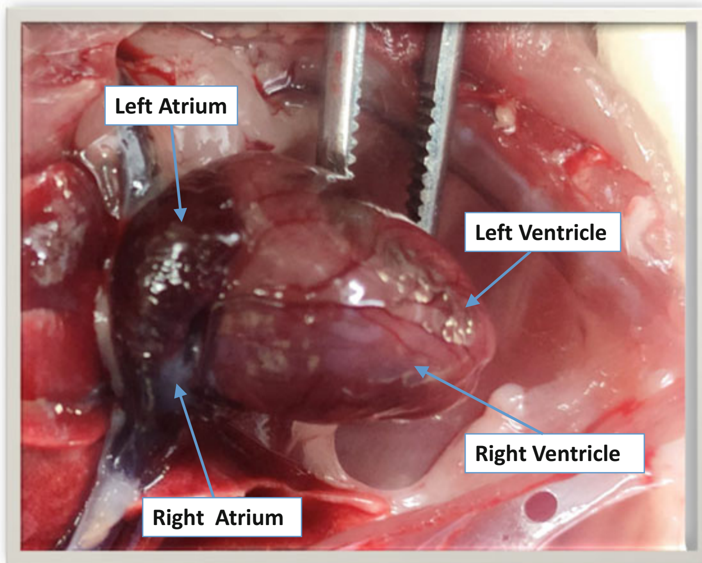


Fig. 9.14 Rat Heart

as pulmonary myocardium [27]. Thus, the infection from the heart easily travels toward the lungs.

Rats among all rodents have the thinnest pulmonary artery and the thickest pulmonary vein. Blood supply to the heart of the rat is primarily from the branches of internal mammary and subclavian arteries and not from coronary arteries as in other mammals and humans. The average systolic blood pressure in rats is 116 mm Hg, and diastolic blood pressure is 90 mm Hg [28] (Table 9.3).

9.8 Urogenital System

The urinary system in rat comprises a pair of kidneys, ureters, urinary bladder, and urethra (Fig. 9.15). The principal function of the urinary system is to excrete out the waste products of metabolism and for control of body fluids and electrolyte balance with some ancillary functions such as the production of certain hormones.

The kidney in the rat is bean-shaped. The kidneys of the rodents are unipapillate with the right kidney slightly cranial as well as heavier as compared to the left. In humans, the kidney is multipapillate which means that each papilla is surrounded by a funnel-shaped "calyx," whereas in rodents, there is a single papilla also called directly as the pelvis corresponding to the unipapillate kidney [30].

The kidneys also accommodate triangular-shaped adrenal glands adjacent to the superior pole of the kidney and weigh around 40–60 mg in rat [31]. The adrenals comprise the medulla which is the site for conversion of amino acid tyrosine into

Table 9.3 Hematological and biochemical parameters of rats

Parameter	Strain and sex			
	Sprague-Dawley rats		Wistar rats	
	Male	Female	Male	Female
Hemoglobin, g/L	135–159	129–154	91–103	88–101
Hematocrit, %	42–49	40–46	42–48	
RBC, 10^{12}/L	6.39–8.01		8.20–9.50	7.80–9.00
MCV, fL	58.01–67.00	55.21–64.80	48–54	48–57
MCH, pg	18.70–21.20		16.10–19.30	17.70–19.30
MCHC, g/L	310–336	318–347	340–361	324–359
Platelet, 10^9/L	923–1580		573–998	591–836
MPV, fL	6.70–8.10		6.70–8.00	7.00–7.80
WBC, 10^9/L	3.00–9.22	2.58–7.34	3.40–9.50	2.20–5.90
Neutrophils, 10^9/L	0.28–1.43	0.19–0.91	0.40–1.50	0.30–1.00
Lymphocytes, 10^9/L	2.45–7.66	2.09–6.39	2.60–7.80	1.70–4.80
Monocytes, 10^9/L	0.17–0.76	0.08–0.43	–	0.10–0.40
Eosinophils, 10^9/L	0.03–0.21		0–0.20	0–0.10
Basophils, 10^9/L	0–0.02 (combined)		0–0.04 (combined)	
ALT, U/L	19–47	14–30	24–4	23–67
Albumin, g/L	26.85–34.55		44.40–58.40	
AST, U/L	59–139		50–96	61–153
Creatinine, μmol/L	32.36–47.990	34.91–59.67	31–48	37–53
Glucose, mmol/L	4.46–7.24	5.31–8.01	5.10–9.20	5.70–8.40
Total protein, g/L	51.10–64.55		40–60	40–80
Total cholesterol, mmol/L	0.68–1.77	0.81–2.03	1.10–2.00	0.70–2.50
Triglycerides, mmol/L	0.23–0.99	0.16–0.89	0.40–2.10	0.40–3.40
Urea, mmol/L	4.32–8.97	5.56–12.67	4.00–9.30	6.80–11.30

Source: Adopted from He Q et al. (2017) [29]

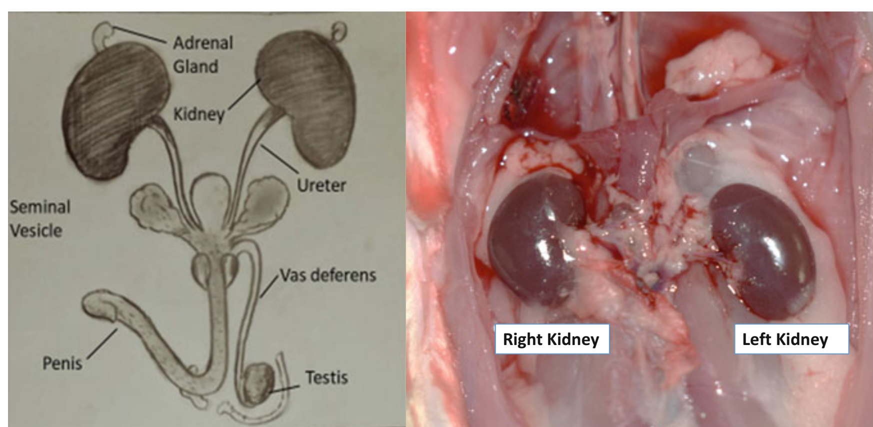


Fig. 9.15 Rat male urogenital system and rat kidney

catecholamines that are released into the bloodstream in response to stressors. The cortex forms the outer part of adrenals which secretes mineralocorticoids, particularly the aldosterone which regulates sodium reabsorption and excretion of potassium by the kidneys.

The adrenal is the most common toxicological target organ in the endocrine system in vivo as it secretes hormones that control many body processes like metabolism and response to stressful circumstances.

In the female rat, the external urinary opening is easily visualized and rests anteriorly to the vaginal opening. The urinary bladder in rats is located in the dorsocaudal area of the abdominopelvic cavity and is a hollow, muscular, distensible organ that serves as a reservoir release of urine. There are approximately 30,000 nephrons in an adult rat kidney with little variation by strain and age and a progressive loss over time with age.

9.9 Reproductive System

9.9.1 Male Reproductive System

The male reproductive system in rats consists of paired testes and extra-testicular ducts, which include the efferent ducts, epididymis, and vas deferens along with the accessory sex glands, consisting of the prostate, seminal vesicles, bulbourethral glands, ampullary glands, and preputial glands alongside the urethra and penis (Fig. 9.15). The ampullary glands and preputial glands are unique to rodents and not present in humans.

The paired testes produce male gametes called spermatozoa. The stromal cells synthesize and secrete hormones such as testosterone. The testes communicate with the abdominal cavity through inguinal canals which remain open throughout life in rats. The testes of the male rat descend by 30th to 40th day of life and lie in two separate thin-walled scrotal sacs that freely communicate with the abdominal cavity. The testis is withdrawn into the abdominal cavity when the rat is sexually inactive. In the human, the inguinal canals are normally closed after the descent of the testes during development. The seminiferous tubules are the main site for the germination, maturation, and transportation of the sperm cells through tubuli recti leading to rete testis and further to the head of the epididymis. Each testis is surrounded by the epididymis which consists of the caput epididymis (along the anterior end of the testis) leading to corpus epididymis, followed by cauda epididymis from which the ductus deferens passes through the inguinal canal to join the urethra.

9.9.2 Female Reproductive System

The female reproductive tract in rat is composed of the ovaries, oviducts, uterus, cervix, and vagina. The paired ovaries of the rat are found in grapelike bunches, and gross appearance may vary depending on the stage of the estrous cycle. The ovary is

covered by modified peritoneal mesothelium known as surface epithelium which is continuous with the broad ligament (mesovarium) that supports the ovary. In sexually mature female rats, the cortex consists of numerous follicles at different stages of development. The estrous cycle in rats lasts 4 days and consists of four stages, namely, proestrus, estrus, metestrus, and diestrus. Rats are continuously polyestrous. Staging of the estrous cycle can be done by making use of vaginal smears also known as vaginal cytology. During ovulation, oviducts attached to the ovaries receive the mature eggs, and fertilization usually occurs in the upper third of the oviduct. Implantation occurs in one of the uterine horns. Rats have a single cervix with two uterine horns. As in humans, the placentation in rats is of the hemochorial type where maternal blood comes in direct contact with the fetal chorion which is the outermost layer of the fetal membrane. In rodents, the walls of the cervix and the vagina are continuous both dorsally and ventrally but not laterally. In the rat, the vagina is formed from the union of the uterine horns. The external opening of the vagina is the vulva; this opening is separate from the urethral orifice through which urine is excreted.

9.10 Nervous System

The nervous system coordinates as well as transmits signals between different body areas. The central nervous system consists of the brain and spinal cord which controls the rest of the body via the peripheral somatic (sensorimotor or voluntary) and autonomic (involuntary) nervous systems. Rat models are extensively used in neuroscience research. However, the rat and human are quite different in terms of size and organization (Fig. 9.16). The brain is covered by meninges, the outermost of which is the dura mater and the innermost is the pia mater. In between these two layers lies the arachnoid layer. The brain is divided into two large cerebral hemispheres via a median fissure. The cortex which is the outer surface of the

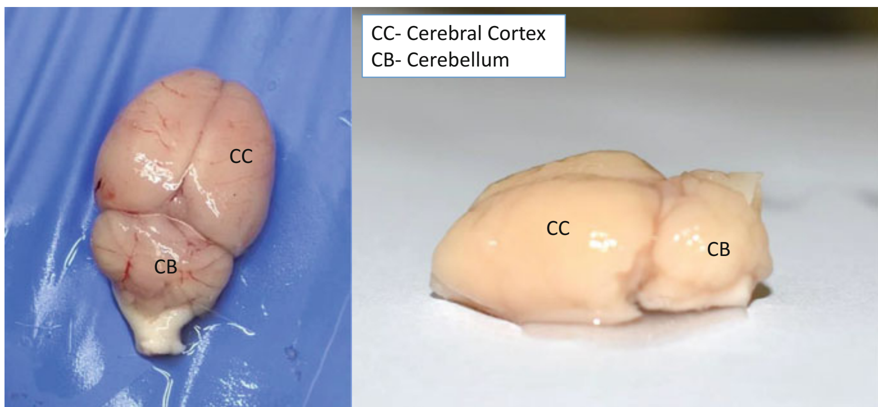


Fig. 9.16 Rat brain

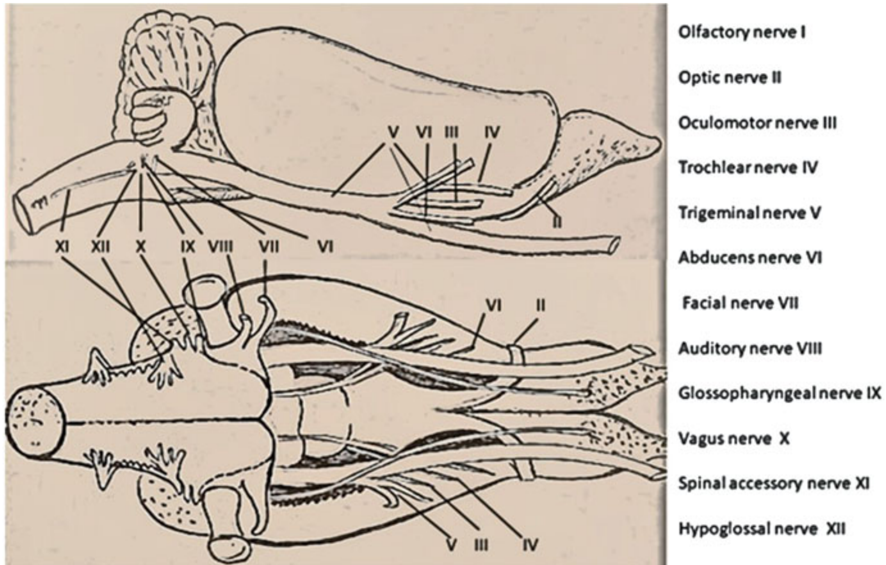


Fig. 9.17 Schematic diagram of cranial nerves in rats

brain is smooth in rats, whereas it is wrinkled up in humans. Thus, the rat brain is lissencephalic which means that it does not have sulci or gyri. The cortex is the largest structure in the brain and is highly specialized and very well developed in humans as compared to rodents. It is responsible for functions such as cognition and language.

Relative to brain size, rats have a larger olfactory bulb in comparison to humans [32]. That is because rats have a very well-developed sense of smell that is used in their interaction with the outer world.

The cerebellum which is located toward the back of the brain is the large lobed structure located behind the cortex and is fairly well developed. The cerebellum is important for motor coordination. The most caudal part of the rat brain is the medulla oblongata which is further connected to the spinal cord. The peripheral nervous system consists of 34 pairs of spinal nerves differentiated into 8 cervical, 13 thoracic, 6 lumbar, 4 sacral, and 3 caudal. There are 12 pairs of cranial nerves which originate from the brain (Fig. 9.17).

9.11 Musculoskeletal System

The musculoskeletal system in rats consists of bones, cartilage, skeletal muscles, and connective tissues. The musculoskeletal system in the rat is immature at birth and develops rapidly during the first 3 weeks.

Rats have 223 bones in total which is 17 more than that in humans. The axial skeleton consists of the skull, mandible, hyoid, ribs, sternum, and vertebrae, whereas

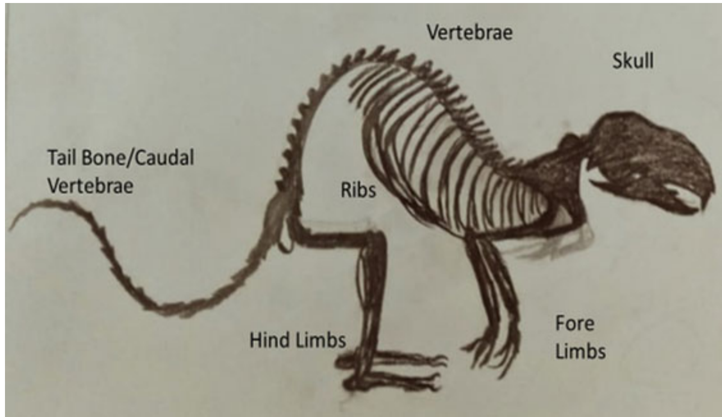


Fig. 9.18 Skeletal system of rat

the appendicular skeleton consists of the limb and feet bones (Fig. 9.18). The vertebral column consists of seven cervical, 13 thoracic, six lumbar, four sacral, and 27–30 caudal vertebra (C_7, T_{13}, L_6, S_4, $Ca_{27–30}$).

9.12 Major Endocrine Glands

The endocrine glands release a chemical messenger/hormone directly into the circulatory system.

9.12.1 Thymus

The thymus gland is a primary lymphoid organ and is attached to the trachea, located in the pericardial mediastinum, anterior to the major vessels of the heart with part of it extending into the cervical region in the rat [33]. The thymus is divided into two distinct lobes which are connected through a connective tissue termed as an isthmus.

The thymus is a primary lymphoid organ responsible for maturation and the production of immunocompetent T cells. The relative weight of the thymus is largest about a week post birth; however, the absolute weight reaches its peak at about the age of 2 months after which it declines with age to a level where it is barely visible in very old animals [34].

Athymic nude rats are a genetic mutant characterized by a lack of normal thymus and functionally mature T cells.

9.12.2 Pineal Gland

The pineal gland is also known as the "pineal body," which is a small endocrine gland (about 0.5 mm of diameter) situated between the cerebral hemispheres and the cerebellum in rats [35]. The pineal gland was considered as a mystical organ for ages, but its role in biological rhythmicity is now well known. Melatonin is produced by the pineal gland and plays an important role in the regulation of seasonal, photoperiodic-dependent reproduction by influencing the pituitary gland's secretion of the sex hormones such as FSH and LH. The production of this hormone is light-dependent which regulates the "biological clock" after converting the neurological activity generated by light in the environment into hormonal information. Thus, the primary function of this hormone is to regulate sleep patterns.

9.12.3 Thyroid

The thyroid gland is first evident at around day 12 of gestation and thus the first of the endocrine tissue to develop in the rat [36]. The thyroid gland in rat consists of a right and left lobe connected by a thin connective tissue known as isthmus. The lobes are placed on the left and right side of the cranial part of the trachea and larynx [37].

The thyroid gland secretes hormones known as triiodothyronine (T_3) and thyroxine (T_4) and a peptide hormone, calcitonin. The T_3 and T_4 hormones play an important role in regulating development, differentiation, and metabolism in all the mammals. Calcitonin is involved in calcium homeostasis.

9.12.4 Hypothalamus and Pituitary Gland

The pituitary gland in rat lies in the midline at the base of the brain on the sella turcica. The hypothalamus is located above the pituitary gland and connected to it by a stalk known as the infundibulum. The pituitary gland in the rat is further divided into the a) pars distalis, b) pars intermedia, and c) pars nervosa [38]. The hypothalamus controls the pituitary which, in turn, helps in regulating the function of other endocrine glands.

References

1. Kalueff A, Stewart A, Song C et al (2015) Neurobiology of rodent self-grooming and its value for translational neuroscience. Nat Rev Neurosci 17:45–59. https://doi.org/10.1038/nrn.2015.8
2. McCormick D (2017) Preclinical evaluation of carcinogenicity using standard-bred and genetically engineered rodent models. A comprehensive guide to toxicology in nonclinical drug. Development:273–292. https://doi.org/10.1016/b978-0-12-803620-4.00012-8
3. Hubrecht RC, Kirkwood J (eds) (2010) The UFAW handbook on the care and management of laboratory and other research animals. John Wiley & Sons
4. Sengupta P (2013) The laboratory rat: relating its age with human's. Int J Prev Med 4(6):624

5. Lashley KS (1932) The mechanism of vision. V. the structure and image-forming power of the rat's eye. J Comp Psychol 13(2):173
6. Artal P, de Tejada PH, Tedó CM, Green DG (1998) Retinal image quality in the rodent eye. Vis Neurosci 15(4):597–605
7. Jacobs G (2009) Evolution of colour vision in mammals. Philos Trans R Soc B: Biol Sci 364:2957–2967. https://doi.org/10.1098/rstb.2009.0039
8. Fay RR, Wilber LA (1989) Hearing in vertebrates: a psychophysics databook. J Acoust Soc America 86(5):2044–2044. https://doi.org/10.1016/b978-0-12-803620-4.00012-8
9. Takahashi N, Kashino M, Hironaka N (2010) Structure of rat ultrasonic vocalizations and its relevance to behavior. PLoS One 5:e14115. https://doi.org/10.1371/journal.pone.0014115
10. Welker W (1964) Analysis of sniffing of the albino rat 1. Behaviour 22:223–244. https://doi.org/10.1163/156853964x00030
11. Rice FL, Mance A, Munger BL (1986) A comparative light microscopic analysis of the sensory innervation of the mystacial pad. I. Innervation of vibrissal follicle-sinus complexes. J Comp Neurol 252(2):154–174
12. Yoshioka I, Muto H (1976) Surface structures of the tongue, palate and buccal mucosa of the rat. Okajimas Folia Anat Jpn 52(6):297–311
13. Wilson DE, Reeder DM (eds) (2005) Mammal species of the world: a taxonomic and geographic reference, vol 1. JHU Press
14. Matsukura N, Shirota A, Asano G (1985) Anatomy, histology, ultrastructure, stomach, rat. In: Digestive System. Springer, Berlin, Heidelberg, pp 281–288
15. Tahvonen R, Salminen S (2004) Functional foods for gut health: an overview. In: Functional foods, ageing and degenerative disease. Woodhead Publishing, pp 295–324
16. Di Sabatino A, Miceli E, Dhaliwal W, Biancheri P, Salerno R, Cantoro L et al (2008) Distribution, proliferation, and function of Paneth cells in uncomplicated and complicated adult celiac disease. Am J Clin Pathol 130(1):34–42
17. Shroyer NF, Kocoshis SA (2011) Anatomy and physiology of the small and large intestines. In: Pediatric gastrointestinal and liver disease. WB Saunders, pp 324–336
18. Gribble FM, Reimann F (2017) Signalling in the gut endocrine axis. Physiol Behav 176:183–188
19. Vdoviaková K, Petrovová E, Krešáková L, Maloveská M, Teleky J, Jenčová J, Jenča A Jr (2016) Importance rat liver morphology and vasculature in surgical research. Med Sci Monit 22:4716
20. Hebel R, Stromberg MW (1986) Anatomy and embryology of the laboratory rat. BioMedVerlag
21. Hofstetter J, Suckow MA, Hickman DL (2006) Morphophysiology. In: The laboratory rat. Academic Press, pp 93–125
22. Nakakuki S (1983) The bronchial tree and blood vessels of the rat lung. Anat Anz 154 (4):305–312
23. Burri PH (1974) The postnatal growth of the rat lung III. Morphology. Anat Rec 180(1):77–98
24. Irvin CG, Bates JH (2003) Measuring the lung function in the mouse: the challenge of size. Respir Res 4:1. https://doi.org/10.1186/rr199
25. Oakes JM, Scadeng M, Breen EC, Marsden AL, Darquenne C (2012) Rat airway morphometry measured from in situ MRI-based geometric models. J Appl Physiol (1985) 112 (11):1921–1931. https://doi.org/10.1152/japplphysiol.00018.2012
26. Krešáková L, Purzyc H, Schusterová I, Fulton B, Maloveská M, Vdoviaková K, Boldižár M (2015) Variability in the cardiac venous system of Wistar rats. J Am Assoc Lab Anim Sci 54 (1):10–16
27. Springall DR, Bhatnagar M, Wharton J, Hamid Q, Gulbenkian S, Hedges M, Polak JM (1988) Expression of the atrial natriuretic peptide gene in the cardiac muscle of rat extrapulmonary and intrapulmonary veins. Thorax 43(1):44–52
28. Heatley JJ (2009) Cardiovascular anatomy, physiology, and disease of rodents and small exotic mammals. Vet Clin North Am Exot Anim Pract 12(1):99–113

29. He Q, Su G, Liu K, Zhang F, Jiang Y, Gao J, Xie H (2017) Sex-specific reference intervals of hematologic and biochemical analytes in Sprague-Dawley rats using the nonparametric rank percentile method. PLoS One 12(12):e0189837
30. Schmidt-Nielsen B (1987) The renal pelvis. Kidney Int 31(2):621–628
31. Mitani F (2014) Functional zonation of the rat adrenal cortex: the development and maintenance. Proc Jpn Acad, Series B 90(5):163–183
32. Soh Z, Saito M, Kurita Y, Takiguchi N, Ohtake H, Tsuji T (2014) A comparison between the human sense of smell and neural activity in the olfactory bulb of rats. Chem Senses 39 (2):91–105
33. Haley PJ (2003) Species differences in the structure and function of the immune system. Toxicology 188(1):49–71
34. Kuper CF, Beems RB, Hollanders VM (1990) Development and aging, thymus, rat. In: Hemopoietic system. Springer, Berlin, Heidelberg, pp 257–263
35. Maganhin CC, Simões RS, Fuchs LFP, Oliveira-Filho RM, Simões M d J, EvêncioNeto J, Baracat EC, Júnior S, Maria J (2009) Rat pinealectomy: a modified direct visual approach. Acta Cir Bras 24(4):321–324. https://doi.org/10.1590/S0102-86502009000400013
36. Boorman GA, Suttie AW, Eustis SL, Elwell MR, MacKenzie WF, Leininger JR, Bradley AE (eds) (2017) Boorman's pathology of the rat: reference and atlas. Academic Press
37. Hadie S, Abdul Manan H, Abdulla S (2013) Thyroid gland resection in euthanized rat. A practical guide. Int Med J 20(1):1–4
38. Osamura RY (1983) Pituitary gland, rat. In: Endocrine system. Springer, Berlin, Heidelberg, pp 121–129

The Anatomy, Physiology, and Husbandry of Laboratory Rabbit

10

Sarita Jena and Saurabh Chawla

Abstract

The laboratory rabbit has a special place in different scientific studies due to some of its unique anatomical and physiological features. The contributions of rabbits toward fundamental knowledge of monoclonal and polyclonal antibody production and testing for therapeutic humanized antibodies are remarkable. There is a need for understanding the biology of laboratory rabbits to address specific scientific questions of biomedical research. This chapter has covered the details of the anatomy and physiology of rabbits along with species-specific requirements and welfare aspects of housing and husbandry measures, aided with key references, to provide comprehensive knowledge to the readers.

Keywords

Laboratory rabbit · Antibody · Humanized · Anatomy · Physiology · Husbandry · Welfare

Abbreviation

CPCSEA	Committee for the Purpose of Control and Supervision of Experiments on Animals
CSM	Colonic separation mechanism
GALT	Gut-associated lymphoid tissue

S. Jena (✉)
Institute of Life Sciences, Bhubaneswar, India

S. Chawla
National Institute of Science Education and Research, Bhubaneswar, India
e-mail: saurabhchawla@niser.ac.in

© The Author(s), under exclusive license to Springer Nature Singapore Pte Ltd. 2021

P. Nagarajan et al. (eds.), *Essentials of Laboratory Animal Science: Principles and Practices*, https://doi.org/10.1007/978-981-16-0987-9_10

HCl	Hydrochloric acid
HDL	High-density lipoprotein
LDL	Low-density lipoprotein
NZW	New Zealand White
SA	Sinoatrial
VFA	Volatile fatty acid
WHHL	Watanabe heritable hyperlipidemic

10.1 Introduction

The laboratory rabbit has descended from the European wild rabbit [1]. Gradually, it was distributed worldwide and has had a place as a laboratory animal in biomedical studies and experiments. Rabbits are mostly bred and maintained commercially for meat, skin, and fur. They are also used as pets and game animals. Besides these uses, it is also used extensively in biomedical research areas including bio-implant studies, cardiovascular diseases, aging, reproductive physiology, infectious diseases, teratological studies, ophthalmological studies, product safety testing (pyrogen testing, skin and vaginal irritation test, etc.), and the production of polyclonal antibodies. The rabbit was the first-ever laboratory animal used for immunological studies like the development of the rabies vaccine and the study on syphilis [2, 3]. Comparatively, larger body size, more total blood volume and longer life span concerning rodents, similar immune system to human [2], and intermediate size between rodents and larger animals are some of the reasons for the researchers to choose rabbit over other experimental animals. There are over 200 breeds worldwide, but the most commonly used laboratory rabbits are New Zealand White (NZW) and Dutch (Dutch Belted). There are a few numbers of inbred rabbit strains and several lines with unique genetic traits specific for certain disease conditions like Watanabe heritable hyperlipidemic (WHHL) used for cardiovascular research.

The taxonomy of the laboratory rabbit is as follows.

10.1.1 Taxonomy

Class: Mammalia
Order: Lagomorpha
Family: Leporidae
Subfamily: Leporinae
Genus: *Oryctolagus*
Species: *O. cuniculus*

10.1.2 Rabbit as a Model for Human Diseases

Although small rodents, such as mice, rats, hamsters, and guinea pigs, are used for various studies of human disease, the use of rabbits has been extensively increased during the last two decades. The rabbit was the first animal model used for immunological studies and was instrumental in the development of the rabies vaccine by Louis Pasteur in 1881 [4]. The rabbit mucosal IgA has 13 C alpha genes, whereas it is one in mice and two in hominoids [5]. Studies of the IgA may reveal many therapeutic opportunities. Rabbits serve as an important model to translate or to extrapolate the research findings to humans for many infectious and noninfectious diseases and health conditions. They are used for the study of noninfectious conditions like cardiovascular diseases, hypercholesterolemia, atherosclerosis, intestinal immunity, cardiovascular surgery, orthopedic surgery, arthritis, human articular lesion, therapeutics, and tumor study [6]. Rabbits act as a reliable model for the development of vaccines and therapeutics and for understanding the cellular and molecular mechanism of many diseases of human importance, such as syphilis, tuberculosis, herpes simplex virus, human immunodeficiency virus, and human papillomavirus [4].

10.2 Anatomy and Physiology

The rabbits are chosen as models of many biomedical research areas due to their unique anatomical and physiological characteristics which sometimes prove to be similar or dissimilar to that of humans and other rodents. For instance, the fasting metabolic rate of rabbits is higher compared to other animals of the same size [7]. The rectal temperature of a healthy adult rabbit is 38–40 °C, and it lacks the sweat gland. Rabbits can adapt to varied environmental temperature but are usually susceptible to high temperature and prefer a cold environment. In rabbits, the ear pinnae represent approximately 12% [7] of the total body surface area and act as important heat-sensing organs [8]. They play a key role in thermoregulation, providing a means to help cool the body. They have a higher level of peripheral circulatory basophils [9] compared to other animals. In rabbits, the thymus is retained in adults [10], and cortisol is the major plasma glucocorticoids [11]. The fasting metabolic rate of an adult healthy rabbit is 44–55 kcal/kg/day [12]. The water consumption rate of rabbits varies from 50 to 150 mL/kg of body weight/day, and if the water is withheld, food consumption decreases [13]. However, they have a remarkable ability to withstand significant levels of dehydration, up to 48% of their body weight [7]. However, pregnant and lactating females require more water [14]. The mature female rabbit is called a doe, a mature male rabbit is called a buck, and the young ones are called as bunnies or kits.

10.2.1 External Features and Musculoskeletal System

The rabbit's body is covered with dense fur. They possess four toes on their hind feet and five toes on their front feet, whereas mice and rat have five toes on hind feet and four toes on the front. In rabbits, shedding of the fur occurs three to four times a year, and the regrowth cycle of hair starts from the ventral side and grows dorsally and posteriorly. But the regrowth of hair after clipping of fur during some experimental procedure occurs in an irregular patchy manner. Mature female rabbits often have a prominent fold of the skin called dewlap beneath their chin. Mature male rabbits normally have larger heads than females. In rabbits, the ear pinnae are comparatively larger and highly vascular (Fig. 10.1) with clearly visible blood vessels, and for this reason, they make ideal sites for blood vascular procedures. Rabbits have three specialized scent glands: inguinal scent glands in the groin region, anal scent glands around the anus, and mental scent gland under the chin. They use the secretions from the scent glands for demarking their hierarchical territory. The territory is marked by placing the feces (act as a vehicle for anal gland secretions) or by pressing the under-chin against inanimate objects in the environment and smearing with mental gland secretions. The dominant animal has a larger anal gland in a group. The males usually spray their urine on females and young ones of their group for marking. Females also mark their offspring and reject or attack other strange young animals of the same age group. Females even abandon their young ones if they are marked or smeared with foreign urine.

The dental formula of the rabbit is I 2/1, C 0/0, PM 3/2, M 2–3/3. A small pair of incisors called "peg" teeth is present along with the primary maxillary incisors. These teeth are located behind the primary incisors and used along with them for biting and shearing the food. The teeth of rabbits continue to grow throughout life and hence require sufficient wear and tear to maintain the normal length of the teeth; otherwise, there will be malocclusion and malalignment of the teeth. That will hamper the normal feeding of rabbits. The molar tooth lacks root and has deep enamel folds. Rabbits use both front-back and lateral-medial side movements of jawbones like a human to masticate the food.

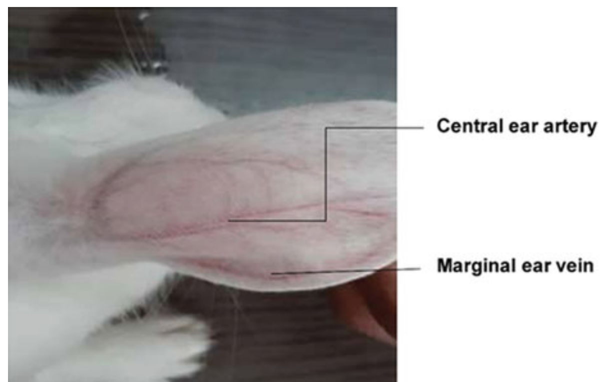


Fig. 10.1 Ear of rabbit (Source: Picture courtesy of Dr. P.K. Sahoo, RMRC, Bhubaneswar)

In rabbits, the skeleton is very light weighted and brittle which contributes only 7–8% to total body weight [7]. But the bones have a higher content of calcium, whereas the muscle mass accounts for half of the body weight. The skeletal system consists of the axial skeleton and appendicular skeleton [14].

The axial skeleton of the rabbit is composed of the spongy textured skull, curved vertebral column, flattened ribs, and sternum. The vertebral formula is C7T12L7S4Cy16. However, the number of vertebrae in each section can vary [15]. The lumbar vertebrae have prominent mammillary processes, where the powerful lumbar muscles (9% of body mass) [14] are attached. The appendicular skeleton comprises the pectoral and pelvic girdle and the limbs. The forelimbs are short, but the hind limbs are long and comprise powerful pelvic limb muscles (13% of body mass) [14, 16]. The ilium, the ischium, and os acetabuli (a small accessory bone) form the acetabulum of the rabbit. Unlike other animals, the pubis is not involved in acetabulum formation. The trochanteric fossa of the femur is deep and well defined in rabbits which is sometimes misinterpreted as a bone lesion in magnetic resonance images [17]. The fossa can easily be located by palpating the prominent greater trochanter and is a good site for intraosseous catheterization [18]. The rabbits must be handled very carefully and securely; otherwise, it may kick with powerful hind limb and lumbar muscles that may result in lumbar vertebral fracture and complete paraplegia. The plantar surface of the tarsus of the hind limb remains in contact with the ground at rest, but it gets digitigrade while running.

10.2.2 Special Sense Organs

10.2.2.1 Eye

The rabbits have large laterally located eyes with a broad field of vision. They have an approximately 300-degree lateral vision field and a 180-degree dorsal vision field [19]. The dorsally placed optic nerve head and dorsoventrally ovoid pupil help in enhancing the range of dorsal vision. A blind spot is there just cranial to the nose. Therefore, rabbits cannot see objects at this place and only can sense by sensitive cleft upper lip and vibrissae. Rabbits have peripheral hypermetropic (long-sighted) and axial myopic (short-sighted) eyesight [19]. The retrobulbar venous plexus is closely placed at the caudal globe. As a stress response, the venous plexus gets engorged leading to exophthalmos and increased axial myopia and peripheral vision. In rabbits, the vision is not exclusively binocular.

Rabbits have merangiotic vascular pattern (retinal blood vessel confined to a horizontal streak), whereas it is holangiotic type (retinal blood vessel distributed throughout) in rats, mice, carnivores, and primates [20]. The rabbit retina is dominantly occupied by rods (95%), and the cones are less in number. Rabbits have dichromatic vision due to the presence of blue cones and green cones and lack of red cones. Rabbits lack tapetum lucidum [14]. A large lacrimal bilobed gland is situated beside the lower eyelid rim. The third eyelid or nictitating membrane is well developed at the medial canthus of rabbits. Superficial nictitans gland is loosely attached behind the nictitating membrane, and the deep Harderian gland is relatively

Table 10.1 Characteristics of the eye of the rabbit

Parameters	Normal values
Inter-blink interval	10 min
Corneal thickness	300–450 μm
Axial globe length	17.12 mm (±0.14 mm)
Globe height	17–18 mm
Globe width	18–20 mm
Anterior chamber depth	2.70 mm (±0.22 mm)
Aqueous volume	0.25–0.3 mL
Pupil diameter	5–11 mm
Axial vitreal chamber length	7.32 mm (±0.45 mm)
Corneal radius (horizontal)	6.5–7 mm
Corneal radius (vertical)	7.5 mm
Corneal radius of curvature	7–7.5 mm

Source: Adopted from Knott (2014) [19]

large, bilobed, and located ventromedial to the globe. The nictitans gland secretes aqueous tear, and the Harderian gland secretion is rich in lipid and porphyrin which is not seen in primates and dogs. Rabbits can resist blinking for a longer period because of stable tear film contributed by the lipid constituent of Harderian gland secretion. The rabbit eye can ideally be used for intracorneal implants as one of the studies found that the tumor does not generate blood vessels for its survival; rather, the body of the rabbit feeds it [21]. The characteristics of the rabbit eye are summarized in Table 10.1.

10.2.2.2 Nose

The rabbits use their nose and olfaction for most of their communications, locating food, attracting mates, demarking territory, and perceiving danger. A remarkable sense of smell is seen in newborn bunnies, by which they can locate the mammary pheromone present in mother's milk [22, 23]. Rabbits wiggle their nose often, to inspire the air as well as to identify the odorants or pheromones in the air [24].

The nasal cavity of a rabbit is divided into two symmetrical parts by the nasal septum. Each part comprises four regions: nasal vestibule, maxilla turbinate region, nasomaxillary region, and ethmoturbinate region [25]. The maxilla turbinate is the branching type, and ethmoturbinate is scroll type in rabbits. The complex structure allows 1.1% of the inhaled air to enter the region for olfaction. Due to the high sensitivity of the olfactory nerves, such a small fraction is sufficient enough for olfaction [26].

10.2.2.3 Ear

The pinnae of rabbits constitute approximately 12% of the total body surface which is highly vascular and act as a thermoregulatory organ. In large-eared rabbits, the auricular blood vessels are easily accessible for venepuncture and act as good sites for noninvasive measurements of arterial blood pressure and blood oxygen saturation. The ears are very sensitive and should not be used to lift or restrain the rabbit.

Rabbit's ear has three parts: external, middle, and inner ear. Unlike other animals, the external acoustic meatus or external ear canal is vertically oriented. In the rabbit's ear canal, there exists a natural diverticulum by a cartilaginous structure called the tragus. Lop-eared breeds (where the ears hang down) have deformed ear canals at the point where the ear canal and tragus meet, and that may predispose to otitis externa. The tympanic membrane or eardrum lies in between the external and middle ear. Within the tympanic bulla of the middle ear, the rabbit lacks any bony ridge or septum, as found in the dog and cat, respectively. The sound is passed from the eardrum to the inner ear through the middle ear. In the inner ear, the organ of Corti is responsible for hearing, and the vestibular apparatus is responsible for maintaining a balance of the body.

10.2.2.4 Skin

The rabbit skin is relatively delicate as compared to dogs and cats. Molting (shedding of hair) is seen in adult rabbits twice a year (usually during spring and fall). Rabbits do not have sweat glands. Rabbits do not have footpads; rather, the coarse fur and the guard hairs cover the toes and plantar surface of the paw. Tactile vibrissae present on the snout region help in food identification, perceiving space information, and navigation. The rabbits have hairless skin at the nose tip, scrotal sacs, and inguinal folds.

10.2.3 Digestive System

Rabbits are coprophagous and hindgut fermenter, and they have a comparatively larger stomach and cecum than that of any monogastric mammal [14]. The gut transit time of ingesta is fast, and the fibers get eliminated from the gut quickly in form of cecotrophs to be utilized again by the rabbit unlike other hindgut fermenters such as horses and ruminants [16].

There are four pairs of salivary glands that exist in rabbits, which include parotid, sublingual, submaxillary, and zygomatic. Humans and other rodents have all three pairs of salivary glands except the zygomatic gland. The parotid gland is the largest among the above four [7], and it is located ventrolateral to the base of the ear. The important constituents of the saliva of the rabbit are potassium and bicarbonate ions [27] along with amylase and galactosidase enzymes that are produced and secreted from the mandibular salivary gland.

The esophagus of the rabbit is devoid of mucous glands and has three layers of striated muscle layer which extends from the pharynx to the cardia of the stomach. Rabbit has a thin-walled stomach which is present toward the left side of the midline. The stomach is divided into three parts, i.e., cardia, fundus, and pylorus. The stomach of the healthy rabbit never remains empty, and it can hold approximately up to 15% of the total volume of the gastrointestinal tract [7]. The cardiac sphincter is well developed in rabbits that separate the esophagus from the stomach and prevent vomiting. The cardiac part is nonglandular and plays no intrinsic role in the churning of food; however, due to large intestinal movement and locomotor movement, some

indirect churning may occur. The rabbits chew their food up to 120 times/min by their jaw movement [12] which is also important for churning of food. The fundus part of the stomach comprises hydrochloric acid-secreting parietal cells and pepsinogen-secreting chief cells. Pylorus has a well-developed muscular sphincter. The stomach never remains empty even if the rabbit remains on 24-h fast, and the contents of the stomach include food, fur, and fluid. The trichobezoars (hair balls) result due to the ingestion of fur (during grooming) and decreased gastric movement [27]. The stomach pH of rabbits is 5.0–6.5 at the pre-weaning period. The digestive enzymes in the suckling bunnies act upon the doe's milk leading to the production of octanoic and decanoic fatty acids inside the stomach content, which is called "stomach oil" or "milk oil." Thus, during the pre-weaning time, the bacterial load is kept at check due to the effect of stomach oil [27]. The bunnies are solely dependent upon doe's milk up to 10 days of age. At about 2 weeks of age, the young rabbits start eating doe's cecotrophs [14] and from that acquire gut flora, and the gut flora colonization occurs at the age of weaning [28]. By that time, the cecotrophy fully develops, and the milk intake reduces, so as the production of milk oil. The adult rabbit's stomach pH is maintained at 1–2 which acts as a barrier against microbiological colonization of the stomach. The gastric transit time is approximately 3–6 h [16]. Hydrolysis of proteins of the ingested food begins in the stomach due to the effect of pepsin and hydrochloric acid (HCl), but due to mucin coating, cecotropes get protected from the adverse effect of acidic gastric juice. Apart from mucin coating, the lactate produced by microbes inside the cecotrophs also protects the cecotrophs by imparting a buffering effect against gastric pH and may increase the pH of the stomach environment to approximately 3. There are two glands associated with the digestive system: the liver and the pancreas.

The liver has four lobes with a gallbladder which lies toward the right side of the midline of the liver. The common bile duct drains the bile from the liver into the duodenum posterior to the pylorus. The production of bile is quite high in rabbits. It is approximately 100–150 mL of bile per kg of body weight each day which is seven times higher than that of dogs [16, 29]. The bile acids convert into deoxycholic acid which plays a role in the breakdown of fat in the feed to small fatty acids that can easily get absorbed along the distal part of the small intestine. Unlike other animals where the majority of bile pigment is bilirubin, in rabbits, 63% of the bile pigment is biliverdin because of the lower activity of biliverdin reductase enzyme, 60 times lower than rat [27]. The rabbit has a small and diffuse pancreas situated within the mesenteric fat between the space formed by the stomach and descending and ascending loop of the duodenum (Fig. 10.2). At a distance of 30–40 cm distal to the common bile duct, the pancreatic duct opens to the duodenum at the starting point of the ascending loop of the duodenum. The pancreatic enzymes include trypsin, chymotrypsin, and carboxypeptidase for protein digestion and lipases for fat digestion. Along with these enzymes, bicarbonate ions are also secreted from the pancreas which helps to increase the pH and neutralizes the acidity of chyme that enters the small intestine.

The small intestine of a rabbit which includes three parts, i.e., the duodenum, jejunum, and ileum, is relatively smaller compared to other laboratory animals and

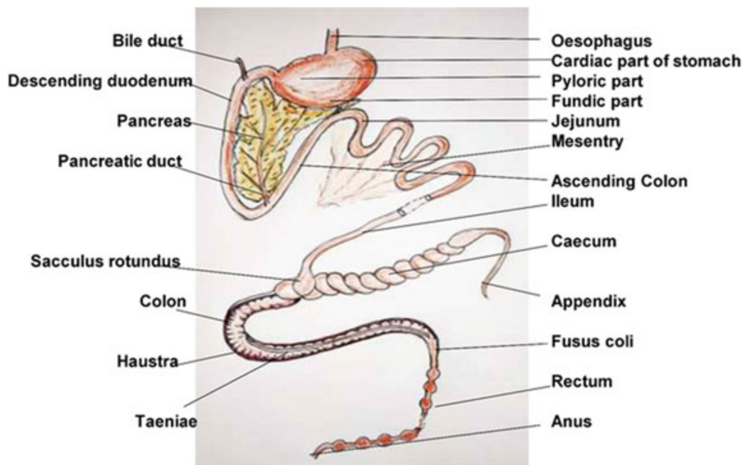


Fig. 10.2 Digestive system of rabbit

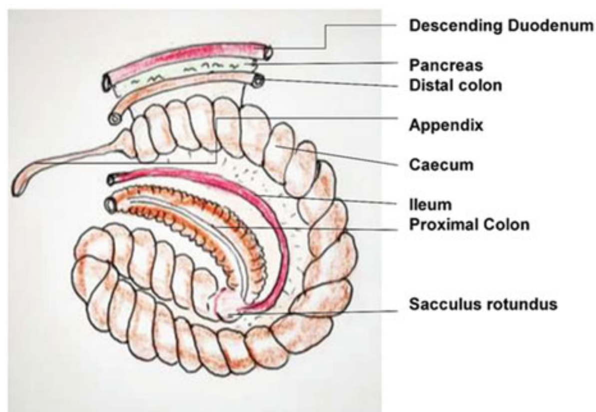


Fig. 10.3 Ileocecocolic complex and position of sacculus rotundus

constitutes 12% of the total length of the gastrointestinal tract [7]. Among the three parts, the jejunum is highly convoluted and the longest part. Both the jejunum and ileum have Peyer's patches, the gut-associated lymphoid tissue (GALT) in the lamina propria. The jejunum and ileum play a key role in electrolyte balance by reabsorbing bicarbonate ions. Transit time through the jejunum is less (10–20 min) as compared to the ileum (30–60 min), and the jejunum is the longest among the three parts of the small intestine [27]. In the small intestine, the carbohydrates and simple proteins get digested, and the end products, i.e., monosaccharides and amino acids, get absorbed across the jejunal brush border or villi. The distal end of the ileum of the rabbit has a unique structure, a spherical thick-walled ball-like structure known as the sacculus rotundus (Fig. 10.3) analogous to the bursa of Fabricius in

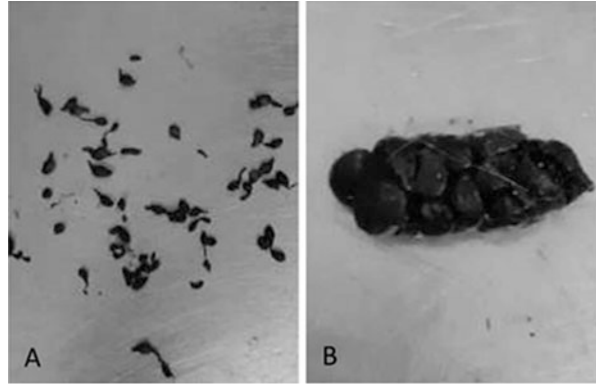


Fig. 10.4 Feces of rabbit. (**a**) Firm, dry daytime feces. (**b**) Soft, mucous-coated nighttime cecotroph (Source: Picture courtesy of Dr. P.K. Sahoo, RMRC, Bhubaneswar)

birds [30]. This is the junction between the ileum, cecum, and colon. The sacculus rotundus is also called "cecal tonsil" due to the abundance of GALT and macrophage in it, and it is thought to be compensating for the small spleen in rabbits. The ileocolic valve separates the ileum and the sacculus that allows only one-way movement of the intestinal content from the ileum to the sacculus.

The large intestine of the rabbit has three parts, namely, the cecum, colon, and rectum. In comparison to other segments of the digestive system, the cecum accounts for approximately 40% of the total tract and with a capacity of ten times the volume of the stomach [31]. The cecum is a thin-walled, self-folded organ (Figs. 10.2 and 10.3). It has long spiral internal folds and then distally ends in a thick-walled narrow 5-inch-long blind vermiform appendix. The appendix has GALT and secretes bicarbonate ions into the lumen of the cecum which helps buffer the effect of volatile fatty acids formed due to cecal fermentation. The cecum plays a very important role in the nutritional health of the animal as the high concentration of the microorganisms [32] converts the substances in the ingesta to a highly nutritive component of cecotrophs. There are two types of feces produced by rabbits: the dry and firm daytime pelleted feces (two-third of the fecal output) composed predominantly of undigested fiber (Fig. 10.4a) and the soft, moist, nutrient-rich, mucous-coated tightly packed nighttime feces called the cecotrophs (Fig. 10.4b) that the rabbit eats again to get the benefit of nutrients [33]. The colon is divided into two portions: proximal and distal colon by fusus coli. The proximal colon is approximately 35 cm in length [14] and has characteristic multiple sacculations called haustra and three longitudinal muscular bands called taenia (Fig. 10.2). The distal end of the proximal colon lacks the haustra and taenia, and there exists thickened mucosa which is the fusus coli or the pacemaker in the colon. The ganglionic aggregation in the fusus coli acts as a pacemaker to regulate peristalsis and mechanical separation of the solid and liquid content of the colon and is responsible for the excretion of two types of feces [16]. The distal colon is approximately 80–120 cm long that extends from fusus coli up to the rectum.

10.2.3.1 Cecotroph Formation and Digestion

Cecotroph digestion is an important part of the digestion process of the rabbit. The large intestine of the rabbit is the principal segment of the gut where the formation and digestion of cecotrophs take place. In the proximal colon, mechanical contractions separate fluid and nonfibrous content of ingesta from fibrous content. The fibers get eliminated as hard feces from the colon. This type of colonic separation mechanism (CSM) is known as washback CSM in rabbits which are proved to be more efficient in the utilization of nutrients [34], whereas in other rodents, it is mucus-trap CSM where two different types of feces are not formed. The antiperistaltic reflex allows the rest of the content to move to the cecum where fermentation occurs, and after 3–8 h of ingestion of soft mucous-coated feces, a bunch of grapelike cecotrophs get expelled. Here, the fusus coli acts differently, and the separation mechanism does not occur as that of during the production of dry hard feces. These cecotrophs at the anus initiate a reflex licking of the anus, and the rabbit ingests upon cecotrophs directly from the anus. The cecotrophs are not chewed but swallowed as a whole. The mucous coating prevents the stomach acid to act upon it. During their course of passage along the large intestine, the digestion of cecotroph microbial protein occurs by the action of the intestinal lysosome. The lysis of cecotroph microbes releases amylase which in turn enhances the process of digestion. The cecotrophs are a rich source of niacin, riboflavin, pantothenic acid, and cyanocobalamin. The volatile fatty acids (VFA) like acetic acid, butyric acid, and propionic acid are also produced due to cecal fermentation and get absorbed across the cecal epithelium, and it is seen that 40% of the total energy requirement is fulfilled by VFAs [14].

10.2.4 Respiratory System

The nostrils of the rabbit are very sensitive to touch due to the presence of sensory pads at the entrance of the nostrils. The nostrils remain relaxed and may twitch up to 150 twitches per minute. Normally, the breathing is characterized by twitching of nostrils at a rate of 20–120 per minute. Rabbits have turbinate bones (branching type) with a vomeronasal organ and olfactory sensory epithelium inside the nasal cavity which contribute to an acute sense of smell in them [35, 36]. The rabbits have relatively larger epiglottis dorsal to the soft palate, which allows air directly from the nasopharynx to the larynx and trachea without the involvement of the oral cavity [16]. Additionally, the rabbits have very long and narrow pharynx and a large tongue making it difficult for the endotracheal intubation process. The rabbits are obligate nasal breathers; they cannot breathe through the mouth and show acute dyspnea when there is any obstruction to nasal passages [10]. The musculature of the thoracic wall does not play an active role in respiration but the diaphragm. For this reason, artificial respiration could not be performed by compressing and relaxing the thoracic cavity and could be achieved by altering the head position in up and down direction.

Table 10.2 Comparative anatomy of the rabbit and human lung

Anatomical structure	Rabbit	Human
Respiratory bronchiole	Absent	Present
Tracheobronchial capillary bed	Five capillaries/mm	Seven capillaries/mm
Branching pattern of the main bronchus	Monopodial type	Dichotomous type
Mucous-producing cells	Goblet cells	Submucosal glands and goblet cells
Lung expansion rate	20 folds	26 folds

Source: Adopted from Kamaruzaman et al. (2013) [37]

Unlike rats and mice, the left lung of the rabbit has three lobes. The rabbit lung consists of a total of six lobes; both right and left lungs have three lobes each, namely, cranial, middle, and caudal lobes, and the right caudal lobe is further divided into lateral and medial parts. In rabbits, the digestive organs are voluminous, and the thoracic cavity is very small. Therefore, lung capacity is also very little. Typically, the lung volume increases with age in the case of rabbits, whereas in humans and dogs, it decreases with age [31]. The trachea bifurcates into right and left bronchi which further branches into the lung lobules in the monopodial pattern of branching where daughter branches are asymmetrical. In humans, it is dichotomous branching where the daughter branches are symmetrical [37] (Table 10.2). The rabbit lung lacks respiratory bronchioles, and the airways terminate in vestibules that contain alveoli. Pulmonary arteries are enveloped in a prominent smooth muscle layer [38]. There is no septal division of lobules in the lungs. For this structural constitution, pneumonia occurs as generalized and not localized [10].

10.2.5 Cardiovascular and Hematological System

Rabbits have a relatively smaller heart accounting for only 0.2% of body weight [39] due to the small thoracic cavity. The rabbit heart is situated slightly left to the midline inside the thoracic cavity and comprises four chambers: two atria and two ventricles. The two ventricular chambers are separated from each other by an interventricular septum and from the atria by valves. Similarly, two atrial chambers are separated from each other by the interatrial septum. The right atrioventricular valve possesses only two cusps, unlike other animals where three cusps exist and the left atrioventricular valve is also bicuspid (mitral valve) which also comprises two cusps. Some valves are present in the ventricles from where the pulmonary artery and aorta originate. The right side of the heart's function is important for pulmonary circulation, whereas the left side of the heart plays an important role in systemic circulation like other mammalian species.

The sinoatrial (SA) node of the rabbit is well developed and relatively long as compared to total heart size [40]. A group of pacemaker cells or P cells plays a key role in generating the impulse of the sinoatrial node in the right atrium of the rabbit.

Rabbit is the first mammal where the SA localization was well defined due to the presence of a large percentage of the P cells [41]. Primarily, the heart of the rabbit gets the supply of blood by the left coronary artery, though both left and right coronary arteries exist. The arterio-ventricular node and bundle of His are relatively small. Another anatomical difference of the cardiovascular system of the rabbits from other lab animals is that the aortic nerve does not respond to any chemoreceptor but only to baroreceptor [30]. The brain receives the blood mainly by a relatively small internal carotid artery and little supply by the vertebral arteries. The primary venous drainage from the head is the external jugular vein unlike in humans, where it is by internal jugular vein. Unlike other species, at some points, anastomosis of both internal and external jugular veins occurs in rabbits. The pulmonary artery is comparatively heavily muscled than other species [16]. If there is any coronary vasoconstriction that occurs, it will lead to cardiac ischemia as there is limited collateral coronary circulation. The Purkinje cells in rabbits are long and cylindrical without admixture of connective tissues. This characteristic feature makes it a choice of animal for Purkinje cell-related studies [16, 42]. Rabbits show low electric potential in the electrocardiogram. The heart of the rabbit is relatively resistant to oxidative damage and the blood pressure increases with an increase in body weight [12]. Rabbits develop hypercholesterolemia within a few days of feeding a cholesterol-rich diet. The rabbit and human lipoprotein profiles are similar, where both of the species exhibit a high level of low-density lipoproteins (LDL), whereas mice show more high-density lipoproteins (HDL). Both rabbits and humans show high-plasma cholesteryl ester protein. Therefore, rabbits are most widely chosen as a model for atherosclerosis research as it shares almost similar lipoprotein metabolism like that of human, over any other rodent.

Like other lab animals, rabbits also have three types of cells in the blood: the erythrocytes (red blood cells), leukocytes (white blood cells), and thrombocytes (platelets). The leucocytes include basophils, eosinophils, neutrophils, lymphocytes, and monocytes. The rabbit red blood cell diameter is around 6.7–7.9 μm [31], and the life span is about 45–68 days [43]. The rabbit's red blood cells are not as deformable as in humans [44] due to the presence of plasma fibrinogens and a higher level of oxygen free radicals [45]. The neutrophils in the rabbit are called pseudo-eosinophils or heterophils because of the presence of intracytoplasmic eosinophilic granules that cause them to resemble eosinophils. Pelger-Huet anomaly may be observed, where the heterophil nucleus is ill-segmented due to incomplete differentiation [31, 33]. True eosinophils possess larger, darker granules than heterophils. The size of the heterophils (10–15 μm in diameter) is smaller than eosinophils (12–16 μm in diameter). Basophils are more common in rabbits than in other mammals. About 2–7% of the leukocyte population is the basophils found in peripheral circulation [9]. Lymphocytes are the predominant leukocyte. The monocytes are the largest cells (15–18 μm in diameter). Similar to the mouse and rat, the rabbit is a steroid-sensitive species in which lymphopenia can be induced by exposure to stressors or exogenous glucocorticoids which cause lympholysis [46]. However, rabbits do not develop leukocytosis even with a chronic bacterial infection. It has been observed that males have higher hematocrit and hemoglobin

Table 10.3 Normal hematological and biochemical values of blood in rabbits

Blood parameters	Values
Erythrocyte ($\times 10^6$/mm^3)	5.4–7.6
Packed cell volume (%)	33–50
Hemoglobin (g/dL)	10–17.4
Leucocyte ($\times 10^3$/mm^3)	5.2–12.5
Lymphocytes (%)	30–85
Neutrophils (%)	20–75
Eosinophils (%)	1–4
Basophils (%)	1–7
Monocytes (%)	1–4
Platelets ($\times 10^3$/mm^3)	250–650

Source: Adopted from Jenkins (2008) [43]

Table 10.4 Normal biochemical values of blood in rabbits

Blood parameters	Values
For indicators of carbohydrate and lipid metabolism	
Glucose (mg/dL)	75–155
Total cholesterol (mg/dL)	10–80
Triglycerides (mg/dL)	15–160
Total lipids (mg/dL)	150–400
For indicators of liver function	
Total bilirubin (mg/dL)	0–0.8
Bile acids (mmol/L)	0–40
Total protein (g/dL)	5.4–8.3
Albumin (g/dL)	2.4–4.6
Globulin (g/dL)	1.5–2.8
For indicators of kidney function	
Blood urea nitrogen (mg/dL)	10–30
Creatinine (mg/dL)	0.5–2.5

Source: Adopted from Washington and Hoosier (2012) [47]

values than females [30]. The normal hematological values and biochemical values of blood are presented in Tables 10.3 and 10.4, respectively. However, these values may vary slightly with different strain and age of rabbits [48, 49].

10.2.6 Urogenital System

In rabbits, the kidney has a single papilla and calyx in comparison to other mammals which are multipapillate in nature. The right kidney is located more cranially than the left kidney. The adrenal gland is located at the craniomedial site of the kidney against the dorsal body wall. It regulates the stress response and thyroid activity by releasing the steroid hormones [47]. In rabbits, cortisol is the predominant steroid hormone [50], and the level of adrenal hormones is in the range of 2.6–3.8 μg/dL of cortisol and 1.54 μg/dL of corticosterone [47]. The number of glomerulus increases in rabbits

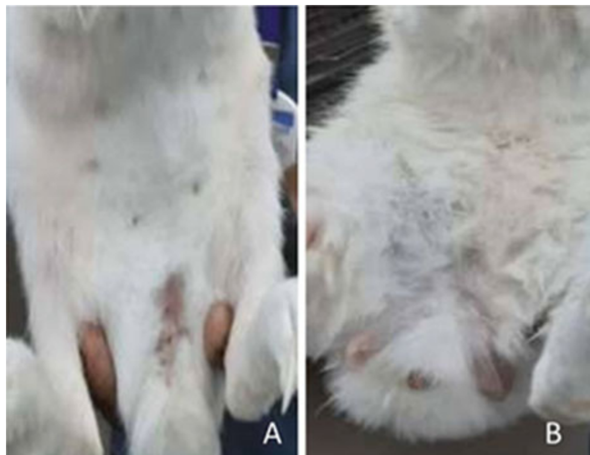


Fig. 10.5 External differentiation of male and female rabbits. (**a**) Female. (**b**) Male (Source: Picture courtesy of Dr. P.K. Sahoo, RMRC, Bhubaneswar)

after birth, whereas in humans, all the glomeruli are developed at birth. Rabbits show the presence of ectopic glomeruli in the medulla [51] and peripelvic region very frequently. The rabbit has a unique feature that its renal tubules can be dissected without disrupting the basement membrane, which makes it useful for renal tubule physiology studies [7]. Rabbits have limited capacity to concentrate urea in the excreted urine. That is why if the urea load increases urine production also increases. As rabbits have the advantage of the presence of a wide range of number of active glomeruli at any one point of time, they can autoregulate the glomerular filtration rate [51]. The young rabbits excrete clear urine but sometimes show albuminuria in normal healthy conditions too. Once it begins eating solid ration, the urine starts becoming turbid due to the presence of a high concentration of ammonium magnesium phosphate and calcium carbonate monohydrate crystals [31]. The calcium and magnesium ions are excreted primarily in the urine rather than in the bile in the case of a rabbit. For this reason, rabbits who are fed a diet rich in calcium, if associated with increased urinary pH, are prone to develop urolithiasis, and prolonged use may result in calcification of the aorta and kidney [52, 53]. The color of urine may vary from reddish or brownish hue due to the alkaline nature of the urine with a pH of about 8.2 and a specific gravity ranging between 1.003 and 1.036 [33]. Few cells, casts, or bacteria may be found in the urine of a normal rabbit [31]. Dietary porphyrins will occasionally cause the urine color to vary from dark red to orange. Differential diagnosis of the pigmented urine must be done from hematuria which may be due to many pathological conditions. Approximately 50–75 mL/kg/day of urine is excreted by a healthy rabbit [33]. The urethral orifice of the doe is slit-like, but it is rounded in the buck. The external differentiation of male and female rabbits is shown in Fig. 10.5. The doe urinates more copiously than the buck. The urine characteristics of the rabbit are summarized in Table 10.5.

The uterine horns of the female rabbit are curled and located dorsal to the urinary bladder. Rabbits have a bicornuate uterus which is connected to the vagina by two separate cervices (Fig. 10.6). The urethra enters the vagina through the urogenital

Table 10.5 Characteristics of urine of rabbits

Parameters	Value/characteristic
Volume (mL/kg/day)	50–75 (20–350 in larger breeds)
pH	7.6–8.8
Specific gravity	1.003–1.036
Urine protein to creatinine ratio	0.11–0.4
Normal color	Yellow/straw colored, occasionally red or brown colored due to the presence of porphyrin pigment
Turbidity	Cloudy
Protein	Traces in young rabbits
Cells	Occasionally leucocytes, erythrocytes, bacteria, or casts
Ketones or occult blood	Absent
Crystals	Ammonium magnesium phosphate, calcium carbonate, calcium oxalate

Source: Adopted from Washington and Hoosier (2012) [47], Mancinelli and Lord (2014) [54], Chen and Quesenberry (2009) [55]

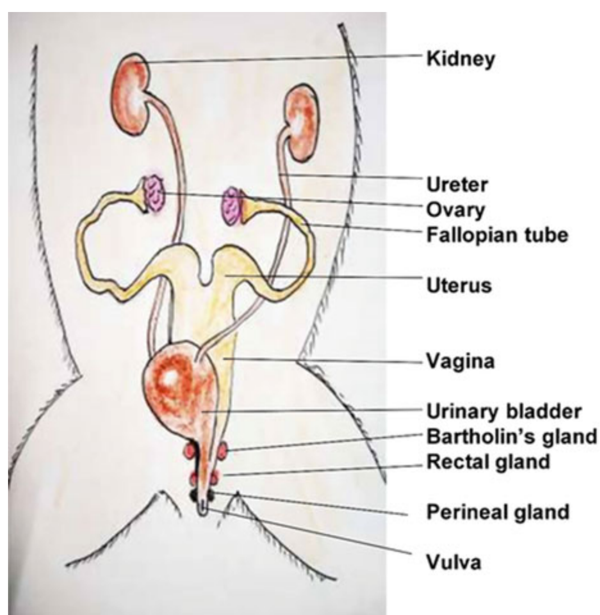


Fig. 10.6 Female urogenital organ of rabbit

sinus or vestibulum. Similar to the human, rabbits possess hemochorial placenta, and it is bidiscoidal (placenta attached to two separate sites on the wall of the uterus) too. The mesometrium is one of the sites for the majority of fat deposition which makes it difficult for the identification of ovaries and blood vessel ligation during ovarian surgeries. The doe has four to five pairs of mammary glands and nipples. The males

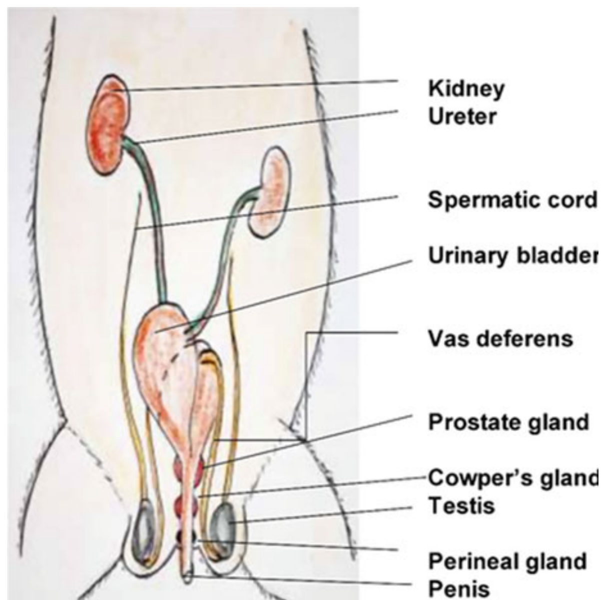


Fig. 10.7 Male urogenital system of rabbit

have only rudimentary nipples visible under their fur. Hairless inguinal pouches (the blind scent glands) present lateral to the genitalia in both sexes.

In the mature buck, the scrotal pouches lie anterolaterally to the penis, whereas in most other mammals, the scrotal sacs are caudal to the penis. Inguinal canals remain open for life. The testes generally descend around 12 weeks of age, and the testes can move between the scrotum and abdominal cavity due to an open inguinal canal. The testes may undergo seasonal involution due to abdominal retraction, and this results in seasonal infertility. The male urogenital system is shown in Fig. 10.7.

10.2.7 Nervous System

The structural anatomy of the rabbit brain and nervous system resembles other mammalian species. But in rabbits, the cerebral cortex is lissencephalic. Rabbits have pigmented and elongated pineal glands which measure about 8 mm long in adults [56], and it projects from the dorsal posterior portion of the third ventricle located midposterior to the cerebrum [57]. The spinal cord extends more caudally and almost covers the whole length of the vertebral canal. The cauda equina is less defined. In rabbits, the spinal cord and vertebral column have a different rate of growth. Therefore, the spinal cord segments, and nerve roots do not essentially correspond to the respective vertebrae. There are two enlargements in the spinal cord: the brachial and lumbar intumescences which give rise to brachial plexus and lumbosacral plexus, respectively. The brachial plexus which provides nerve supply to the forelimb is constituted by the spinal cord segments of the 4th–8th cervical

nerve and 1st thoracic nerve, whereas the lumbosacral plexus that provides nerve supply to the hind limb is constituted by the spinal segments of the 4th–7th lumbar nerve and 1st–3rd sacral nerve [47].

10.3 Housing and Husbandry

10.3.1 Housing and Husbandry

The housing facility of rabbits should include adequate heating, ventilating, and air-conditioning facilities to regulate the temperature and humidity; adequate but not intense lighting; and easily sanitizable surfaces such as the floor, walls, and ceiling. Normally, the rabbits are housed in cages or pens. The cage housing is done singly or in groups with compatible rabbits of the same sex. Three types of cages are used for housing the rabbits, i.e., wire mesh type (Fig. 10.8a), metal sheet type with wire mesh front and barred window, and polycarbonate or polypropylene plastic with the slatted floor with wire front and barred window to allow the animals to view each other (Fig. 10.8b) and communicate using senses and pheromones. The material of the cage should be durable and corrosion-free, should resist the effect of detergent and disinfectants, and should not have sharp edges to injure both the animals and personnel. The provision of space should be sufficient to allow the rabbits to execute normal body movements like hopping, stretching, and sitting upright. The requirement varies with the size and stage of life of the animal. As per the *Guide for the*

Fig. 10.8 Different type of hosing system of rabbit. (**a**) Wire mesh cage with catch pen. (**b**) Solid bottom cage with wire mesh window. (Source: Picture courtesy of **a**, Dr. P.K. Sahoo, RMRC, Bhubaneswar; **b**, Dr. S. Mallick, ICFMD, Bhubaneswar, **c**, Dr. A. Mohanty, Pharmaceutical College, Barpali)

Table 10.6 Cage space recommendation for rabbits

Weight (kg)	Floor area per animal (cm^2)	Height (cm)
<2	1400	40.5
Up to 4	2800	40.5
Up to 5.4	**3700**	40.5
>5.4	≥4600	40.5

Source: Adopted from *Guide for the Care and Use of Laboratory Animals* (2011) [59]

Table 10.7 Space recommendation for one or two socially harmonious rabbits or doe with litter in cage housing

Weight	Floor area (cm^2)	Height (cm)	Extra area for nest box (cm^2)	Optimum shelf/raised platform size (cm^2)	Height of shelf/ raised platform (cm)
<3	3500	45	1000	55 × 25	25
3–5	4200	45	1200	55 × 30	25
>5	5400	60	1400	60 × 35	30

Source: Adopted from Lidfors and Edstrom (2010) [1]

Care and Use of Laboratory Animals (2011) and the guidelines of Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) [58], India, the floor space requirement is 3 ft^2, and the height requirement should be 14 inches for 2–4 kg-weighing rabbit (Table 10.6). In the cages, raised area should be provided to enable more upright sitting, to hop, and to hide. The boxes or shelves inside the cage will act as a source of enrichment. The wire mesh or slatted floor of the cage should be provided with a catch pan to hold the urine and droppings. Rabbits excrete urine with a high concentration of protein and minerals that get deposited at the cage bottoms or catch pans which should be treated with an acid solution before or during washing [59]. Rabbits should be provided with wooden sticks, wooden rings, hiding boxes, and hay as enrichment material. The young siblings of same sex and older females can be housed in groups based on their compatibility, and the space requirement is presented in Table 10.7. However, caution shall be exercised while group housing the females as well, since dominance can develop eventually resulting in unexpected fights and dominant females can injure other submissive ones. The adult males are housed singly due to their territorial behavior.

For pen housing, the floor area should be sufficient enough to allow the rabbit to do their basic behavior such as locomotion, rearing, and grooming. As per the European Commission's (2007) recommendation, the floor area should be the same as per cage dimensions for each rabbit with an additional floor area of 3000 cm^2 per rabbit for up to three to six rabbits and 2500 cm^2 above additional rabbit over six. The height of the floor pen should be 1.25 m, and the pen should be enriched with objects like hiding box, wooden rings, raised platforms, and hay. The floor should be perforated with small holes or slatted with a provision to collect the excreta and urine or solid covered with bedding material such as straw, paper

shredding, and sawdust. The pen should be cleaned completely once in 15 days. Wire floors or grid floors should not be used, and if used, there should be the provision of the resting area with a solid bottom with bedding or perforated bottom.

The rabbit is a social animal, and it prefers social housing or group housing. For social or group housing of rabbits, some important factors should be taken into consideration which include compatibility of individual animals, the appropriate size of pen or cage, environmental enrichment, and stocking density. But for pen housing, the rabbit numbers in a group should be limited to six to eight adult individuals. The best option is to form a group of females that are littermates and can be kept together from the time of weaning. In the case of males, they should be housed individually from the age of 10 weeks to avoid territorial aggressive behavior [1].

The feed and water should be provided ad libitum. The rabbits should be essentially provided with a fiber source, grass, and hay to complement the fiber requirement as well as enrichment material. The water requirement for rabbits is 10% of the body weight or approximately 100 mL water/kg body weight in 24 h [60]. However, lactating does consume more water. In rabbits, the gut flora has an important role, and change in diet should be made gradually over 4–5 days to allow the gut flora to adapt.

10.3.2 Environment

The rabbits prefer cooler temperatures in the range of 16–22 °C [60]. Rabbits have large, highly vascular ears that can function as heat radiators. The sweat glands on their lips have a little role, and they rarely ventilate through the mouth. The relative humidity for the rabbits should be in the range of 30–70% [60]. The rabbit colonies should be maintained at 12:12 or 14:10 light and dark cycle, whereas the breeding females require 14–16 h light in the light cycle [31]. There may be the provision of the introduction of artificial dawn and dusk of 30 min to avoid the adverse effect of sudden illumination or sudden darkness. The maximum light intensity should be 200 lux at 1 m above ground level or floor [1]. The rabbits can hear in the range of 75–50,000 Hz with sensitive hearing in the range of 2000–9000 Hz. The rabbits cannot tolerate high-pitched noise and get startled and scared. They should be housed in a noise-free environment away from noise-generating areas. The sound level should be maintained below 85 dB. Ammonia production is a major problem in rabbit cages; the level of ammonia should not exceed 10 ppm, or else, there may be a chance of upper respiratory tract problems. Therefore, the room should be well ventilated with 10–15 air changes per hour [59]. The excreta pan also needs to be cleaned regularly to avoid ammonia buildup. Cages should be washed and sanitized every week. The rabbit cages or pens should be provided with environmental enrichment material as a measure of welfare and to decrease the stress and associated abnormal behavior. The enrichment material may be hay, straw, hay blocks, grass, wooden chew stick or ring, small cardboard hiding boxes, and raised platforms. The racks having rabbit cages should be placed in such a way that can enable visual

Table 10.8 Recommended environmental requirement

Environmental parameters	Values
Temperature	16–22 °C
Relative humidity	30–70%
Air changes per hour	10–15
Light cycle (light/dark)	12:12 or 14:10 14–16:8–10 in breeding females
Light intensity	200 lux, 1 m above ground level
Noise	<85 dB
Ammonia	<10 ppm

contact among the rabbits. The recommended environmental parameters are mentioned in Table 10.8.

10.3.3 Management-Associated Health Conditions

The housing, husbandry, environment, hygiene, sanitation, feed, and water affect the health and well-being of rabbits. The rabbits develop certain diseases or health conditions due to ill-management and improper handling. The common conditions seen are enlisted in Table 10.9.

10.3.4 Reproduction and Breeding Management

The sexual maturity of the rabbits occurs at the age of 5–7 months, and the breeding life lasts approximately 1–3 years. The does do not show any distinct estrous cycle, and vaginal cytology is not much useful for determining the estrus; rather, the vulvar changes during estrus, i.e., swollen, moist, and dark pink- or red-colored vulva, and the behavioral changes like restlessness and rubbing of the chin on the cage can be taken into account for determining the estrus or receptivity. In rabbits, ovulation is induced. It occurs 10–13 h after copulation. Artificially, by administering luteinizing hormone, human chorionic gonadotropin, or gonadotropin-releasing hormone, ovulation can also be induced [31].

For mating to happen, the doe is taken to the buck's cage because the does are more territorial and may attack the buck and inflict injury. A period of 15–20 min is usually allowed for the pair of animals to see compatibility and mating. If the doe does not allow the buck to mount, it is introduced to another buck. The does exhibit postpartum receptivity immediately after parturition. Getting up to 11 litters from a single doe is possible with the advantage of postpartum breeding, but it is advisable to breed the doe after the weaning of kits. This will yield only four litters per year [31].

The gestation period of rabbits is 30–33 days. The pregnancy can be detected at 14 days of gestation by palpating the fetus externally. Doe starts to prepare nest by pulling its hair 3–4 days before parturition, and it remains off fed 2–3 days before

Table 10.9 Management-associated health conditions

Name of the disease	Managemental cause	Clinical signs
Lumbar spinal fracture and luxation	Rabbit's fall from a height Handler's failure to support hindquarter while handling	Sudden onset of posterior paresis or paralysis
Hair balls (gastric trichobezoars)	Excessive self-grooming due to boredom Low-fiber feed Dehydration, stress, and pain	Acute pyloric obstruction and rabbits suddenly stop eating and rapid clinical decline of health Acute hepatic lipidosis Many times found in necropsy
Ulcerative pododermatitis or sore hocks	Inadequate hygiene Wire floor housing Foot stamping Rough and sharp wood-shaving bedding material	Bleeding and chronic wound of ventral metatarsal region of hind feet, circumscribed ulcerated area of the skin covered by a dry crusty scab, and rabbit sit in a hunched position or seen shifting weight on hind legs
Heatstroke	High environmental temperature over 29.5 °C High humidity (>70%) Poor ventilation and overcrowding	Young, pregnant, older, and obese rabbits are affected Lethargy, panting, salivating, weakness, and reddening of the ears
Tracheal injury following intubation	Faulty tracheal intubation, Long-term and/or repeated intratracheal intubation	Stridor, mild cyanosis, and ulcerative tracheitis
Deficiency associated with the dental disorder	Pellet feed deficient in calcium and vitamin D	Drooling of saliva, overgrowth, and ridging of incisor, malocclusion, and distorted growth of premolar and molar
Lead toxicity	Accidental chewing or licking of painted objects	Mild anemia, tremors, and posterior ataxia
Hutch burn	Filthy and unhygienic condition	Urine scalding of the perineal region and serous exudation from the skin
Physical injury and trauma	Group housing of incompatible rabbits	Skin abrasion, hair loss, laceration around the genitalia, and amputation of the tip of the ears
Moist dermatitis	Hypersalivation due to malocclusion Use of water bowl for watering High humidity Prolonged contact with urine	Dermatitis in the area ventral to dewlap or anogenital region
Splay leg	Inappropriate flooring Too slippery flooring in pen housing without provision of bedding material	One or more limbs abducted and rabbits sit with legs splayed out sideways

Source: Adopted from Lidfors and Edstrom (2010) [1], Suckow et al. (2002) [31], Colby et al. (2019) [33], and Barthold et al. (2016) [38]

parturition or "kindling." The pregnant doe's cage must be provided with a nesting box with some nesting material several days before kindling. Pseudopregnancy may occur in rabbits due to mounting by other does or sterile buck. The kindling usually

Table 10.10 Biological and reproductive values of the laboratory rabbit

Parameters	Values
Adult body weight	2–5 kg
Life span	5–7 years
Body temperature	38.5–39.5 °C
Food consumption	5/100 g per day
Water consumption	5–12 mL/100 g per day
Breeding onset: male	6–10 months
Breeding onset: female	4–9 months
Estrous cycle/ovulation	No defined estrous cycle/induced ovulation
Gestation period	29–35 days
Postpartum estrus	48 h after kindling
Litter size	4–10
Birth weight	30–70 g
Weaning age	4–6 weeks
Breeding activity	1–3 years
Chromosome number (diploid)	44
Respiratory rate	32–60 breaths/min
Lung capacity	111 ± 14.7 ml
Minute volume	0.6 L/min
Tidal volume	4–6 mL/kg body weight
Mean alveolar diameter	93.97 μm
Heart rate	200–300 beats/min
Arterial systolic pressure	90–130 mmHg
Arterial diastolic pressure	80–90 mmHg
Arterial blood pH	7.2–7.5

Source: Adopted from Wolfensohn and Lloyd (2013) [60], Colby et al. (2019) [33], Suckow et al. (2002) [31]

happens during the early morning hours, and it takes 30–60 min. The average number of kits born per litter is 7–9. After cleaning the kits, the doe eats upon the placenta. Sometimes, cannibalism of kits may happen due to stress factors. At birth, the kits are altricial, furless, closed ears, and closed eyes [1]. The weight varies between 40 and 50 g at birth, and there will be a weight gain of 30 g each day. The fur starts to grow after 7 days of age. The hearing ability and the opening of the eyes happen at 10–11 days of age [61]. Does have 4–5 pairs of mammary glands, and the milk yield ranges from 160 to 220 g/day. But the doe nurses the kits only once a day. The milk yield is at a peak of 2 weeks following parturition and gradually declines up to 4 weeks. Orphaned kits may be nursed with commercially available milk replacers for lagomorphs that do not accept or nurse kits that are not their own. Kits start consuming solid food by the age of 3 weeks and are weaned at 5–6 weeks of age [31]. The biological and reproductive values of the rabbit are presented in Table 10.10. These values may vary with breeds and strains.

10.3.5 Transportation, Barrier, and Quarantine

Rabbits require transportation from the breeder to the laboratory, maybe internationally or within a country, by road, by train, or by airplane. The transport container should be made up of fiberboard, fiberglass, wire mesh, and half wooden board-half wire mesh. A grid on the floor should be provided to separate the excreta. Sufficient bedding material should be provided to maintain dryness, sanitation, and thermoregulation. The food and water supply should be adequate. To avoid spillage of water, solid water sources such as a hydrogel, tomatoes, apple, and carrot can be provided. The rabbits may suffer from transit-related stress, and there may be altered cardiac and pulmonary physiology and reduced food and water intake, and there may be some weight loss. Gradually, the rabbits come to normal if provided with a more stable environment after the transit. Some points to be taken into consideration before transportation are the following:

- The health of the animal
- Design of transport box/container for the comfort of the animal
- The environmental condition of the transporting vehicle
- Feed, water, and bedding supply in the container during transit
- Duration and type of transport
- Handling of animals during loading and unloading of animal boxes
- The density of animals per container

Following transport, when rabbits are introduced to a laboratory animal facility, quarantine procedures should be followed as a precautionary measure to rule out any doubt about the health status of new animals. As most of the time, the animal's health problem is subclinical; serological sampling of more commonly occurring diseases of that particular state may be followed.

To achieve high health standards and the welfare of the animals, the biosafety and barrier system of housing is most essential. This includes entry of properly trained authorized persons; sterilized goods like bedding, feed, and other materials; sterilized personal protective equipment; regular education; and training of staff.

References

1. Lidfors L, Edstrom T (2010) The laboratory rabbit. In: Hubrecht R, Kirkwood J (eds) The UFAW handbook on the care and management of laboratory and other research animals, 8th edn. Wiley Blackwell, Iowa, pp 399–417
2. Esteves PJ, Abrantes J, Baldauf H et al (2018) The wide utility of rabbits as models of human diseases. Exp Mol Med 50(5):1–10. https://doi.org/10.1038/s12276-018-0094-1
3. Hicks DJ, Fooks AR, Johnson N (2012) Developments in rabies vaccines. Clin Exp Immunol 169(3):199–204. https://doi.org/10.1111/j.1365-2249.2012.04592.x
4. Burnett RC, Hanly WC, Zhai SK, Knight KL (1989) The IgA heavy-chain gene family in rabbit: cloning and sequence analysis of 13 C alpha genes. EMBO J 8(13):4041–4047

5. Shiomi M (2009) Rabbit as a model for the study of human diseases. In: Houdebine LM, Fan J (eds) Rabbit biotechnology: rabbit genomics, transgenesis, cloning and models. Springer, Netherlands, pp 49–63. https://doi.org/10.1007/978-90-481-2227-1
6. Peng X, Knouse JA, Hernon KM (2015) Rabbit models for studying human infectious diseases. Comp Med 65(6):499–507
7. Brewer NR (2006) Biology of the rabbit. J Am Assoc Lab Anim Sci 45(1):8–24
8. Gonzalez RR, Kluger MJ, Stolwijk JA (1974) Thermoregulatory responses to thermal transients in the rabbit. Am J Phys 227(6):1292–1298. https://doi.org/10.1152/ajplegacy.1974.227.6.1292
9. Kozma C, Macklin W, Cummins LM, Mauer R (1974) Anatomy, physiology and biochemistry of the rabbit. In: Weisbroth SH, Flatt RE, Kraus AL (eds) The biology of the laboratory rabbit. Academic, New York, pp 50–72
10. Johnson-Delaney CA, Orosz SE (2011) Rabbit respiratory system: clinical anatomy, physiology and disease. The veterinary clinics of North America. Exotic Animal Practice 14(2):257–266, vi. https://doi.org/10.1016/j.cvex.2011.03.002
11. Hampton A, Cotroneo T, Colby LA (2017) The laboratory rabbit. In: Kurtz DM, Travlos GS (eds) The clinical chemistry of laboratory animals, 3rd edn. CRC Press, Boca Raton, pp 79–111
12. Morimato M (2009) General physiology of rabbits. In: Houdebine LM, Fan J (eds) Rabbit biotechnology: rabbit genomics, transgenesis, cloning and models. Springer, Netherlands, pp 27–35. https://doi.org/10.1007/978-90-481-2227-1
13. Cizek LJ (1961) Relationship between food and water ingestion in the rabbit. Am J Phys 201:557–566. https://doi.org/10.1152/ajplegacy.1961.201.3.557
14. Meredith A (2014) Biology, anatomy and physiology. In: Meredith A, Lord B (eds) BSAVA manual of rabbit medicine. British Small Animal Veterinary Association, Gloucester, pp 1–12
15. Greenaway JB, Partlow GD, Gonsholt NL, Fisher KR (2001) Anatomy of the lumbosacral spinal cord in rabbits. J Am Anim Hosp Assoc 37(1):27–34. https://doi.org/10.5326/15473317-37-1
16. Sohn J, Couto MA (2012) Anatomy, physiology and behaviour. In: Suckow MA, Stevens KA, Wilson RP (eds) The laboratory rabbit, guinea pig, hamster, and other rodents, American College of Laboratory Animal Medicine Series, edited by Academic Press
17. Wang HH, Wang YX, Sheng H et al (2009) Fossa trochanterica of the proximal femur in rabbits: an anatomic structure for potential misinterpretation on magnetic resonance images. Acta Radiol 50(2):212–216. https://doi.org/10.1080/02841850802635208
18. Briscoe JA, Syring R (2004) Techniques for emergency airway and vascular access in special species. Semin Avian Exotic Pet Med 13(3):118–131. https://doi.org/10.1053/j.saep.2004.03.007
19. Knott T (2014) Ophthalmology. In: Meredith A, Lord B (eds) BSAVA manual of rabbit medicine. British Small Animal Veterinary Association, Gloucester, pp 191–204
20. De Schaepdrijver L, Simoens P, Lauwers H, De Geest JP (1989) Retinal vascular patterns in domestic animals. Res Vet Sci 47(1):34–42
21. Folkman J, Merler E, Abernathy C, Williams G (1971) Isolation of a tumor factor responsible for angiogenesis. J Exp Med 133(2):275–288. https://doi.org/10.1084/jem.133.2.275
22. Schaal B, Coureaud G, Langlois D, Giniès C, Sémon E, Perrier G (2003) Chemical and behavioural characterization of the rabbit mammary pheromone. Nature 424(6944):68–72. https://doi.org/10.1038/nature01739
23. Melo AI, González-Mariscal G (2010) Communication by olfactory signals in rabbits: its role in reproduction. Vitam Horm 83:351–371. https://doi.org/10.1016/S0083-6729(10)83015-8
24. Kromin AA, Ignatova YP (2014) Objective method for registration of the sniffing component of the search behavior in rabbits subjected to food deprivation. Bull Exp Biol Med 156 (4):522–525. https://doi.org/10.1007/s10517-014-2389-0
25. Xi J, Si XA, Kim J, Zhang Y, Jacob RE, Kabilan S, Corley RA (2016) Anatomical details of the rabbit nasal passages and their implications in breathing, air conditioning, and olfaction. Anat Record (Hoboken, N.J.: 2007) 299(7):853–868. https://doi.org/10.1002/ar.23367

26. Corley RA, Minard KR, Kabilan S, Einstein DR, Kuprat AP, Harkema JR, Kimbell JS, Gargas ML, Kinzell JH (2009) Magnetic resonance imaging and computational fluid dynamics (CFD) simulations of rabbit nasal airflows for the development of hybrid CFD/PBPK models. Inhal Toxicol 21(6):512–518. https://doi.org/10.1080/08958370802598005
27. Davies RR, Davies JA (2003) Rabbit gastrointestinal physiology. Vet Clin North Am Exot Anim Pract 6(1):139–153. https://doi.org/10.1016/s1094-9194(02)00024-5
28. Bennegadi N, Fonty G, Millet L, Gidenne T, Licois D (2003) Effects of age and dietary fibre level on caecal microbial communities of conventional and specific pathogen-free rabbits. Microb Ecol Health Dis 15(1):23–32. https://doi.org/10.1080/08910600310015574
29. Varga M (2014) Clinical pathology. In: Textbook of rabbit medicine, pp 111–136. https://doi.org/10.1016/B978-0-7020-4979-8.00002-9
30. Butler JE, Sinkora M (2013) The enigma of the lower gut-associated lymphoid tissue (GALT). J Leukoc Biol 94(2):259–270. https://doi.org/10.1189/jlb.0313120
31. Suckow MA, Brammer DW, Rush HG, Chrisp CE (2002) Davisson. Biology and diseases of rabbits. In: Fox JG, Anderson LC, Lorw FM, Quimby FW (eds) Laboratory animal medicine. Elsevier Academic Press, San Diego, pp 329–364
32. Michelland RJ, Combes S, Monteils V, Cauquil L, Gidenne T, Fortun-Lamothe L (2010) Molecular analysis of the bacterial community in digestive tract of rabbit. Anaerobe 16 (2):61–65. https://doi.org/10.1016/j.anaerobe.2009.05.002
33. Colby LA, Nowland MH, Kennedy LH (2019) Rabbits. In: Clinical laboratory animal medicine: an introduction, 5th edn. Wiley Blackwell, Iowa, pp 286–332
34. Franz R, Kreuzer M, Hummel J, Hatt JM, Clauss M (2011) Intake, selection, digesta retention, digestion and gut fill of two coprophageous species, rabbits (Oryctolagus cuniculus) and guinea pigs (Cavia porcellus), on a hay-only diet. J Anim Physiol Anim Nutr (Berl) 95(5):564–570. https://doi.org/10.1111/j.1439-0396.2010.01084.x
35. Xi J, Si XA, Kim J et al (2016) Anatomical details of the rabbit nasal passages and their implications in breathing, air conditioning, and olfaction. Anat Rec (Hoboken) 299(7):853–868. https://doi.org/10.1002/ar.23367
36. Mahdy EAA, El Behery EI, Mohamed SKA (2019) Comparative morpho-histological analysis on the vomeronasal organ and the accessory olfactory bulb in Balady dogs (Canis familiaris) and New Zealand rabbits (Oryctolagus cuniculus). J Adv Vet Anim Res 6(4):506–515. https://doi.org/10.5455/javar.2019.f375
37. Kamaruzaman NA, Kardia E, Kamaldin N, Latahir AZ, Yahaya BH (2013) The rabbit as a model for studying lung disease and stem cell therapy. Biomed Res Int 2013:691830. https://doi.org/10.1155/2013/691830
38. Barthold SW, Griffey SM, Percy DH (2016) Pathology of laboratory rodents and rabbits, 4th edn. Wiley Blackwell, Iowa, pp 253–324. https://doi.org/10.1002/9781118924051
39. Hew KW, Keller KA (2003) Postnatal anatomical and functional development of the heart: a species comparison. Birth Defects Res B Dev Reprod Toxicol 68(4):309–320. https://doi.org/10.1002/bdrb.10034
40. James TN (1967) Anatomy of the cardiac conduction system in the rabbit. Circ Res 20 (6):638–648. https://doi.org/10.1161/01.res.20.6.638
41. Bleeker WK, Mackaay AJ, Masson-Pévet M, Bouman LN, Becker AE (1980) Functional and morphological organization of the rabbit sinus node. Circ Res 46(1):11–22. https://doi.org/10.1161/01.res.46.1.11
42. Cordeiro JM, Spitzer KW, Giles WR (1998) Repolarizing K+ currents in rabbit heart Purkinje cells. J Physiol 508(Pt 3):811–823. https://doi.org/10.1111/j.1469-7793.1998.811bp.x
43. Jenkins JR (2008) Rabbit diagnostic testing. J Exot Pet Med 17(1):4–15. https://doi.org/10.1053/j.jepm.2007.12.003
44. Amin TM, Sirs JA (1985) The blood rheology of man and various animal species. Q J Exp Physiol 70(1):37–49. https://doi.org/10.1113/expphysiol.1985.sp002895

45. Hirayama T, Folmerz P, Hansson R et al (1986) Effect of oxygen free radicals on rabbit and human erythrocytes. Studies on cellular deformability. Scand J Thorac Cardiovasc Surg 20 (3):247–252. https://doi.org/10.3109/14017438609105933
46. Toth LA, January B (1990) Physiological stabilization of rabbits after shipping. Lab Anim Sci 40(4):384–387
47. Washington IM, Hoosier GV (2012) Clinical biochemistry and hematology. In: Suckow MA, Stevens KA, Wilson RP (eds) The Laboratory Rabbit, Guinea Pig, Hamster, and Other Rodents, American College of Laboratory Animal Medicine Series, edited by Academic Press, pp 57–116
48. Laird CW, Fox RR, Mitchell BP, Blau EM, Schultz HS (1970) Effect of strain and age on some hematological parameters in the rabbit. Am J Phys 218(6):1613–1617. https://doi.org/10.1152/ajplegacy.1970.218.6.1613
49. Jeklova E, Leva L, Knotigova P, Faldyna M (2009) Age-related changes in selected haematology parameters in rabbits. Res Vet Sci 86(3):525–528. https://doi.org/10.1016/j.rvsc.2008.10.007
50. Kellie A, Fecteau KA, Deeb BJ, Rickel JM, Kelch WJ, Oliver JW (2007) Diagnostic endocrinology: blood steroid concentrations in neutered male and female rabbits. J Exotic Pet Med 16 (4):256–259. https://doi.org/10.1053/j.jepm.2007.09.003
51. Steinhausen M, Endlich K, Wiegman DL (1990) Glomerular blood flow. Kidney Int 38 (5):769–784. https://doi.org/10.1038/ki.1990.271
52. Clauss M, Burger B, Liesegang A et al (2012) Influence of diet on calcium metabolism, tissue calcification and urinary sludge in rabbits (Oryctolagus cuniculus). J Anim Physiol Anim Nutr (Berl) 96(5):798–807. https://doi.org/10.1111/j.1439-0396.2011.01185.x
53. Hampton A, Cotroneo T, Colby LA (2018) The laboratory rabbit. In: Kurtz DM, Travlos GS (eds) The clinical chemistry of laboratory animals, 3rd edn. CRC Press, New York, pp 79–112
54. Mancinelli E, Lord B (2014) Urogenital system and reproductive disease. In: Meredith A, Lord B (eds) BSAVA manual of rabbit medicine. British Small Animal Veterinary Association, Gloucester, pp 191–204
55. Chen S, Quesenberry KE (2006) Rabbits. In: Saunders manual of small animal practice, pp 1858–1880. https://doi.org/10.1016/B0-72-160422-6/50178-9
56. Romijn HJ (1973) Structure and innervation of the pineal gland of the rabbit, Oryctolagus cuniculus (L.). I. A light microscopic investigation. Z Zellforsch Mikrosk Anat 139 (4):473–485. https://doi.org/10.1007/BF02028389
57. García-Mauriño JE, Sicilia C, Boya J, Calvo JL (2001) Embryonic development of the rabbit pineal gland (Oryctolagus Cuniculus) (Lagomorpha). Eur J Anat 5(2):55–66
58. http://cpcsea.nic.in/WriteReadData/userfiles/file/Compendium%20of%20CPCSEA.pdf. Accessed 18 Sept 2020
59. Environment, Housing, and Management (2011) National Research Council (US) Committee for the update of the guide for the care and use of laboratory animals, guide for the care and use of laboratory animals, 8th edn. National Academies Press (US), Washington, DC
60. Wolfensohn S, Lloyd M (2013) Handbook of laboratory animal management and welfare. Blackwell Publishing Ltd., Hoboken, pp 209–246
61. Kersten AMP, Meijsser FM, Metz JHM (1989) Effects of early handling on later open-field behaviour in rabbits. Appl Anim Behav Sci 249(2):157–167. https://doi.org/10.1016/0168-1591(89)90043-9. issn:0168-1591.

The Laboratory Guinea Pig

11

Saurabh Chawla, Sarita Jena, and Sunita Nayak

Abstract

Guinea pig is considered as a symbol of a scientific experiment. Guinea pig is more popular as a pet animal than a research model these days. However, these animals are still popular among the research community as an excellent model for tuberculosis, asthma, scurvy, and otology-related research. This chapter covers the important as well as unique anatomical as well as physiological details of guinea pigs useful for researchers, students, and veterinarians. The important features of the digestive, respiratory, cardiovascular, urogenital, and nervous systems have been covered. This chapter also covers the housing and husbandry requirement of guinea pigs. The husbandry part covers the important and key aspects of reproduction, housing, and nutrition. Major anatomical and physiological differences with other species of laboratory animals have also been explained under different sections.

Keywords

Guinea pigs · Anatomy · Physiology · Nutrition · Housing

S. Chawla (✉)
National Institute of Science Education and Research, Bhubaneswar, India
e-mail: saurabhchawla@niser.ac.in

S. Jena
Institute of Life Sciences, Bhubaneswar, India

S. Nayak
Department of Anatomy, All India Institute of Medical Sciences, Patna, India
e-mail: drsunitanayak@aiimspatna.org

© The Author(s), under exclusive license to Springer Nature Singapore Pte Ltd. 2021
P. Nagarajan et al. (eds.), *Essentials of Laboratory Animal Science: Principles and Practices*, https://doi.org/10.1007/978-981-16-0987-9_11

11.1 Introduction

Guinea pigs are considered as a symbol of scientific experiments on living beings, a term also used for human beings exploited. The general public considers guinea pigs as one of the most commonly used animals in biomedical research. However, the guinea pig has now been replaced by other laboratory animals such as rats and mice. Guinea pigs have been most widely used for tuberculosis research and the study of infectious disease and the role of vitamins. Guinea pigs are now more popular as pet animals due to their small size, cleanliness, docile nature, and relatively easy maintenance [1]. Guinea pigs are a monogastric herbivorous, hystricomorphic rodent, and a member of the Caviidae family. The dentition of the guinea pig is described as a radicular hypsodont (e.g., all teeth have a comparatively long crown and are "open rooted") [2]. The incisors grow throughout life (open rooted) and are white in appearance in contrast to other rodents where the same are pigmented yellow. Young ones are fully haired when born and eat solid food from birth (precocial species). Guinea pigs like humans require a dietary source of vitamin C, lack of which leads to scurvy. Hence, vitamin C was discovered in 1907 using guinea pigs. The species continues to be used for studying collagen biosynthesis, a process that needs vitamin C and is important in wound healing, bone remodeling, and atherosclerosis [3].

11.2 Taxonomic Classification: Guinea Pigs

Class—Mammalia
Order—Rodentia
Family—Caviidae
Subfamily—Caviinae
Genus—*Cavia*
Species—*C. porcellus*

11.3 Anatomy and Physiology

11.3.1 External Features

Guinea pigs have a compact body with an elongated head bearing a pair of the large rounded hairless pinna, short neck, trunk, and short limbs. The tail is absent, but rudimentary tailbone is present toward the posterior end of the body beneath which lies the anus. A pair of nipples (inguinal) is present on the ventral surface toward the caudal end of the abdomen. Undeveloped mammary glands with small teats are present in males. Limbs are the pentadactyl type with forelimbs shorter than hind limbs. The life span of guinea pigs is usually 5–6 years. The forelimb bears four digits, whereas the hind limb bears three digits (Fig. 11.1). Guinea pigs may have coats varying in length, color, and quality but usually large guard hair with an

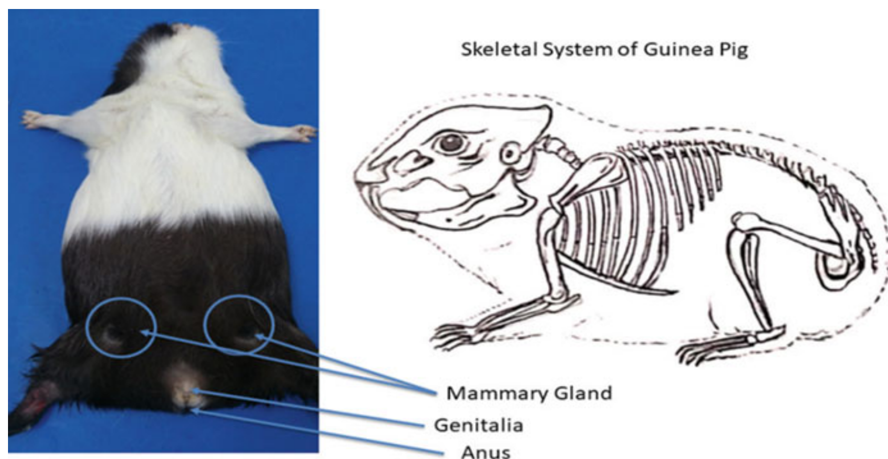


Fig. 11.1 The skeletal system, external features, mammary gland, genitalia, and anus in guinea pig

undercoat of fine hair. The guinea pig features a short, flat nose; laterally placed eyes; and hairless external pinnae. Their skin sensitivity has led to their widespread use in testing for allergic skin reactions. But this practice has now been replaced by the utilization of local lymph node assays in mice [4, 5].

11.3.2 Digestive System

The guinea pigs have a pair of incisors, premolars, and a pair of three molars. Canines are absent in guinea pigs. The dental formula of guinea pigs is 2[I1/1, C0/0, PM1/1, M3/3] = 20. The cheek teeth composed of molar and premolar are different from other rodents in having curved reserve crowns. The occlusal plane in the case of the guinea pig is oblique which is horizontal.

In guinea pigs, molar malocclusion is a common feature where the maxillary cheek teeth arch laterally into the buccal mucosa while the mandibular cheek teeth overgrow medially, causing tongue entrapment and subsequent anorexia.

There are five pairs of salivary glands, parotid, mandibular, zygomatic, and major and minor sublingual, unlike human beings who have numerous minor salivary glands, but three pairs of major salivary glands: parotid, submandibular, and sublingual.

The soft palate is a soft tissue flap-like structure “hinged” to the rear end of the hard palate. The nasopharynx and oropharynx are separated by the soft palate, which communicates with the mouth cavity via a narrow opening in the posterior part of the pharynx referred to as the palatal ostium (not found in humans). This makes intubation difficult in guinea pigs and oral gavage challenging [6]. A lateral slip could harm the vascular velopharyngeal folds.

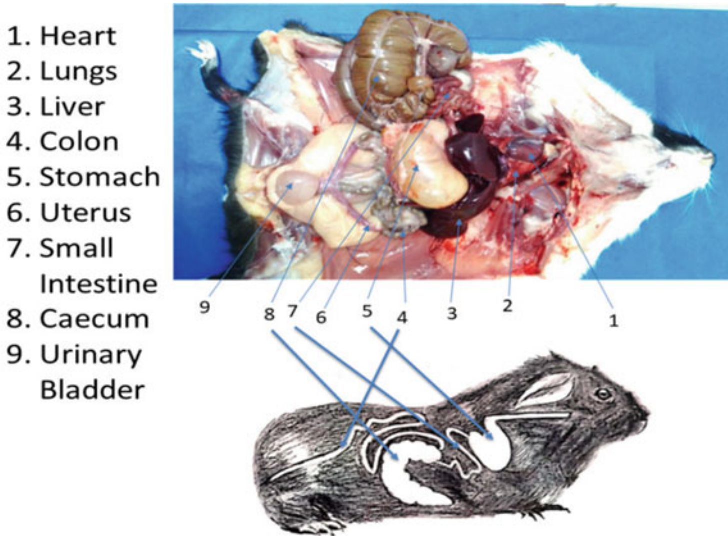
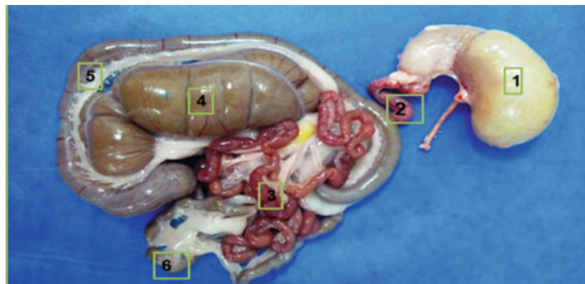


Fig. 11.2 Internal organs of guinea pigs



1. Stomach
2. Duodenum
3. Part of Small Intestine
4. Caecum
5. Ascending Colon
6. Descending Colon

Fig. 11.3 The gastrointestinal tract in guinea pig

Guinea pigs don't have tonsils but have lymphoid nodules in folds within the wall of the pharynx. The stomach in guinea pigs is monogastric and completely glandular unlike other rodents and thus completely lined by glandular epithelium [7]. Guinea pigs rarely vomit due to strong cardiac sphincter. The colon lacks taenia which is a ribbonlike longitudinal muscle on the outside of the colon [8]. The caecum is large and voluminous and possesses taenia which consists of three bands running longitudinally (medial, ventral, and lateral) (Figs. 11.2 and 11.3). Taenia causes

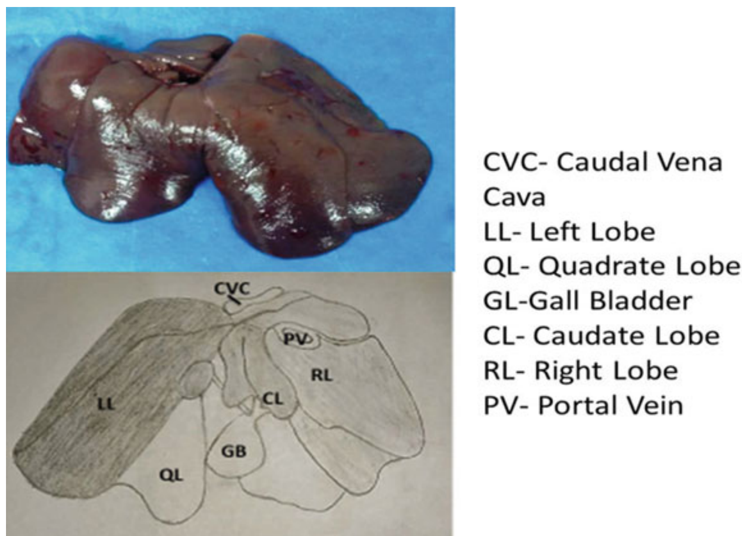


Fig. 11.4 Liver – guinea pig

sacculations called haustra and is responsible for the constriction in the wall of the caecum [9].

The microflora in the caecum of guinea pigs is a predominantly gram-positive type. Antibiotics such as penicillin having a predominantly gram-positive bactericidal action result in an imbalance of microbiota with a shift toward the gram-negative population of microbes [10]. Thus, guinea pigs are sensitive to antibiotics such as penicillin which may eventually result in lethality.

The liver in guinea pigs consists of six separate interconnected lobes (right, medial, left lateral, left medial, caudate, and quadrate) with the left lateral lobe being the largest [11] (Fig. 11.4). The gallbladder is well-developed, thin-walled, oval attached to the fossa of the quadrate liver lobe with less contractile ability as compared to other species such as dogs [1, 12].

While the human liver is grossly divided into right, left, caudate, and quadrate lobes based on the peritoneal ligamentous attachment, the right lobe is the largest in volume and contributes to all surfaces of the liver.

The pancreas is triangular and located retroperitoneally [13]. It has three lobes and lies in contact with the descending duodenum (Fig. 11.5a). The spleen is larger and broader in size as compared to other rodents and rabbits [1] (Fig. 11.5b). It lies on the left side, lateral to the greater curvature of the stomach and attached by the gastrosplenic ligament [14].

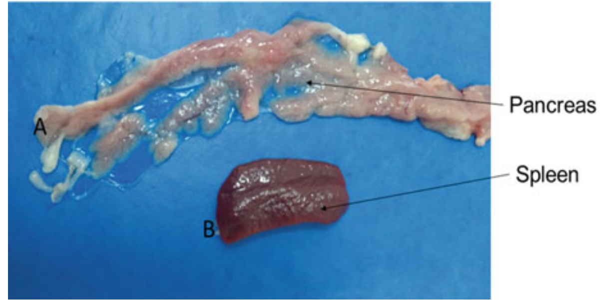


Fig. 11.5 (**a**) Pancreas. (**b**) Spleen

11.3.3 Respiratory System

The nasal cavity is lined by sensitive olfactory epithelium, and this provides the guinea pig a keen sense of smell. The larynx is typical of all mammals; therein, it has five cartilages, but there is no laryngeal ventricle. Despite their wide vocal variety, guinea pigs have small and poorly developed vocal cords [14].

Rabbits and guinea pigs are valuable models in asthma research due to their bronchial hyperresponsiveness which is a major feature of bronchial asthma characterized by an exaggerated response to stimuli that can induce an increased resistance to airflow in the lung [15].

The airways display hypersensitivity to histamine which is absent in rats and mice. Both rabbits and guinea pigs are nasal breathers; they cannot breathe through the mouth and show acute dyspnea when there is any obstruction to nasal passages.

The thymus surrounds the trachea, located in the cervical region in contrast to other rodents where it is located in the thoracic cavity [16]. Thus, the anatomical location is quite accessible as compared to other rodents. The cervical thymus is very readily accessible that makes the guinea pigs to be used extensively for immunological research. In immature animals, it is located within the cranial mediastinum and subcutaneously within the neck where it surrounds the trachea ventrally and laterally. It is composed of two yellow-brown, oval lobes extending from the angle of the mandible to halfway to the thoracic inlet. In adults, it is mainly replaced by fat [14].

The right lung has four lobes (cranial, middle, accessory, and caudal) that are separated by a deep fissure. The left lung has three lobes: cranial, middle, and caudal (Fig. 11.6). The newborn have completely developed alveoli, whereas the same is absent in rats and mice at birth.

11.3.4 Cardiovascular System

In guinea pigs, the heart occupies most of the thoracic cavity and lies mostly on the midline but slightly left in the caudal direction approximately 1 cm above the xiphoid process [13] (Fig. 11.7). It lies in the midline at the level of the 2nd–4th intercostal space. The pericardium has two layers: an outer fibrous layer and a

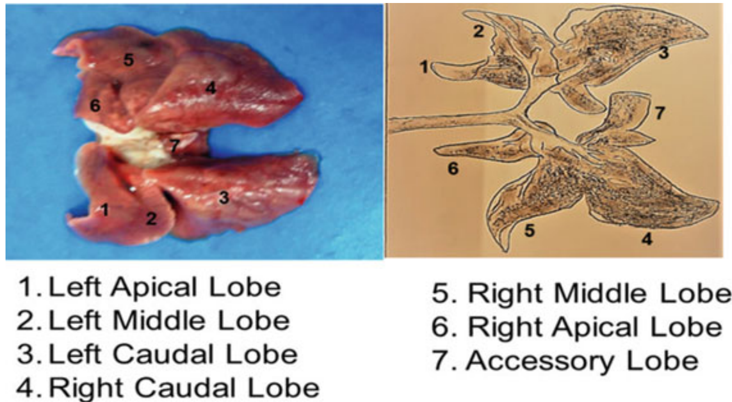


Fig. 11.6 Lungs—guinea pig

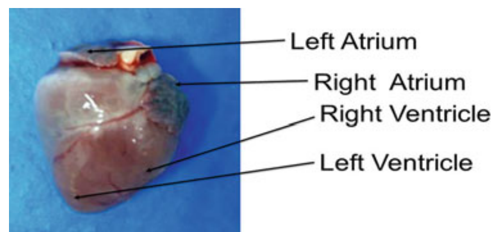


Fig. 11.7 Heart—guinea pig

thinner serous layer. Guinea pigs are the model of choice to study cardiac arrhythmia and also the pharmacological effect of drugs on the heart due to its similar electrocardiogram pattern as compared to humans. Guinea pig's heart has been traditionally used as an ex vivo model for various pharmacological and physiological studies, first used by Oscar Langendorff in the year 1895, also popularly known as the Langendorff apparatus [1].

11.3.5 Urogenital System

The male has lateral scrotal swellings on each side of the anus. The cranial orifice is the penile urethra, which is covered by preputial folds. The penis can be easily retracted upon gentle pressure above the prepuce. An intromittent sac located ventrally to the urethra was described in a study by Stan in 2015 [17]. The vesicular glands, prostate glands, coagulating glands, and bulbourethral glands are also a part of the reproductive system in males. The vesicular gland is long and coiled lying ventral to the urethra. The perineal sac glands are present in both sexes but are more extensive in the male. They lie on either side of the anus and contain extensive caseous secretions, hair, and skin debris [18]. Os penis (or the baculum) is a small,

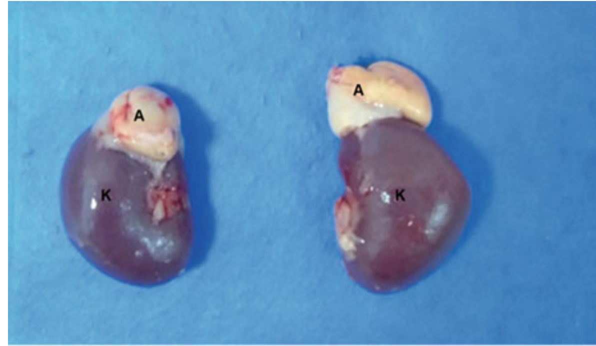


Fig. 11.8 Guinea pig kidney

thin rodlike bone that lies within the glans of the penis. It is absent in the human penis. Peculiar penis horns, two in number, are present caudoventral to the urethral opening in males [19]. These horns project externally during erection. Males are sexually mature at 3 months of age. The inguinal ring is permanently open. Like other mammals, the kidneys in guinea pigs are bean-shaped located toward the posterior part of the abdomen on each side of the vertebral column (Fig. 11.8). The kidneys lie retroperitoneally on either side of the midline and are often surrounded by dense fat on the caudal and medial borders. In females, the broad ligament containing abundant fat is attached to the ventral aspect of the kidney. The ovaries are located caudolateral to the kidneys and are supported by a short mesovarium. The oviduct lies in close contact with the ovarian bursa. The left kidney is more caudal than the right, whereas, in humans, the right kidney is pushed downward because of the liver. The renal pelvis is large, and there's one longitudinal papilla [14, 18]. Urolithiasis occurs commonly in pet guinea pigs, and therefore, the common clinical signs related to the disease include stranguria and pollakiuria, vocalizing while urinating, and hematuria. The underlying cause of this condition isn't completely understood but is probably associated with a genetic predisposition and/or the presence of a high-calcium diet. Calcium carbonate and ammonium phosphate crystals excreted in the urine of guinea pig make its appearance cloudy and white.

In guinea pigs, the percentage of short- and long-loop nephrons is almost similar, whereas in mice and rat, the percentage of short-loop nephrons is higher, and the opposite is true for hamsters and gerbils [19]. The adrenal glands in guinea pig are very prominent and comparatively large (about 1/5th the size of the kidney) when matched to the size of other rodents and rabbits. In humans, the adrenal gland is only one-thirtieth the size of the kidney.

Guinea pigs have a bicornuate uterus with a litter size of 1–6 [20]. The uterine body is short and a single os cervix. The vaginal orifice is U-shaped and covered by a vaginal covering membrane that opens either during estrus or at day 26 or 27 of

gestation or during parturition [21]. Estrous cycles range from 13 to 21 days with an average of approximately 16 days. Estrus lasts for 8–11 h during which females become active, chase fellow mates, wag their hindquarters, display lordosis, and make guttural sounds [1]. The genitalia in females is seen as typically Y-shaped. The urethral opening is located cranially between branches of Y, the vulva in the middle, and the anus at the base of the Y.

11.3.6 Nervous System

Most of the gross anatomical features of the central nervous system are similar to other rodents. The shape of the cerebrum is diamond-shaped in guinea pigs, whereas the same is triangular in the rabbit. The cerebrum lacks prominent gyri and sulci; thus, guinea pigs like rabbits are placed in the lissencephalic group. The medulla entirely covers the cerebellum, whereas in rabbits, it is visible as a conical-shaped structure [22]. Olfactory bulbs are visible from the dorsum of the brain.

11.3.7 Special Senses

The differential anatomy of the ear in rat and guinea pig has been very well described in a study conducted by Albuquerque et al. in 2009 [23]. The guinea pigs are popular as models for auditory research. The external auditory canal is much smaller as compared to rats, thus making visualization of the tympanic membrane easy.

The size and strength of temporal bones allow easy access to the tympanic bulla which is also bigger as compared to rat. Thus, tympanic membrane and the malleus handle are better visible. The malleus and incus bones are fused in guinea pigs; thus, access to stapes and the internal ear is easy. Guinea pigs are best suited for studies related to the administration of drugs through an oval or round window into the internal ear which is further supported by the fact that cochlea has three and a half turns whereas that of rat has two and a half, thus providing better surface area. The vibrissae are sensitive to touch as in other rodents. Olfaction is well developed and plays a crucial role in reproductive behavior. The vision is dichromatic and well developed at birth.

11.4 Housing and Husbandry

11.4.1 Reproduction

The mature female is called a sow and a mature male is called boar. Sexual maturity occurs at around 2 months in females and 3 months in males. The females are continuously polyestrous, nonseasonal breeders. The pelvic bones get fused with age at around 7 months of age, thus predisposing females to dystocia. Thus, older females should not be used for breeding. The copulatory plug is visible after the

act of mating. Estrous cycles range from 13 to 21 days with an average of approximately 16 days. Estrus lasts for 8–11 h during which males will "purr" and fuss over the females and also lick the genital area. Pregnancy diagnosis by manual palpation can be made 2–3 weeks after mating. The gestation length can vary between 59 and 72 days. Litter size varies from 1 to 6. Animals are fully haired, mobile, and active at birth with open eyes and ears. Animals can be weaned at 3 weeks of age.

Both monogamous (one male and one female) and polygamous (one male with several females) system of mating can be followed. Continuous cohabitation of males and females results in around five litters per female.

11.4.2 Nutrition

Guinea pigs are crepuscular and thus feed at dawn and dusk. They are strict herbivores with molar teeth fitted to grinding vegetative matter and, like rabbits, exhibit cecotrophy. They are also fastidious eaters that learn early in life what to eat.

Supplementation of an external source of vitamin C is necessary due to the absence of the enzyme L-gulonolactone oxidase, which synthesizes ascorbic acid from glucose. The feed should be high in fiber. The commercially available chow contains around 18–20% crude protein and 9–18% crude fiber. Water consumption is around 10% of the body weight, and feed consumption is around 6% of the body weight. Vegetables and fruits may be added to the diet if not contraindicated with the ongoing research. When supplementing guinea pigs with vitamin C, it's important to avoid any human multivitamin products as these are often too high in vitamin D and cause metastatic calcification

11.4.3 Housing

Guinea pigs are social animals who live in burrows in the wild. Animals should be housed in groups as much as possible. The dry bulb temperature recommended for a guinea pig is 20–26 °C. The enclosure of guinea pigs should be large enough for exercise and exhibit normal social behavior. The space requirement as per the guide is given in the table. The urine in guinea pig contains high concentrations of proteins and minerals which may adhere to cage surfaces. Thus, acid treatment before washing may be necessary to remove the stains from cages. Nesting material may be provided but not necessary. Guinea pigs are neophobic; thus, new objects or feed items should be introduced with caution. Providing forage encourages natural grazing behavior. The lighting pattern of a 12-h day-night cycle is followed under laboratory conditions. They are housed in floor pens or open-top metal pens or inappropriately built cages under laboratory conditions. Bedding material should be

Table 11.1 Cage space recommendation for guinea pig

Weight (kg)	Floor area/animal, in^2 (cm^2)	Height (cm)
Up to 350 g	60 (387.0)	7 (17.8)
>350	≥101 (≥651.5)	7 (17.8)

Adopted from *Guide for the Care and Use of Laboratory Animals* [24]

Table 11.2 Biological and reproductive data of guinea pigs

Parameters	Values
Adult body weight	700–850 g for females and 950–1200 g for males
Life span	4–8 years
Body temperature	37.2–39.5 °C
Food consumption	6/100 g per day
Water consumption	10 mL/100 g per day
Breeding onset: Male	2 months
Breeding onset: Female	3 months
Oestrous cycle	13–21
Gestation period	59–72 days
Litter size	1–6
Birth weight	42–150 g
Weaning age	3 weeks
Breeding activity	1–3 years
Chromosome number	64
Respiratory rate	69–104 breaths/min
Minute ventilation	100–382.2 mL/min
Tidal volume	1.75 mL
Heart rate	280.0 ± 6.7 beats/min
Arterial systolic pressure	94.0 ± 2.2 mmHg
Arterial diastolic pressure	48.4 ± 1.6 mmHg

Adopted from Suckow et al. (2012), Ediger (1976), Czarnecki & Adamski (2016), and Guyton (1947) [1, 25–27]

provided to satisfy burrowing needs and cover the young ones. The guinea pigs appear nervous but are docile and can be handled easily as they rarely bite. Guinea pigs are quite vocal and produce a variety of sounds which helps with communication. Guinea pigs can hear frequencies ranging from 200 to 2000 kHz (Tables 11.1, 11.2, and 11.3).

Table 11.3 Hematological and biochemical parameters of guinea pig (percentile, 2.5–97.5)

Parameter	Strain and sex	
	13/N guinea pig	
	Female	Male
Hemoglobin, g/dL	12.8–16.3	11.5–15.6
RBC, 10^{12}/L	5.3–6.9	4.7–6.3
MCV, fL	66–83	69–87
MCH, pg	20.2–27.5	21.5–29
MCHC, g/dL	29.2–36.5	29.2–36.9
Platelet, 10^{9}/L	164–417	193–427
WBC, 10^{9}/L	4.7–10.1	4.6–9.1
Neutrophils, %	30–70	24–62
Lymphocytes, %	23–66	31–70
Monocytes, %	2.5–9.3	1–6
Eosinophils, %	0.25–2.5	0.5–5.75
Basophils, %	0–0.5	0–0.625
ALT, U/L	24–67	19–39
Albumin, g/dL	2.2–3.2	2.7–3.3
AST, U/L	27–162	27–99
GGT, U/L	6–16	2.5–21
Creatinine, mg/dL	0.1–0.8	0.1–0.9
BUN, mg/dL	14–26	17–29
Total bilirubin, mg/dL	0.3–0.4	0.3–0.4
Glucose, mg/dL	115–179	80–218
Total protein, g/L	4.7–5.9	4.8–5.6
Amylase, U/L	850–1380	964–1519
Calcium, mg/dL	10.4–12.2	10.5–12.2

Adopted from Genzer et al. (2019) [28]

References

1. Hargaden M, Singer L (2012) Anatomy, physiology, and behavior. In: The laboratory rabbit, guinea pig, hamster, and other rodents. Academic, Cambridge, pp 575–602
2. Verstraete, F. J. (2003). Advances in diagnosis and treatment of small exotic mammal dental disease. In Seminars in Avian and exotic pet medicine (Vol. 12, 1, pp. 37-48). WB Saunders, Philadelphia
3. Barnes MJ (1975) Function of ascorbic acid in collagen metabolism. Ann N Y Acad Sci 258 (1):264–277
4. Basketter DA, Kimber I (2010) Skin sensitization, false positives and false negatives: experience with guinea pig assays. J Appl Toxicol 30(5):381–386
5. Legendre LF (2002) Malocclusions in guinea pigs, chinchillas and rabbits. Can Vet J 43(5):385
6. Timm KI, Jahn SE, Sedgwick CJ (1987) The palatal ostium of the guinea pig. Lab Anim Sci 37 (6):801–802
7. Zaldívar R (1965) Hyperplastic and infiltrating lesions of the stomach in guinea pigs after intramural injection of 3-Methylcholanthrene. Oncology 19(6):433–452

8. Spines RL (1982) Anatomy of the guinea-pig cecum. Anat Embryol 165(1):97–111
9. Potter GE, Rabb EL, Gibbs LW, Medlen AB (1956) Anatomy of the digestive system of guinea pig (Cavia porcellus). Bios 27:232–234
10. Farrar WE, Kent TH, Elliott VB (1966) Lethal gram-negative bacterial superinfection in guinea pigs given bacitracin. J Bacteriol 92(2):496–501
11. Stan FG (2017) Macroscopic anatomy of the gallbladder and extrahepatic biliary tract in the guinea pig (cavia porcellus). Change case scientific works. Series C. Vet Med 63(2):25–29
12. Shaw HM, Heath TJ (1974) The gallbladder of the guinea pig: its concentrating and contractile abilities. Comp Biochem Physiol A Physiol 49(2):231–240
13. Stan FG (2018) Macroscopic anatomy of pancreas in rats, guinea pigs, chinchillas and rabbits. Scientific works. Series C. Vet Med 64(1):15–20
14. Breazile JE, Brown EM (1976) Anatomy. In: Wagner JE, Manning PJ (eds) Biology of the guinea pig. Academic, New York, pp 53–62
15. Florio C, Martin JG, Styhler A, Heisler S (1994) Antiproliferative effect of prostaglandin E2 in cultured guinea pig tracheal smooth muscle cells. Am J Phys Lung Cell Mol Phys 266(2):L131–L137
16. O'Malley B (2005) Guinea pigs. In: Clinical anatomy and physiology of exotic species. Elsevier Saunders, Philadelphia, pp 197–208
17. Stan F, Crișan M, Damian A, Dezdrobitu C, Martonoș C, Gudea A (2015) Heart topography and pericardic ligaments of guinea pigs. Scientific works. Series C. Vet Med 61(1):57–64
18. Cooper G, Schiller AL (1975) Anatomy of the guinea pig. Harvard University Press, Cambridge
19. Ichii O, Yabuki A, Ojima T, Matsumoto M, Suzuki S (2006) Species specific differences in the ratio of short to long loop nephrons in the kidneys of laboratory rodents. Exp Anim 55 (5):473–476
20. Quesenberry KE (1994) Guinea pigs. Vet Clin N Am Small Anim Pract 24(1):67–87
21. Harrell MI, Burnside K, Whidbey C, Vornhagen J, Adams Waldorf KM, Rajagopal L (2017) Exploring the pregnant guinea pig as a model for group B Streptococcus intrauterine infection. J Infect Dis Med 2(2):109. https://doi.org/10.4172/2576-1420.1000109
22. Musa SA, Yahaya FM, Omoniyi AA, Timbuak JA, Ibegbu AO (2016) Comparative anatomical studies of the cerebrum, cerebellum, and brainstem of males guinea pig (Cavia porcellus) and rabbit (Oryctolagus cuniculus). J Vet Anat 9(2):1–14
23. Albuquerque AAS, Rossato M, De Oliveira JAA, Hyppolito MA (2009) Understanding the anatomy of ears from guinea pigs and rats and its use in basic otologic research. Braz J Otorhinolaryngol 75(1):43–49
24. National Research Council (2010) Guide for the care and use of laboratory animals. National Academies Press, Washington, DC, p 55
25. Ediger RD (1976) Care and management. In: The biology of the guinea pig. Academic, Cambridge, pp 5–12
26. Czarnecki R, Adamski M (2016) Factors influencing litter size and birthweight in the newborn long-haired guinea pigs (Cavia aperea f. porcellus). J Appl Anim Res 44(1):71–76
27. Guyton AC (1947) Measurement of the respiratory volumes of laboratory animals. Am J Physiol Legacy Content 150(1):70–77
28. Genzer SC, Huynh T, Coleman-McCray JD, Harmon JR, Welch SR, Spengler JR (2019) Hematology and clinical chemistry reference intervals for inbred strain 13/N guinea pigs (Cavia porcellus). J Am Assoc Lab Anim Sci 58(3):293–303

The Laboratory Hamster

12

Padmakar Tambare and Yogeshkumar Murkunde

Abstract

Hamsters are one of the commonly used animal species in research related to virology and infectious agents. Hamsters are similar to human beings in many metabolic and physiological processes. Though the use of hamsters in research has declined in the last few decades, the genetically engineered Syrian hamster (GESH) model is seeing increased popularity due to advancements in gene-editing technologies like CRISPR/Cas9. The presence of immunologically privileged large highly distensible non-glandular cheek pouches makes hamsters a preferred model for oral carcinogenesis, microvascular investigations of inflammation, ischemia-reperfusion investigations, tumor development, and vascular smooth muscle function. Due to the unique immune system of hamsters, skin allografts are not rejected to the degree as compared to other models and have a higher susceptibility to certain infections. It is already known that for, cell culture experiments, Chinese hamster ovary (CHO) cells are extensively used. This chapter will briefly discuss general anatomy, physiology, husbandry, and reproduction, followed by references to reported research uses.

Keywords

Hamsters · Anatomy · Physiology

P. Tambare (✉)
Translational Health Science and Technology Institute (THSTI), NCR Biotech Science Cluster, Faridabad, Haryana, India
e-mail: padmakar.tambare@thsti.res.in

Y. Murkunde
Centre for Toxicology and Developmental Research (CEFTE), Sri Ramachandra Institute of Higher Education and Research, Chennai, Tamil Nadu, India

© The Author(s), under exclusive license to Springer Nature Singapore Pte Ltd. 2021

P. Nagarajan et al. (eds.), *Essentials of Laboratory Animal Science: Principles and Practices*, https://doi.org/10.1007/978-981-16-0987-9_12

12.1 Introduction

Hamsters are one of the most commonly used animal models in virology and infectious agent research. The usage has been found to decline since 1970 owing to the introduction of a genetically manipulable mouse. Hamsters exhibit many characters that are similar to the physiology and metabolism of humans, e.g., low levels of hepatic low-density lipoprotein (LDL) receptor activity, cholesteryl ester transport protein, intestinal-only ApoB editing [1], a high glycemic response to dietary fructose [2], and increased susceptibility for diabetes and atherosclerosis [3], to name a few. Hamster is becoming increasingly popular due to advancements in gene-editing technologies like CRISPR/Cas9. The genetically engineered Syrian hamster (GESH) model is found to be critical in infectious disease research. The commonly known hamster species include the Syrian, the Chinese, the Armenian, the European, and the Djungarian. Hamsters are characterized by thick fur, big cheek pouches, dark eyes, big ears, long whiskers, sharp claws, short tail, and loose skin, continuously growing incisors, cuspidate molars, and short legs. In their natural habitat, hamsters are nocturnal and live in burrows with cold temperatures and high humidity. The nickname "father of the saddlebags," a Syrian name, is given to hamsters because of incredibly flexible membranes of "cheek pouches" [4], as it extends beyond cheeks to the scapula below the skin along with the body. Large cheek pouches allow them to gather food from various sites and store it in the burrow for later consumption. In the laboratory cage setup, too, hamsters prefer to collect food and hoard it, instead of consuming it directly [5].

Several distinctive characteristics related to anatomy and physiology make hamsters a suitable animal model for research. Hamsters are vulnerable to a wide range of cancer-causing agents and respond to specific neoplasia that does not develop in other animal models. They also respond to multiple congenital disorders, like humans. Moreover, they are also susceptible to disorders related to metabolism, and metabolic disorders can be incited by nutritional manipulation, exhibiting several relevant clinical syndromes.

12.2 Taxonomy

Taxonomy of hamsters is classified as follows [6]:

Order: Rodentia
Suborder: Myomorpha
Family: Cricetidae
Subfamily: Cricetinae

Genus species:

Mesocricetus auratus: Syrian (golden) hamster
Cricetulus griseus: Chinese (stripped-back) hamster

Cricetulus migratorius:	Armenian (gray) hamster
Cricetus cricetus:	European hamster
Phodopus campbelli:	Djungarian hamster (Russian dwarf)
Phodopus sungorus:	Djungarian hamster (Siberian dwarf)

12.3 General Biology/Physiology

12.3.1 Syrian Hamster

The Syrian or golden hamster *(Mesocricetus auratus)* (Fig. 12.1) originated in Syria. All Syrian hamsters available today in the laboratory are considered to come from one litter picked from Syria in 1930 and its descendants that reached the United States in 1938. Saul Adler who was looking for an animal model vulnerable to Leishmania infection initiated the use of the golden hamster [7]. Their vulnerability to infections induced experimentally continues to make them the most suitable animal model for research on diseases caused by infectious agents.

A newborn *M. auratus* pup is hairless, with closed eyes and ears and visible incisor teeth. The ears open on day 4–5, the hair growth are first observed on day 9, and the eyes will open between days 14 and 16 [8]. At birth, the pup weighs 2–3 g, grows to 35–40 g by weaning at day 21, and reaches 85–110 g in males and 95–120 g in females at maturity at 6–8 weeks. The adult Syrian hamster grows 6–8 inches in length. The gender is recognized by the assessment of the distance between the anus and genital papilla; it is longer in male than in female, and distinct scrotum is present in male (Fig. 12.2). This can be best accomplished by lifting the tails of littermates and comparing the perineum. The adult male appears smaller than the female. The males are easily differentiated by noticeable scent or flank glands that appear as dark patches on both flank region and big size testicles.

Fig. 12.1 Syrian (golden) hamsters

Fig. 12.2 Gender identification in hamsters. Note the greater anogenital distance and the noticeable scrotal sac in the male hamster

The hair coat of the hamster is smooth with short hair. The hamster has a small blunt tail. The normal hair coat color is golden reddish, with grayish-white ventrum. Other hair coats are also seen, e.g., albino, cinnamon cream, and piebald; likewise, the hair length can also change [9]. The ears of the hamster are pointed, with dark pigmentation, and the eyes are small, dark, and bright. The average life span is 2 years; males live longer than females [10].

12.3.2 Chinese Hamster

The Chinese hamster (*Cricetulus griseus*), otherwise called the stripped-back hamster, was first utilized as a lab animal model in 1919 [11]. The newborn pup of a Chinese hamster weighs 1.5–2.5 g, reaching the weight of 39–46 g and the length of close to 9 cm when adult.

12.3.3 Armenian Hamster

The Armenian hamster (*Cricetulus migratorius*), otherwise called the gray hamster, was first utilized as a lab animal model in the 1960s due to its vulnerability to mutagens as well as cancer-causing agents. They are almost similar in size and weight to those of Syrian/Chinese hamster [12].

12.3.4 European Hamster

European hamsters are the biggest hamster species, which is nominally three times in size as compared with Syrian hamster. At 25 days, the average body weight is 75 g, approaching 300 and 400 g in females and males, respectively, by 6 months [13]. The average body length excluding the tail in adult males and females is between 27 and 32 cm and 22 and 25 cm, respectively [14]. Their life span is about 3 years in a laboratory-bred condition [15] but 4–10 years in the wild [16].

12.3.5 Djungarian Hamster

The Djungarian hamsters include two separate species: *Phodopus campbelli* (Russian dwarf) and *P. sungorus* (Siberian dwarf). Russian dwarf does not respond to short photoperiod and retains its gray hair coat, while Siberian dwarf changes to pure white hair coat [17]. The body length of Djungarian hamsters varies from 50 to 100 mm, excluding the tail. The weight of these hamsters ranges from 18 to 25 g, which reaches up to 40–50 g in adult males. Their average life span is between 9 and 15 months [18] (Tables 12.1 and 12.2).

Table 12.1 represents normal hematological values in three different strains of hamsters. Table 12.2 represents normal serum blood chemistry in Syrian hamster.

12.4 Management and Husbandry

12.4.1 Housing and Environment

Hamsters prefer to stay in groups during the young, but separate caging is preferred due to fighting behavior in adults. A floor space of 10 in^2 is recommended for hamsters weighing less than 60 g and 13–19 in^2 for a hamster with more than 60 g

Table 12.1 Normal hemogram in hamster

Hemogram	Syrian (golden) hamster [19]	Chinese hamster [20–22]	European hamster [23, 24]
Leukocytes ($10^3/\mu l$)	7.62	5.5	7.4–8.3
Neutrophils (%)	21.9	19.3	23.2–34.6
Lymphocytes (%)	73.5	76.1	60.0–74.0
Monocytes (%)	2.5	2.1	1.0–2.6
Eosinophils (%)	1.1	1.7	0.07–1.13
Basophils (%)	1.1	0.1	0.00–0.02
RBC ($10^6/\mu l$)	7.50	7.1	7.45–7.64
PCV (%)	32.9–59.0	42.1	49.2
Hemoglobin (g/dl)	16.8	12.4	18.0
Sedimentation rate (mm/h)	1.64	3.5	–

Table 12.2 Normal serum blood chemistry in Syrian Hamster

Serum analyte	Units	Syrian hamster [25]	
		Male Mean ± SD	Female Mean ± SD
Protein, total	g/l	63 ± 3.2	59 ± 3.4
Sodium	mEq/l	148.0 ± 3.70	148.0 ± 3.70
Albumin	g/l	43 ± 2.2	41 ± 2.8
Alanine aminotransferase	IU/l	44.7 ± 25.9	50.3 ± 18.3
Urea nitrogen	mg/dl	23.2 ± 4.1	27.5 ± 4.6
Creatinine	mg/dl	0.40 ± 0.89	0.50 ± 0.15
Bilirubin, total	mg/dl	0.3 ± 0.09	0.3 ± 0.13
Glucose	mg/dl	84.0 ± 18.5	100.0 ± 16.6
Uric acid	mg/dl	4.6 ± 0.5	4.4 ± 0.5
Potassium	mEq/l	6.50 ± 0.75	6.40 ± 0.73
Chloride	mEq/l	104.0 ± 3.10	104.0 ± 3.60
Calcium	mg/dl	12.6 ± 0.59	13.2 ± 1.38
Phosphorus	mg/dl	5.40 ± 1.00	5.50 ± 1.09
Creatinine kinase	IU/l	469 ± 174	520 ± 184
Lactate dehydrogenase	IU/l	257 ± 63.6	208 ± 54.7
Alkaline phosphatase	IU/l	–	126 ± 6
Aspartate aminotransferase	IU/l	61.2 ± 39.1	53.3 ± 22.7
Triglycerides	mg/dl	209 ± 53.3	212 ± 52.7
Bile acids	μmol/l	–	0.9 ± 0.2
Cholesterol	mg/dl	143 ± 23.5	158 ± 35.3

body weight. The cage height must be 12 cm from the floor of the cage [26, 27]. Nesting females and pre-weanlings should be provided with a solid bottom cage. The most appropriate bedding materials comprise processed corn cob, hardwood chips, shavings, and sawdust. Generally, corn cob and aspen shavings are used. Hamsters housed in bedding material of larger depth showed profoundly minimal bar biting and exhibited an increase in burrow development than those housed in the lesser depth of bedding material [28]. In hamsters, the usual excretion of urine is very minimal, and it tends to utilize one cage corner constantly for excretion. Bedding materials can be replaced routinely once a week or twice a week and can be kept unchanged up to 2 weeks, specifically if the litter is present inside the cage to avoid unwanted disturbances. Normally, fighting is observed in males and females housed in groups; however, when hamsters were housed together beginning at a younger age, compatible groups have been reported. The cage mates aggression can be reduced by employing environmental enrichment inhoused cages [29]. Suitable nesting material, tubes, pipes, shelters, or burrows that simulate the natural housing environment can be used for providing environmental enrichment for hamsters [29] (Fig. 12.3). Housed cage environment must be kept at around 20 °C–26 °C (68 °F–79 °F) with humidity ranging from 30% to 70% [27]. Hamsters are reasonably adjustable to a cold environment, with one investigation demonstrating that pre-hibernating hamsters feel more comfortable with an environment having a temperature of approximately 8 °C (46 °F), while post-hibernating

Fig. 12.3 Hamster cage with corn cob bedding, enrichments, and availability of food on the cage floor

hamsters exhibit an inclination toward environment having greater temperature approximately 24 °C (75 °F) [30]. The recommended photoperiod is 12–14 h. For breeding animals, it is necessary to provide an extended photoperiod of 14 h. An illumination of 323 lux (30 ft-candles) assessed around 1 m above the floor level is essential for hamsters [27].

12.4.2 Handling and Restraint

Hamsters are naturally aggressive, and chances of biting attacks are expected if they are startled/surprised, awakened, injured, or handled roughly.

Females are more aggressive than males, but regular handling can reduce aggression. European hamsters are way more aggressive, get easily frightened, and threaten to bite. Hamsters are deep sleepers and commonly found in sleep in a daytime and, if disturbed in these circumstances, may roll onto their back and voice their disapproval. To avoid startling them, it is good to wake them up gently before handling. The following are routine methods employed to handle and restraint the hamsters [31].

To take the hamster out from the cage, a small container with a broad mouth can be placed inside the housed cage; the hamster may typically get into the container, and once the hamster is inside the container, it can be swiftly taken out (Fig. 12.4). The hamster may be scooped up with one or both hands (Fig. 12.5). While using

Fig. 12.4 Use of broad mouth container for hamster handling

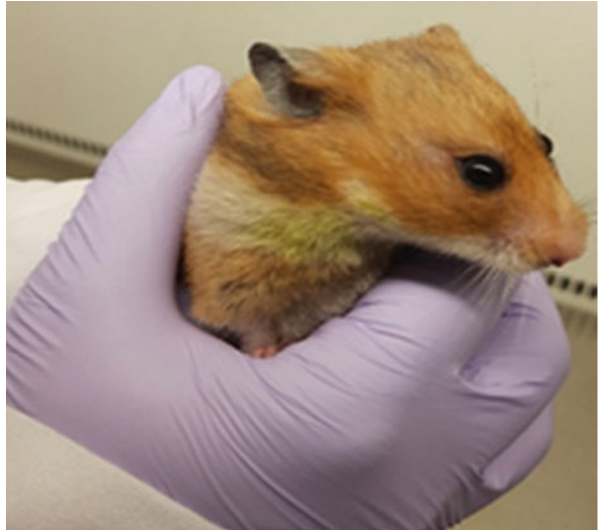


Fig. 12.5 Scooping of a hamster with both hands

these handling methods, care must be taken to avoid the jumping of hamster which can result in injuries. The hamster may be picked up by the scruff (by grasping loose skin across the dorsal cervical area) (Fig. 12.6) by making sure that sufficient skin is held while lifting a hamster to avoid turning around and biting. This method may be used to transfer the hamster from one cage to another or for other purposes for a short

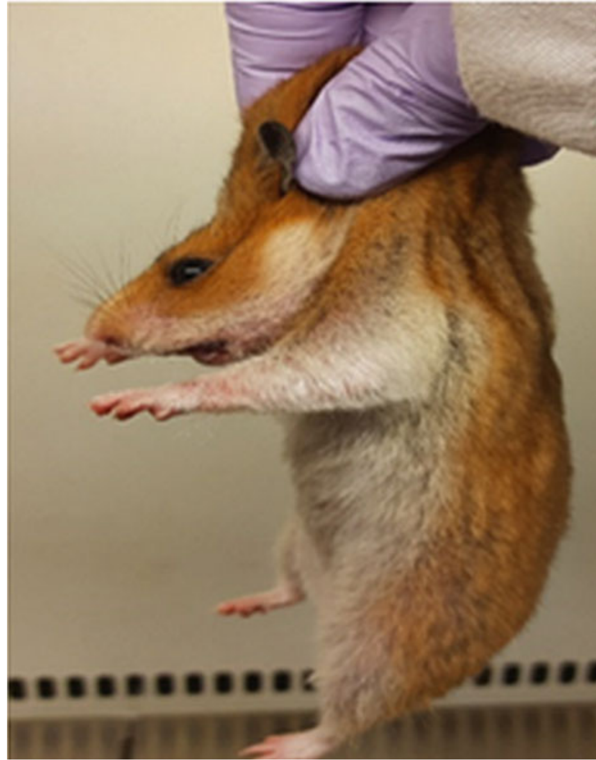


Fig. 12.6 Handling by scruff hold method

duration. Another approach to handling the hamster is by grasping around the head and shoulder with the back lying on the palm of the person holding the animal (Fig. 12.7).

For restraint, approach the hamster similarly, but grasp only the loose skin securely in the hand. After lifting the hamster, the support to the body of the hamster can be provided by rotating the hand in which the hamster is held (Figs. 12.8 and 12.9).

Another restraint method is to gently grasp the loose skin across the back. This can be done by placing the hamster on a cage top or other flat surface and gently pressing the palm down against the hamster's back by keeping the fingers straight. The fingers and thumb are then curled around opposite sides of the animals to gather in as much loose skin as possible (Fig. 12.10). By using the holding hand, the loose skin gathered tightly, and the skin shall be stretched against the abdomen and thorax. The hamster should not be held so tightly that it is unable to breathe nor should it be held so loosely that it can bite the handler.

12.5 Nutrition

Because of the existence of non-glandular forestomach and preliminary digestion using fermentation, nutritional requirements for Syrian hamsters may vary from other rodents. However, when placed on commercial rodent feed, they reported normal growth and reproduction and are considered the basic diet for hamsters.

Fig. 12.7 Hamster grasped around the head and shoulder

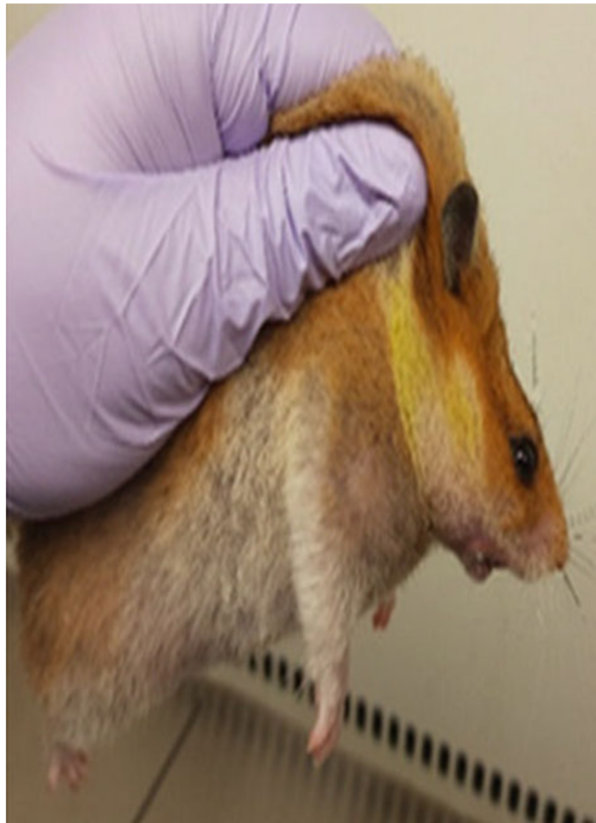


Fig. 12.8 Step 1: Hamster is gently grasped by securely holding loose skin at the back

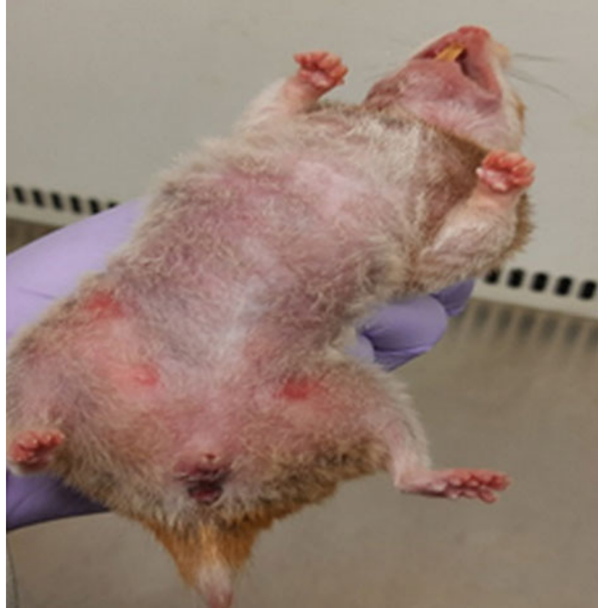


Fig. 12.9 Step 2: Hamster's body is supported by the holding hand

Fig. 12.10 Hamster is held by gently grasping the loose skin across the back, fingers and thumb are curled around the opposite side to gather in as much loose skin as possible

Thus, standard rodent diets as used for rats and mice can be used. European hamsters eat mainly seeds. The wheat germ is reported as a good supplement for Chinese hamster breeders. Djungarian dwarf hamsters are omnivores [32] and may be kept on a diet similar to Syrian hamsters.

During development and growth, the daily average food consumption of Syrian hamsters is between 5 and 9 g, whereas of European hamsters, it is between 1.8 and 2.9 g/100 g body weight [24].

Because of the broad muzzle, hamsters cannot eat from routine food hoppers. If these are utilized, the size of the feed pellets should be small so that the feed pellets fall from the available spaces on the floor of the cage [33]. Placing the food on the floor of the cage, with or without a feeder, is also preferred [26]. A specially designed food hopper may be used for providing food to the hamsters. Hamsters are also coprophagic like other rodents.

Soybean meal is nutritionally more efficient than a fish meal in hamsters. Syrian hamster has high requirements for minerals such as zinc, copper, and potassium compared with the rat [34]. Syrian hamsters also require sources of nonnutritive bulk and the B complex vitamins [35].

Syrian hamsters require fluid at the rate of 8.5 ml/100 g body weight [36], while European hamsters require fluid at the rate of 5 ml/100 g body weight [24] and the Chinese hamsters require 11–13 ml per 100 g body weight [37]. Sipper tubes made of stainless steel are recommended for providing water to the hamsters for drinking purposes. The sipper must be kept low enough to be accessible to the smallest animal.

12.6 Mating and Reproduction

The hamster may exhibit sexual activeness at the earliest when they are 4 weeks old, but pregnancy may not happen until they become 8 weeks old. Sexual maturity occurs when the males attend the weight of 90 g. Estrus in females commences by 6–8 weeks of age [38]. The estrus cycle lasts for 4–6 days with variations between strains [39, 40]. The end of ovulation (day 2) is noticeable by a postovulatory discharge which is opaque, creamy white, and viscous with a distinct odor from the vagina (Fig. 12.11). After this postovulatory discharge, the female can be mated in the evening of the third day (Table 12.3).

Subsequently, on the third day after the postovulatory discharge, a female can be placed inside the male cage roughly 1–2 h before the initiation of the dark cycle. It is proven that the female remains responsive to mating for roughly 16 h from early evening up to mid-afternoon on the next day [41]. A female assuming a lordosis posture with extended hind legs and erected tail is considered ready for mating. The female of the European hamster is responsive to mating only during estrus for a shorter duration and remains in aggression during the remaining period of the cycle [13]. If the female is aggressive or in absence of mating in less than 5 min, the female can be taken out of the male cage; other females can be used for mating and placed inside the male cage. In case when mating takes place, then duos shall be kept

Fig. 12.11 Postovulatory discharge from the vagina

Table 12.3 Reproductive indices of hamsters

Index	Syrian (golden) hamster [19]	Chinese hamster [48–50]	European hamster [13]
Age at sexual maturity	Male, 6–8 weeks (90 g) Female, 8–12 weeks (90–100 g)	8–12 weeks	Males, 60 days Females, 80–90 days
Duration of estrus cycle	4 days	4 days	4–6 days
Length of estrus	6–8 h	6–8 h	–
Gestation	15–18 days	20.5 days	15–17 days
Average litter size (pups)	4–12	4.5–5.2	7–9
Weaned	21 days (35–40 g)	21–25 days	25–28 days

together up to the next light cycle. During the usual dark cycle, ovulation and fertilization normally take place during the early hours of the morning, and this separation day becomes day one of the gestation period. Immediately after mating, females may become aggressive and possibly kill the male. The female is provided with a dedicated cage along with nesting material not less than 2 days in advance and until 10 days post-parturition to reduce litter refusal or cannibalization by the mother. The alternate breeding method allows trios (one male with two females) kept together inside the cage for 7–14 days; subsequently, females can be separated and housed individually for delivery. After mating, if the female hamster exhibits postovulatory discharge on days 5 and 9, then it is considered nonpregnant.

Confirmation of pregnancy may be done at day 4 post-mating by visual examination, a closure of vaginal opening comprising of pale, dry, and scaly perineal tissues [42]. Maternal behavior sets in abruptly during late gestation in female Syrian hamsters, unlike other rodents, where it is exhibited throughout gestation [43]. Just

before delivery, the female exhibits restlessness and frequently shifts between nest building, eating, and grooming. An increased rate of respiration is also an additional indication of impending parturition within the next few hours. Most hamsters will deliver on day 16th of the gestation period, and delivery typically persists for longer than 3 h. Dystocia may occur because of fetal wedging. If the mother with litter undergoes any potential stress or is frightened, then it may result in the cannibalization of pups. On the other hand, a mother usually hides her litter inside the cheek pouch because of transitory stress and takes them out when calmed down.

The gestation period in the Syrian hamster ranges from 15 and 18 days. The litter size is four to twelve, with an average of six to eight pups. The gestation period in Armenian and Djungarian hamster ranges between 18 and 19 days, with an average litter size of five to seven pups. The gestation period in a European hamster ranges between 15 and 19 days, with an average litter size of seven to nine pups.

Hamster pups live with the mothers until at least the first 19 days. The typical weaning period ranges between 21 and 28 days. Usually, the estrus cycle of the mother resumes only after 1–8 days postdelivery [44], except for dwarf hamsters which can mate on the day of parturition and deliver again while weaning the first litter, within 36 days [45].

Phodopus campbelli is known for monogamous parental care by both parents [46, 47], while the males of *P. sungorus* abstain from caring for a litter.

A female's vaginal opening remains sealed, and the size of the male's scrotum decreases with the testes positioned inside the abdomen during the nonbreeding season [16]. In both males and females, the reproductive capability declines roughly around the age of 14 months.

12.7 Genetics

Syrian hamsters have 44 diploid chromosome numbers. Multiple mutations are developed in this animal model since the 1930s [51]. Eighteen mutations are about the coat and eye color. Six mutations are about the neuromuscular system and six for the quantity or texture of hairs. Inbred strains of hamsters were also developed for research on genetically transmissible conditions and particular vulnerability to teratogens and cancer-causing agents [52]. The first useful transgenic hamsters were developed in 2014, which promises the utilization of genetically engineered hamsters as disease models [53].

12.8 Anatomy

The important anatomical features of hamsters which are characteristic and distinguishing are explained below [54].

Harderian glands: These are pigmented lacrimal glands situated posterior to the ocular globes.

Flank glands: The flank glands are cutaneous structures comprised of sebaceous glands that generate secretions in response to androgens. These glands are prominent in males.

Salivary glands: Four sets of salivary glands exist in hamsters, viz., parotid, mandibular, sublingual, and zygomatic glands. The parenchyma of the mandibular and parotid gland is similar to other rodents.

Brain: The brain is characterized by well-developed cerebral hemispheres without sulci, large olfactory bulbs, and cerebellum of about half the size of the cerebrum. The pons and medulla oblongata are longer than wide.

Cheek pouches: These are highly distensible, bilateral invaginations of the oral mucosa lined with stratified squamous epithelium, abundant in mast cells, and extremely vascular, situated in the lateral buccal walls. This is used for temporarily storing the food and bedding materials [55] (Figs. 12.12, 12.13, and 12.14). The pouches are considered "immunologically privileged" as they deficit an intact lymphatic drainage pathway.

Teeth: Incisors and molars in hamsters are continuously growing, but the canines and premolars are lacking. Only one set of permanent teeth is present in hamsters. The dental formula is 2 × (incisors 1/1, canines 0/0, premolars 0/0, and molars 3/3) and consists of a total of 16 teeth.

Lungs: The right lung is divided into five lobes (cranial, middle, caudal, intermediate, and caudal accessory lobes), while the left lung has only one lobe (Fig. 12.15).

Liver with gallbladder: The liver of a hamster is usually brown. It consists of four lobes, two median lobes (dorsal bipartite and ventral) and two dorso-caudal lobes (right and left). The gallbladder is present in the ventral median lobe (Fig. 12.16).

Stomach: The stomach is divided by a distinct constriction into the larger forestomach lined with squamous cell epithelium and a smaller glandular stomach

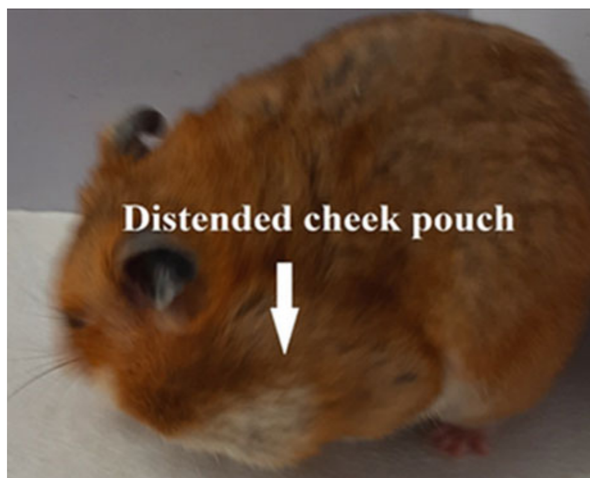


Fig. 12.12 Left cheek pouch distended with feed inside

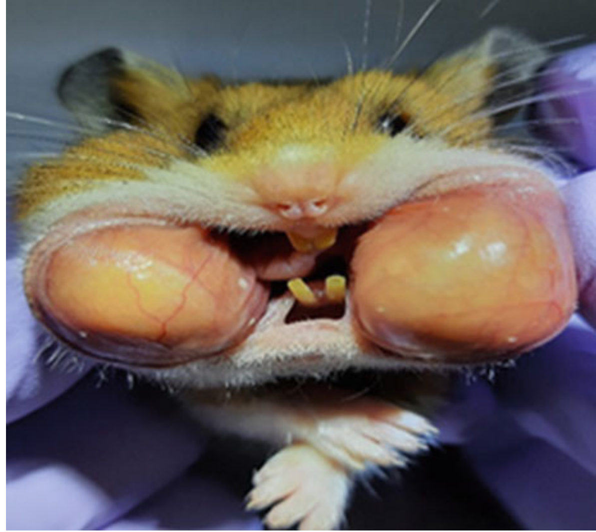


Fig. 12.13 Bilateral cheek pouches have been manually everted for the explanation. The minute blood vessels present in the cheek pouch are visible

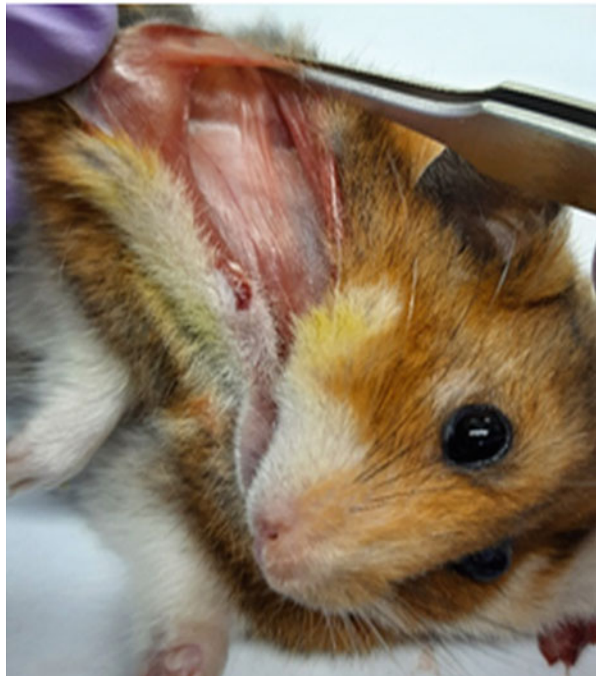


Fig. 12.14 The exposed right cheek pouch. Note the length of the cheek pouch extending up to the scapula

lined with the gastric mucosa. The non-glandular forestomach functions similarly to that of ruminants and contains higher pH levels and microflora which helps in digestion employing fermentation (Fig. 12.17).

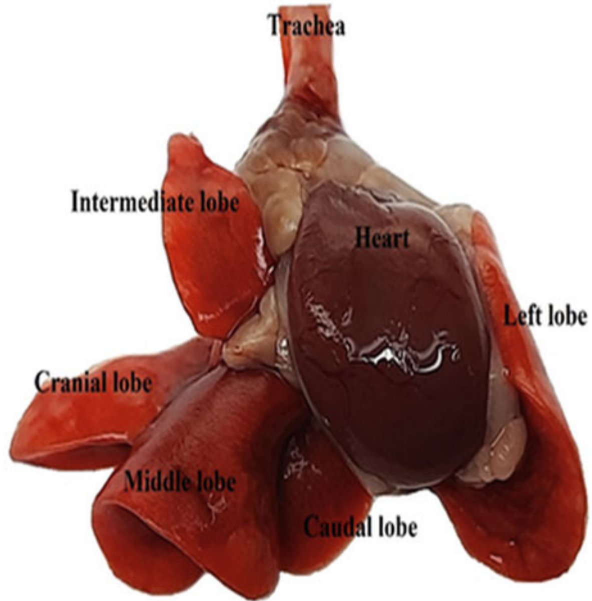


Fig. 12.15 Gross image of the lung and heart

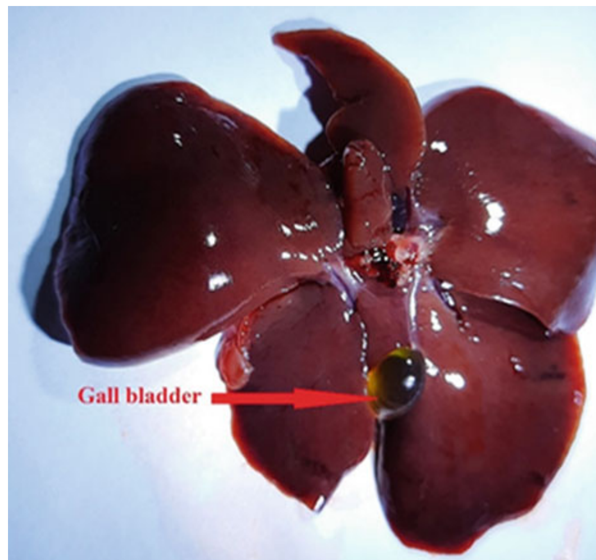


Fig. 12.16 Gross image of the liver with gallbladder

Duodenum: In the duodenum, the entry of a common duct as a combination of bile and major pancreatic ducts is seen about 30 mm from the pylorus.

Jejunum: This has histology similar to the duodenum, with fewer and smaller villi.

Ileum: The ileum is characterized by frequent goblet cells at the broad base of the villi.

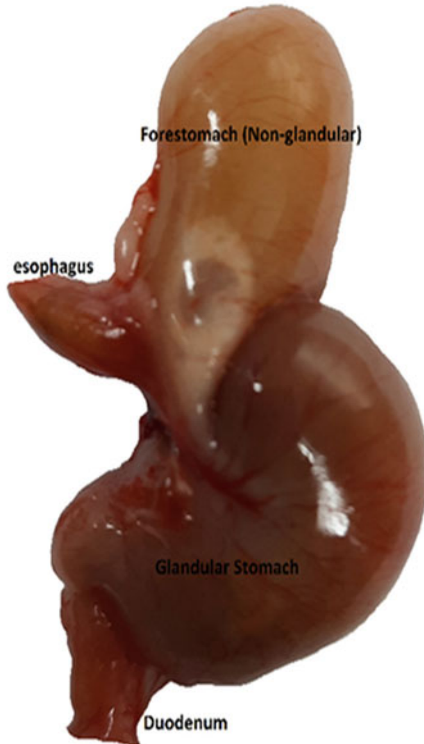


Fig. 12.17 Gross image of the stomach

Cecum: The cecum has a distinct apex, distinct body, and a free mobile base.

Colon: In the colon, the crypts are deeper than the small intestine. The colonic epithelium consists mostly of goblet cells. The lymphatic tissues are spread diffusely.

Rectum: The rectum is longer in males than in females. Many lymph nodes are lined along the lamina propria and submucosa. It has an increased thickening of tunica muscularis and is characterized by fewer goblet cells than the colon.

Pancreas: This has three well-defined, elongated parts (gastric, duodenal, and splenic). The main pancreatic duct and bile duct form a common duct of 15mm length before it enters the duodenum.

Kidneys: The kidneys are bilateral, with a smooth surface, and are reddish-brown. The cortex and medulla have no distinct border.

Adrenal glands: These are located bilaterally at the cranial pole of the kidneys. The adrenal glands in males are approximately double the size of females as they contain reticular cells in large numbers than females within the adrenal cortex [56].

Urinary bladder: The bladder is lined by transitional epithelium. The urethra discharges urine separately from the vagina and anus in females.

Male genital organs: The testes are found externally during the breeding season. However, all organs involute during hibernation. The epididymis is firmly attached to the testes.

Female genital organs: The uterus is bipartite with an undivided body and paired horns and firm cervix. The epithelium closing the vagina disappears seasonally.

Table 12.4 represents the use of hamsters as animal models in various research areas.

Table 12.4 Distinct models of hamsters in research

Research/models	Methods
Cholesterol cholelithiasis	By providing a diet containing excess cholesterol or high sucrose levels [57–59]
Diabetes mellitus	1. Streptozotocin (STZ) is more reliable than alloxan 2. Type 2 diabetes induced by feeding high-fat (15%) diet containing moderate cholesterol level (0.12%) for 3 weeks [60–63]
Oral tumor	Cheek pouch carcinogenesis studies [64–67]
Pancreatic ductal adenocarcinoma	Dispensing of nitrosamines [68, 69]
	Transplantable cell line, PGHAM-1 [69, 70]
Non-small cell lung carcinoma	Carcinogen 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), with or without inclusion of hyperoxia [71, 72]
Simian virus 40 (SV40), polyomavirus	SV40 develop a variety of tumors [73–76]
Renal tumor studies	Administration of estrogens [77, 78]
Vaccinations and immunotherapy treatments	Human adenoviruses, an experimental model for hantavirus pulmonary syndrome, severe acute respiratory syndrome (SARS); [12]
Pulmonary disease model – COPD and emphysema	Intratracheal dose of porcine pancreatic elastase or Feeding a diet deficient in copper [79–81]
Infectious diseases	*Histoplasma* spp., *Mycoplasma pneumonia*, *Clostridium difficile*, *Treponema pallidum*, leprosy, atypical tuberculosis, leptospirosis, scrapie, and *Leishmania infantum* [19]
Naturally occurring disease	Dilated cardiomyopathy (DCM) and hypertrophic cardiomyopathy (HCM). Amyloid-like deposits in their brains Susceptibility to scrapie [82]
Study of prion disease	Transmissible mink encephalopathy (TME), Creutzfeldt–Jakob disease, and Gerstmann–Straussler syndrome (GSS) [12]
Anatomical distinction in disease models	Cheek pouch—Microvascular investigations of inflammation, cancerous growth, vascular smooth muscle function, and ischemia-reperfusion investigations [83–85]
	Lung—Less glandular, chronic bronchitis [86]
	Kidneys are highly responsive to estrogen—Chemical carcinogens on the urinary bladder [87]

References

1. Briand F (2010) The use of dyslipidemic hamsters to evaluate drug-induced alterations in reverse cholesterol transport. Curr Opin Investig Drugs 11:289–297
2. Naples M, Baker C, Lino M et al (2012) Am J Physiol Gastrointest Liver Physiol 302:1043–1052
3. Jove M, Pamplona R, Prat J et al (2013) Nutr Metab Cardiovasc Dis 23:84–93
4. Lea SEG, Tarpy RM (1986) Hamsters' demand for food to eat and hoard as a function of deprivation and cost. Anim Behav 34(6):1759–1768
5. Jones CH, McGhee R, Wilkie DM (1990) Hamsters (Mesocricetus auratus) use spatial memory in foraging for food to hoard. Behav Process 21:179–187
6. ICTV (2013) ICTV master species list 2013—International Committee on Taxonomy of Viruses. http://www.ictvonline.org/virusTax-onomy.asp
7. Adler S (1948) Origin of the golden hamster, Cricetus auratus, as a laboratory animal. Nature 162:256–257
8. Mulder G (2012) Anatomy, physiology, and behavior. In: Suckow MA, Stevens KA, Wilson RP (eds) The laboratory rabbit, Guinea pig, hamster, and other rodents. Academic Press, New York
9. Harkness JE, Turner PV, VandeWoude S, Wagner JE (2010) Harkness and Wagner's biology and medicine of rabbits and rodents. Williams and Wilkins, Media, Pennsylvania, PA
10. Bernfeld P, Homburger F, Adams RA, Soto E, Van Dongen C (1986) Base-line data in a carcinogen-susceptible first generation hybrid strain of Syrian golden hamsters: FID Alexander. J Natl Cancer Inst 77:165–171
11. Yerganian G (1985) The biology and genetics of the Chinese hamster. In: Gottesman M (ed) Molecular cell genetics. Wiley, New York, pp 3–36
12. Miedel EL, Hankenson FC (2015) Biology and diseases of hamsters. In: Fox J, Anderson L, Loew F (eds) Laboratory animal medicine, 2nd edn. Elsevier, pp 209–245
13. Mohr U, Schuller H, Reznik G, Althoff J, Page N (1973) Breeding of European hamsters. Lab Anim Sci 23:799–802
14. Mohr U, Ernst H (1987) The European hamster-biology, care, and use in research. In: Van Hoosier GL Jr, McPherson CW (eds) Laboratory hamsters. Academic Press, New York, pp 351–366
15. Ernst H, Kunstyr I, Rittinghausen S, Mohr U (1989) Spontaneous tumours of the European hamster (Cricetus cricetus L.). Zeitschrift für Versuchstierkunde 32(2):87–96
16. Reznik-Schüller H, Reznik G, Mohr U (1974) The European hamster (Cricetus cricetus L.) as an experimental animal: breeding methods and observations of their behavior in the laboratory. Zeitschrift für Versuchstierkunde 16:48–58
17. Claire HF, Gerald L, Hoosier V (2002) Biology and diseases of hamsters. In: Fox JG, Anderson LC, Loew FM, Quimby FW (eds) American college of laboratory animal medicine. Laboratory animal medicine, 2nd edn. Academic Press, pp 167–202
18. Lawrie AM, Megahy IW (1991) Tumours in Russian hamsters. Vet Rec 128:411–412
19. Heatley JJ, Harris MC (2009) Hamsters and gerbils. In: Mitchell MA, Tully TN (eds) Manual of exotic pet practice. W.B. Saunders, pp 406–432
20. Feeney WP (2012) The Chinese or striped-back hamster. In: Suckow MA, Stevens KA, Wilson RP (eds) The laboratory rabbit, Guinea pig, hamster, and other rodents. Academic Press, Boston, pp 907–922
21. Moore W Jr (1966) Hemogram of the Chinese hamster. Am J Vet Res 27:608–610
22. Yerganian G, Klein E, Roy A (1955) Physiological studies on Chinese hamster, Cricetulus griseus. II. A method for study of bleeding time in hamsters. Federation of American Societies for. Exp Biol 14:424
23. Emminger A, Reznik G, Reznik-Schuller H, Mohr U (1975) Differences in blood values depending on age in laboratory-bred European hamsters (Cricetus cricetus L.). Lab Anim 9:33–42

24. Silverman J, Chavannes JM (1977) Biological values of the European hamster (Cricetus cricetus). Lab Anim Sci 27:641–645
25. Lowenstein DH, Butler DA, Westaway D, McKinley MP (1999) The clinical chemistry of laboratory animals. In: Loeb W, Quimby F (eds), Taylor and Francis, Philadelphia
26. Code of Federal Regulations (CFR) (2013) Title 9; animals and service; Part 3: standards; Subpart B: specifications for the humane handling, care, treatment and transportation of guinea pigs and hamsters. Office of the Federal Register, Washington, DC
27. Institute of Laboratory Animal Resources (2011) Guide for the care and use of laboratory animals. National Academy Press, Washington, DC
28. Hauzenberger AR, Gebhardt-Henrich SG, Steiger A (2006) The influence of bedding depth on behaviour in golden hamsters (Mesocricetus auratus). Appl Anim Behav Sci 100:280–294
29. Arnold C, Westbrook RD (1998) Enrichment in group-housed laboratory golden hamsters. Anim Welf Inform Center Newslett 8:22–24
30. Gumma MR, South FE, Allen JN (1967) Temperature preference in golden hamsters. Anim Behav 15:534–537
31. Silverman J (2012) Biomedical research techniques. In: Suckow MA, Stevens KA, Wilson RP (eds) American College of Laboratory Animal Medicine, the laboratory rabbit, guinea pig, hamster, and other rodents. Academic Press, New York, pp 779–795
32. Sawrey DK, Baumgardner DJ, Campa MJ, Ferguson B, Hodges AW, Dewsbury DA (1984) Behavioral patterns of Djungarian hamsters: an adaptive profile. Anim Learn Behav 12:297–306
33. Harkness JE, Wagner JE, Kusewitt DF, Frisk CS (1977) Weight loss and impaired reproduction in the hamster attributable to an unsuitable feeding apparatus. Lab Anim Sci 27:117–118
34. Newberne PM, McConnell RG (1979) Nutrition of the Syrian golden hamster. Prog Exp Tumor Res 24:127–138
35. Warner RG, Ehle FR (1976) Nutritional idiosyncrasies of the golden hamster (Mesocricetus auratus). Lab Anim Sci 26:670–673
36. National Research Council (1995) Nutritional requirements of laboratory animals, 4th edn. National Academy Press, Washington, DC
37. Thompson R (1971) The water consumption and drinking habits of a few species and strains of laboratory animals. J Anim Sci Technol 22:29–36
38. Slater GM (1972) The care and feeding of the Syrian hamster. Prog Exp Tumor Res 16:42–49
39. Reznik G, Reznik-Schuller H, Emminger A, Mohr U (1979) Comparative studies of blood from hibernating and nonhibernating European hamsters (Cricetus cricetus L.). Lab Anim Sci 25:210–215
40. Erb GE, Edwards HE, Jenkins KL, Mucklow LC, Wynne-Edwards KE (1993) Induced components in the spontaneous ovulatory cycle of the Djungarian hamster (Phodopus campbelli). Physiol Behav 54:955–959
41. Ciaccio LA, Lisk RD (1971) Hormonal control of cyclic estrus in the female hamster. Am J Physiol 221(3):936–942
42. Sato T, Komeda K, Shirama K (1984) Plasma progesterone concentrations during the estrous cycle, pregnancy, and lactation in the Chinese hamster, Cricetulus griseus. Jikken Dobutsu 33:501–508
43. Buntin JD, Jaffe S, Lisk RD (1984) Changes in responsiveness to new born pups in pregnant, nulliparous golden hamsters. Physiol Behav 32:437–439
44. Battles AH (1985) The biology, care, and diseases of the Syrian hamster. Compend Contin Educ Veterin 7:815–825
45. Newkirk KD, Mcmillan HJ, Wynne-Edwards KE (1997) Length of delay to birth of a second litter in dwarf hamsters (Phodopus): evidence for post-implantation embryonic diapause. J Exp Zool 278:106–114
46. Wynne-Edwards KE, Lisk RD (1984) Djungarian hamsters fail to conceive in the presence of multiple males. Anim Behav 32:626–628

47. Wynne-Edwards KE, Lisk RD (1987) Male–female interactions across the female estrous cycle: a comparison of two species of dwarf hamster (Phodopus campbelli and Phodopus sungorus). J Comp Psychol 101:335–344
48. Yerganian G (1958) The striped-back or Chinese hamster. J Natl Cancer Inst 20:705–727
49. Festing M (1972) Hamsters. In: University Federation for Animal Welfare, The UFAW handbook on the care and management of laboratory animals. Churchill-Livingstone, Edinburgh, pp 242–256
50. Moore W Jr (1965) Observations on the breeding and care of the Chinese hamster, Cricetulus griseus. Lab Anim Care 15:95–101
51. Yoon CH, Peterson JS (1979) Recent advances in hamster genetics. Prog Exp Tumor Res 24:157–161
52. Homburger F (1972) Disease models in Syrian hamsters. Prog Exp Tumor Res 16:69–86
53. Gao M, Zhang B, Liu J, Guo X, Li H, Wang T et al (2014) Generation of transgenic golden Syrian hamsters. Cell Res 24:380–382
54. Murray KA (2012) Anatomy, physiology, and behavior. In: Suckow MA, Stevens KA, Wilson RP (eds) American College of Laboratory Animal Medicine, the laboratory rabbit, guinea pig, hamster, and other rodents. Academic Press, pp 753–763
55. deArruda MSP, Montenegro MR (1995) The hamster cheek pouch: an immunologically privileged site suitable to the study of granulomatous infections. Rev Inst Med Trop Sao Paulo 37:303–309
56. Militzer K, Herberg L, Buttner D (1990) The ontogenesis of skin and organ characteristics in the Syrian golden hamster. II. Body and organ weights as well as blood glucose and plasma levels. Exp Pathol 40:139–153
57. Cohen BI, Matoba N, Mosbach EH, McSherry CK (1989) Dietary induction of cholesterol gallstones in hamsters from three different sources. Lipids 24:151–156
58. Khallou J, Riottot M, Parquet M, Verneau C, Lutton C (1991) Biodynamics of cholesterol and bile acids in the lithasic hamster. Br J Nutr 66:479–492
59. Trautwein EA, Siddiqui A, Hayes KC (1999) Characterization of the bile acid profile in developing male and female hamsters in response to dietary cholesterol challenge. Comp Biochem Physiol A Mol Integr Physiol 124:93–103
60. Phares CK (1980) Streptozotocin-induced diabetes in Syrian hamsters: new model of diabetes mellitus. Experientia 36:681–682
61. Fararh KM, Atoji Y, Shimizu Y, Takewaki T (2002) Isulinotropic properties of nigella sativa oil in streptozotocin plus nicotinamide diabetic hamster. Res Vet Sci 73:279–282
62. Masiello P, Broca C, Gross R, Roye M, Manteghetti M, Hillaire-Buys D et al (1998) Experimental NIDDM: development of a new model in adult rats administered streptozotocin and nicotinamide. Diabetes 47:224–229
63. Van Heek M, Austin TM, Faarley C, Cook JA, Tetzloff GG, Davis HR (2001) Ezetimibe, a potent cholesterol absorption inhibitor, normalizes combined dyslipidemia in obese hyperinsulinemic hamsters. Diabetes 50:1330–1335
64. Vairaktaris E, Papageorgiou G, Derka S, Moulavassili P, Nkenke E, Kessler P et al (2007) Expression of ets-1 is not affected by N-ras or H-ras during oral oncogenesis. J Cancer Res Clin Oncol 133:227–233
65. Vairaktaris E, Papakosta V, Derka S, Vassiliou S, Nkenke E, Spyridonidou S et al (2008) H-ras and c-fos exhibit similar expression patterns during most stages of oral oncogenesis. In Vivo 22:621–628
66. Vairaktaris E, Spyridonidou S, Papakosta V, Vyllliotis A, Lazaris A, Perrea D et al (2008) The hamster model of sequential oral oncogenesis. Oral Oncol 44:315–324
67. Gimenez-Conti IB, Slaga TJ (1993) The hamster cheek pouch carcinogenesis model. J Cell Biochem 17:83–90
68. Konishi Y, Tsutsumi M, Tsujiuchi T (1998) Mechanistic analysis of pancreatic ductal carcinogenesis in hamsters. Pancreas 16:300–306

69. Uchida E, Matsushita A, Yanagi K, Hiroi M, Aimoto T, Nakamura Y et al (2008) Experimental pancreatic cancer model using PGHAM-1 cells: characteristics and experimental therapeutic trials. J Nippon Med Sch 75:325–331
70. Fukuhara M, Uchida E, Tajiri T, Aimoto T, Naito Z, Ishiwata T (2005) Re-expression of reduced VEGF activity in liver metastases of experimental pancreatic cancer. J Nippon Med Sch 72:155–164
71. Oreffo VI, Lin HW, Padmanabhan R, Witschi H (1993) K-ras and p53 point mutations in 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone-induced hamster lung tumors. Carcinogenesis 14:451–455
72. Sunday ME, Willett CG, Graham SA, Oreffo VI, Linnoila RI, Witschi H (1995) Histochemical characterization of non-neuroendocrine lung tumors and neuroendocrine cell hyperplasia induced hamster lung by 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone with or without hyperoxia. Am J Pathol 147:740–752
73. Rollison DE, Page WF, Crawford H, Gridley G, Wacholder S, Martin J et al (2004) Case control study of cancer among US army veterans exposed to simian virus 40-contaminated adeno- virus vaccine. Am J Epidemiol 160:317–324
74. Allison AC, Chesterman FC, Baron S (1967) Induction of tumors in adult hamsters with simian virus 40. J Natl Cancer Inst 38:567–572
75. Cicala C, Pompetti F, Carbone M (1993) SV40 induces mesotheliomas in hamsters. Am J Pathol 142:1524–1533
76. Sroller V, Vilchez RA, Stweart AR, Wong C, Butel JS (2008) Influence of the viral regulatory region on tumor induction by simian virus 40 in hamsters. J Virol 82:871–879
77. Li JJ, Li SA (1996) Estrogen carcinogenesis in the hamster kidney: a hormone-driven multistep process. Prog Clin Biol Res 394:255–267
78. Liehr JG (1997) Hormone-associated cancer: mechanistic similarities between human breast cancer and estrogen-induced kidney carcinogenesis in hamsters. Environ Health Perspect 105 (3):565–569
79. Borzone G, Liberona L, Olmos P, Saez C, Meneses M, Reyes T et al (2007) Rat and hamster species differences in susceptibility to elastase-induced pulmonary emphysema relate to differences in elastase inhibitory capacity. Am J Phys Regul Integr Comp Phys 293:1342–1349
80. Soskel NT, Watanabe S, Sandberg LB (1984) Mechanisms of lung injury in the copper-deficient hamster model of emphysema. Chest 85(6):70–73
81. Kleinerman J (1972) Some aspects of pulmonary pathology in the Syrian hamster. Prog Exp Tumor Res 16:287–299
82. Czub M, Braig HR, Diringer H (1986) Pathogenesis of scrapie: study of the temporal development of clinical symptoms, of infectivity titers and scrapie-associated fibrils in brains of hamsters infected intraperitoneally. J Gen Virol 67:2005–2009
83. Svensjo E (1990) The hamster cheek pouch as a model in microcirculation research. Eur Respir J Suppl 12:595–600
84. Hedqvist P, Raud J, Dahlen SE (1990) Microvascular actions of eicosanoids in the hamster cheek pouch. Adv Prostaglandin Thromboxane Leukot Res 20:153–160
85. Bertuglia S, Reiter RJ (2007) Melatonin reduces ventricular arrhythmias and preserves capillary perfusion during ischemia-reperfusion events in cardiomyopathic hamsters. J Pineal Res 42:55–63
86. Hayes JA, Christensen TG, Snider GL (1977) The hamster as a model of chronic bronchitis and emphysema in man. Lab Anim Sci 27:762–770
87. Van Hoosier GL, Ladiges WC (1984) Biology and diseases of hamsters. In: Fox JG, Cohen BJ, Loew FM (eds) Laboratory animal medicine. Academic Press, New York, pp 121–147

The Housing and Husbandry of Zebrafish (*Danio rerio*) in a Laboratory Environment

13

Kalidas Kohale

Abstract

The international research community has witnessed the substantial contribution of the zebrafish, from its origins as a low-cost, model system for developmental biology research that has now been extended to a broad number of scientific disciplines including genetic analysis, gene regulation, angiogenesis, cancer biology, drug discovery, and toxicology. A high degree of genetic homology with humans and the advent of genome-editing CRISPR/Cas9 techniques allow for the creation of zebrafish knockouts that have helped elucidate gene function and identify disease genes in humans. Access to large-scale research resources, genetic markers, and mutant inventory that accumulated over the decades, combined with the availability of commercial aquatic housing systems, has led many investigators to choose zebrafish as a model system. Given its wide-scale usage, understanding new approaches and methods of zebrafish husbandry is essential to ensure efficient propagation and maintenance of healthy and genetically diverse fish lines. This chapter provides basic information about facility design, modern aquatic systems, water quality, husbandry, nutrition, breeding, and health of the zebrafish. Along with present-day resources, updates on the basic requirements for raising the zebrafish in laboratory conditions will benefit the zebrafish research community, facility managers, veterinarians, and technicians.

Keywords

Zebrafish · Aquatic · Larvae · Mutant · Filtration

K. Kohale (✉)
Department of Biological Sciences, TIFR, Mumbai, India
e-mail: kalidas@tifr.res.in

© The Author(s), under exclusive license to Springer Nature Singapore Pte Ltd. 2021
P. Nagarajan et al. (eds.), *Essentials of Laboratory Animal Science: Principles and Practices*, https://doi.org/10.1007/978-981-16-0987-9_13

Abbreviations

DPF Days postfertilization
HPF Hours postfertilization
WRS Water recirculation system

13.1 Introduction

The use of the zebrafish (*Danio rerio*) model has grown in numerous scientific disciplines over the past few decades. This growth is due to its many inherent biological features, including external fertilization, rapid embryonic development, visible organogenesis, higher fecundity, and low-cost maintenance. These traits make it most suitable for studying embryonic development and genetics. It has been used in research since the 1950s [1–3]; however, its potential as a model for genetics was explored by Professor George Streisinger in the early 1980s [4]. Two large-scale screens for zebrafish mutants performed in Tubingen, Germany, and Boston, USA, generated 1500 mutations in more than 400 new genes, involved in a wide range of mechanisms that govern development and organogenesis [5]. This development set the stage for the use of the zebrafish in developmental genetics and, eventually, many other disciplines. Now, the importance of zebrafish as a vertebrate model organism for the study of embryogenesis, neuronal networks, regeneration, and disease is well documented [6–13]. The development of genome-editing CRISPR/Cas9 technologies has revolutionized the field of biomedical sciences, particularly concerning animal model development. CRISPR/Cas9 targets specific DNA sequences in zebrafish and is now the most effective gene-targeting tool that facilitates the modelling of zebrafish for human gene functions and diseases [14].

Zebrafish husbandry and care have evolved along with the growth of the model system. Zebrafish facilities have undergone a dramatic transformation from early fish rooms with glass tanks, beakers, and rodent cages to sophisticated aquaculture systems equipped with water filtration, monitoring, and alarming apparatus. The large-scale zebrafish facility with recirculating aquaculture that was originally developed for the Tubingen 1993 mutagenesis screen [15] set a standard for the commercial zebrafish housing and proved to be the foundation for present-day zebrafish housing systems.

Zebrafish nutrition is another facet of management that has evolved considerably although it still lags far behind other important animal model organisms. The problems associated with the lack of nutritional information and standardization in the zebrafish research community have been addressed [16]. Analysis of four commercial zebrafish diets for iron content revealed higher concentration (0.6–4.6 g/kg) than the maximum requirement of 0.2 g kg^{-1} dry feed, and these higher concentrations could be toxic to fish [17]. New studies are focused on zebrafish nutrition, but the long-term goal should be to identify the daily nutritional

requirements of wild-type and mutant zebrafish so that standardized dietary formulations with sources of good-quality raw materials can be developed.

In general, husbandry and management practices for laboratory zebrafish have evolved over the years; however, regular refinement with new methods and approaches will be better and more efficient use of the model going forward. Data on the external environment, filtration management, husbandry, nutrition, and health of the zebrafish can all be used to achieve this goal.

13.2 Natural Habitat

The zebrafish is a member of the Cyprinidae family of freshwater fishes [18] that were originally found in slow streams, paddy fields, and the Ganges River in East India and Burma. In 1822, zebrafish was originally described by Francis Hamilton in his report [19]. The distribution of this species was recorded from Nadhave and Kalauma rivers in Kumaon Himalayas and also from Rajmahal [20, 21], Balang district, Orissa [22], and Sikkim [23]. In the Western Ghats, distributions have been reported from the Thunga River in Karnataka and Kabini River in Wynand district of Kerala [24]. In 1878, this species was reported from Bengal to Coromandel Coast [25]. Distribution of this species from the Brahmaputra, Gandak, Rapti, Karnal, Ramganga, Ganges, Yamuna, and Indus river drainages was also documented in 1962 [26]. The wild habitation of *Danio rerio* was surveyed in Bangladesh in 2006 [27]. Whitley and colleagues studied wild zebrafish (*Danio* rerio) populations from Nepal, India, and Bangladesh for population genomics [28]. Based on several surveys and studies [29–31], the distribution of the wild population of *Danio rerio* was confined to rivers, small streams, channels, and paddy fields from India, Pakistan, Bangladesh, Nepal, and Myanmar. Fang [32] reported 44 danionin species scattered throughout South and Southeast Asia. Studies of Barman [33] revealed the highest species diversity in northeastern India, Bangladesh, and Myanmar. Most recently, 21 wild populations of *Danio rerio* were reported from streams/rivers of the Western Ghats of Peninsular India to those of the Western and North-Eastern Himalayas. These authors found that *Danio rerio* were mostly associated with habitats of low flow with sandy substrates in secondary and tertiary channels connected with the main channel of a stream/river or habitats adjacent to wetlands with abundant vegetation and paddy fields [34]. The name *Danio* originates from the Bengali term for “rice field” known as “dhani” [35]. At the beginning of monsoon when the water levels rise in rivers, streams, and irrigation channels (home during dry months), zebrafish are thought to move laterally to the flooded areas such as paddy fields where they find nutrient-rich water to spawn. At the end of the monsoon when the water levels subside, adults and juveniles migrate out of the flooded areas into rivers, streams, and channels [36]. Reports from the field studies [37] revealed populations of zebrafish found in mosaic habitat types within the floodplains such as major rivers and their secondary channels, canals, permanent lakes, and floodplain depressions (beels) excavated fish pits (kuas) and household ponds (mathels). Spence and colleagues reported natural distributions of zebrafish extensively in

Fig. 13.1 Wild-type zebrafish (*Danio rerio)* and their natural habitat. (**a**–**d**) Typical wild-type zebrafish collected from the paddy field, Ziro Valley (**e**), and paddy field pond, Hari Village (**f**), in Arunachal Pradesh, India

floodplains of Ganges and Brahmaputra rivers as well as man-made habitats such as canals, fish pits, household ponds, and rice fields [38]. Streams, drainage ditches, and ponds close to the rice fields are other typical habitats of zebrafish reported by McClure and colleagues [39]. From the studies of Junk and colleagues, fish abundance is noticed at the border of channels where plant beds create varying habitats and availability of food is plentiful [40]. In one such survey, zebrafish have been found in tributaries of main rivers such as the Ganges and Brahmaputra but have not been found in main rivers [41] revealing slow stream fish. Observations of Paul have been supported by the findings of Jackson and colleagues where zebrafish tend to move into shallow water or complex habitats or leave the site to avoid predators in the rivers [42]. Recently, Dani Rupa collected wild-type zebrafish from rice field and pond in Arunachal Pradesh, India (Fig. 13.1) for the assessment of genetic diversity of zebrafish (*Danio rerio*) [43].

13.3 Zebrafish Facility Design

As the zebrafish has become a major model organism for research, there is an ever-increasing demand to design and establish modern and often centralized facilities. For any fish research program, thoughtful facility design is critical, with a layout that maximizes the use of the available space, facilitates smooth traffic flow, ensures personnel safety, holds up to environmental challenges, and allows for future

expansion. All interested parties (i.e., researchers, veterinarians, technicians, etc.) must be represented during the planning and design stages for a given facility, since their respective needs all must be met when it is operational.

While common principles will be important in any facility, regardless of its size, research goals and available space will largely dictate its design. Facilities can range from a small room that accommodates a few large tanks or several stand-alone units to large, centralized facilities holding multi-linked systems with hundreds or even thousands of tanks.

If space and resources are available, a fish facility layout should be composed of discrete spaces for quarantine, a nursery for larval rearing, and an area for adult fish holding. These areas should be compartmentalized to the greatest extent possible. Fixed or mobile shelving units (trolleys) can help make efficient use of available space, especially for breeding cages and other procedures. It is important to ensure the rooms are designed with sufficient space between rows of fish tanks to allow movement of people and trolleys. Long benchtops with a sink or sinks are required for handling the fish tanks and the collection of embryos. A battery of conical flasks (imhoff cones) for brine shrimp and/or rotifer culture can be arranged in its own discrete space or, if necessary, within the fish housing area.

The design of the zebrafish facility should accommodate space for the main fish holding and supporting activities (Table 13.1). Rooms for HVAC/AHU, return water treatment assembly, and RO water purification are physically sited away from the main fish aquaria and provide separate entry for ease of servicing. This is also crucial to minimize vibration, heat, and undesirable effects associated with the mechanical equipment [44] which may negatively affect aquatic life [45]. It is when looking at options for the physical layout of the facility that the designer must consider the substantial mass of the water (water weight: 8.3 pounds per gallon) that will be stored in the aquaria. Given this reality, basement locations are often preferred, but the upper-level sites can also be utilized if the structural strength of the slab is reinforced and a good drainage system is put in place to prevent leakages [46].

Considering the nature of the fish room environment with the presence of a large volume of water, high humidity, and elevated temperatures, the selection of impermeable and oxidation-free material is a key for base construction and fixtures. The selection of the appropriate materials will reduce the likelihood of fungal infestations and water damage in these components.

Aquatic life support systems require an ample, on-demand supply of water, and thus, the storage of the water inside the facility is of paramount importance. It is recommended that dedicated pipelines supply the RO purification system and sanitation areas, along with the provision of backup storage.

Multi-linked centralized zebrafish systems are custom-made, designed, and fabricated by commercial vendors within the available area provided by the institution. Depending on the locations of life support systems and the aquaria, points for the floor drains will be identified. For such facilities, multiple floor drains or even trenches are preferable at the lowest point in the room but will require additional maintenance to reduce pests. It is important to ensure that drain covers are easily removed for inspection and cleaning and access for pest control measures. It may not

Table 13.1 Zebrafish facility components and associated activities

Components	Activities carried out
Fish quarantine	(A) Isolated tank (s) within fish holding area in small fish setup. (B) Separate room with one or more stand-alone units in large-scale facility, for quarantine of newly procured fish, larvae and embryos, breeding, embryos bleaching, and raising the larvae. In both the arrangements (A and B), sharing of water between the main fish holding area and quarantine room is avoided. Provision of a separate set of fish housing accessories
Breeding	Isolated space in small setup or a separate room in a large-scale facility. Stand-alone or multi-linking units for holding adult breeder stock, setting breeding crosses, obtaining embryos, bleaching, embryo incubation, and larval raising. Independent water supply
Nursery	Isolated space in small setup or a separate room in a large-scale facility. Stand-alone or multi-linking units for raising larvae and juveniles. Independent water supply
Fish housing	Separate area with the stand-alone or multi-linking system for holding adult breeders, surplus fish, and potential old adults
Fish experiments	Separate setup for holding the fish during chemical screens, behavioral studies, or other experimental purposes depending on the research program.
Live food preparation room	Culture and harvesting of artemia, paramecium, or rotifer. Storage of foods/diets, salts, and buffers
Tank sanitation station	Central cage washing station for cleaning dirty tanks, disinfection, sterilization, tanks drying, nets sterilization, and temporary storage of carcasses
Fish laboratory	Handling and observation of embryos during experiments, embryo manipulation, treatment, autopsy, sample collection, and water testing
RO water plant	Isolated space in small setup or a separate room in multi-linking systems for setting RO/DI purification plant, storage tanks for RO water, and municipal water
Effluent treatment and sterilization	Life support system including assembly of filters (mechanical, biological, and chemical filtration), UV sterilization, aeration, degassing of the fish water, and servicing of the system
HVAC/AHU	Installation of air handling units (AHU) for ventilation, temperature, humidity control units, and maintenance
Office	Administrative activities, records keeping, and storage of stationary
Filters accessories stores	Storage of filters, surplus tanks, lids, baffles and plumbing materials, floor disinfectants, and bleaching solutions

be a good idea to include floor drains in the design especially when the aquatic facility is planned at the basement site in low-lying areas and water lodging is often an issue. On rainy days, floor drains may prove a gateway to the backflow of the rainwater causing a flood-like situation in the facility. Under such circumstances, facilities without floor drains should have a system of removing spillage water either by sump pumps or by frequent mopping. It would be ideal to install sensor-based

alarming devices to detect containment of water bodies in the facility if a flood-like situation arises.

Given the wet environment in an aquaculture facility, special attention should be given to electrical infrastructure: lighting, outlets, and any electrical components [44, 46]. All fixtures and equipment must be ground faulted and sealed to prevent moisture damage.

13.3.1 Water Source

Water is a living substrate for the fish and is a critical infrastructural requirement in aquaculture systems. The water supply should be from a reliable source. The aquatic facility can receive water from a bored well, river, lake, or other water body; however, in all cases, it must be conditioned and decontaminated before it enters the aquaria. In general, municipal water piped from the lake or dam for human consumption is usually treated as per the national drinking water standards before it reaches the sink (Indian Standards, IS, 10500:2012). Such water can be used without much purification, but the removal of chlorine or chloramine by charcoal filters and decontamination of living organisms (bacteria, viruses, and fungi) by UV sterilization should be the minimum requirement. In most of the groundwater, water hardness is achieved naturally from the crumbling of limestone, sedimentary rocks, and calcium-bearing minerals; however, the total hardness (very hard $>$180 ppm, mg/L, $CaCO_3$) will vary from place to place and is dependent on the geographical location [47]. Knowing this fact, the groundwater should be pre-treated with reverse osmosis or deionization to reduce total dissolved solids to near zero. This will require re-addition of salts and sodium bicarbonate to adjust conductivity and pH, respectively. Pre-treatment of source water using reverse osmosis will thus ensure a high level of water quality and consistency [48]. It is also practical to mix hard water with deionized or reverse osmosis treated water to adjust the hardness between 100 and 150 ppm.

13.3.2 Water Purification, Effluent Treatment, and Sterilization

Good-quality water is essential for the physiological and behavioral well-being of the fish. In a large-scale facility, a dedicated water purification plant with regular servicing is elemental to ensure good-quality water. In the first stage, freshwater from the main supply is purified before entry into the aquarium. Various methods employed in the water purification process include distillation, reverse osmosis (RO), or deionization. In the second stage, fish aquaria effluent is treated and disinfected continuously as it recirculates throughout the aquaculture systems. Schematic representation of the cleaning and sterilization process, i.e., biological, mechanical, and chemical filtration and UV lamps sterilization, is outlined (Fig. 13.2). In the water recirculation system (WRS), the whole filtration assembly is integrated as a part of the system (fish housing system). Significant expenditure is

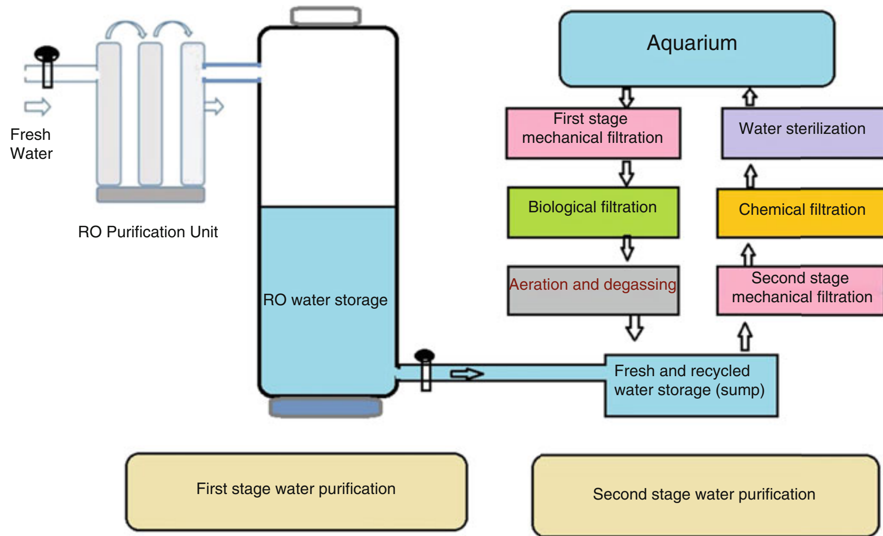


Fig. 13.2 Purification of raw water, effluent treatment, and sterilization process. In the first stage, water from the municipal source is purified using reverse osmosis/deionization, to remove natural hardness and other impurities. RO/DI water enters into the main aquaria after re-adding with ultrapure Red Sea salts. In the second stage, fish aquaria water (effluent) is treated by passing through mechanical filters (first step, filter pads, mats), biological filters (siporax media, plastic beads), mechanical filters (second step, 50 μm filter bags, cartridges), and chemical filters (activated charcoal). Finally, this water is sterilized through UV exposure and reenters the fish aquaria

incurred toward consumables and filter servicing, so researchers must plan a yearly budget to support the running cost.

13.3.3 Water Chemistry

In the aquaculture system, water chemistry includes pH, salinity, alkalinity, hardness, dissolved oxygen, carbon dioxide, phosphate, and nitrogenous compounds. These factors and the interactions between them exert both direct and indirect impacts on the physiology of fish. These vital parameters must be maintained stably within a specific range that is favorable to the health and well-being of zebrafish [48].

pH refers to hydrogen ion (H+) concentration in the culture water that ranges from 0 (strongest acid) to 14 (strongest base) with 7 representing a neutral solution. In natural habitats, zebrafish exist in slightly basic water, ranging in pH between 6.6 and 8.2 with seasonal fluctuation [27, 39]. Contrary to these observations, when the zebrafish were exposed to varying levels of pH for 2 h in acute studies, the median LC50 was found at pH 3.9 and 10.8 [49], without known optimal pH [50]. Aquaculture researchers prefer to maintain pH levels between 7 and 8, as it is found to be optimal for fish health and suitable for the metabolism of nitrifying bacteria in the biological filters [51–53]. The conversion of total ammonia nitrogen (TAN) into less

toxic nitrate by nitrifying bacteria in biological filters drives down pH [53]. Therefore, the buffering capacity of the system water needs to be increased by the addition of sodium bicarbonate. This counteracts acid production and helps to maintain an optimal pH level [54]. Buffering of the system water is also essential for the nitrifying bacteria in the biological filter. Failure to buffer the water will cause the pH to drop below the threshold required for the metabolism of nitrifying bacteria, resulting in high ammonia in the water that can potentially stress, injure, or even kill the fish [44]. An episode of acute mortality was reported in zebrafish when the pH level dropped below 4 [55].

Conductivity/salinity refers to the concentrations of dissolved salts and ions in the freshwater and is usually measured in microsiemens (uS). The sum of dissolved ions such as sodium (Na+), potassium (K+), calcium (Ca^{2+}), magnesium (Mg^{2+}), chloride (Cl−), bicarbonate (HCO_3-), and others determines the conductivity value in the solutions. The term salinity is generally used for solutions with high ion concentrations and is measured in units of part per thousand (ppt) [54]. Osmoregulation is an important, energy-dependent process in freshwater fish through which their internal body salts and water are balanced against the salinity of the surrounding water [50]. Zebrafish housing systems monitor and regulate salts (sodium chloride) and may be maintained at levels that reduce the energetic cost of osmoregulation, which helps to conserve energy for growth and reproduction purposes [50]. In natural habitats, zebrafish are adapted to survive in a freshwater environment with the conductivity of the water range between 100 and 2000 uS (0.006–1.3 ppt), whereas in culture systems, they are typically maintained within a broad range of 200–3000 uS (0.5–2 ppt) [52]. Salinity is measured by the change in water density, optical properties, or conductivity. In WRS, conductivity in the fish water is maintained by adding sea salt solution automatically. Salt concentration at a higher level may be beneficial to fish health [56]. One study found that fish showed depressed larval survival and breeding rates in soft water [54].

Alkalinity can be best described as the concentrations of titratable ions that exist in the solutions. In other words, alkalinity is the buffering capacity of the water that is important for balancing pH in the culture water and also incorporates bicarbonate ions essential for the nitrification process in the biological filtrations [53]. High alkalinity thus affects the metabolism of the nitrifying bacteria and inhibits the conversion of the nitrogenous compounds. The alkalinity is measured in units of parts per millions (ppm) of calcium carbonate (CaCO3). In WRS, the alkalinity is most frequently regulated through the addition of sodium bicarbonate, the buffer used for maintaining the ph. The recommended level of alkalinity in the system water ranges between 50 and 75 ppm and may not be problematic at even higher concentrations if the pH is stable [53].

Hardness represents the measurement of divalent ions such as calcium (Ca^{2+}), magnesium (Mg^{2+}), ferrous ion (Fe^{2+}), strontium (Sr^{2+}), and manganous manganese (Mn^{2+}) in solutions [57, 58]. These ions are involved in several physiological processes, including brain functions, bone formation, blood clotting, muscular function, neurological function, and osmoregulation [58, 59]. The fish also receive these ions from their diet. In natural water, hardness is achieved from the crumbling

of limestone, sedimentary rocks, and calcium-bearing minerals [47], which is dependent on the location. When raising zebrafish in a laboratory environment, pre-treatment of the sourced water is often necessary to remove natural hardness and other impurities. Knowing this fact, municipal or groundwater should be treated to remove total dissolved solids using reverse osmosis (RO) or deionization (DI) system before it reaches aquarium. Re-addition of high-quality sea salts in RO water is essential because the process of RO removes natural salts from the water. To match the natural requirement of zebrafish that is originally found in freshwater, the hardness in the system water should be maintained between 75 and 200 ppm (mg/L) [53].

Dissolved oxygen (DO) is essential for the survival of fish and also important for the metabolism of the nitrifying bacteria in biological filters. Under standard conditions, the solubility of oxygen in water is very low (10.08 ppm) which is further reduced by elevated water temperature, altitude, and salinity [54]. Along with these limiting factors, the demand of the fish and aerobic bacteria for DO increases with the increased water temperature, higher fish density, and high feeding rates. In tanks, uneaten food and decomposed organic matter provide a rich substrate for the bacterial proliferation that consumes more DO. Under such situations, DO can fall below the threshold level [54]. Studies indicate that aquaculture water with low DO can result in more mortalities than any other parameters [44]. Adverse effects have been reported when DO levels fall below 5.0 mg/L in a warm water system [60]. For normal fish respiration and bacterial metabolism, it is recommended that DO levels are maintained at or near saturation (6–8 ppm) at all times, although a lower standard of 5–6 ppm is set by the traditional food aquaculture systems [53].

Carbon dioxide (CO_2) is produced in the aquarium as a by-product of the fish and bacterial metabolism, as well as the result of acid-base reactions in the recirculating culture system. It is found that higher levels of CO_2 in water reduce the ability of hemoglobin to bind and transport oxygen molecules, which leads to a reduction in blood pH [61]. At the extreme concentrations, CO_2 has a deleterious effect on fish health that can result in unconsciousness (15–20 ppm) [53] and even death [62]. Measures such as increasing aeration, removing solid waste, reducing fish density, increasing water exchange, and room ventilation are effective at bringing down elevated CO_2 levels in system water [54].

Total dissolved gas, nitrogen (N_2) gas has the highest concentration (78%) in atmospheric air with 16.36% solubility in water [53]. It is a common phenomenon that the supersaturation in aquarium water occurs when the equilibrium between total gas pressure and the atmospheric gases is disturbed. This generally happens as a result of pressurized water in plumbing, rapid warming of cold water in the system, and a small leak on the intake (impeller) side of a water pump [62]. In supersaturated water, excess gas, mostly N_2, may leak into the blood circulation of the fish and cause illness or death. Generally, saturation levels of 110% or more have deleterious effects on the fish health [59], and N_2 gas in supersaturated water is a major cause of gas bubble disease in zebrafish [54].

WRS systems are generally equipped with a set of sensors (temperature, pH, conductivity, DO, total gas pressure), monitoring devices, and dosing pumps for the

Table 13.2 Recommended water parameters, normal values, testing kits, and frequency in zebrafish [44]

Parameters	Target range	Available testing methods	Testing frequency
pH	Stable, 6.8–8.5	Colorimetric kit, automated monitoring system	Continuous daily
Salinity, g/L	Stable up to 0.5	Refractometer, automated monitoring system	Continuous daily
Alkalinity, mg/L	Stable, 50–150	Colorimetric kits	Monthly
Hardness, g/L	Stable, 75–200	Colorimetric kits	Monthly
Total ammonia nitrogen, mg/L	Zero	Colorimetric kits	Daily[a] weekly
Nitrite, mg/L	Zero	Colorimetric kits	Daily[a] weekly
Nitrate, mg/L	Up to 200	Colorimetric kits	Daily[a] weekly
Dissolved oxygen, mg/L	Not less than 4	Colorimetric kits, automated monitoring system	Continuous monthly
Carbon dioxide, mg/L	Not more than 20	Colorimetric kits	Monthly
Temperature, °C	Stable, 24–30	Handheld thermometer, automated monitoring system	Continuous daily

[a]At the system startup

salt and buffer solutions. Sensors detect the concentrations/levels of the given parameters, and that information may be relayed to a data log that can be displayed on monitoring devices for quick visual check-ins. Sensors may also relay information to dosing pumps that are enabled if salt and pH values of the water drop below set thresholds. If systems are equipped with heating and chilling equipment, they may also communicate with sensors to help maintain optimum temperature. It is advisable to cross-check sensor-measured parameters using portable instruments or test kits available in the market. Common water parameters, normal values, available testing kits, and recommended testing frequencies are presented in Table 13.2.

13.4 Zebrafish Housing Systems

Maintaining the physical and chemical parameters of the water within a favorable range for fish health and well-being is the most elemental and often challenging management goal in any aquaculture system. Aquatic animals may be easily stressed due to poor-quality water, and this can directly affect growth, metabolism, development, immune function, physiology, behavior, stress, reproduction, and many other parameters [54]. The toxic elements in fish excreta and decomposing feed exert major impacts on water quality. In static water housing situations where the exchange of the water is done manually to keep the nitrogenous compounds in water at acceptable levels, water quality can be ensured by housing fish at lower

density, avoiding excess feeding, and performing regular water exchanges. The rate of water exchange can be flexible depending on the levels of ammonia, nitrite, or nitrate in the fish water, however; 5–10% water exchange per day will help to remove the traces of these compounds [44]. In WRS, effluent water is detoxified by passing it through a series of filters. This is achieved via an effective biological filtration process that oxidizes highly toxic ammonia into nitrite and then to a less toxic nitrate compound. Further, with the flexible water exchange rates, the nitrate compounds are removed from the fish water to the traces that are acceptable to the fish.

In *static water tanks*, *fish* are maintained in non-flowing water using glass tanks, beakers, or rodent cages. Fish are generally kept at very low density (1–3 fish/L), and up to 30% of the tank water is replaced daily to keep total ammonia nitrogen (TAN) at zero or trace levels acceptable to aquatic life. However, the percentage of water exchange will vary with the number of fish in a container, size of the container, and amount of feed put into the tank. This is a relatively simple, low-cost setup to establish but only accommodates a small number of fishes in a given space footprint with high labor requirements (Fig. 13.3a). There are also questions about how well fish tolerate frequent disturbances and handling, as well as swings in water quality that may occur under these conditions.

In *flow-through tanks*, fish are kept in aquaria with an adjustable inlet for water inflow and an outlet covered with a net or mesh to prevent fish from escaping with the exiting water (Fig. 13.3b). The rate of daily water exchange between 5% and 10% is adjusted to maintain TAN at zero or trace values in fish water [44]. In this type of system, one should avoid overfeeding because uneaten feed decomposes quickly and leads to the proliferation of aerobic bacteria that consume DO in the water [61] and decrease its concentration in the water. Tanks may be manually cleaned by siphoning fish excreta and surplus feed regularly. Municipal authorities routinely use chlorine and chloramine to disinfect drinking water. Chlorine is less stable and easy to remove from the water [54]. As a minimum requirement, chlorine is removed using charcoal filters, and water is sterilized by UV exposure before it is used in the fish systems. Under standard conditions, DO is poorly soluble in water, and its solubility decreases with the increasing water temperature, altitude, and salinity. In addition to its low solubility, fish and aerobic bacteria consume more DO with the elevated water temperature, excess feeding, and high fish density. DO levels are maintained between 6 and 8 mg/L in the water at all times [53]. Contrary to the static aquaria, a large volume of water is issued in a flow-through system. On the other hand, a static system is space-intensive and high maintenance and is more viable under circumstances where good-quality water is available at a low cost [56].

WRS, these systems are equipped with a series of filters that allow for the recycling of water after cleaning and purification (Fig. 13.3c). These provide much cleaner water and high-water exchange to remove ammonia and other waste in the fish water [44]. Biological filtration is a critical process in WRS that converts toxic nitrogenous materials generated from the fish excreta and decomposing feed (ammonia) and nitrite (NO_2) into far less toxic nitrate (NO_3). Biological filters may consist of plastic beads, gravel, or siporax media, packed in the cassettes or bags to harbor

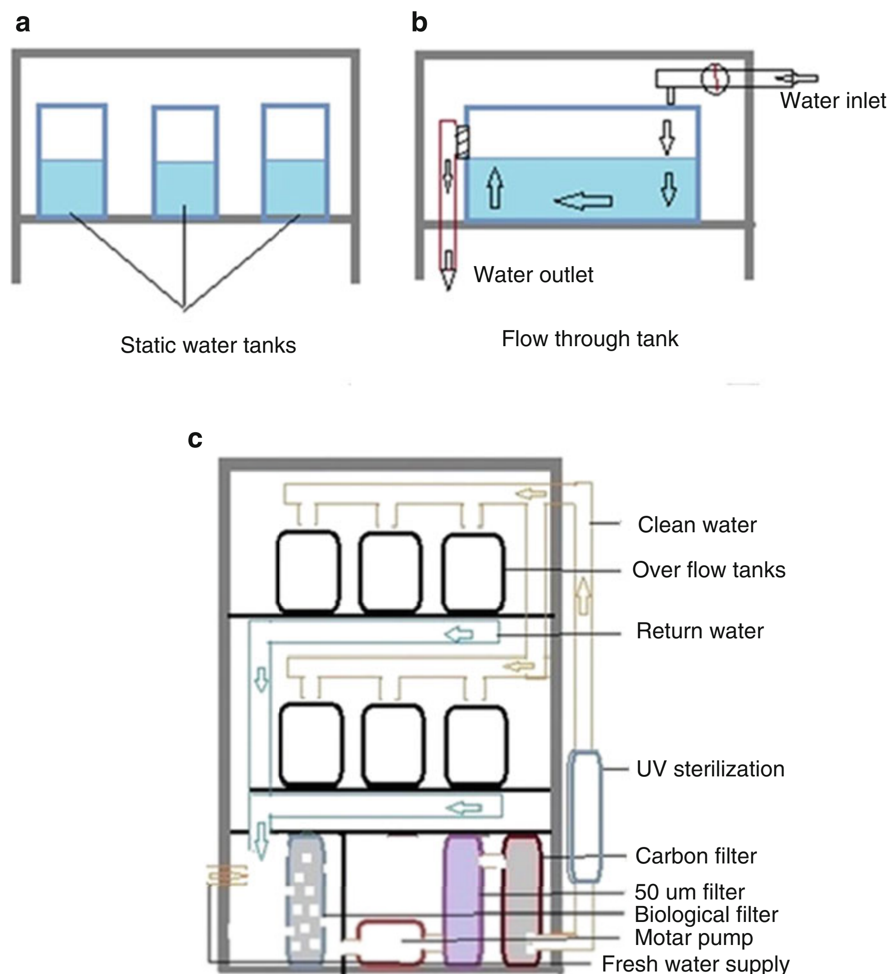


Fig. 13.3 Type of aquaria systems available for raising the laboratory zebrafish. (**a**) Static water system includes glass tanks, rodent cages, or beakers. These containers can be stacked on racks or table top. (**b**) Flow-through system with continuous water inflow or regulated water inflow using adjustable valve. Tanks are receiving fresh water from one end and drained out from another end. (**c**) Stand-alone unit with continuous water recirculation. Effluent water treated by passing through series of filters (mechanical, biological, and chemicals), sterilization, and recycled to the fish tanks

colonies of the nitrifying bacteria. The bacteria *Nitrosomonas* sp. oxidizes toxic ammonia into nitrite [63, 64], which is further converted into less toxic nitrate (NO_3) by *Nitrobacter* sp. [65]. The process of nitrification takes reasonable time to establish itself in a new system, often up to 50 days, after which point the biological filter would be considered to be fully functional [53, 66, 67]. Therefore, care should be taken while raising the fish in a newly installed system. If possible, the new systems

should be loaded with less fish initially, and nitrogenous compounds in the water must be monitored frequently. After ensuring the activation of biofilters, systems can be housed with full strength. At least 1 month is required for the colonization of a healthy population of nitrifying bacteria (capable of removing the ammonia and nitrite) at normal feeding rates [60].

Mechanical filtration is essential to remove uneaten food particles and fish feces which constitute solid waste in the fish water. Solid wastes reduce the efficiency of biological filtration by lowering the oxygen concentration in the system as heterotrophic bacteria consume oxygen during the process of their metabolism [60]. While the breakdown of solid waste generates significant amounts of ammonia nitrogen, it also stimulates the growth of heterotrophic bacteria. These bacteria may occupy significant space on the biological filters, thus reducing the substrate for the growth of nitrifying bacteria, involved in the process of biological filtration [44]. Settleable, suspended and fine, or dissolved solids are three types of waste, generated in the recirculating aquaculture system [68]. Removing settleable and suspended solids from the water is critical before it enters the biological filter. The process of the mechanical filtration in the recirculating fish systems helps to remove these solids. In the first step, the effluent water flowing from the multiple aquaria runs into a common gutter and passes through a filter pad, usually made up of polyester fiber that catches the settleable solids and removes them from the waste stream. The suspended particles that are not removed during the first stage of filtration move along with the water flow and are then trapped in the secondary mechanical filter that has a small pore size (<50 μm). Various types of mechanical filters, including sand/bead filters, drum filters, filter bags, or cartridges, are commonly employed in recirculating aquaculture systems. During normal operation, these filters eventually become clogged with solid waste during the process of effluent filtration, and this reduces their efficiency. Therefore, regular cleaning or replacement of the filters is necessary, which can either be automated or be done manually.

Biological filtration in recirculating aquaculture system refers to the conversion of total ammonia nitrogen, built up during the fish metabolism, and breakdown of the solid waste such as uneaten food and fecal matter [44] into less toxic forms of nitrogenous waste. In water, unionized ammonia (NH_3) and ionized ammonia (NH_4) constitute total ammonia nitrogen (TAN) [69] of which the unionized ammonia is much more toxic to the fish and should be removed from the system [59, 60]. Removal of ammonia from fish water is achieved during the process of biological filtration, which involves oxidation of ammonia into nitrite and then to nitrate by the nitrifying bacteria. Biological filters are specially designed substrates with large surface area (fluidized media, siporax bags or panels, and plastic beads) placed in the water filtration unit in a manner that facilitates nitrifying microbes to attach and grow on it. These microbes, commonly present in the air, water, and soil, are colonized and grow on the surface of biological filters [44]. As effluent water passes through the treatment unit, certain species of nitrifying bacteria, e.g., *Nitrosomonas* sp., present on biological filters convert ammonia into nitrite followed by the conversion of nitrite into nitrate by *Nitrobacter* sp. [70]. The efficiency of any given biological filters is highly dependent on healthy nitrifying microbes colonizing

it [44]. Water temperature, pH, dissolved oxygen, salinity, and alkalinity are all critical to support the growth of nitrifying microbes on the biological filters. In WRS, the end product of the biological filtration process—nitrate—is acceptable to most fish species at higher levels (up to 200 ppm); however, regular water exchange at the rate of 5–10% of system water is typically enough to keep the concentration of nitrate well below these levels [44, 70].

Chemical filtration is another important process in WRS that is employed to capture dissolved solids, volatile organics, and other potentially hazardous materials (e.g., chlorine). Activated carbon (charcoal), ion exchange resins, and ammonia binders are all commonly used to sequester organic compounds in the effluent water that remain even after solid removal and biological filtration [44]. Activated carbon removes the concentration of dissolved salts through the process of adsorption and ensures optimal water clarity. The high clarity of the water is important to receive the proper function of ultraviolet (UV) sterilizers during the process of water disinfection if employed [71].

Large-scale systems enable housing and maintenance of large numbers of zebrafish in comparatively small space. WRS systems have integrated components for the treatment of effluent water, sterilization of water, and automation of water quality monitoring that allows for the maintenance of zebrafish in a controlled microenvironment.

Aquaria, High-grade glass, acrylic, polycarbonate, or polysulfone materials can be used to cast fish housing containers (tanks or aquaria). Commercially available rodent cages that are made from polycarbonate or polysulfone materials and modified to allow plumbing can be used as an alternative in small zebrafish setups (Fig. 13.4a). High-grade plastic materials are preferred over glass because they tend to be safer, more lightweight, and resistant to scratching. These tanks can withstand repeated autoclaving or tunnel washing. Alternatively, glass tanks should be chosen over most plastics for toxicology studies because glass minimizes the absorption of toxic compounds. Serial tanks may be created by partitioning a larger tank into multiple small tanks, allowing the flow of water from the first to the last tank in the series through the interconnected slit (1–2 mm) at the bottom and a drain out from the last tank (Fig. 13.4b). Commercially available fish aquaria typically include a tank body, baffle, and cover. The tanks are often made in a rectangular shape with rounded inside corners, a groove for fitting the baffle, and an overflow vent at the backside (Fig. 13.4c). Transparent tanks are good to observe fish during the day-to-day activities. Any tank material will be subject to the growth of biofilms and algae that reduce their transparency and functionality. Lids should be used to prevent the fish from jumping out. Small holes (2–3 nos.) of 1 cm diameter are often provided within the lids to allow feeding and water/air inputs.

Water temperature in natural aquatic habitats often varies widely. Zebrafish are poikilothermic animals that adjust their body temperature in accordance with the external environment. Zebrafish are known to have an extremely broad temperature tolerance. Laboratory studies show that wild-type zebrafish have a lower lethal temperature of 6.20 °C and an upper lethal temperature of 41.70 °C [72]. Large-scale husbandry data suggests that the housing of zebrafish at

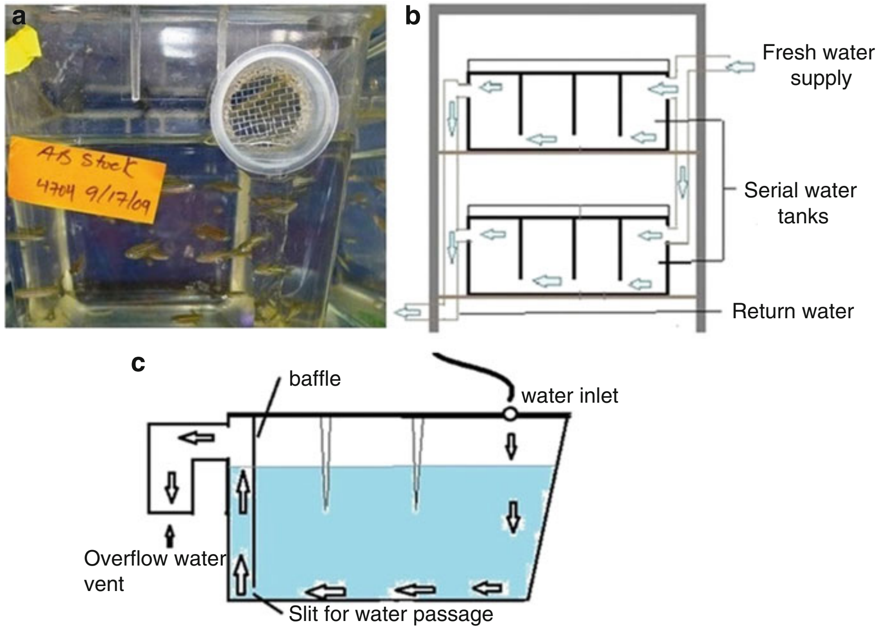


Fig. 13.4 Types of fish tanks used to raise the laboratory zebrafish. (**a**) Commercially available rodent cage made up of polycarbonate or polysulfone material, modified to allow plumbing. A hole at the backside of the cage, covered with wire mesh to drain overflow water. (**b**) Serial tanks created by partitioning, a larger tank into multiple small tanks that allow inflow of water from the first to the last tank through the interconnected slit (1–2 mm) at the bottom. The overflow water is drained out from the last tank. (**c**) Commercially available tank made in rectangular shape with rounded inside corners, a groove for fitting the baffle and an overflow vent at the backside. Water enters through inlet at the front and drains out through overflow vent at the backside

28.50 °C is an optimal temperature for their usage in biomedical research [73]. It is important to maintain stable temperature with minimum fluctuations, because high water temperature increases oxygen consumption and heart rates in zebrafish [74], reduces the oxygen content in the fish water, and increases the toxic fraction of ammonia [53, 59]. Moreover, a warmer environment may not be comfortable for the personnel working in the aquaculture facilities. Maintaining a temperature in the macroenvironment that is closer to the desired set point within the microenvironments often desirable as this typically eases the demands placed on equipment to maintain optimum temperatures. An HVAC system typically is used to maintain the room temperature and circulating air in and out of a given space. Many modern aquatic systems have built-in heating elements under thermostat control that adjust water temperature when it moves outside the desired range. The ability to rapidly cool the water when temperatures are elevated during hot weather or in the event of a breakdown of air conditioning systems is critical and can be achieved by chillers, especially in tropical climates.

Lighting: Zebrafish is very sensitive to photoperiod and usually spawn within the first 2 hours after sunrise in the natural habitat [62]. Given the fact that they are photoperiodic breeders, zebrafish should be maintained on consistent light cycles (12-h light, 12-h dark). It is well documented that interruptions in photoperiods depress or even completely stop breeding in zebrafish. If warranted, researchers may alter light cycles of small subsets of fish in a given facility without altering the photoperiod for the entire population.

The facility can be illuminated using white fluorescent lights or light-emitting diodes (LED). Placing light fixtures on the ceiling, not immediately above the fish units, but targeting space between rows of the fish units, will allow more uniform illumination from the top to the bottom tanks. The upper tanks usually receive light of higher intensity which often increases the growth of algae.

A recommended light intensity of 300 lux is required at a one-meter height above the floor in the zebrafish facility. Exposure to the light above 300 lux for a longer period can be detrimental to zebrafish [75]. Too much light will lead to excess growth of the algae in the tanks and reduce the visibility of the fish. Interruptions of photoperiod should be avoided.

13.5 Zebrafish Breeding

The zebrafish research community has witnessed exponential growth in the availability of mutant lines over the years after the ethyl nitrosourea (ENU) mutagenesis and then reverse genetics like TALENS, ZFNS, and of course CRISPRs. With the availability of molecular biology tools and recently discovered genome-editing techniques, clustered regularly interspaced short palindromic repeats (CRISPR) and CRISPR-associated protein 9 (CRISPR-Cas9), an expansion in the inventory of zebrafish mutants can be foreseen. Given this, there is an immediate need to define breeding strategies that allow for the expansion and continued propagation of these mutant genotypes. Inbred rodent strains are very useful in immunology, cancer biology, and genetic research [56]; however, inbred strains are not available in the zebrafish model for various reasons. When researchers first began using zebrafish, the primary focus for the production of all standard zebrafish wild-type lines was to provide genetic backgrounds for the induction, manipulation, mapping, and identification of mutations. Typical hallmarks of inbreeding depression such as poor viability, reduced fecundity, and susceptibility to disease are the major obstacles while working with inbred lines in zebrafish. Therefore, it is a general practice to avoid full-sib mating and to maximize effective population sizes while propagating zebrafish lines [62]. A few isogenic zebrafish lines have been established through the process of gynogenesis, including SJD [76] and C32 [77]; however, these lines are not robust and natural mating is difficult. Shinya and Sakai propagated two lines (IM12m and IM14m) up to the 16th generation through full-sib mating [78] and subsequently completed the 20th generation of inbreeding [79]. Analysis of breeding data revealed greater inbreeding depression in zebrafish than in medaka but likely less than in mice [78].

Zebrafish lines are usually maintained in a manner more consistent with an outbred stock. Increasing effective population sizes are typically maximized by deriving lines from many parents during propagation events [56]. Brand and colleagues have proposed a breeding method designed to prevent the spread of spontaneous mutations in wild-type stock that involves the setting of multiple pair crosses, collecting 50 embryos each from at least 10–12 individual breeding pairs, and monitoring them for the first 5 days of the development. Embryos showing abnormal phenotypes should be discarded, while the rest of the normal embryos is grouped and raised for colony propagation [56].

An updated list of wild-type zebrafish lines and their current locations in 2019 by Zebrafish International Resource Center (ZIRC) includes a total of 31 lines (Table 13.3). There are no commercial suppliers of the fish, therefore many lines are maintained within individual laboratories in small closed populations. Without programmatic exchange, this drives differentiation between all of these lines, which could be an issue for reproducibility.

Propagation of mutant lines is a real challenge and sometimes may not produce the required genotype if a defined breeding strategy is not followed. Many of the existing zebrafish mutants are caused by recessive, zygotic lethal mutations [56]. Mortality in homozygous mutants often occurs before the animals attain reproductive age, so the mutant stock must be maintained and propagated using adult heterozygous carriers of the mutation(s). Brand and colleagues [56] suggest that breeding of heterozygous mutant stock should include (1) outcrossing of heterozygous mutant carriers with wild type, (2) growing F1 embryos to reproductive age, and (3) in-crossing of F1 adults to recover heterozygous mutant carriers (Fig. 13.5).

Mating crosses, zebrafish embryos are required for experimental usage or propagation of the fish lines. Embryos are obtained by mating reproductively mature adult zebrafish. Studies have shown that gonadal development in males is initiated within 5–7 weeks after hatching, and normal testes are fully developed by the approximately third month of development [80, 81]. Meanwhile, environmental factors such as food supply, temperature, and growth rate may affect this process. In one study, faster and slower growing individuals were more likely to develop as females and males, respectively [82]. Our experience shows the differentiation of male and female zebrafish in 8–10 weeks postfertilization under rearing conditions defined by our laboratory (unpublished data).

Zebrafish lines used for breeding are usually ready to spawn by 3–4 months of age. The body of males is typically slender in shape with light orange coloration on the abdomen and fins. The females have a round body with whitish coloration on the abdomen and fins. Males and females are most often paired in the evening after the day's last feeding and kept overnight in non-flowing water. In wild habitats, stagnant water bodies such as ponds, rivers, slow-streaming flow, and rice crops are preferred spawning sites for the zebrafish [38]. The density of fish during a crossing event will depend on the size of the breeding tank, but a typical number would be 2–3 fish in a ~1 L spawning tank. It has been observed that wild-caught fish tended to spawn in pairs rather than in groups when housed in larger tanks (1100 L), and similar

Table 13.3 Updated list of wild-type zebrafish strains and the current source, ZIRC/EZRC

Strains	Current location
AB (AB)	European zebrafish resource Center (EZRC), zebrafish international resource Center (ZIRC)
AB/C32 (AB/C32)	
AB/TL (AB/TL)	
ABO	European zebrafish resource Center (EZRC)
AB/Tubingen (AB/TU)	
C32 (C32)	
CB	Zebrafish international resource Center (ZIRC)
Cologne (KOLN)	
Darjeeling (DAR)	
EkkWill (EKW)	
HK/AB (HK/AB)	
HK/sing (HK/SING)	
Hong Kong (HK)	
India (IND)	
Indonesia (INDO)	
SPF 5-D	
SPF AB	
Nadia (NA)	Zebrafish International Resource Center (ZIRC)
NHGRI-1	European Zebrafish Resource CENTER (EZRC), Zebrafish International Resource Center (ZIRC)
RIKEN WT (RW)	
SAT	Zebrafish International Resource Center (ZIRC)
Singapore (SING)	
SJA (SJA)	
SJD (SJD)	European Zebrafish Resource Center (EZRC)
SJD/C32 (SJD/C32)	
Tubingen (TU)	European Zebrafish Resource Center (EZRC), Zebrafish International Resource Center (ZIRC)
Tupfel long fin (TL)	European Zebrafish Resource Center (EZRC), Zebrafish International Resource Center (ZIRC)
Tupfel long fin nacre (TLN)	Baier Lab
WIK (WIK)	European Zebrafish Resource Center (EZRC), Zebrafish International Resource Center (ZIRC)
WIK/AB (WIK/AB)	
WT	

behavior was expressed by the laboratory zebrafish when housed in a 600 L tank [83, 84]. Contrary to the previous reports, group spawning was seen in 70% of the spawning events when fish were housed at higher densities with access to limited spawning sites [84, 85]. Fish tend to consume their eggs, so breeding cages must be designed to protect spawned eggs from the adults. There are many versions of

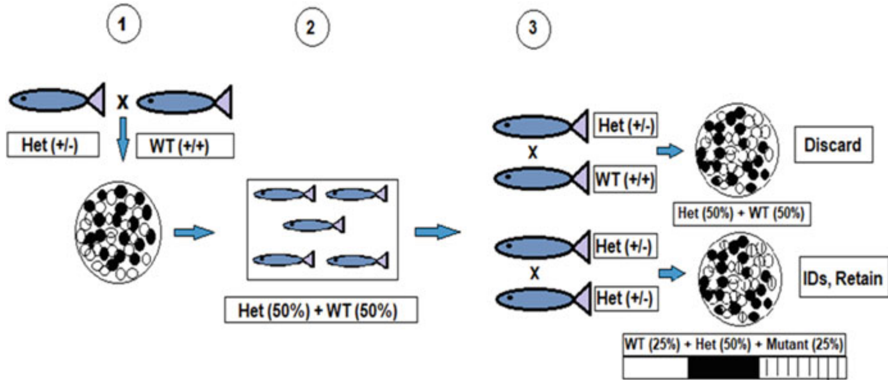


Fig. 13.5 Breeding protocol for the propagation of heterozygous mutant stock: (1) Adult heterozygous mutant carriers are outcrossed with wild-type partners to produce F_1 progeny. The F_1 embryos are raised to the parenthood. (2) F_1 progeny with 50% heterozygous (Black) and 50% wild-type (Empty) population ready to cross after reaching reproductive age. (3) In-crossing between F_1 siblings to produce F_2 progeny. The embryos from individual pairs are monitored for the development of mutant phenotype. All the pairs mated for the egg production are numbered and maintained in separate tanks until the observation of mutant phenotype. Embryos obtained from cross between wild-type and heterozygous will not produce mutant phenotype and should be discarded; however, embryos obtained from cross between two heterozygous mutant carriers will show 25% mutant phenotype (vertical strip). These parents will be labeled as IDs and will be retained for the experiments or propagation

mating cages that can be utilized. Marbles, suspended meshes, or closely spaced rods are all versions of structural enrichment that have been shown to encourage spawning in some experiments [86]. Commercially produced breeding cages often consist of two tanks of 1–1.5 L capacity, with an inner tank with a mesh or perforated bottom nesting inside the outer tank. When fish spawn, the spawned eggs fall through the floor of the inner tank and settle at the bottom of the outer tank and are therefore protected from the parents that would consume them. This type of apparatus is scalable; for example, the iSpawn (Tecniplast) is a large vessel that can hold up to 150 adult fish and is capable of collecting as many as 10,000 developmentally synchronized embryos per event [87]. It has been noticed that zebrafish do spawn frequently at irregular intervals if a zebrafish pair is housed together continuously [88]. There is a balance between spawning too frequently and not often enough; female zebrafish tend to become "egg bound" if not allowed to spawn regularly [38]. The most appropriate interspawning interval will depend on environmental conditions, including nutrition, density, and housing situation.

Collection and incubation of embryos, zebrafish spawn in the morning, immediately after illumination with two more peaks in the early afternoon and last hour of light [89, 90]. Fish are removed from the breeding containers and transferred to original tanks before embryo collection. Embryos from the spawning tank can be siphoned using a Pasteur pipette and then transferred to Petri plate, but this method is time-consuming. The commonly used approach is to pour water containing eggs

from the breeding container into a tea strainer. The embryos are retained on the surface of the mesh as the water is poured through it. The embryos can then be flushed with E3 buffer into a Petri dish [91]. Embryos are examined under a stereomicroscope, and dead, abnormal, unfertilized embryos and fecal wastes are removed from the solution. Zebrafish clutch sizes are highly variable; an average number can range between 100 and 200 eggs, with clutches of over 1000 eggs possible from a single pair of zebrafish [92]. In our observations, breeding data from 50 females from each of four wild types and one mutant line revealed 42–58% viability, with a clutch size that ranged from 162.36 to 242.42 embryos with an average of 179.04 [93]. In another report, the clutch size obtained from one female ranged from 1 to 700 embryos with a mean of 185 [94].

Embryos should be washed with E3 buffer [56] before transferring to an incubator that maintains a consistent temperature, e.g., 28.5 °C. Our experience shows that up to 150 embryos can be conveniently incubated in a 90 mm Petri dish for the first 24 h under refined laboratory protocol. Optimal development has been recorded when 60 embryos were incubated in a 90 mm Petri dish, although nonsynchronous growth has been observed when 200 embryos are incubated in a 90 mm Petri dish [95]. Embryos should be monitored daily up until the introduction of food (day 5). Dead or nonviable embryos tend to be easily contaminated with fungus and should be removed immediately. Hatching of zebrafish larvae typically occurs between 48 and 72 hpf at 28.5 °C. The inflation of the posterior chamber of the swim bladder occurs at 4.5 dpf [45, 96]. Once this occurs, the fish are ready to begin exogenous feeding and must be presented with feed [54]. Studies have shown that water temperature may have irreversible effects during the early stage of development and can influence sex differentiation [97, 98]. Findings of Kimmel and colleagues, however, established 28.5 °C as an optimal temperature for zebrafish embryo development and hatching efficiency [99].

Embryo bleaching, embryos may be surface sanitized by bleaching [100] or povidone-iodine exposure [101] before hatching to minimize the transmissions of pathogens on the outside of the chorion or in associated water. The outer surface of the embryo, i.e., the chorionic membrane, can be sanitized by a brief exposure to a 0.59 ml/L solution of sodium hypochlorite (5.65–6%) [95]. Newly spawned embryos are often mixed with fecal waste and other detritus. Several fish pathogens (i.e., *Pseudoloma neurophilia* and *Pseudocapillaria tomentosa*) are often associated with and can be transmitted by exposure to this mixture [102, 103]. In many facilities, an important practice is to bleach embryo between 24 and 30 hpf, before hatching. This is also commonly done when fish are exchanged between laboratories. Published SOPs for the zebrafish embryo bleaching can be referred to for details [45, 56, 95].

Larval and juvenile rearing, a wide range of factors affect larval growth and development. Access to highly nutritious, digestible prey items (alive or inanimate) in small amounts during the early feedings without altering the quality of water is important [98]. To meet this challenge, several larval rearing protocols shaves been developed over the years [97, 104–106]. In our laboratory, we have refined protocols

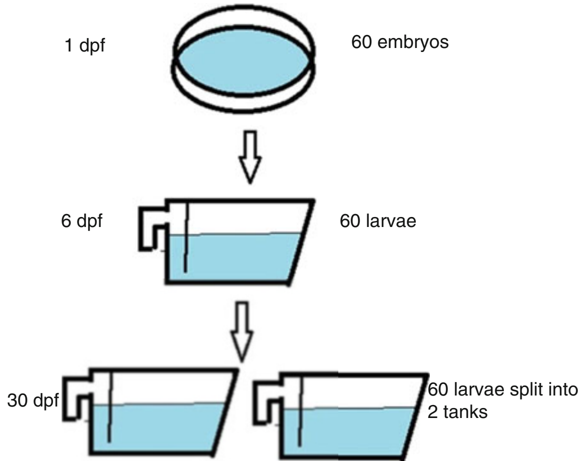


Fig. 13.6 Typical larval rearing protocol refined at the zebrafish facility, TIFR. On 1 dpf, 60 surface-sanitized embryos incubated in 90 mm Petri plate. On 6 dpf, 50–60 swim bladder bloated larvae (5%–10% mortality), transferred into 1.5 L water (Red Sea salt: 3 g/L) in 3 L-size tank and reared till 12 dpf without system water. On 13th dpf, tanks receive system water at the rate of 60 drops per minutes and with increased rate as the age advances. On 30 dpf, 50–60 larvae split into two 3 L tanks (25–30 juvenile/tank) and grow in the same tank till 60–120 dpf. After 120 dpf, 60–70 adults pull together and hold in 10 L-size tanks

Table 13.4 Foods, feeding frequencies, life stages, and timings for larval and adult zebrafish at TIFR

Food/diets	Feeding frequency	Life stage	9.00 h	12.00 h	16.00 h	19.00 h
Larval diet, fine powder, (50–100 μm), novo tom	4	6–12 dpf	√	√	√	√
Live artemia, INVE (400–500 μm)	4	13–60 dpf	√	√	√	√
Live artemia, INVE (400–500 μm)	3	61–120 dpf	√	X	√	√
Juvenile diet, pellet, Aquafin, (150–300 μm)	1	61–120 dpf	X	√	X	X
Live artemia, INVE (400–500 μm)	1	>120 dpf	√	X	X	X
Adult zebrafish diet, Zeigler, pellet (300–500 μm)	1	>120 dpf	X	X	√	X

√—Food offered, X—Food not offered

for larval rearing (Fig. 13.6) and feeding regimes (Table 13.4) that were found to be appropriate to support the growth and survival of the larvae.

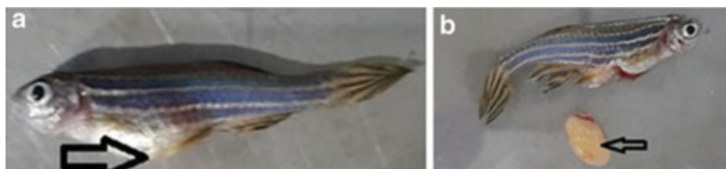


Fig. 13.7 Typical "egg bound syndrome" in zebrafish female. (**a**) Female with enlarged ventral abdomen (*arrow*). (**b**) Dissected zebrafish female with solidified mass of gonad (*arrow*) removed from the abdomen

Adult fish maintenance and density, zebrafish are social animals, and understanding their behavioral requirements is necessary to maintain their well-being in laboratory conditions. Data from studies of animals in their natural habitats provide important clues about their social, physiological, and environmental requirements and should inform the design of the artificial housing [27, 34]. It has been observed that zebrafish seem to prefer being housed in groups [107, 108] and are typically maintained together in mixed-sex populations. It appears that shoaling behavior is innate and commences soon after hatching [109]. The reproductive behavior of zebrafish shows that females need exposure to males to oviposit. When the females are not exposed to males for longer periods, they are at risk of becoming "egg bound" [38] (Fig. 13.7). Factors such as the efficiency of the filters, the age of the fish, and the amount of feeding are prerequisites to decide maximum stocking density [56]. In the static systems with manual water exchange, fish are kept at lower densities, usually at 1–2 fully grown fish per liter [56]. In large-scale recirculating systems with efficient filtration and water exchange, adult fish are often kept at higher densities. Commercially manufactured aquaria are available in a wide range of different volumes. Although there are no fixed numbers for stocking, 5–10 adult fish per liter can be conveniently housed [110]. Small-size aquaria (i.e., 1 L) are often employed to house individual fish or pairs for identification purposes. These situations are often sub-optimal for welfare, particularly when fish are kept in pairs, as zebrafish are prone to develop aggressive behavior [56]. The unwanted consequences of this chronic aggression can be reduced by adding some enrichment materials to the containers to allow subordinate fish to hide from the dominant animal [56].

13.6 Zebrafish Nutrition

With the domestication of wild zebrafish as a hobby species over the decades, entrepreneurs associated with the ornamental fish market did come up with some traditional diets. The formulated diets were mostly fish meal based, with variable and often undefined ingredients. Field studies from the Indian subcontinent revealed that the gut contents of wild zebrafish often consist of a broad range of materials, including terrestrial arthropods and aquatic insects [34], zooplankton and insects,

phytoplankton, filamentous algae and vascular plant material, spores and invertebrate eggs, fish scale, arachnids, detritus, sand, and mud [85].

Despite widespread usage of zebrafish in research, little progress has been made in understanding their nutritional requirements. No commercially available diets have been made specifically for laboratory-bred zebrafish, so diet made for other species is often used instead. Zebrafish diets available in the market come mostly from the existing fish farming industry. Diet is important to support fish growth, health and productivity. Nutrition is also a major variable in any study, given its impacts on health, physiology, behavior, etc. It is imperative to develop zebrafish-specific diets, with formulations based on an understanding of the nutritional requirement of the species at various life stages. Feeding protocols that include a mixture of defined and undefined diets tend to produce experimental variability, so defined dietary standards should be developed to support common utility in research programs [111]. Studies of Helena and colleagues targeted the dietary protein requirement for juvenile zebrafish at 37.6% and 44.8% for maximum weight gain and maximum protein retention, respectively [112].

There are no universally accepted defined diets or standard feeding protocols available to the zebrafish research community. Dietary requirements of zebrafish change over their development. Early-stage larvae ideally must begin feeding before depleting the yolk sac reserves, usually before 6 dpf. At this stage, fine--powdered larval diets (50–100 μm particle size) or live zooplankton (rotifers, paramecium) will be suitable because larvae may not be able to eat larger granules or flake due to their small mouths. Items that are too large may not be available to the fish in the water column, where they are most effective at feeding. Mid-stage larvae (13–29 dpf) and juvenile (30–60 or 90 dpf) are large enough to consume micro-pellets along with live feed (artemia in addition to rotifers or paramecium). In the majority of the research facilities, multiple feedings are offered at variable times. The importance of frequent feedings on the outcome of nutritional experiments is well known in other fish species, but the effects of these factors had not been investigated in zebrafish until relatively recently [111]. Studies on the effects of multiple feeding in zebrafish revealed differences in the growth and subsequent reproductive performances when the fish was presented 5% of the body weight/day with feeding frequency ranging from on every other day to five times daily [48, 91, 112, 113]. In a similar study, growth was promoted at the higher feeding frequencies (three or five per day), whereas feeding every other day or once a day showed good reproductive performance [114]. The concept of offering frequent small feedings over fewer larger feedings helps to ensure proper feed utilization and avoid overfeeding, but it is labor-intensive and time-consuming. Based on these data, a majority of research groups have adopted the practice of multiple feedings, although some laboratories feed pelleted diets once per day [115]. Overfeeding should be discouraged to avoid deteriorations in water quality and fish health. Zebrafish will usually consume feed within a short period after it is introduced, so whatever remains in the tank after 5–10 min are typically excess. The various types of foods, availability (dry or live), pellet size, and suitability for the life stages of zebrafish are presented (Table 13.5).

Table 13.5 Different types of zebrafish foods, presentations, pellet size, and feeding stages

Types of foods	Presentation	Size of pellets or live foods	Culture and harvesting	Life stage of zebrafish
Micro-pellet	Dry powder	50–100 μm	No	Early and mid-larvae
Micro-pellet	Dry powder	100–200 μm	No	Mid-larva, juvenile
Micro-pellet	Dry powder	200–500 μm	No	Juvenile, adults
Micro-pellet	Dry powder	400–700 μm	No	Adults
Brine shrimp Artemia	Dry cysts	400–500 μm (length)	Yes	Larval, juvenile, and adults
Rotifers	Culture	100–210 μm and 130–340 μm	Yes	Larval, juvenile, and adults
Paramecium	Culture	50 × 180 μm (width × length)	Yes	Larval, juvenile, and adults

13.7 Fish Health and Disease

Fish pathogens that are capable of infecting zebrafish colonies are well documented. The most commonly reported pathogens in zebrafish facilities include *Pseudoloma neurophilia*, a microsporidian organism that infects the central nervous system, and *Mycobacterium chelonae*, which is a bacterial pathogen that typically causes subclinical infections [102]. Infection by *Pseudoloma neurophilia* either results in subclinical infection or in some cases may lead to clinical signs such as emaciation, spinal deformities, and reductions in growth and reproduction [116]. Less commonly encountered agents include *Pseudocapillaria tomentosa*, which is a nematode that causes proliferative enteritis, and *Myxidium streisingeri* which is often found in the urinary tract [102]. Fish pathogens such as *Mycobacterium marinum* [117–119] and *Aeromonas hydrophila* [120, 121] are known to cause potential zoonosis, and their occupational health risk is well established. Another bacterial pathogen, *Edwardsiella ictaluri*, has recently emerged as an agent that is highly pathogenic to zebrafish [122]. It is well-documented that subclinical infections caused by the common zebrafish diseases are often only revealed by histopathological examinations of normal healthy-looking animals [102]. Researchers and facility supervisors must be informed about the common zebrafish diseases and health status of their fish colonies to help prevent disease outbreaks, protect staff from zoonotic disease, and improve data reproducibility [117, 118]. Subclinical infections in zebrafish can produce confounded experimental outcomes; misinterpreted, non-reproducible research data; and unwanted utilization of more animals to substantiate statistical significance [123]. Given these realities, it is recommended to have routine colony health monitoring programs in zebrafish facilities [123]. Implementation of a health monitoring program will be dependent on the type of research being conducted in a given facility, availability of a good laboratory setup, and a

funding source. As a minimum requirement, zebrafish health monitoring programs must address the needs of the research being carried out at the institution and should accommodate changing needs. For example, many pathogens can be excluded from the program when research involves the usage of immunocompromised animal models or toxicology studies [123].

Healthy zebrafish can be raised by providing optimal environmental conditions and practicing good quarantine procedures. Various environmental stressors, such as poor water quality, inadequate nutrition, and adverse social conditions (low-density housing or overcrowding), can lead to disease outbreaks in zebrafish [56].

Zebrafish should be examined on a daily basis for clinical manifestations such as lethargy, anorexia, edema, abnormal swimming behavior, skeletal abnormality, wounds on the body, and raised scales [124]. Fish displaying these symptoms and other clinical signs should be removed from the system and euthanized immediately. In live animals, clinical examinations are limited, and anesthesia of the fish is required for a more detailed physical assessment. Dead fish should be examined for lesions on the body as well as on internal organs. Minimum preliminary examinations include microbiological cultures of the skin, fin, and gill scrapings. Histopathological examination is the most informative and in-depth diagnostic technique that should be carried out to provide more details of the health status of the animals. The small size of the fish allows them to be fixed and sectioned as a whole mount. This permits examination of all primary parts on a single slide. PCR techniques have been standardized for the diagnosis of a few fish diseases [125].

The concept of fish quarantine is to prevent the introduction of new pathogens into the population from outside sources. Newly procured fish are quarantined in a room marked for the purpose and should not be introduced directly into the main facility. The fish are maintained in the quarantine room for their entire life, and only sanitized embryos that are obtained from these animals are taken into the main facility. When the outside sources provide embryos/larvae, these offspring are raised and maintained in the quarantine room. After attaining sexual maturity, embryos are obtained from these fish and introduced into the main facility after proper surface sanitization. Any dead fish should be considered as biomedical waste and disposed of accordingly. A small number of fish could also be preserved for laboratory analysis. Fresh mortalities are often a good diagnostic sample for PCR and/or histology if it is not degraded. A small facility may reserve one isolated tank or rack within its primary fish housing space for quarantine, but this is not ideal. If at all possible, a physically separate quarantine room should be established with multiple tanks or racks. However, in both the arrangements, a discrete life support system and equipment (tanks, nets, breeding apparatus, etc.) should be provided.

In a quarantine area, fish may be held for a longer time and bred to obtain embryos that can be surface disinfected before introduction into the main population [56, 86]. Once it has been confirmed that the genetic material of interest (i.e., transgene, genetic mutation, etc.) has been successfully introduced into the main facility, the parents in the quarantine may be euthanized. General practice is to procure surface-disinfected embryos from breeders or collaborators. Because bleaching does not kill all of the possible incoming pathogens, it is advisable to

rear these offspring in quarantine up until breeding age, so they can produce embryos that can be surface disinfected before introduction into the main population. The quarantine period will allow for the observation of animals for clinical signs and to screen parents for the pathogens if the resources are available.

Sanitation and sterilization, fish housing equipment such as tanks, lids, baffles, nets, and breeding containers can be a potential source of disease transmission in the facility. Regular cleaning and sterilization of these materials should be undertaken. When tanks contain fish and recirculating water, they will naturally accumulate debris and grow algae, cyanobacteria, and other microorganisms, such as bryozoans [126]. All of these things will decrease the visibility of the fish tanks and may impede daily observations that are vital to the maintenance of welfare. The suspended particles in the system are removed with the water flow; however, the dirt such as mat, crust, or biofilm that attaches to surfaces requires manual removal [127]. Breeding containers used for setting the crosses are cleaned after every use. Cleaning, sanitization, and sterilization can be done using sinks, large vats, manual cleaning, washing machines, or tunnel washers and autoclaves [127]. Commonly used cleaning agents include citric and acetic acids, alkalis, bleach solution 1.98% (sodium hypochlorite), hydrogen peroxide soap and detergents, and 70% ethanol [127]. Existing ecosystems in fish aquaria are critical, and cleaning procedures and disinfectants should be used carefully. Thin biofilms formed on the inner walls of the fish tanks harbor colonies of nitrifying bacteria and are an essential part of the biological filtration in a recirculating system. Vigorous cleaning procedures or non-tested disinfectants may damage this ecosystem. The common problem with the usage of detergents or chemical disinfectants is often the presence of residues which in the long run could negatively affect biofilms and the fish themselves, so the aquaria should be thoroughly rinsed after exposure to chemicals. Chemical usage and exposure should be completely compartmentalized from living active systems where fish are housed to ensure that chemicals are not introduced into systems [56, 125]. In WRS, the gutters, sumps, and plumbing require periodic cleaning for optimal performance (Table 13.6).

Transmission of infections between tanks can be inhibited by routine handling of fish with only sterilized nets. Nets should be cleaned and disinfected after each use. A study conducted by Garcia and colleagues [126] at one facility revealed a 96.6% reduction in relative light units (RLU), as an expression of ATP amount when nets were soaked for 1 hour in commercial disinfectant (benzalkonium chloride and methylene blue; 4.93 ml/3.8 L RO water). The material quality of the nets should be such that it can withstand chemical exposures or high temperatures. It is advisable to have a separate set of nets for each rack. In larger facilities, it may be helpful to have a small number of net stations located inside the fish room for holding sterilized nets. Nets are used once and then sent for sterilization before they are used again. In such an arrangement, a large number of nets will be required to ensure that this system is sustainable.

Table 13.6 Cleaning and sanitation of the fish tanks, baffles, filters, and other accessories [126, 127]

Items	Common dirt and cleaning techniques employed (replacement/servicing)
Fish holding and spawning racks	These items attract dust; dirt includes water stains and food spills. These items are either washed or wiped down by hand using nonvolatile chemicals (NaOCl, 70% ethanol)
Supply and return plumbing	Inner surfaces stockpiled with fine dirt, bacterial colonization, detritus, and crust formation. Cleaning and sanitization can be carried out (when no fish in the system) using tested disinfectants
Water sump	Settleable solids, detritus, and fine dirt slurry. Cleaning by siphoning solid waste and fine dirt slurry using suction pumps. Sanitization before repopulation of fish with tested disinfectants
Fish tanks, lids, siphon, and baffles	Algal growth, crust formation by bryozoans, biofilms, and detritus are common contaminants. Cleaning and sanitization manually or using industrial cage washers and autoclaving. Alkalis primarily remove dirt, and acids are used to neutralize alkalis (citric, acetic acids, alkalis, NaOCl, NaOH, H_2O_2, detergents)
Spawning tanks and traps (artificial plants, mesh-screen panels, or marbles)	Food and fecal waste and fine dirt. Cleaning and sanitization manually or washing machine after every use. Autoclave if material quality approves it (NaOCl, NaOH, H_2O_2, detergents)
Mobile carts, trolleys	Dust, spilled fish water, food, manual cleaning, and sanitizing before moving to a new place (NaOCl, NaOH)
Paramecium/rotifer/artemia culture flask	Culture residues, discoloration, and fine dirt. Manual cleaning and sanitization after every cycle (NaOCl)
Mechanical filters (filter pads, bags, cartridges)	Suspended solids. Disposable filters are replaced. Reusable or permanent are cleaned and sanitized (when tanks received water with low pressure)
Biological filters	Settleable solids, fine dirt, and detritus. Cleaning with system water. Sanitization procedure may damage nitrifying bacteria and need tested agents
Chemical filters (activated charcoal)	Dissolved particles in water or fine dirt. Disposal after ensuring saturation
UV-C bulbs and quartz sleeves	UV-C bulbs. Disposal after completion of exposure period as specified by the manufacturers (9000–16,000 h). Quartz sleeves are reused after cleaning and salinization
Handling nets	Fine dirt, fish scales, and fecal matter. Cleaning and sanitization by immersion into the chemical bath (NaOCl, benzalkonium chloride, and methylene blue)
Room floors, walls, ceilings	Dust, water spills, and dirt. Surface disinfection and rinsing with tested sanitization agents

(continued)

Table 13.6 (continued)

Items	Common dirt and cleaning techniques employed (replacement/servicing)
Aeration	Air diffusers in water may attract fine dirt and detritus. Replace if disposable or proper cleaning and disinfection as per instruction by the manufacturer

13.8 Summary

Based on the considerable amount of data generated from fish-related research over the last few decades, the zebrafish has become one of the most important animal models in science. The research community has witnessed a wide-scale adoption of the zebrafish model in diverse fields of science. With this growth, there are emerging challenges associated with refining control of the aquatic environment, fish husbandry, and health. Development of new validated methods and approaches is necessary to meet these demands, and the development of well-defined diets, standards for fish health and reporting, and trained experts to manage all of these resources will only contribute further to the success and utility of the model system.

Acknowledgments The author would like to express gratitude to Professor Mahendra Sonawane, Department of Biological Sciences, TIFR, Mumbai, for continuous encouragement and guidance. The cooperation of research scholars (Prof. Sonawane's group) in managing the fish facility is highly appreciated. Sincere thanks to Mr. Christian Lawrence, Aquatic Resources Program, Boston Children's Hospital, USA, for critical review, useful comments, and recommendations that improved the quality of this chapter. The author also would like to thank Dr. M A Pathan, ICAR-Central Institute of Fisheries Education, Mumbai, India, for sharing original photographs of recently collected wild zebrafish from the natural habitat.

References

1. Hisaoka KK, Battle HI (1958) The normal developmental stages of the zebrafish, *brachydanio rerio* (Hamilton-Buchanan). J Morphol 102:311–327. https://doi.org/10.1002/jmor.1051020205
2. Weis JS (1968) Analysis of the development of nervous system of the zebrafish, *Brachydanio rerio*. I. The normal morphology and development of the spinal cord and ganglia of the zebrafish. J Embryol Exp Morphol 19:109–119
3. Weis JS (1968) Analysis of the development of the nervous system of the zebrafish, *Brachydanio rerio*. II. The effect of nerve growth factor and its antiserum on the nervous system of the zebrafish. J Embryol Exp Morphol 19:121–135
4. Streisinger G, Walker C, Dower N, Knauber D, Singer F (1981) Production of clones of homozygous diploid zebra fish (*Brachydanio rerio*). Nature 291:293–296
5. Volhard CN (2012) The zebrafish issue of development. Development 139:4099–4103. https://doi.org/10.1242/dev.085217

6. Donizetti A, Fiengo M, Gaudio RD, Iazzetti G, Pariante P, Minucci S, Aniello F (2015) Expression pattern of zebrafish rxfp2 homologue genes during embryonic development. J Exp Zool B Mol Dev Evol 324:605–613. https://doi.org/10.1002/jez.b.22637
7. Machluf Y, Gutnick A, Levkowitz G (2011) Development of the zebrafish hypothalamus. Ann N Y Acad Sci 1220:93–105. https://doi.org/10.1111/j.1749-6632.2010.05945.x
8. Whitfield TT, Riley BB, Chiang MY, Phillips B (2002) Development of the zebrafish inner ear. Dev Dyn 223:427–458. https://doi.org/10.1002/dvdy.10073
9. Wang X, Wang X, Yuan W, Chai R, Liu D (2015) Egfl6 is involved in zebrafish notochord development. Fish Physiol Biochem 41:961–969. https://doi.org/10.1007/s10695-015-0061-x
10. Monk KR, Voas MG, Franzini AC, Hakkinen IS, Talbot WS (2013) Mutation of sec63 in zebrafish causes defects in myelinated axons and liver pathology. Dis Model Mech 6:135–145. https://doi.org/10.1242/dmm.009217
11. Sonawane M, Martin MH, Schwarz H, Volhard CN (2009) Lgl2 and E-cadherin act antagonistically to regulate hemidesmosome formation during epidermal development in zebrafish. Development 136:1231–1240. https://doi.org/10.1242/dev032508
12. Xu M, Ye Y, Ye Z, Xu S, Liu W, Xu J, Zhang Y, Liu O, Huang Z, Zhang W (2020) Human BCR/ABL1 induces chronic myeloid leukemia-like disease in zebrafish. Haematologica 105:674–686. https://doi.org/10.3324/haematol.2019.215939
13. Grunwald DJ, Eisen JS (2002) Headwaters of the zebrafish—emergence of a new model vertebrate. Nat Rev Genet 3:717–724
14. Doughman E (2015) The use of CRISPR/Cas9 on zebrafish could speed human gene function. Discovery http://www.alnmag.com. Accessed 12 June 2015
15. Brand M, Deuchle D, Endres F, Haffter P, Hammerschmidt M, Mullins M, Schulte-Merker S, Volhard CN, Luck R, Jurgen K, Schwarz S (1995) Keeping and raising zebrafish (*Danio rerio*) in Tubingen. The Zebrafish Sci Mon 3:2–7
16. Watts SA, Powell M, D'Abramo LR (2012) Fundamental approaches to the study of zebrafish nutrition. ILAR J 53:144–160
17. Penglase S, Moren M, Hamre K (2012) Standardize the diet for zebrafish model. Nature 491:333. https://doi.org/10.1038/491333a
18. Nelson JS (1994) Fishes of the world, 3rd edn. Wiley, New York
19. Hamilton F (1822) An account of the fishes found in the river Ganges and its branches. Constable, Edinburgh and London
20. Hora SL (1937) Notes on fishes in the Indian museum XXXI. On a small collection of fish from Sandoway, lower Burma. Rec Ind Mus 39:323–331
21. Hora SL (1938) Notes on fishes in the Indian museum XXXI. On a small collection of fish from the Rajmahal Hills, Santhalparganes, Bihar. Rec Ind Mus 40:169–181
22. Chauhan BS, Ramakrishna G (1953) Fauna of the Balangir district (formerly Patna state), Orissa. Rec Ind Mus 51:395–416
23. Tilak R (1968) On a collection of fish from Sikkim. Rec Zool Surv Ind 66:277–286
24. Arunachalam M, Johnson JA, Sankaranarayanan A (2003) Fishes of rain forest stream/rivers of India. A research overview. ENVIS. Bulletin on conservation of rain forests in India. Wild Life Institute of India (ed), pp 153–172
25. Day F (1878) The fishes of India: being a national history of the fishes known to inhabit the seas and fresh waters of India, Burma and Ceylon. Indian reprint by Jagmander book agency, New Delhi
26. Menon AGK (1962) A distributional list of fishes of Himalayas. J Zool Soc Ind 2:23–32
27. Spence R, Fatema MK, Reichard M, Huq KA, Wahab MA, Ahmed ZF, Smith C (2006) The distribution and habitat preferences of the zebrafish in Bangladesh. J Fish Biol 69:1435–1448. https://doi.org/10.1111/j10958649
28. Whitely AR, Bhat A, Martins EP, Mayden RL, Arunachalam M, Heikkilä SU, Ahmed ATA, Shrestha J, Clark M, Stemple D, Bernatchez L (2011) Population genomics of wild and laboratory zebrafish. Mol Ecol 20:4259–4276. https://doi.org/10.1111/j.1365-294X.2011.05272.x

29. Munshi JS, Srivastava MP (1988) Natural history of fishes and systematics of freshwater fishes of India. Narendra Publishing House, Delhi
30. Sen N (1995) Pisces. In: Ghosh AK (ed) Fauna of Meghalaya: part 1, vertebrates. Zool Surv India, Calcutta, pp 483–592
31. Menon AGK (1999) Check list-fresh water fishes of India. Rec Zool Surv India Occ Pap 175:366
32. Fang F (2001) Phylogeny and species diversity of the South and Southeast Asian cyprinid genus Danio Hamilton (Teleostei, Cyprinidae). PhD Thesis, Stockholm University, Stockholm, Sweden
33. Barman RP (1991) A taxonomic revision of the indo-Burmese species of Danio rerio. Rec Zool Surv Ind Occ Pap 137:1–91
34. Arunachalam M, Manickam R, Chinnian V, Malaiammal P, Mayden RL (2013) Natural history of zebrafish (*Danio Rerio*) in India. Zebrafish 10:1–14. https://doi.org/10.1089/zeb.2012.0803
35. Talwar PK, Jhingran AG (1991) Inland fishes of India and adjacent countries. Oxford & IBH Publishing, Calcutta, p 1158
36. Engeszer RE, Patterson LB, Rao AA, Parichy DM (2007) Zebrafish in the wild: a review of natural history and new notes from the field. Zebrafish 4:21–40. https://doi.org/10.1089/zeb.2006.9997
37. Hoggarth DD, Dam RK, Debnath K, Halls AS (1999) Recruitment sources for fish stocks inside a floodplain river impoundment in Bangladesh. Fish Mang Ecol 6:287–310. https://doi.org/10.1111/j.1365-2400.1999.tb00081.x
38. Spence R, Gerlach G, Lawrence C, Smith C (2008) The behaviour and ecology of the zebrafish, *Danio rerio*. Biol Rev Camb Philos Soc 83:13–34. https://doi.org/10.1111/j.1469-185X.2007.00030.x
39. McClure MM, McIntyre PB, McCune AR (2006) Notes on the natural diet and habitat of eight danionin fishes, including the zebrafish *Danio rerio*. J Fish Biol 69:553–570. https://doi.org/10.1111/j.1095-8649.2006.01125.x
40. Junk WJ, Bayley PB, Sparks RE (1989) The flood-pulse concept in river-floodplain systems. In: Dodge DP (ed) Proceedings of the international large river symposium (LARS), Canadian journal of fisheries and aquatic sciences special publication 106. NRC Research Press, Ottawa, pp 110–127
41. Paull GC (2008) Improving the welfare of laboratory-kept fish: available from Winston Churchill Memorial Trust. https://wcmt.org.uk/users/gregorypaull
42. Jackson DA, Peres-Neto PR, Olden JD (2001) What controls who is where in freshwater fish communities — the roles of biotic, abiotic, and spatial factors. Can J Fish Aquat Sci 58:157–170. https://doi.org/10.1139/cjfas581157
43. Dani R (2020) Assessment of genetic diversity of zebrafish (Danio rerio) from Arunachal Pradesh, MFSc Dissertation, ICAR-Central Institute of Fisheries Education (University Established Under Section 3 of UGC Act 1956) Panch Marg, Off Yari Road, Versova, Andheri (W), Mumbai, India
44. Lawrence C, Mason T (2012) Zebrafish housing systems: a review of basic operating principles and considerations for design and functionality. ILAR J 53:179–191
45. NRC [National Research Council] (2011) Guide for the care and use of laboratory animals. National Academies Press, Washington
46. Sanders E (2013) Considerations and reflections on design and construction of a centralized zebrafish facility. Ani Lab News Mag https://www.alnmag.com. Accessed 10 May 2013
47. Hardness in groundwater (2007) Health Canada, Guidelines for Canadian Drinking water quality supporting documents. http://www.hc-sc.gc/ewh-semt/pubs/water-eau/doc
48. Lawrence C (2016) New frontiers for zebrafish management. Methods in cell biology. In: Detrich HW, Zon L, Westerfield M (eds) The zebrafish; genetics, genomics, and transcription, 4th edn. Academic Press, Elsevier Inc, pp 483–508

49. Zahangir MM, Haque F, Mostakim GM, Islam MS (2015) Secondary stress responses of zebrafish to different pH: evaluation in a seasonal manner. Aquacul Rep 2:91–96. https://doi.org/10.1016/J.AQREP.2015.08.008
50. Lawrence C (2007) The husbandry of zebrafish (Danio rerio): a review. Aquaculture 269:1–20. https://doi.org/10.1016/j.aquaculture.2007.04.077
51. Chen S, Ling J, Blacheton JP (2006) Nitrification kinetics of biofilm as affected by water quality factors. Aquac Eng 34:179–197
52. Harper C, Lawrence C (2011) The laboratory zebrafish. A volume in the laboratory animal pocket reference series. CRC Press Taylor and Francis Group, 6000 Broken Sound Pathway NW, Suite 300, Boca Raton, FL 33487-2742
53. Timmons MB, Ebelings JM (2013) Recirculating aquaculture (3rd ed.). Ithaca Publishing Company, LLC, Ithaca, NY
54. Hammer HS (2020) Water quality for zebrafish culture. In: Cartner SE et al (eds) Zebrafish in biomedical research. Biology, husbandry, diseases and research applications. Academic Press, pp 321–335
55. Kohale KN (2019) An incidence of acute mortality in laboratory zebrafish-a case report. J Lab Ani Sci 5:13–15
56. Brand M, Volhard CN, Dahm R (2002) Keeping and raising zebrafish. In: Volhard CN (ed) Zebrafish: a practical approach. Oxford University Press, New York, pp 1–37
57. Boyd CE, Tucker CS, Somridhivej B (2016) Alkalinity and hardness: critical but elusive concepts in aquacul. J World Aquac Soc 47:6–41. https://doi.org/10.1111/jwas.12241
58. Wurts WA (2002) Understanding water hardness. Kentucky State University Cooperative Extension Program. WKY-192
59. Noga EJ (2010) Fish disease: diagnosis and treatment, 2nd edn. Wiley-Blackwell, Ames, IA
60. Masser MP, Rakocy J, Losordo TM (1999) Recirculating aquaculture tank production systems management of recirculating systems. Southern Regional Aquaculture Center. Southern Regional Aquaculture Center Publication No 452
61. Summerfelt ST, Sharrer MJ (2004) Design implication of carbon dioxide production within biofilters contained in recirculating salmonid culture system. Aqua Eng 32:171–182
62. Trevarrow B (2004) Zebrafish facilities for large and small laboratories. In: Detrich HW, Zon L, Westerfield M (eds) Methods in cell biology. The zebrafish: genetics, genomics, and informatics, vol 77. Elsevier, pp 565–591. https://doi.org/10.1016/s0091679x
63. Moe MA (1989) The marine aquarium reference, system and invertebrate. Green Turtle Publication, Plantation FL
64. Timmons MB, Ebeling JW, Wheaton FW, Summerfelt ST, Vinci BJ (2001) Recirculating aquaculture system. Publication No. 01-002, Northwest Regional Aquaculture Center. Cayuga Aqua Ventures, Ithaca, NY
65. Burrell PC, Phalen CM, Hovanec TA (2001) Identification of bacteria responsible for ammonia oxidation in freshwater aquaria. Appl Environ Microbial 57:5781–5800
66. DeLong DP, Losordo TM (2012) How to start a biofilter. Publication ID SRAC 4502
67. Manthe DP, Malone RF (1987) Chemical addition for accelerated biological filter acclimation in closed blue crab shedding systems. Aquac Eng 6:227–236. https://doi.org/10.1016/0144-8609(87)90006-9
68. Losordo TM, Masser MP, Rakocy JE (1999) Recirculating aquaculture tank production systems. Southern Regional Aquaculture Center, Southern Regional Aquaculture Center Publication No 453
69. Timmons MB, Ebeling JM, Wheaton FW, Summerfelt ST, Vinci BJ (2002) Recirculating aquaculture system, 2nd edn. Cayuga Aqua Ventures LIc, Ithaca, NY
70. Hrubec T, Smith SA, Robertson JL (1996) Nitrate toxicity: a potential problem of recirculating systems. Aqua Eng Soc Proc 1:41–48. https://www.researchgate.net/ publication/267377185
71. System manual and as built instructions (2011) Mechanical filtration RM8 filter assembly, Chemical filtrations, Section V-D. Aquatic Habitat

72. Cortemeglia C, Beitinger TL (2005) Temperature tolerances of wild-type and red transgenic zebra danios. J Trans Ame Fish Soc 134:1431–1437. https://doi.org/10.1577/T04-197.1
73. Schaefer J, Ryan A (2006) Developmental plasticity in the thermal tolerance of zebrafish Danio rerio. J Fish Biol 69:722–734. https://doi.org/10.1111/j.1095-8649.2006.01145.x
74. Barrionuevo WR, Burggren WW (1999) O2 consumption and heart rate in developing zebrafish (*Danio rerio*): influence of temperature and ambient O2. Am J Phys RegulInteg Comp Phys 276:506–513. https://doi.org/10.1152/ajpregu.1999.276.2.R505
75. Zynda JR (2020) Aquatics facility design considerations: incorporating aquatics into an Animal Facility. In: Cartner SE et al (eds) Zebrafish in biomedical research. Biology, husbandry, diseases and research applications. Academic Press, pp 265–277
76. Johnson SL, Africa D, Horne S, Postlethwait JH (1995) Half-tetrad analysis in zebrafish: mapping the ros mutation and the centromere of linkage group I. Genetics 139:1727–1735
77. Krause HJ (1992) Hand bucha quarien technik. bedeverlag, Kollngurg, Germany
78. Shinya M, Sakai N (2011) Generation of highly homogeneous strains of zebrafish through full sib-pair mating. G3 (Bethesda) 1:377–386. https://doi.org/10.1534/g3.111.000851
79. Shinya M (2016) Construction of the inbred strain. In: Kawakami K (ed) Zebrafish: methods and protocols. Methods in molecular biology, vol 1451, pp 107–118
80. Devlin RH, Nagahama Y (2002) Sex determination and sex differentiation in fish; an overview of genetic, physiological, and environmental influences. Aqua 208:191–364
81. Maack G, Segner H (2003) Morphological development of the gonads in zebrafish. J Fish Biol 62:895–906
82. Lawrence C, Ebersole JP, Kesseli RV (2007) Rapid growth and out-crossing promote female development in zebrafish (Danio rerio). Env Biol Fish 81:239–246. https://doi.org/10.1007/s10641-007-9195-8
83. Delaney M, Follet C, Ryan N, Hanney N, Lusk-Yablick J, Gerlach G (2002) Social interaction and distribution of female zebrafish (*Danio rerio*) in a large aquarium. Biol Bull 203:240–241. https://doi.org/10.2307/1543418
84. Hutter S, Penn DJ, Magee S, Zala SM (2010) Reproductive behavior of wild zebrafish (Danio rerio) in large tanks. Behaviour 147:641–660. https://doi.org/10.1163/000579510X12632972473944
85. Spence R, Fatema MK, Ellis S, Ahmed ZF, Smith C (2007) Diet, growth and recruitment of wild zebrafish in Bangladesh. J Fish Biol 71:304–309. https://doi.org/10.1111/j.1095-8649.2007.01492.x
86. Westerfield M (1995) The zebrafish book. A guide for the laboratory use of zebrafish (*Danio rerio*), 3rd edn. University of Oregon Press, Eugene, p 385
87. Adatto I, Lawrence C, Thompson M, Zon LI (2011) A new system for the rapid collection of large numbers of developmentally staged zebrafish embryos. PLoS One 6:1–7
88. Eaton RC, Farley RD (1974) Spawning cycle and egg production in zebrafish. B*rachydanio rerio,* Reared in the laboratory. Copeia 1:195–204
89. Baganz D, Siegmund R, Staaks G, Pflugmacher S, Steinberg CEW (2005) Temporal pattern in swimming activity of two fish species (*Danio rerio* and Leucaspius delineatus) under chemical stress conditions. Biol Rhyt Res 36:263–276. https://doi.org/10.1080/09291010500103112
90. Plaut I (2000) Effects of fin size on swimming performance, swimming behaviour and routine activity of zebrafish Danio rerio. J Exp Biol 203:813–820
91. Castranova D, Wang C (2020) Zebrafish breeding and colony management. In: Cartner SE et al (eds) Zebrafish in biomedical research. Biology, husbandry, diseases and research applications. Academic Press, pp 357–364
92. Castranova D, Lawton A, Lawrence C, Baumann DP, Best J, Coscolla J (2011) The effect of stocking densities on reproductive performance in laboratory zebrafish (Danio rerio). Zebrafish 8:141–146. https://doi.org/10.1089/zeb.2011.0688
93. Kohale KN (2015) Zebrafish breeding in laboratory environment at TIFR. J Lab Ani Sci 3:25–30

94. Spence R, Smith C (2006) Mating preference of female zebrafish, Danio rerio, in relation to male dominance. Behav Ecol 17:779–783
95. Wilson C (2012) Aspects of larval rearing. ILAR J 53:169–178
96. Harper C, Lawrence C (2010) The laboratory zebrafish (laboratory animal pocket reference). CRC Press, Boca Raton, FL
97. Penman DJ, Piferrer F (2008) Fish gonadogenesis. Part I: genetic and environmental mechanisms of sex determination. Rev Fish Sci 16:14–13
98. Villamizar N, Ribas L, Piferrer F, Vera LM, Javier F, Vazquez S (2012) Impact of daily thermocycles on hatching rhythms, larval performance and sex differentiation of zebrafish. PLoS One 12:1–9
99. Kimmel CB, Ballard WW, Kimmel SR, Ullmann B, Schilling TF (1995) Stages of embryonic development of the zebrafish. Dev Dyn 203:253–310
100. ZIRC (2020) Embryo surface sanitation (egg bleaching) protocol. https://zebrafish.org. Accessed 30 May 2020
101. Chang CT, Amack JD, Whipps CM (2016) Zebrafish embryo disinfection with povidone-iodine: evaluating an alternative to chlorine bleach. Zebrafish 13:96–101. https://doi.org/10.1089/zeb.2015.1229
102. Kent ML, Harper C, Wolf JC (2012) Documented and potential research impacts of subclinical diseases in zebrafish. ILAR J 53:126–134
103. Murray KN, Dreska M, Nasiadka A, Rinne M, Matthews JL, Carmichael C, Bauer J, Varga ZM, Westerfield M (2011) Transmission, diagnosis, and recommendations for control of *Pseudoloma neurophilia* infections in laboratory zebrafish (*Danio rerio*) facilities. Comp Med 61:322–329
104. Carvalho AP, Araújo L, Santos MM (2006) Rearing zebrafish (*Danio rerio*) larvae without live food: evaluation of a commercial, a practical and a purified starter diet on larval performance. Aquacult Res 37:1107–1111. https://doi.org/10.1111/j.1365-2109.2006.01534.x
105. Hensley MR, Leung YF (2010) A convenient dry feed for raising zebrafish larvae. Zebrafish 7:219–231. https://doi.org/10.1089/zeb.2010.0652
106. Verga ZM (2011) Aquaculture and husbandry at the zebrafish international resource center. Meth Cell Biol 104:453–478. https://doi.org/10.1016/B978-0-12-374814-0.00024-0
107. Larson ET, O'Malley DM, Melloni RH Jr (2006) Aggression and vasotocin are associated with dominant-subordinate relationships in zebrafish. Behav Brain Res 167:94–102
108. Gerlach G (2006) Pheromonal regulation of reproductive success in female zebrafish: female suppression and male enhancement. Anim Behav 72:1119–1124
109. Engeszer RE, Albericida BL, Ryan MJ, Parichy DM (2007) Timing and plasticity of shoaling behavior in the zebrafish, Daniorerio. Anim Behav 74:1269–1275. https://doi.org/10.1016/j.anbehav.2007.01.032
110. Matthews M, Trevarrow B, Matthews J (2002) A virtual tour of the guide for zebrafish users. Lab Anim 31:34–40
111. Fowler LA, Williams MB, Dennis-Cornelius LN, Farmer S, Barry RJ, Powell ML, Watts SA (2019) Influence of commercial and laboratory diets on growth, body composition, and reproduction in the zebrafish *Danio rerio*. Zebrafish 16:508–521. https://doi.org/10.1089/zeb.2019.1742
112. Fernandes H, Peres H, Carvalho AP (2016) Dietary protein requirement during juvenile growth of zebrafish (Danio rerio). Zebrafish 13:548–556. https://doi.org/10.1089/zeb.2016.1303
113. Best J, Adatto I, Cockington J, James A, Lawrence C (2010) A novel method for rearing first-feeding larval zebrafish: polyculture with type L saltwater rotifers (Brachionus plicatilis). Zebrafish 7:289–295. https://doi.org/10.1089/zeb.2010.0667
114. Fowler LA, Williams MB, D'Abramo LR, Watts SA (2020) Zebrafish nutrition-moving forward. In: Cartner SE et al (eds) Zebrafish in biomedical research. Biology, husbandry, diseases and research applications. Academic Press, pp 379–401

115. Gemma micro, the complete nutrition for all life stages of zebrafish. www.zebrafish.skrettingusa.com
116. Ramsay JM, Watral V, Schreck CB, Kent ML (2009) Pseudoloma neurophilia infections in zebrafish Danio rerio: effects of stress on survival, growth, and reproduction. Dis Aquat Org 88:69–84
117. Ang P, Rattana AN, Goh CL (2000) Retrospective study of *Mycobacterium marinum* skin infection. Int J Derm 39:342–347
118. Ostland VE, Watral V, Whipps CM, Austin FW, St-Hilaire S, Westerman ME, Kent ML (2008) Biochemical, molecular, and virulence characteristics of select *Mycobacterium marinum isolate*s in hybrid striped bass Morone chrysops x M saxatilis and zebrafish *Danio rerio*. Dis Aquat Organ 79:107–118. https://doi.org/10.3354/dao01891
119. Watral V, Kent ML (2007) Pathogenesis of Mycobacterium spp. in zebrafish (*Danio rerio*) from research facilities. Com Bioch Phy Tox Phar CBP 145(1):55–60. https://doi.org/10.1016/j.cbpc.2006.06.004
120. Cipriano RC (2001) Aeromonas hydrophila and motile aeromonad 5 septicemias of fish. Fish Dis Leaflet 68
121. Pullium JK, Dillhay DL, Webb S (1999) High mortality in zebrafish (Danio rerio). Cont Top Lab Ani Sci 38:80–83
122. Hawke JP, Kent M, Rogge M, Baumgartner W, Wiles J, Shelley J, Peterson TS (2013) Edwadsiellosis caused by *Edwardsiella ictaluri* in laboratory populations of zebrafish Danio rerio. J Aquat Ani Hel 25:171–183. https://doi.org/10.1080/08997659.2013.782226
123. Collymore C, Crim MJ, Lieggi C (2016) Recommendations for health monitoring and reporting for zebrafish research facilities. Zebrafish 13:138–148. https://doi.org/10.1089/zeb.2015.1210
124. ZIRC (2015) Daily monitoring of fish morbidity and mortality SOP. https://zebrafish.org. Accessed 30 May 2020
125. ZIRC (2010) Health monitoring protocols. https://zebrafish.org. Accessed 30 May 2020
126. Garcia RL, Sanders GE (2011) Efficacy of cleaning and disinfection procedures in a zebrafish (Danio rerio) facility. J Am Assoc Lab Anim Sci 50:895–900
127. Sanders E (2020) Cleaning and disinfection of life systems. In: Cartner SE et al (eds) Zebrafish in biomedical research. Biology, husbandry, diseases and research applications. Academic Press, pp 299–307

14 Nonhuman Primates in Biomedical Research

Surender Singh, B. G. Roy, S. G. Ramachandra, and P. Nagarajan

Abstract

Nonhuman primates (NHP) are phylogenetically closer to humans and hence considered an ultimate animal model for various diseases in biomedical research. NHPs are often used in concurrence with other animal models. The data obtained from NHPs are used to validate any new molecule or vaccine before progressing to human clinical trials. The cost of maintenance and stringent ethical issues constrain researchers from using this animal model. Among the nonhuman primates, rhesus macaque (*Macaca mulatta*) is the most commonly used species. Considering the importance of NHP in the preclinical trial for various biomedical studies, this chapter discusses care, management, breeding, common infectious, and zoonotically important diseases of nonhuman primates with particular emphasis on rhesus macaque.

Keywords

Nonhuman primates · *Macaca mulatta* · Physiology · Microbiology · Preventive medicine · Zoonosis

S. Singh
Primate Research Center, National Institute of Immunology, New Delhi, India
e-mail: surenders@nii.ac.in

B. G. Roy
Institute of Nuclear Medicine and Allied Sciences, Delhi, India

S. G. Ramachandra
Central Animal Facility, Indian Institute of Science, Bangalore, India
e-mail: sgr@iisc.ac.in

P. Nagarajan (✉)
National Institute of Immunology, New Delhi, India
e-mail: nagarajan@nii.ac.in

© The Author(s), under exclusive license to Springer Nature Singapore Pte Ltd. 2021

P. Nagarajan et al. (eds.), *Essentials of Laboratory Animal Science: Principles and Practices*, https://doi.org/10.1007/978-981-16-0987-9_14

14.1 Introduction

Nonhuman primates (NHPs) are phylogenetically close to human beings and are considered as most comparable to the human-animal model for translational research. The research on nonhuman primates and the results obtained could support various discoveries relevant to human application. Nonhuman primates, in particular rhesus macaque, have helped to understand various diseases that affect human beings. This chapter will provide a short outline of husbandry practices, standard procedures, and microbial diseases of rhesus macaque (*Macaca mulatta*) used in captivity for biomedical research.

During the initial phase of biomedical research, wild-caught NHPs were quarantined and used in animal trials/research. In recent decades, they are bred and reared under laboratory conditions. As per the latest census, the number of macaques used in research has declined in the last few decades due to ethical concerns, the high cost of maintenance, and regulatory stringency. However, the need for NHPs cannot be undermined due to frequent reports of toxicity of existing drugs in humans. The emergence of newer diseases and the need for their control bring the role of NHPs to the fore. It is well recognized that NHPs fill the gap between rodents and humans in the areas of biomedical research.

14.2 Classification

Kingdom:	Animalia
Phylum:	Chordata
Class:	Mammalia
Order:	Primates
Suborder:	Haplorrhini
Infraorder:	Catarrhini (old-world anthropoids)
Superfamily:	Cercopithecoidea
Family:	Cercopithecidae (old-world monkeys)
Subfamily:	Cercopithecinae
Genus:	*Macaca*
Species:	*M. mulatta* (rhesus macaques)

14.3 Biology

Rhesus macaques (*Macaca mulatta*) are old-world nonhuman primates from Cercopithecidae family. Macaques are medium-sized monkeys with a robust body type, variable tail length, and moderately elongated nose [1]. The characteristic features of rhesus macaques include tough keratinized ischial callosities overlying and firmly adherent to the underlying ischial tuberosities at the hip bone; catarrhine

nasal morphology with forward-facing, closely spaced nares; and a nonprehensile tail. Macaques have cheek pouches and nails in all digits instead of claws. The skin may be patchily pigmented. Rhesus macaques display moderate sexual dimorphism. The average body weight of the rhesus male is 7.7 kg, with an average height of 53.1 cm. The average weight for a female is 5.34 kg, with an average height of 46.9 cm [2]. Rhesus reaches adult size at 5–7 years of age, and the males mature later than females. The average life span in captivity is 25–27 years. The maximum life span may approach 40 years [2–6]. Rhesus macaques are of either Indian or Chinese origin. These types have wide variances in morphology, behavior, ecology, physiology, reproduction, and disease progression. Hence, careful consideration must be taken into account when used in biomedical research.

14.3.1 Digestive System

Rhesus macaques are mainly herbivores, and their food includes leaves, plants, insects, and seeds. In laboratory conditions, the diets are formulated as per the standard nutritional requirements. The food is temporarily stored in the cheek pouches, containing amylase that helps in the digestion of starch [7]. The dental formula of adult macaque is 2-1-2-3/2-1-2-3 (Incisor/Canine/Premolar/Molar) [8]. The stomach has three-part body, fundus, and pylorus that aid in the digestion of protein. The small intestine comprises the duodenum, jejunum, and ileum, for digestion of lipid and simple carbohydrates. The digestion of complex carbohydrates is primarily by cecocolic segment of the large intestine with microbial activity and fermentation. The descending colon helps in water absorption and storage of waste for defecation.

14.3.2 Respiratory System

The upper respiratory system includes the nasal cavity, paranasal sinuses, nasopharynx, larynx, trachea, and bronchi. Macaques lack a vomeronasal organ. There are four lobes in the right lung and three lobes in the left lung [9]. The rhesus macaque has a higher lung mass than humans. The lung mass comprises 8% of its body weight compared to 6% in humans and 30% in mice. Rhesus macaque has a comparatively stiff chest wall like a human chest wall. They have enhanced lung volume, which gives a larger total lung capacity [9, 10]. The respiratory rate is approximately 38 breaths per minute for males and 40 for females [11, 12].

14.3.3 Urinary System

The kidney is multilobular with a simple medullary structure. The gross and microscopic anatomy of the kidney, ureter, urinary bladder, and urethra in nonhuman primates are similar to humans [13]. Each kidney weighs12–13 g on average,

and the size of the kidney correlates weakly with body size among adults. Captive animals may exhibit polydipsia without other abnormal behavior [14]. The daily water consumption is approximately 726.70 ± 308.50 ml, and the daily urine volume is 538.70 ± 376.60 ml. The specific gravity of rhesus macaque urine is 1.013, with pH 6.4 ± 0.99 and osmolality 47 ± 22.5. Sterile urine samples can be collected by sterile urinary catheterization and cystocentesis. The glomerular filtration rate (GFR) is usually measured to know the proper functioning of the urinary system. The inulin clearance rate in young rhesus macaques is 3.30 ± 0.69 ml/min/kg body weight, and creatinine is 4.30 ± 0.9 ml/min/kg body weight, and the ratio of creatinine to inulin clearance is 1.30 in healthy monkeys [14].

14.3.4 Olfactory, Visual, and Auditory Senses

The macaques have an excellent color vision similar to or even better than human beings to visualize few colors. To judge spatial relationships, they have overlapping visual fields, owing to the frontal positioning of the orbits and a reduced muzzle size. The tail has pressure pads with flexion creases and ridges on the ventral surface. The auditory sound frequency ranges from 125 Hz to 40 kHz [15, 16]. Like other primate species, vocalizations are the primary mode of communication in rhesus, especially when visual contact is disturbed. Various humans' sound tones are well distinguished by primates, which help in their training. Most primates, including rhesus, have a good sense of smell [17, 18].

The important biological, reproductive, serum biochemistry, and hematological parameters are given in Tables 14.1, 14.2, and 14.3.

14.4 Nutrition

Energy requirement depends on age, sex, and other factors. Juvenile macaque weighing 2 kg requires approximately 80–90 g of feed having 200 kcal/kg/day, whereas an adult rhesus macaque weighing 8 kg needs 110–190 g of feed having 100 kcal/kg/day. The lactating and pregnant females have a higher daily energy requirement of 125 kcal/kg/day [19].

14.4.1 Maintenance Energy

For calculating the maintenance energy in captive nonhuman primates, NRC (2003) [20] recommends using general formula 140 × (body mass in kg)$^{0.75}$. Young, growing, pregnant, and lactating females need additional energy. It is essential to ensure that all animals receive adequate amounts of food when they are housed in a group. Macaques may eat more than their requirement in group housing. Hence, frequent monitoring of body weight is more important to prevent obesity.

Table 14.1 Biological and reproductive data for rhesus macaques [6]

Parameters	Rhesus macaques
Adult body wt. male	6–11 kg
Adult body wt. female	4–9 kg
Life span	25+ years
Body temp.	36–40 °C (96.8–104 °F)
Rectal temp.	37–39 °C (98.6–103.1 °F)
Heart rate	150–333 (beats per minute)
Respiratory rate	10–25 (breaths per minute)
Breeding onset	Male, 3–5 years; females, 2–4 years
Maturity	Male, 4–6 years; female, 3–5 years
Estrous cycle length	28 days
Mensuration days	4.6 days
Gestation period	165–170 days
Weaning age	9 months
Newborn initiation of solid food consumption	1.5–3 months
Birth weight	450–475 g
Chromosome number (diploid)	42
Type of breeding	Seasonal

14.4.2 Protein

The protein requirement in the total diet ranges from 15% to 22% of dry matter (NRC 2003) [20] with a daily requirement of 3 g/kg BW. Protein deficiency will lead to weight loss, muscle weakness, lethargy, and neurological effects [21].

14.4.3 Fiber

As recommended by NRC (2003) [20], the primate diet should have neutral detergent fiber (NDF) and acid detergent fiber (ADF) at 10% and 5% of the total feed, respectively.

Nonhuman primates, in general, requires feed around 3–4% of their body weight. A captive rhesus macaque should be provided dry pelleted feed that should contain a minimum of 15–25% of crude protein, 4–5% of crude fat, 4% of crude fiber, and a maximum of 10% of moisture, for its daily maintenance along with fortified vitamins and minerals, in particular vitamins C and D. Along with pelleted feed, fresh vegetables and fruits are to be added to make it a balanced diet. Moreover, additional food and water access points should be provided to docile animals during group housing. The diet should be formulated according to the age, sex, and physiological status (pregnancy, lactation, and growth) of the animals. Furthermore, feeding and watering devices should be designed in such a way to prevent food contamination and infections. Dominance behavior should be taken care of during feeding

Table 14.2 Serum biochemistry parameters (mean ± SD) in rhesus macaque [45–47]

Analyte	Values
Liver profile	
Alanine aminotransferase (IU/L)	53.42 ± 25.26
Aspartate aminotransferase (IU/L)	38.67 ± 9.62
Total protein (g/L)	78.05 ± 3.59
Indirect bilirubin (μmol/L)	2.11 ± 0.68
Gamma glutamyl transferase (IU/L)	73.67 ± 15.73
Alkaline phosphatase (IU/L)	546.53 ± 171.43
Kidney profile	
Urea nitrogen (mmol/L)	7.83 ± 1.24
Creatinine (μmol/L)	69.64 ± 10.24
Uric acid (μmol/L)	5.30 ± 5.33
Albumin (g/L)	53.87 ± 2.67
Lipid profile	
Triglyceride (mmol/L)	0.84 ± 0.22
Cholesterol (mmol/L)	2.95 ± 0.52
High-density lipoprotein cholesterol (mmol/L)	1.28 ± 0.30
Low-density lipoprotein cholesterol (mmol/L)	1.91 ± 0.39
Electrolytes	
Glucose (mmol/L)	5.72 ± 1.20
Sodium (mmol/L)	151.72 ± 3.82
Potassium (mmol/L)	4.78 ± 0.69
Chloride (mmol/L)	107.14 ± 3.31
Calcium (mmol/L)	2.67 ± 0.16
Magnesium (mmol/L)	0.92 ± 0.07

Table 14.3 Hematological values (range) of rhesus macaques [45–47]

Parameter	Units	*M. mulatta*
Hematocrit	%	37–40
RBC	10^6/ml	5.1–5.6
WBC	10^3/ml	4.2–8.1
Hemoglobin	g/dl	12–13.1
Neutrophils	%	26–52
Lymphocytes	%	39–72
Eosinophils	%	0–4
Basophils	%	0–0.4
Monocytes	%	1–4
Platelets	10^3/ml	260–361
MCV	Fl	71–75
MCH	pg	22.8–24.5
MCHC	g/dl	31–33.4

Ref. [45]

macaques in captivity. Transition to new commercial feed formulation should be gradual with continuous monitoring to avoid any gut upsets [19].

14.5 Housing of NHP

14.5.1 Location

The primate facility should be located on a relatively elevated landscape. It should preferably be away or at a corner from the main laboratory area with good transport. Two main types of housing are prevalent: (1) indoor housing and (2) outdoor housing. The micro- and macroenvironment for indoor housing of rhesus macaques is given in Table 14.4 [22].

14.5.2 Building Material

The building materials should be resistant to the effects of water, fire, and chemicals with flawless design. The painting should be nontoxic and able to withstand high-pressure cleaning.

14.5.3 Corridors

To facilitate the easy movements of housing equipment and caretakers, corridors should be wide enough (≥180 cm). The floor-wall junction shall be finished well. Electric and water supply/drainage pipes need to be operated through an operation panel, preferably located outside the housing.

Table 14.4 Micro- and macroenvironment for indoor housing of rhesus macaques

Microenvironment and macroenvironment	Acceptable range
Temperature	27 °C (summer) and 20 °C (winter)
Relative humidity	40–70%
Ventilation frequency	10–15 times/hour (100% fresh air)
Airflow velocity	13–18 cm/s (direct wind exposure to the animals should be avoided)
Light intensity	150–300 lux
Noise level	<60 dB
Ammonia	<20 ppm

14.5.4 Doors

Modern facilities include doors to open inward for the safety of the personnel with an anteroom in each animal housing room to prevent animal escape. Metal doors with an observation window are preferred. Doors should preferably have a width of ≥90 cm for single-swing doors or ≥120 cm for double-swing doors and a height of ≥200 cm for easy transport of equipment. Doors should have sealing provisions for pests and insects and should be lockable. Considering the welfare and environmental enrichment, the room should have exterior view windows.

14.5.5 Floors

The flooring should be smooth but not slippery. It should be resistant to erosion by acid and alkali and must bear the weight of cages and equipment. Suitable floor materials include epoxies, hard concrete with a smooth surface, very hard rubber, and glass fibers.

14.5.6 Walls

The material used for the wall should withstand cleaning with detergents or disinfectants and sustain high water pressure.

14.5.7 Ceilings

Ceilings may be constructed of smooth materials or metallic grids and humidity resistant. Suspended ceilings should be avoided. The pipes or equipment should not be exposed directly to the ceiling.

14.5.8 Drainage System

A good drainage system with easy drying is essential to prevent humidity. The floor slope should have a minimum pitch of 2.1 cm/m with a diameter of drain pipes preferably ≥15 cm. The pipes from the drain should be connected to the waste treatment unit. Drain pipes should be short and steeply sloped connecting to the main pipe. The drain holes should be properly closed to prevent the backflow of sewage gas.

14.5.9 Individual Cages

The advantage of individual housing helps to maintain the physical health of a primate with clear observation and regular sanitization. The individual caging would prevent injury due to fighting in the open caging system. For an individual caging system, the cage height should accommodate the brachiating movements of the brachiating species. Full leg and tail length should be accommodated in cage height. As there are fewer social activities in an individual caging system, proper environmental enrichment is needed. Chronic self-injuring behavior and stereotypic behavior can be prevented by using enrichment. The minimal cage space requirements for nonhuman primates as per European Council and other guidelines are given in Table 14.5 [23].

Individual cage for each monkey needs to be provided with 24 h water supply with a nozzle system. A seat or perch to rest and enough space to stand to its full length inside the cage are recommended. A stainless steel cage with a provision for a squeezing device to restrain the animal is recommended.

14.5.10 Outdoor Housing/Run Activity/Open Seminatural Housing

Preferably, outdoor housing should be connected with a passage to transport the NHPs to the procedure room/treatment room whenever needed or in big facilities; there can be a separate procedure room for outdoor macaques. Outdoor housing is best preferred for social housing of macaques for growing infants, rehabilitation after experiments, and group matings. There should be an adjacent indoor housing facility within the outdoor system to protect animals from adverse climates. Peripheral and open-top fencing with galvanized metal wire/fiber sheets helps to avoid contact with wild monkeys and other species. A double-door design, along with a security area locking system, is provided in outdoor housing to prevent possible escapes. The inside gate is always designed smaller than the outside gate to discourage the escaping tendency of monkeys.

The restraining of NHPs from outdoor housing is facilitated with the help of squeeze traps in the confinement region of the security area under a double-door gate system. Multiple open enclosures for group housing are preferred to avoid dominance in a relatively larger group. Portable drinking water with push nipples must be available 24 h × 7 days. Food items must be made available at regular intervals for

Table 14.5 Cage size requirement

Adult female rhesus weighing below 10 kg	Floor area	Height	Maximum cage volume
US guidelines (USDA 1991)	4.3 ft^2 (0.4 m^2)	30 in. (76.2 cm)	10.7 ft^3 (0.30 m^3)
European Union (EU) parliament	21.5 ft^2 (2.0 m^2)	70.9 in. (180.1 cm)	127 ft^3 (3.6 m^3)

Fig. 14.1 An outdoor housing for free ranging activity along with environmental enrichment (swings, rocks, and plantation)

Fig. 14.2 An outdoor housing for free ranging activity along with environmental enrichment (swings, rocks, and plantation)

good health and to keep them busy. A wall of 2–3 ft. height along with pavements is made to avoid the entry of pests and reptiles in an outdoor housing. The floor slope should have a minimum pitch of 2.1 cm/m for the easy flow of liquid waste while washing the floor(s). Swings and other enrichment devices like wooden logs and rocks are preferred for outdoor housing for their well-being (Figs. 14.1 and 14.2).

14.5.11 Other Rooms Needed in a Primate Facility

There should be sufficient space for the office and staff room; feed storage room; clinical laboratory including biochemistry, pathology, and microbiology; surgery room; x-ray facility; necropsy room; etc.

The following points are recommended by the International Primatological Society for primate housing in captivity [24]:

- The outside enclosure facility must have secondary containment barriers if a primate escapes from the primary enclosure.
- The housing system should facilitate physical, physiological, and psychological well-being.
- The open housing system should be large enough for group housing so that the animals have free movement and postural adjustments and should contain adequate environmental enrichment for foraging, exploration, grooming, and play, with easy access to food and water and adequate ventilation.
- Housing and caging systems should be furnished with durable materials.
- Sharp edges with wires protruding should be avoided.
- Rusted or deteriorating parts should be regularly renovated or replaced.

14.6 Breeding of Rhesus Macaques

In rhesus macaques, the first menstrual period starts approximately 1 year before the regular cycles. The initial cycles are anovulatory and irregular [25]. There will be an increase in estrogen concentration with uterine growth and secondary sexual characteristics like the coloration of sexual skin [26]. Females start bearing the first offspring approximately at 4–5 years of age. They are seasonal breeders, and the majority of breeding occurs from October to December. Conceptions often happen in the first cycle of the mating season. The mating period lasts about 9–11 days. During the breeding season, the female will have sexual skin at the perineal region that gets reddened. Rhesus females have lesser swelling of the perineal skin as compared to other macaques. The menstrual cycle in female rhesus macaques is 28–29 days, and they are more receptive to copulation on days 9–11 of the cycle. The female reproduces until about 20 years of age.

Males have red sexual skin on the face and scrotal area that brightens in color during the mating season. Usually, the size of the testis increases during the breeding season. Fertile sperm will be produced by 4 years of age, but they reproduce when they reach adult body size at 6–8 years of age. The males mount several times before ejaculating.

Females usually produce only one offspring and are uniparous animals. The captive rhesus macaques attain maturity sooner than the wild. During the breeding season, females will have multiple partners and spend more time in mating with males [27]. Gestation averages 164 ± 5 days, and the interbirth interval is 12 to 24 months [28]. There are reports that during the first pregnancy, they have early

abortion or death of infants after their first delivery. However, during the next breeding season, the success rate of pregnancy and survival of the infants will be better than the first pregnancy [29].

14.7 Procedures Performed on NHPs/Rhesus Macaque in Biomedical Research

14.7.1 Identification

Different types of identification can be performed in macaques. Individually caged animals can have cage cards, collars with/without tags, dye marking on hairs, hair clipping, etc. The invasive permanent marking system includes thigh/chest tattoos and electronic microchips. For group or social housing, the macaques can be easily identified from a distance by applying loose tags around their necks. Dyes and markers on hair coats and a unique pattern of clipped hair are another way of temporary identification in an open outdoor housing. Ideally, noninvasive methods of identification should be used, particularly for pre-weaned animals. A combination of highly visible temporary and permanent identification method is preferred [30].

14.7.2 Handling and Restraint

The rhesus macaques are restrained using squeeze cages, manual restraint, restraint boxes, and primate restraint chairs/pole-and-collar method. However, adequate training is needed to restrain the macaques for restraining using chairs and pole-and-collar methods. Squeeze cage system is adopted for short-duration restraints like single blood samples and administration of injections. For a longer duration, chemical methods are adopted. Ketamine hydrochloride is commonly used for the chemical restraining of macaques. However, careful consideration should be given before performing chemical restraint. It should not create the following:

a. Pain and/or distress while restraining.
b. It should not cause harm to the animal and the handler [31].

The positive reinforcement training or habituation to the procedure can help in stress-free proper restraint.

The handlers should be well trained and experienced. The duration of restraint should be minimum, and the monitoring of animals is essential during restraint. Patience and positive reinforcement significantly reduce the stress to both the handler and the primate. Prior training of larger macaques in open social housing is needed for restraining. Young animals can be manually restrained by holding both arms and then captured from the back. Care should be taken by the handlers, and make sure that the head and face of the monkey are away from the handler to avoid scratches, bites, etc. Proper inspection of the restraining devices has to be done

periodically so that the parts are secure and function efficiently without causing injury to the animal or the handler. Ideally, restraint chairs that allow primates free use of the arms and legs should be equipped with a guard to protect handlers from exposure [32].

14.7.3 Blood Collection

The approximate circulating blood volume in a rhesus macaque is 44–67 ml/kg body weight with total blood volume in a normal adult male monkey of 420–770 ml and for females 280–630 ml [33]. A maximum of 10% of the circulating blood volume can be withdrawn at a single time at 3–4-week intervals with minimum adverse effects. The shorter the interval, the lesser would be the volume of blood that can be withdrawn. The maximum permissible blood volume is 10 ml/kg for adult macaques and 6 ml/kg for macaques less than 4 months of age at single time. The maximum blood volume for collection depends on hematocrit and/or hemoglobin, health status, age, time of the female cycle, etc. Animals that are aged, stressed, or with the spontaneous or experimental disease or females that are menstruating may not tolerate blood collections at maximum prescribed volume [34].

Blood collection is usually performed after restraining with anesthesia. The animals should be trained so that they would cooperate by presenting a limb or other site for conscious sampling. The blood collection sites are femoral, saphenous, and cephalic veins [35]. Smaller volumes (less than 5 ml) can be collected from cephalic and saphenous veins after proper restraint and sterlization sterilization of the collection site(s). For larger blood volumes, the femoral vein is preferred. For arterial blood, the femoral triangle is a suitable site. The person collecting the blood samples must be well trained and experienced to know the anatomy and behavior of the primates. Both the technical person and primate should be conditioned with regular training to obtain maximum cooperation for the procedure. Restraining a macaque to facilitate venipuncture will cause stress that may results in hematological alterations. This must be taken into account when assessing normal values and when interpreting experimental data.

For long-term blood collections and multiple continuous drug dosing, surgically placed vascular access ports (VAPs) are implanted using aseptic principles. VAPs can be maintained for months together by proper use of anticoagulants and sterile techniques. Sometimes, tethering of VAPs can be extended to adjoining remote sampling rooms to avoid any physical disturbances to the macaque, particularly in hormonal studies.

14.7.4 Urine Collection

Urine can be collected either by a free catch method where a metabolic cage or a collection pan with a wire grid is used to separate the urine and feces. For sterile urine collection or minimizing the contaminants, cystocentesis is performed under

anesthesia. During cystocentesis, the urinary bladder is palpated to ensure that the urine is present; the lower abdominal site is cleaned and sterilized with 70% alcohol and/or disinfectant like povidone-iodine. A 22 G needle is inserted perpendicular to the abdomen just cranial to the pubic bone, into the bladder, and the urine sample is slowly withdrawn. Care should be taken not to redirect the needle as it may cause a laceration of the viscera. A sterile 3.0–9.0 French catheter can also be used to collect the urine through urethra [36].

14.7.5 Fecal Collection

The feces can be collected by metabolic cages or a clean pan placed under the housing cages at a slant to avoid mixing of urine in a gross fecal sample. For anaerobic cultures, the deep rectal swabs can be taken and immediately transferred to a suitable container. This is preferably performed under chemical restraint [37].

14.7.6 Drug Administration

14.7.6.1 Oral Route

Like other large animals, drug administration in NHPs is done by oral and parenteral routes. Oral dosing is not very easy in macaques. It is accomplished by placing the drug in a piece of fruit or on the bread piece covered with fruit jelly or butter. Macaques tend to pick out the drug or medicine and eat the rest of the fruit or bread piece. Therefore, most palatable and favorite diets and their flavors are often tried for such a purpose.

14.7.6.2 Nasogastric Intubation

For intubation, a 5–8 French nasogastric tube can be used for the administration of oral fluids and medication. Nasogastric intubation also helps in relieving acute gastric dilation (bloat) and for performing gastric lavage [37].

14.7.6.3 Subcutaneous (SC) Route

Subcutaneous injections are usually done under aseptic precautions on loose skin folds at dorsal neck regions. The maximum volume that should be administered is 5 ml/kg per site [37]. Care should be taken not to puncture the vein.

14.7.6.4 Intramuscular (IM)

This route is most preferred for any small-volume injections. IM injection is usually given in the thigh region. Care should be taken to avoid sciatic nerve that may induce paralysis. In larger macaques triceps and gluteal muscles can be used for IM injections.

14.7.6.5 Intravenous (IV)

For IV injections, cephalic vein and saphenous veins are mostly preferred. The jugular vein is also used for IV injections, but it is not common. Small doses can be safely given by an experienced person in squeeze-back cages by proper restraining. A larger dosage should be given with intravenous infusions after chemical restraining/sedation. For repeated collection and dosing, needles or catheters are secured with a micropore tape at the site of the femoral vein. More sophisticated systems can be used by employing a jacket with tether or backpack equipment [38]. Vascular access ports (VAPs) are used for multiple bleeding based on sampling time points [39].

14.7.6.6 Intradermal (ID)

ID Injections are usually performed for antigen testing like tuberculin and some vaccine response. Hairs are clipped, and the skin area is rendered aseptic or normal saline as desired by research protocol and drug compatibility, and the injection is given in the dermis region. The maximum dosing volumes (ml/kg) in macaque are given in Table 14.6 [40, 41].

14.8 Anesthesia in Primates

Anesthesia is a reversible condition where the patient undergoes the suppression of pain perception by the central nervous system and produces a sufficient level of unconsciousness in general anesthesia. Sometimes, a localized body part can be made insensitive as in local anesthesia. Muscle relaxation and suppression of reflex are two other important features of general anesthesia.

14.8.1 General Anesthesia

Macaques are usually anesthetized or sedated with ketamine hydrochloride injection for all major and minor procedures. Dose ranges from 5 to 20 mg/kg body weight depending on duration and level of sedation or anesthesia required. The anesthesia lasts for 15–30 min, and complete recovery occurs within 1–2 h. For a longer duration, the dose can be increased to 15–20 mg/kg body weight spread in longer period as a sole agent along with the use of anticholinergics and fluids and temperature monitoring. Ketamine will not be able to provide sufficient analgesia in major

Table 14.6 Maximum dosing volumes (ml/kg) in macaque

Route	Macaque
Oral	Ideal 5–8 ml/kg, max 15.0 ml
SC	Ideal 1 ml/kg, max 2 ml (divided in 2–3 sites)
IM	Ideal 0.25 ml/kg, max 2 ml limit
IV	Ideal 1–5 ml/kg bolus, max 10 ml (infusion)
IP	Ideal 3 ml/kg, max 10 ml

surgeries. Therefore, analgesics and other suitable anesthetic combinations are preferred.

14.8.2 Inhalant Anesthesia

Isoflurane is a potent inhalant anesthetic agent that produces rapid induction and rapid recovery and is safe and effective in most species, including macaques. Isoflurane is nonflammable and nonexplosive. Isoflurane does not undergo biotransformation, and most of it is removed from the lungs by exhalation. Liver enzymes are not affected with the minimum risk of drug interactions. It is a potent anesthetic used with a properly calibrated vaporizer. It produces hypotension and tachycardia. The depth of anesthesia is accompanied by a reduction in respiratory rate, heart rate, palpebral reflexes, pedal reflexes, and jaw tension. Due consideration to all these parameters is beneficial to avoid anesthetic accidents and their adverse effects like respiratory and cardiac rate depression. Other complications like regurgitation, hypothermia, and dehydration should be taken care of during general anesthesia.

14.8.3 Local Anesthesia

Lignocaine and bupivacaine are the main local anesthetic drugs used alone or in combination with epinephrine to enhance their effect. Surface anesthesia and local infiltration techniques are mainly used to reduce the dosage of the main drug in general anesthesia and also as pain management postoperatively. Local anesthesia has less influence on normal physiological functions. Local nerve block and regional anesthesia are rarely indicated in primates.

In surface anesthesia, the local anesthetic drug is directly applied to the mucous membranes and on the skin to have the desired desensitization. This is commonly used in the correction of rectal and vaginal prolapse, catheterization of the urethra, or superficial skin incisions. Local infiltration is indicated for superficial tissue biopsy.

The details of dose and drug combinations for various anesthesia are given in Table 14.7.

14.8.4 Preparation for Surgery Preoperative and Postoperative Care

Generally, the macaques fasted 12–16 h before any major surgery or deep anesthesia. This helps to avoid the occurrence of regurgitation and aspiration pneumonia. But fasting may not be necessary for sedation and light anesthesia. For proper surgical anesthesia, thermoregulation is taken care of using thermal pads, room heaters, blowers, or warm circulating water pads.

Endotracheal intubation: Inhalation anesthesia may be delivered either by face mask or by endotracheal intubation. Macaques can be intubated using a laryngoscope using lubricated endotracheal tube sizes 3–5 mm. Endotracheal tubes need to

Table 14.7 Preanesthetics and anesthetics used in nonhuman primates [48]

Drugs anticholinergic	Dose	Dosage route: IM, intramuscular; SC, subcutaneous; IV, intravenous
Atropine	0.05 mg/kg	SC
Sedatives		
Diazepam	1 mg/kg	SC
Acepromazine	0.2 mg/kg	IM
Anesthesia		
Ketamine	5–25 mg/kg	IM
Ketamine + diazepam	15 mg/kg 1 mg/kg	IM IM
Ketamine + medetomidine	2–5 mg/kg 0.03–0.05 mg/kg	IM IM
Ketamine + xylazine	10 mg/kg 0.5 mg/kg	IM IM
Propofol	7–8 mg/kg	IV
Thiopentone	15–20 mg/kg short duration, 5–10 min	IV
Inhalant anesthesia		
Isoflurane	3–4% induction inhalation 1.5–2.0% maintenance	
Sevoflurane	4–8% induction; 1.25–4% maintenance inhalation	

have a cuff, and it needs to be inflated during anesthesia. For most macaque species (>5 kg), a rebreathing system is used. Overinflation of the endotracheal tube cuff can damage the trachea. Ophthalmic ointments are used for the eyes to prevent corneal drying and trauma.

14.8.5 Surgical Preparations and Postoperative Care

Necessary legal and ethical permissions must be obtained before the start of the experimental surgery. The person carrying out the procedure needs surgical skill, anatomical knowledge, and adequate experience gained under the guidance and training by well-qualified professionals. Hygiene and asepsis are the basis of sterility, and proper sterilization procedures must be adopted strictly.

14.8.6 Preoperative Consideration

Scrubbing of the planned surgical wound site is done after proper shaving of hairs. The animal should be appropriately positioned. The limb flexion may compromise peripheral circulation and contribute to the formation of intravascular thrombosis, local ischemia, or postoperative edema [42]. The surgeons should wear necessary

sterile surgical clothing before the procedures. The surgical site should be cleaned thrice using antiseptics in a circular outward pattern from the center of the surgical site to the periphery at the preparatory room itself. It is first done with a 2% chlorhexidine solution, followed by 70% alcohol and then finally with a povidone-iodine 5% solution. Then, the animal is shifted to the surgical area room, and final scrubbing is done with the povidone-iodine solution before applying the sterile surgical drapes. The surgical room should have surgical lights that provide cool, shadowless, and color-corrected light; prevent direct glare; and allow easy monitoring of the vital parameters like pulse rate, heart rate, and respiratory rate. The body temperature (98.6–99.8 °F, which can be slightly increased to 100–101 °F) of the animal should be recorded during and after the surgery. Hemorrhages and tissue trauma should be taken care of by the surgical team to avoid postoperative complications of inflammation and infection. Fluid therapy (0.9% NaCl or lactated Ringer's 5–10 ml/kg/h i.v.) and thermoregulation during the perioperative periods are essential. Fluids can offset blood pressure decreases that may be mild to moderate [42]. The preferable suture materials used are chromic gut or polyglactin 910 (vicryl) as absorbable suture material and nylon; polyester is used as nonabsorbable sutures. The use of silk sutures results in excessive tissue reaction and increased susceptibility to infection and wicking and is not recommended.

14.8.7 Postoperative Consideration

Recovery from anesthesia should be well monitored by the perioperative team by providing an ambient environment and postsurgical pain management. Some animals will try to disturb the implants or stitches by scratching the area. Opioids can be used for calming but not for a prolonged period. Tranquilizers such as acepromazine maleate at 0.1–1 mg/kg SC or IM, SID, or BID may be effectively used [42]. Empirical antibiotic therapy should also be started to control opportunistic bacterial infections. The animal should also be monitored for any abnormal bleeding, discharges, or unwarranted events. When the animal comes to sternal recumbency and the righting reflex is restored, it can be safely shifted from the recovery room to the suitable holding area. Water and food withdrawals can be continued for the next 4–6 h. Following that, water and soft and palatable diets can be provided.

Postoperatively, the animal and wound are monitored every day for any abnormal swelling (edema), redness (erythema), discharges, bleeding, wound dehiscence, and abnormal postures due to pain. Food and water intakes, as well as urine and fecal outputs, need to be monitored daily. Any deviation from normal observations should be attended promptly. The suture removal is usually done around 10–14 days or after proper healing. After recovery, the animals should be properly taken care of for 4–6 weeks and should not be used for any procedures. Some commonly used antibiotics and analgesics are given in Table 14.8.

Table 14.8 Common antibiotics and analgesics used in nonhuman primates and their doses [52]

Antibiotics	Dose and route of administration
Amikacin	5 mg/kg IM TID
Amoxicillin	6.7–13.3 mg/kg IM/PO TID
Amoxicillin/clavulanic acid	6.7–13.3 mg/kg PO TID
Ampicillin	25–50 mg/kg/day IV/IM div TID-QID
Cefotaxime	50 mg/kg IM TID
Cefazolin	20 mg/kg IV/IM TID
Ceftriaxone	50 mg/kg IM SID
Cephalexin	30 mg/kg PO BID
Ciprofloxacin	10 mg/kg PO BID
Doxycycline	2.5 mg/kg PO BID 1st day; 2.5 mg/kg PO SID day 2
Enrofloxacin	5 mg/kg IM/PO SID/BID
Erythromycin	35 mg/kg PO TID; 15–20 mg/kg/day IM BID
Furazolidone	10 mg/kg PO BID
Gentamicin	2–4 mg/kg IM BID
Metronidazole	50 mg/kg PO SID
Streptomycin	2.5–5 mg/kg IM BID
Sulfadimethoxine	50 mg/kg 1st day and then 25 mg/kg IM SID
Sulfamethoxazole/trimethoprim	Trimethoprim: 4 mg/kg SQ/PO TID
Tetracycline	20 mg/kg PO TID
Analgesics/anti-inflammatory	
Acetaminophen	6 mg/kg PO TID
Buprenorphine	0.01–0.03 mg/kg IM BID
Carprofen	2 mg/kg PO BID
Dexamethasone	Shock, 2–4 mg/kg IV once; CNS, 0.25–2 mg/kg IM BID; anti-inflam, 0.25–1 mg/kg IM SID; CNS, 0.25–2 mg/kg IM BID
Fentanyl	7–10 mcg/kg/h IV CRI; 0.05–0.15 mcg/kg IM; patch: 25 mcg/5–10 kg, 50 mcg/10 kg animal q48-72 h
Flunixin meglumine	2 mg/kg IM BID
Ibuprofen	7 mg/kg PO BID
Ketoprofen	2 mg/kg IV/IM SID
Ketorolac	15–30 mg/animal initially; then 10–15 mg/animal q8h
Lidocaine	0.7–1.4 mg/kg IV PRN
Prednisolone sodium succinate	10 mg/kg IM/IV
Prednisone/prednisolone	0.5–2 mg/kg PO
Triamcinolone	0.02–2 mg/kg IM

SID once daily, *BID* two times a day, *TID* three times a day, *PO* orally, *IM* intramuscular, *PRN* as needed

The drug doses listed in this formulary were taken from several references and the authors' personal experience. As always, exercise professional judgement when selecting a dose. While every effort has been made to ensure the accuracy of information contained herein, the publisher, editor and contributors are not legally responsible for errors or omissions

14.9 Euthanasia/Humane Endpoints

Early euthanasia is done to prevent, alleviate, or reduce pain, distress, and suffering of nonhuman primates. Euthanasia should provide a rapid death with no or minimal pain and stress. Primates have a highly evolved brain, so they are euthanized at an isolated place that is away from other primates. This will avoid stress and anxiety among group mates. Nonhuman primates must be handled very carefully, safely, and humanely during euthanasia. Vocalization and release of pheromones lead to stress and anxiety among other primates. The personnel involved in doing this must be well qualified and trained. The person must be aware of biohazard dangers and should take all possible precautions thereof. Euthanasia is advised when macaque suffers from a non-recoverable state of health conditions such as major organ failure, nonresponsive medical conditions, conditions like paresis, paralysis due to spinal injury, or lateral recumbency due to other primary diseases. Other factors considered for euthanasia include anorexia and considerable loss of body weight due to illness, loss of consciousness, persistent irreversible hypothermia, or if an animal is found to be or is suspected to be infected with a highly dangerous pathogen.

Primates are first given the sedative or anesthesia with ketamine hydrochloride. It is then removed from the place safely, and euthanasia is performed by an intravenous injection of barbiturate in a designated place. In general, a 20% solution of sodium thiopental/pentobarbital injection via intravenous route as a bolus of 100 mg per kg body weight is considered an instant and effective drug for euthanasia. The confirmation of death is done by observing the cardiac and respiratory arrest with a stethoscope for a minimum of three consecutive times for 8–10 min to avoid any reversal from the deep plane of anesthesia. Cessation of pulse and respiration and a loss of blink reflexes are taken as confirmation of death. The carcass is disposed of as per the guidelines of the regulatory authorities and local municipal laws. No biomedical waste should contaminate the premises as well as the personnel. It must be packed leakproof. The autopsy room or other designated place for this procedure must be sanitized with suitable disinfectants like sodium hypochlorite, detergents, ionophore disinfectants, and UV light exposures. When the procedures involve exposure of body cavities, there are chances for viruses and other pathogens to exit the carcass. During these procedures, strict safety measures should be adopted to prevent the risk of infection.

14.10 Preventative Health Program

14.10.1 Pre-Shipment

Before pre-shipment, each animal should be given a physical examination and their body conditions scored. Serum biochemistry and hematological parameters should be performed and evaluated by a veterinarian. Necessary bacterial culture from nasal and fecal swabs and stool examination should be performed for checking the presence of infection/infestation. Tuberculin skin testing (two times 2 weeks apart)

and serology to rule out viral diseases should be performed before shipment to the primate colony. At entry into the facility, it should undergo a proper quarantine period of 45–90 days so that any underlying diseases could be identified and could be prevented from entering/spreading into the colony [43].

14.10.2 Disease Surveillance

A strict quarantine with good husbandry and trained staff will help to reduce the occurrence of disease and the introduction of new diseases. Besides, balanced nutrition, planned sanitation programs, and an ideal environment are important factors that provide the optimal health and well-being of a primate colony. For a good disease surveillance program, daily health observation of animals by animal care staff, veterinary technical staff, and veterinarians plays a significant role in preventing and controlling diseases in a colony. However, in macaques, identifying disease in its early stages particularly infectious and metabolic diseases can be challenging. The animals should be checked for external injuries at the early stages in captivity. Social and open housings predispose them to greater exposure to pathogens and injuries due to fights. But social housing is needed for better growth and development. Some of the pathogens affecting primates are fatal and zoonotic, thus causing a great loss in terms of morbidity and mortality both in NHP populations and in human personnel attending them. Hence, proper health monitoring systems must be in place for disease prevention.

The macaques should be observed discretely daily for locomotion, activity, and interaction with others. Further, any abnormal fecal, bloody, or body discharges and their dietary intakes should be noted. Lethargy and lack of feed and water intake should be reported to the attending veterinarian. On physical examinations, attention should be given to body condition, body weight, lymph nodes, mouth, and palpable reproductive organs as well as limbs and digits. Apart from seasonal alopecia, the skin and hair coat are good indicators of health status. Coughing, swollen or draining lymph nodes, and progressive body weight loss are suggestive of tuberculosis. Mouth ulcers in macaque are suggestive of herpes B virus. Hemorrhages of gums and gingivitis are often due to vitamin C deficiency. Any trauma, abnormal tissue growth, or neoplasia should be noticed and reported to the veterinary team. The details of the diseases and clinical signs are given in Annexure 1.

Intradermal tuberculin skin test (TST) is the primary screening for diagnosis of tuberculosis in macaques. After injecting the tuberculin antigen intradermally, usually at the upper eyelid, the tissue reaction is observed at 24, 48, and 72 h post injection for redness/erythema/swelling/edema. The reactive individuals are considered positive and are separated from the colony mates. The test is repeated at 2–3-week interval. During this period, hematological examination and other tests like chest x-ray, PCR, culture, and acid-fast staining of mycobacteria should be done to confirm the disease. Positive animals, if any, are generally euthanized.

14.10.3 Vaccination

Vaccination in nonhuman primates depends on various factors like species, colony risk, safety, research needs, and regulatory needs. Prophylactic vaccines for measles, tetanus, and rabies are commonly practiced in primates [43, 44].

14.11 Infectious Diseases of Nonhuman Primates

Infectious and zoonotic diseases are the major causes of concern for rhesus macaques in captivity. The details of diseases and causative organisms, their clinical signs, and their control are tabulated in Annexure 2. They can be effectively controlled by judicious health monitoring programs, preventative programs, proper education, and training of the staff involved in the primate research center.

14.12 Zoonotic Importance

There is an immense possibility for the zoonotic transmission of pathogens from NHPs to humans in captive NHPs. This happens during the handling of primates, tissues, or fluids and during the handling of sick or dead animals. The increased proximity to humans invites greater risk for NHPs because of their susceptibility to human diseases. An emerging disease resulting from cross-species transmission from NHPs could result in fatalities among human populations. Therefore, all nonhuman primates are considered a potent source of zoonotic disease, and strict precautions must be taken while working with macaque species [44]. In addition to the major bacterial and viral zoonotic diseases (Annexure 2) caused by nonhuman primates, some of the parasites like *Entamoeba histolytica* and *Enterobius vermicularis* and mycotic diseases like dermatophytosis, systemic phytosis, and nocardiosis are commonly transmissible to humans from NHPs.

14.13 Conclusion

Among the nonhuman primates, the rhesus macaque is mostly used in biomedical research. Macaques are genetically very close to human beings. Its use in research has helped us to understand various diseases that occur in human beings and their mechanisms. For maintaining rhesus macaques in captivity, a fair knowledge and understanding of their anatomy, physiology, behavior, environment, housing, diseases, and their control measures are of utmost importance.

Annexure 1: Bacterial and Viral Diseases in Nonhuman Primates [49, 50]

Disease	Causative agent	diagnosis	Clinical signs and symptoms	Treatment/remarks
Bacterial				
Tuberculosis	*Mycobacterium tuberculosis*	Tuberculin skin testing, acid-fast staining and culture of sputum, PCR, chest x-ray	Body weight loss, body condition loss, lymph node (LN) swelling, fever, coughing, partial or complete anorexia, yellowish caseous nodules in the lungs, and hilar LN at necropsy	Isoniazid, rifampicin, ethambutol, streptomycin. Quarantine test in and culling of reactors or diseased individuals
Pneumonia	*Streptococcus pneumoniae*, *Streptococcus* sp. *Klebsiella pneumoniae*, *Pasteurella multocida*, *Haemophilus influenzae*, *Staphylococcus aureus*	Clinical signs and symptoms, culture and isolation of causative organism, necropsy findings—consolidated and red to gray lungs	Fever, coughing, tachycardia, dyspnea, sneezing, mucopurulent nasal discharge, lethargy, anorexia, cyanosis, and prostration in severe cases in *Streptococcus pneumoniae* meningitis with CNS signs complications	Culture and sensitivity (c/s) test helps to select effective antibiotic. Penicillins, cephalosporins, enrofloxacin, tetracycline, and chloramphenicol are effective antibiotics and support complicated individuals with fluid therapy, antipyretics, anti-inflammatory drugs, decongestants, and bronchodilators
Bloody nose syndrome in macaques	*Moraxella catarrhalis*	Isolation of diplococcal organism from nasopharynx and response to select antibiotics	Epistaxis, occasionally periorbital edema	Amoxicillin and clavulanic acid, broad-spectrum cephalosporin, tetracycline, trimethoprim/ sulfamethoxazole, and fluoroquinolones
Bacterial gastroenteritis	*Campylobacter jejuni* in OW primate's transmission is fecal-oral	Fecal culture on 5–10% CO_2 environment, serology	Watery diarrhea without mucus or blood causing dehydration	Fluid therapy to correct dehydration and electrolyte imbalances. The use of antibiotics is debated
	Shigella spp. *Salmonella* spp.	Culture and isolation, sometimes mixed with *Salmonella* spp.	Asymptomatic to acute severe dysentery, depression weakness, blood-tinged mucoid diarrhea, dehydration and emaciation,	Antibiotics based on c/s test, supportive fluid and electrolyte therapy, strict environmental decontaminations. Exposed individuals with infected ones should be evaluated for

(continued)

			abdominal pain, folded hand sitting position to comatose condition, and eventually death. In *Salmonella* spp., similar signs with vomiting also	infection and indicated for treatment
Tetanus	*Clostridium tetani*	Clinical signs and symptoms	Tonic muscle spasms, fever, trismus (lockjaw), seizures, opisthotonos, dysphagia respiratory paralysis, and death	Antipyretics, fluid therapy, procaine penicillin, and tetanus antitoxin can be an effective treatment for tetanus cases. Vaccine – Tetanus toxoid is recommended for primates
Gastritis and vomiting in rhesus	*Helicobacter pylori* in rhesus stomach	Gastric cultures and biopsy results. Histologically lymphocytic, plasmocytic gastritis is seen	In primates, occasional vomiting In appetence	Treatment is similar to humans, multidrug regimen. Complete eradication is not successful
Pseudotuberculosis	*Yersinia pseudotuberculosis* Wild rodents and birds are reservoir hosts. Transmission is by contaminated feed	Microscopic liver or lung lesions show central area of necrosis surrounded by neutrophils, clumps of bacteria, and zone of macrophages	Diarrhea, enteritis depression, dehydration, and death in acute cases. In chronic cases, septicemia and mucopurulent lesions in the liver, spleen, and other organs may be confused with tuberculosis	Isolation, symptomatic treatment with antibiotics for valuable individuals
Melioidosis	*Burkholderia pseudomallei*, a latent infection, may be transmitted via contact with a contaminated environment	Gross findings—suppurative or caseous nodules in tissues or organs—the lung, liver, spleen, and lymph nodes – And or pneumonia	Nonspecific or depends on affected organ system, bronchopneumonia, subcutaneous abscesses, lymphadenitis, and swollen joints	Most infections are resistant to treatment
Viral diseases				
Monkey pox	Orthopoxvirus	Clinical symptoms like vesicular exanthema in the skin, face, hands, and feet. PCR	Viremia develops in 3–4 days and later the virus in the skin, lung, mucous membranes, spleen, and gastrointestinal tract. In the skin, it initially appears as	No specific treatment and animal may recover after several weeks

(continued)

			papules and progress to vesicles	
B virus	*Macaca* herpesvirus It is an alphaherpesvirus	By PCR, ELISA, immunohistochemistry, and histopathology	Increases rapidly with sexual maturity, approaching 80%–90% in some colonies by 3–4 years of age Characteristic vesicular lesions in the oral and genital mucosa, leading to ulcers and recovery within 2 weeks. Macaques are often asymptomatic carriers throughout life	Antiviral drugs like acyclovir may help. Human is the reservoir host Proper PPE kit should be worn by workers, cleaning of exposed parts with detergent water for 15 min Antiviral treatments by expert physicians. Avoid activity of virus shedding/ contaminations to other human beings
Measles virus	*Morbillivirus*	ELISA for deducting measles, immunoglobulin G antibody, real-time PCR	The incubation period varies from 6 to 10 days. Usually, asymptomatic with mild, conjunctivitis, macula papular exanthema is observed, mild upper respiratory infection in macaques—nasal and ocular discharges, facial edema	Human is reservoir host for macaques. Prevent contamination through caretakers and other support staff Necessary vaccinations to animal caretakers Macaques will have immunosuppression when given the measles vaccine
Simian Retrovirus-1	Simian retrovirus (SRV) and simian immunodeficiency virus (SIV)	Serology and viral isolation	SIV is closely related to human AIDS	Recurrent serological and PCR health monitoring of primates to prevent infections
Simian hemorrhagic fever virus	Simian hemorrhagic fever virus	Serology (viral RNA in the blood)	Symptoms include bleeding diathesis and rapid progression to death	Periodic health monitoring of primates However, elimination of clinically ill and exposed animals, disinfection of premises, diagnostic for the etiologic agent
Hepatitis A virus	HAV naturally occurring infection in NHPs	Serology	NHPs are asymptomatic	Personnel working with NHPs should be vaccinated with the hepatitis A vaccine
Hepatitis B virus	HBV hepadnavirus	Serology	NHPs are asymptomatic, but approx. 8% of infected people	Working staff should get HBV vaccine

(continued)

			become chronic carriers	
Simian T-cell leukemia	Delta retrovirus family	By serology and identification of the virus in tumor cells by molecular biological techniques. PCR	Leukemia and lymphoma in baboons, macaques, and African green monkeys	Immunohistochemical diagnosis of neoplastic lymph nodes using antibodies to CD3 and CD20 antigens to rule out the diseases
Rabies		Clinical signs and symptoms; outdoor housed are at risk	Aggression, irritability, hypersalivation, self-mutilation, paralysis, and sudden death	Only killed anti-rabies vaccines advised. A live modified virus vaccine may induce the disease

Annexure 2: Common Zoonotic Diseases Transmitted from Rhesus Macaques [51]

Disease	Mode of transmission	Symptoms
Tuberculosis	By aerosols, infected animals, or tissues	Symptoms include cough, sputum production, and eventually hemoptysis
B virus	By bites and scratches	Symptoms include myalgia, fever, headache, and fatigue. It leads to neurological disorders, including paralysis
Shigellosis	By direct or indirect fecal-oral route	An early symptom includes bloody diarrhea, fever, and stomach cramps
Measles	By fomites and nasal or throat secretions from animals	Symptoms include fever followed by conjunctivitis, coryza, cough, and Koplik's spots inside the mouth
Ebola hemorrhagic fever	By needles or syringes	The symptoms include fever, sore throat, headache, joint and muscle aches, and weakness and, later, diarrhea, vomiting, and stomach pain. A rash, red eyes, hiccups, and internal and external bleeding may be seen in some patients
Marburg virus hemorrhagic fever	Droplets of body fluids, contaminated with infectious blood or tissues	The symptoms include fever, chills, headache, and myalgia Maculopapular rashes develop in the chest, back, and stomach
Hepatitis A	By fecal-oral route and by contaminated feed and water	The symptoms include fever, malaise, anorexia nausea, and abdominal discomfort with jaundice

References

1. Nowak RM, Walker EP (1999) Walker's mammals of the world, vol 1. JHU Press
2. Cawthon Lang K (2005) Primate factsheets: rhesus macaques (Macaca mulatta) taxonomy, morphology & ecology. http://pin.primate.wisc.edu/factsheets/entry/rhesus_macaque
3. Smith DG (2012) Taxonomy of nonhuman primates used in biomedical research. In: Nonhuman primates in biomedical research. Academic Press, pp 57–85
4. Colman RJ, Anderson RM, Johnson SC, Kastman EK, Kosmatka KJ, Beasley TM et al (2009) Caloric restriction delays disease onset and mortality in rhesus monkeys. Science 325:201–204
5. Lambert JE (1998) Primate digestion: interactions among anatomy, physiology, and feeding ecology. Evolution Anthropol Issues News Rev 7(1):8–20
6. Lewis AD, Prongay K (2015) Basic physiology of Macaca mulatta. In: The nonhuman primate in nonclinical drug development and safety assessment. Academic Press, pp 87–113
7. Lambert JE (1998) Primate digestion: interactions among anatomy, physiology, and feeding ecology. Evolution Anthropol Issues News Rev 7(1):8–20
8. Hartman CG (1965) Fifty years of research on mammalian reproduction: a bibliography of the scientific publications of Carl G. Hartman (No. 1281). US Department of Health, Education, and Welfare, Public Health Service, National Institutes of Health
9. Hartman WL, Carl G, Straus (1961) The anatomy of the rhesus monkey, New York, NY, Hafner, pp 22–27
10. Pare PD, Boucher R, Michoud MC, Hogg JC (1978) Static lung mechanics of intact and excised rhesus monkey lungs and lobes. J Appl Physiol 44(4):547–552
11. Miller LA, Royer CM, Pinkerton KE, Schelegle ES (2017) Nonhuman primate models of respiratory disease: past, present, and future. ILAR J 58(2):269–280
12. Binns R, Clark G, Simpson C (1972) Lung function and blood gas characteristics in the rhesus monkey. Lab Anim 6:189–198
13. Cline JM, Brignolo L, Ford EW (2012) Urogenital system. Nonhum Primat Biomed Res:483–562
14. Tischer CC (1975) Structure and function of the rhesus kidney. In: Bourne GH (ed) The rhesus monkey. Academic Press, New York, p 107e143
15. Heffner RS (2004) Primate hearing from a mammalian perspective. Anatom Rec A Dis Mol Cell Evol Biol 281(1):1111–1122
16. Pfingst BE, Laycock J, Flammino F, Lonsbury-Martin B, Martin G (1978) Pure tone thresholds for the rhesus monkey. Hear Res 1(1):43–47
17. Jacobs GH, Neitz J, Neitz M (1993) Genetic basis of polymorphism in the color vision of platyrrhine monkeys. Vis Res 33(3):269–274
18. Laska M, Seibt A, Weber A (2000) 'Microsmatic'primates revisited: olfactory sensitivity in the squirrel monkey. Chem Senses 25(1):47–53
19. Knapka J (2003) Nutrient requirements of nonhuman primates. Lab Anim 32(10):26–26
20. National Research Council (2003) Nutrient requirements of nonhuman primates: second revised edition. The National Academies Press, Washington, DC. https://doi.org/10.17226/9826
21. Riopelle AJ, Hill CW, Li S-C, Wolf RH, Weibold HR, Smith JL (1974) Protein deprivation in primates. I. Nonpregnant adult rhesus monkeys. Am J Clin Nutr 27:13–21
22. National Research Council (US) Committee on Well-Being of Nonhuman Primates (1998) The psychological well-being of nonhuman primates. National Academies Press (US), Washington, DC. Available from: https://www.ncbi.nlm.nih.gov/books/NBK232192/
23. Abee CR, Mansfield K, Tardif SD, Morris T (eds) (2012) Nonhuman primates in biomedical research: biology and management, vol 1. Academic Press, pp 251–268
24. McCann C, Buchanan-Smith H, Jones-Engel L, Farmer K, Prescott M, Fitch-Snyder H, Taylor S (2007) IPS International guidelines for the acquisition, care and breeding of nonhuman primates. International Primatological Society. Available at http://www.Internationalprimatologicalsociety.org/docs/IPS. International Guidelines for the Acquisition Care and Breeding of Nonhuman Primates Second Edition

25. Saltzman W, Tardif SD, Rutherford JN (2011) Hormones and reproductive cycles in primates. In: Hormones and reproduction of vertebrates. Academic Press, pp 291–327
26. Dixson AF, Anderson MJ (2004) Effects of sexual selection upon sperm morphology and sexual skin morphology in primates. Int J Primatol 25(5):1159–1171
27. Lindburg DG (1971) The rhesus monkey in North India: an ecological and behavioral study. Primate Behav Dev Field Lab Res 2:1–106
28. Fooden J (2000) Systematic review of rhesus macaque, Macaca mulatta (Zimmermann, 1780). Fieldiana Zool 96:1–180
29. Seth PK (2000) Habitat, resource utilization, patterns and determinants of behaviour in rhesus monkeys. J Hum Ecol 11(1):1–21
30. Wolfensohn SE (1993) The use of microchip implants in identification of two species of macaque. Anim Welf 2(4):353–359
31. Toth LA, Compton S, Tolwani R, Farrar J (2019) Association of primate veterinarians guidelines for nonhuman primate restraint. J Am Assoc Lab Anim Sci 58(3):276–278
32. Wolfensohn S, Lloyd M (2003) Conduct of minor procedures. In: Wolfensohn S, Lloyd M (eds) Handbook of laboratory animal management and welfare, 3rd edn, pp 150–181
33. Hobbs TR, Blue SW, Park BS, Greisel JJ, Conn PM, Pau FK (2015) Measurement of blood volume in adult rhesus macaques (Macaca mulatta). J Am Assoc Lab Anim Sci 54(6):687–693
34. Diehl KH, Hull R, Morton D, Pfister R, Rabemampianina Y, Smith D, Vorstenbosch CVD (2001) A good practice guide to the administration of substances and removal of blood, including routes and volumes. J Appl Toxicol Int J 21(1):15–23
35. Everds NE (2017) Deciphering sources of variability in clinical pathology—it's not just about the numbers: preanalytical considerations. Toxicol Pathol 45(2):275–280
36. Kurien BT, Everds NE, Scofield RH (2004) Experimental animal urine collection: a review. Lab Anim 38(4):333–361
37. Wolf RF, White GL (2012) Clinical techniques used for nonhuman primates. In: Bayne K, Turner PV (eds) Nonhuman primates in biomedical research: biology and management, 2nd edn. Academic Press (Elsevier), San Diego, CA, pp 323–336
38. McNamee GA, Wannemacher RW Jr, Dinterman RE, Rozmiarek H, Montrey RD (1984) A surgical procedure and tethering system for chronic blood sampling, infusion, and temperature monitoring in caged nonhuman primates. Lab Anim Sci 34:303–307
39. Graham ML, Mutch LA, Rieke EF, Dunning M, Zolondek EK, Faig AW et al (2010) Refinement of vascular access port placement in nonhuman primates: complication rates and outcomes. Comp Med 60:479–485
40. Gad SC, Spainhour CB, Shoemake C et al (2016) Tolerable levels of nonclinical vehicles and formulations used in studies by multiple routes in multiple species with notes on methods to improve utility. Int J Toxicol:1–84
41. Morton DB, Jenning M, Buckwell A, Ewbank R, Godfrey C, Holgate B, Inglis I, James R, Page C, Sharman I, Verschoyle R, Westall L, Wilson AB (2001) Refining procedures for the administration of substances. Report of the BVAAWF/FRAME/RSPCA/UFAW joint working group on refinement. Lab Anim 35:1–41
42. Niekrasz MA, Wardrip CL (2012) Surgery in nonhuman primates. Nonhum Primat Biomed Res:339–358
43. Kramer JA, Ford EW, Capuano S (2012) Chapter 12: Preventative medicine in nonhuman primates. In: Abee CR, Mansfield K, Tardif S, Morris T (eds) American College of Laboratory Animal Medicine, nonhuman primates in biomedical research, 2nd edn. Academic Press, pp 293–321
44. Locatelli S, Peeters M (2012) Non-human primates, retroviruses, and zoonotic infection risks in the human population. Nat Educ Knowl 3(10):62
45. Chen Y, Qin S, Ding Y, Wei L, Zhang J, Li H et al (2009) Reference values of clinical chemistry and hematology parameters in rhesus monkeys (Macaca mulatta). Xenotransplantation 16 (6):496–501

46. Kessler MJ, Rawlins RG, London WT (1983) The hemogram, serum biochemistry, and electrolyte profile of aged rhesus monkeys (Macaca mulatto). J Med Primatol 12(4):184–191
47. Smucny DA, Allison DB, Ingram DK, Roth GS, Kemnitz JW, Kohama SG, Black A (2004) Changes in blood chemistry and hematology variables during aging in captive rhesus macaques (Macaca mulatta). J Med Primatol 30:161–173. (2001) J Med Primatol 33(1):48–54
48. Flecknell P (2016) Chapter 5: Anaesthesia of common laboratory species: special considerations. In: Flecknell P (ed) Laboratory animal anaesthesia, 4th edn. Academic Press, pp 193–256
49. Gibson SV (1998) Chapter 2: Bacterial and Mycotic diseases. In: Bennett BT, Abee CR, Henrickson R (eds) American College of Laboratory Animal Medicine, nonhuman primates in biomedical research. Academic Press, pp 59–110
50. Mansfield K, King N (1998) Chapter 1: Viral diseases. In: Bennett BT, Abee CR, Henrickson R (eds) American College of Laboratory Animal Medicine, nonhuman primates in biomedical research. Academic Press, pp 1–57
51. Burgos-Rodriguez AG (2011) Zoonotic diseases of primates. Vet Clin North Am Exot Anim Pract 14(3):557–575
52. Lee R, Doane CJ (2003) APV primate formulary. APV primate formulary – European primate veterinarians. http://euprimvets.eu/uploads/publications/public/Nonhuman%20Primate%20Formulary.xls

Further Reading

National Research Council (2003) Occupational health and safety in the care and use of nonhuman primates. The National Academies Press, Washington, DC. https://doi.org/10.17226/10713
Revised Recommendations for Health Monitoring of Nonhuman Primate Colonies (2018) FELASA working group report. Lab Anim 53(5):429–446
Sasseville VG, Mansfield KG (2010) Overview of known nonhuman primate pathogens with potential to affect colonies used for toxicity testing. J Immunotoxicol 7(2):79–92. https://doi.org/10.3109/15476910903213521
Christian Abee et al (2012) Nonhuman primates in biomedical research. Biology and management, vol 1, 2nd edn. Academic Press
Christian Abee et al (2012) Nonhuman primates in biomedical research. Diseases, vol 2, 2nd edn. Academic Press
Fortman JD, Hewett TA, Halliday LC. The laboratory non human primate, 2nd edn. CRC press, Taylor & Francis group

Mouse Genetics and Breeding

15

Satheesh Kumar Panneer, Shailendra Kumar Arindkar, and
Perumal Nagarajan

Abstract

The laboratory mouse (*Mus musculus*) has been employed in biomedical research for its amenability to genetic modification. The genetic modifications have enabled the development of new mouse strains for studying diverse diseases. This chapter will provide information on mouse genetics, their nomenclature, and different strategies of breeding. This chapter will also describe the genetic monitoring program and quality control program associated with it. For ease of learning, we have classified the chapter into four broad categories—selection aids, selection methods, mating aids, and mating methods to facilitate a deeper understanding of mouse genetics and breeding.

Keywords

Mouse strains · Breeding system · Genotyping · Quality control

S. K. Panneer
Tamil Nadu Veterinary and Animal Science University, Chennai, Tamil Nadu, India

S. K. Arindkar
Experimental Animal Facility, National Institute of Immunology, New Delhi, India
e-mail: arindkar@nii.ac.in

P. Nagarajan (✉)
National Institute of Immunology, New Delhi, India
e-mail: nagarajan@nii.ac.in

© The Author(s), under exclusive license to Springer Nature Singapore Pte Ltd. 2021
P. Nagarajan et al. (eds.), *Essentials of Laboratory Animal Science: Principles and Practices*, https://doi.org/10.1007/978-981-16-0987-9_15

Abbreviation

GEM	Genetically engineered mice
MGI	Mouse genome informatics
MPD	Mouse phenome database
NCBI	National center for biotechnology information
PCR	Polymerase chain reaction
SNP	Single-nucleotide polymorphism

15.1 History and Introduction of Mouse Genetics

The word mouse originally comes from the Sanskrit word "Mus" which means "to steal" – food from the human food storage areas. The adaptation of mouse colonies around the human habitat started early in history. The mention of a spotted mouse in the Eh Yah lexicon dates to 1100 BC. It is believed that the domestication of the mouse was first done by the Chinese and Japanese. The Greeks and Romans worshipped mice, and different physicians used mice for medical experiments. In India, rodents were regarded as the holy descendants of "Karni Mata," and mice were worshipped as a vehicle of Lord Ganesha in 600 BC. The domestication of various varieties of mice started in the 1700s, and these were used as pets in China and Japan. The Europeans imported them and bred them locally for fancy purposes and as pets. The breeding activity led to the conception of laboratory mice as hybrids among *Mus musculus domesticus*, *Mus musculus musculus*, and other subspecies. In 1900, after the discovery of Mendel's laws of inheritance, scientists considered fancy mice used as pets to test the new theories of inheritance. Different types of breeding programs using fancy mice paved the way to create many inbred strains for research. French biologist Lucien Cuénot in 1902 using Mendel's principle showed the way to the discovery of a lethal mutation in mice. William C. C. Little began to breed mice in the USA and studied the genetics of mouse coat colors. With the help of mouse breeders in the USA, he bred the mice for a particular characteristic for modeling human diseases. He developed the first "lab mouse," the dilute brown non-agouti (DBA) mouse, in 1909. Further, albino mouse strains were developed by Bagg. Strong, another researcher, developed A strain and C3H strains using mice developed from Bagg and C. C. Little. E. C. MacDowell from Cold Spring Harbor used Bagg's albino mice to develop BALB/c mice. In 1921, C57BL strain was developed by C. C. Little from the mating of female 57 with male 52 from Miss Abbie Lathrop's stock [1–4].

15.2 Mouse Genetics

The mouse genome has 3.1 billion base pairs, out of which 5% of the sequence nucleotides are coding regions comprising genes or exons and over 90% are noncoding DNA, including introns, that have no known function. The human genome is 85% identical to the mouse genome in the conserved coding regions. Consequently, mice and humans have many functionally similar genes. Mice are similar to humans in terms of functional genes, anatomy, and physiology. Using mice in research has many advantages such as ease of handling, shorter reproduction life, large litter size, and low cost of production and maintenance. Therefore, scientists predominantly use mice for studying human disorders and some other diseases of domestic animals. A gene of interest or gene combinations are introduced into a mouse that is related to a particular human condition to develop mouse models of human disease and to support the discovery of new therapeutic interventions for human illnesses.

15.3 Mouse Breeding

Breeding of mice for research needs is guided by considerations such as, but not limited to, aptness and availability of a mouse strain or model for the intended research, accessibility of information regarding the strain or model including its genetic background and biological characteristics, adaptability to experimental methods and protocols, ecological consequences, and ethical issues. The breeding system is aimed at preserving or dealing with the genetic causes of variation in the traits of interest. Combinations of selection and mating systems provide geneticists a wide variety of methods for controlling the inherited characteristics of mouse research models. The mouse breeding and quality control programs are closely consistent processes and involve different complex subprograms. However, the specific goal is to produce quality mice for prospective research use. The flow diagram mentioned below illustrates how the two complex processes work for specific goals of a successful mouse breeding program. In the next part of this chapter, we will describe all the topics mentioned in the flow diagram (Fig. 15.1).

15.3.1 Selection Aids

Some Reproductive Characteristics in Mice

1. Mating age: 6–8 weeks.
2. Gestation period: 19–21 days.
3. The weaning age: Average 21 days and based on the weight of the pups.
4. Litter size: 2–12 pups (depends on strain and stock).
5. Replace breeders: Females at 7–8 months of age and males at 1 year of age.

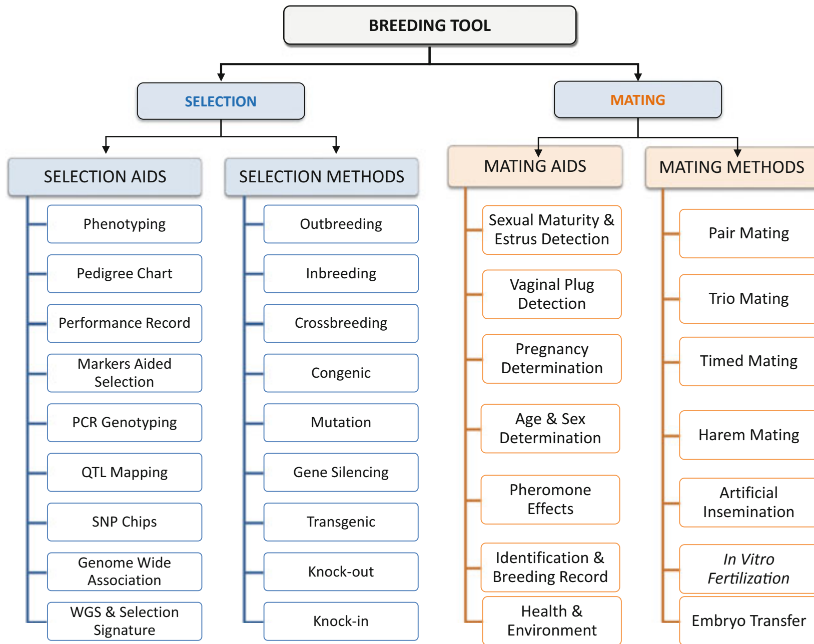


Fig. 15.1 Illustrated flow diagram for an overall outlook of mouse breeding programs

Based on mouse strain, either one or more "selection aids" listed in the flow diagram are used for selecting the breeding pairs. These selection aids are the superior tools for breeders to determine the attributes of animals or judge or rank each animal. Based on this data, the breeder can choose the best breeding individuals for future breeding.

15.3.1.1 Phenotyping

Phenotyping includes characterizing the external qualities of the mice, and the measurable attributes (normal features, traits, and abnormalities) are assessed. Phenotyping also includes evaluating the physiological and biochemical attributes of a mouse biological system. The aim is to define phenotypes that can be recognized by a breeder and used in mouse model development (selective breeding). Knowing the inherent morphological phenotype of mouse strains helps to understand and interpret mouse models that are developed using these strains. This systemic phenotyping encourages us to understand the primary and secondary effects of genes. Systemic phenotyping also aids to analyze the phenotypes of all available mouse models. In the case of mutants, examining (systemic phenotyping) all the organ systems and analysis of mutant mice lines are required for a productive breeding program [5]. Many breeders provide systemic details of the strains they develop that helps to start active breeding programs. In the present times, research involving mouse models requires the investigator to use lines of different genetically

Table 15.1 Names of the online databases and its websites for mouse phenotype and genotype information access

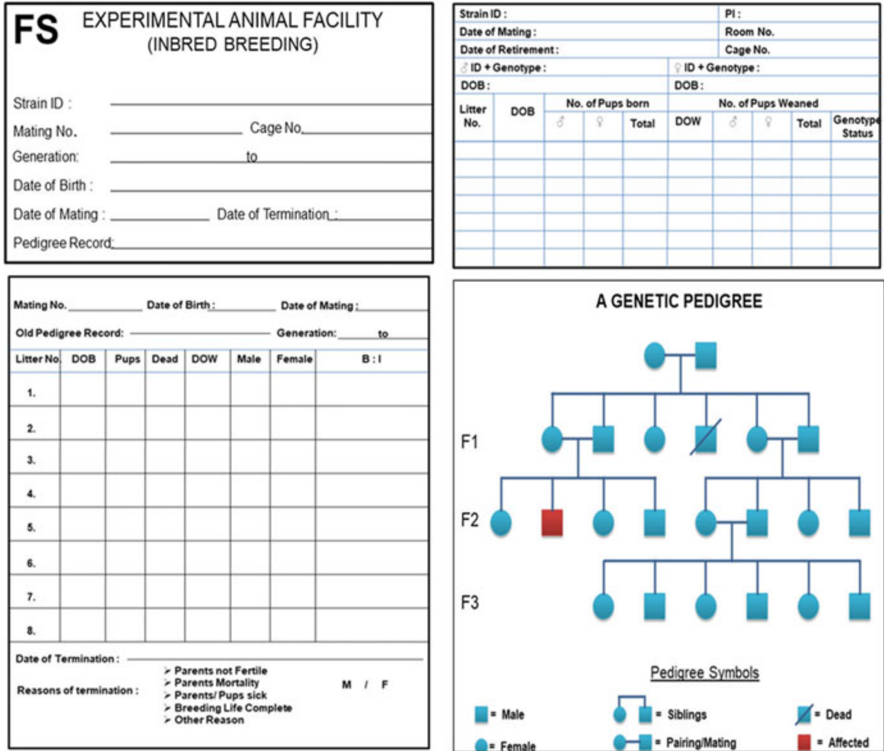
Mouse Genome Informatics	http://www.informatics.jax.org/
Ensembl Genome Browser	http://useast.ensembl.org/index.html
Mouse Genomes Project	https://www.sanger.ac.uk/data/ensembl-mouse/
Mouse Microsatellite Japan	https://shigen.nig.ac.jp/mouse/mmdbj/top.jsp
Mouse Phenome Database	http://phenome.jax.org/
International Mouse Phenotyping Consortium	https://www.mousephenotype.org/impress/
Federation of International Mouse Strain Resources	http://www.fimre.org
International Mouse Strain Resource	http://www.findmice.org/
The Jackson Laboratory	https://www.jax.org/
Riken Bioresource Center	http://mus.brc.riken.jp/en/
European Mouse Mutant resources	https://www.infrafrontier.eu/resources-and-services/access-emma-mouse-resources
Europhenome mouse phenotyping	http://www.europhenome.org/
Mouse Mine Strain information	http://www.mousemine.org/mousemine/templates.do
Mutant Mouse Resource and Research Centers (MMRRC)	https://www.mmrrc.org/
Rat Resource and Research Center	http://www.rrrc.us/
The National BioResource Project Japan	http://www.anim.med.kyotou.ac.jp/nbr/Default.aspx
Rat Genome Database Strain and markers information	http://rgd.mcw.edu/

modified mice that leads to new mouse lines or strains with complex genetic background. Hence, systemic phenotyping is of paramount importance in mutant mice breeding. Since mutant mice are developed using two strains of mice, maintaining proper records of the modified animals is important and must include the correct strain name, the mutation, the background strain, genotyping protocol, and phenotypic changes. A Mammalian Phenotype (MP) Ontology Browser designed by The Jackson Laboratory helps to acquaint researchers with apparent morphological, physiological, behavioral, and other features of the mouse during their development and lifetime (http://www.informatics.jax.org/vocab/mp_ontology/).

Apart from MP, few databases for details of various strains are available on publicly accessible repositories and databases that provide a better understanding of the strain and allow the user to select accordingly (Table 15.1).

15.3.1.2 Pedigree Records

Accurate documentation of lineage and genotype is essential when crossing or backcrossing is done. This can be done using a cage card, and the cage card should provide the basic minimum information regarding cage number, strain name or ID, mating number, line number, gender, date of birth, genotype, generation number,



FS EXPERIMENTAL ANIMAL FACILITY
(INBRED BREEDING)

Strain ID : ____________________
Mating No. ____________ Cage No.________
Generation: ____________ to ________
Date of Birth : ____________________
Date of Mating : ________ Date of Termination : ________
Pedigree Record: ____________________

Strain ID :									PI :	
Date of Mating :									Room No.	
Date of Retirement :									Cage No.	
♂ ID + Genotype :					♀ ID + Genotype :					
DOB :					DOB :					
Litter No.	DOB	No. of Pups born			No. of Pups Weaned					
		♂	♀	Total	DOW	♂	♀	Total	Genotype Status	

Mating No. ________ Date of Birth : ________ Date of Mating : ________
Old Pedigree Record: ________________ Generation: ________ to ________

Litter No	DOB	Pups	Dead	DOW	Male	Female	B : I
1.							
2.							
3.							
4.							
5.							
6.							
7.							
8.							

Date of Termination : ____________________

Reasons of termination :
- Parents not Fertile
- Parents Mortality
- Parents/ Pups sick
- Breeding Life Complete
- Other Reason

M / F

Fig. 15.2 Illustrated samples format for a cage card and Pedigree chart

and date of termination. Besides, a record on breeding setup, successive litters with dates of birth, the number of pups born, and the number of pups weaned, their gender, and genotype should be maintained [6]. Many online breeding database programs are available to help the colony and pedigree maintenance. The pedigree record retraces the pathway of each breeder pair to their ancestors. The pedigree record also helps to identify unwanted mutations affecting fertility and sterility and any changes in the phenotype. A simple pedigree chart system also helps to detect spontaneous mutations and to preserve maximum genetic diversity in outbred strains (Fig. 15.2).

Some of the valuable colony management software are provided in Table 15.2.

15.3.1.3 Performance Record

The following indices can be calculated to test the performance of individual mouse or mouse strains. The breeding indices mentioned below are decisive on the performance of individual mice but based on the performance can help the investigator decide about the replacement of particular mouse breeders as breeding stock. However, each strain has a different reproductive performance, and the records of

Table 15.2 Names of the few mouse colony management software and its website information

Software	Link
MausDB	www.nervenet.org/
JCMS	http://colonymanagement.jax.org/
mLIMS	https://bioinforx.com
Facility	www.locustechnology.com/
SoftMouseDB	https://softmouse.net/
MouseJ	http://mousej.org/
Mouse Colony	http://mousecolony.com/Home.html
PyRAT	www.scionics.com/pyrat.html
ezColony	https://ezcolony.com/
Mosaic vivarium	https://mosaicvivarium.net/

each strain help to select proper breeders. Therefore, consideration of the following parameters as performance records is of paramount importance in selection.

15.3.1.3.1 Important Parameters of Female Performance Record

Litter size = Average number of pups born per litter by a breeding pair.

Wean rate = Average number of pups weaned per litter.

Whelping interval = Average time between births of litters.

Weaning interval = Average time between weaning of litters.

Production index = Average number of weaned pups per life of females.

Sex ratio = Ratio of weaned males to weaned females.

15.3.1.3.2 Important Parameters of Male Performance Record

Plug rate = Number of plugged females/numbers of matings.

Pregnancy rate = Number of pregnancy/plugged females.

15.3.1.4 Genotyping

15.3.1.4.1 Biochemical Markers

Fewer Biochemical markers help to characterize inbred strains. Biochemical markers such as isoenzymes are used to differentiate strains and are identified using blood, tissue extracts, and urine from the mouse strains to be evaluated. There are standard biochemical markers that are used in combination with coat color. The markers are polymorphic and located on chromosomes throughout the genome. This fact is mentioned already in the second sentence above. Few biochemical markers used for genotyping inbred and substrains are as follows: Idh, isocitrate dehydrogenase; Pep-3, peptidase-3; Car2, carbonic anhydrase 2; G6PD, glucose-6-phosphate dehydrogenase; PGM1, phosphoglucomutase 1; Ldr-1, lactate dehydrogenase regulator 1, Gpi-1, glucose phosphate isomerase-1; Hbb, hemoglobin beta-chain; Es-1, esterase-1; Mod-1, malic enzyme 1; Es-3, esterase-3; and Es-10, esterase-10, Hbb, Car1, Car2, and Gpi-1 from RBC lysate, Apoa-1, Trf-1, and Es-1 from plasma, and Es-3, Mod-1, Idh-1, and PGM1 from tissues.

15.3.1.4.2 Immunological Genetic Markers

Immunological markers help to interpret expected phenotypes. They also help to study individual components of the immune system in genetically engineered mice (GEM). They help in identifying the immune cell variation based on the gene of interest and also the effect of background strains used for developing a particular GEM. In immunophenotyping, many selective antibodies are employed for identifying specific leukocyte subsets in each unique cell lineage. Flow cytometry can be used to get quantitative data. Specific fluorescent-tagged antibodies have been employed that allow for the direct examination of a blood sample by multicolor flow cytometry. Multiple cell types can be identified and quantified in a single blood sample, helping to limit sample volume. Evaluation of immune cell composition by immunophenotyping also helps in genetic quality control of inbred and GEM strains [7].

15.3.1.4.3 PCR Genotyping

In this method, a small bit of tissue from the tip of the tail or ear is used for DNA extraction, performing PCR with appropriate primers, and gel electrophoresing the PCR products to determine the genotype. As per the experimental requirement, the investigator can choose the breeding pairs with a suitable genotype combination to obtain heterozygote or homozygote animals in the subsequent generations. Further, the genotypes of each mouse in the colony are helpful to the investigators in identifying the desired progenies in the next generation.

15.3.1.4.4 Restriction Fragment Length Polymorphisms (RFLPs)

A restriction fragment length polymorphism is defined by the existence of alternative alleles associated with restriction fragments that differ in size from each other. It involves fragmenting of DNA with a restriction enzyme that will recognize and cut DNA wherever the specific short restriction sequence occurs. Restriction endonucleases are used as enzymes that cleave the DNA molecules at specific nucleotide sequences characteristic to a particular enzyme being used. The sizes and numbers of cleaved DNA fragments and their patterns on gels vary between strains of mice or even within a strain. The latter is possible if the animal is a heterozygote and the two alleles have variations or a trait under consideration has multiple alleles or there are spontaneous mutations at one of the alleles within the same strain. RFLP can be used to differentiate homozygous, heterozygous, and wild type using restriction enzymes and select the appropriate breeding program. Mouse breeding for *lepr* mutation in db/db mice and mdx4cv mutation in muscular dystrophy mice is done using RFLP. This method is also used in differentiating MHC haplotypes between two strains using various restriction enzymes (Fig. 15.3).

15.3.1.4.5 Random Amplified Polymorphic DNA-Polymerase Chain Reaction (RAPD-PCR)

In this method, the random segments of genomic DNA are PCR amplified by using a series of single primers of arbitrary nucleotide sequences. The amplified products are analyzed by electrophoresis on agarose gels. A small set of random primers is used in

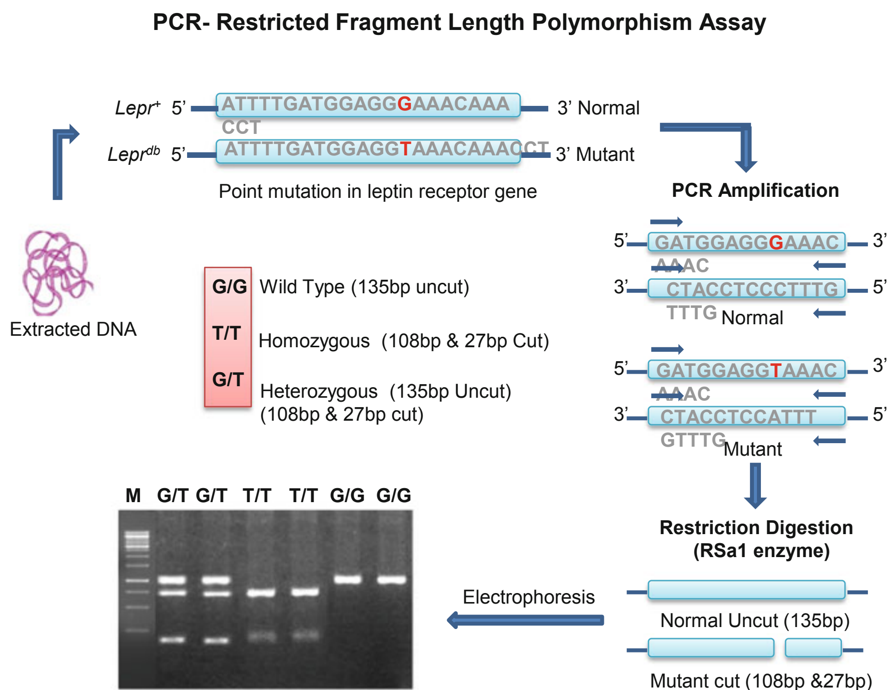


Fig. 15.3 Illustrated flow diagrams of a restriction fragment length polymorphisms (RFLPs) assay

RAPD, and it can identify many polymorphisms because they can be used individually or in pairs [8]. This method can be used to review the DNA variation between the recombinant inbred strains of interest.

15.3.1.4.6 Amplified Fragment Length Polymorphisms (AFLPs)

Amplified fragment length polymorphism (AFLP) is performed when an investigator needs to identify polymorphism. It is a PCR-based technique where restriction enzymes are used to cleave the genomic DNA. The digested/restricted DNA fragments are amplified by PCR, electrophoresed, and then detected by autoradiography or fluorescent sequencing equipment [9]. AFLP is a very sensitive technique for detecting genetic polymorphisms but requires relatively large amounts of high-quality DNA. AFLP markers help to estimate levels of inbreeding and inbreeding coefficient when inbred strains are bred for many generations.

15.3.1.4.7 Single-Nucleotide Polymorphisms (SNPs)

Single-nucleotide polymorphisms (SNPs) are point mutations or natural variations and are noticed in all mouse strains, and they occur at greater than 1% frequency in a genomic population. SNPs are changes in a single base at a specific position in the genome, in most cases with two alleles. Therefore, SNP genotyping is used for the genetic analysis of the mouse because biallelic markers help to characterize and

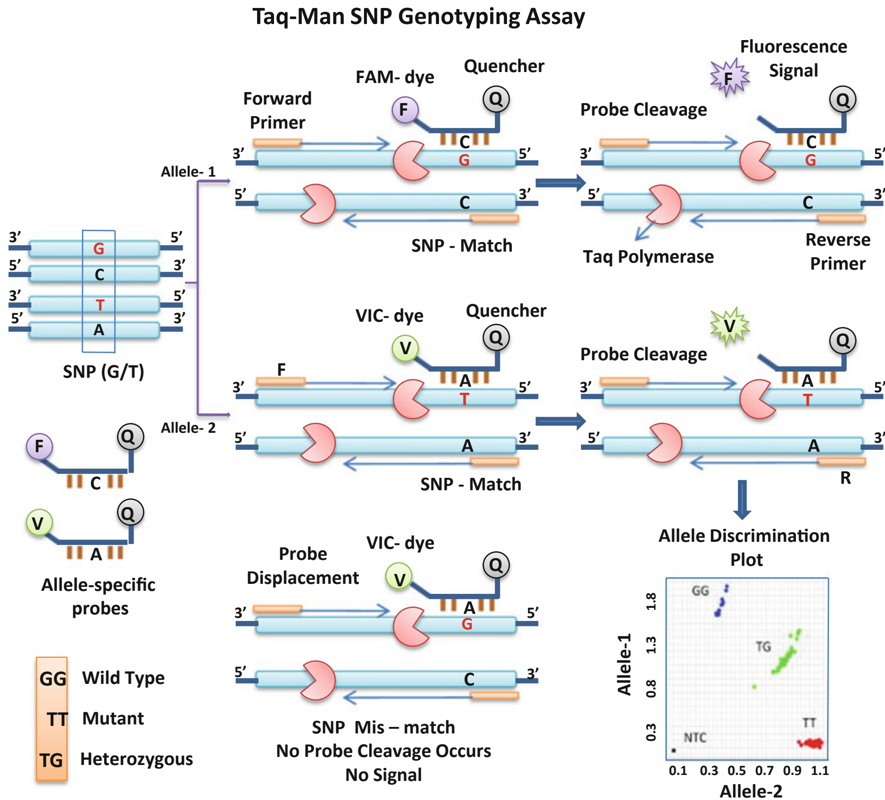


Fig. 15.4 Illustrated flow diagrams of a single-nucleotide polymorphisms (SNPs) assay

provide more information on a particular strain or about crosses between inbred strains. SNP query for various mouse strains can be done (https://phenome.jax.org/genotypes) [10]. Besides, a variety of diagnostic SNP panels are available from commercial vendors (Fig. 15.4).

15.3.1.4.8 Simple Sequence Repeats (SSRs or Microsatellites)

SSRs are short tandem repetitive sequences, which are codominant, abundant, multi-allelic, and uniformly distributed, and can be detected by microsatellite markers. The mouse strains have variable CA repeat lengths, and using PCR, these CA stretches of different lengths can be identified. Detected sequence length varies between mice strains and within the strain and so is useful for linkage and genetic diversity studies among the population. Microsatellites are useful for detecting deletions, insertions, and point mutations in inbred mouse strains. Presently, this method is practiced in genetic quality control of inbred strains (Fig. 15.5).

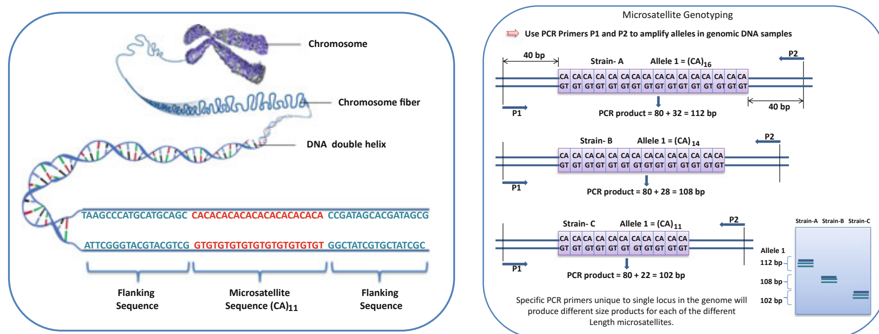


Fig. 15.5 Illustrated flow diagrams of a simple sequence repeats (SSRs) or microsatellites markers assay

15.3.1.4.9 Quantitative Trait Locus (QTL) Mapping

QTL mapping helps to set and find the effect of genetic loci that regulate quantitative traits. QTL mapping requires two or more different strains for the same trait of interest and genetic markers that distinguish between these parental lines. QTL mapping was initially performed using phenotypic markers and then using RFLP, followed by microsatellite markers and at present by SNP markers. A QTL study involves the selection of appropriate parental inbred strains, the establishment of the backcross, collaborative cross with F2 intercross, phenotyping and genotyping of progeny for polymorphic markers that are spaced throughout the genome, and regression analysis [11]. The goal is to determine genomic regions in which allele variation is associated with phenotypic variation. For detecting modifiers of a specific disease phenotype, one of the parental strains should carry a disease-causing allele for QTL analysis.

15.3.1.4.10 SNP Chip (the Mouse Diversity Genotyping Array)

The microchips used in this process contain varying numbers of markers and are referred to as unique “SNP panels.” To test the differences between the substrains of a mouse model, for example, a panel of 200–300 SNPs may be tested. However, to mostly characterize the background of a mouse, SNP panels comprising two thousand SNPs may be required. SNP chips are designed by different companies using the Illumina Infinium HD platform. This helps to genotype the various inbred strains and their collaborative crosses.

15.3.1.4.11 Genome-Wide Association Study (GWAS)

Genome-wide association studies in mouse models provide multiple advantages over large-scale analyses in human populations, including cost-effectiveness, reproducibility of results, and reducing the impact of environmental-related factors. The hybrid mouse diversity panel (HMDP) is one such resource that consists of many characterized inbred, outbred, or recombinant inbred strains. These strains have been analyzed for the genetic and environmental factors underlying complex traits. Traits

relevant to many diseases are available using HMDP that have been determined through various technologies and under various environmental conditions [12, 13].

15.3.1.4.12 Whole-Genome Sequencing (WGS) and Selection Signature

WGS is the sequencing of the entire genome of a particular species. These sequenced genomic data are called "reference genome" which are available in the online database, for example, *MGI* and *NCBI*. The high cost of shotgun sequencing and the large demand for computing resources and storage have limited WGS implementation in non-model species with limited genomic resources and conservation. WGS is a powerful tool for constructing a reference genome that offers unprecedented marker density and surveys a wide diversity of genetic variations unlike GWAS that were limited to single-nucleotide polymorphisms [14]. It helps in the detection of selection signatures, local adaptation, the genetic basis of phenotypic traits, and diseases. Selection signatures are detecting contiguous sequences of identical homozygous by descent haplotypes [15]. It helps to conserve the unique characteristic of parental mice through backcrossing with one of its offspring which is having its haplotype gene sequences.

15.3.1.5 Based on the Selection Aid

There are six distinct ways mice can be selected for breeding, which may further be grouped into five types, viz., incross, cross, backcross, test cross, and intercross. The six different mating combinations based on the genotypes of parental mice that can be selected for breeding, as per the investigator's experimental interests, are detailed below:

1. *Dominant homozygous (+/+) × dominant homozygous (+/+)*—selection of like homozygote (incross)—100% F1 dominant homozygotes.
2. *Recessive homozygous (−/−) × recessive homozygous (−/−)*—selection of like homozygote (incross)—100% F1 recessives homozygotes.
3. *Recessive homozygous (−/−) × dominant homozygous (+/+)*—selection of unlike homozygotes (cross)—100% F1 heterozygotes.
4. *Heterozygous (+/−) × dominant homozygous (+/+) (backcross)*—50% dominant homozygote and 50% heterozygote dominant genotypes in F1 (but 100% dominant phenotype).
5. *Heterozygous (+/−) × recessive homozygous (−/−) (test cross)*—50% dominant heterozygote and 50% recessive phenotypes, in F1—used for identification of a carrier trait or parent.
6. *Heterozygous (+/−) × heterozygous (+/−)*—selection of heterozygotes (intercross).

   Monoallelic cross—phenotypic ratio in F1 is 3:1 and genotype ratio is 1:2:1.

   Diallelic cross—phenotypic ratio in F1 is 9:3:3:1 and GR is 1:2:1:2:4:2:1:2:1.

Similarly, for mutant or lethal condition:

1. *Dominant* mutant or lethal *(+/+)* × *Dominant* mutant or lethal *(+/+)*—selection of like homozygote (incross)—100% F1 dominant mutant or lethal.
2. *Recessive* mutant or lethal *(−/−)* × *recessive* mutant or lethal *(−/−)*—selection of like homozygote (incross)—100% F1 recessives mutant or lethal.
3. *Recessive carrier (+/−)* × *recessive* homozygous mutant or lethal *(−/−)*—in F1 50% carrier and 50% recessive mutant or lethal. Example: nude mice—heterozygous female and homozygous males are used for breeding as female homozygous mutants do not breed very well. The ovulation starts at 10 weeks, and the female ceases ovulating when it reaches 16 weeks [16].
4. *Recessive carrier (+/−)* × *normal dominant homozygous (+/+)*—50% dominant homozygote and 50% *recessive carrier* in F1 (phenotype also 50% dominant homozygote and 50% *recessive carrier*).
5. *Recessive carrier (+/−)* × *normal recessive homozygous (−/−)*—50% carrier and 50% *normal recessive homozygous*.
6. *Carrier (+/−)* × *carrier (+/−)*—in F1, genotype ratio is 1:2:1.
   Monoallelic cross—phenotypic ratio also is 1:2:1 (mutant) or 1:2 (lethal).
   This system of breeding is practiced in transgenic or mutant models. Homozygous mutant male and female mice are severely impaired, infertile, and embryonic lethal or die before reaching sexual maturity. For example, db/db mice: in this strain, the homozygous female mutants are infertile, and homozygous male mutants have a reduced ability to mate. In such a case, heterozygous male should be bred with a heterozygous female, and the resulting offspring will comprise 25% homozygous mice that will have impaired reproductive capacity.
   In maintaining some transgenic lines that result in lethality or strain inviability and infertility, hemizygous mutants are bred with noncarrier (of a trait) or wild-type breeding partner. This results in 50% of hemizygous transgenic and 50% of noncarrier mice in the F1 generation. Proper genotyping is needed to distinguish hemizygous from noncarrier mice [16].

One or more (in combination) of the above mating schemes are used for preserving the particular variant/mutant allele(s) or genetically engineered allele or transgenic allele or target mutated allele in the mouse strain.

15.3.2 Selection Methods

The analysis results obtained using the above selection aids help the investigator (s) to choose mice generated from one or more of the following selection methods and subsequently use the chosen mice for downstream mating to accomplish the intended research objective(s).

15.3.2.1 Inbreeding

Inbreeding (brother × sister or parent × offspring mating) is continued for over 20 consecutive generations to develop an inbred strain, in which 98.6% of genetic loci are homozygous. This strain can be regarded as genetically identical and is

called "isogeneic." By inbreeding, genetic variability is eliminated, and results obtained using inbred mice between laboratories, in any part of the world, can be compared. However, there is some residual heterozygosity about 3–4% during inbreeding where at a given generation of inbreeding there is still heterozygous at a specified locus.

15.3.2.1.1 Nomenclature of Inbred Mice

Inbred strain names contain all capital (uppercase) letters, numbers only, or a combination of letters and numbers, beginning with a letter, e.g., BALB, NZB, NOD, 129, and C57BL. Some preexisting strains do not follow this nomenclature, e.g., mouse strain 129P1/J. Inbreeding generation can also be indicated, e.g., NZB/J (F30).

A substrain may arise for various reasons after 20 generations of inbreeding, and such substrains are designated as a symbol of the original strain, followed by a forward slash and a substrain designation. The designation is the laboratory code of the individual investigator or laboratory developing the strain; e.g., C57BL/6J and C57BL/6N mice J and N are substrains originating from The Jackson Laboratory and National Institute of Health.

15.3.2.1.2 Different Types of Inbreeding

Close inbreeding: after 20 generations of close breeding, over 98.6% of genetic loci are homozygous, but such strains are prone to inbreeding depression after the 20th generation of close inbreeding. There are two types of inbreeding:

1. Full-sib mating: sister and brother mating of littermates from the same parents.
2. *Line breeding*: Over 40 generations are required for attaining over 98.6% of genetic loci and having less inbreeding depression.

   Types:

(a) Half-sib: mating of sister and brother having either one of the common maternal or paternal parent.
(b) Half cousins: having half of the common ancestors.
(c) Double cousins: having twice of the common ancestors.

15.3.2.1.3 Recombinant Inbred Strains

This involves the crossing of two or more pure inbred mouse strains. The F1 progeny from the two inbred strains is brother-sister mated for 20 or more generations, and resulting lines are recombinant inbred (RI) lines. The resulting inbred strains are 100% homozygous at all loci, each of which has a different combination of genes from the same original progenitor strains [17]. RI strains should be designated by uppercase one- or two-letter abbreviations of both parental strain names, with the female strain written first, and separated by an uppercase letter X with no intervening

spaces; e.g., BXH.BXH strains are derived from the C57BL/6J and C3H/HeJ progenitor strains [17].

15.3.2.1.4 Coisogenic Strains

The new strain, which differs from the original strain at a single-gene locus, is called a coisogenic strain. The nomenclature of coisogenic strains is indicated by the strain (substrain, subline) symbol, followed by a hyphen and the gene symbol in italics, i.e., the CBA/CA-*nu* mouse strain is a coisogenic strain of the CBA/Ca strain, which carries the nu (nude) gene mutations determining hairless and athymic phenotype instead of the “normal” alleles of the appropriate genes [17–19].

15.3.2.1.5 Congenic Strains

In these kinds of strains, the selected gene of interest or genetic region of the donor strain is introduced into a recipient strain. The breeding of this strain depends on dominance, codominance, or recessive gene. This is usually done by backcrossing. Initially, after crossing two inbred strains, the F1 offspring are backcrossed with the recipient strain. The F1 offspring carrying the donor gene is selected and backcrossed with the recipient strain. At least ten backcrossings are done, and after the last backcross, a male and a female animal, which are heterozygotes for the donor gene, are selected and mated. Subsequently, from the offspring, two homozygous mice are selected and bred to develop a congenic strain. The new congenic strain carries the donor gene, and due to the backcrosses, all the background genes are similar to the recipient strain. Congenic strains help to minimize genetic background effects for any mutation studies based on the background used. By congenic methodology, it is possible to transfer the segments of the gene of interest from one strain environment to another strain, and the investigator can study the effect of a gene of interest that is carried by the other mouse strain [17, 18].

The nomenclature of congenic strains involves designating by a full or abbreviated symbol of the recipient strain with a period of separation and an abbreviated symbol of the donor strain followed by a hyphen that separates the strain name from the symbol (in italics) of the differential allele(s) [19]:

E.g., B6.129P2-*Apoe*^{tm1Unc}/J: B6, recipient strain; 129P2, donor strain; (.) period of 129P2; strain backcrossed with B6 mice, Apoe; gene symbol.

(In this congenic strain, 129P2 is the donor strain, where E14TG2a ES cell line was used to create a targeted mutation and is transferred onto the B6 mice using ten or more backcrosses.)

15.3.2.1.6 Consomic Strain or Chromosome Substitution Strains

It is an inbred strain that contains a single entire chromosome introduced from another strain. Such a strain is derived by backcrossing to a parental inbred strain for at least ten generations while selecting for retention of a specific whole chromosome from the donor strain.

For example, C57BL/6J-Chr 1A/J Chr 3DBA/2J. In this consomic mouse strain, chromosome 1 from the A/J strain and chromosome 3 from the DBA/2J strain have been backcrossed onto C57BL/6J [17]. The consomic mice provide a sort of

midpoint, in that a whole chromosome is isolated from its usual genetic background and placed in another background [20].

15.3.2.1.7 Conplastic Strain

In recent years, attention has increased toward the relationship of mitochondrial DNA (mtDNA) to metabolic risk factors for diabetes and other common diseases [21]. The substitution of different mitochondrial genomes on the same nuclear genetic background (conplastic strains) may help in the isolation of the effects of the mitochondrial genome on complex disease traits. Conplastic strains can be developed from a traditional backcrossing technique; the nuclear genome from one strain has been introduced into the cytoplasm of another by backcrossing. Here, the mitochondrial donor is always the female parent. For example, C57BL/6J-mt$^{PWD/Ph}$/Fore J is a strain that was developed by backcrossing PWD/Ph donor female to the C57BL/6J host male. This conplastic strain carries the mitochondrial genome of the donor strain [22]. The development of conplastic mouse strain can also be achieved by direct nuclear transfer into another strain's enucleated zygote and is called supersonic conplastic strain.

15.3.2.1.8 Supersonic Conplastic Strains

Production of conplastic strains by a traditional backcross breeding method is a time-consuming process that usually takes more than 3 years [21]. This process can be expedited by a selection of breeding pairs with a similar genetic background using marker-assisted selection. The female is superovulated and backcrossed with a selected male. This is followed by the collection of the embryos at two-cell stages and subsequently transferring the embryos into different pseudopregnant foster mothers. Recently, SHR/Ola-mtBN/Crl conplastic strain was developed to analyze the effects of different mitochondrial genomes on metabolic and hemodynamic parameters in the spontaneously hypertensive rat (SHR—an animal model of the human metabolic syndrome). In each of the ten backcrossed generations, embryos obtained from 4-week-old superovulated females that were mated with SHR males were transferred to pseudopregnant foster mothers. Each backcross generation takes approximately 7 weeks (superovulation in 4 weeks +3 weeks of pregnancy). Therefore, within one and a half years duration, a supersonic conplastic strain can be developed [21] (Fig. 15.6).

15.3.2.2 Outbreeding

Outbred rodent stocks are usually maintained as large breeding colonies within which there is less inter-individual genetic variation. Each animal is genetically different. Breeding is done by random mating of individuals from the same strain but those individuals not having a common ancestor at least for the past 4–6 generations. Outbred stocks exhibit hybrid vigor with longer life spans, higher disease resistance, early fertility, large and frequent litters, low neonatal mortality, rapid growth, and large size. Outbred mice are useful in experiments where the precise genotype of animals is not important [23]. Outbred colonies are bred by a systematic breeding program with unrelated animals of the same strain. Using SNP panels, an

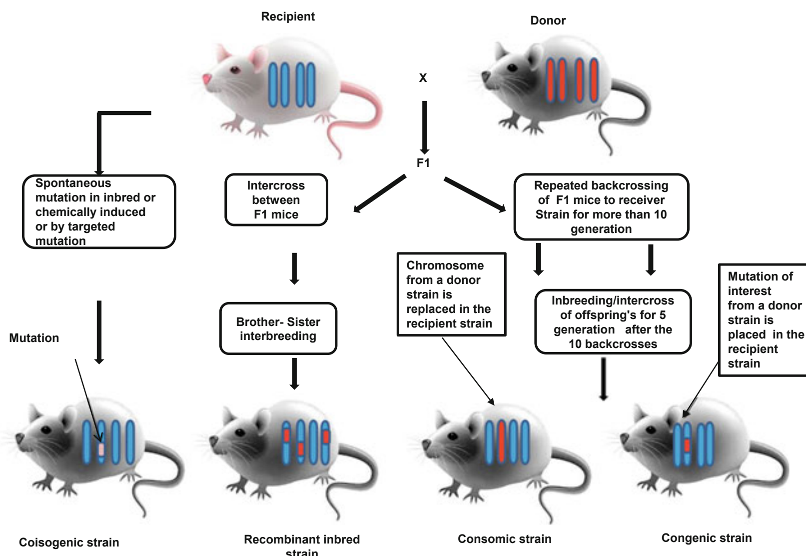


Fig. 15.6 Illustrated flow diagrams to develop mouse strains using multiple inbred strains

investigator can identify genetic heterozygosity. This type of breeding needs large independent breeding lines with many breeding units for each line. The breeder selections should be from the different independent maintained colonies and cryopreserved stocks. Outbred mice are often ideal as a source of material for biochemical purification and as stud males for the stimulation of pseudopregnancy. They are often used in toxicology and pharmacology research, considering the heterogeneity observed in the human population. The nomenclature of outbred stocks includes the company or laboratory designation followed by a colon, the stock designation, for example, Crl: CD1 (ICR) [17–19].

15.3.2.3 Crossbreeding or Hybrid or F1 Hybrids

Crossbreeding is mating of two different pure inbred pairs and F1 hybrids are the first generation progeny of these two inbred parents. All individuals are isogeneic except being heterozygous for all genetic loci at which the two parental strains differ, e.g., histocompatibility genes. The hybrid vigor or heterosis or genetic superiority over the parents can be observed in the F1 hybrids. For getting F1 progeny, repeated mating of two inbred strains is consistently needed. The crossbred strain is designated using uppercase abbreviations of the two parents followed by F1 [17–19]; e.g., C57BL/6 female x C3H/Hej males are denoted by B6C3F1.

15.3.2.4 Genetically Modified Mice

Mice carrying genetic mutations are often used to determine the role of a specific gene in physiology and pathology. The three main mouse models most frequently employed are genetically engineered mutants (knockout, transgenic, knockin, etc.),

chemically induced mutants, and spontaneously occurring mutants [23]. Mice are genetically modified to either have the mutation(s) on the same genetic background or to separate the effects of the allele(s) of interest from the differences caused by the genetic dissimilarities between any two backgrounds. The recent advent of high-throughput sequencing and gene targeting and engineering has made it possible to identify the causative mutant gene in spontaneous mutant models, and many new induced mutants are being developed at an unprecedented rate [24].

15.3.2.5 Transgenic Mice

Transgenic mice have been created by introducing the foreign gene(s) in the mouse genome. These transgenic mice developed using various new genetic approaches have helped to create different mouse models of many human diseases. Many mouse transgenic lines having the tissue-specific expression of foreign genes have been created and characterized using various transgenic mouse technologies. The transgenic or altered gene construct can be introduced into the genome by four different methods:

- Microinjection.
- Transfection and transfer of targeted embryonic stem cells.
- Virus (adenovirus, retrovirus)-mediated gene transfer.
- CRISPR-Cas9-based genome editing.

These methods are used to create genetically engineered mice described below.

15.3.2.6 Knockout Animals

In the knockout mice, the gene of interest that needs to be studied is deleted or silenced so that there is a loss of function of the particular gene. This is commonly achieved by gene targeting or homologous recombination and by gene trapping. There are two types of knockouts. (1) Constitutive knockout mice: In such mouse models, the gene of interest is permanently inactivated in every cell of the animal. (2) Conditional knockout mice: In this model, a particular gene can be silenced in particular cell types in a certain tissue, while other cell types and tissues will have the normal functional gene expression. This can be developed by crossing genetically modified mice that have the gene of interest flanked by specific (flox) sequences that are recognized and excised by an enzyme Cre. Cre mouse strains can be crossed to flox strains, and the Cre expression is brought under spatial control by the use of tissue-specific promoters and/or temporal control by using tamoxifen-inducible Cre, to conditionally ablate the gene(s) of interest.

15.3.2.7 Knock-in

A Knock-in (KI) mouse is created by adding gene sequences that are not found within the locus or in the species to alter a mouse gene of interest. The insertion of a transgene or a new DNA sequence of interest is done at a particular mouse locus/gene of interest. This method is used when the expression of the transgene should be regulated by an endogenous promoter or enhancer regions. The Knock-in of a

mutant allele in the mouse genome can be achieved using homologous recombination (HR) in embryonic stem (ES) cells [25]. The mouse gene or transgene of interest is knocked in or introduced into a specific region of the genome, while other cell types and tissues are unmodified nor change in functional gene expression.

15.3.3 Mating Aids

Mating aids are composed of information and tools for understanding the mating behavior of different strains and species. These aids are very important in selecting animals and choosing the selection method to be followed by the investigator. Therefore, to decide the mating method to use in the closed controlled environment, the investigator must understand the following mating aids.

15.3.3.1 Sexual Maturity and Estrus Cycle Detection

15.3.3.1.1 Determination of Sexual Maturity of Male Mice

Males are typically placed into breeding at 6–8 weeks of age. Male mice are usually housed with one or more females for breeding. Male mice from the same litter may be housed together, but fighting often occurs as the animals reach sexual maturity. Adult males from separate litters should not be co-housed as the mixing of adult males commonly results in severe fight wounds. Males should be housed alone in their cage for several days before breeding. This allows the male to mark the cage and establish his territory by pheromones that are present in the urine of male mice. Females are introduced to the male's cage for breeding. Aggressive male mice may attack the females disrupting breeding or the female's ability to conceive. Once a male mouse has been used for breeding, he should not be returned to group housing as fighting will frequently ensue. The breeding life of a male mouse is highly strain-dependent. Typically, older males continue to successfully breed, and spermatogenesis continues throughout life although litter size and frequency may decline. Obesity may negatively affect their ability to breed. It is important to set up new males for breeding on a rotational basis to maintain high levels of production [26].

15.3.3.1.2 Determination of Sexual Maturity of Female Mice

Females reach sexual maturity at approximately 4–6 weeks of age. Inbred strains tend to mature slower than outbred stocks. Thus, the typical time to initiate breeding in females is ~6 weeks of age. Female mice (dams) are polyestrous and spontaneous ovulators. They have a 4–5-day estrus cycle that is divided into four phases: proestrus, estrus, metestrus, and diestrus. Proestrus is the period during which ovarian follicular development occurs. Estrus, or heat, is the period during which the dam is sexually receptive to the male. Estrus usually occurs 3–5 h after the onset of the dark cycle with ovulation occurring shortly thereafter. Estrus lasts approximately 12 h. During metestrus, the corpora lutea form, and mature eggs move through the oviduct into the uterus. Diestrus is the period during which follicles undergo rapid development in preparation for the subsequent ovulation. Each phase

of the estrus cycle can be identified by the changes in the appearance of the vaginal epithelium and vulva [26].

15.3.3.1.3 Determination of the Stage of Estrus Cycle in Females

To identify females in estrus by examining the color, moistness, and degree of swelling of the vagina:

(a) Proestrus—The vagina is gaping; tissues are reddish pink and moist; numerous longitudinal folds or striations are visible on the dorsal or ventral lips.
(b) Estrus—The vagina appears as above, but tissues are lighter pink and less moist; striations are more pronounced.
(c) Metestrus—Vaginal tissues are pale and dry; dorsal lip is less edematous; whitish cellular debris may line the inner walls and/or partially fill the vagina.
(d) Diestrus—The vagina has a small opening; tissues are pale and very moist.

Vaginal lavage is performed by inserting a glass pipette filled with a few drops of saline—3 mm into the vagina. The saline is administered and immediately withdrawn back into the pipette. A drop of the liquid is then deposited onto a glass slide, and with the help of another empty glass slide, the drop is tightly moved across the length of the slide to form a thin layer of a smear. For the collection of cells via vaginal swab, the female is placed on a flat surface and gently restrained by the tail. A saline moistened swab is inserted 3–4 mm into the vaginal orifice, then rotated, and gently moved in and out 1–2 mm. Cells are collected from the roof of the vagina and immediately transferred onto a clean glass slide containing a drop of sterile saline. Slides are evaluated by light microscopy. The four stages of the mouse's estrus cycle can be distinguished by the nature and frequency of specific cell types in the sample collected. The cytologic characteristics of each of the four stages are as follows (Fig. 15.7):

A. Proestrus—100% epithelial cells
B. Estrus—100% cornified cells
C. Metestrus—50% cornified cells and 50% leukocytes
D. Diestrus—100% leukocytes

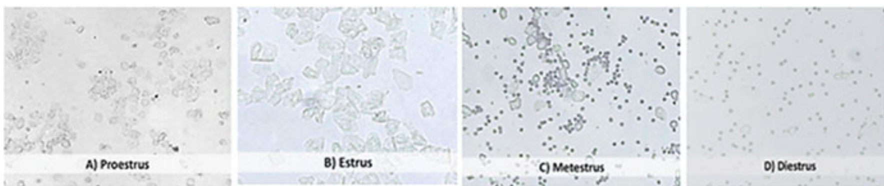


Fig. 15.7 Microscopic views of vaginal smears with the different cells at each stage of the mouse estrous cycle

15.3.3.2 Vaginal Plug Detection

The estrus cycle is mainly affected by photoperiod or light cycle (hours of light and dark). Interruption of the photoperiod can adversely affect reproductive performance. In general, breeding mice should be maintained on a 12:12 or 14:10 light/dark cycle. Males generally copulate with a female in estrus at the midpoint of the dark period. The male ejaculate forms a white to yellowish vaginal plug that is visible in the vagina for about 12 h after mating. The best time to check for plugs is early morning. The vaginal plug or the copulatory plug is a mixture of sperm, ejaculate fluid, and mucus. The presence of a vaginal plug could confirm mating had occurred but does not guarantee conception. External visualization of the vulva generally reveals the plug. Deeper plugs may be missed and may be observed by palpating to help in distending and expelling the plug. However, the lack of a vaginal plug does not mean mating did not occur as the plug may have fallen out or dissolved.

15.3.3.3 Pregnancy Determination

The gestation period of a mouse is between 19 and 21 days. Pregnancy can be confirmed by visual inspection by gestation day 15 (GD15) or earlier, depending on the strain and age of the dam and the number of previous pregnancies [27]. Although palpation can be used as early as GD10, the associated stress to the dam might represent a confounding variable, particularly for teratology studies. Moreover, many studies require chemical, environmental, or surgical manipulation of the dam or harvesting of embryos at earlier stages [27]. Initial litters are generally smaller, and it is not unusual for first or second litters to be either abandoned or cannibalized by the dam until she gains more experience giving birth. Inbred strains and transgenic lines generally have smaller litters (6–8 pups) than outbred stocks. Mouse pups are altricial or poorly developed at birth. They are hairless, have poorly developed eyes and ears, and weigh between 0.5 and 1 g at birth. Weight and growth rate depend on litter size; the less the pups, the greater the share of the dam’s milk and the larger the pups. Prenatal mortality in inbred strains can be up to 10–20%.

15.3.3.4 Fostering

Fostering is required when the dam exhibits poor maternal behavior, maternal neglect, excessively large litters, poor milk production, rederivation, dystocia (difficult labor), and/or maternal death. Foster dams should be selected from a strain having good maternal behavior; outbred stocks are good foster dams. Foster dams should have their litter within a few days of the age of the pups to be fostered. Prefer mothers with different coat colors and having litters of the same age as pups. It is better to remove maximum pups from foster mothers, and the remaining pups should be intermingled with soiled beddings from foster mother cages and then placed with foster mother. Weaning is typically done at 21 days of age but may be delayed up to 28 days based on weight and other factors.

15.3.3.5 Postpartum Estrus

A nursing mother will normally go into a postpartum estrus within 28 h of giving birth that allows her to become pregnant again, immediately. There is a tendency to ovulate 12–18 h after the time of birth, but this can be countered by the tendency to ovulate nocturnally [28]. The embryos resulting from the postpartum mating may undergo developmental arrest and delayed implantation, and hence, delivery can be delayed by 4–5 days. Intensive mouse breeding programs can benefit from the postpartum estrus. The use of postpartum mating yields high numbers of offspring and frequently results in overcrowding. Multiple litters demanding for the dam's milk place the younger litter at a nutritional disadvantage. Overcrowding is unacceptable and may result in a violation of institutional policies regarding the cage population. Intensive monitoring of cages in which a breeding pair is maintained throughout pregnancy, which is without removal of the pregnant female to take benefit of postpartum estrus, is essential.

15.3.3.6 Sex Determination

The sex of the mice is determined by visual comparison of the anogenital distance. The distance between the genital papillae and the anus of the female is shorter and usually shows a hairless line of flesh. This anogenital distance is greater in males, and often, the scrotum is visible in this area. A vaginal closure membrane is present in young mice. The membrane usually opens by 35 days of age.

15.3.3.7 Effect of Pheromones

The reproductive life span of a mouse is approximately 12 months. The litter size at birth and weaning are useful indicators to measure the utility of breeders. Decreasing litter sizes and unthrifty pups signify that the existing breeding pair has to be retired. Breeding information must be recorded on a breeding card for culling or retirement of breeding pairs. The reproductive cycle of mice can be altered by pheromones. Pheromones are volatile chemicals emitted by animals that cause behavioral, physiological, and possibly psychological changes in other members of the same species. Pheromones can significantly influence reproductive performance in mice and can be used to an investigator's advantage. Some of the pheromone-induced reproductive effects are described below [29]:

- Lee-Boot *effect*—This is characterized by suppressed or prolonged estrus when a group of females is reared without males in the colony.
- Whitten *effect*—In this, a colony of female mice reared separately and exhibiting anestrus initiates and has a synchronized estrus cycle, when a male is introduced into the colony. After exposure, the majority of females will enter estrus in 72 h. Synchronized groups of females ensure that a maximum number of naturally ovulated eggs are available for harvesting or mating will occur at a given time yielding synchronized pregnancies.
- Bruce *effect*—In this, a pregnant female suddenly undergoes pregnancy block during preimplantation by exposure to a strange male or his urine. This can occur during both a normal pregnancy and during pseudopregnancy. Exposure to a male

of a different strain causes implantation failure, followed by a return to estrus in 4–5 days.

15.3.3.8 Hormonal Manipulation

Hormonal manipulation of the murine reproductive cycle is now a common practice. Synchronization of the estrus cycle and parturition may be desirable to generate age-matched experimental animals. Synchronization can be accomplished by several methods. The methods include adopting the Whitten effect, as discussed above; hormonal synchronization by the administration of methoxy progesterone followed by pregnant mare's serum gonadotropin (PMSG); or using a luteinizing hormone-releasing hormone (LHRH) agonist.

15.3.3.9 Superovulation

This regimen is commonly used to maximize the number of ova released at particular ovulation. This is commonly done for generating large numbers of embryos for uterine transfer, the collection of ova in the production of transgenic animals, or in vitro fertilization. A combination of pregnant mare's serum gonadotropin (PMSG) and human chorionic gonadotropin (HCG) is used.

15.3.3.10 Factors Affecting Reproduction

Various factors affect reproduction and breeding. The most important parameter is the feed. The feed must have the recommended crude protein and fat percentage. Besides, both macro- and microenvironmental factors like lighting cycle, noise, temperature, relative humidity, and air exchange in a breeding cage and room should be properly maintained. Also handling methods and procedures followed in breeders will have an impact on breeding and reproduction. Hence, proper environmental, feed, and handling procedures has to be taken care of for a proper breeding program.

15.3.4 Mating Methods

Based on reproductive nature, mating behavior, and required number of population size following different types of mating methods practiced in selected populations from the stock by the above selection methods.

15.3.4.1 Pair Mates

This term refers to the establishment of stable long-term pairs of a single male and female. This system takes advantage of the postpartum estrus, thus decreasing the time between litters and increasing the production of offspring per female. This type of mating is practiced to maintain pedigree. It can be easily performed in a standard mouse cage and requires less careful and frequent observation of females, as females are not removed from the cage before delivery, but requires periodic monitoring to avoid overcrowding by multiple successive litters. In some cases, males from pair cages can be used in rotation mating when large numbers of similarly aged mice are needed. The disadvantage of pair mating is the requirement of more males. It can

also be physically demanding for females to produce repeated young without rest between litters.

15.3.4.2 Trio Mating

A mating system that employs a single male and two females housed together in one cage is known as trio mating. If a standard small shoebox-sized cage is used, one female must be removed when pregnant to reduce overcrowding when both females give birth. The advantages of trio mating are that it can be used to expand a line in a short period as it takes advantage of postpartum estrus in one dam, thus potentially decreasing the time between litters. The disadvantages are that it requires frequent and careful observation to remove one pregnant female. To reduce the stress on females of continually producing young, the females mated at postpartum estrus should be alternated between litters. Besides, there is a chance of overcrowding of littermates that affects the health and causes stress to the animals.

15.3.4.3 Timed Mating or Hand Mating

It involves manually placing a regular cycling estrus female with a male for a short time interval, usually allowing for mating overnight and removing the male from the cage in the morning the next day. This is advantageous as it provides a precise estimation of the time of mating or conception, assigning accurate embryonic ages, and a method for timed deliveries. The disadvantage of timed mating is that it may require large numbers of males and can be time-consuming and labor-intensive.

15.3.4.4 Harem Mating

In this mating system, one male is placed with several (typically 2–4) females for a short period and hence is known as harem breeding. The females are removed when they are visibly pregnant or have been with the male for a set period, 12–14 days. The advantages of this system are that it requires relatively few males and can be used to expand a strain in a short period. The disadvantages are that it does not take advantage of postpartum estrus. It requires a system for identifying individual pregnant females and the need to remove them frequently at appropriate times from cages; otherwise, it results in postpartum mating and overcrowding of pups.

15.3.4.5 Artificial Insemination (AI)

It is one of the emerging areas of mouse breeding. AI in the mouse may be especially useful for breeding transgenic or mutant mice with fertility problems and expansion of mouse colonies and as an alternative to in vitro fertilization. Both surgical and nonsurgical techniques are in use in mice. For the nonsurgical method of embryo transfer in mice, nonsurgical embryo transfer (NSET) device is used. Forty μl of fresh sperm is required, which is transferred by using a mini AI gun to the uterine horns of the female mice [30, 31]. This mating method doesn't require post-procedure monitoring.

15.3.4.6 In Vitro Fertilization (IVF)

In vitro fertilization (IVF) refers to a reproduction technique in which fertilization takes place outside the living animal in an artificial environment, i.e., in vitro condition. The process involves the recovery of oocytes followed by culturing the oocytes, maturation, grading, and selection. It also involves sperm collection, capacitation, and their introduction into oocyte for fertilization. The zygotes are then incubated and observed for evidence of division. The mouse embryos are cultured to the blastocyst stage and at the 2–8 cell embryo stage transferred into the oviducts of 0.5-day pseudopregnant recipient females.

15.3.4.7 Embryo Transfer (ET)

To prepare the recipient mice, it must be ensured that they are free from contamination and other infectious agents. Surgical techniques and anesthetic equipment are required to transfer embryos from dams of the desired strain to clean pseudopregnant females. Both IVF and ET are used to generate transgenic mouse models and also for obtaining a large number of offspring much more rapidly than the standard breeding strategies. Embryo transfer which includes the transfer of embryos into the oviduct of pseudopregnant females is the starting point for several downstream applications such as embryo transfer rederivation after in vitro fertilization, harvesting transgenic embryos for cryopreservation, and cryopreservation recovery.

15.4 Breeding and Genetic Quality Control Programs for Best-Quality Mice Production

15.4.1 Breeding Colony

Breeding animals are kept in a three-tier system (nuclear, multiplier, production), and each tier is separated by a closed environment. The movement of animals from one tier to another is by the unidirectional flow. This kind of system is mainly adopted for better colony management called a “closed nuclear breeding system (CNBS).”

15.4.1.1 Phase 1

Nuclear breeding colony or foundation colony/stock (FC/FS)—It consists of pure parental lines that are maintained at minimal numbers and also produce the breeding pairs for the multiplier’s tier. All the pedigree, performance, breeding, reproduction, phenotyping, and genotyping data are maintained for all the breeding pairs. Mostly paired mating is practiced.

15.4.1.2 Phase 2

Pedigree expansion colony/stock (PES)—It comprises breeding pairs from the nuclear tier that are mated as per the requirement of future in vivo studies. Breeding and reproduction data are maintained for all the breeding pairs. In some cases, non-pedigree in production (NPIP) can be maintained based on the requirements

of the investigators. In NPIP, mostly trio or harem mating is practiced, and no pedigree records are maintained.

15.4.1.3 Phase 3

Production colony (PC)—It contains newly weaned young ones from multiplier tiers that are transferred to the production tier. These production stocks are maintained until the animals attain the required bodyweight and/or age for future in vivo studies. Bodyweight, growth rate, and other phenotypic data are collected. No breeding is practiced in this tier (Fig. 15.8).

15.4.2 Genetic Quality Control

Genetic quality control should be an essential component in laboratory animal facilities to prevent contamination between strains and the maintain quality of the mouse strains [30, 31]. It also helps rule out contamination in inbred strains or any mutation in the background strain of the genetically engineered mouse [32, 33].

Phenotyping of the mouse helps identify the first noticeable change indicative of contamination of a strain. In the case of potential contamination, the coat color phenotype with other phenotypic variations including body size, weight, skeletal structure, behavior, reproductive performance, tumor susceptibility, and life span helps in identifying contamination.

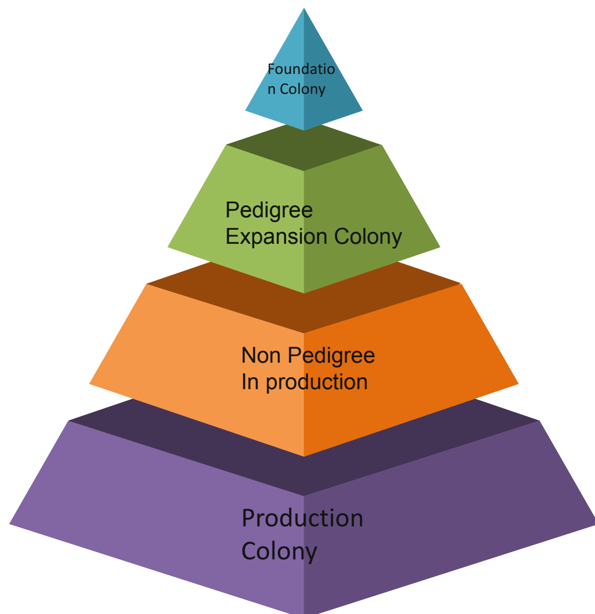


Fig. 15.8 Illustration of mouse colony management represented by Pyramid system

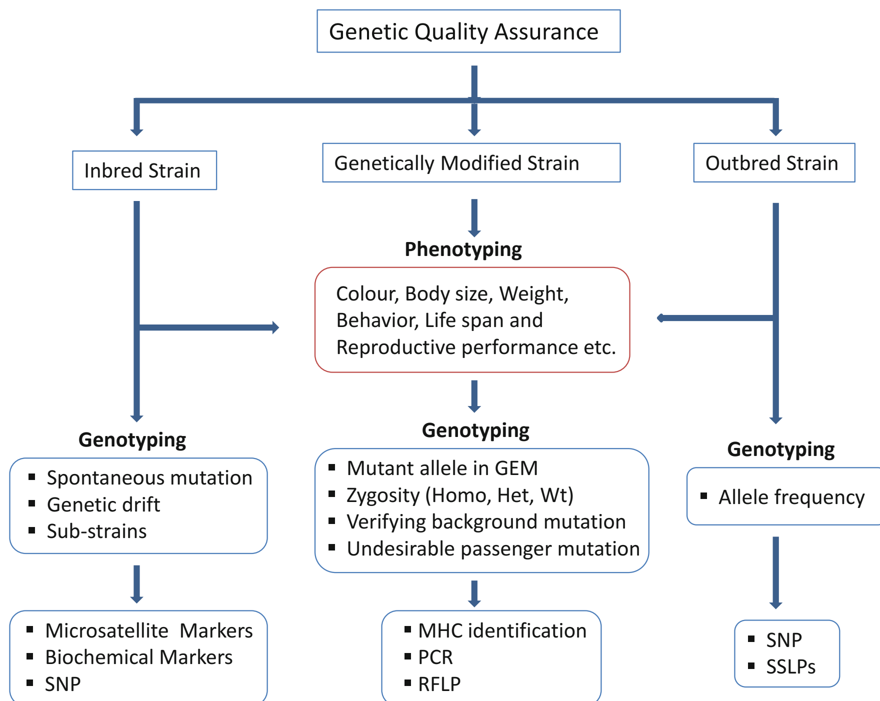


Fig. 15.9 Genetic quality assurance for genetic quality control on laboratory animals

Further, to identify any spontaneous mutations, polymorphisms, and genetic drift in an inbred strain, various genetic quality control programs using different methods should be adopted that may not be limited to the following:

1. Microsatellite markers.
2. Biochemical markers.
3. SNP chips.

In the case of genetically engineered mice (GEM), in addition to the above methods, other methods of genotyping such as RFLP and immune markers are needed to detect mutant allele(s); to distinguish homozygote, heterozygote, and wild-type animals; and to identify mutant alleles transferred to a different genetic background.

Three important levels of protocols should be followed for the quality control program (Fig. 15.9):

- Level 1. Visual phenotypic characterization of strains
- Level 2. Careful recordkeeping, line segregation, and strict pedigree record
- Level 3. Genetic monitoring using microsatellite markers or SNP chips

In addition to the genetic quality control program, a good proper health monitoring program should also be in place to screen regularly for pathogens of the breeders/ colony either by the sentinel program. The details of health monitoring and quality control are discussed elsewhere in the book under the chapter "Microbiological Quality Control of Lab Animals." Many laboratories maintain mouse strain repositories through cryopreservation as a part of the quality control program. Cryopreservation of either sperm, ova, or embryos helps in rederivation of the known genotype of mouse strain in event of a loss of the mouse strain, accidental contamination, infections, mutations, and genetic drifts. It also conserves finances, space, and labor resources in a laboratory animal facility.

15.5 Conclusion

Since laboratory mouse is a vital model for biomedical research, an efficient breeding program is desired to appreciate the rationale of the breeding colony and its role in meeting the production needs. Breeding strategies involve breeding of founders, expansion of a homozygous colonies, and backcrossing if needed to develop a congenic strain. Further, based on the requirement of the investigator, the production of both small- and large-scale colonies can be planned. Efficient colony maintenance is feasible only if the genetic background of the strain, age, sex, reproductive history, genotype, breeding methods, and health status of the mice are scientifically done. Appropriate breeding vigil and proper management could help in evaluating and controlling animal health, breeding performance, and quality of the colony so that good quality animals can be obtained for research.

References

1. Keeler CE (2014) The laboratory mouse: its origin, heredity, and culture. Harvard University Press
2. Eisen EJ (2005) The mouse in animal genetics and breeding research. World Scientific
3. Morse HC (1981) The laboratory mouse-a historical perspective. Mouse Biomed Res:1–16
4. Russell ES (1985) A history of mouse genetics. Annu Rev Genet 19(1):1–29
5. Beckers J, Wurst W, De Angelis MH (2009) Towards better mouse models: enhanced genotypes, systemic phenotyping and envirotype modelling. Nat Rev Genet 10(6):371–380
6. Ayadi A, Ferrand G, Cruz IGD, Warot X (2011) Mouse breeding and colony management. Curr Protocol Mouse Biol 1(1):239–264
7. Petkova SB, Yuan R, Tsaih SW, Schott W, Roopenian DC, Paigen B (2008) Genetic influence on immune phenotype revealed strain-specific variations in peripheral blood lineages. Physiol Genomics 34(3):304–314
8. Cheah YC, Nadeau JH, Pugh S, Paigen B (1994) New murine polymorphisms detected by random amplified polymorphic DNA (RAPD) PCR and mapped by use of recombinant inbred strains. Mamm Genome 5(12):762–767
9. Hong Y, Chuah A (2003) A format for databasing and comparison of AFLP fingerprint profiles. BMC Bioinformat 4(1):7
10. Petkov PM, Cassell MA, Sargent EE, Donnelly CJ, Robinson P, Crew V, Wiles MV (2004) Development of a SNP genotyping panel for genetic monitoring of the laboratory mouse. Genomics 83(5):902–911

11. Peters LL, Robledo RF, Bult CJ, Churchill GA, Paigen BJ, Svenson KL (2007) The mouse as a model for human biology: a resource guide for complex trait analysis. Nat Rev Genet 8 (1):58–69
12. Pirie E, Ray S, Pan C, Fu W, Powers AF, Polikoff D, Crooke RM (2018) Mouse genome-wide association studies and systems genetics uncover the genetic architecture associated with hepatic pharmacokinetic and pharmacodynamic properties of a constrained ethyl antisense oligonucleotide targeting Malat1. PLoS Genet 14(10):e1007732
13. Lusis AJ et al (2016) The Hybrid Mouse Diversity Panel: a resource for systems genetics analyses of metabolic and cardiovascular traits. J Lipid Res 57(6):925–942. https://doi.org/10.1194/jlr.R066944
14. Pearson TA, Manolio TA (2008) How to interpret a genome-wide association study. JAMA 299 (11):1335–1344
15. Almeida OAC et al (2019) Identification of selection signatures involved in performance traits in a paternal broiler line. BMC Genomics 20:449
16. Lambert R (2009) Breeding strategies for maintaining colonies of laboratory mice. TJ Laboratory
17. Festing MFW (1979) Inbred strains in biomedical research. Macmillan International Higher Education
18. Hedrich HJ (2020) Taxonomy and stocks and strains. In: The laboratory rat. Academic Press, pp 47–75
19. Festing MFW (1987) Introduction to laboratory animal genetics. UFAW handbook on the care and management of laboratory animals/edited by Trevor B. Poole; editorial assistant, Ruth Robinson
20. Rogner UC, Avner P (2003) Congenic mice: cutting tools for complex immune disorders. Nat Rev Immunol 3(3):243–252
21. Landa V, Zídek V, Pravenec M (2010) Generation of rat "supersonic" congenic/conplastic strains using superovulation and embryo transfer. In: Rat genomics. Humana Press, pp. 267–275
22. Festing M, Staats J (1973) Standardized nomenclature for inbred strains of rats: fourth listing. Transplantation 16(3):221–245
23. Ackert-Bicknell CL, Rosen CJ (2016) Passenger gene mutations: unwanted guests in genetically modified mice. J Bone Miner Res 31(2):270–273
24. Davisson MT, Bergstrom DE, Reinholdt LG, Donahue LR (2012) Discovery genetics: the history and future of spontaneous mutation research. Curr Protocol Mouse Biol 2(2):103–118
25. Rappaport A, Johnson L (2014) Genetically engineered knock-in and conditional knock-in mouse models of cancer. Cold Spring Harb Protoc (9):pdb-top069799
26. Allen E (1922) The oestrous cycle in the mouse. Am J Anat 30(3):297–371
27. Heyne GW et al (2015) A simple and reliable method for early pregnancy detection in inbred mice. J Am Assoc Lab Anim Sci 54(4):368–371
28. Berry ML, Linder CC (2007) Breeding systems: considerations, genetic fundamentals, genetic background, and strain types. In: The mouse in biomedical research. Academic Press, pp 53–78
29. Bind RH et al (2013) The role of pheromonal responses in rodent behavior: future directions for the development of laboratory protocols. J Am Assoc Lab Anim Sci 52(2):124–129
30. Green MA, Bass S, Spear BT (2009) A device for the simple and rapid transcervical transfer of mouse embryos eliminates the need for surgery and potential post-operative complications. BioTechniques 47(5):919–924
31. Stone BJ, Steele KH, Fath-Goodin A (2015) A rapid and effective nonsurgical artificial insemination protocol using the NSET™ device for sperm transfer in mice without anesthesia. Transgenic Res 24(4):775–781
32. Strobel MC, Reinholdt LG, Malcolm RD, Pritchett-Corning K (2015) Genetic monitoring of laboratory mice and rats. In: Laboratory animal medicine, pp. 1403–1416
33. Benavides F, Rülicke T, Prins JB, Bussell J, Scavizzi F, Cinelli P, Wedekind D (2020) Genetic quality assurance and genetic monitoring of laboratory mice and rats: FELASA Working Group Report. Lab Anim 54(2):135–148

Laboratory Animal Nutrition

16

Suresh Kumar, Manoj Kumar Rajput, and Pradeep Kumar Yadav

Abstract

High-quality diets are essential for the growth, maintenance, and reproduction of laboratory animals. It is one of the most independent variables in the experimental design. Various specialized diet formulations are used to make different disease models by altering the gut microbiome in laboratory animals. Any imbalance in diet affects animal health, animal welfare, and reproducibility of experimental data. Besides diet, the drinking water provided to laboratory animals should also be taken into consideration in an experimental design, and it should be free from waterborne pathogens and chemical contaminants. Modern units of diet formulation follow the hazard analysis and critical control point (HACCP) principles and European Union Good Manufacturing Practice (EU-GMP) to ensure quality as well as batch to batch consistency of diet. Animal Research: Reporting of In Vivo Experiments (ARRIVE) guidelines stress that the specifications of diet should be provided along with the experimental results while publishing research papers. This review gives an overall view of the nutrient requirements of laboratory rodents and lagomorphs, which form the bulk of laboratory animals in research. The types of the animal diet and their importance in giving consistent results in animal experimentation are also discussed. It is also intended to sensitize the scientists on the importance of quality laboratory animal feed and drinking water in getting consistent results.

S. Kumar (✉) · M. K. Rajput
National Institute of Biologicals, Noida, UP, India
e-mail: suresh.kumar@nib.gov.in

P. K. Yadav
All India Institute of Medical Science, New Delhi, India

© The Author(s), under exclusive license to Springer Nature Singapore Pte Ltd. 2021

P. Nagarajan et al. (eds.), *Essentials of Laboratory Animal Science: Principles and Practices*, https://doi.org/10.1007/978-981-16-0987-9_16

Keywords

Diets · Nutrition · Rodents · Drinking water · Contaminants · Reproducibility and variability

16.1 Introduction

Diet is an essential entity that is a primary requirement as well as a prerequisite to a healthy human life. The animal body requires the major diet constituents (carbohydrates, fats, and proteins) and the minor constituents (vitamins and minerals) for the physical growth, development and maintenance of normal body function, and physical activity and health. An unbalanced diet has adverse impacts on health and development and may lead to the progression of diseases inducing metabolic disorders, immune-related disorders, and neurobehavioral traits. A balanced diet is required for survival and productive processes such as growth, lactation, and reproduction. The type of diets, dietary composition, and even feeding schedules have direct impact on the experimental results as well as animal health and welfare [1–3].

Initially, the role of diet on the physiological response as well as the variability they might contribute in the experimentation had been overlooked. The researchers gradually have realized that reproducibility is challenged by many factors and diet is one of the most influential factors [4]. Many researchers have started designing their studies well by minimizing dietary variability in control and the experimental diets while studying the variable of interest. Unfortunately, there are still many studies wherein the control and experimental diets are not matched, and it creates a problem in reproducibility because there is a possibility of inducing undesirable variables owing to unmatched diets. Of late, researchers have started specifying the constituents of the diet in the publications [5]. Moreover, the importance of the nutritionally complete diet that will provide all essential nutrients in such a proportion that maintain the optimal growth and maintenance of the species of interest comprising species-specific dietary requirements for gestation and lactation is being stressed upon [6].

Sensitive methods of analytical measurement with affordable cost and high-throughput techniques have provided a deep insight into the role of diet on critical physiological functions. The impact of diet on the alterations of the microbiome and epigenetics has become a subject of interest [7–10]. The researchers should be aware of the concentration of ingredients and publish it with experimental results when required. Furthermore, researchers should ensure that the diet ingredients do not change from batch to batch. The primary aim is to present the most important issues to the researchers related to the diet which could be a source of extrinsic variability. Further, the impact of diet on rodent health, rodent welfare, study design, and experimental outcomes that provide suggestions on how to address these effects are also covered in this chapter.

16.2 Diet Intake in Rodents

Laboratory animals are usually provided ad libitum. The food intake mainly depends upon their physiological state. Accumulating scientific evidence shows the increase in feed intake with the onset of pregnancy. At lactation, there is bound to be a substantial increase in dietary intake. A rat is likely to consume nearly three times the diet during lactation as compared to a non-lactating rat. The approximate voluntary daily requirement of diet in the different physiological states in laboratory animals fed on a commercial diet has been summarized in Table 16.1. In general, the metabolizable energy content of these diets usually prevails in the range of 8–12 MJ/kg diet. Some studies manifested that diet intake in all species is directly proportional to basal metabolic rate (BMR) with a ratio of 4:1. These predictions may provide some indication of diet intake; the exactness of these estimated values is profoundly doubtful as the balance between energy intake and metabolic live body weight is not constant during growth or at the maintenance level [11, 12].

16.3 Coprophagy

Rodents are known as hindgut fermenters. The small species need more energy and protein based on body mass as compared to large animals. The rodents require more quantity to meet their energy demands than the large species as the passage rate of these small species is much faster while the fermentation rate is almost similar. To curb this limitation, they have evolved a characteristic strategy in their digestive system known as coprophagy. This practice has nutritional advantages and acts as a source of amino acids, vitamins, and other nutrients which get excreted with feces. Several species among coprophagous mammals excrete feces of two types termed as soft feces and hard feces. The soft feces, composed of more water, more protein, and less fiber, are eaten again by the animals. This behavior is possible in herbivorous hindgut fermenters because of a colonic separation mechanism (CSM) in the digestive tract. This mechanism operates by the retrograde transport of the fluid and fine particles from the indigestible matter in the enlarged cecum for fermentation and microbial reproduction. The washback and mucus trap are mainly two types of CSM in hindgut fermenter small animals [13, 14]. The overview of these two types of

Table 16.1 Approximate voluntary daily requirement of diet in the different physiological state in laboratory animals [1]

S. no.	Species	Estimated average food intake (g/day)			
		Growing	Adult	Pregnant	Lactating
1.	Mouse	3–5	5–7	6–8	7–15
2.	Rat	8–25	25–30	25–35	35–65
3.	Hamster	6–12	10–12	12–15	20–25
4.	G. pig	35–45	45–70	70–80	100–130
5.	Rabbit	120–200	200–300	300	300–400

Table 16.2 Types of colonic separation mechanism (CSM) and concentration of crude protein (percentage of dry matter) in soft feces and hard feces in small hindgut fermenters [14]

S. no.	Animal species	Type of CSM	Commercial diet	Soft feces	Hard feces
1.	Mouse	Mucus trap	26.2	22.9	18.1
2.	Guinea pig	Mucus trap	18.6	19.4	12.5
3	Rabbit	Wash trap	17.3	35.5	13.6

colonic separation mechanism (CSM) and crude protein (percentage of dry matter) concentration in soft feces and hard feces in some small hindgut fermenters is shown in Table 16.2.

16.4 Role of Nutrients in Rodents

In rodents, the dietary macronutrients including protein, carbohydrate, and fat provide energy by catabolism. A brief overview of these nutrients is provided in Table 16.3 [15]. Nonessential amino acids can be synthesized by gut microbiota, whereas essential amino acids are required in the diet. Most of the animal-derived proteins provide all essential amino acids, while the plant sources like corn are deficient in specific amino acids, such as lysine and tryptophan, and soy lacks methionine. Similarly, the essential fatty acids like linoleic (omega-6) and alpha-linolenic (omega-3) acids are required in the diet because they are not synthesized by rodents [10]. The dietary requirements of these fatty acids are expressed in terms of lineolate, and the amount equivalent to 1–1.5% of the metabolizable energy (ME) of the diet is adequate for most monogastric animals [1].

Mineral micronutrients act as cofactors for diverse cellular processes. The five crucial macro-minerals indispensable for rodent species include calcium, phosphorus, sodium, potassium, and magnesium. These macro-minerals are commonly required at more than 0.01% of total body weight. The trace minerals needed less than 0.01% of the total body weight include iron, iodine, zinc, manganese, copper, cobalt, molybdenum, selenium, sulfur, chloride, boron, silicon, vanadium, nickel, arsenate, and chromium [16]. A brief overview of major nutrient requirements in rodents, their main roles, and the cardinal deficiency symptoms is provided in Table 16.4. Vitamins are vital to the maintenance of the health of rodents as they serve as catalysts of various cellular processes. Based on solubility, these organic compounds are listed as fat- and water-soluble vitamins. Most of the vitamins can be synthesized in animals by their gut microbiome. However, a few animal species cannot synthesize some specific vitamins in sufficient amounts and are added as supplements in rodent diets [25, 26]. The sterilization of diet is a prime requirement for maintaining the health of rodents. However, the most pressing issue is the postproduction degradation of vitamins during sterilization (steam autoclave and gamma irradiation) and storage [10]. Therefore, the heat-labile vitamins including A, E, pantothenic acid, thiamine, pyridoxine, and B12 are supplemented in rodent diets.

Table 16.3 Overview of major nutrient requirement in the rat and mouse, their main roles, and cardinal symptoms observed in their deficiencies [1, 10, 15]

Nutrients	Requirement (amount per kg)			Main function	Cardinal symptoms in deficiency
	Maintenance	Growth	Reproduction		
Protein	50 g	150 g[a]	150 g[a]	Energy, growth, reproduction, and various physiological functions	Reduced growth, hypoproteinemia, muscular wasting, emaciation, and infertility
Fat	50 g	50 g	50 g	Excellent source of energy and essential fatty acids (absorption and carrier for fat-soluble vitamins) and enhances diet acceptability. Linoleic acid primarily helps in the building of cell membranes	Dermal lesions, alopecia, growth retardation, reproductive failure, reduced myocardial contractility, increased susceptibility to bacteria, and also affect inflammatory pathways. Linoleic deficiency caused premature death
Carbohydrate				Energy, growth, and tissue repair	Minimal consequence, increase susceptibility to pathogenic bacteria
Fiber (crude)	52 g	52 g	38 g	Fermented by the intestinal microflora and produced short-chain fatty acids (SCFAs) that help in de novo synthesis of glucose body fat and activate the microbial synthesis of several vitamins	Damage to the colonic mucus barrier reported in gnotobiotic mice fed on fiber-deficient diet
Calcium	5 g	5 g	6.3 g	Bone calcification and sustained of reproduction	Growth retardation, osteoporosis, reduced appetite, reduced activity and sensitivity, increased basal metabolic rate, rear leg paralysis, and reproductive failure
Phosphorus	3 g	3 g	3.7 g		
Potassium[b]	3.6 g	3.6 g	3.6 g	Adequate growth and reproduction	Lethargic, untidy appearance, comatose, cyanosis, reduces appetite and growth, short furs like hair, diarrhea, distended abdomen, and ascites
Sodium	0.5 g	0.5 g	0.5 g	Numerous physiological activities	Growth retardation, corneal lesions, soft bones, infirmity in males, and delay of sexual maturity in females

(continued)

Table 16.3 (continued)

Nutrients	Requirement (amount per kg)			Main function	Cardinal symptoms in deficiency
	Maintenance	Growth	Reproduction		
Magnesium	0.5 g	0.5 g	0.6 g	Numerous physiological activities	Hyperirritability, vasodilation, spasticity, cardiac arrhythmias, and reproductive failure
Iron	35 mg	35 mg	75 mg	Adequate growth and reproduction	Anemia, increased metabolic rate, hyperlipidemia, and growth failure
Manganese	10 mg	10 mg	10 mg	Numerous physiological activities	Reduced appetite, poor growth, impairment in reproduction, ataxia, severe bone diseases, and early postnatal death in litters
Copper	5 mg	5 mg	8 mg	Cofactor for platelet cytochrome c oxidase activity, serum ceruloplasmin activity, and zinc superoxide dismutase activity	Abnormalities in the nervous, cardiovascular, skeletal, reproductive, hematopoietic, and immune systems
Iodine	150 µg	150 µg	150 µg	Reproduction, adequate growth, and synthesis of thyroid hormone	Impaired in reproduction, enlargement of the thyroid glands, and coarse and less dense hair
Zinc[c]	12 mg	12 mg	25 mg	Numerous physiological activities especially the homeostasis of calcium and phosphorus	Anorexia, growth retardation, alopecia, reproductive failure, and behavioral abnormalities in offspring
Vitamin A[d](retinol)	0.7 mg	0.7 mg	0.7 mg	Cellular differentiation processes, signal transduction in B lymphocytes, and vision	Defect in vision and bones, growth failure, keratinization of epithelial tissues, and impairment of the immune system. Reproductive failure in rats
Vitamin D[e]	0.025 mg	0.025 mg	0.025 mg	A precursor of hormone 1, 25-dihydroxycholecalciferol	Effect on the growth rate, irritability, tetany, decreased bone calcification, and rickets
Vitamin E[f]	18 mg	18 mg	18 mg	Antioxidant activities	Kyphoscoliosis, muscular dystrophy, reproductive failure, increased hemolysis, hyaline degeneration, and lipofuscin that accumulates in the tissues

Vitamin B1[g] (thiamine)	4 mg	4 mg	4 mg	A precursor of thiamin pyrophosphate, coenzyme for oxidative decarboxylation, and other oxidative reactions	Anorexia, poor growth, severe convulsions, cartwheel or circular movements, brain hemorrhages, weight loss, and early mortality
Vitamin B2 (riboflavin)	3 mg	3 mg	4 mg	A precursor of the flavin coenzymes required for normal metabolism of vitamin B_6 and folate coenzyme	Hyperkeratotic epidermis, myelin degeneration in the spinal cord, corneal vascularization with ulceration, loss of weight in adult, and poor growth in young mice
Vitamin B6[h] (pyridoxine)	6 mg	6 mg	6 mg	Coenzymes for amino acid decarboxylases, racemases, transaminases, and other enzymes involved in amino acid, glycogen, and fatty acid metabolism	Dermatitis, alopecia, convulsions, posterior paralysis, hyperirritability, necrotic degeneration of the tail, progressive hypochromic microcytic anemia, and poor growth
Pantothenic acid	10 mg	10 mg	10 mg	A constituent of coenzyme A and as a component of the acyl carrier protein in fatty acid synthesis	Dermatosis, partial posterior paralysis, neurological abnormalities, achromotrichia, loss of weight and hair
Biotin	0.2 mg	0.2 mg	0.2 mg	Required for carboxylase enzymes in mammalian systems for fatty acid synthesis, leucine methionine, threonine, and valine catabolism	Alopecia, achromotrichia, growth failure, decreased reproduction, and lactation efficiency
Folic acid	1 mg	1 mg	1 mg	Transfer of one-carbon units such as purine synthesis, thymidylate synthesis, serine synthesis from glycine, and methionine synthesis	Decreased organ growth especially the brain, decrease in white cell count, impaired reproduction and antibody response
Vitamin B12	50 μg	50 μg	50 μg	Required for transmethylation of homocysteine to methionine, the conversion of methyl malonyl-CoA to succinyl-CoA, and in utilizing 5-methyl-tetrahydrofolic acid	Exfoliative dermatitis, retarded growth, achromotrichia, general alopecia, and renal atrophy

(continued)

Table 16.3 (continued)

Nutrients	Requirement (amount per kg)			Main function	Cardinal symptoms in deficiency
	Maintenance	Growth	Reproduction		
Choline	750 mg	750 mg	750 mg	Important role in one-carbon metabolism, component of lecithin and of sphingomyelin, and the neurotransmitter acetylcholine	Fatty liver, lower conception rates with low viability of offspring

The mouse has a similar nutrient value except for protein requirements. In mouse, 180 g (equivalent to 20% casein supplemented with 0.3% DL-methionine or 24% casein) for growth or 200 g (casein) and 180 g (natural ingredients) for reproduction

Rat: Digestible energy for maintenance and growth, 3.6 kcal ME/g (15.0 kJ ME/g), and for lactation, 311 kcal/$BW_{kg}^{0.75}$ (1300 kJ/$BW_{kg}^{0.75}$)

[a]Estimates based on the highly digestible protein of balanced amino acid composition (e.g., lactalbumin)

[b]Estimate represents an adequate amount, rather than a true requirement

[c]The higher concentration is required when ingredients that contain phytate (such as soybean meal) are included in the diet

[d]Equivalent to 2300 IU/g. The requirement may also be met by 1.3 mg β-carotene/kg diet. Higher vitamin A concentration is needed under conditions of stress (e.g., surgical recovery)

[e]Equivalent to 1000 IU/kg

[f]Equivalent to 27 IU/kg. Higher concentration may be required if high-fat diets are fed

[g]Higher concentration may be required with low-protein, high-carbohydrate diets

[h]Estimate represents the adequate amount, rather than true requirements

Table 16.4 Non-nutrient factors affecting the composition of nutrients in diets

S. no.	Non-nutrient factors	Example	References
1.	Phytoestrogens	Coumestrol from alfalfa meals Isoflavones from soybean meal	[10, 17]
2.	Synthetic contaminants	Genetically modified organisms, pesticides, pollutants including polychlorinated dibenzo-p-dioxins, polychlorinated biphenyls, and dibenzofurans	[18]
3.	Heavy metals	Cereal grains and animal by-products that contain lead, arsenic, and cadmium	[18, 19]
4.	Mycotoxins	Toxic metabolites of fungi present in cereal grains including ochratoxin A, deoxynivalenol, and zearalenone	[20]
5.	Endotoxins	Lipopolysaccharides present in the outer cell membrane of gram-negative bacteria	[21]
6.	Fibers (insoluble and soluble)	Insoluble fibers obtained from plant cell walls of cereal grains (wheat middlings, beet pulp, ground wheat, and corn) Insoluble fibers obtained from plant cell walls include cellulose, hemicellulose (xylans, mannans, glucans), and lignin Soluble fiber includes pectin	[22–24]

These vitamins should be added in such concentration that the reduction after steam autoclaving at 12 °C for 15–25 min still departs the optimal level to the animals [27].

16.5 Dietary Choices in Rodents

Many animal facilities procure laboratory animal diets from commercial manufacturers. These manufacturers have good experience in making high-quality diets. These modern units adopt the HACCP concept and EU- GMP guidelines to assure quality and safety in various diet formulations. The rodent diets can be classified broadly in two types of categories: grain-based or cereal-based rodent chows (commonly referred to as "chows or natural ingredient diet") and purified diets. A third class is known as chemical-defined diets which use entirely synthetic ingredients and are not routinely used in research due to high cost.

16.5.1 Natural Ingredient Diets

Natural ingredient diets are formulated by using of raw agriculture material such as whole grain (wheat, corn, soy, oats, etc.) or grain by-products (wheat middling, oatmeal, soybean meal, cornmeal, alfalfa meal), plant fat (plant oils), animal fats (animal lard, fish), fish meal, and mineral/vitamin mixture (bone meal, limestone, purified mixture). These diets are also known as chow or standard chow diets or

grain-based diets (GB). These formulations are often used for laboratory animals owing to low cost and easy availability. Natural ingredient diets are made by using open or closed formulas. However, these diets mostly use "closed formulas or proprietary." The analysis of nutrients in both open and closed formulations as rendered by the manufacturer should only be used as a guide. Finally, the direct testing of the final feed product is imperative to corroborate the diet composition [2, 15].

The closed formula also has natural ingredients, but the actual levels of the ingredients are not revealed. These diets can be low-cost, variable, and fixed formulations. Often, the variability in formulations may not be informed to the researchers. In low-cost formulations, the manufacturers substitute the diet with lower-cost ingredients by keeping the same composition of basic nutrients. In the fixed-formula diets, the manufacturers maintain a similar percentage of the mixture of their proprietary ingredients from batch to batch. While in variable formulations also termed as "constant nutrition" diets, there may be chances of variation in the composition based on the batch nutrient analysis [1, 10].

The grain-based ingredients usually comprise the nutrients and non-nutrients, which are likely to change with geographic location, climate, soil conditions, and timing of harvest. Therefore, the variations in these types of diets can be necessary to maintain a given nutrient concentration. The consistency in nutrient concentration may be accomplished by employing modern quality-control measures. However, other potential factors such as bioavailability of nutrients, potential nutrient interactions, and nutrient losses during manufacturing and storage need to be taken into account [28]. The common non-nutrient factors that will directly affect the nutrient feed quality are shown in Table 16.4.

The most common source of protein utilized in chow diets is soybean meal (around 50% protein). Soybean meal, besides the protein, also comprises other nutrients (like fiber, fat, minerals) as well as the non-nutrients including the phytoestrogens. The phytoestrogens in soybean meal are genistin and daidzin, which are similar to the hormone estrogen. The level of these phytoestrogens in soybean can change significantly based on soy variety, location of harvest, and time of year. As a result, the soybean meal, which is altered intentionally in some chows to maintain the desired concentration of protein, caused unintentional changes to levels of phytoestrogens [10, 29]. However, conversely, a study carried out to compare the quality of soybean meal from the United States with that of other countries revealed that there were only minor differences in the quality of protein and lipid concentration [30]. These slight differences did not significantly affect the nutrient content of finished, natural ingredient diets [31]. Other studies related to trace minerals such as selenium (Se) in laboratory animal diets showed a wide fluctuation in their concentration ranging from negligible to toxic concentrations [32]. Thus, there are mixed opinions on whether the nutrient composition of individual ingredients varies significantly in the final formulation.

Open-formula diets could be replicated by any diet manufacturer. These diets describe the complete formulation of ingredients employed for their manufacturing. These diets were prepared as standardized laboratory animal diets at the National

Institutes of Health (NIH) in the early 1970s with the intent of decreasing the variability in diet formulations. Consequently, about 13 open formulations have been developed and published by the NIH [33]. Nephropathy was frequently confronted owing to the high protein content of the NIH-07 diet in aging F344 rats which were used in rodent bioassay. This problem was addressed by reducing the protein content in early 1996 by developing an open-formula rodent diet termed as NTP-2000. The use of the NTP-2000 diet had reduced the incidence of leukemia and gaining of weight [34] in F344 rats due to higher fat content (8.2% vs 5.3% in NIH-07). The open-formula diets are advantageous since they can be prepared by any manufacturer and offer open competition in terms of product quality and pricing. However, it is difficult to maintain the uniformity in the non-nutrient and nutrient contents in the fixed formula and open chow from batch to batch. Of late, the manufacturers of chow diet also provide a phytoestrogen-free diet by replacing soybean meal or both soybean meals with casein (containing around 87% protein) or combinations of other plant protein sources. These changes helped reduce the phytoestrogen content [17]. However, the influence of a toxicological phenotype of interest can be masked by other confounders or endocrine disruptors present in other ingredients [35].

16.5.2 Purified Diets

Purified diets are "open and fixed formulas" formulated with separate refined ingredients. Each ingredient contains the "main" nutrient. Examples are soybean meal or casein as the protein source, cornstarch or sucrose as carbohydrate source, and corn or soybean or corn oil as a fat source. The synthetic forms of vitamins are added to these diets [15]. The terms semisynthetic, semi-purified diets are also interchangeably used with purified diets. Around the 1920s, the role of the individual dietary compounds such as minerals and vitamins was begun to be understood [6]. These diets are most consistent from batch to batch in terms of their nutrient profile compared to natural ingredient diets. However, the cost is the main constraint in using these as the standard diet. Until the 1970s, the researchers formulated their diet in the lab. As a result, the chances of variation of essential nutrients were significant. In 1976, the American Institute of Nutrition (AIN) realized the need to formulate a standard purified rodent diet by using the "open formula." Consequently, the AIN-76 diet was formulated, which could be fed to laboratory rats and mice for all stages of life (growth, reproduction, and lactation). Another diet termed as AIN-76A was formulated to address the issue of loss of vitamin K concentration, thus fulfilling the nutrient requirements of rodents established in 1972 by the Committee on Animal Nutrition, National Research Council [36]. However, issues such as nephrocalcinosis in female rats and liver periportal lipidosis in male CD and F344 rats were noticed. The reassessment of AIN-76A diet by AIN based on the understanding of rodent nutritional requirements was in the early 1990s, and subsequent modifications led to the AIN-93 series of diets [6, 37, 38]. Now, there

are several specialized purified diets available from several manufacturers across the world.

Purified diets are advantageous since the researchers can report the ingredient composition due to "open formulas" that remain constant in the diet. Further, the nutrient concentration of both micro- and macronutrients is maintained consistently by using the refined ingredients that limit the contaminants as well as the variability of nutrients [39]. These diets also give freedom to the researcher to manipulate the desired nutrient according to their advantage. Though a specific purified-ingredient diet may not be suited for every research need, they provide the researcher with the opportunity to study the influence of nutrients such as excess or deficiency on the study. Further, AIN-76A or AIN-93 series diets permitted the toxicologists to study the effects of compounds without the influence of contaminants which are mainly present in grain-based (GB) diets.

These diets also help in understanding the specific role of nutrients by excluding the one ingredient. Such specific diets also paved the way for studying the development of metabolic disease in mice and rats by increasing the calories from carbohydrate adding excess amounts of calories from the carbohydrate source (corn, starch) or fat like lard. However, control diets (containing low fat and fiber) used for higher-fat diet studies may cause mild metabolic syndrome such as glucose intolerance or alter other phenotypes compared to GB diets having high amounts of fiber. A drawback of purified-ingredient diets is that they contain appreciable amounts of sucrose and lack soluble fiber. This can be altered by adding soluble fiber such as inulin or substituting sucrose with other sources such as cornstarch and dextrose to minimize fructose. These modifications help in reducing the development of metabolic disease, including glucose intolerance, insulin resistance, and hypertriglyceridemia [10, 40]. It is also crucial for researchers to identify the difference in the composition of GB diets and the impact of these differences on the rodent phenotype. A summary of the source of nutrient and contaminant sources of purified and GB diets is presented in Table 16.5.

16.5.3 Chemically Defined Diets

Chemically defined diets are composed of "basic ingredients" such as specific sugars, amino acids, essential fatty acids, purified minerals, and vitamins. These diets are rarely used owing to high costs [24]. Chemically defined diets were usually applied for deficiency studies of dietary components like amino acids in the past. Now, chemically defined diets are being used in the studying of intestinal homeostasis, immune function, and immunological tolerance. Besides, these diets have also been used for the study of nonalcoholic steatohepatitis and nonalcoholic fatty liver disease [10, 42]. These diets are less palatable; hence, the rigorous monitoring of feed intake is required.

Table 16.5 Summary of the source of nutrient and contaminant sources in purified and grain-based diets[a]

Nutrients	Grain-based diet	Purified-ingredient diet
Carbohydrate	Wheat, ground corn, and wheat middlings	Sucrose, cornstarch, and maltodextrin
Protein	Ground corn, soybean meal, wheat, oats, whey, meat meal, alfalfa, and fish meal	Casein
Fat	Fish meal, meat meal, and porcine animal fat	Corn oil and soybean oil
Fiber	Ground corn, oats, wheat, dried beet pulp, alfalfa, and wheat middlings	Refined cellulose
Micronutrients	Most ingredients and extra micronutrients added	Mainly vitamin and mineral premixes
Non-nutrients/contaminants		
Heavy metals	Grains and animal by-products (lead, arsenic, lead, cadmium, cobalt)	Trace/not detectable
Phytoestrogens	Alfalfa meal (coumestrol) and soybean meal (daidzein, genistein)	Absent[b]
Pollutants, mycotoxins, pesticides, nitrosamines, endotoxins[c]	Animal by-products (pollutants, nitrosamines) and grains (pollutants, mycotoxins)	Trace/not detectable

[a]Table adapted from Ref. [41] with slight modifications
[b]Unless soy protein isolate is used
[c]Endotoxin source unknown but high in grain-based diet

16.6 Physical Forms of Diets

Diets for rodents are commonly available in two forms, pelleted and extruded. Both are compounded meals. In both forms of diets, the individual raw ingredients are ground and mixed with vitamin and mineral supplements. The carboxymethyl cellulose (0.1–1%) may be added as a binding agent in the raw ingredient mixture. The diet pellets are formed by compressing ingredient paste through a metal die under steam and pressure. The size of the pellet may vary according to the size of the metal die. In the case of the extrusion process, the ground mixture is subjected to higher temperatures than in the pelleting process. The heating during the pelleting and extruding process reduces the microbial contaminants. The uniformity of nutrient composition, adequate digestibility, and reduction in feed waste has been observed in the case of pelleted feed. The greater gelatinization of starches takes place in extruded diet form because ingredients of extruded diets are usually ground finer than in pelleted diets. The heated mixture of ingredients of extruded diets is forced out through a die in semisolid form. After getting out of die, the extruded diet cools to ambient temperature and gets dried. During vaporization, water vapors soften the diet by creating air pockets [43]. Pelleted or extruded meals are ground

to make fine granules or powder called meal or powdered diet. The nutritional additives which cannot withstand the process of pelleting or extrusion can be added in a meal or powdered diets. During stress or anorexia such as post-experimental manipulations, post-surgery, or transport, soft and moist diets are provided to laboratory animals. The soft diets are also given to young animals during the transition from milk to solid foods. It encourages caloric intake in lab animals [27].

16.7 Rodent Diet as a Source of Experimental Variation

16.7.1 Formulation of Diet

The role of diet as a source of variation that can hinder the replicability of the experiment is seldom recognized. Diet may lead to physiological changes in rodents. Therefore, if the diet is not formulated with care keeping in mind the requirements of an experiment, it may lead to extrinsic variability. Diet formulation affects the bioavailability of nutrients and may be responsible for significant changes in physiological responses.

Natural ingredient diets are cost-effective. These are used for the breeding and maintenance of most laboratory animals. Natural ingredient diets should not be considered for experiments where the study is related to physiological and biochemical changes since there is a high probability of variation from lot to lot and batch to batch, in terms of nutrients and chemical contaminants in comparison to formulated or designed diets. While investigating the role of a particular dietary component, the researcher often mistakenly uses natural ingredient diets that influence the study outcome or as a control diet [44]. This has been observed in natural ingredient diets containing nonnutritive, natural chemicals as raw components.

Most of the studies on dietary fat use formulated and purified diets to vary the concentration of fat up to 60% of caloric sources with the natural ingredient diet as a control. In these studies, it is stated that the phenotype may be the result of the fat alone, but the inherent variability of components in natural diets has been ignored. The diet for experimental and control animals should have identical components except for the component which is under study. Although the purified or the formulated feed reduces the variability of diet components in comparison to natural ingredient diets, these may also have few drawbacks; e.g., the formulated low-fat diets used for control are added with high levels of sucrose to balance it calorically. In this case, fructose may affect insulin signalling and lipid metabolism because fructose contains 50% sucrose. The gut microbiome composition and lipid metabolism are affected and decided by the concentration and type of dietary fiber. Though cellulose is included as a fiber source in most of the purified diets, cellulose cannot be fermented in most animals and does not favor gut bacterial growth. Therefore, their use can restrict animals from the beneficial effects of the gut microbiome [44].

The impact of natural chemicals present in the natural ingredient diet, on physiological responses, is not limited to a particular species but has been seen in many species. The well-known natural chemicals as raw components of natural diets are

the phytoestrogen or isoflavone compounds that have estrogen-like activity. Natural ingredient diets containing alfalfa and soybean meal are the primary source of phytoestrogens such as genistein and daidzein. Phytoestrogens are similar to sex hormones and have been shown to affect the studies related to reproductive biology, neurological disease, bone mineral density, obesity, toxicology, and oncology [17, 45, 46].

To maintain the uniformity of diet components, National Toxicology Program (NTP), National Institute of Health (NIH), formulated specifications for a diet for rodents, and it was named as NTP-2000 diet. The other diet specification was named as NIH-07 and was formulated for reproduction and lactation in rodents. But NIH-07 formulation of rodents was replaced in long-term studies due to the occurrence of diseases unrelated to the chemical under study [34, 47].

16.7.2 Contaminants in Animal Diet

The effects of feed contaminants have been revealed by conducting various toxicology and pharmacology studies on animals. Many regulatory agencies have framed guidelines for animal feed in toxicology and pharmacology studies. The first guideline about animal feed and water was published by the US Food and Drug Administration (USFDA) in 1978 under Good Laboratory Practices (GLP). It fixed the limits for the contaminants in animal feed and water. The animal feed and water should be checked regularly to eliminate the possibility of contaminants above the prescribed or the specified limits. The contaminants expected to interfere with the study should be analyzed with great care [48]. Rodent feeds formulated with natural ingredients such as animal and plant products may contain biotic and abiotic contaminants. The biotic contaminants are generally microbes and their toxins. The abiotic contaminants in plant products may be residues of chemical fertilizers and pesticides. The formulated diets are devoid of such contaminants. But their high cost precludes their wide use. The USFDA GLP suggested the need for analysis of feed and water for experimental animals but did not give any recommendation and left it to the discretion of the investigator to decide the appropriate maximum contaminant levels (MCLs). MCLs for contaminants such as pesticides and minerals were published by the Environmental Protection Agency (EPA) in test standards in 1978 [49]. These MCLs were updated in 1984 [50]; (Table 16.6). G. N. Rao, a veterinarian and toxicologist, published test and analysis data of 94 batches of the NIH-07 rodents' open-formula diets. These diets were being used in toxicology studies by NTP [51]. Based on his findings, he recommended MCLs for diets to be used in NTP. To date, these MCLs are followed by the NTP and the National Institute of Environmental Health Sciences (NIEHS) as standards for feed contaminants (Table 16.6). The guidelines for MCLs in feeds for laboratory rodents have been published in 1992 by the British Research Quality Association and in 2002 by the German Society for Laboratory Animal Science [52]. Recently, for developing standards for contaminants in rodent diets, the Federation of European Laboratory Animal Science Associations (FELASA) convened the Working Group on Rodent Diets and Nutrition [53].

Table 16.6 Contaminant level standards in laboratory animal diet

Maximum contaminant level (MCL) standards						
Contaminant	Units	NIH-31[a]	Certified diets[b]	NIEHS[c]	NTP[c]	EPA 1979 [49]/1984[d]
Organophosphates						
Chlorpyrifos-methyl	ppm	<0.010	–	–	0.10	–
Diazinon	ppm	<0.010	0.5	0.2	0.2	–
Disulfaton	ppm	<0.010	0.5	–	–	–
Ethion	ppm	<0.010	0.5	0.02	0.02	–
Malathion	ppm	0.012 ± 0.003	0.50	0.50	0.50	2.5/0.50
Methyl parathion	ppm	<0.010	0.5	0.02	0.03	–
Parathion	ppm	<0.010	0.5	0.02	0.03	–
Phorate	ppm	<0.010	0.5	–	–	–
Ronnel	ppm	<0.010	–	0.02	0.03	–
Trithion	ppm	<0.010	0.5	0.05	0.05	–
Chlorinated hydrocarbons						
Aldrin	ppm	Not tested	0.03	–	–	–
Benzene hexachloride (BHC)	ppm	<0.010	0.05	0.02	0.02	–
Chlordane	ppm	<0.010	0.05	0.05	0.05	–
Dichlorodiphenyltrichloroethane (DDT)	ppm	<0.010	0.15	–	–	0.10/–
Dieldrin	ppm	<0.010	–	0.02	0.02	0.02/–
Endosulfan	ppm	<0.010	0.05	0.02	0.02	–
Endrin	ppm	<0.010	–	0.02	0.02	–
Heptachlor	ppm	<0.010	0.03	0.02	0.02	0.02/–
Hexachlorobenzene	ppm	<0.010	0.03	–	0.02	–
Lindane	ppm	<0.010	0.05	0.02	0.02	0.02/–
Polychlorinated biphenyls (PCBs)	ppm	<0.010	0.15	0.20	0.20	0.05/–
Toxaphene	ppm	<0.010	0.15	0.10	0.10	–

Heavy metals						
Arsenic	ppm	0.412 ± 0.070	1	0.6	0.5	1/–
Cadmium	ppm	0.046 ± 0.003	0.5	0.15	0.5	0.16/–
Lead	ppm	0.186 ± 0.032	1.5	1	1	1.5/–
Mercury	ppm	<0.02	0.20	0.05	0.05	0.1/–
Selenium	ppm	0.373 ± 0.060	0.5	0.5	0.5	0.6/0.4
Miscellaneous contaminants						
Aflatoxins	ppb	< Limit of detection	5	5	5	5/20
Estrogenic activity	ppb	Not tested	–	–	–	1/4
Ethoxyquin	ppm	7.2 ± 6.3	–	–	–	–/10
Nitrosamines	ppb	15.8 ± 7.5	–	15	15	10/–
Nitrate	ppm	6.4 ± 1.3	–	20	20	–
Nitrite	ppm	0.5 ± 0.4	–	5	5	–
Butylated hydroxyanisole (BHA)	ppm	3.7 ± 5.6	–	10	10	20
Butylated hydroxytoluene (BHT)	ppm	–	–	5	5	–
Daidzein and genistein	ppm	155.0 ± 22.4	–	200	–	–
Bacteria	CFU/g	Not tested	–	200,000	1000	–
Coliforms	MPN/g	0[e]	–	2000	10	–
E. coli	MPN/g	0[e]	–	25	10	–
Salmonella	Per g	0[e]	–	<1	0	–

CFU colony forming units, *EPA* US Environmental Protection Agency, *g* gram, *ppb* parts per billion, *ppm* parts per million, *NTP* National Toxicology Program, *NIEHS* National Institute of Environmental Health Sciences

[a]Contaminant levels in 23 batches of NIH-31 open-formula rodent feed used at NIEHS from 2016 to 2019. Values are mean ± SD

[b]LabDiet Certified Diets https://www.labdiet.com/cs/groups/lolweb/documents/web_asset/ndjf/mdi3/~edisp/36142_027288.pd) and Teklad Certified Diets (https://www.envigo.com/resources/data-sheets/teklad-certifieddiet-sheet.pdf)

[c]Adapted from [51]

[d]Concentrations listed are suggested revisions to 1979 standards; otherwise, 1979 concentrations were still in place

[e]NIH-31 is autoclavable, *E. coli* and *Salmonella* spp. Tested but not quantified

16.7.2.1 Phytoestrogens

The well-known natural chemicals as raw components of natural diets are the phytoestrogen or isoflavone compounds that have estrogen-like activity. Natural ingredient diets containing alfalfa and soybean meal are the primary source of phytoestrogens such as genistein and daidzein. Phytoestrogens are similar to sex hormones and have been shown to affect the studies related to reproductive biology, neurological disease, bone mineral density, obesity, toxicology, and oncology [17, 45, 46].

Epigenetic effects and alteration in drug-metabolizing enzymes have been reported due to dietary phytoestrogens. Though physiological effects of phytoestrogens are known for more than four decades, studies on estrogen function and environmental endocrine-disrupting chemicals (EDCs) using natural ingredient diets containing genistein and daidzein are still going on [35, 45]. The studies for the assessment of the pharmacological or toxicological effects of EDCs should use natural diets free from soy and alfalfa because these formulated natural diets have insignificant levels of phytoestrogen [17, 29, 45, 46]. It is critical for the studies involving the assessment of the physiological effects of a defined dietary component to use a formulated diet. For many years, diets containing significant levels of fat have been studied for its role in diabetes, obesity, and other metabolic disorders. Alteration in the gut microbiome by varying diet also plays an important role in the study [7–10]. The laboratory animal feed contaminant standards and NIH-31 feed contaminant levels for phytoestrogen are provided in Table 16.6.

16.7.2.2 Pesticides

Most of the agricultural products contain pesticide residues, and these pass to the animals through the food chain. The EPA in the United States fixed the limits of maximum pesticide residue levels in foods, and the FDA enforced these limits in animal food. In 2012, the EPA published a list of the top 25 most widely used pesticides in the US agricultural practices with atrazine, glyphosate, S-metolachlor, dichloropropene, and 2,4-dichlorophenoxyacetic acid (2,4-D) topping the list [54]. As per the report published by FDA in 2016, 43% of animal feeds were found to have non-detectable levels of pesticide residues. The animal feeds were found to have 74 different pesticide residues, and out of these 74, the top five were ethoxyquin, malathion, piperonyl butoxide, deltamethrin, and chlorpyrifos-methyl [55]. The presence of the residual concentrations of heavy metals and several pesticides in 13 natural ingredient diets used in some countries on five different continents was reported by Mesnage et al. in 2015 [18]. But the concentrations of pesticides in all these natural ingredient diets were below the limits recommended by European and US regulators. The EPA does not recommend screening animal feed for these pesticides except malathion [50], and the current NTP and NIEHS feed specifications are based on these recommendations. Ethoxyquin at an MCL of 150 ppm is approved for animal feed by USFDA. It is the most common pesticide residue found in animal feed, but it is not allowed in food for human consumption. In standard rodent feed (NIH-31), the residues of only two pesticides have been detected, and these were ethoxyquin at 6.3 ppm and malathion at 0.003 ppm. But

NIH-31 has not been tested for glyphosate. Glyphosate has been found at the average concentrations of 0.68 ppm in several natural ingredient diets used at the NIH [55, 56]. The laboratory animal feed contaminant standards and NIH-31 feed contaminant levels for various pesticides are provided in Table 16.6.

16.7.2.3 Microbial Contaminants

Numerous animal disease outbreaks have occurred due to microbial contamination of animal feed. Disease outbreaks due to microbes in animals have been responsible for the significant animal and economic losses. The microbial contaminants survive in storage conditions in raw agricultural feed components. Unsterilized animal feeds formulated with raw agricultural or natural ingredients can have various environmental microbes as contaminants. Mouse parvovirus outbreak has been recorded due to an unsterilized diet. The animal products added to the animal feed may also be the source of microbial contamination. The bacteria of Enterobacteriaceae family such as *Escherichia coli*, *Salmonella*, and *Yersinia* are the main pathogenic microbial contaminants in animal feed [57]. All microbes (e.g., bacteria, fungi, and viruses) are not eliminated or killed during the milling and pelleting or extrusion process. Unsterilized natural ingredients and purified pelleted feeds have been found to have spore-forming bacilli and sometimes enteric pathogens such as *Salmonella*, *Escherichia coli*, or *Clostridium perfringens*. It was observed that mouse parvovirus and mouse norovirus survived the pelleting process and infected mice fed with pellets [58]. Therefore, animal feeds should be sterilized before use. The laboratory animal feed contaminant standards and NIH-31 feed contaminant levels for various microbes are provided in Table 16.6.

16.7.2.4 Heavy Metals

Natural ingredient diets, which include raw agricultural products or by-products, are expected to have varying concentrations of heavy metals depending on the soil used to grow agricultural crops. These may contain essential heavy metals such as iron, cobalt, manganese, molybdenum, and zinc and nonessential heavy metals such as arsenic, cadmium, lead, and mercury. The concentration of these heavy metals cannot be controlled in natural ingredient diets. These inorganic heavy metals are ubiquitous and are natural components of the soil. The unusually high concentrations of all heavy metals can be toxic, but nonessential heavy metals are considered toxic even at very low concentrations. The maximum tolerable levels for many animal species have been published by the National Research Council (NRC) [59]. Generally, nonessential metals such as lead (Pb), arsenic, cadmium (Cd), and mercury (Hg) are found in animal feed as contaminants [19]. Laboratory animal feed manufacturers should indicate the concentrations of certain heavy metals in their feed analysis report. The laboratory animal feed contaminant standards and NIH-31 feed contaminant levels for various heavy metals are provided in Table 16.6.

16.7.2.5 Organofluorides

Organofluorides are used in various products such as food packaging, nonstick and water-resistant coatings, and aqueous film-forming foams used in firefighting. The

most commonly used organofluorides are perfluorooctanoic acid and perfluorooctane sulfonate. As per the advisory published by the EPA, limits for combined exposure for perfluorooctane sulfonate and perfluorooctanoic acid are 70 ppm. Bioaccumulation of organofluorides has been reported, but the biological effects at exposure levels from the environment are unknown and are still under investigation. The immunological effects of these chemicals in rodents have been published by NTP in its report. These chemicals are also reported to affect the endocrine system of animals. Organofluorides are very stable under typical environmental conditions and have half-lives of 40–90 days [60]. Therefore, these chemicals are considered persistent organic pollutants. The laboratory animal feed contaminant standards and NIH-31 feed contaminant levels for organofluorides are provided in Table 16.6.

16.7.2.6 Mycotoxins

Mycotoxins are metabolites secreted by various fungi. These mycotoxins are toxic to animals and are responsible for economic loss by causing diseases. Mycotoxins are mostly produced by members of the genera *Aspergillus*, *Alternaria*, *Fusarium*, and *Penicillium* sp. These metabolites are stable and resistant to decomposition at normal environmental conditions. The common classes of mycotoxins are aflatoxin, ergot alkaloids, fumonisins, ochratoxins, trichothecenes, and zearalenone. The contamination of feed can occur during both pre- and post harvest processes. The disease due to mycotoxins may vary depending on the type of toxin, age, sex, species, and nutritional requirements of the animal. The mycotoxin aflatoxin B1 is a potent mutagen, carcinogen, and teratogen. It may impact both in vivo and ex vivo research studies. The physiological implications of mycotoxins have been studied extensively [61]. The laboratory animal feed contaminant standards and NIH-31 feed contaminant levels for mycotoxins are provided in Table 16.6.

16.7.2.7 Nitrosamines

The most common nitrosamines found in food are N-nitrosodimethylamine and N-nitrosopyrrolidine. These chemicals are added as a preservative in food. The most common source of nitrosamine contamination in natural ingredient diets is cured fish meal. Mortality in animals was observed in Norway in the 1950s and 1960s, due to hepatotoxicity as a result of feeding fish meals. Sodium nitrite was used to preserve the fish meal, and it was found to have elevated levels of N-nitrosodimethylamine. These compounds are carcinogenic and are found in cured meats, tobacco smoke, and rubber products. Nitrosamines are formed when nitrosating agents such as oxides of nitrogen react with secondary or tertiary amines. The studies in rodents have demonstrated the tumorigenic effects of these compounds on various organs such as the liver, lung, and stomach [39]. Nitrosamines induce carcinogenicity by mutating DNA via alkylation. The compounds such as ascorbic acid, alpha-tocopherol, and sulfur dioxide are reported to inhibit nitrosation and are usually added to animal feed to eliminate the formation of nitrosamine. However, in vivo, ascorbic acid along with a high-fat diet can increase the formation of nitrosamine [62]. The laboratory animal feed contaminant standards and NIH-31 feed contaminant levels for nitrosamines are provided in Table 16.6.

16.7.3 Methods of Diet Sterilization

The demand for sterilized diets has increased to eliminate microbial contamination in the diet. It is also required to get specific pathogen-free (SPF) animals required for specific research. Currently, steam autoclaving and gamma irradiation methods are used for animal feed sterilization. These methods sterilize the diet up to satisfactory levels. At the same time, they may reduce the nutrient levels, thus reducing the quality of diet. During steam autoclaving, the concentrations of heat-labile vitamins such as A, B1, and D are reduced. The heating also affects the availability and quality of proteins available in animal diet [63]. Manufacturers increase the levels of heat-labile nutrients to compensate for their loss during autoclaving, but the quantity of loss may not be uniform. It becomes difficult for the manufacturer to decide the additional quantity of nutrients to be added to the diet. The natural ingredient diets containing higher concentrations of starches have been reported to produce acrylamide during autoclaving. Acrylamide is a neuro- and genotoxin. Feeding acrylamide to mice has been shown to elevate liver DNA-adduct formation which may lead to carcinogenesis [64].

The gamma irradiation sterilization method generally uses doses of 10–30 kGy. The animal facilities may irradiate the diet themselves but most rely on the feed suppliers or manufacturers. Though irradiation has little effect on the physical characteristics of diets, it may affect the chemical composition. The levels of glucosinolates and peroxidation of lipids have been reported to increase in natural ingredient diets upon irradiation. The chemical changes due to irradiation in diets are supported by the fact that when the irradiated diet is fed to rats, the incidence of mammary tumors increases in comparison to rats fed with nonirradiated diet. In another study, the mortality of mice was found to increase by up to 50% when fed with an irradiated, purified diet (AIN-76) supplemented with vitamin K [64, 65]. Both steam autoclave and gamma irradiation do not guarantee complete sterilization. The efficiency of sterilization may be affected by various reasons such as faulty equipment, improper loading, or overloading of the sterilizer. The performance of sterilization should be checked by biological and chemical indicators.

16.7.4 Hardness of Diet

Animal diets generally come as pelleted or extruded. The diet becomes hard due to the gelatinization of the starches during pelleting and extrusion. The degree of gelatinization during the manufacturing of diet depends on processes such as grinding and mixing. The hardness of diet also depends on water content and heating temperature during manufacturing. The pressure during pelleting or extrusion also adds additional hardness to the feed. Diet hardness may not have much impact in the case of adult rodents, but reduced intake of hard diet has been reported in young ones. Diet hardness can be tested by methods such as mechanical tumbling [66]. The heating of feed during autoclaving is also known to increase the hardness of diet. Therefore, the hardness of the diet should be tested after autoclaving.

16.7.5 Discrepancies in Diet Manufacturing

Manufacturing discrepancies are less reported in diet formulations but are significant. These errors include either presence of an additional component or escape of the required diet component. During 2001–2002, rats fed with a diet containing a racemic mixture of choline bitartrate developed urolithiasis. The reason was found to be the change in raw material supplier which resulted in the altered quality of choline bitartrate. The feed manufacturer generally buys feed components from different sources, and the concentration of components varies from one source to another. A similar case was observed when L-tartaric acid isomer which had been in use for many years changed to synthetic DL-tartaric acid mixture [67]. In 2013, vitamin D toxicosis was reported due to manufacturing errors. The investigators should be vigilant about these errors especially when a new lot of feed or feed from a different source is received [68]. The investigators should store frozen feed samples from all batches of diet until the results of the study are analyzed. This will help in arriving at any conclusion if any deviation from the expected results has happened.

16.7.6 Storage of Diet

Proper storage of diet is necessary to maintain its quality and shelf life. The methods for proper storage of feed have been published in detail [43]. The continuity of proper storage begins at feed manufacturing sites. This may further be strengthened by distributors and end users. The stored diet should be inspected periodically for any decay or degradation. The quality of all organic products present in the diet decreases over time. The components of diet degrade due to oxidation. The storage conditions may cause a loss of nutrients. The degradation of nutrients reduces both feed intake and palatability. The diet infested with pests or vermin can be responsible for causing disease in laboratory animals. Moist storage conditions can result in fungal growth and the production of mycotoxins. The diet should be stored in a moisture-free and clean place. The recommended storage temperature and humidity for natural ingredient diets should be below 21 °C (70 °F) and 50%, respectively. Special care should be taken for perishable items like fruits, vegetables, meats, fruits, high-fat diet, and medicated diet because exposure to elevated temperature and humidity may lead to deterioration of diet quality. The shelf life of purified and chemically defined diets is generally less than 6 months, and these are less stable than natural ingredient diets. Therefore, the storage temperature for these diets should be at 4 °C (39 °F) or below [69].

16.7.7 Restricted Diet vs Ad Libitum Feeding

Both restricted feeding and ad libitum feeding have their pros and cons. Rodents are social animals and are kept in colonies. The dominant rodents in the diet restriction feeding will eat more than other cage mates, and in this way, there will be differences

in their growth. This will also create a caloric intake imbalance among the rodents housed within a cage. Ad libitum feeding attempts to offset this imbalance. However, ad libitum feeding has been a cause of conditions such as obesity, chronic renal disease, and reduced life span [34, 70, 71]. In rodent colonies, diet restriction or daily ration type of feeding is labor intensive. The rodents are generally kept in a group and provided feed for 7 days. The NTP has developed the NTP-2000 diet to solve the issues with ad libitum feeding. The NTP-2000 diet contains less protein and higher fat compared to the NIH-07 diet. In the F344 rat, NTP-2000 diet has been shown to reduce chronic renal lesions and increased life span. Many toxicology investigators prefer to use restricted feeding for rodents. It has been observed that the rodents fed 70–75% of ad libitum diet have shown increased life span and less incidence of chronic disease [34, 72]. It is to be noted that diet restriction may affect the results of studies since diet-restricted rodents tend to eat aggressively when fed after a gap, and this may alter biochemistry and physiology. While restricting diet, the practice of feeding during the daytime shifts their circadian rhythm. The various methods of feeding such as diet restriction, ad libitum, modified ad libitum (e.g., 70–75% ad libitum or fixed amount), modified diet restriction, and intermittent fasting have been described for rodents. These modified methods of feeding are difficult to implement on a large scale [73].

16.8 Drinking Water Quality for Laboratory Animals

The quality of drinking water for experimental animals is an important factor in eliminating experimental variations. As per the Guide for the Care and Use of Laboratory Animals and the US Department of Agriculture Animal Welfare Act and Regulations, all laboratory animals should have access to purified and uncontaminated drinking water [74]. All microbial and chemical contaminants are not covered by regulatory standards of EPA. The drinking water for experimental laboratory animals comes from various sources such as surface water (rivers, lakes, reservoirs) and groundwater. The water from different sources may contain different types of contaminants. The National Primary Drinking Water Regulations (NPDWRs) of EPA has published MCLs for 90 microbial, chemical, and radiological contaminants in drinking water for human consumption in the United States. Many other countries and the WHO have adopted similar water quality standards [75].

16.8.1 Microbial and Chemical Contaminants in Water

Laboratory animal drinking water may contain microbial and chemical contaminants that affect physiological responses and may introduce the experimental variability. Surface water is most likely to be contaminated with pathogenic microorganisms due to the mixing of animal feces, animal by-products, and/or plant waste. Water is treated to eliminate microbial contamination. But microbial contamination may

occur post treatment due to the rupture of the pipes used for distribution. The most common microbes found in water are *Salmonella*, *Cryptosporidium*, fecal coliforms such as *E. coli*, *Giardia* spp., and enteric viruses [76].

Due to the increased use of pesticides and nitrogen-based fertilizers, the chemical contamination of water is increasing. Due to these contaminations, cytogenetic damages have been observed in splenocytes of F344 rats and B6C3F1 mice [77]. Chemical contaminants in water may vary from location to location. The commonly found chemical contaminants in drinking water are pesticides, disinfectants, antibiotics, hormones, halogenated organic compounds, heavy metals, and radionuclides. Most of these chemicals affect the physiological parameters of laboratory animals [78, 79]. The chlorine disinfectants react with an organic contaminant in the water and form trihalomethanes which are carcinogenic in rats and mice at high doses. Nitrosamines formed as a by-product of chloramine disinfectants are found to disrupt the microbiome and increase obesity in the rats [80, 81]. Organofluorides and heavy metals are common contaminants of water and are not efficiently removed in municipal water treatment plants [82]. Arsenic at a low dose of 10 ppb has been reported to affect female fertility in mice which leads to an increase in body weight, body fat, and glucose intolerance [83]. The gut microbiome of perinatal mice is affected by the presence of lead in the drinking water. Low-level exposure to lead in the early postnatal period has been found to impede hippocampal development in rats [84].

16.8.2 Purification of Laboratory Animal Drinking Water

Several methods for water purification are available. The physical method, filtration, removes unwanted substances with the application of resins, granular media, and membranes. The charcoal in granular media filters adsorbs organic contaminants, chlorination by-products, heavy metals, and certain pesticides. Deionization resin filters do not remove non-ionized organic substances or microbes. Membrane filters are made up of various materials such as woven fibers, ceramics, and polymers [85]. Membrane filters are classified based on their pore size as microfiltration ($\geq$0.5 μm), ultrafiltration ($\geq$5.0 nm), nanofiltration ($\geq$0.7 nm), and reverse osmosis ($\geq$0.275 nm).

Water filtration may lead to demineralization, and lack of minerals decreases water palatability and water intake. Demineralized water has been reported to decrease hemoglobin content, thyroid hormone secretion, and fetal skeletal ossification in rats [86]. The water filtration methods, especially nanofiltration and reverse osmosis, do not remove all microorganisms. Therefore, an additional step for complete water disinfection should be used. This can be done by physical methods such as steam sterilization and ultraviolet (UV) irradiation or by chemical methods such as chlorination and acidification. The chemical method for disinfection reduces the pH of the water, and that reduces water intake by mice. Acidified water (pH 2) has been reported to cause dental abnormalities in rats [78]. The acidic water is also known to alter the gut microbiota in laboratory animals [87].

The most common method used for the supply of water in modern rodent facilities is water bottles. These water bottles are reusable and inexpensive. The capacity of the water bottle is selected as per the requirements of animal species. They are generally made up of plastic or glass. Water bottles are capped with stoppers having sipper tubes with a small opening. Water bottles provide the flexibility for alterations of the water for target individuals or a group of rodents such as the addition of test chemicals or antibiotics. Water bottles should be sterilized before use.

16.9 Unique Features of Diets of Guinea Pigs and Rabbits

The guinea pigs and rabbits are herbivorous hindgut fermenters. They follow the practice of coprophagy to ensure maximum nutrient absorption; particularly, vitamin B12 and vitamin K are produced by gut microbiota [88, 89]. They have a voluminous cecum that breaks down even the cellulose content of the diet and derives an energy source [90]. Dietary fiber has a significant role in the guinea pig diet to maintain normal physiological function in the gut. Therefore, a minimum level of dietary fiber has to be added to the diet. Similar to humans, guinea pigs lack the enzyme L-gulonolactone oxidase required for vitamin C synthesis. Guinea pigs need 10 mg/kg of vitamin C daily which can be supplied through a balanced pellet diet enriched with a higher amount of vitamin C or providing it in the drinking water [90]. Unfortunately, vitamin C is quite unstable and degrades faster from the pellet diet at higher temperatures and over time. The best practice is to use a vitamin C tablet, and a quarter of a 100 mg tablet is the recommended daily dose for an adult guinea pig. The addition of vitamin C in water requires changing the water every day to ensure an appropriate concentration for guinea pig. However, the addition of vitamin C in water may reduce the intake of water due to change in the taste.

In addition to pellets, the vegetables and fruits are good sources of vitamin C, which include roots like beetroot, carrots, and leafy greens (kale, cabbage, spinach, parsley, romaine lettuce, dandelions, and turnip greens) [88, 90]. The cruciferous vegetables like cabbage, cauliflower, broccoli, bok choy, and collards can cause gas production in the digestive tract of guinea pigs. Like rodents, rabbits can also synthesize vitamin C and do not require the extra addition in their pelleted diet. Therefore, guinea pigs require an exclusive diet, and the feed of rabbits and other rodents is unsuitable for guinea pigs [91]. The exclusive feeding of a pelleted rat diet to a guinea pig can lead to death due to vitamin C deficiency within 14 days [20]. Guinea pigs also need a higher concentration of folic acid and lower amounts of vitamin D as compared to rodents and rabbits [92]. The breeding female guinea pigs require a higher amount of vitamin C (twofold or threefold) in pregnancy due to the abolition of coprophagy as the abdomen expands [90, 91]. The overview of the major nutrient requirements for the growth of guinea pigs and rabbits has been provided in Table 16.7.

Table 16.7 Overview of major nutrients (amount/kg) for growth of g. pigs and rabbit [15, 93]

S. no.	Nutrients	G. pigs	Remarks	Rabbits	Remarks
1.	Protein	180 g	Growth is equivalent with 300 g casein plus 3 g L-arginine per kg or 200 g soybean protein plus 10 g DL-methionine per kg	160 g	–
2.	Essential fatty acids/fat	1.33–4 g	10 g corn oil/kg diet is satisfactory	20 g	May not be minimum but known to be adequate
4.	Fiber	150 g	Used cellulose and/or materials of low digestibility to supply bulk	100–120 g	May not be minimum but known to be adequate
5.	Calcium	8 g	Requirements for calcium, phosphorus, magnesium, and potassium seem to reflect interactions among them	4 g	–
6.	Phosphorus	4 g	–	2.2 g	–
7.	Magnesium	1 g	–	0.3–0.4 g	–
8.	Potassium	5 g	–	6 g	–
9.	Sodium	0.5 g	–	2 g	May not be minimum but known to be adequate
10.	Chloride	0.5 g	From the estimate for rats fed a purified diet	3 g	
11.	Iron	50 mg	Estimate	Quantitative requirement not determined, but dietary need demonstrated	
12.	Manganese	40 mg	–	8.5 mg	Converted from amount per rabbit per day using an air-dry feed intake of 60 g per day for a 1 kg rabbit
13.	Copper	6 mg	Minerals measured in mg/kg correspond to ppm	3 mg	
14.	Iodine	150 μg	Based on rat requirement	0.2 mg	May not be minimum but known to be adequate
15.	Zinc	20 mg	–	Quantitative requirement not determined, but dietary need demonstrated	

16.	Vitamin A (retinol/β-carotene)	6.6/ 28 mg	Used 40% as efficiently as preformed vitamin A	0.83 mg as carotene	May not be minimum but known to be adequate
17.	Vitamin D (cholecalciferol)	0.025 mg	Adequate; no quantitative data		Probably required, amount unknown
18.	Vitamin E (RRR-α-tocopherol	26.7 mg	Adequate	40 mg	Estimated
19.	Vitamin K (phylloquinone)	5 mg	Adequate; dietary deficiency has not been produced	–	Intestinal synthesis probably adequate
20.	Ascorbic acid	200 mg	–	–	–
21.	Vitamin B1 (thiamine)	2 mg	–	–	–
22.	Vitamin B2 (riboflavin)	3 mg	Estimate	–	–
23.	Vitamin B6 (pyridoxine)	2–3 mg	–	39 mg	–
24.	Pantothenic acid	20 mg	–	–	–
25.	Biotin	0.2 mg	–	–	–
26.	Folic acid	3–6 mg	–	–	–
27.	Vitamin B12	50 μg	–	–	–
28.	Choline	1800 mg	Adequate; simple dietary deficiency has not been produced	–	–
29.	Niacin	10 mg	–	180 mg	–

16.10 Conclusion

A balanced diet is essential for optimal growth, reproduction, and welfare of laboratory animals. Unbalanced diets harm animal health as well as compromise animal welfare. Diet is considered as a potential factor which affects research outcome. The development of modern techniques and analytical methods has helped in defining standard nutrient requirements in laboratory animals. These standards have been published as guidance documents by the various regulatory agencies. The limit of various contaminants in diets has been established.

ARRIVE guidelines advocated for providing the essential information on the diet used for experimental animals to replicate the research findings [94, 95]. The modern diet manufacturing units strictly adhere to various quality management systems like HACCP principles and also follow the EU-GMP guidelines to maintain quality assurance system (QAS). Most of the international diet manufactures provide complete analysis report from the accredited laboratories for each lot and ensure consistency from batch to batch. From a research perspective, the area of nutrigenomics and gut microbiota in laboratory animals are areas that have garnered attention. There is also a need to generate dietary standards for many of the other species used in research.

References

1. Drewnowski A, Popkin B (1997) The nutrition transition: new trends in the global diet. Nutr Rev 55:31–43
2. Curfs JAJ, Chwalibog A, Savenije BS (2011) Handbook of Laboratory Animal Science, Nutrient requirements, experimental design, and feeding schedules in animal experimentation. CRC Press, pp 306–335
3. Bryda EC (2013) The Mighty Mouse: the impact of rodents on advances in biomedical research. Mo Med 110:207–211
4. Pellizzon MA, Ricci MR (2020) Choice of laboratory rodent diet may confound data interpretation and reproducibility, current developments in nutrition. Curr Dev Nutr 4:nzaa031
5. Ricci M (2015) Laboratory animal control diets: very important, often neglected. Lab Anim 44:240–241
6. Knapka JJ (1988) Animal diets for biomedical research. Lab Anim 17:17–18
7. Turnbaugh PJ, Backhed F, Fulton L et al (2008) Diet-induced obesity is linked to marked but reversible alterations in the mouse distal gut microbiome. Cell Host Microbe 3:213–223
8. Turnbaugh PJ, Ridaura VK, Faith JJ et al (2009) The effect of diet on the human gut microbiome: a metagenomic analysis in humanized gnotobiotic mice. Sci Transl Med 1:6ra14
9. Barko PC, McMichael MA, Swanson KS et al (2018) The gastrointestinal microbiome: a review. J Vet Intern Med 32:9–25
10. Kurtz DM, Feeney WP (2020) The influence of feed and drinking water on terrestrial animal research and study replicability. ILAR J:1–22
11. McDonald P, Edwards RA, Greenhalgh JFD (2002) Animal nutrition. Addison Wesley Longman, London
12. Kleiber M (1961) The fire of life. Wiley, New York
13. Kerrin G (2014) Rodent nutrition: digestive comparisons of 4 common rodent species. Vet Clin Exot Anim Pract 17:471–483
14. Sakaguchi E (2003) Digestive strategies of small hindgut fermenters. Anim Sci J 74:327–337

15. National Research Council (1995) Nutrient requirements of laboratory animals, 4th rev edn. The National Academies Press, Washington, DC
16. Mertz W (1986) Trace elements in human and animal nutrition, 5th edn. Academic Press, San Diego, CA
17. Jensen MN, Ritskes-Hoitinga M (2007) How isoflavone levels in common rodent diets can interfere with the value of animal models and with experimental results. Lab Anim 1–18(36):41
18. Mesnage R, Defarge N, Rocque LM et al (2015) Laboratory rodent diets contain toxic levels of environmental contaminants: implications for regulatory tests. PLoS One 10:e0128429
19. Kozul CD, Nomikos AP, Hampton TH et al (2008) Laboratory diet profoundly alters gene expression and confounds genomic analysis in mouse liver and lung. Chem Biol Interact 173:129–140
20. Waldemarson AH, Hedenqvist P, Salomonsson AC et al (2005) Mycotoxins in laboratory rodent feed. Lab Anim 39:230–235
21. Hrncir T, Stepankova R, Kozakova H et al (2008) Gut microbiota and lipopolysaccharide content of the diet influence development of regulatory T cells: studies in germ-free mice. BMC Immunol 9:65
22. Wise A, Gilburt DJ (1980) The variability of dietary fibres in laboratory-animal diets and its relevance to the control of experimental conditions. Food Cosmet Toxicol 18:643–648
23. Kwiatkowski S, Thielen U, Glenney P (2009) A study of Saccharomyces cerevisiae cell wall glucans. J Inst Brew 115:151–158
24. Pellizzon MA, Ricci MR (2018) Effects of rodent diet choice and fiber type on data interpretation of gut microbiome and metabolic disease research. Curr Prot Toxicol 77:e55
25. McDowell LR (1989) Introduction and historical considerations. In: McDowell LR (ed) Vitamins in animal nutrition. Academic Press, San Diego, CA, pp 1–9
26. Ammerman CB, Baker DH, Lewis AJ (1995) Bioavailability of nutrients for animals-amino acids, minerals, and vitamins. Academic Press, San Diego, CA
27. Carter R, Lipman N (2018) Feed and bedding. In: Weichbrod RH, Thompson G, Norton JN (eds) Management of animal care and use programs in research, education and testing, 2nd edn. CRC Press/Taylor & Francis, Boca Raton, FL, pp 639–654
28. National Research Council (1995) Subcommittee on laboratory animal nutrition. Committee on Animal Nutrition, Board on Agriculture, Nutrient requirements of laboratory animals. National Academies Press, Washington, DC
29. Brown NM, Setchell KD (2001) Animal models impacted by phytoestrogens in commercial chow: implications for pathways influenced by hormones. Lab Investig 81:735–747
30. Thakur M, Hurburgh CR (2007) Quality of US soybean meal compared to the quality of soybean meal from other origins. J Am Oil Chem Soc 84:835–843
31. Mickelson B (2009) Formula for success. In: Laboratory equipment (ed). Advantage Business Marketing
32. Greenman DL, Oller WL, Littlefield NA et al (1980) Commercial laboratory animal diets: toxicant and nutrient variability. J Toxicol Environ Health 6:235–246
33. NIH Division of Veterinary Resources (2019) Quality assurance plan and information on NIH diets. https://www.ors.od.nih.gov/sr/dvr/drs/nutrition/Pages/Diets.aspx
34. Rao GN, Morris RW, Seely JC (2001) Beneficial effects of NTP-2000 diet on growth, survival, and kidney and heart diseases of Fischer 344 rats in chronic studies. Toxicol Sci 63:245–255
35. Heindel JJ, vom Sall F (2008) Meeting report: batch-to-batch variability in estrogenic activity in commercial animal diets - importance and approaches for laboratory animal research. Environ. Health Perspect 116:389–393
36. American Institute of Nutrition (1977) Report of the American Institute of Nutrition ad hoc committee on standards for nutritional studies. J Nutr 107:1340–1348
37. Medinsky MA, Popp JA, Hamm TE et al (1982) Development of hepatic lesions in male Fischer-344 rats fed AIN-76A purified diet. Toxicol Appl Pharmacol 62:111–120
38. Cockell KA, Belonje B (2004) Nephrocalcinosis caused by dietary calcium: phosphorus imbalance in female rats develops rapidly and is irreversible. J Nutr 134:637–640

39. Sebranek JG, Cassens RG (1973) Nitrosamines: a review. J Milk Food Technol 36:76–91
40. Thresher JS, Podolin DA, Wei Y et al (2000) Comparison of the effects of sucrose and fructose on insulin action and glucose tolerance. Am J Physiol Regul Integr Comp Physiol 279:R1334–R1340
41. Sumiyoshi M, Sakanaka M, Kimura Y (2006) Chronic intake of high-fat and high-sucrose diets differentially affects glucose intolerance in mice. J Nutr 136:582–587
42. Kim KS, Hong S-W, Han D et al (2016) Dietary antigens limit mucosal immunity by inducing regulatory T cells in the small intestine. Science 351:858–863
43. Behnke KC (1996) Feed manufacturing technology: current issues and challenges. Anim Feed Sci Technol 62:49–57
44. Pellizzon MA, Ricci MR (2018) The common use of improper control diets in diet-induced metabolic disease research confounds data interpretation: the fiber factor. Nutr Metab 15:3–3
45. Thigpen JE, Setchell KD, Padilla-Banks E et al (2007) Variations in phytoestrogen content between different mill dates of the same diet produces significant differences in the time of vaginal opening in CD-1 mice and F344 rats but not in CD Sprague-Dawley rats. Environ Health Perspect 115:1717–1726
46. Westmark CJ, Westmark PR, Malter JS (2013) Soy-based diet exacerbates seizures in mouse models of neurological disease. J Alzheimers Dis 33:797–805
47. National Toxicology Program (NTP) (2011) Specifications for the conduct of studies to evaluate the toxic and carcinogenic potential of chemical, biological and physical agents in laboratory animals for the National Toxicology Program (NTP). National Institutes of Health
48. US Food and Drug Administration (1978) Good laboratory practices for nonclinical laboratory studies. US Department of Health and Human Services, Washington, DC
49. US Environmental Protection Agency (EPA) (1978) Proposed health effects test standards for toxic substances control act test rules, 44 Federal Register 145, Office of Toxic Substances 441979, pp 44054–44093
50. Pal BC, Ross RH, Milman HA (1984) Nutritional requirements and contaminant analysis of laboratory animal feeds. Oak Ridge National Lab, Oak Ridge, TN
51. Rao GN, Knapka JJ (1987) Contaminant and nutrient concentrations of natural ingredient rat and mouse diet used in chemical toxicology studies. Fundam Appl Toxicol 9:329–338
52. Ritskes-Hoitinga M, Tobin G, Jensen TL et al (2012) Nutrition of the laboratory mouse. In: Hedrich HJ (ed) The laboratory mouse, 2nd edn. Elsevier, London, pp 567–593
53. Federation of European Laboratory Animal Science Associations (FELASA) (2018) Nutrition of laboratory rodents. http://www.felasa.eu/working-groups/working-groupspresent/nutrition-of-laboratory-rodents/
54. US Environmental Protection Agency (EPA) (2016) Pesticides industry—sales and usage—2008–2012 market estimates. In: Office of chemical safety and pollution prevention. Washington, DC
55. US Food and Drug Administration (2016) Pesticide residue monitoring program—fiscal year 2016 pesticide report. Washington, DC
56. International Agency for Research on Cancer (IRAC) (2017) Some organophosphate insecticides and herbicides. International Agency for Research on Cancer, Lyon
57. Crump JA, Griffin PM, Angulo FJ (2002) Bacterial contamination of animal feed and its relationship to human foodborne illness. Clin Infect Dis 35:859–865
58. Adams SC, Myles MH, Tracey LN et al (2019) Effects of pelleting, irradiation, and autoclaving of rodent feed on MPV and MNV infectivity. JAALAS 58:542–550
59. National Research Council (NRC) (2005) Mineral tolerance of animals, 2nd rev edn. The National Academics Press, Washington, DC
60. National Toxicology Program (NTP) (2016) Monograph on immunotoxicity associated with exposure to perfluorooctanoic acid (PFOA) and perfluorooctane sulfonate (PFOS). Department of Health and Human Services, National Institute of Environmental Health Sciences, Research Triangle Park, NC

61. Haschek WM, Voss KA (2013) Mycotoxins. In: Haschek WM, Rousseaux CG, Wallig MA (eds) Haschek and Rousseaux's handbook of toxicologic pathology, 3rd edn. Academic Press, Boston, MA, pp 1187–1258
62. Combet E, Paterson S, Iijima K et al (2007) Fat transforms ascorbic acid from inhibiting to promoting acid-catalysed N-nitrosation. Gut 56:1678–1684
63. Collins TF, Hinton DM, Welsh JJ et al (1992) Evaluation of heat sterilization of commercial rat diets for use in FDA toxicological studies. Toxicol Ind Health 8:9–20
64. Kurtz DM, Glascoe R, Caviness G et al (2018) Acrylamide production in autoclaved rodent feed. J Am Assoc Lab Anim Sci 48:709–713
65. Caulfield CD, Cassidy JP, Kelly JP (2008) Effects of gamma irradiation and pasteurization on the nutritive comparison of commercially available animal diets. J Am Assoc Lab Anim Sci 47:61–66
66. Miladinovic D (2013) Strength and durability of feed pellets influenced by different particle size distribution, pellet volume and dehydration techniques. Ann Trans Nordic Rheol Soc 21:107–115
67. Kankesan J, Vanama R, Renlund R et al (2003) Source of a micronutrient in a semi-synthetic basal diet as a causative factor in inducing urinary calculi in rats and its inhibition by PSC 833, a potent inhibitor of P-glycoprotein. Comp Med 53:444–447
68. US Food and Drug Administration (2018) FDA alerts pet owners about potentially toxic levels of vitamin D in several dry pet foods. https://www.fda.gov/animal-veterinary/news-events/fda-alerts-pet-owners-about-potentially-toxiclevels-vitamin-d-several-dry-pet-foods
69. Carter RL, Lipman N (2018) Feed and bedding. In: Weichbrod RH, Thompson G, Norton JN (eds) Management of animal care and use programs in research, education, and testing, 2nd edn. CRC Press/Taylor & Francis, Boca Raton, FL, pp 639–654
70. Rao GN (2002) Diet and kidney diseases in rats. Toxicol Pathol 30:651–656
71. Keenan KP, Laroque P, Soper KA et al (1996) The effects of overfeeding and moderate dietary restriction on Sprague-Dawley rat survival, pathology, carcinogenicity, and the toxicity of pharmaceutical agents. Exp Toxicol Pathol 48:139–144
72. Hubert MF, Laroque P, Gillet JP et al (2000) The effects of diet, ad libitum feeding, and moderate and severe dietary restriction on body weight, survival, clinical pathology parameters, and cause of death in control Sprague-Dawley rats. Toxicol Sci 58:195–207
73. Vaughan KL, Kaiser T, Peaden R et al (2017) Caloric restriction study design limitations in rodent and nonhuman primate studies. J Gerontol A Biol Sci Med Sci 73:48–53
74. US Department of Agriculture (2017) Animal welfare act and animal welfare regulations service. Animal and Plant Health Inspection Service. USDA, Washington, DC
75. World Health Organization (2017) Guidelines for drinking-water quality. Incorporating the first addendum, 4th edn. World Health Organization, Geneva
76. Cabral JPS (2010) Water microbiology. Bacterial pathogens and water. Int J Environ Res Public Health 7:3657–3703
77. Kligerman AD, Chapin RE, Erexson GL et al (1993) Analyses of cytogenetic damage in rodents following exposure to simulated groundwater contaminated with pesticides and a fertilizer. Mutat Res 300:125–134
78. Baker DG, Lipman NS (2015) Factors that can influence animal research. In: Fox JG, Anderson LC, Otto GM, Pritchett-Corning KR, Whary MT (eds) Laboratory animal medicine, 3rd edn. Academic Press, Boston, MA, pp 1441–1496
79. Reynolds KA, Mena KD, Gerba CP (2008) Risk of waterborne illness via drinking water in the United States. Rev Environ Contam Toxicol 192:117–158
80. National Toxicology Program (1976) Report on the carcinogenesis bioassay of chloroform (CAS no. 67-66-3). Natl Cancer Inst Carcino Tech Rep Ser:1–60
81. Zhu J, Kong Y, Yu J et al (2019) Consumption of drinking water nitrosamines mixture alters gut microbiome and increases the obesity risk in young male rats. Environ Pollut 248:388–396
82. Takagi S, Adachi F, Miyano K et al (2011) Fate of perfluorooctane sulfonate and perfluorooctanoate in drinking water treatment processes. Water Res 45:3925–3932

83. Rodriguez KF, Ungewitter EK, Crespo-Mejias Y et al (2016) Effects of in utero exposure to arsenic during the second half of gestation on reproductive end points and metabolic parameters in female CD-1 mice. Environ Health Perspect 124:336–343
84. Wu J, Wen XW, Faulk C et al (2016) Perinatal lead exposure alters gut microbiota composition and results in sex-specific bodyweight increases in adult mice. Toxicol Sci 151:324–333
85. Sagle A, Freeman B (2003) Fundamentals of membranes for water treatment in. Texas Water Development Board, Austin, TX
86. Kozisek F (2005) Health risks from drinking demineralised water. In: Water, sanitation and health protection and the human environment. WHO, Geneva. http://www.who.int/water_sanitation_health/dwq/nut-rientsindw.pdf
87. Bidot WA, Ericsson AC, Franklin CL (2018) Effects of water decontamination methods and bedding material on the gut microbiota. PLoS One 13:e0198305
88. Meredith A, Johnson-Delaney C (2010) BSAVA manual of exotic pets, 5th edn. Wiley
89. Wenk C (2001) The role of dietary fibre in the digestive physiology of the pig. Anim Feed Sci Technol 90:21–33
90. Richardson VGC (2000) Diseases of domestic guinea pigs, 2nd edn. Wiley
91. Terril LA, Clemons DJ, Suckow MA (1998) The laboratory guinea pig. CRC Press
92. Harkness JE, Turner PV, VandeWoude S et al (2010) Harkness and Wagner's biology and medicine of rabbits and rodents. Wiley
93. National Research Council (1977) Nutrient requirements of rabbits, 2nd rev edn. The National Academies Press, Washington DC
94. Kilkenny C, Browne W, Cuthill IC et al (2010) Animal research: reporting in vivo experiments: the ARRIVE guidelines. Br J Pharmacol 160:1577–1579
95. Percie du Sert N, Hurst V, Ahluwalia A et al (2019) The ARRIVE guidelines 2019: updated guidelines for reporting animal research. bioRxiv2019. https://doi.org/10.1101/703181

17 Bacterial and Parasitological Diseases in Laboratory Animals

Jerald Mahesh Kumar and Yadav Govind

Abstract

The laboratory animals harbor a variety of bacterial and parasitic pathogens that cause various diseases. However, many of the pathogens may not cause overt disease but may alter the hematological, biochemical, gross, and histopathological data rendering the host animal unsuitable for many experimental uses. An intensive health monitoring program in the animal facility with effective therapeutic procedures will improve the health status of the vivarium. This chapter will discuss the common bacterial and parasitic diseases affecting laboratory animals and their diagnostic approaches with gross and histopathological observations that would prevent and control the outbreak of various infectious and zoonotic diseases.

Keywords

Pathology · Bacterial diseases · Diseases rodents · Hamsters · Rabbits

17.1 Introduction

Many spontaneous diseases of laboratory animals occur at different ages and vary with sex, genetic background, immune status, microbial status, diet, and other environmental factors. Despite good sanitation practices, infectious agents may

J. M. Kumar (✉)
CSIR—Centre for Cellular and Molecular Biology, Hyderabad, Telangana, India
e-mail: mahesh73@ccmb.res.in

Y. Govind
CSIR—Indian Institute of Integrative Medicine, Jammu, Jammu and Kashmir, India
e-mail: gyadav@iiim.res.in

© The Author(s), under exclusive license to Springer Nature Singapore Pte Ltd. 2021

P. Nagarajan et al. (eds.), *Essentials of Laboratory Animal Science: Principles and Practices*, https://doi.org/10.1007/978-981-16-0987-9_17

appear periodically in the colony. Some of the agents are highly contagious and pathogenic to lab animals. Some produce asymptomatic conditions without any observable pathological changes in systemic organs and some are opportunistic with secondary complications. One of the major concerns of infectious agents is that they affect the health status of the breeding colony and importantly pose a major zoonotic health risk to animal users. Research of evidence also point to the interference of infectious agents with the experimental outcomes. Housing methods including open cages, ventilated cages, and micro-isolator cages significantly influence the spread of infection to the colony. Clinically ill animals or dead animals are a potential source of spread of active pathogens. Unrestricted movement of personnel or biological materials including cancer cells and ES cells can act as a possible source of infection. Diagnosis of the active stage of infections in the animal colony can be done by the reading of clinical examination and necropsy of dead animals. Special stains are useful for the detection of various bacteria. Effective serological methods and microbiological culture will improve the diagnosis of infection status in the colony. Histopathology and special techniques such as electron microscopy and immunohistochemistry provide vital support in the diagnosis but these methods are expensive to carry out in a surveillance program. Serological methods are used to detect antibodies against specific antigens. However, serology may give a positive result even when the infectious agent is absent in the host system because the antibodies detected could be either due to previous exposure or due to the transfer of maternal antibodies. PCR is highly sensitive to rule out infectious agents. For the detection of endoparasites, fecal flotation method is used to detect eggs of the parasites. Direct microscopy can be used for ruling out mites, ticks, lice, and various metazoan parasites. Skin scraping digested with 1% KOH or 1% NaOH helps in the identification of ectoparasites such as fur mites and follicular mites.

This chapter will discuss about common bacterial and parasitic diseases affecting lab animals and their diagnostic methods with special emphasis on their gross and histopathological changes in internal organs.

17.2 Bacterial Diseases

17.2.1 Mycoplasmosis

17.2.1.1 Epidemiology

Mycoplasma pulmonis and *Mycoplasma arthritidis* infect mice and rats. These are Gram-negative bacteria that cause murine respiratory mycoplasmosis. Mycoplasma infection is highly contagious and spreads through aerosol in adult mice and in utero transmission to neonatal pups and suckling mice. It produces disorders of the respiratory and reproductive systems. *Mycoplasma arthritidis* infection under natural condition is nonpathogenic, but experimental induction may cause respiratory disease following intranasal inoculation, and the main concern that animals are infected with *Mycoplasma arthritidis* can cause seroconversion to *M. pulmonis* during investigations.

17.2.1.2 Significance

Susceptibility is very high especially in animals maintained for a long duration. The level of susceptibility may differ in a different host. Mouse strains like C57BL/6 are reported to be resistant, and LEWIS rat is comparatively more susceptible to genital mycoplasma infection and immunocompetent mice for severe arthritis.

17.2.1.3 Transmission

Transmission of *Mycoplasma pulmonis* happens horizontally by direct contact with infected secretions and aerosol route and vertically by in utero transmission. Venereal transmission is also possible. *Mycoplasma pulmonis* disease outcome depends on the complex interaction of factors related to host, pathogen, and environment. Typical sites of colonization for *Mycoplasma pulmonis* are the middle ear and nasopharynx [1].

17.2.1.4 Pathology

Mice are commonly asymptomatic. During clinical infection suppurative rhinitis, otitis media, weight loss, ruffled hair coat, dyspnea, and chronic pneumonia are observed. Intravenous inoculation of *Mycoplasma pulmonis* produces chronic suppurative arthritis in immunocompetent mice. *M. pulmonis* colonizes in the apical cell membranes of the respiratory epithelium causing mucopurulent exudation in nasal passages and trachea that interferes with mucociliary clearance. This leads to secondary bacterial and viral complications. In chronic cases, atelectasis, bronchiectasis, bronchopneumonia, with raised yellow-tan nodules with abscessation, gray and red hepatization may be evident on gross examination (Fig. 17.1).

Histologically, the respiratory epithelium of the nasal passages and upper respiratory systems shows the loss of cilia and flattening of epithelial lining cells. In chronic infection, syncytia are noticed in the affected nasal mucosa and larynx. In the lower respiratory tract, peribronchiolar lymphoid-associated tissue hyperplasia (Fig. 17.2), and perivascular lymphocytic infiltration and plasma cell infiltrations,

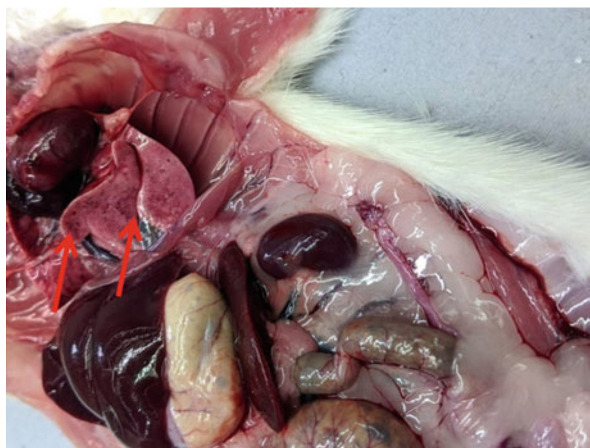


Fig. 17.1 Early stage of gray and red hepatization of lung (arrow) with the consolidation of lobes of lungs

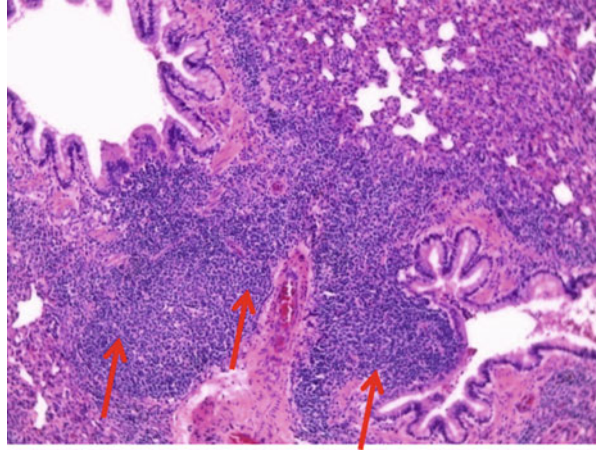


Fig. 17.2 Hyperplasia of bronchial associated lymphoid tissue

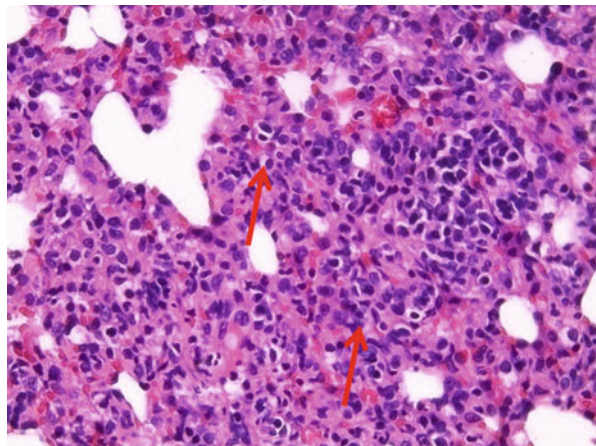


Fig. 17.3 Inflammation of alveolar interstitium with infiltration lymphocytes and plasma cells—arrow

inflammation in alveolar interstitium with infiltration of alveolar macrophages and lymphocytes and plasma cells are usually seen (Fig. 17.3). In advanced cases, squamous metaplasia of respiratory epithelium, bronchiectasis, and abscess are observed. Intense peribronchial lymphocytic infiltrates and severe bronchiectasis that are seen in rats are not seen in mice [2, 3].

17.2.1.5 Diagnosis

Gross and microscopic lesions in the upper and lower respiratory tract confirms the disease. Culturing nasopharyngeal flushing and trachea bronchial lavages with *Mycoplasma* broth or phosphate-buffered saline are recommended procedures to confirm the disease. Differential diagnosis includes bronchopneumonia associated with cilia-associated respiratory bacillus and Sendai virus. PCR using tracheal or nasal lavages confirms the disease. The PCR is done using species-specific primers for *Mycoplasma pulmonis* (sense primer, 5′-AGCGTTTGCTTCACTTTGAA-3′;

antisense primer, 5′-GGGCATTTCCTCCCTAAGCT-3′), which generates a 266-bp amplification product specific to *M. pulmonis* [4].

Tracheal wash through the nasopharynx and grounded lungs is the most preferred method for isolation of mycoplasma. However, tympanic chamber in case of otitis media. Sample inoculation is done in PPLO broth and agar plates, both supplemented with horse serum, DNA, glucose (1%), phenol red (0.1%), and arginine (1%). Incubation temperature for growth is 37 °C, humid and microaerophilic atmosphere in a candle jar. Growth of mycoplasma is monitored daily up to 21 days under the stereo microscope. Mycoplasma colonies are characterized by Dienes staining and typed [5]. *Mycoplasma pulmonis* can be biochemically characterized by glucose fermentation and the non-utilization of arginine.

17.2.1.6 Interference with Research

M. pulmonis infection may cause interference in research by clinical disease or death. It may alter the immunological responsiveness. It affects the T and B lymphocytes (mitogenic effect) and increases natural killer cell activity in mice. It reduces the antibody response to sheep RBC and collagen in rats. It also delays the onset and affects the severity of adjuvant and collagen-induced arthritis. In experiments involving the respiratory tract (treatment via inhalation or inhalation toxicity) and genital tract (histology and reproductive efficiency), *M. pulmonis* may cause alteration of the results.

17.2.2 Tyzzer's Disease

17.2.2.1 Epidemiology

It is the most prevalent bacterial disease of mice and rats. Hamsters, guinea pigs, and rabbits are also susceptible. The disease is caused by spore-forming, Gram-negative, filamentous bacterium of *Clostridium piliforme*. Natural infection occurs due to the ingestion of infected fecal materials contaminated with feed, water, and bedding materials. The vegetative form of bacteria is unstable but the spores are viable up to 1 year at room temperature, and it is the most common source of outbreaks in rodent colony. Bacterial spores enter into the gastrointestinal tract and reach the liver and heart. The disease may cause low morbidity and high mortality; the affected rabbits show profuse watery diarrhea, anorexia, dehydration, lethargy, and staining of the hindquarters with feces. Rabbits may die within 1–2 days after exhibiting clinical signs [6].

17.2.2.2 Significance

Unknown, it has been reported that C57BL/6 mice are more resistant than DBA2 mice.

17.2.2.3 Transmission

Transmission through the ingestion of spore or contaminated material with animal feces. Spores can survive up to a year in an animal colony and facility. Proper sterilization of feed, water and area can be done using chemical disinfectants such as chlorine dioxide or formalin. Discarding the infected colony and embryo transfer and rederivation of the embryo may help to stop further transmission.

17.2.2.4 Pathology

Grossly, mice ileum shows hemorrhagic enteritis with the dilated cecum and colon (Fig. 17.4) and also reddened mucosal surface with watery, fetid contents, and white to gray foci of necrosis may be noticed in the liver, mesenteric lymph nodes, and heart.

In rats, dilation of the cecum and ileum and enlargement of Peyer's patches are observed (Fig. 17.5) in the terminal small intestine (megaloileitis), and necrotic foci appear on the surface of the liver in immunodeficient mice.

In rabbits, hemorrhages may be noticed on the serosal surface of the cecum and thickened edematous bowel wall and foci of necrosis in the cecal mucosa. Histologically, foci of degeneration, inflammation, edema, and necrosis are evident in the intestinal mucosal epithelial layer and muscularis layer of the ileum, cecum, and colon, and mesenteric lymph node. Multifocal hepatic necrosis with polymorphonuclear leukocyte and mononuclear cell infiltration may be noticed in the liver of rats (Fig. 17.6). Myocardial degeneration with myocarditis and infiltration of inflammatory cells specifically lymphocytes may be present in the heart (Fig. 17.7).

The cluster of organisms enters into the enterocytes of the mucosal layer of the intestine to smoothen the muscular layer and neurons of Auerbach's plexus. Bundles of long, slender rods occur in the cytoplasm of viable cells bordering necrotic foci, especially in the liver and intestine. In rats, hepatic lesions are characterized by fibrosis, with multinucleated giant cells, and mineralized debris in reparative foci may be noticed [7].

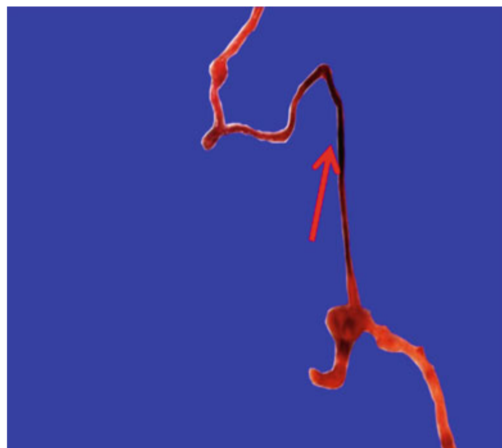


Fig. 17.4 Hemorrhagic enteritis in ileum (arrow) with dilatation of caecum was observed

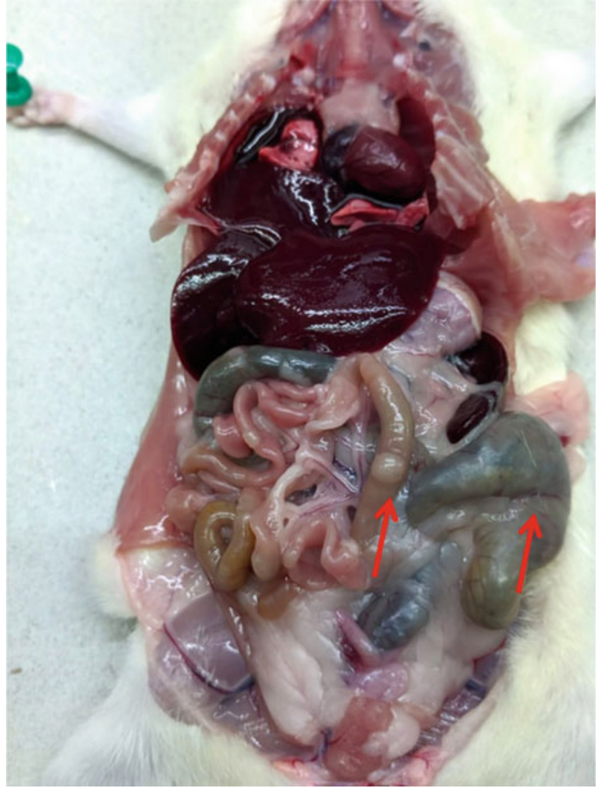


Fig. 17.5 Enlargement or dilation of cecum and Peyer's patches in the colon of rats

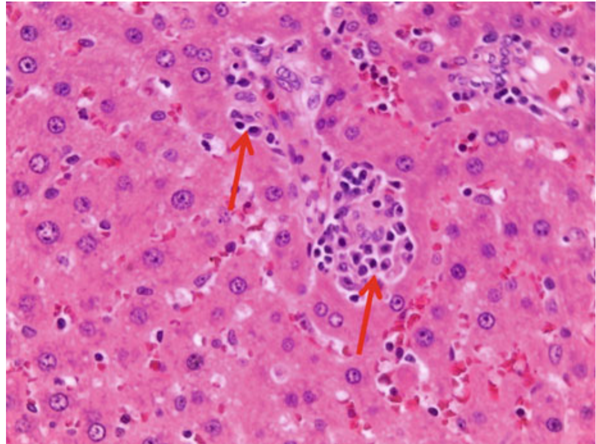


Fig. 17.6 Multifocal necrosis with infiltration of plasma cells and lymphocytes—arrow

17.2.2.5 Diagnosis

Tyzzer's disease can be diagnosed in tissue sections of the liver and intestine by impression smears of these tissues stained with appropriate stains such as Giemsa,

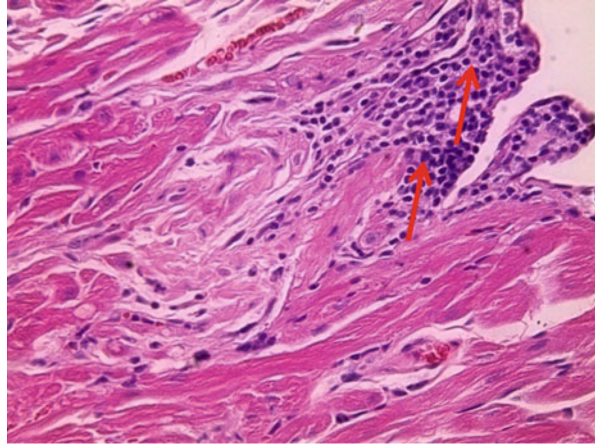


Fig. 17.7 Myocardial degeneration and inflammation along with infiltration of inflammatory cells were observed—arrow

Warthin-Starry, or periodic acid-Schiff (PAS) stains. The typical bacilli may be visualized in impression smears. Serologic assays and PCR amplification can be used. DNA isolation can be done from fecal samples or mesenteric lymph nodes. PCR reaction is done using species-specific primers of 16S ribosomal DNA from the organism of F: 5′GTGCTAGGTGTTGGGAAG-3′ and R: 5′TACTTTACGTAGCCTGTCAA-3′ [8]. Gross and histopathological lesions of Tyzzer's disease must be differentiated from other corona viral hepatitis, helicobacteriosis, and salmonellosis. Differential diagnosis for necrotizing hepatitis in the rat include *Corynebacterium kutscheri*, *Helicobacter hepatitis*, and salmonellosis. Isolation of *C. piliforme* from liver tissue homogenates by repeated passaging in 6–9-day-old embryonated hen's eggs through yolk sac route or by primary cultures of hepatocyte cell collected from mice. The isolation of *Clostridium piliforme* (*Bacillus piliformis*) can be done by passaging of suspensions of infected tissue in mouse strains (CBA/N-xid or C3.CBA/N-xid) with or without administration of cortisone acetate (100–200 mg/kg).

17.2.2.6 Interference with Research

Animals infected with *Clostridium piliforme* are unfit for use and interfere through termination of research due to morbidity and mortality. Loss of weanlings and sub-adult can happen. Recovered rabbits may have necrosis and fibrosis in the liver and fibrosis in the intestine. Immunodeficient or immune-compromised mice are at greater risk. It is reported that infection elevates the level of the selected cytokines.

17.2.3 Helicobacteriosis

17.2.3.1 Epidemiology

It is a common pathogen in the conventional animal facility, and it causes inflammatory bowel disease. *H. hepaticus*, *H. bilis*, and *H. muridarum* are found in mice, *H. bilis*, *H. trogontum*, *H. muridarum*, *H. typhlonius*, and *H. rodentium* are identified in rats, and *Helicobacter pylori* species is identified in a guinea pig. Infection is spread through contaminated feces mixed with feed, water, and bedding materials, and these infections are persistent, with long-term fecal shedding of organisms. Most of the immune-competent mice and rats are asymptomatic. Chronic infection leads to the progressive reduction in body weight (wasting), and feces may be sticky, mucoid, and hemorrhagic. A/J, SCID, BALB/c, C3H/He, and SJL mice are susceptible to *H. hepatitis*, whereas C57BL6 and B6C3F1 mice strains are resistant [7, 9].

17.2.3.2 Transmission

Primary transmission of *Helicobacter* is fecal-oral spread, but the infectivity heavily depends on the strain involved, husbandry, and environmental conditions. Once the natural infection is acquired, long shedding of *Helicobacter* in feces is observed. Effective eradication is done by depopulation and establishment of new infection-free colonies. Embryo transfer is also an effective way of eradicating the organism.

17.2.3.3 Pathology

Rectal prolapsed may be observed frequently with eroded rectal mucosa with hemorrhagic and marked inflammation (Fig. 17.8). *Helicobacter* spp. colonizes the crypts of the cecum and colon and causes inflammation in the gastrointestinal tract and enters in to the liver. Thickening of the cecum wall and large bowel develops proliferative typhlitis (inflammation in cecum), colitis (inflammation in colon) (Fig. 17.9), and proctitis (inflammation in rectum). The bacteria also colonize the liver with extensive nonsuppurative hepatitis and cause hepatic necrosis. Inflammation originates from portal triads and spreads to adjacent hepatic parenchyma.

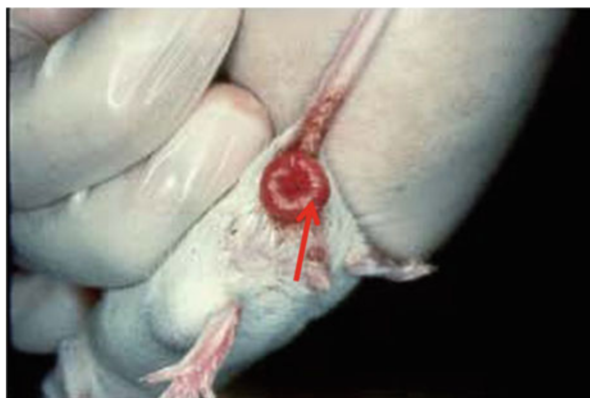


Fig. 17.8 Rectal prolapsed with hemorrhagic rectal mucosa with marked inflammation—arrow

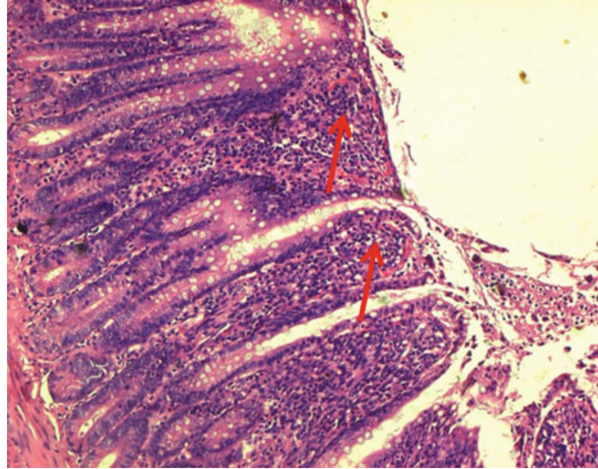


Fig. 17.9 Inflammation noticed in mucosal folds of the colon with infiltration of lymphocytes were observed—arrow

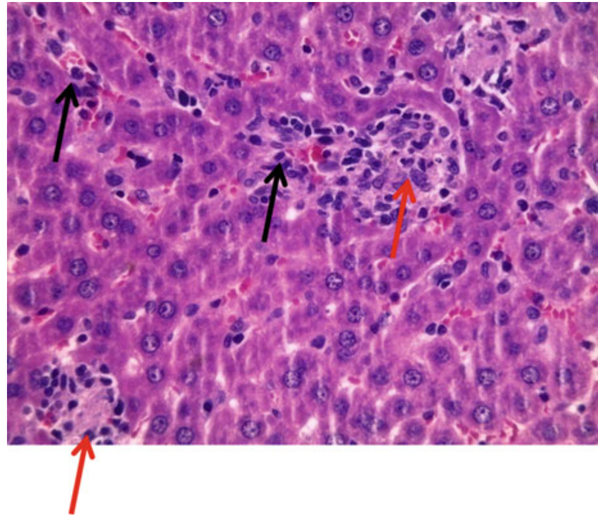


Fig. 17.10 Hepatic necrosis may occured adjacent to the intralobular venules and develop phlebitis

Hepatic necrosis may occur adjacent to intralobular venules and develop phlebitis (Fig. 17.10).

Marked hypertrophy and hyperplasia of Kupffer cells, hepatic stellate cells and oval cells, and hyperplasia of bile duct with the increased mitotic activity of hepatocytes may be observed during infection. Hepatomas and hepatocellular carcinomas in aged mice may be the sequelae from infected colonies.

17.2.3.4 Diagnosis

The organism can be identified by the impression smear of the liver with silver stains. Diagnosis of *Helicobacter* infection is done by polymerase chain reaction (PCR). Various primers are used to differentiate *Helicobacter* spp., *H. bilis*, or

H. hepaticus. DNA samples isolated from fecal pellets, cecal mucosal scrapings, or tissue may also be used. Pieces of the liver and intestine are inoculated in brain heart infusion (BHI) broth and incubated overnight at 37 °C and centrifuge for 10 min at 2000 × *g* to collect the supernatant. The filtered supernatant in different concentrations should be used for isolation in a selective agar medium (Columbia blood agar plate) and incubated at 37 °C under microaerophilic and anaerobic conditions. The single pure colony should be utilized for the biochemical test. It rapidly hydrolyzes urea and catalase, remains oxidase-positive, reduces nitrate to nitrite, is resistant to cephalothin and nalidixic acid, but is sensitive to metronidazole. The pure bacterial colony may be further confirmed by PCR, 16S rRNA and 23S rRNA gene sequence, and RFLP.

17.2.3.5 Interference with Research

Inflammation of the liver and intestine causes untoward effects on study outcome and the studies may need to be terminated. It affects T lymphocyte receptors by targeted deletions. *H. hepaticus* stimulates a strong Th1 proinflammatory cell response and also causes hepatic neoplasia in A/J, B6C3F1, A, B6F1, and CAR KO strains of mice.

17.2.4 *Pasteurella pneumotropica* Infection

17.2.4.1 Epidemiology

P. pneumotropica is a Gram-negative rod-shaped bacterium that affects mice, rats, and hamsters. The clinical form of the disease may not be seen in these animals; the bacteria shed in respiratory secretions and feces and spread to other animals by direct contact. The vagina and uterus are often colonized with this bacterium in mice leading to the primary source of spread of infection to litters. *P. pneumotropica* do not survive for a long period in the environment and can be effectively eliminated by the disinfection of working surfaces and fomites.

17.2.4.2 Significance

It is commonly reported in the laboratory animal population. It has a high prevalence in infected colonies.

17.2.4.3 Transmission

Transmission of *Pasteurella pneumotropica* is by direct contact with diseased animals or secretions of diseased animals [10].

17.2.4.4 Pathology

Conjunctivitis, panophthalmitis, dacryoadenitis (inflammation in lacrimal glands), periorbital abscessation, rhinitis, otitis, cervical lymphadenitis, abscesses of preputial glands and bulbourethral glands, necrotizing dermatitis, subcutaneous abscesses, mastitis, and metritis are observed in mice and rats. It produces disorder of the urinary tract and the reproductive system that leads to abortion and infertility.

17.2.4.5 Diagnosis

P. pneumotropica is closely related to *Pasteurellaceae*, and culture of this organism from an oral and fecal swap with blood agar and colonies can be confused with *Pasteurella multocida* and *Pasteurella gallinarum*. Several biochemical tests (indole, urease, and ornithine decarboxylase positive) are used to confirm the bacteria. For isolation and diagnosis of *P. pneumotropica*, samples from the infected or suspected animals should be taken from the nasopharynx, vagina, intestine, and conjunctiva according to clinical signs. The samples can be inoculated on Columbia 5% sheep blood agar and incubated at 37 °C for 24–48 h under a 7% CO_2 environment. Smooth nonhemolytic 0.5–1 mm gray to yellow bacterial colonies can be seen. Biochemical characterization and PCR can be done for further confirmation.

17.2.4.6 Interference with Research

Infection in immunodeficient animals and those affected by clinically severe infection causes major interference in research. In subclinical or silent infection, proinflammatory cytokines are stimulated in immunocompetent mice for 7 days and their increase detectable up to 28 days.

17.2.5 Pasteurellosis

17.2.5.1 Epidemiology

Pasteurellosis is a common disease of rabbits in India and is caused by *Pasteurella multocida*. It is a Gram-negative coccobacillus and causes a variety of clinical syndromes in chronic cases. *P. multocida* may produce acute septicemia with no clinical manifestation and sudden death without any gross pathological changes. Transmission may occur via direct contact with infectious secretions or excretions from the infected animals. Major clinical signs include serous to mucopurulent ocular, nasal discharge, sneezing, coughing, exudate on the fur of the forepaws, and head-tilting with otitis interna.

17.2.5.2 Significance

This infection is uncommon in well-managed colonies.

17.2.5.3 Transmission

Transmission of *P. multocida* is mostly through direct contact, fomites, and aerosol, and if vaginal infection, newborn is infected at the time of birth.

17.2.5.4 Pathology

Grossly, conjunctival reddening, suppurative conjunctivitis, swollen eyelids, and hair loss around the eyes may be noticed. *P. multocida* first colonizes in the pharynx and later spreads to the nares and other organs [11]. Pathological changes include chronic rhinitis with catarrhal to mucopurulent exudate with sinusitis (snuffles), pneumonia, suppurative otitis media, otitis interna, conjunctivitis, abscess formation, genital infection, and septicemia [11]. Nasal passages become edematous,

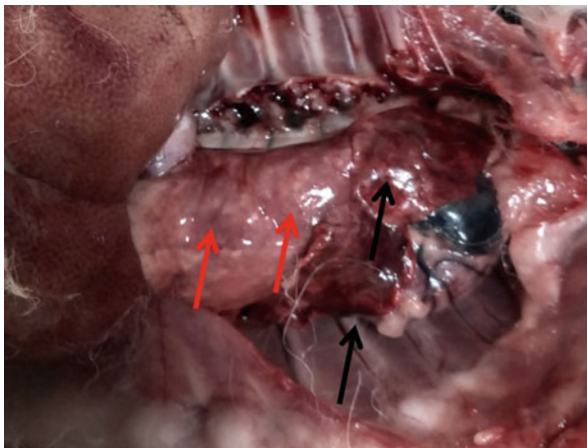


Fig. 17.11 Consolidation of lungs (red arrow) along with patchy hemorrhages (black arrow) was observed

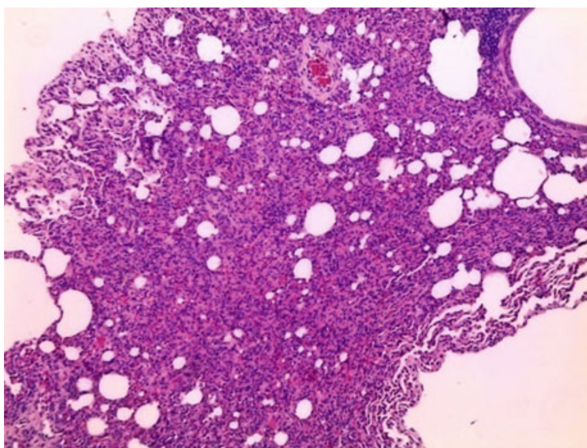


Fig. 17.12 Bronchopneumonia with alveolar interstitial fibrosis

congested, and inflamed, with mucosal ulceration and atrophy of turbinate bones. The lungs may show consolidation (Fig. 17.11), atelectasis, hemorrhage, and fibrinopurulent exudates. Acute necrotizing fibrinous bronchopneumonia with hemorrhages is observed (Fig. 17.12).

Toxigenic strain can exhibit hepatic necrosis and splenic lymphoid atrophy associated with pleuritis, pericarditis, and pneumonia. Systemic and visceral organ abscesses with a necrotic center and infiltration of polymorphonuclear neutrophils and fibrous capsule may be noticed. In the female rabbit, the genital tract may show serous, mucous, or mucopurulent vaginal discharge and pyometra, with chronic suppurative salpingitis and peri-oophoritis or localized suppurative lesions in the

genital system leading to abortion and infertility. Male rabbits may develop orchitis or epididymitis with enlarged testicles and decreased fertility.

17.2.5.5 Diagnosis

Culture of the organism and serological assays such as ELISA and indirect hemagglutination assays can be used for confirmation of pathogen. DNA isolated from nasal swabs or culture samples with PCR reaction using species-specific primers of KMT1SP6: GCTGTAAACGAACTCGCCAC and KMT1T7: ATCCGCTATTTACCCAGTGG [12] is confirmatory. Isolation of *Pasteurella multocida* is done by inoculating clinical samples on blood agar or chocolate agar or HS agar at 37 °C for 24 h. Colonies grown are mucoid with mousy odor are seen. Further confirmation can be made by biochemical tests for catalase, oxidase, and fermentation of several carbohydrates.

17.2.5.6 Interference with Research

Infected animals with *P. multocida* greatly affect the outcomes of the research. Carriers may become clinically ill which leads to termination of the study.

17.2.6 Salmonellosis

17.2.6.1 Epidemiology

S. enteritidis and *S. typhimurium* are Gram-negative major organisms that induce a clinical form of the disease in mice, rats, hamsters, and guinea pigs. Salmonella is tropic to the intestinal tract, and infection spreads through the contaminated fecal material in food, water, and bedding material to other animals, and the organism has a zoonotic potential to humans. Mice that recover from acute infection can become carriers and constantly shed of the pathogen from feces. Though modern animal husbandry practices can control the spread of infection, the organism is still widespread [13].

17.2.6.2 Significance

The disease is of zoonotic importance. Under stress, the subclinical infection becomes clinical and leads to illness among rodents. In a well-managed modern facility, the incidences are low.

17.2.6.3 Transmission

Transmission of *Salmonella* spp. is through fecal-oral route or fomites. Vertical transmission is also possible.

17.2.6.4 Pathology

Young mice that are exposed to acute infection suffer from severe gastroenteritis, diarrhea, weight loss, lethargy, and dullness and have humped posture. In subacute conditions, distended abdomen with hepatomegaly and splenomegaly and catarrhal enteritis are seen (Fig. 17.13). In chronic cases, gradual weight loss may be

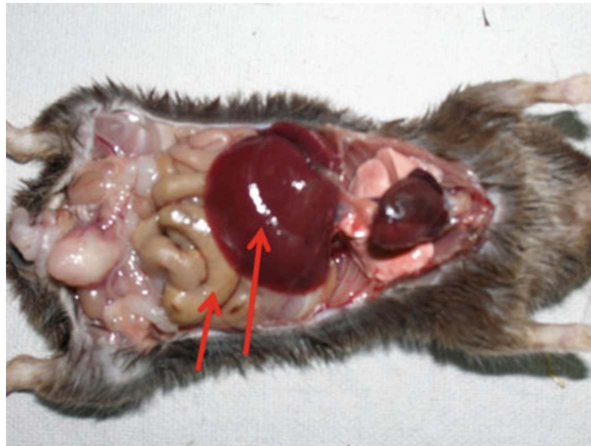


Fig. 17.13 Hepatomegaly with catarrhal enteritis was observed—arrows

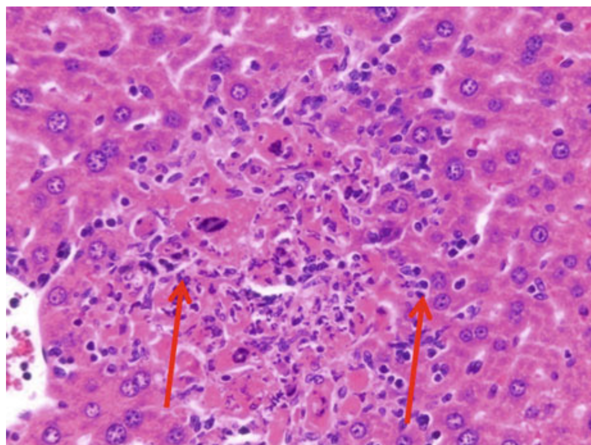


Fig. 17.14 Multifocal necrosis was observed in centrilobular region of the liver

observed. *S. enteritidis* and *S. typhimurium* enter the intestinal tract and penetrate the intestinal wall and lymphatic vessels, Peyer's patches, and mesenteric lymph node and reach different organs such as the liver and lymph nodes. In the acute case, deaths may occur with hyperemia, enlarged liver, and catarrhal enteritis. The intestine of mice that survived for several weeks may be distended and reddened. The liver, spleen, and lymph nodes will be enlarged having yellow-gray foci of necrosis with inflammation in the myocardium.

Histologically, multifocal necrotic foci are found in the intestine, mesenteric lymph nodes, liver, and spleen (Fig. 17.14) with inflammation in myocardium and joints. In the intestine, mucosal ulcers with leukocyte infiltration and reactive hyperplasia of crypt epithelial cells may be observed in rats. Infiltration of neutrophils and histiocytes is present in lymphoid tissue and granulomatous lesions in the liver.

17.2.6.5 Diagnosis

The approach to diagnosis includes isolation and identification of the organism from the intestinal contents, liver, spleen, mesenteric lymph nodes, and intestine by MacConkey agar along with gross and histopathological evaluations. Differential diagnosis has to be made with other diseases such as Tyzzer's disease and *Helicobacter*-induced hepatitis.

Isolation and culture of *Salmonella* spp. are done by inoculating feces, intestinal contents, and mesenteric lymph nodes in salmonella-selective media. In culture, characteristic pale colony (lactose non-fermenter) on MacConkey agar, red colonies in Brilliant green agar (BG) and red colony with a black center in xylose lysine and deoxycholate medium (XLD). The biochemical reaction on Triple sugar iron agar is R/Y/H2S+. sugar fermentation xylose (+) and lysine (+) positive but do not ferment lactose (−) and sucrose (−). Further confirmation by complete biochemical test and PCR is required.

17.2.6.6 Interference with Research

Under stress, carrier rodents become clinically ill and therefore unsuitable for use in research. Carrier rodents mostly have lesions, and those reflect in histology and may also affect the immune system.

17.2.7 Colibacillosis

17.2.7.1 Epidemiology

Colibacillosis is caused by *Escherichia coli*, a Gram-negative rod and a normal inhabitant of the intestine of mice, rats, hamsters, guinea pigs, and rabbits. Transmission of infection is from contaminated fecal material with food, water, and bedding materials and direct contact from infected animals. Acute infection in mice and hamsters may cause watery diarrhea with moist, matted fur on the tail, perineum, and ventral abdomen. RDEC-1 (rabbit diarrhea *E. coli*) is a more virulent strain in rabbits. The weanlings are mostly affected by having neonatal yellowish diarrhea, anorexia, weight loss, and stunted growth with high mortality, and in the guinea pig, yellow fluid and gas and bloating may occur in the lower intestine [14].

17.2.7.2 Significance

Colibacillosis has to be differentiated from other diarrheal diseases of rodents.

Transmission: By the fecal-oral route and fomite contamination. Virulence factors are important from adherence to invasion. Stress and lack of fresh drinking water are predisposing factors to the disease.

17.2.7.3 Pathology

Gross lesions consist of segmental thickening of the colon or cecum with blood-tinged feces, enlargement of the mesenteric lymph nodes, and peritonitis. Microscopically, hyperplasia of columnar mucosal epithelial cells in the terminal ileum, proliferation of the glandular epithelium, lymphadenitis with lymphoid hyperplasia, and leukocytic infiltration in the colon and focal necrosis in the liver are common

pathological findings. In hamsters, lengthening of intestinal crypts with mitosis in crypt-villus with decreased numbers of goblet cells and atrophy of villi of the colon may be observed. In rabbits, the ileal, cecal, and colonic walls may be thickened, with edema and ulcers. Focal necrosis in mucosal epithelium with infiltration of neutrophils in lamina propria may be observed.

17.2.7.4 Diagnosis

Diagnosis may be done by gross and histopathological changes and culturing of fecal samples with a specific agar medium. The disease must be differentiated from proliferative and inflammatory intestinal disease caused by *Citrobacter rodentium* or by enterotropic mouse hepatitis virus. Diagnosis of colibacillosis depends on the presence of lesion and isolation of non-lactose fermenting *E. coli*. For the isolation of pathogenic *E. coli* from intestine samples, the sample should be inoculated on MacConkey agar medium and incubated at 37 °C for 18–24 h. The pure colony may further be incubated at 45 °C to propagate stable toxin strain. Further, biochemical identification and PCR should be conducted for confirmation.

17.2.7.5 Interference in Research

Immunodeficient mice may be clinically ill which affects the results of the study. The study may have to be terminated due to mortality.

17.2.8 Cilia-Associated Respiratory (CAR) Bacillus Infection

17.2.8.1 Epidemiology

CAR bacillus is a Gram-negative, non-spore-forming rod-shaped bacillus that causes chronic respiratory disease in mice, rats, and rabbits. Natural primary infection is very rare, and it is associated with Sendai and pneumonia virus in mice as a secondary pathogen. In general, infection is asymptomatic but may cause weight loss and dyspnea in rats.

17.2.8.2 Significance

CAR bacillus causes respiratory disease in mice and rats as an opportunistic organism rather than as a primary pathogen.

17.2.8.3 Transmission

Infection of CAR bacillus is transmitted by direct contact. Control of infection in the colony is done by culling. Embryo rederivation is the best possible means of eradication of infection from the colony [15].

17.2.8.4 Pathology

Grossly, translucent gray cystic lesions with dilated mucus-filled airways may be observed on the pleural surface. Co-infection with mycoplasma leads to bronchopneumonia in the lungs. Histologically, hyperplastic peribronchial and peribronchiolar mononuclear cell cuffs are observed in the lungs of rats. Cilia will appear more basophilic than normal epithelium.

17.2.8.5 Diagnosis

Impression smears of the lung stained with Warthin-Starry may visualize argyrophilic bacilli adherent to the apical membranes of bronchial respiratory epithelium. CAR bacillus infection must be distinguished from murine respiratory mycoplasmosis and pneumonia caused by *Streptococcus pneumoniae*, *Corynebacterium kutscheri*, and respiratory viruses. DNA samples can be isolated from a nasal swab, and CAR bacillus can be readily detected by PCR [16, 17]. Routine serological assays such as ELISA and IFAs are used to diagnose the pathogen in the serum or plasma. Isolation of CAR bacillus requires cell culture or culture in embryonated hen's eggs. CAR bacillus does not grow in cell-free media.

17.2.8.6 Interference with Research

The infection is mostly subclinical in mice, but studies are affected particularly when mice are immunocompromised. CAR bacillus contributes to morbidity and mortality in rats and mice due to murine respiratory mycoplasmosis (MRM).

17.2.9 Staphylococcal Infections

17.2.9.1 Epidemiology

Staphylococci are Gram-positive bacteria commonly found in the skin and mucous membranes of rodents, guinea pigs, and rabbits. *S. aureus*, *S. xylosus*, and *S. epidermidis* are the major organisms producing infections. Staphylococci produce various toxic proteins like hemolysins, nucleases, proteases, lipases, hyaluronidase, collagenase, and exotoxins, enterotoxin A, enterotoxin B, enterotoxin C, and toxic shock syndrome toxin-1 during entry into the skin. This toxin causes burn-like lesions on the surface of the skin.

17.2.9.2 Significance

The significance is low. This is a causative agent of naturally occurring skin lesions in mice and rats. Mice can be infected with the human phase type of staphylococci, but the zoonotic importance of this is not clear.

17.2.9.3 Transmission

Transmission is by direct contact or through cages, room surfaces, contaminated environments, or the personnel involved. Staphylococci are ubiquitous. Staphylococcus can be carried on the skin, nasopharynx, and gastrointestinal tract. The mouse strains that are susceptible are C57BL/6, C3H, DBA, and BALB/c.

17.2.9.4 Pathology

S. aureus causes chronic suppurative inflammation and necrotizing dermatitis in the skin, conjunctiva, periorbital tissue, preputial glands, and regional lymph nodes. *S. aureus* is mostly found in athymic nude mice and causes suppurative lymphadenitis, suppurative conjunctivitis, ophthalmitis, preputial adenitis, and periocular abscessation and abscess in regional lymphadenitis; the lymphadenitis is

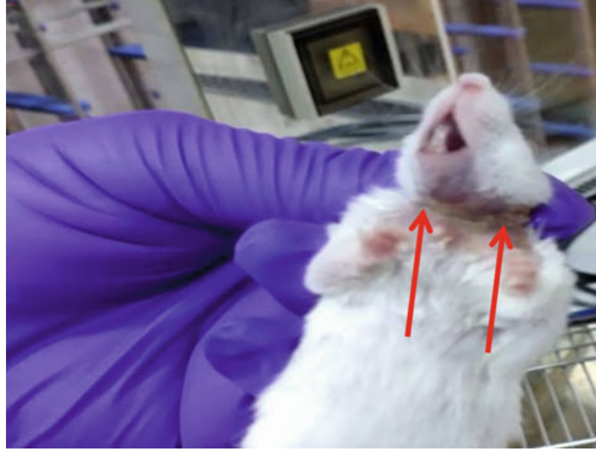


Fig. 17.15 Ulcerative necrotizing dermatitis observed below the neck (arrow)

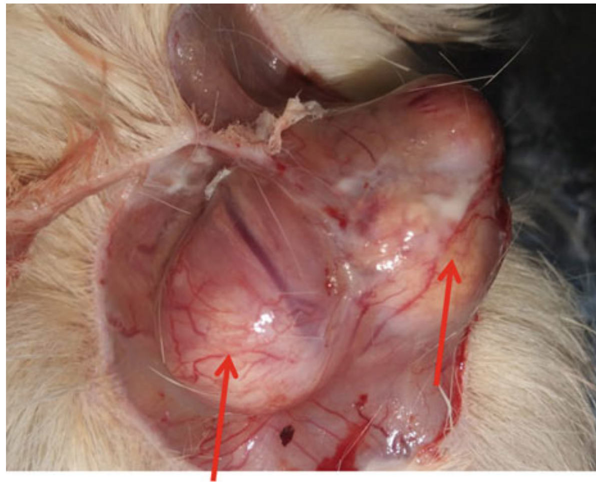


Fig. 17.16 Multifocal abscesses in the lymph node of guinea pig—arrow

characterized by necrotizing suppurative inflammation with a central core of necrotic neutrophils observed as a focal or diffused pattern. Necrotizing dermatitis in the skin appeared as small to large foci of ulcerative lesions (Fig. 17.15) found in the face, head, neck, and footpads.

High prevalence of staphylococcal infections in nude mice will lead to furunculosis around their snout, lacrimal gland abscesses, and preputial gland infections.

In guinea pigs, chronic *Staphylococcus aureus i*nfection causes suppurative inflammation in the dermal region of skin, abscess formation in lymph nodes (Fig. 17.16), ulcerative pododermatitis ("bumblefoot"), hyperkeratosis in the foot, and amyloid accumulation in the liver, adrenal glands, spleen, and pancreatic islets. *S. aureus* also causes pneumonia, mastitis, conjunctivitis, cheilitis, and osteoarthritis

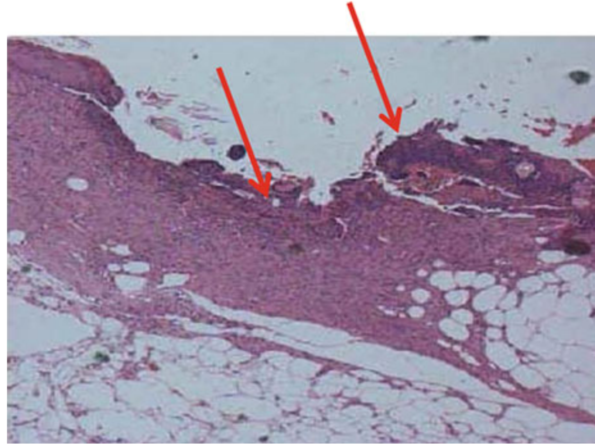


Fig. 17.17 Multifocal necrosis with inflammation in the epidermis to the dermal region of the skin—arrow

in the guinea pigs. It may also cause exfoliative dermatitis characterized by alopecia, erythema, scabs, and epidermal cracks.

Microscopically, multifocal necrosis from the epidermis and dermis that extends up to the subcutaneous layer in the skin in mice with severe infiltration of neutrophils and granulation tissues may be present (Fig. 17.17). Chronic lesions may lead to systemic amyloidosis, splenomegaly, and extra medulla hematopoiesis in the liver, spleen, and plasmacytosis in regional lymph nodes. In guinea pigs, parakeratosis of dermal lesions with inflammation may be noticed [18, 19].

17.2.9.5 Diagnosis

Gram positive Cocci bacteria can be identified by impression smear from infected organs stained with Gram's stain appearing as spherical cells in clusters of grapes and can also be isolated with selective agar medium. Isolation of *staphylococcus* can be done by inoculating samples of dermal lesions and swabs from the nasopharynx and intestine on blood agar (yellow pigmented colony), with 15 mg/liter nalidixic acid and 10 mg/liter colistin sulfate in medium to inhibit the growth of Gram-negative bacteria. Mannitol salt agar and Baird Parker medium are specifically selective for staphylococci. The inoculated plate should be incubated aerobically for 24–48 h at 37 °C. Based on the coagulation of rabbit plasma, *Staphylococcus* spp. is identified as coagulase-positive (*S. aureus*, *S. intermedius*) or coagulase-negative (*S. epidermidis*, *S. xylosus*). DNase test and test for protein A are indicators of pathogenicity of *Staphylococcus* spp. Colony characteristics, pigmentation, hemolysis (alpha, beta, delta, gamma), phase typing, antibiotic susceptibility testing, biochemical tests, and PCR are appropriate for confirmation of specific *Staphylococcus* spp.

17.2.9.6 Interference with Research

Staphylococcus can cause illness; specially immunodeficient mice are at higher risk due to alterations in immune responses. The study can be affected due to morbidity

and mortality. Staphylococci produces enzymes and toxins that play an important role in pathogenesis which reflects in the results of the study. *S. aureus* in rats causes renal abscesses in prolonged immunosuppression with corticosteroids.

17.2.10 Streptococcus Infection

17.2.10.1 Epidemiology

Streptococci are Gram-positive organisms that are commensal but can be pathogenic. It attaches to the nose, mouth, intestine, genital tract, and skin of mice. Pathogenic streptococci produce beta-hemolysin and polysaccharide surface antigens which can be differentiated into Lancefield groups A, B, C, and G.

17.2.10.2 Transmission

The transmission is by direct contact and via contaminated cage, bedding, and water. Immunocompromised (SCID) and immunodeficient (athymic) mice are highly susceptible to different groups of streptococci infections.

17.2.10.3 Pathology

Group A *Streptococcus* can cause pharyngitis and cervical lymphadenitis in mice. Group B *Streptococcus* (*S. agalactiae*) produces lesions in systemic organs with meningoencephalitis, epididymitis, and periventriculitis in nude mice, and the infection spreads from the nose to brain. *S. agalactiae* can produce pyelonephritis in DBA/2 mice and suppurative lesions in the heart, spleen, liver, thorax, lymph node, and lungs. Group C *Streptococcus dysgalactiae* may cause subcutaneous, hepatic, and abdominal abscesses in ICR Swiss mice. Group G *Streptococcus* may cause ulcerative necrotic dermatitis in the surface of the skin in the neck and throat regions (Fig. 17.18) of mice.

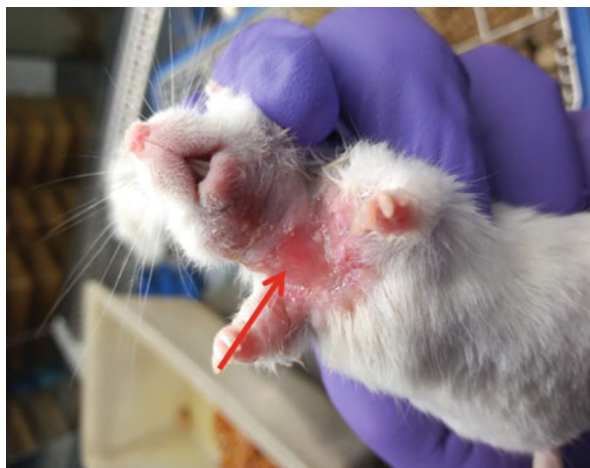


Fig. 17.18 Ulcerative dermatitis was observed below the neck of mice—arrow

Streptococcus zooepidemicus is Lancefield's group C which is a capsulated coccus that causes suppurative lymphadenitis in guinea pig. The organism enters through the nasopharynx and conjunctiva leading to systemic infections and enlargement of the cervical lymph and mesenteric lymph nodes. It may cause retro-orbital abscessation, exophthalmos, and otitis media in guinea pigs. In chronic systemic infection, fibrinopurulent bronchopneumonia, pleuritis, pericarditis arthritis, and abortion are noticed.

Microscopically, central necrosis in cervical lymph nodes and peripheral fibrosis with market infiltration of neutrophils are observed. In guinea pigs, during an acute case of systemic infection, *Streptococcus pyogenes* leads to bleeding from the nose, mouth, and vagina before death. At necropsy, pneumonia with the consolidation of one or both lungs, hemopericardium and hemothorax, fibrinopurulent pericarditis, focal myocardial degeneration in the heart, focal hepatitis in the liver, and bronchopneumonia in the lungs can be seen.

17.2.10.4 Diagnosis

Gram-positive coccoid bacteria can be identified in impression smear from infected organs stained with Gram stain. It appears as spherical or ovoid cells in chains or pairs. The organisms can be isolated in a selective agar medium [20, 21].

17.2.11 Diplococcal (Pneumococcal) Infection

17.2.11.1 Epidemiology

Streptococcus pneumonia is a Gram-positive coccus that appears round and pairs as short chains. This infection is prevalent in guinea pigs, mice, and hamsters. It produces upper respiratory infection and is mostly spread through aerosol from infected animals. It may cause high mortality, abortion, and stillbirth.

17.2.11.2 Significance

The significance is low. The septicemic condition causes the distribution of bacteria which causes suppurative lesions in organs that lead to death.

17.2.11.3 Transmission

Transmission is mostly by aerosol infection. Infection begins from the bronchopulmonary segment and spreads to the lung to the pleura pericardium. The spread to the rest of the body is by septicemia. Virulence depends on bacterial components, mostly pneumolysin, multifunctional toxins with cytolytic, and complement activating activity.

17.2.11.4 Pathology

Grossly, diplococcal infection causes fibrinopurulent pleuritis, pericarditis, peritonitis, and consolidation of lungs in guinea pigs. Histologically, acute bronchopneumonia with fibrinous exudation and polymorphonuclear cell infiltration is observed in the lungs with thrombosis of pulmonary blood vessels in acute conditions.

Fibrinopurulent pleuritis, pericarditis and epicarditis, splenitis, fibrinopurulent meningitis, metritis, focal hepatic necrosis, lymphadenitis, and ovarian abscessation may also be observed [3, 7].

Due to infection by *Streptococcus pneumoniae*, IL10 production is induced that attenuates the response of proinflammatory cytokines within the lungs and therefore hampers the effective clearance of the infection. This leads to a shortening of the survival of an animal.

17.2.11.5 Diagnosis

Direct impression smears from infected tissues or surfaces stained with Gram stain show Gram-positive diplococcic organisms. The organism can be isolated from infected tissues or exudates by a selective medium such as blood agar or enrichment media. Isolation of *Streptococcus pneumonia* can be done by inoculating specimens *on* sheep blood agar plates for 24–48 h. *S. pneumoniae* is alpha-hemolytic; colonies are mucoid or flat with smooth borders. A central concavity (draughtsman colonies) appears after 48–72 h of incubation at 37 °C on blood agar. Blood smear from animals showed pairs of cocci that are characteristic of *S. pneumoniae.* Biochemically *S. pneumoniae* is characterized as inulin (+), lactose (+), raffinose (+), salicin (v), trehalose (+), aesculin hydrolysis, mannitol (−), sorbitol (−), and sodium hippurate (−), with growth recorded in 6.5% NaCl.

17.2.11.6 Interference with Research

S. pneumoniae infection causes septicemia in rats and alters the hepatic metabolism, biochemistry, blood pH, electrolytes and thyroid functions, and respiratory parameters.

17.2.12 Corynebacteriosis

17.2.12.1 Epidemiology

Corynebacterium kutscheri and *Corynebacterium bovis* are short Gram-positive diphtheroid bacilli that cause corynebacteriosis or pseudotuberculosis in mice and rats and skin infection in immunodeficient mice. Organisms enter directly through the oral mucosa and enter regional lymph nodes to other organs by hematogenous routes. Normally it colonizes in the oral cavity, cecum, and colon of mice and rats. Immunodeficient mice, CBA, and BALB/c mice are the most susceptible, and C57BL/6 and ICR mice strain are resistant to this infection.

17.2.12.2 Significance

The significance is uncertain. The infection with *Corynebacterium kutscheri* in rat and mice in the conventional animal facility is subclinical or latent, and in immunosuppressed conditions, animals become clinically ill (virulent).

17.2.12.3 Transmission

Transmission of infection is by the fecal-oral route.

17.2.12.4 Pathology

Corynebacterium kutscheri causes conjunctivitis and raised gray or white nodules without abscessation in the lymph nodes, liver, kidney, lungs, and subcutaneous tissues of mice. Sometimes, suppurative arthritis may be noticed in carpal/metacarpal or tarsal/metatarsal joints with swelling and erythema. *Corynebacterium bovis* grows in the keratin layer of the skin and causes diffuse scaling dermatitis with hyperkeratotic dermatitis in athymic nude mice (Fig. 17.19). Histologically epidermal hyperplasia, orthokeratotic hyperkeratosis, and infiltration of mononuclear and polymorphonuclear cells infiltrate in the dermis (Fig. 17.20) can be seen in *C. bovis* infection.

Histologically C. *Kutscheri* infection shows coagulation to cassation necrosis in the lymph node, liver, kidney, and lungs with the peripheral aggregation of neutrophils. Suppurative thrombosis and emboli aggregation were observed in the blood vessels of the lung, liver, and lymph nodes. The cluster of microorganisms

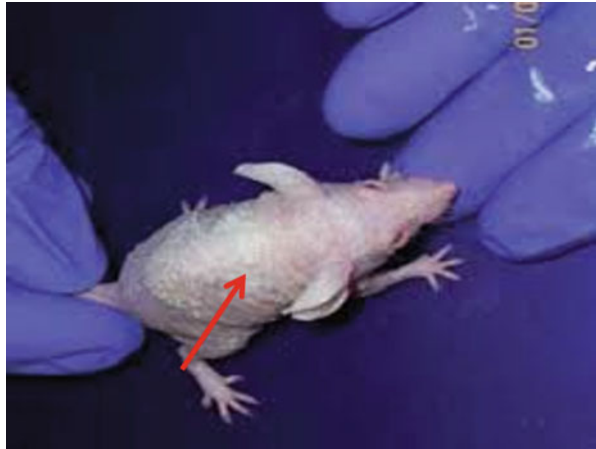


Fig. 17.19 Diffuse scaling and hyperkeratotic dermatitis on the dorsal surface of the skin—arrow

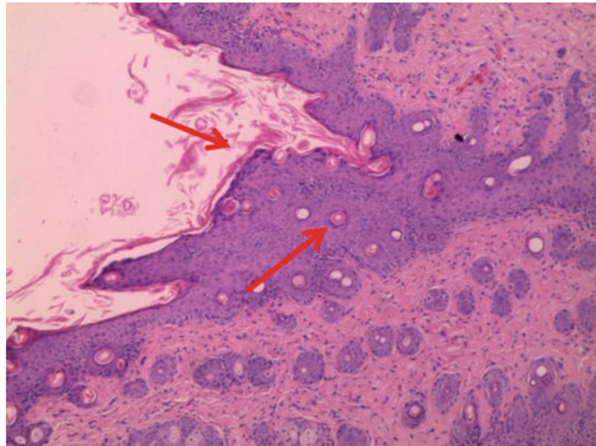


Fig. 17.20 Severe epidermal hyperplasia with hyperkeratosis was observed—arrow

was observed between the necrosis and the zone of the infiltration of inflammatory cells.

17.2.12.5 Diagnosis

Gross and histopathological observations give first-hand information about the infection. Besides, culture from the skin and peripheral lymph nodes with the selective medium is useful for isolation and diagnosis of the infection. Impression smears or scrapping from the keratin layer of skin stained with Gram stain shows Gram-positive rod-shaped organism. Isolation can be done by using selective agar medium such as Columbia agar with 5% sheep blood. Differential diagnosis of hyperkeratosis has to be made with Proteus infection and low environmental humidity. Differential diagnosis of abscesses in the lymph node, liver, kidney, and lung has to be done from other bacterial infections such as *Staphylococcus*, *Streptococcus*, and mycoplasmosis.

The organism is difficult to isolate from the nasopharynx and mucosal surface. The colony resembles that of other bacteria. Isolation of *Corynebacterium kutscheri* is by inoculation of specimens (abscesses, exudates) from the liver, kidney, lungs, and lymph nodes on sheep blood agar and incubation for 24–48 h at 37 °C. Identification is based on colonial morphology (small, whitish, nonhemolytic colonies that resemble C. *pseudotuberculosis*). *Corynebacterium kutscheri* shows a positive reaction with aesculin hydrolysis, nitrate reduction, urease, fermentation of glucose, maltose, and sucrose but negative for casein digestion. Further confirmation of isolates by PCR is appropriate for diagnosis [22–24].

17.2.12.6 Interference with Research

The infection with *C. kutscheri* can interfere in the experimental study in immunocompromised mice and rats and ultimately termination of the study. Further *C. kutscheri* infection can mask the infection of experimental infection with Sendai virus, sialodacryoadenitis virus, or Kilham virus in the rat.

17.2.13 *Bordetella bronchiseptica* Infection

17.2.13.1 Epidemiology

Bordetella bronchiseptica is a common pathogen of the upper respiratory tract, and the infection mainly spread through nasal secretion from the infected guinea pigs, and the infection rate is high during winter. The pregnant guinea pig during infection may cause abortion or produce stillborn offspring, and mortality occurs frequently in young guinea pigs during winter.

17.2.13.2 Significance

There is a high prevalence of seropositivity among rabbits. Often infections are asymptomatic and become complicated in association with *Pasteurella multocida*.

17.2.13.3 Transmission

The transmission of *Bordetella bronchiseptica* is through aerosol, fomites, and direct contact. The infection usually occurs in early life.

17.2.13.4 Pathology

Grossly, nasal passages and trachea may show mucopurulent or catarrhal exudates, swelling of eyelids, and conjunctivitis. The lung develops red to gray areas of consolidation in guinea pigs. Histologically, suppurative rhinitis, multifocal bronchopneumonia with infiltration of polymorphonuclear cell and lymphocytic infiltration, and parabronchial lymphoid hyperplasia are seen. In chronic cases, fibrous tissue proliferation in the inflamed lungs along with mononuclear cell infiltration may be observed.

17.2.13.5 Diagnosis

The organism can be isolated from blood agar cultures from the respiratory tract lavages or secretions. Gross and histopathological lesions help in the differentiation of the infection from acute pneumococcal infection, *Klebsiella* and staphylococcal infections, or systemic form of *Streptococcus zooepidemicus* infection. The infection is frequently observed as co-infection with coronavirus infection in rats.

17.2.13.6 Interference with Research

Infection with *Bordetella bronchiseptica* causes interference in research through typical pathogenic changes in the lower respiratory tract like suppurative bronchopneumonia and interstitial pneumonia and peribronchial lymphocyte cuffing, which activate the defense cells and inflammatory cells that will interfere in the outcome of the research [3, 7, 11].

17.3 Microbiology and Pathology of Parasitic Diseases

17.3.1 Ectoparasitic Infections: Fur Mite Infestation—Acariasis

17.3.1.1 Epidemiology

Common fur mites of mice are *Myobia musculi*, *Radfordia affinis*, and *Myocoptes musculinis.* Mice are commonly infected with a mixed population of mites. Mice are also susceptible to follicle mite *Demodex musculi* which is more commonly seen in transgenic and *SCID* mice. The outbreak of mite infestation during summer is common in conventional animal facilities. Mite infestation is very rare in laboratory rats except for *Radfordia ensifera* (*Myobia ratti*). *Sarcoptes scabiei* is a burrowing mite of rabbits and causes sarcoptic mange, and affected rabbits will show intense pruritus, hair loss, and abrasions.

Mites are localized in dorsal anterior regions of the body, such as the head, neck, and withers as these areas are less prone to grooming activities. In severe cases, it can spread all over the body. These Mites feed on skin secretions and interstitial fluid of mice and not on the blood of mice, which results in immune sensitization. C57Bl/6,

BALB/c, and substrains are highly susceptible, and nude mice are resistant to mite infestations. The non-*H-2*-linked haplotypes shared by all B6 background strains are responsible for high susceptibility. Transmission of ectoparasite infection occurs by direct transfer of adult mites to other animals either by direct contacts or through the air and feed and also through bedding materials, grills, and cages.

17.3.1.2 Pathology

In mice, early stages of mites' infestation start with continuous itching specifically the neck region which turns brown in albino mice owing to the presence of bedding material in the scratching limbs (Fig. 17.21). Erythema, pruritus, and hair loss follow. Elevated serum IgE levels are seen. Scruffiness and, in severe cases, ulceration, suppurative abscesses, ulcerative dermatitis, and pyoderma or self-inflicted trauma (Fig. 17.22) have been observed.

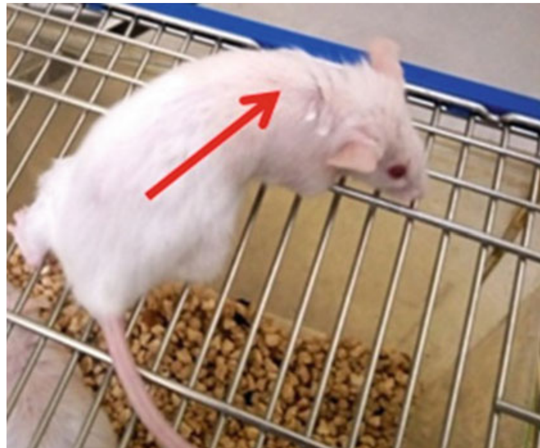


Fig. 17.21 Early stage of mites infestation: Brownish discoloration of neck region (arrow)

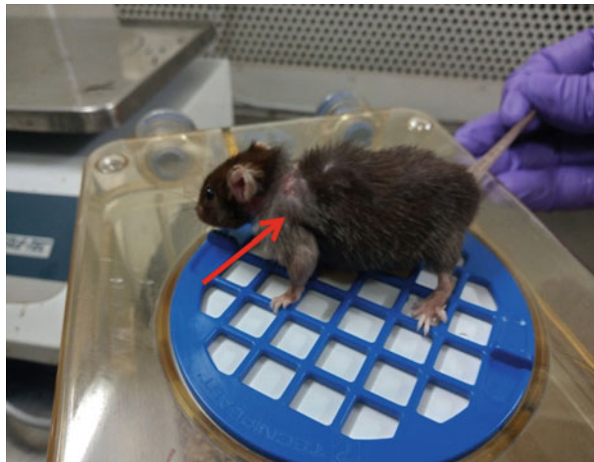


Fig. 17.22 Late stage of mite infestation: ulceration and ulcerative dermatitis in the neck—arrow

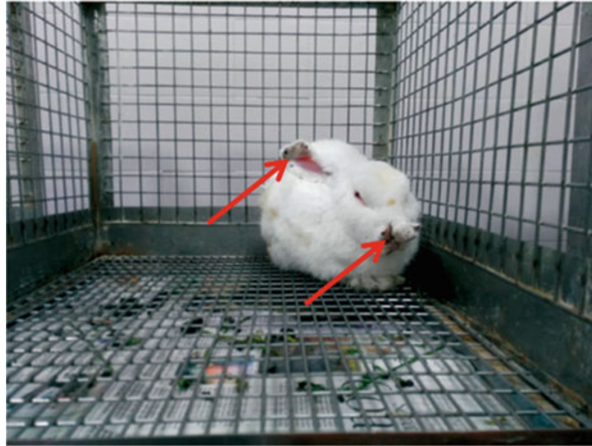


Fig. 17.23 Heavy mite infestation in the ear and nose of rabbits with ear canker formed—arrow

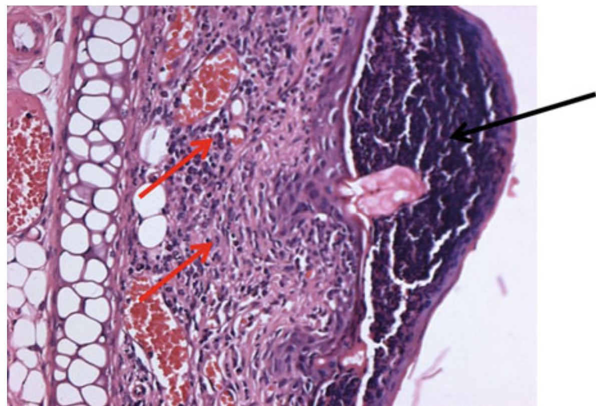


Fig. 17.24 Multiple abscesses in the keratin layer (black arrow) with epidermal hyperplasia and dermal inflammation (red arrow)

Psoroptes cuniculi mite infestation in rabbits is very common in a conventional facility. The mite pierces the epidermal layers of the external ear and makes a marked inflammatory response (ear canker). In a heavy infestation, the infested ear may show foul-smelling, bran like crusts with pus fill the external ear canal and extend up to the inner ear and some time on the dorsal surface of the ears (Fig. 17.23). The affected ear becomes thick and edematous; sometimes hemorrhagic erythematous reaction may be visible on the inner side of the ear.

Histologically, epidermal hyperplasia, hyperkeratosis, acanthosis, and chronic ulcerative dermatitis and infiltration of mononuclear leukocytes and mast cells in the dermal region will occur (Fig. 17.24). In chronic cases, severe skin inflammation associated with secondary bacterial infection (*Staphylococcus* and *Streptococcus*) is seen. Skin trauma leads to fibrosis and proliferation of granulation tissues associated with fibrosis.

17.3.1.3 Diagnosis

Skin scraping and cellophane taping of hairs on the surface of the neck and head area are sampled for microscopic evaluation to identify the mites. *Myobia* and *Radfordia* are similar in morphology with elongated bodies and bulging between their legs. Identification of *Myobia* includes single terminal tarsal claw in the second pair of legs, and *Radfordia* have two tarsal claws with unequal length. *Myocoptes musculinus* is an oval-shaped mite with pigmented third and fourth legs and suckers on its tarsal (Fig. 17.25). Under a microscope, *Myocoptes* mites can be seen along with a large number of eggs attached to the hair shaft.

17.3.2 Endoparasite Infections: Protozoal Infections

17.3.2.1 Giardiasis

17.3.2.1.1 Epidemiology

Giardiasis is caused by the protozoa *Giardia muris* which is a pear-shaped flagellate and normal inhabitant of the duodenum of mice, rats, and hamsters. Normally they are asymptomatic during mild infection. However, during a heavy infestation, abdominal dilatation without diarrhea, sluggish movement, weight loss, and rough hair coats are seen. Transmission of infection is by fecal-oral transmission by contaminated fecal material, and the infection spreads to other animals by feed, water, and bedding materials. Cross infection from mice to hamsters has been reported, but rats do not seem to pick up the infection from mice [25, 26]. C57BL/6 and BALB/c mice are resistant to infection, but C3H/He is highly susceptible. Female mice are more resistant to infection than male mice.

Fig. 17.25 *Myocoptes musculinus* with eggs under the microscope 100×

17.3.2.1.2 Pathology

Grossly, yellowish watery fluid may be observed in the small intestine. Histologically, organisms (trophozoites) adhere to the microvilli of enterocytes or mucosal surfaces. The crypt to villi ratio may be reduced. Higher infiltration of inflammatory cells was observed in lamina propria.

17.3.2.1.3 Diagnosis

Diagnosis can be made by the detection of trophozoites in the enterocytes of small intestine mucosa or wet mounts of fecal material visualized under microscopy. Diagnosis is by tumbling and rolling movements of organisms and ellipsoidal giardia cyst containing four nuclei inside the cyst. PCR amplification of DNA isolated from giardia cyst or fecal samples with giardin gene-specific primer sequences of (5′-AAGTGCGTCAACGAGCAGCT-3′), (5′-TTAGTGCTTTGT GAC CATCGA-3′) and amplicon size of 171 bp [27].

17.3.2.2 Coccidiosis

17.3.2.2.1 Intestinal Coccidiosis

Epidemiology

Coccidiosis is rare in mice, and it is caused by *Eimeria* species, and 18 species of *Eimeria* were identified. Among them, *E. falciformis*, *E. vermiformis*, *E. papillata*, and *E. ferrisi* are pathogenic to mice. In rabbits, coccidiosis is very common.

Intestinal coccidiosis is caused by *E. intestinalis*, *E. flavescens*, *E. magna*, *E. irresidua*, *E. piriformis*, *E. perforans*, *E. neoleporis*, and *E. media* in the rabbit. Oocysts released from the infected rabbits require 1–2 days to sporulate at room temperature and become infectious to other animals. Sporulated oocysts (sporocysts) will release sporozoites and enter into enterocytes and mucosal of duodenum and ileum of the small intestine. This leads to extensive destruction of the mucosal and lamina propria layer of the duodenum and ileum.

Pathology

Clinically, watery diarrhea and dark green to brown feces surrounding the anal region is observed. Grossly, the ileal mucosa is congested, edematous, and hemorrhagic with minute milky color secretions flooding the entire duodenum and ileum (Fig. 17.26). The cecum and colon contain dark green to brown watery fluid with foul-smelling material. Dilatation of cecum with greenish yellowish diarrhea was observed (Fig. 17.27). Impression smear of fecal contents or milky fecal secretions from the ileum shows sporulated oocytes (Fig. 17.28).

Histologically, villous atrophy in affected areas of the small intestine and extensive leukocytic infiltration (Fig. 17.29) along with gametocytes and oocysts may be observed in the intestinal mucosa.

Fig. 17.26 Duodenum and ileum of the rabbit are edematous, congested, and hemorrhagic with milky secretion inside the lumen—arrow

17.3.2.3 Hepatic Coccidiosis

17.3.2.3.1 Epidemiology

Hepatic coccidiosis is caused by *Eimeria stiedae*, *Coccidium oviforme*, and *Coccidium cuniculi*, and it is prevalent in weaning rabbits. Transmission is by contamination of oocytes from the feces of an infected animal through the feed, water, and bedding materials.

Ingested sporulated oocysts from fecal-oral route invade into duodenal mucosa, enter lamina propria prior, and enter into systemic migration. It also enters the regional lymph node specifically the mesenteric lymph nodes. Sporozoites may move to the liver through mononuclear cells via lymphatics. Live sporozoites may be visible in peripheral blood and bone marrow. In the liver, sporozoites invade into epithelial cells of the bile ducts. Schizogony followed by the gametogeny occurs, leading to the formation of oocysts that are released into the bile ducts and enter into the intestine.

17.3.2.3.2 Pathology

Clinically the protozoa block the function of the liver and bile duct that leads to enlargement of the liver (hepatomegaly). Serum bilirubin levels will increase manifold. Affected animals show gradual loss of body weight, potbellied appearance, and lack of body fat reserves. Dark brown to green color fecal materials that stick to the perineal region is seen. Sometimes ascites may be observed. Enlarged liver with

Fig. 17.27 Dilatation of the cecum with greenish yellowish diarrhea was observed—arrow

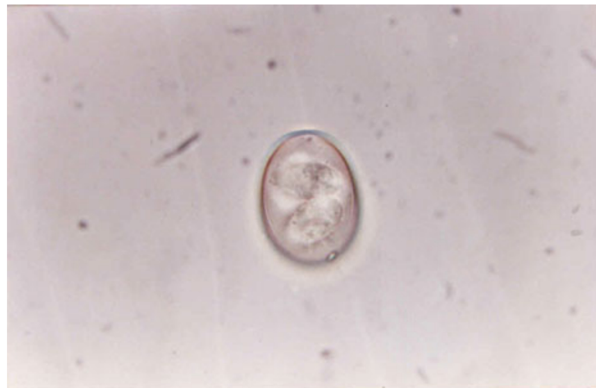


Fig. 17.28 Impression smear of ileum contents showing coccidia oocysts—arrow

raised, yellow to pearl gray circumscribed lesions 0.5–2 cm in diameter scattered throughout the surface of the hepatic parenchyma is seen.

Microscopically, dilation of bile ducts, hyperplasia of the epithelium of the bile duct, and periductular infiltration of inflammatory cells specifically lymphocytes and macrophages and a large number of gametocytes and oocysts are present in the bile

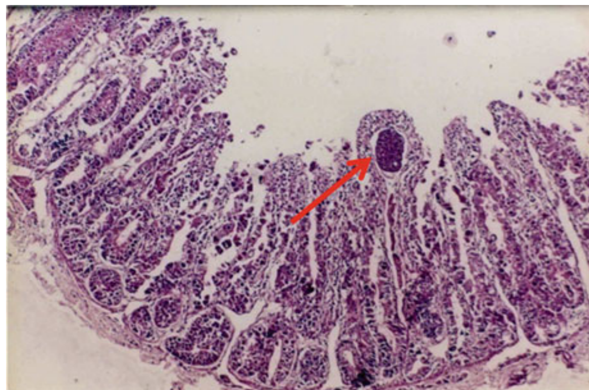


Fig. 17.29 Extensive villous atrophy with gametocyte is observed in the ileum mucosa—arrow

duct. Periportal fibrosis and infiltration of mixed inflammatory cells surrounding periportal regions are also seen.

17.3.2.3.3 Diagnosis

Diagnosis is by fecal examination or from impression smears from bile duct or aspiration from gall bladder under a microscope to observe the oocysts. PCR with generic amplification of ITS1 gene sequences specific to *Eimeria* species can be identified [28, 29].

17.3.2.4 Encephalitozoonosis [3, 7]

17.3.2.4.1 Epidemiology

Encephalitozoon cuniculi is an intracellular microsporidian parasite commonly found in conventional rabbit and guinea pig colonies but rarely in mouse and rat colonies. It is transmitted by ingestion of spores that are shed in urine from infected animals contaminating the feed, water, and bedding materials. Following ingestion, spores enter systemic circulation via mononuclear inflammatory cells and reach the lung, liver, kidney, heart, and brain. The infected spores may shed through urine and feces.

17.3.2.4.2 Pathology

The major clinical signs include torticollis and other neurological symptoms. Renal lesions such as multiple irregular pitted surfaces on the cortical surface are also seen. Histologically, *E. cuniculi* infection may produce interstitial nephritis and nonsuppurative or granulomatous meningoencephalitis in the brain and spinal cord. Granulomatous lesions may be produced in the interstitium of the lung, kidney, and liver. In the lungs, focal to diffuse interstitial pneumonitis, mononuclear cell infiltration and granulomatous lesions with infiltration of lymphocytes in the periportal region of the liver may be observed. The infiltration of inflammatory cells in myocardium and focal to segmental granulomatous interstitial nephritis with tubular degeneration and mononuclear infiltration may be observed in the kidneys.

Besides, spores are seen in tubular epithelial cells, in macrophages of inflammatory foci, and in collecting tubules. In the brain, focal nonsuppurative granulomatous meningoencephalomyelitis with astrogliosis and perivascular cuffing may be noticed in the cerebral hemisphere. In the eye there is a rupture of the lens, and inflammation in the cornea and infiltration of macrophages and lymphocytes and multinucleated giant cells may be observed.

17.3.2.4.3 Diagnosis

Impression smears of the eyes and kidneys of infected animals may show the spores of *E. cuniculi*. It must be differentiated from other protozoal diseases such as toxoplasmosis by tropism of tissues and Gram staining of this organism. It takes a Gram-negative stain because it cannot stain with carbol fuchsin.

17.3.2.5 Toxoplasmosis

Toxoplasmosis is a zoonotic disease caused by *Toxoplasma gondii.* Rats, rabbits, and guinea pigs act as an intermediate host for toxoplasmosis, while cats and other felines are the definite hosts. Transmission of oocysts takes place through infected fecal materials from the definite host to the intermediate host. Normally *Toxoplasma gondii* infection is asymptomatic, and clinical active infection cases may produce multifocal hepatitis and pneumonitis with cysts in the myocardium and brain.

17.3.2.6 Spironucleosis

17.3.2.6.1 Epidemiology

Spironucleosis is caused by flagellated protozoan *Spironucleus muris.* It is normally present in the gastrointestinal tract of mice, rats, and hamsters. Interspecies transmission between the mice and hamster has been reported, but not with rats. Mice become infected by the ingestion of trophozoites or cysts and are released from infected faces. Later, it enters the villi of the small intestine. The organism normally colonizes the small intestine in the crypts of the duodenum. Heavy infestations with *Spironucleus* impair immune response and macrophage function in athymic nude mice, alter the progression of tumor, and shorten the life span in nude mice.

17.3.2.6.2 Pathology

Clinical signs include diarrhea, hunched posture, dehydration, and mortality noticed in young animals. Distended small intestine with red to brown contents filled with gas may be noticed. Microscopically, elongated pear-shaped trophozoites may appear in crypts of the villi of duodenal mucosa with edematous lamina propria and infiltration of inflammatory cells such as lymphocytes and neutrophils. Sometimes organisms may enter in between enterocytes and lamina propria with cellular infiltration specifically plasma cells.

17.3.2.6.3 Diagnosis

On direct wet mount, smears from intestinal contents may have typical “Easter egg” cysts.

17.3.2.7 Cestodiasis: Tapeworms

17.3.2.7.1 Epidemiology

Mice, rats, and hamsters may be infected with *Hymenolepis nana*, *Hymenolepis diminuta*, and *Rodentolepis microstoma*. These threadlike tapeworms can utilize arthropods such as fleas and beetles as their intermediate host, but *H. nana* has a direct life cycle by which parasites enter the mucosa, develop into the cysticercoids stage, further develop into the adult stage, and released into the intestinal lumen. *R. microstoma* also has a direct life cycle in immunodeficient nude and NOD-*scid*, NOD-*scid-IL-2R gamma* null mice.

17.3.2.7.2 Pathology

Microscopically, the presence of cysticercus (larvae) may be observed in the lamina propria and the presence of adult stage with prominent serrated edges in the lumen of mice. Occasionally cysticercus is found in the mesenteric lymph node.

17.3.2.7.3 Diagnosis

Fecal examination shows *H. nana* (threadlike morphology with 1 mm size), while other species are of a larger size of more than 4 mm. *H. nana has* scolex with hooks, and its ova has polar filaments.

17.3.2.8 *Taenia taeniaeformis* Infection

17.3.2.8.1 Epidemiology

It is highly prevalent in a conventional animal facility in India and has been commonly reported in rats and mice [30]. *Cysticercus fasciolaris* is the larval stage of *T. taeniaeformis* of cat tapeworm, and the cat is the final host or definite host. The main transmission of infection is by ingestion of infected cat feces that contain the eggs of *T. taeniaeformis* spread through the feed, water, and bedding materials. After ingestion of the eggs, the shells of the eggs are digested in the rat intestine, and the freed oncospheres gain access to the blood vessels of the intestinal wall and are carried to the liver and form a cyst in the liver called *Cysticercus fasciolaris*.

17.3.2.8.2 Pathology

Normally one or two cysts in the liver and even seven cysts are reported in the single liver [30]. The liver cysts will appear creamy white, and in most of the cases, the cyst is found deeply attached in the caudal and lateral lobe of the liver with a size of 0.8–1.6 cm in diameter (Fig. 17.30). Each cyst has a thick fibrous capsule surrounding one loosely coiled white-colored cestode larva with creamy colored thick fluid. The viable larva is 18–32 cm in length and has a big scolex with a long neck (strobila, 3–4 cm). The pseudosegmentation of the entire body length is seen with a terminal bulged portion (Fig. 17.31). The larva when stained with acetic alum carmine shows armed rostellum characterized by two rows of hooks and four suckers (Fig. 17.32).

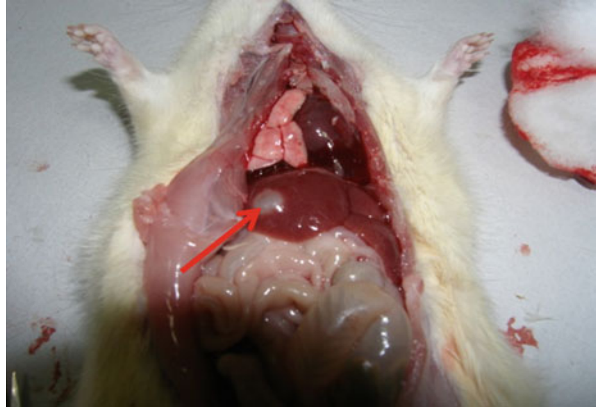


Fig. 17.30 Creamy white in color of cyst *T. taeniaeformis* in the liver of rats—arrow

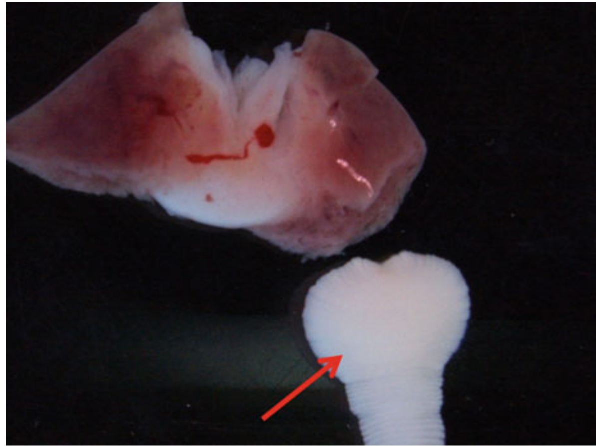


Fig. 17.31 Larvae of *T. taeniaeformis* inside the cyst in the liver—arrow

Fig. 17.32 Carmine stained larvae armed rostellum characterized by two rows of hooks and four suckers

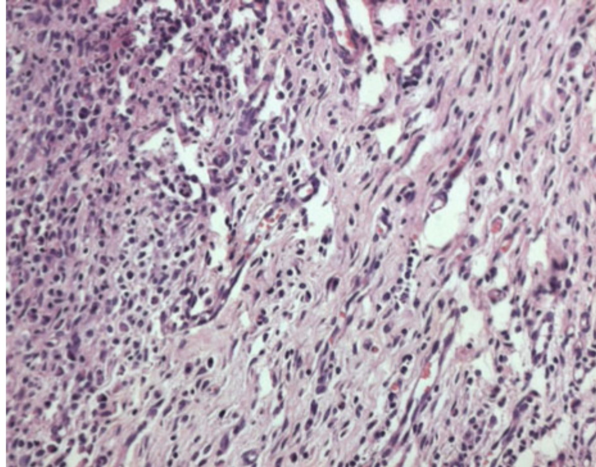


Fig. 17.33 Fibrosarcoma of the liver with infiltration of plasma cells, macrophages, and eosinophils

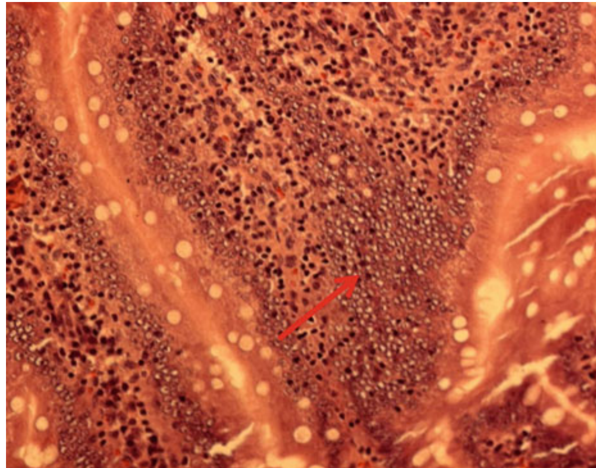


Fig. 17.34 Intestinal mucosal epithelial hyperplasia (arrow) with infiltration of inflammatory cells

Microscopically, heavy infestation of the larva in the liver leads to fibrosarcoma with infiltration of inflammatory cells specifically plasma cells, macrophages, and eosinophils (Fig. 17.33). In the stomach and intestine, gastroenteropathy with gastric mucosal hyperplasia, dilation of gastric glands with secretion, intestinal mucosal cell hyperplasia with infiltration of inflammatory cells, and proliferation of duodenal submucosal glands are seen (Fig. 17.34).

17.3.2.9 Pinworms Infections

17.3.2.9.1 Epidemiology

Syphacia obvelata, *Syphacia muris*, and *Aspicularis tetraptera* are the common pinworms in mice, rats, and hamsters. Young mice are highly susceptible, while

adults are resistant [31]. Syphacia infection may occur along with other pinworms such as *Aspicularis tetraptera* or with a bacterial infection such as *Citrobacter freundii*. Transmission of pinworm infestations is by the airborne dissemination of eggs or eggs contaminated through feed, water, and bedding materials. The life cycle of the parasites is direct and takes 11–15 days. The eggs liberate larvae in the intestine which migrate into the cecum and colon. Larvae of *S. obvelata* and *S. muris* mature and mate in the cecum and deposit their eggs to reach the rectum and perianal region. In the case of *Aspicularis tetraptera*, the egg deposition is found in the colon. Rats may become infected by direct ingestion of embryonated eggs in the contaminated feed, water, and bedding materials from mice.

17.3.2.9.2 Pathology

The condition may remain asymptomatic, but heavy infestation leads to severe enteritis, rectal prolapse, intussusception, and fecal impaction.

17.3.2.9.3 Diagnosis

Microscopic examination of fecal contents, perianal fecal contents, or perianal adhesive tape method will help. The detection of embryonated eggs with banana-shaped being flattened at one end and sharpened at other ends is confirmatory of *Syphacia obvelata* and *Syphacia muris*. The eggs of *Aspicularis tetraptera* is ellipsoidal and can be detected in the feces. The adult worms are found in the colon, and threadlike mature adult worms may appear in the cecum or colon [32].

17.3.2.10 *Trichosomoides crassicauda*: Bladder Worm Infestation

17.3.2.10.1 Epidemiology

T. crassicauda is a common nematode in rats. *T. crassicauda*-infected rats continuously pass the embryonated eggs in the urine, and the ingestion of the eggs by other rats leads to the transmission of infection. Embryonated eggs hatch in the stomach, and larvae migrate through the walls of the stomach into the peritoneal cavity and enter into the systemic circulation and reach the kidney and bladder causing hemorrhages. The larvae reaching the kidney and bladder may survive and reach maturity and release embryonated eggs. The entire life cycle may take 8–9 weeks; hence, eggs may not be present in the urine of rats until 8–12 weeks.

17.3.2.10.2 Pathology

Adult female parasites enter into the mucosal folds of the urinary bladder or the lumen of the urinary bladder (Fig. 17.35). Adult female parasites may cause hyperplasia of bladder mucosal epithelial cells, desquamation of bladder epithelial cells, and infiltration of inflammatory cells in the submucosal region. The male parasites degenerate and may be located in the vagina or uterus of the female's parasites.

17.3.2.10.3 Diagnosis

Direct examination of rat urine for embryonated eggs of *T. crassicauda* and urinary bladder for adult worms under a microscope will help in diagnosis.

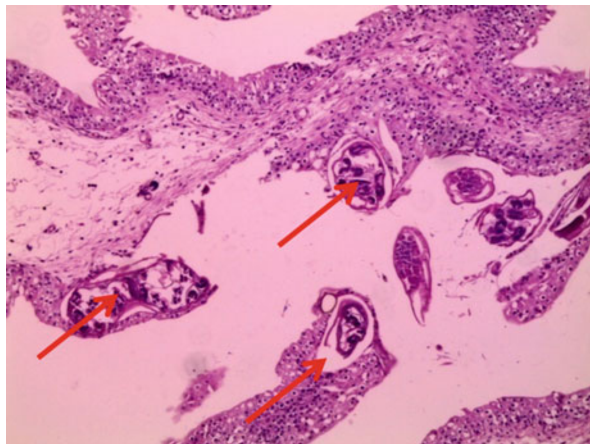


Fig. 17.35 *T. crassicauda* enter into mucosal folds or lumen of the urinary bladder of rats—arrow

17.3.2.11 Pneumocystosis

17.3.2.11.1 Epidemiology

Pneumocystosis is caused by *Pneumocystis carinii in* mice, rats, and rabbits. This organism is non-filamentous yeast-like trophic forms that adhere to skin and type I pneumocytes of alveoli with clusters of developmental stages extending into the alveolar lumen.

17.3.2.11.2 Pathology

Major clinical signs include dyspnea, hunched posture, and dry, scaly skin. It may develop into cysts with an ascosporic in the alveoli and leads to a severe inflammatory reaction in the alveoli. Grossly, the lung may appear as patchy consolidation with thick rough surfaces [33].

Histologically, severe inflammation of interstitium between the alveoli along with proteinaceous exudation into the alveolar lumen is seen. Thickening of alveolar septa along with the infiltration of mononuclear cells may be observed. In mice, alveolar filling with the typical foamy honeycomb material may be observed. In the case of rabbits, pulmonary edema, congestion of alveolar vessels, thickening, and hypercellularity of alveolar septa may be observed.

17.3.2.12 Mycotic Infections

17.3.2.12.1 Epidemiology

Trichophyton mentagrophytes and *Microsporum canis* are predominant dermatophytes in mice and rats that cause dermatophytosis. *Cryptococcus neoformans* and *Candida tropicalis* may cause systemic and pulmonary mycosis. It is rare in immunocompetent mice. It is commonly observed in immunodeficient mice.

17.3.2.12.2 Pathology

These dermatophytes produce favus, and it is characterized by scalp with the presence of dull yellow cuplike crusts and severe alopecia on the muzzle, head, ears, face, and tail. The crusts are mainly composed of epithelial debris, exudates, mycelia, and masses of arthrospores, with underlying inflammation of the dermis.

17.3.2.12.3 Diagnosis

Definitive *diagnosis* can be made on culture and identification of the organisms by using Sabouraud's or other *dermatophytes* media.

References

1. Chawla S, Jena S, Venkatsan B, Mahara K, Sahu N (2007) Clinical, pathological, and molecular investigation of Mycoplasma pulmonis-induced murine respiratory mycoplasmosis in a rat (*Rattus norvegicus*) colony. Vet World 10(11):1378–1382
2. Lindsey JR, Cassell GH, Baker HJ (1978b) Diseases due to mycoplasmas and rickettsias. In: Benirschke K, Garner FM, Jones TC (eds) Pathology of laboratory animals, vol II. Springer-Verlag, New York, pp 1481–1550
3. Lindsey JR, Cassell GH (1973) Experimental mycoplasma pulmonis infection in pathogen free mice. Models for studying mycoplasmosis of the respiratory tract. Am J Pathol 72:63–90
4. Kuppeveld FJ, Melchers WJ, Willemse HF, Kissing J, Galama JM, van der Logt JT (1993) Detection of Mycoplasma pulmonis in experimentally infected laboratory rats by 16S rRNA amplification. J Clin Microbiol 31(3):524–527
5. Barreto ML, Nascimento ER d, Campos CA d M, Nascimento M d GF d, Lignon GB, Lira MLF, Silva RG (2002) Detection of mycoplasma pulmonis in laboratory rats. Braz J Microbiol 33(3):260–264
6. Franklin CL, Motzel SL, Besch-Williford CL, Hook RR Jr, Riley LK (1994) Tyzzer's infection: host specificity of Clostridium piliforme isolates. Lab Anim Sci 44:568–572
7. Percy DH, Barthold SW (2013) Pathology of laboratory rodents and rabbits. John Wiley & Sons, New York
8. Furukawa T, Furumoto K, Fujieda M, OKADA E (2002) Detection by PCR of the Tyzzer's disease organism (Clostridium piliforme) in feces. Exp Anim 51(5):513–516
9. Fox JG, Rogers AB, Whary MT, Taylor NS, Xu S, Feng Y, Keys S (2004) Helicobacter bilis-associated hepatitis in outbred mice. Comp Med 54:571–577
10. Scharmann W, Heller A (2001) Survival and transmissibility of Pasteurella pneumotropica. Lab Anim 35:163–166
11. DeLong D, Manning PJ (1994) Bacterial diseases. In: Manning PJ et al (eds) The biology of the laboratory rabbit. Academic Press, Orlando, FL, pp 131–170
12. Townsend KM, Frost AJ, Lee CW, Papadimitriou JM, Dawkins HJ (1998) Development of PCR assays for species-and type-specific identification of Pasteurella multocida isolates. J Clin Microbiol 36(4):1096–1100
13. Caseboldt DB, Schoeb TR (1988) An outbreak in mice of salmonellosis caused by Salmonella enteritidis serotype enteritidis. Lab Anim Sci 38:190–192
14. Luperchio SA, Newman JV, Dangler CA, Schrenzel MD, Brenner DJ, Steigerwalt AG, Schauer DB (2000) Citrobacter rodentium, the causative agent of transmissible murine colonic hyperplasia, exhibits clonality: synonymy of C. rodentium and mouse-pathogenic Escherichia coli. J Clin Microbiol 38:4343–4350
15. Cundiff DD, Besch-Williford CL, Hook RR Jr, Franklin CL, Riley LK (1994) Characterization of cilia-associated respiratory bacillus isolates from rats and rabbits. Lab Anim Sci 44:305–312

16. Franklin CL, Pletz JD, Riley LK, Livingston BA, Hook RR Jr, Besch-Williford CL (1999) Detection of cilia-associated respiratory (CAR) bacillus in nasal-swab specimens from infected rats by use of polymerase chain reaction. Lab Anim Sci 49:114–117
17. Goto K, Nozu R, Takakura A, Matsushita S, ITOH T (1995) Detection of cilia-associated respiratory bacillus in experimentally and naturally infected mice and rats by the polymerase chain reaction. Exp Anim 44(4):333–336
18. Dietrich HM, Khaschabi D, Albini B (1996) Isolation of Enterococcus durans and Pseudomonas aeruginosa in a scid mouse colony. Lab Anim 30:102–107
19. Duignan PJ, Percy DH (1992) Diagnostic exercise: unexplained deaths in recently acquired C3H3 mice. Lab Anim Sci 42:610–611
20. Amao H, Komukai Y, Sugiyama M, Saito TR, Takahashi KW, Saito M (1993) Differences in susceptibility of mice among various strains to oral infection with Corynebacterium kutscheri. Jikken Dobutsu 42:539–545
21. Amao H, Komukai Y, Sugiyama M, Takahashi KW, Sawada T, Saito M (1995) Natural habitats of Corynebacterium kutscheri in subclinically infected ICGN and DBA/2 strains of mice. Lab Anim Sci 45:6–10
22. Amao H, Moriguchi N, Komukai Y, Kawasumi H, Takahashi K, Sawada T (2008) Detection of Corynebacterium kutscheri in the faeces of subclinically infected mice. Lab Anim 42:376–382
23. Baker DG (1998) Natural pathogens of laboratory mice, rats, and rabbits and their effects on research. Clin Microbiol Rev 11(2):231–266
24. Csiza CK, Abelseth MK (1973) An epizootic of protozoan enteritis in a closed mouse colony. Lab Anim Sci 23:858–861
25. Hawash Y, Ghonaim MM, Al-Shehri SS (2016) An improved PCR-RFLP assay for detection and genotyping of asymptomatic Giardia lamblia infection in a resource-poor setting. Korean J Parasitol 54(1):1
26. Fox JG, Barthold S, Davisson M, Newcomer CE, Quimby FW, Smith A (2006) The mouse in biomedical research: normative biology, husbandry, and models, vol 3. Elsevier, Amsterdam
27. Mahbubani MH, Bej AK, Perlin M, Schaefer FW, Jakubowski W, Atlas RM (1991) Detection of Giardia cysts by using the polymerase chain reaction and distinguishing live from dead cysts. Appl Environ Microbiol 57(12):3456–3461
28. Oliveira UC, Fraga JS, Licois D, Pakandl M, Gruber A (2011) Development of molecular assays for the identification of the 11 Eimeria species of the domestic rabbit (Oryctolagus cuniculus). Vet Parasitol 176(2–3):275–280
29. Oliveira UC, Fraga JS, Licois D, Pakandl M, Gruber A (2011) Development of molecular assays for the identification of the 11 Eimeria species of the domestic rabbit (Oryctolagus cuniculus). Vet Parasitol 176(2–3):275–280
30. Kumar JM, Reddy PL, Aparna V, Srinivas G, Nagarajan P, Venkatesan R et al (2006) Strobilocercus fasciolaris infection with hepatic sarcoma and gastroenteropathy in a Wistar colony. Vet Parasitol 141(3–4):362–367
31. Jacobson RH, Reed ND (1974) The thymus dependency of resistance to pinworm infections in mice. J Parasitol 60:976–979
32. Lytvynets A, Langrova I, Lachout J, Vadlejch J (2013) Detection of pinworm eggs in the dust of laboratory animals breeding facility, in the cages and on the hands of the technicians. Lab Anim 47:71–73
33. Henderson KS, Dole V, Parker NJ, Momtsios P, Banu L, Brouillette R, Simon MA, Albers TM, Pritchett-Corning KR, Clifford CB, Shek WR (2012) Pneumocystis carinii causes a distinctive interstitial pneumonia in immunocompetent laboratory rats that had been attributed to “rat respiratory virus”. Vet Pathol 49(3):440–452

18 Microbiology and Pathology of Viral Diseases in Laboratory Animals

M. J. Mahesh Kumar and Sachin Singh

Abstract

Laboratory animal vivarium is susceptible to various pathogens. Frequently, these pathogens cause clinical disease and interfere with haematology and biochemical and histopathological data. Viral diseases are a common menace in the colonies. The occurrence of spontaneous viral diseases has been a major problem in laboratory animal colonies. Understanding viral diseases becomes important in the maintenance of colony health. Strains of animals within species vary with resistance and susceptibility to viral infection. In this chapter, common viral diseases affecting laboratory animals and their epidemiology, diagnostic methods and necropsy findings will be discussed.

Keywords

Pathology · Viral diseases · Rodents · Hamsters · Rabbits · Serology · PCR

Abbreviations

ELISA	Enzyme-linked immunosorbent assay
IFA	Immunofluorescence assay
PCR	Polymerase chain reaction

M. J. M. Kumar (✉) · S. Singh
CSIR - Centre for Cellular and Molecular Biology, Hyderabad, Telangana, India
e-mail: mahesh73@ccmb.res.in; sachin@ccmb.res.in

© The Author(s), under exclusive license to Springer Nature Singapore Pte Ltd. 2021
P. Nagarajan et al. (eds.), *Essentials of Laboratory Animal Science: Principles and Practices*, https://doi.org/10.1007/978-981-16-0987-9_18

18.1 Introduction

The advancement of biological research had led to enhancement in animal husbandry practices, identification of various pathogens, health surveillance and improvement of therapeutic measures in laboratory animals. The infection of laboratory animals can have serious adverse effects on research. It can alter an animal's response to the experimental procedure even in the absence of clinical signs of disease [1].

The disease transmission and expression are mostly influenced by the age of the animals, their genotypes, environment conditions and immunodeficiency status of the strains. Some of the viral diseases have limited clinical signs in the animals even though they are highly pathogenic. Diagnostic pathology helps us to identify the possible pathogens in a colony, and their pathological changes in systemic organs help us to understand the disease and to eradicate the diseases. The diagnosis is vital and is arrived at taking into consideration various factors such as clinical history, microbial status, diagnostic tests, the strain of animals and their susceptibility and the type of tests carried out.

The presence of microorganisms in the colony is an important factor that influences animal experiments [2]. It is important to know the transmission of disease, its effects on the systems and complications. This chapter will highlight important viral diseases that commonly affect laboratory animals in research. This will help the readers to understand the pathology of the important viral diseases.

18.2 Important Viral Diseases Affecting Laboratory Animals

The viruses that affect the laboratory animal species are classified as either DNA or RNA virus. The details of the virus and systems affected are given in Tables 18.1 and 18.2.

18.3 Parvoviral Infections

Parvoviruses are non-enveloped and single-stranded DNA viruses. Under this family, minute virus of mice (MVM) and mouse parvovirus type-1 (MPV-1) commonly affect the mouse colony.

18.3.1 Minute Virus of Mice (MVM)

18.3.1.1 Epidemiology

MVM infects the gastrointestinal tract and is excreted through urine and faeces. By oral and nasal routes, they are transmitted to other animals. The natural hosts are laboratory wild mice. Rats and hamsters are susceptible to experimental infections.

Table 18.1 List of DNA and RNA viruses infecting laboratory animals

	DNA viruses	RNA viruses
Mice	(a) Adenovirus: MAdv1 and MAdv2 (b) Herpes virus: Mouse cytomegalovirus, mouse thymic virus (c) Parvovirus: Mouse parvovirus, minute virus of mice (d) Pox virus: Ectromelia virus (e) Papovavirus: Polyoma virus, K virus	(a) Arenavirus—Lymphocytic choriomeningitis virus (b) Arterivirus—Lactate dehydrogenase-elevating virus (c) Astrovirus (d) Coronavirus—Mouse hepatitis virus (e) Calicivirus—Mouse norovirus (f) Paramyxovirus—Pneumonia virus of mice (g) Sendai virus (h) Picornavirus—Mouse encephalomyelitis virus (i) Reovirus—Epizootic diarrhoea of infant mice (j) Retrovirus—Murine mammary tumour virus, murine leukaemia virus
Rats	(a) Rat adenovirus (b) Rat cytomegalovirus (c) Rat papilloma virus (d) Rat polyomavirus (e) Rat parvovirus	(a) Rat coronavirus (b) Hantavirus (c) Sendai virus (d) Parainfluenza virus 2 (e) Rat theilovirus (f) Rat rotavirus
Hamsters	(a) Adenovirus (b) Cytomegalovirus (c) Papillomavirus (d) Parvovirus (e) Polyoma virus	(a) Sendai virus (b) Pneumonia virus
Guinea pigs	(a) Guinea pigs adenovirus (b) Guinea pig's cytomegalovirus (c) Guinea pig herpes-like P virus	(a) Lymphocytic choriomeningitis virus (b) Coronavirus-like infection (c) Parainfluenza (d) Picornavirus (e) Rabies virus
Rabbits	(a) Adenovirus (b) Leporid herpes virus 1 and 3 (c) Rabbit papilloma virus (d) Rabbit oral papilloma virus (e) Myxoma virus (f) Pox virus	(a) Astrovirus (b) Borna virus (c) Calicivirus—Rabbit haemorrhagic disease (d) Rabbit enteric coronavirus (e) Hepatitis E virus (f) Rabies virus (g) Rabbit rotavirus

18.3.1.2 Pathology

The neonates of various mice strains (BALB/c SWR, CBA, C3H, SJL) develop cerebellar lesions with renal papillary haemorrhages and infarction [3]. In DBA 2 mice, intestinal haemorrhage and hepatic haematopoiesis were observed. Growth retardation, reduced fecundity and premature deaths were shown to occur in NOD mice infected with MVM [4]. Experimental intranasal inoculation of a lethal dose of MVM is shown to significantly reduce platelet count (thrombocytopenia) and

Table 18.2 Viruses and systems affected

Digestive system	Respiratory system	Skin and dermal	Haematopoietic system	Miscellaneous organs
(a) Mouse adenoviruses (b) Mouse hepatitis virus (c) Mouse and rat parvovirus (d) Mouse and rat rotavirus (e) Mouse thymic virus (f) Mouse and rat cytomegalovirus (g) Reovirus type 3 (h) Sialodacryoadenitis virus	(a) Pneumonia virus of mice (b) Sendai virus (c) K virus (d) Rat coronavirus	(a) Ectromelia virus (b) Mouse mammary tumour virus	(a) Lactate dehydrogenase-elevating virus (b) Lymphocytic choriomeningitis virus (c) Murine leukaemia virus	(a) Mouse adenoviruses (b) Hantavirus (c) Kilham rat virus (d) Minute virus of mice (e) Mouse hepatitis virus (f) Polyoma virus (g) Sialodacryoadenitis virus (h) Toolan’s virus

decrease colony-forming units of megakaryocytes (CFU—MK) on 14 days of post-infection and on 35 days of post-infection, respectively. Additionally, a decrease in the bone marrow and splenic cellular content with a survival rate of less than 1% [5].

18.3.1.3 Diagnosis

The virus can be detected in faeces, spleen and mesenteric lymph nodes from day 1 to 30, and later on the viral load tends to decrease, and seroconversion of viral antigen can be detected from day 7 onwards [6]. ELISA is useful in detecting viral antigen after 7 days of infection. However, the most used assay to detect the viral antigen is PCR by amplifying the conserved region of NS gene (nonstructural protein) of MVM. The mesenteric lymph node is the target tissue for the deduction of virus by PCR.

The primer sequence was MVM-1458f (5′ACC AGC CAG CAC AGG CAA ATC TAT3′) and MVM-1791r (5′CAT TCT GTC TCT GAT TGG TTG AGT3′); the annealing temperature was set to 61.5 °C with a product size of 310 bp. Another primer sequence for MVM virus capsid protein (VP2) gene-specific to MVM-2929f is (5′AAA TTA CTG CAC TAG CAA CTA GAC3′) and MVM-3713r (5′CTT CAG GAA AGG TTG ACA GCA 3′), with an annealing temperature of 60 °C product size 735 bp. This sequence could differentiate MVM from MPV [6].

18.3.2 Mouse Parvovirus (MPV)

18.3.2.1 Epidemiology

Three important parvovirus strains infect the mice: MPV-1, MPV-2 and MPV-3. MPV-3 is similar to hamster parvovirus (HaPV) (98.1% homology), and MPV-2 is similar to MPV-1 (95.3% homology). The genetic similarity of MPV-3 and HaPV suggests cross-species transmission though mice are the natural host for this virus [7]. Mouse parvoviruses are distinctly different from the parvovirus of rats. MPV replicates in the intestinal epithelium and lymphoid organs. It is transmitted primarily by faecal excretion and ingestion of contaminated material. Transmission occurs from adults to naive cage mates intermittently for up to 6 weeks. Prenatal transmission, however, is not observed.

18.3.2.2 Pathology

MPV has a high tropism for endothelial cells, haematopoietic cells and lymphoid cell cells. During acute infection, cytolysis of mucosal epithelial cells of the small intestine and germinal centre of the lymph node is seen. Orally inoculated virus replicates in the intraepithelial lymphocytes, lymphocytes in the lamina propria and endothelium of the small intestine and then disseminates to multiple organs, including the kidney, lymphoid tissue and liver.

18.3.2.3 Diagnosis

Faecal shedding of MPV is probably the primary mechanism of virus transmission, and viral DNA is detected in faeces by PCR for at least 2 weeks after infection and

sporadically for 24 weeks after infection in immunocompetent mice [7]. Virus capsid protein (VP-2), gene-specific for MPV, helps to differentiate it from MVM by PCR. DNA from faecal samples, mesenteric lymph node and spleen are used for diagnosis by PCR. The primers for MPV-specific PCR are forward primer: ATGAATTTGCTACTGGAACTTAC and reverse prime GTAAGGTTTGGTGCAAGTCTAAC to yield a 731 bp amplicon corresponding to MPV-1 which is subsequently digested with the restriction enzymes NlaIV and BfuCI to differentiate MPV-1, MPV-2 and MPV-3 [7].

18.3.3 Rat Parvovirus

18.3.3.1 Epidemiology

Many serotypes of Rat parvovirus are reported viz. Kilham rat virus (RV), H-1 (Toolan's) virus, rat parvovirus 1 and RPV-2, and rat minute virus (RMV-1a, RMV-1b, RMV-1c). Rat is the natural host for RV, and it causes clinical disease in rats. Adult rats are asymptomatic and transmit to neonates by oral, nasal and faecal routes. Wild rats and contaminated bedding materials and equipment are major sources of transmission to the colony. RV and RPV are shredded in milk, urine and faeces. The virus is transmitted by the aerosol route through direct contact or fomites [8]. RV may persist in the colonies for many years.

18.3.3.2 Pathology

RPV has a tropism for the small intestine and lung. However, RV causes decreased fertility, foetal resorption, tiny litters and ranting of pups. Infected neonates show tremors, ataxia, jaundice, stunted growth and oily hair coats. It affects young adult male rats resulting in lethal disease from haemorrhage and necrosis of the brain and testicles. Subclinical infection of RV in post-weanling or adult rats causes paralysis resulting from multifocal necrosis and haemorrhages in the cerebral hemisphere

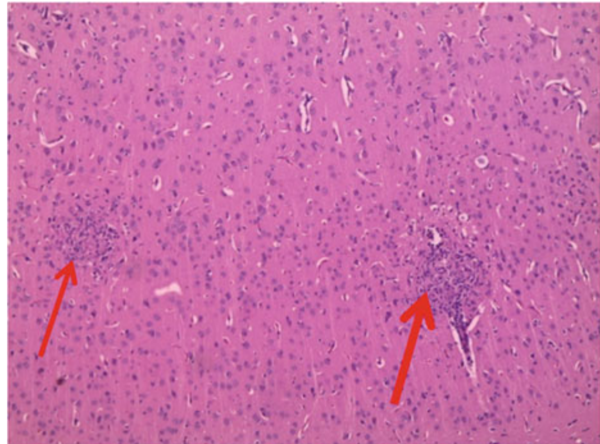


Fig. 18.1 Multifocal necrosis in the cerebral hemisphere—arrow

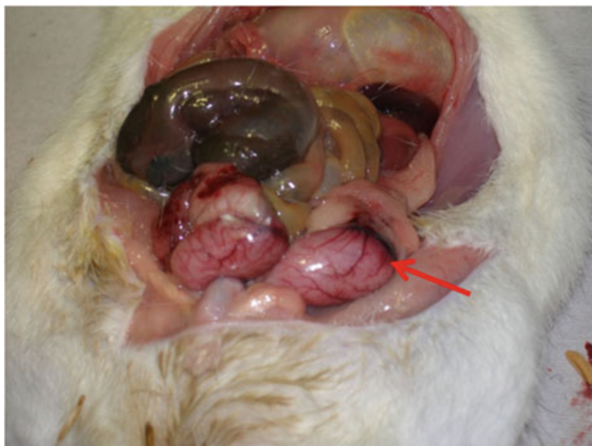


Fig. 18.2 Cyanosis and patchy haemorrhages in the testes of the rat—arrow

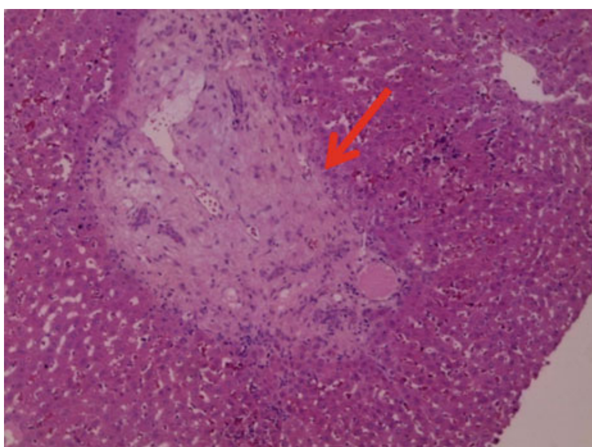


Fig. 18.3 Focal fibrotic nodule in the liver—arrow

(Fig. 18.1) and haemorrhagic infarction in the spinal cord. Cyanosis or patchy haemorrhages are commonly seen in testicles of juvenile or adult rats with typical patchy haemorrhage in between the seminiferous tubules of testes (Fig. 18.2). Subacute to chronic stage of infection in rats results in hepatocytomegaly, nodular hepatic fibrosis, nodular regenerative hyperplasia, multinucleated hepatocytes (Figs. 18.3 and 18.4) and bile duct hyperplasia and peliosis hepatis [8]. Intranuclear inclusion bodies are commonly seen in hepatocytes, Kupffer cells, bile duct epithelium and vascular endothelial cells of the cerebral cortex [8].

18.3.3.3 Diagnosis

Diagnosis of RV, RPV and H-1 viruses are commonly performed by detecting antibody levels in sera by ELISA or IFA using RV, H-1 or recombinant NS1 antigens. ELISA or IFA positive serum samples are subject to analysis with haemagglutination inhibition (HAI) for RV and H-1 antigen. Samples negative for

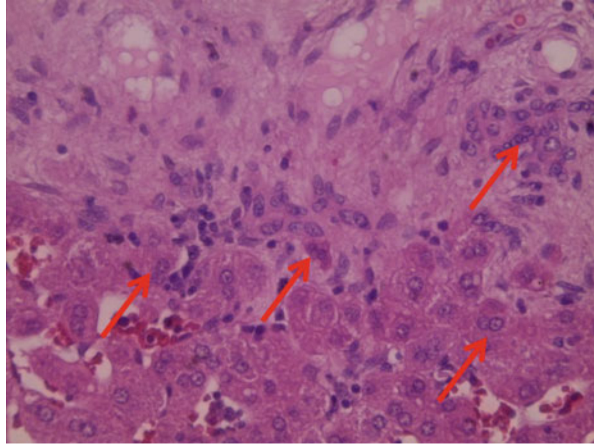


Fig. 18.4 Hepatic fibrosis associated with multinucleated hepatocytes [arrow] were observed

HAI tests are considered to be positive for RPV [8]. PCR assays for RV, H- 1, RPV and RMV provide a rapid, specific and sensitive method of detecting viral DNA in tissues. Viral DNA can be amplified from total DNA isolated from the kidney, liver, spleen, intestine, uterus and brain using H-1-specific primers.

Forward primer CTAGCAACTCTGCTGAAGGAACTC and reverse primer TAGTGATGCTGTTGCTGTATCTGATG that yield a 245 bp fragment.

Besides, detection of RV can be done with specific forward primers GCACAGACAACCAAACAGGAACTCTCC and reverse primers AGTCTCACTTTGAGCGGCTG yielding a PCR product size of 281 bp [9]. Detection of RPV-1 virus in PCR assay with the primer for nonstructural protein (NS) consisting of forward primer sequences of CGCACATGTAGAATTTTTGCTG and reverse primer sequence of CAAAGTCACCAGGCAATGTGTT giving a product of size 487 bp. Detection of RMV-1 can be done with primers designed for capsid protein (VP) region with the forward primer of ACTGAGAACTGGAGACGAATTC and reverse primer sequence of GGTCTCAGTTTGGCTTTAAGTG with a product size of 843 bp [10].

18.3.4 Hamster Parvovirus (HaPV)

18.3.4.1 Epidemiology

The virus affecting hamsters closely relates to MPV-3 and is rare in hamsters. Hamsters are contacted by virus shed in faeces or urine. HaPV and MPV-3 are parvovirus variants, and interspecies transmission to the hamster is uncommon. HaPV initially isolated from Syrian hamsters causes high morbidity and mortality, up to 100%, among suckling and weanling hamsters. Major clinical presentations in Syrian hamsters includes a potbellied appearance with the domed cranium, the incisor teeth discoloured, malformed or absent and smaller testicles [11].

18.3.4.2 Pathology

Neonatal hamsters inoculated with a high dose of HaPV uniformly succumb to a lethal haemorrhagic disease affecting the kidneys, gastrointestinal tract, testicle or uterus and brain after 7–8 days after inoculation. Thrombosis in blood vessels and basophilic intranuclear inclusion bodies in endothelial cells with subsequent ischemic necrosis are commonly seen [12]. The incisor teeth have enamel hypoplasia, periodontitis and haemorrhage in the dental pulp. In some cases, cerebral malacia and testicular hypoplasia are seen.

18.3.4.3 Diagnosis

Diagnosis is based on quantitative PCR and serological assays such as multiplex fluorescent immunoassay (MFI) and ELISA specific for hamster parvovirus.

18.4 Mousepox Viral Infection (Ectromelia): ECTV

18.4.1 Epidemiology

Ectromelia infections are caused by *Orthopoxvirus*, a DNA virus that belongs to *Poxviridae*. DBA/1J, DBA/2J, BALB/c, C3HeJ and immunodeficient mice are highly susceptible, and C57BL/6J and AKR mice strains are resistant to acute lethal systemic infection. ECTV enters through the broken skin and undergoes local replication and then extends into the regional lymph nodes and finally escapes into blood circulation. In the liver and spleen, it causes multifocal necrosis resulting in massive secondary viraemia. The virus is highly resistant to heat, and various disinfectants and effective disinfectants are vapour-based formaldehyde and sodium hypochlorite.

18.4.2 Pathology

The infection occurs in three forms: (1) acute asymptomatic, (2) acute lethal form (systemic infection) and (3) subacute to chronic form (cutaneous form). Systemic forms are characterized by facial oedema, conjunctivitis, multi-organ necrosis and high mortality. This form is less contagious than a cutaneous form because the animal dies before virus shedding. In the cutaneous form, the animals have ruffled fur, hunched posture, swelling of the face, conjunctivitis and skin lesions with papules; these animals will have typical pox lesions with multiple skin papules without scab formation in the dorsal surface of the skin (Fig. 18.5). In acute cases, necrotic amputation of limbs (ectromelia) and tails is observed [13].

Gross lesions include enlarged lymph node, splenomegaly and hepatomegaly with multiple miliary focal necrosis on the dorsal surface of the liver (Fig. 18.6). Occasionally, necrosis is also observed in other organs such as the vagina, ovaries, uterus, intestine and lungs. The animals that survived show characteristic scarring of the spleen with a mosaic pattern of white and red-brown areas. The cutaneous form

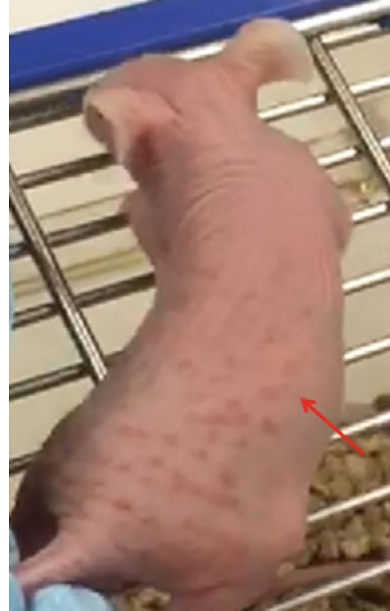


Fig. 18.5 Typical pox lesion with multiple skin papules without scab formation were observed in the dorsal surface of the skin—arrow

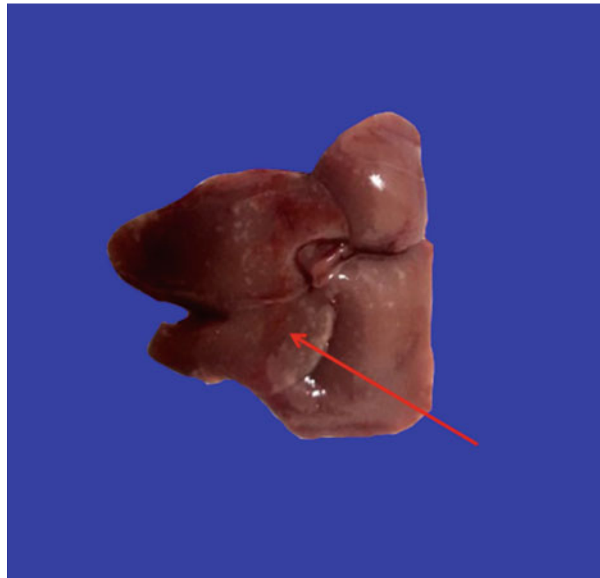


Fig. 18.6 Multiple miliary necroses [arrow] on the dorsal surface of the liver

shows localized swelling with inflammatory oedema and necrosis of dermal epithelium, leading to scab and pustule formation with secondary skin rashes after 2–3 days. Histologically skin shows epidermal hyperplasia (Fig. 18.7) with degeneration of epithelial cells that frequently contain numerous large intracytoplasmic

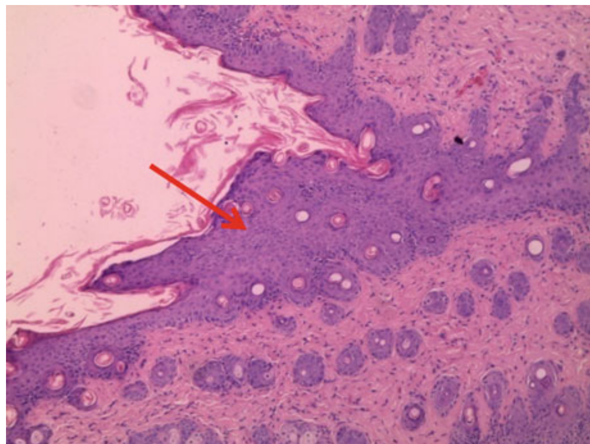


Fig. 18.7 Severe epidermal hyperplasia was observed—arrow

eosinophilic inclusion bodies. Spleen shows multifocal diffuse lymphoid necrosis in the white pulp region, and the liver shows scattered foci of hepatocellular necrosis.

18.4.3 Diagnosis

Primary diagnosis can be made based on clinical signs, gross and histopathology from moribund and dead animals. MFIA, ELISA and IFA can be used to detect viral antigens in the early stages. PCR is used as a confirmatory test [14].

18.5 Murine Adenovirus Infection (MAdV)

18.5.1 Epidemiology

MAdV is a non-enveloped double-stranded DNA virus from the family *Adenoviridae*. Two different strains were isolated from mice, FL-1 isolated from Friend leukaemia (FL) and Kl87 isolated from mouse faeces [15, 16] currently known as MAdV1 and MAdV2 strains, respectively. Naturally, infected immunocompromised mice do not show any clinical disease, but the experimentally infected suckling pups show retarded growth, and death occurs within 10 days of infection [15, 16]. It is reported that MAdV1 is shed in urine and faeces for up to 2 years after infection.

18.5.2 Pathology

Experimental infection of MAdV1 in adult C57BL/6 and DBA mouse strain causes fatal haemorrhagic encephalomyelitis with tremors, seizure and paralysis. Grossly,

haemorrhages are noticed in the brain and spinal cord. Duodenal haemorrhages are seen in FOXN1 nude mice and Prkdc Scid mice. The liver appears pale yellow. In suckling mice, multifocal necrosis is seen in the liver, adrenal gland, heart, kidney, salivary gland, spleen, brain and pancreas. Basophilic intranuclear inclusion bodies are common findings in the affected organs. In some cases, haemorrhagic encephalomyelitis and multifocal petechial haemorrhages are seen throughout the brain and spinal cord due to damage to the vascular epithelium of the central nervous system.

MAdV2 strain has tropism to the intestinal epithelium. In naturally or experimentally infected mice, intranuclear inclusion in enterocytes, especially in the ileum and caecum, is commonly seen. Virus is usually shed in the faeces.

18.5.3 Diagnosis

Based on clinical signs, gross and histopathology of infected animals and routine MFIA, ELISA and IFA may be used to rule out the prevalence of infection in the colony. A nested PCR to amplify a portion of the MAV-1 E1A coding sequence helps in the diagnosis of lung tissue [17].

18.6 Paramyxovirus Infections

18.6.1 Pneumonia Virus of Mice (PVM) Infection

18.6.1.1 Epidemiology

PVM is an enveloped RNA virus in the genus *Pneumovirus* of the *Paramyxoviridae* and agglutinates erythrocytes of several rodent species including mice. PVM causes natural infection in mice, rats, hamsters and rabbits and is transmitted through aerosol. Natural PVM infection in mice is asymptomatic in syngeneic mice, but dyspnoea, listlessness and wasting may develop in immunodeficient mice. When inoculated with a pathogenic strain of PVM, SJL mice were highly resistant, BALB/c and B6 mice displayed intermediate susceptibility, and DBA/2, C3H/HeN and 129Sv were most susceptible based upon virus titre and disease. Athymic nude mice develop pneumonia and wasting and die later [18].

18.6.1.2 Pathology

PVM replicates in the respiratory tract especially in the lungs after 6–7 days. Intranasal inoculation of pathogenic PVM strain into BALB/c mice results in nonsuppurative, perivascular and interstitial inflammation and bronchiolar desquamation and inflammation in lungs within 2 weeks. However, during natural infection, no observable lesions were seen in the lungs. Histologically, necrotizing rhinitis, necrotizing bronchiolitis and interstitial pneumonia with infiltration of mononuclear cells in the lungs are seen. Severe pulmonary oedema with interstitial pneumonia and inflammatory exudates with desquamated alveolar pneumocytes (Fig. 18.8) are seen in immunodeficient mice.

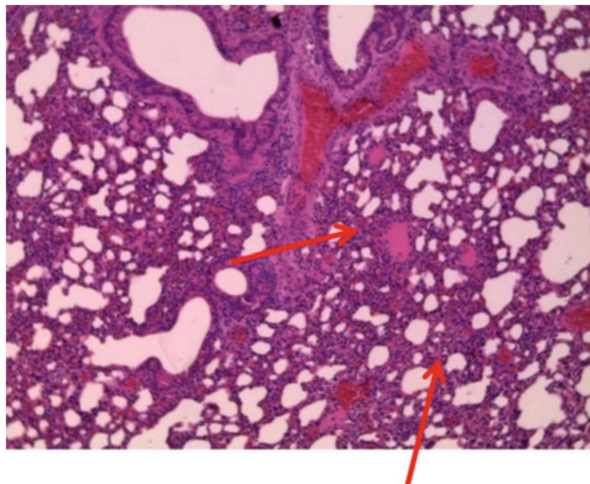


Fig. 18.8 Severe pulmonary oedema with interstitial pneumonia and inflammatory exudates inside the alveoli—arrow

18.6.1.3 Diagnosis

Diagnosis of PVM is done by serological assays such as ELISA and IFA supported by histopathology and immunohistochemistry. Virus can also be detected in tissues using RT-PCR [18]. PVM lung lesions must be differentiated from lesions caused by Sendai virus and *Pneumocystis carinii*. PVM also coexists with *P. carinii* infection in immunodeficient mice [19].

18.6.2 Sendai Virus Infections (SeV): Murine Parainfluenza Virus-1

18.6.2.1 Epidemiology

SeV is an enveloped single-stranded RNA virus that belongs to *Paramyxoviridae*, parainfluenza-1, and the virus is named after where it was first isolated from mice, at Sendai, Japan. Infection is common in mice and rats and is also reported in hamsters, guinea pigs and rabbits. The route of infection is aerosol or direct contact with infected animals.

In mice, the infection is subclinical and persistent for 2 weeks followed by seroconversion and is persistent for a year. In acute cases, mice show rough hair coat, hunched posture, respiratory distress and death of neonates and suckling mice. DBA and 129 mouse strains are susceptible to this virus, whereas SJL/J and C57BL6/J are resistant [13].

18.6.2.2 Pathology

During natural infection, otitis media/ear infection is also reported. Grossly there is dark consolidation of the lungs with plum colouration on individual lobes, and the cut surface may exude a frothy serosanguinous fluid. Pleural adhesions or lung abscesses are seen occasionally, and fluid may accumulate in the pleural and pericardial cavities due to secondary bacterial infections. Histologically, sloughed

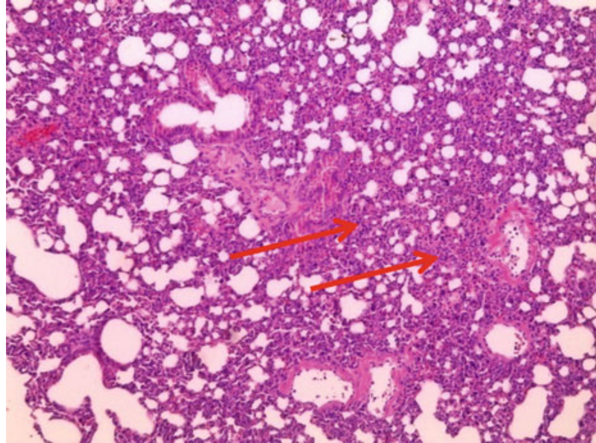


Fig. 18.9 Severe interstitial pneumonia with infiltration of lymphocytes in interstitium—arrow

necrotic epithelium and infiltration of fibrin, neutrophils and mononuclear cells, bronchitis leading to bronchopneumonia and emphysema in the lungs are seen. Alveoli are infiltrated with leukocytes leading to interstitial pneumonia (Fig. 18.9). Lymphoid cells also invade epibronchial and perivascular spaces. Adenomatous hyperplasia and squamous metaplasia (multilayer of flat epithelial cells instead of columnar epithelial cells) in bronchioles and alveoli of the lungs are a hallmark of SeV infection. In some cases, hypertrophy and hyperplasia of bronchial epithelial cells are observed. These lesions exacerbate and become more severe if concurrently infected with *Mycoplasma pulmonis*.

18.6.2.3 Diagnosis

ELISA or IFA serology is an effective means to detect infection in all strains of immunocompetent mice, and their viral antibody titre can be detected 7 days post-infection. RT-PCR assays are now the standard for rapid testing and confirmation of isolates [20]. Differential diagnoses should be considered with another respiratory pathogen such as *Mycoplasma* and *Corynebacterium kutscheri*.

18.6.3 Sendai Virus Infection (SeV): Rats

18.6.3.1 Epidemiology

Natural infection in rats is asymptomatic. However, there is a decrease in body weight and breeding efficiency. The morbidity rate is high; however, mortality is rare. SeV infection is more severe in athymic nude rats and is highly contagious. The transmission occurs through the respiratory tract either by aerosol or direct contact.

18.6.3.2 Pathology

SeV is tropic for respiratory epithelium, specifically bronchi, bronchiole, alveoli and alveolar macrophages. Grossly, grey to red foci are randomly distributed on the

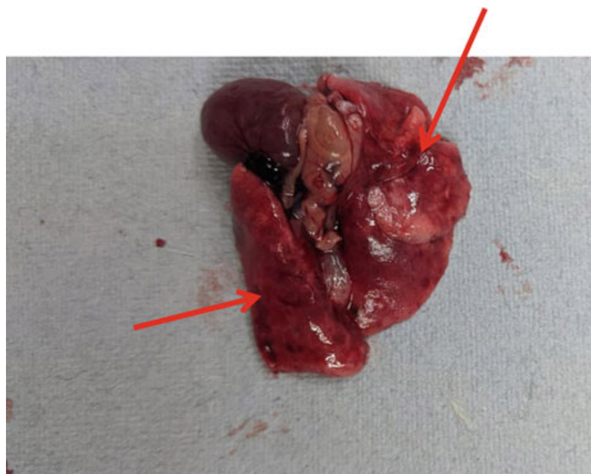


Fig. 18.10 Dark red coloration of the colour lung lobes with severe consolidation—arrow

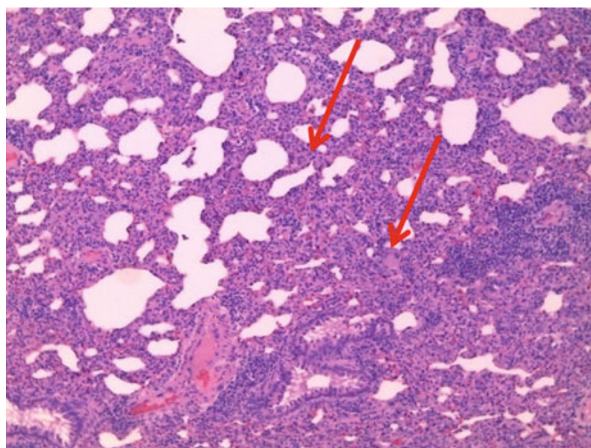


Fig. 18.11 Broncho-interstitial pneumonia with infiltration of multinucleated syncytial cells—arrow

surface of the lung and diffusely reddened (tan red) with the consolidation of lungs and individual lobes with plum colour (Fig. 18.10). Other changes include retarded embryonic development and vaginal discharges in experimentally infected animals [20]. Histologically, in the acute stage of infection, epithelial necrosis and inflammation in the nasal cavity, trachea, larynx, bronchi, bronchioles and alveoli of the lungs are seen. Infiltration of inflammatory cells that are composed of lymphocytes, alveolar macrophages, plasma cells and neutrophils results in necrotizing bronchointerstitial pneumonia. Besides, hyperplasia and squamous metaplasia along with the infiltration of multinucleated syncytial cells are seen in the bronchial epithelium (Fig. 18.11). In some cases, bronchiolitis obliterans may be present.

18.6.3.3 Diagnosis

RT-PCR assay is a standard method for the diagnosis of active infections. RNA isolated from the trachea and lungs are used. Nucleoprotein gene (NP) of Sendai virus will be detected in the lung samples of rats by touchdown RT-PCR using a nucleic acid sequence of HVJ1S—ACCCTTTGCTTTGCTGA and HVJ1AS—CTGTCAACTCCCCTAT with a product size of 684 bp and HVJ2S—ACCTTCGATACATTTAGCTC and HVJ2AS—TGTCATCAGGTCTTAACATG with a product size of 248 bp [21]. Common organisms that co-infect during SV infections include *Mycoplasma pulmonis*, *Flexibacter* sp. (CAR bacillus), *Pasteurella pneumotropica* and pneumonia virus of mice (PVM).

18.6.4 Sendai Virus Infection in Hamsters

SeV infections are subclinical in adult animals. However, it causes clinical signs and mortality in newborn animals. The consolidation of lung lobes is a common finding at necropsy. On experimental infection of hamster, hyperplasia of nasal mucosal epithelium, bronchial epithelium and peribronchial lymphocytic infiltration are seen [22]. A differential diagnosis should be made from *Corynebacterium* sp. and *Streptococcus pneumonia*.

18.7 Coronavirus Infections

18.7.1 Mouse Hepatitis Virus (MHV)

18.7.1.1 Epidemiology

MHV is an enveloped RNA virus that shares cross-reactivity with Group 2 coronaviruses, which include human coronavirus, bovine coronavirus, rat coronavirus and haemagglutinating encephalomyelitis virus of swine. There is no evidence of the source of MHV transmission other than mice and mouse products. Five strains are more prevalent viz. JHM (MHV-4), MHV-1, MHV-3, MHV-S and MHV-A59.

MHV infection is one of the major pathogens and frequent outbreaks in conventional animal facilities. MHV is classified into two important strains based on its tropism. One strain has respiratory tropism and replicates in the upper respiratory tract and disseminates into a variety of organs because of polytrophic nature such as blood vessels and lymphatic systems, liver and central nervous system. The second strain of MHV has tropism in enterocytes of the intestine. MHV strains of 1, 3, A59, JHM and S have respiratory tropism, and MHV-Y and wt-2 are heterotrophic strains. BALB/c and DBA/2 mice are susceptible to MHV, whereas SJL mice are remarkably resistant. The prevalence and severity of clinical signs depend primarily on the age, strain and immunological status of the infected mouse and strain and tropism of the virus [23]. Suckling immunodeficient mice develop diarrhoea, inappetence, dehydration, weight loss, lassitude, ruffled pelage, progressive wasting and death. Immunocompetent mice are asymptomatic, and seroconversion usually begins about

a week after infection, and mice recover fully within 3–4 weeks. Mice that have developed immunity to one strain of MHV are susceptible to one or more genetically and antigenically divergent strains resulting in reinfection [24]. MHV is highly contagious and shed in faeces, nasal secretions and by aerosol. Direct contact and vertical transmission (in utero) have been demonstrated in experimental conditions.

18.7.1.2 Pathology

Grossly, necrotic foci is yellowish with depression on the surface of the liver (Fig. 18.12). The liver is accompanied by icterus and peritonitis. The stomach is often empty, and the intestine is filled with watery to mucoid yellowish, haemorrhagic or gaseous contents with rupture of the intestine. Histologically, multifocal confluent necrosis with syncytia (multinucleated cells) are noticed at the margin of necrosis (Fig. 18.13). The formation of syncytia is the hallmark of MHV infection in many tissues, including the intestine, lung, liver, mesenteric lymph

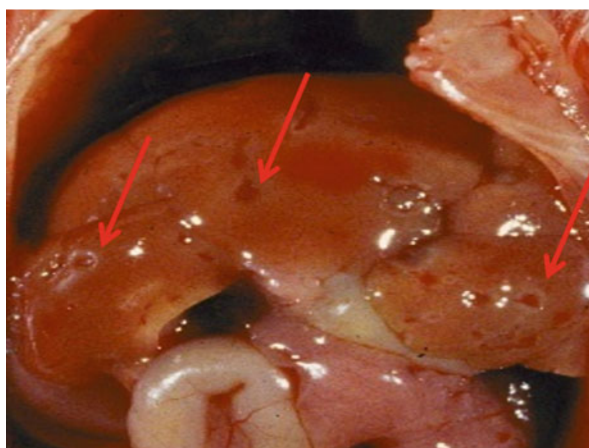


Fig. 18.12 Multiple foci of yellowish necrotic foci with depression on the surface of the liver—arrow

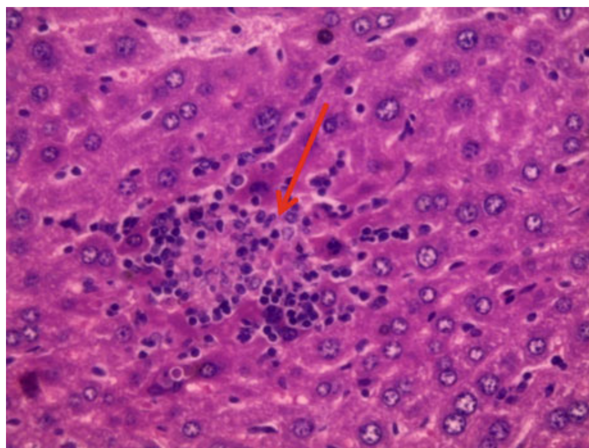


Fig. 18.13 Multi focal confluent necrosis with syncytial (multinucleated cells) at the margin of necrosis in the liver—arrow

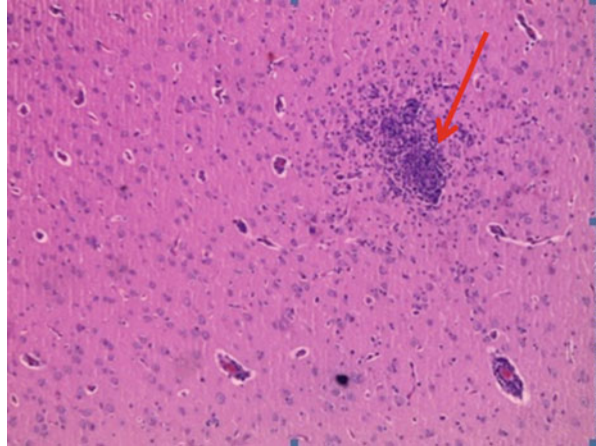


Fig. 18.14 Multifocal necrotizing encephalitis in the brain—arrow

nodes, spleen, thymus, brain, bone marrow and vascular endothelium. Polytrophic strain enters the brain and causes acute necrotizing encephalitis or meningoencephalitis, with demyelination in the brain stem (Fig. 18.14). Enterotropic strains produce severe necrotizing enterocolitis causing high mortality within 48 h after inoculation.

18.7.1.3 Diagnosis

Serological testing of ELISA and IFA is the most reliable diagnostic method for routine monitoring. Molecular diagnostics are widely used with RT-PCR protocols available to detect the viruses in various tissues and excretes. Primers for PCR from the region of the MHV genome that encodes the membrane (M) protein, the most conserved structural protein among antigenically different strains, are 5-′-AATGGAACTTC TCGTTGGG-3′ and 5′-TAGTGGCTGTTAGTGTATGG-3′, with an amplicon size of 375 bp in length [24]. MHV must be differentiated from other diseases that induce diarrhoea or cause wasting syndromes such as Tyzzer's disease, salmonellosis and reovirus.

18.7.2 Coronavirus Infection: Rats

18.7.2.1 Sialodacryoadenitis Virus (SDAV) and Rat Coronavirus (RCV)

18.7.2.1.1 Epidemiology

SDAV and RCV are very common pathogens in laboratory rats and wild rats, and these are transmitted through direct contact, aerosol and fomites and from infected animal house materials. SDAV and RCV are highly tropic and multiply in the respiratory epithelium, lachrymal glands, salivary glands and Harderian glands.

18.7.2.1.2 Pathology

Pre-weaning animals show conjunctivitis, and post-weaned or adult animals show swelling in the cervical (neck) region because of enlarged cervical and submaxillary lymph node. Nasal and ocular red discharge is seen with porphyrin pigmentation. In some affected animals, blepharospasm, photophobia, keratoconjunctivitis, corneal opacity, and corneal ulcers and necrosis in Harderian glands are seen.

Histologically, affected salivary and lacrimal glands have coagulation necrosis of ductal and acinar epithelial cells associated with inflammation with infiltration of mononuclear and polymorphonuclear cells. Sialodacryoadenitis (SDA) is observed during the acute stages of the disease and squamous, metaplasia is observed after 7–10 days post-infections.

Besides, inflammation of the respiratory system predominantly in the mucous membrane of the nose (rhinitis), trachea (tracheitis) and bronchi (bronchitis) along with hyperplasia of the respiratory epithelial cells with loss of cilia and focal inflammation of alveoli (alveolitis) are seen. The pathological changes are usually seen during the acute stage of the disease.

18.7.2.1.3 Diagnosis

Serological method of ELISA and histological examination of the Harderian glands and the submaxillary and parotid salivary glands are the most reliable diagnostic methods for routine monitoring. RT-PCR can also detect the virus using RNA from the lung, Harderian gland or the submandibular salivary gland from infected rats [25]. Differential diagnosis should be made from mycoplasma, Sendai virus, pneumonia virus of mice (PVM) and stress-induced chromodacryorrhea.

18.8 Lymphocytic Choriomeningitis Virus (LCMV)

18.8.1 LCMV Infection in Mice

18.8.1.1 Epidemiology

LCMV is an enveloped single-stranded RNA virus that belongs to *Arenaviridae*. Mice serve as the natural reservoir, whereas Syrian hamsters, guinea pigs and rabbits are also susceptible to natural infection. The zoonotic concerns make this disease serious. LCMV causes two types of infections in mice, persistent tolerant form and acute nontolerant form. Persistent forms are immunotolerant and transmitted in utero or after birth. Infected mice will shed the virus through urine, milk and secretions throughout their life. The nontolerant acute form occurs in immunocompetent mice, and these animals become viraemic and do not shed the virus, but these mice may die within 1 or 2 days.

18.8.1.2 Pathology

LCMV binds to specific antibodies and complements and accumulates in the renal glomerulus, choroid plexus of the brain, synovial membrane of joints and blood leading to the formation of immune complex nephritis with ascites and death.

Grossly, there is an enlargement of the spleen, kidney and lymph node due to lymphoid hyperplasia and glomerulonephritis. Microscopically, generalized lymphoid follicular hyperplasia, inflammation of glomerulus, tubules and perivascular lymphocytic infiltration of most of the visceral organs can be seen.

18.8.1.3 Diagnosis

LCMV is commonly diagnosed by serological methods such as ELISA, FIA and MFIA methods. Quantitative RT-PCR technique assays have been developed for the detection of viral antigen-specific viral glycoprotein (GP) primers GP-R (S pos. 970–991), GCAACTGCTGTGTTCCCGAAAC and GP-F (S pos. 877–901) and CATTCACCTGGACTTTGTCAGACTC [26].

18.8.2 LCMV Infection in Hamsters

The mode of transmission is either by direct contact, congenital or aerosol route, and the virus is also excreted through the urine for a prolonged period [27]. Major clinical signs include weight loss and impairment of reproductive performance. The presence of viral antigen and γ globulin in glomerulus leads to the formation of an immune complex leading to glomerulonephropathy [27] similar to mice. The renal lesion in hamsters must be differentiated from glomerular amyloidosis.

18.9 Murine Norovirus (MNV) Infection

18.9.1 Epidemiology

MNV is an RNA virus from *Caliciviridae* and is highly contagious in humans. It is not pathogenic in immunocompetent mice. But clinical disease occurs in immunodeficient mice. However, recent studies have shown that natural MNV infection is widespread in laboratory mouse colonies and MNV antibodies were detected in 67.3% of conventional mice and 39.1% of SPF mice from Japan and 62.5% of conventional mice from the USA [28]. STAT null and RAG null mice and interferon alpha, beta and gamma null mice are highly susceptible to infection. Transmission mainly occurs through the oral-faecal transmission.

18.9.2 Pathology

The virus has a tropism for the spleen, lymph node, and small intestine. Tissue-specific macrophages in the lung, liver, spleen and lymph nodes are the primary sites of virus replication. Pathological changes in the immunodeficient mice include pulmonary oedema with alveolitis and splenic necrosis. Histologically, multifocal hepatitis with mononuclear inflammatory cells along with scattered

polymorphonuclear cell infiltration, multifocal interstitial pneumonia, pleuritis, peritonitis, degeneration and focal fibrosis in the mesenteric lymph node is seen [29].

18.9.3 Diagnosis

Serology-based diagnosis is performed using MNV-1 capsid protein and immunohistochemistry to demonstrate viral antigen in target tissues. A one-step SYBR Green I real-time RT-PCR assay was developed for the detection and quantification of a broad range of murine noroviruses (MNVs) [30].

18.10 Rotavirus Infection: Epizootic Diarrhoea of Infant Mice (EDIM)

18.10.1 Epidemiology

EDIM is a highly contagious disease. It is caused by an RNA virus from *Reoviridae* belonging to genus *Rotavirus*. It affects all age groups of mice from 2-week-old mice to adult animals, and BALB/c mice are highly susceptible, whereas C57BL/6 mice are resistant.

18.10.2 Pathology

Mouse rotavirus shows tropism for enterocytes of villi of the small and large intestine. Clinically affected mice appear potbellied, with loose, mustard-coloured faeces that often stick to the perineum. Flaccid bowel that is distended with fluid and gas is commonly seen during necropsy. Histologically vacuolar degeneration of enterocytes in the villi and mucosal surface of the intestine is seen.

18.10.3 Diagnosis

The diagnosis is based on clinical signs and intestinal lesions. Differential diagnosis of intestinal lesions should be made from other pathogens such as MHV, MAdV, reovirus, salmonellosis, and Tyzzer's disease. EDIM infection must be differentiated from normal lipoprotein vacuoles seen in suckling mice with a pink proteinaceous droplet in enterocytes of the villi of the intestine. Rotavirus antigen can be detected in RNA from faeces by RT-PCR methods.

18.11 Herpes Virus: Mouse Cytomegalovirus and Mouse Thymic Virus

18.11.1 Mouse Cytomegalovirus (MCMV)

18.11.1.1 Epidemiology and Transmission

Mouse cytomegalovirus (MCMV) is DNA virus and mouse-specific beta-herpesvirus and is transmitted through urine, faeces and saliva from infected animals for many months. The suckling pups are protected from maternal immunity. The infection occurs in both young and adult mice. As age advances, the pathogenicity changes. Neonates are highly susceptible to lethal infection, but weanlings are resistant to the disease.

18.11.1.2 Pathology

The animals have no clinical signs, and the primary site of lesions are the salivary glands. Viraemia followed by viral replication in many tissues is seen. The salivary gland has eosinophilic intranuclear and/or cytoplasmic inclusions with lymphoplasmacytic interstitial inflammation. When there is a persistent infection, it affects the kidney leading to glomerulonephritis.

18.11.1.3 Diagnosis

Histopathologically enlarged cells with intranuclear inclusions in salivary glands are seen. Serological diagnosis using ELISA and IFA is employed. Real-time PCR using forward primer 5′-AGGGCTTGGAGAGGACCTACA-3′, reverse primer 5-′-GCCCGTCGGCAGTCTAGTC-3′ and probe 5-′-AGCTAGACGACAGCCAACGCAACGA-3′ is also employed. The probe carried a 5′ FAM reporter and a 3′ TAMRA quencher group [31].

18.11.2 Mouse Thymic Virus

Mouse thymic virus (MTV) is a herpesvirus (murid herpesvirus 3). Though MTV can infect mice at any age, lesions develop only in mice infected perinatally. Infected mice and adults develop a persistent infection in salivary glands for several months or more. The virus is spread through saliva.

18.11.2.1 Pathology

MTV causes severe, diffuse necrosis of the thymus and thymocytes. The severity of thymic and lymph node necrosis depends on mouse strain. Gross lesions are also seen in the smaller thymus. The thymocytes display MTV-positive intranuclear inclusions. Necrosis is followed by granulomatous inflammation and syncytium formation.

18.11.2.2 Diagnosis

Thymic necrosis with intranuclear viral inclusions is specific in MTV. Viral antigen can be detected by immunohistochemistry, ELISA and PCR.

18.12 Polyomaviruses: Polyoma Virus (PyV) and K Virus

18.12.1 Polyoma Virus

Natural transmission is by the respiratory route. Infected neonatal mice result in persistent infection and shedding of the virus in urine, faeces and saliva. The infection of adult mice is temporary with minimal virus shedding.

18.12.2 Pathology

Neonates inoculated with high doses of oncogenic strains result in multisystemic infection and multiple hyperplastic and neoplastic foci in a variety of tissues (polyoma). Natural infection is seen in young mice multisystemic, but tumours are exceedingly unlikely. Renal tubules are affected with mild necrosis, inclusions and interstitial inflammation. In athymic nude mice, posterior paresis occurs during natural and experimental infections. This is due to vertebral tumours and progressive multifocal leukoencephalopathy (PML). C57BL/6 mouse strain is resistant to PyV oncogenesis.

18.12.3 Diagnosis

The diagnosis is done by ELISA and immunohistochemistry. PCR can also be used to detect the virus [32].

18.13 Lactate Dehydrogenase-Elevating Virus

18.13.1 Epidemiology

Mostly by bite wounds. Natural transmission is rare even though infected mice may excrete virus in faeces, urine, milk and probably saliva.

18.13.2 Pathology

The serum biochemistry shows elevated lactate dehydrogenase enzyme, and lesions are not seen in naturally infected mice. On experimental infection, significant lesions

that are noticed is poliomyelitis. Inoculated viruses in susceptible mouse (AKR and C58) strains have pathological lesions like spongiosis, neuronal necrosis, and astrocytosis of the ventral spinal cord and brain stem, with axonal degeneration of ventral roots.

18.13.3 Diagnosis

Biochemistry analysis of serum lactate dehydrogenase enzyme, RT-PCR methods and ELISA could detect the virus [33].

18.14 Retrovirus Infection (Mammary Tumour Virus and Mouse Leukaemia Virus)

18.14.1 Epidemiology

MMTV and MLV are primarily transmitted through the milk of lactating females and saliva.

18.14.2 Pathology

MMTV causes mammary carcinomas in mammary cancer-susceptible strains. MLV causes lymphomas and is strain-specific. For example, AKR mice develop thymic lymphoma, whereas aging BALB/c mice develop multicentric lymphoma. They also cause precancerous glandular hyperplasia to adenomas and various carcinomas. The glands are generally enlarged, firm and often circumscribed. The primary lesions are usually seen in the mammary gland regions.

18.15 Mouse Encephalomyelitis Virus (MEV): Theiler's Murine Encephalomyelitis Virus (TMEV)

18.15.1 Epidemiology

The transmission is by the faecal-oral route. After ingestion, the virus replicates primarily in the intestinal mucosa. The enteric infection causes intermittent excretion of the virus in faeces over several months [34].

18.15.2 Pathology

The weaned mice are usually affected and recover at 6 months of age. Neurologic infection in the brain and spinal cord for at least 1 year. Often infections are

subclinical. The virus usually affects CNS, and the signs include convulsions and posterior paresis (flaccid) in young mice. The virus also infects enterocytes with minimal effect. CNS infection causes acute encephalomyelitis (neuronolysis), and the severity of CNS disease is the strain-, host-, age- and genotype-dependent. In natural infections, lesions like acute necrosis of ganglion cells, neurons and perivascular inflammation and neuronophagia in the ventral horn of spinal cord grey matter are observed. It is also involved in higher centres such as the hippicampus, thalamus and brain stem.

18.15.3 Diagnosis

The virus is usually detected serologically or by PCR in caecum and faeces [35].

Annexure 1: Important Viral Diseases of Mouse

Family	Virus	Incidence	Prevalence	Symptoms/ pathological lesions
Adenoviridae	Mouse adenovirus (MAdV1 MAdV2)	Subclinical in older mice	Rare	Runting, dehydration, focal necrosis in the liver and spleen, haemorrhages in the heart and other organs, intranuclear inclusion in focal necrotic areas
Herpesviridae	Mouse cytomegalovirus (MCMV)	Subclinical	Rare in lab mice; common in wild mice	Lesions in submandibular and parotid salivary glands
Herpesviridae	Mouse thymic virus (MTV)	Subclinical in adult mice	Rare in lab animals, wild mice common	Intranuclear inclusion bodies and necrosis in thymocytes, spleen and lymph nodes
Parvoviridae	Mouse parvovirus (MPV)	Natural infections are subclinical	Common	Intestinal haemorrhage, renal papillary infarction
Parvoviridae	Minute virus of mice (MVM)	Natural infections are subclinical	Common	Intranuclear inclusion bodies in the spleen
Papovaviridae	Polyoma virus (PyV)	Subclinical	Rare	Intranuclear inclusion bodies in

(continued)

				renal tubular epithelium
Poxviridae	Ectromelia virus	The natural disease varies from subclinical to high mortality	Rare	Conjunctivitis, cutaneous erythema and erosion externally. The liver is swollen, multiple pinpoint white coalescing haemorrhagic foci
Arenaviridae	LCMV	LCMV is non-cytolytic and does not directly cause disease	Rare in laboratory mice, common in wild mice	Chronic wasting, infiltration of lymphocytes in many tissues like the brain, kidney and lung
Arteriviridae	LDV—Lactate dehydrogenase-elevating virus	Subclinical	Rare in laboratory mice, common in wild mice	Necrosis in T cell areas of lymphoid tissues, splenomegaly and lymphadenomegaly
Coronaviridae	Mouse hepatitis virus (MHV)	High mortality in pups and subclinical in adults	Very common	Focal acute necrosis, severe multifocal coalescing hepatic necrosis, syncytia of parenchymal cells and vascular endothelium of multiple organs
Caliciviridae	Murine norovirus (MNV)	Subclinical in immunocompetent mice	Common	Severe multifocal coalescing hepatic necrosis in the liver, multifocal interstitial pneumonia, pleuritis and peritonitis
Paramyxoviridae	Pneumonia virus of mice (PVM)	Subclinical with upper respiratory tract infections	Common	Necrotizing rhinitis, bronchiolitis. Interstitial pneumonia. Alveolar septa thickened with oedema, alveolar space is collapsed and filled with blood and fibrin

(continued)

Paramyxoviridae	Sendai virus	Clinical symptoms include pneumonia, dyspnoea, chattering of teeth	Common	Necrotizing bronchiolitis, nonsuppurative interstitial pneumonitis, hyperplasia and hypertrophy of bronchiolar epithelial cells, plum-coloured consolidated of lung lobes
Picornaviridae	Mouse encephalomyelitis virus (MEV); Theiler's murine encephalomyelitis virus (TMEV)	Usually subclinical, some CNS symptoms like convulsions and flaccid paresis are seen	Common	Posterior paresis, demyelination of the spinal cord, brain stem and cerebellum, sometimes acute myositis
Reoviridae	Epizootic diarrhoea of infant mice (EDIM) virus; murine rotavirus-A/EDIM (MuRV-A/EDIM)	Clinical signs are seen in infant mice. Lesions and diarrhoea in mice infected at 12 or less days of age runted and potbellied, mustard coloured faeces, flaccid, dilated small intestine	Common	Cytoplasmic swelling of enterocytes
Retroviridae	Murine mammary tumour virus (MMTV)		Very common	Mammary neoplasia

Annexure 2: Important Viral Diseases of Rats

Rat parvovirus	RV or Kilham rat virus	Subclinical	Very common	Congestion of lymph node, loss of body fat, scrotal haemorrhages. Focal haemorrhages in the brain, intranuclear inclusion bodies in the liver
Rat coronavirus	Sialodacryoadenitis	Clinical signs include ocular signs associated	Common	Excessive lacrimation, oedema of the

(continued)

		with conjunctivitis		neck and salivary glands
Paramyxovirus	Parainfluenza virus 1, Sendai virus	Asymptomatic however reduced production of litter, retarded growth of young pups	Rare	Rhinitis, focal to diffuse necrosis of the respiratory epithelium
Rotavirus	Infectious diarrhoea of rats	Poor growth in suckling rats with poor growth and perianal dermatitis	Rare	The stomach usually contains milk curd, the distal intestine contains yellow-brown to green gas, necrosis of enterocytes
Rat theilovirus	RT	Usually asymptomatic	Rare	No prominent lesions noticed—Usually enteric infection
Bunyaviridae	Hanta virus	Asymptomatic	Rare	Lesions have not been observed in reservoir hosts infected with hantaviruses. Lungs and other tissues may contain large amounts of the virus without morphologic lesions
Rat cytomegalovirus	RCMV	Subclinical	Rare in laboratory rats and common in wild rats	Infects salivary and lacrimal glands, intracytoplasmic and intranuclear inclusion bodies in ductal epithelium
Rat polyomavirus	Rat PyV	Subclinical	Low	Wasting, pneumonia, parotid sialadenitis, intranuclear inclusion bodies in salivary gland
Rat adenovirus		Subclinical	Rare	Intranuclear inclusion bodies in intestinal villi epithelium

Annexure 3: Important Viral Diseases of Rabbits

Myxomatosis	*Poxviridae*	Subcutaneous oedema, nodular skin tumours, splenomegaly, large, eosinophilic, cytoplasmic inclusion bodies in the conjunctival epithelial cells with conjunctivitis
Rabbit fibroma virus	*Poxviridae*	The subcutaneous tissue is thickened having intracytoplasmic inclusion bodies
Rabbit pox	Vaccinia virus	Rashes are seen in the skin, oedema in the mouth and subcutaneous tissue
Papillomatosis	Papillomavirus	Warts on the neck, shoulders, ears or abdomen
Rotavirus infection	*Reoviridae*	The animal will have diarrhoea and dehydration. The small intestine and caecum are distended, fluid-filled. Histopathologic lesions include short, fused villi with attenuated epithelium and an increase in the size of crypt depth.
Rabbit calicivirus	Viral haemorrhagic disease	Haemorrhages are present in multiple organs. Necrosis in hepatocytes and lymphoid with massive coagulopathy
Herpes virus	*Herpesvirus hominis*	Anorexia, restlessness, circling, and tonic-clonic spasms. Histopathology lesions include nonsuppurative meningoencephalitis with neural cell necrosis and intranuclear inclusion bodies
Coronavirus infections	Rabbit enteric coronavirus	Anorexia, abdominal distension, watery diarrhoea and sudden death. On histopathology, necrosis of villous epithelial cells of the small intestine is seen

Annexure 4: Details of Sample Collection Required for DNA Isolation for PCR Assays

Name of the viral agents	Collection of samples for DNA isolation
Minute virus of mice (MVM)	Mesenteric lymph node, faeces, spleen
Mouse parvovirus (MPV)	Mesenteric lymph node, faeces, spleen
Rat parvovirus	Kidney, liver, spleen, intestine, uterus, brain
Hamster parvovirus	Kidney, faeces, testicle, uterus
Ectromelia virus (mousepox)	Skin lesions, liver, oropharyngeal secretion
Adeno virus	Lung
Mouse cytomegalovirus (MCMV):	Lung, liver, salivary gland, spleen, heart

Annexure 5: Details of Sample Collection Required for RNA Isolation Followed with RT-PCR Assays

Name of the viral agent	Collection of samples for RNA isolation
Pneumonia virus of mice (PVM)	Trachea, lung
Sendai virus	Lung, trachea
Mouse hepatitis virus (MHV)	Liver, mesenteric lymph node, faeces, spleen
Rat coronavirus Sialodacryoadenitis virus (SDAV)	Salivary gland, lacrimal gland, Harderian gland, lung
Lymphocytic choriomeningitis (LCMV)	Liver, spleen
Murine norovirus	Spleen, lymph node, small intestine
Epizootic diarrhoea of infant mice EDIM (rotavirus)	Small intestine
Mouse encephalomyelitis virus (MEV)	Intestine, brain, faeces
Murine mammary tumour virus (MMTV)	Mammary gland

References

1. Nicklas W (2008) International harmonization of health monitoring. ILAR J 49:338–346
2. Baker DG (1998) Natural pathogens of laboratory mice, rats, and rabbits and their effect on research. Clin Microbiol Rev 11:231–266
3. Brownstein DG, Smith AL, Jacoby RO, Johnson EA, Hansen G, Tattersall P (1991) Pathogenesis of infection with a virulent allotropic variant of minute virus of mice and regulation by host genotype. Lab Investig 65:357–364
4. Naugler SL, Myles MH, Bauer BA, Kennett MJ, BeschWilliford CL (2001) Reduced fecundity and death associated with parvovirus infection in B-lymphocyte deficient mice. Contemp Top Lab Anim Sci 40:66
5. Lamana ML, Albella B, Bueren JA, Segovia JC (2001) In vitro and in vivo susceptibility of mouse megakaryocytic progenitors to strain i of parvovirus minute virus of mice. Exp Hematol 29(11):1303–1309
6. Janus LM, Mähler M, Köhl W, Smoczek A, Hedrich HJ, Bleich A (2008) Minute virus of mice: antibody response, viral shedding, and persistence of viral DNA in multiple strains of mice. Comp Med 58(4):360–368
7. Besselsen DG, Romero MJ, Wagner AM, Henderson KS, Livingston RS (2006) Identification of novel murine parvovirus strains by epidemiological analysis of naturally infected mice. J Gen Virol 87:1543–1556
8. Otto GM, Franklin CL, Clifford CB, Fox JG, Anderson LC, Pritchett-Corning KR (2015) Chapter 4: Biology and diseases of rats. In: Laboratory animal medicine, 3rd edn. Academic Press, Boston, pp 151–207
9. Besselsen DG, Besch-Williford CL, Pintel DJ, Franklin CL, Hook RR, Riley LK (1995) Detection of newly recognized rodent parvoviruses by PCR. J Clin Microbiol 33:2859–2863
10. Wan CH, Bauer BA, Pintel DJ, Riley LK (2006) Detection of rat parvovirus type 1 and rat minute virus type 1 by polymerase chain reaction. Lab Anim 40(1):63–69
11. Besselsen DG, Gibson SV, Besch-Williford CL, Purdy GA, Knowles RL, Wagner JE, Riley LK (1999) Natural and experimentally induced infection of Syrian hamsters with a newly recognized parvovirus. Comp Med 49(3):308–312

12. Christie RD, Marcus EC, Wagner AM, Besselsen DG (2010) Experimental infection of mice with hamster parvovirus: evidence for interspecies transmission of mouse parvovirus 3. Comp Med 60(2):123–129
13. Brayton C, Mähler M, Nicklas W (2004) Viral infections. Lab Mouse 357
14. Neubauer H, Pfeffer M, Meyer H (1997) Specific detection of mousepox virus by polymerase chain reaction. Lab Anim 31(3):201–205
15. Richter CB (1986) Mouse adenovirus. K virus, pneumonia virus of mice. In: Bhatt PN, Jacoby RO, Morse HC, New AE (eds) Viral and mycoplasmal infections of laboratory rodents: effects on biomedical research. Academic Press, Orlando, FL, pp 137–192
16. Porterfield PD, Richter CB (1994) Mouse adenovirus. In: Waggie K, Kagiyama N, Allen AM, Nomura T (eds) Manual of microbiological monitoring of laboratory animals, 2nd edn. National Institutes of Health, Bethesda, MD, pp 47–51
17. Weinberg JB, Stempfle GS, Wilkinson JE, Younger JG, Spindler KR (2005) Acute respiratory infection with mouse adenovirus type 1. Virology 340(2):245–254
18. Percy DH, Barthold SW (2013) Pathology of laboratory rodents and rabbits. John Wiley & Sons, New York
19. Wagner AM, Loganbill JK, Besselsen DG (2003) Detection of Sendai virus and pneumonia virus of mice by use of fluorogenic nuclease reverse transcriptase polymerase chain reaction analysis. Comp Med 53(2):173–177
20. Bray MV, Barthold SW, Sidman CL, Roths J, Smith AL (1993) Exacerbation of Pneumocystis carinii pneumonia in immunodeficient (scid) mice by concurrent infection with a pneumovirus. Infect Immun 61(4):1586–1588
21. Coid CR, Wardman G (1971) The effect of para-influenza type 1 (Sendai) virus infection on early pregnancy in the rat. J Reprod Fertil 24(1):39–43
22. Hayase Y, Tobita K, Kii M, Hakamada Y, Arai T (1997) Detection of nucleoprotein gene of Sendai virus in the lungs of rats by touchdown nested reverse transcription polymerase chain reaction. Exp Anim 46(4):307–310
23. Percy DH, Palmer DJ (1997) Pathogenesis of Sendai virus infection in the Syrian hamster. Lab Anim Sci 47(2):132–137
24. Barthold SW, Beck DS, Smith AL (1993) Enterotropic coronavirus (mouse hepatitis virus) in mice: influence of host age and strain on infection and disease. Lab Anim Sci 43(4):276–284
25. Homberger FR, Smith AL, Barthold SW (1991) Detection of rodent coronaviruses in tissues and cell cultures by using polymerase chain reaction. J Clin Microbiol 29(12):2789–2793
26. Compton SR, Vivas-Gonzalez BE, Macy JD (1999) Reverse transcriptase polymerase chain reaction-based diagnosis and molecular characterization of a new rat coronavirus strain. Lab Anim Sci 49(5):506–513
27. McCausland MM, Crotty S (2008) Quantitative PCR technique for detecting lymphocytic choriomeningitis virus in vivo. J Virol Methods 147(1):167–176
28. Miedel EL, Hankenson FC (2015) Biology and diseases of hamsters. Lab Anim Med:209–245
29. Ohsugi T, Matsuura K, Kumar JM (2013) Natural infection of murine norovirus in conventional and specific pathogen-free laboratory mice. Front Microbiol 4:12
30. Hsu CC, Piotrowski SL, Meeker SM, Smith KD, Maggio-Price L, Treuting PM (2016) Histologic lesions induced by murine Norovirus infection in laboratory mice. Vet Pathol 53 (4):754–763
31. Vliegen I, Herngreen S, Grauls G, Bruggeman C, Stassen F (2003) Improved detection and quantification of mouse cytomegalovirus by real-time PCR. Virus Res 98(1):17–25

32. Zhang S, McNees AL, Butel JS (2005) Quantification of vertical transmission of Murine polyoma virus by real-time quantitative PCR. J Gen Virol 86(10):2721–2729
33. Wagner AM, Loganbill JK, Besselsen DG (2004) Detection of lactate dehydrogenase-elevating virus by use of a fluorogenic nuclease reverse transcriptase-polymerase chain reaction assay. Comp Med 54(3):288–292
34. Brownstein D, Bhatt P, Ardito R, Paturzo F, Johnson E (1989) Duration and patterns of transmission of Theiler's mouse encephalomyelitis virus infection. Lab Anim Sci 39 (4):299–301
35. Yuan W et al (2016) Development of a duplex real-time RT-PCR for the simultaneous detection and differentiation of Theiler's murine encephalomyelitis virus and rat theilovirus. J Virol Methods 236:139–146

Laboratory Animal Behaviour and Its Applications in Biomedical Research

19

V. S. Harikrishnan

Abstract

Even after a century or more in captivity and being perpetuated under stringent confinement and controlled conditions, the rodents and rabbits still retain the behaviour of their counterparts living in natural habitats. Ethology, the study of behaviour makes use of the observed behaviour enlisted in detail known as ethograms, enabling the analysis of the normal behavioural repertoire of each species and its deviations. Providing an environment that simulates and promotes the expression of natural behavioural patterns in the wild to its closest degree can play a positive role in the care and welfare of animals in confinement. Behavioural studies enable scientists to "impart a culture of care" by generating data to improve animal care and use programs and hastening the process of finding better housing standards. Behavioural analysis in animals also finds its use in drug discovery research and also in studying specific pathologies of diseases especially in the area of neurobehavioral research. This chapter reveals the secrets of measuring the animal's mental state from a practical point of view for the benefit of both laboratory animal species and mankind.

Keywords

Ethology · Preference testing · Anxiety · Maze · Operant conditioning

V. S. Harikrishnan (✉)
Division of Laboratory Animal Science, BioMedical Technology Wing, Sree Chitra Tirunal Institute for Medical Sciences and Technology, Thiruvananthapuram, Kerala, India
e-mail: harikrishnan@sctimst.ac.in

© The Author(s), under exclusive license to Springer Nature Singapore Pte Ltd. 2021

P. Nagarajan et al. (eds.), *Essentials of Laboratory Animal Science: Principles and Practices*, https://doi.org/10.1007/978-981-16-0987-9_19

19.1 Introduction

Laboratory animal science can be broadly classified into two entities, namely, "basic" and "applied". Veterinarians and researchers who work in the area of basic laboratory animal science focus on improving the standards of care, health status, and welfare requirements. Ethology, the study of behaviour and its analysis in the captive species, gives a true narrative of their welfare states and fulfilment of specific behavioural requirements. The term "welfare" deceives to be self-explanatory, and it is believed that the term is understood and interpreted by scientists uniformly. The issue is more complex and a bit philosophical as well. An example adopted with modifications [1] is the case of a fictitious lonely dog named *Snowy* left alone in the house by his owner, turning aggressive, destructive and noisy during his owner's absence. *Snowy* can be assigned to be having a reduced state of welfare and can be treated with antipsychotics, anxiolytics and mood elevators (first view), whereas it could also be considered that being species naturally living in packs, and making it live in solitude despite it being calm with the aid of medication, is yet compromised in welfare (second view). The first view, termed as "Hedonistic view", considers that it is the feelings and the mental state of the animals that matters and accepts treating the dog and letting it be in a seemingly peaceful state as welfare. In contrast, the beholder of the second view, the "perfectionist", considers the dogs as pack animals and to express its anxiety while in solitude is natural and the state of altered behaviour observed as calmness is not natural and hence the animal is in a reduced status of welfare even when it doesn't express anxiety, owing to the treatment with drugs. Attempts to define what is good or bad for an animal is based on values and measurements from the scientist's point of view. And there are many measurables (like corticosteroid levels in serum or faeces) based on which the conclusions can be drawn. Animals can neither communicate using words about their mental state nor can be measured using a scale, and this is when ethology can offer clues. However, like body temperature or blood pressure values, it is not possible to express the level of welfare in numerical units, and so attempts are made to quantify the data, to make the assumptions look more trustworthy. The animal behavioural study design is getting evolved in this direction. In addition to the previously stated views, "preference-hedonism" is another view on welfare that gathers data on "which pleasure is more desirable" and "which pain is more aversive" based on animal's preferences, thereby forming the basis of preference-based behavioural tests. Conventionally, the areas of prime focus as part of quality control in laboratory animal facilities included barrier maintenance and housing systems to maintain microbiological status quo, health and genetic monitoring, maintaining environmental standards using heating, ventilation and air conditioning (HVAC) systems and nutritional standards. However, in the recent past, the addition of species-specific behavioural requirements and its fulfilment into the array to ensure welfare has gained momentum.

Applied biomedical researchers are particularly interested in animal models as the tool for their research. They use animal behaviour to draw clues for their biomedical research queries especially in the fields of drug discovery, basic neurobehavioral and neuropsychiatry. Moreover, experimentation and assessment of underlying

anatomical and physiological processes behind the targeted behaviour are not possible in humans. Further, rather than owing to physiological or pathological processes, behavioural processes owe their explanations towards the evolution of animals, and more insights can be obtained by assessing animal behaviour to draw inferences on the targeted human behaviour.

19.2 Activity, Interspecies Interactions, Anxiety and Fear

Rodents, in their natural habitat, live near water sources in social groups, move around foraging, spend time for borrowing, nest building, avoidance or evading the predators and express territorial behaviour and a considerable party also spent for self-grooming and other physiological activities. Both the species avoid open spaces to avoid predators and are nocturnal. They exhibit fear towards new objects attracting the term "neophobic". Even while being neophobic, the rodents are exploratory as a part of finding feed and resources as well as to feed their curiosity. However, there is evidence that domesticated rats are less neophobic and that the laboratory animals are much tamed and gentler than their wilder counterparts [2]. The mice more readily explore new spaces, environments and objects and show lesser neophobic tendencies in comparison to rats. Neophobia can be against new types of food, bait boxes and monitoring devices in the wild which play a big role in avoiding poisoned food baits and rodent traps as a mechanism of survival. Further, they exhibit "thigmotaxis", a property of exhibiting affinity to the touch and feel of a wall or a vertical object in contact with their body, giving a sense of security. Even after a century of domestication and usage in laboratories, both these species still evidently retain their basic natural behaviour in the wild. Rats are natural predators of mice and will kill and or prey on mice. A set of pheromones termed as "alarm pheromones" secreted by rodents on exposure to a stressful situation can play a major role in alarming conspecifics about the presence of a threat, without exposing their conspecifics to the situation [3, 4]. In comparison to the activities they engage in, in the wild, the cage-bred animals in laboratories have a lot of leisure time, which makes them resort to vices or aberrant behaviour. It is also established that abnormal brain function can also lead to behavioural abnormalities [5], hence providing the animals with well-evaluated enrichment to ensure their welfare is of importance.

Before moving to the next section on aberrant behaviours, some commonly applied behavioural tests that find their origin from fear, anxiety, neophobia, thigmotaxis and exploration and physical activeness are discussed in detail here.

19.2.1 Open Field Test

The procedure involves letting the rodent tread on a flat surface with adequate grip (as a hard-plastic floor) within surrounding opaque walls of metal or plastic of 1.5–2 feet height for 5 min. The arena can be either round or square, and the area

Fig. 19.1 Model for open field test

can also vary as per different research groups. As an example, the diameter could be around 100 cm for both rats and mice [6] (Fig. 19.1). The animals are left at the central point, and a 5-min video recording is done, ideally without the presence of the operator. The analysis is done by arbitrarily splitting the full arena into a central area and periphery, and peripheral areas can be subdivided into several quadrants. The time of rest and activity (definition of rest has to be predefined, e.g. will grooming be considered as an activity or not), the number of visits and time spent in the central arena and the number of crossings in total between arenas [6] are noted. The total distance travelled can also be measured if the analysis is automated. The less anxious animals are likely to spend more time in the centre and be more active in general.

19.2.2 Dark-Light Box

A box that has dark and light areas and the dark side painted black and having a roof is used. An opening through which the animal can get access to both sides is present at the interface. The test starts by placing the animal on one side of the box. Several

variations in the initial positioning, dimensions of chambers and their comparative sizes have been reported by many groups in rodents. The test is also reported to be conducted in a range of light intensities from 2 Lux or 8 Lux in the dark box and the light side-lit brightly with 390 Lux or even 600 Lux. The number of transitions the animals perform between the two sides and the latency (time taken for emerging from the dark compartment to the lit side) shall be noted. The more the number of transitions and the lesser the latency to emerge into the lighter side, the less anxious or less fearful behaviour the animal is exhibiting. Animals with lower anxiety and neophobia will exhibit lesser latency and spend more time in the lit area.

19.2.3 Elevated Plus Maze (EPM)

This maze has two closed and two open arms connected by a common central stage with high walls with an open topside to provide darkness and at the same time permit observation (Fig. 19.2). The maze is erected at about 2 feet high from the floor to prevent the animals from leaving it by choice, and the dimensions vary as reported by many groups in both the rodent species. During the test, the animal will be left free at the central stage, and the operator leaves the room allowing a 5-min video recording, and the analysis includes the number of entries made and the total time spent in the covered and the open arms. The time spent in the open central area and the number of attempts to place their front paws and face to the open area and to hesitantly attempt exploring the open arms even without making a complete entry are termed as risk assessments and are also calculated [6]. The animals that are with lower anxiety, fear or depression will spend more time in open spaces, will be more active and perform more risk analyses.

Fig. 19.2 Model for elevated plus maze test

19.3 Aberrant Behaviour

Normal behavioural adjustments play a major role in the homeostasis of the biological organism, enabling them to maintain, control and modify its environment, whereas the scope for making these behavioural modifications is limited in captivity, leading to the expression of modified or aberrant behaviour [5].

Barbering, bizarre fighting, stereotypic behaviour, unusual aggression towards being handled and bar biting are some commonly observed aberrant behaviour in laboratory rodents. Barbering is chewing away or eating of hairs, seen in group-housed mice and rats where the animals will be seen as "cleanly shaven" in localized areas of their body in general. Mostly, the areas over the back and on the sides of the muzzle and whiskers (whisker trimming or eating) are being barbered, and usually the cage mate who exhibits this behaviour (partner-directed) can be identified as the one with an intact fur coat. Barbering is considered as an indicator of an environmental stressor and as a sign of neural impairment [7], whereas it has also been proposed as an expression of social dominance and behavioural adjustment to cope with the stressful environment of the animals being housed [8]. However, there is no evidence whether the barbers get a stress relief or could have lowered anxiety levels after the act of barbering. It has also been noticed that placing toys that are routinely replaced can reduce barbering; however, complete elimination of the behaviour is not reported [9].

Aggression and conspecific fighting are commonly observed in group-housed male mice, and strain differences are also reported in the degree of this behaviour. Being social animals, it is recommended to group house mice and from a management perspective; males will be housed together. In this scenario, aggression is normally observed because never in the wild male mice share territories [10] that lead to fighting, serious wounds and even death in cages indicating compromised welfare. It is also reported that many management practices can aggravate fighting in male mice. Ear-notched male mice showed more aggression in comparison to tail-tattooed mice, and the male mice kept on the rack facing the active side of the room exhibited more aggression in comparison to animals housed in racks facing the other side [11]. Rats housed in increased cage densities exhibited fighting, and in juvenile rats, this was more of a play-fight [12].

Stereotypic behaviour in laboratory animals can be defined as repeated meaningless action. One of the most commonly observed stereotypic behaviours in rodents is the repeated and continuous circling movement around the perimeter of the cage floor. Another version of the same is noticed as the animal assumes a rearing position, and it rotates the upper part of its body in circles over and over (mostly observed in rats). Some stereotypes after climbing on the cage top-grill (more commonly seen in mice) circle on the grill and bite the bars of the top grill.

19.4 Senses and Communication

Both the species perceive and emit ultrasound and can communicate using ultrasound vocalizations, which is above the audible range of humans. The auditory range for humans is 20 Hz and 20 kHz [13], whereas for mice and rats, it is 1 kHz to 80–90 kHz [14, 15]. High decibel noises above 85 dB can cause eosinopenia, increased weights of adrenal glands and reduced fertility in rodents. Audiogenic seizures are observed in rats and mice as well.

Rats cannot see things clearly and perceive objects very vaguely beyond a few feet distance. Mouse retina has two types of cones which serve them to sense the yellow-green side of the spectrum and the UV region which humans and many other mammals are unable to perceive. The rodents cannot see through the red end of the colour spectrum; hence, they perceive red colour as opaque or as a dark shade. So, it could be naturally hypothesized as the animals being nocturnal and aversive towards bright light might prefer red shades of cages. But there are studies in which this hypothesis was proven not to be accurate. A study on cage colour preference was set up, and the study showed that white cages were preferred mostly as a choice by the CBA mice and red cages the least, suggestive of avoidance of the environment which could have induced a negative mental state [16]. It is also reported that the usage of coloured enrichment devices [17], as well as different colour-tinted cages [18], can affect the scientific outcomes of the study significantly by altering the levels of plasma measurements of metabolism and physiology in rats.

Whiskers or vibrissae in rodents offer them a tactile sense of objects around their immediate vicinity even though tactile sensory inputs can be perceived from all over the body. Pheromones are another major tool for communication in these rodent species. Rodent urine, porphyrin, semen and saliva fluoresce in ultraviolet light. In the wild, their ultraviolet-sensing vision detects fluorescing urine which is used for territorial marking which plays a part in communication as well.

A wide range of terrestrial mammals use olfactory cues for various purposes, and among laboratory species, fishes, rats, mice rabbits, dogs and pigs use pheromones for communication, and the presence of a vomeronasal organ in the nasal cavity as a sensory organ plays an important role in the olfactory communication. From the detection of nipples of their dams and suckling of milk by the day-old bunnies and pups, while the eyes have not opened, to courtship, aggression and using the trails of other rats to find the source of water, olfactory cues play a huge role in the biology and survival of these animals [19, 20]. Influence of season on behaviour like investigatory behaviour and blood corticosteroid concentrations in laboratory mice within the environmentally controlled laboratory animal facility maintained in 12:12 L:D cycles has been reported [21] which points towards the existence of an endogenous biological clock in these animals.

19.5 Learning and Memory

Rats are known to accomplish even complex tasks and to solve mazes and also learn and memorize the ways they solved the puzzles earlier. Interestingly, studies indicate that learning and behaviour can vary between strains. C57BL/6 and DBA mice were proven to have well-differentiated spatial learning abilities [22]. Differences in neuronal micro-anatomy and protein kinase-c concentration in the hippocampus between C57 and DBA mice strains are also reported [23, 24]. Rodents depend a lot on the sense of smell and communication as a mechanism of survival, the reason why naturally fear-based learning is highly evolved in them.

The limbic system is a set of structures in the brain dealing with emotions and memory and the hippocampus is an important part regulating motivation, emotions, learning and memory in both humans and rodents. The amygdala is involved in emotional responses, emotionally driven learning and memories in rodents and human beings, and it is important to use a fear conditioning challenge to test this limbic region. At localized levels, the visual cortex, primary sensory cortex and auditory cortex also play a role in learning. So, to comprehensively test all these areas, a well-designed protocol is required. Parahippocampal regions of the temporal lobe including the perirhinal, entorhinal and inferior temporal lobe cortex facilitate object recognition and memory in rodents and monkeys [25, 26]. The intactness of memory can be used to analyse the pharmacological efficacy of drugs but also to understand the pathogenesis of Alzheimer's disease and spinal cord injury.

Broadly, the tests can be divided into tasks, firstly, requiring a stimulus (either positive or negative) to elicit a change in behaviour and, secondly, preference-based tests to investigate novel stimuli. Learning can be studied using a spectrum of operant tests in which automated sensor-based or mechanical mazes can be used to assess the animals based on their abilities to perform certain prefixed goals. There are reward-based tests in which learning to push on and operate an inbuilt lever gives a food treat or a punishment-based test in which an aversive stimulus results as a result of stepping on an area, like an electric shock on paws. The animals after the first exposure to aversive stimuli learn to avoid the particular area. Automated touchscreen-based rewards systems to learn cognitive deficits are also used to study traumatic conditions of the brain and also for other similar purposes where the animals have to touch a button on a touchscreen to obtain a food reward.

Memory and learning can be tested using positive reinforcement using tests like "Y" and "T" mazes, and radial arm maze [27]. The basis of all these tests is the ability in terms of several trials and errors the animals commit while trying to locate a food treat offered in one arm in T, Y or radial arms, on a definitely and systematically alternating pattern. For testing memory and spatial learning, the following tests are widely used.

19.5.1 Novel Object Recognition (NOR) Test

The animals are familiarised with two newly placed similar objects for some time (familiarisation phase) in an arena, and then after an interval, animals are reintroduced into the same arena in which one of the two objects is replaced using a novel object of entirely different shape and dimension (test phase). The animals with intact memory will ignore the familiar object and explore the novel object more. The definition of exploration shall be predefined, as closely looking at the object, biting, touching using paws or snout, licking, rearing on it or sniffing. Short- and long-term memory can be tested by adjusting the time between the familiarization phase and the test phase. The usually tested parameter as a measure of memory is discrimination index (DI), measured as $DI = TN - TF/TN + TF$, where TN is total time spent with the novel object and TF is total time spent with the familiar object [28]. As in the other behavioural tests, variations are widely used in the testing set up and the objects used to perform NOR as well.

19.5.2 Morris Water Maze

This is a widely used test since its inception in 1980 to test spatial learning and memory in rats and mice. The test has the potential to assess discrimination learning, latent and cued learning. The apparatus includes a circular container with water and sufficiently high walls to prevent the animals from not stopping swimming, and somewhere in the apparatus, just below the level of water, is a raised platform that, when discovered, can be used to perch on safely. The water is made opaque using inert chemicals to prevent the animal from seeing the raised platform. Once the animals are tested initially, using some spatial cues, they learn the position of the platform, and every subsequent attempt takes lesser time. It should be worthy to apply caution that even blind rats can successfully use reference memory and complete the cued task, hence questioning the utility of the test to screen animals with visual sensory deficits [29].

19.6 The Role of Environment on Animal Behaviour and Homoeostasis

A much familiar figure in the field, “22 ± 2 °C”, is generally considered to be the standard range for temperature settings in rodent housing and well-being. Mice and rats housed in laboratories at 20–24 °C are being housed way below their lower critical temperature which is approximately 30 °C where the range of their thermoneutral zone (TNZ) is from 26 °C to 34 °C. Hence, housing these animals at lower temperature ranges will increase the thermal stress and may alter scientific outcomes which in turn makes the mice consume about 60% more food than in warmer temperatures and will lead to alterations in behaviour [30]. In a preference test, C57BL/6J mice, during their inactive, maintenance and resting phases of the

day preferred 30 °C from a range of home cage temperatures, offered to choose from 20, 25 or 30 °C, whereas no preference was observed during active phase [31]. The study concluded that C57BL/6J mice housed at 20–24 °C are not being housed based on their comfort as per evidence obtained from the preference tests and is indicative of the fact that mice preferences altered based on their gender as well as activity levels. This example illustrates the importance and utility of behavioural tests in the design of care and use programs and welfare assessments.

Published data suggest that an increase of temperatures leads to cage mate aggression, and hence temperatures much below species-specific TNZ is used as the housing standard. In the wild during colder months, the aggression between the males decreases, and the non-compatible animals share the same shelter space, and animals cuddle for conserving heat, thereby maintaining harmony [32]. This is important while considering denser housing in facilities which is a prerequisite for practical reasons and efficient experimental resource management. In the wild, both the species burrow subterranean holes to live in, to tackle the rise of environmental temperatures and to avoid being preyed upon. Further, in colder weather, they are reported to use a variety of material from nature to build highly complex nests, mice in particular. It is scientifically proven that nesting material can alleviate thermal discomfort in mice [30]. Departure from wellness and normal ethological homeostasis can be tested using nest building in mice and sucrose preference and dark-phase home cage activity in rodents.

19.6.1 Nest Building Test

Nesting behaviour of mice is used to assess the level of normal ethological activity in mice [33]. The nesting is also sensitive to lesions in the brain, certain pharmacologic agents and some mutations [33]. The use of commercially available nestlets and a translucent mouse igloo with three openings can be used for this test. Once nestled, each is offered per cage of group-housed mice and the percentage shredded out, and the number of openings in a mouse igloo covered by nesting material is assessed on an hourly basis over 24 h (excluding observations during 12 h of dark phase) and graded on a 5-point assessment scale. Many modifications of the basic test find applications in various areas of research like in schizophrenia [34] and in blood collection routes and welfare [6]. The mice will be deprived of any nesting material and will be provided with mouse igloos for acclimatization for 2 weeks before the introduction of nestlets (Fig. 19.3).

19.6.2 Sucrose Preference Test to Assess Hedonic Behaviour

The natural tendency to seek pleasure or “hedonic behaviour” is tested in rodents by simultaneously offering sweetened sugar solution (1.5%) and plain water in two different bottles (Fig. 19.4). Normally the animals selectively drink sweetened water. The bottle weights are recorded before being offered to the animals, and after 24 h,

Fig. 19.3 Model for nest building test

Fig. 19.4 Model for sucrose preference test

again the weight difference is noted and compared between groups. As a matter of caution, there had been many papers publishing data where groups did not show differences in the sucrose preference tests, and specific automated apparatus to bring in precision to the test has been demonstrated [35]. Sucrose preference as a measure to assess depression and motivation to move around and seek the pleasure from sweetened water in rodents [36] also assesses and differentiates anergia and fatigue between animals.

19.6.3 Dark-Phase Home Cage Activity and Rearing

The animals can be observed in their home cages for their activity and rest in total, social interaction, deviant behaviours and for specific signs of pain if any using recorded videos using night-vision cameras or by direct observation with the aid of

Table 19.1 Effects of environmental parameters and routine variables in animal facilities on laboratory animal behaviour

Environmental factor/conditions	Effects on behaviour	References (nonexhaustive)
Transport	Aggression, overall stress	[39]
Light (colour intensity and temperature)	Reduced playfulness, increased aggression, lower spontaneous activity, raised anxiety, reduced locomotion	[40]
Noise	Food intake, acoustic startling, fear, reduced grooming activity, impaired cognition, suppressed exploration, increased aggression	[41–44]
Vibrations (audible and infrasound)	Altered locomotion and grooming	[45]
Blood collections (responses vary with various routes and techniques and between species)	Anxiety-like behaviour, reduced activity, nest building	[6]

red bulbs during the dark phase. Removal of nesting materials, dwelling tubes and other enrichment materials well beforehand during the rest phase will aid the observation of the animals. However, this may cause some alterations due to changes in the environment, but randomization and handling all the groups similarly can hopefully address the confounding variable. Many researchers also reverse the dark-light cycle to suit their convenience, but it shall be remembered that the chances of interruption of the dark and active phase are more in this type of setting. In a home cage activity monitoring, the pre-set definition of what all are considered as activity shall be stated. However, it is important to note that long-term, low-intensity red light exposure during the scotophase can adversely affect the circadian rhythm and also alter the metabolic and physiologic parameters of well-being in rats [37]. Dim light exposure will stimulate tumour production and alter melatonin release by pineal glands, thereby affecting the health and circadian physiology considerably and is equally damaging as a constant light would have done during the scotophase (Tables 19.1 and 19.2) [38].

19.7 Pain-Associated Behaviour

Pain in rats is generally characterised by observing freezing, aggression to handlers, jumping and rarely by vocalization. Specifically, in case of abdominal pain, rats exhibit twitching, abdominal press, stagger, writhe, hop, partial or complete falling and back arch. Identification of abdominal pain in mice is by observing back arch, circle, fall, flinch, abdominal press, rear leg lift, raised tail, stagger, twitch and writhe [49]. Quantification or measuring in a scale is done by video analysis of these signs from 5-min videos and affixing 1 point each for each sign and summing up in each animal so that animals from different groups can be compared [50]. More details on

Table 19.2 Some of the commonly employed tests which use rodent behaviour to study specific targeted human diseases/systems/pharmacological agents

Behavioural test used	Behaviour studied	References (nonexhaustive)
Elevated plus maze	Anxiety, activity	[6]
Open field test	Anxiety, activity	[6]
Nest building test	Overall welfare, activity, depression and anxiety	[6]
Sucrose preference	Hedonic behaviour	[6]
Dark-light box	Anxiety, activity	[46]
Forced swimming test	Behavioural despair, depression-like behaviour	[47]
Inverted flower pot	Sleep	[48]
Novel object recognition	Memory	[28]
Morris water maze	Impairment in learning and memory abilities, spatial learning	[47]

all these symptoms can be read in the chapter on anaesthesia, analgesia and euthanasia.

For detecting acute pain of various origins, Grimace scales have established that it makes use of four action units in rats and five in mice. The action units are orbital tightening, nose/cheek flattening, ear changes and whisker changes in rats [51], and in mice, the action points are orbital tightening, nose bulge, cheek bulge, ear position and whisker changes [52]. In practice, acrylic boxes that are longer than wide with the longer surfaces obscured with opaque black paint or plastic from the outer surface to motivate the animals face the transparent ends where video cameras are being placed on both ends. Baseline values from each animal before the infliction of the painful stimulus (the experiment) can act as the control. The control value is subtracted from the post-test value to obtain the differential Rat Grimace Scale (RGS) score, for each action unit. The scoring can range from zero (pain not present), one (moderate pain) and two (extreme/obvious pain). For chronic pain, this method is not sensitive.

19.8 General Considerations in Behavioural Testing

Rats recognize their handlers and get more docile and pet-like on repeated handling and habituation. A technique known as gentling or tickling of rats is recommended to bring down stress due to handling well before animal studies commence. In comparison to the rats, mice are less docile. Lifting rats by their tails for cage changing can turn the temperament to aggressive.

Variability in behavioural studies tends to be high, and in studies and so, it is important to also include a quantitative parameter like serum glucose or faecal corticosteroid concentration or histopathology to ascertain the results obtained. Including baseline observations from the same animal before the experiment is

performed to keep track of individual variations as control values are advised, and this helps to exclude animals that are outliers.

Studies show that male researchers induce more stress in mice in comparison to female staff and thereby clearly indicating the ability of rodents to identify the handlers. Male staff, or their T-shirts or secretions from axilla when presented to a group of mice, produced stress-induced analgesia, and when female staff handled them, they did not produce the reaction [53]. It is important to notice the lower reproducibility in behavioural research considerably owes to failure in detailing of materials and methods including the gender of the animal handler. Differences could also occur between the studies depending upon whether they were conducted during the dark or light period. Circannual changes can also have an impact on stress levels, which could have an indirect impact on results obtained and in turn a possible interstudy variability of results [21]. It is important to note that there are many variations reported in the dimensions of the setup where even cleaning of the maze between the tests or leaving the animals in a soiled maze resulted in variable results [54]. So, reporting of variables and details of the study setup is as equally important as conducting an artefact-free study incorporating animal behaviour as a parameter.

Removal of faecal pellets between tests and cleaning and removing the scent of the previous animal from the arena using alcohol wipes and also randomizing the animals to be tested are of importance to avoid confounding of results in the test, and this applies to all the mazes. It is also important to make sure the spirit used for wiping has evaporated from the surfaces before introducing the animals, since ethanol is proven to modulate exploratory behaviour in mice by inducing avoidance and decreased locomotor activity [55].

Automatization of the memory test assessment with software is being done to avoid bias and to achieve precision in the assessment of parameters like total distance travelled. In automated software, total distance travelled and time can also be obtained which is not feasible with manual analysis. But this will also increase the cost of setting up tests. The locomotor ability of animals plays a major part in performing many of the tasks, and this reflects in the results obtained, and the fact shall be considered during the designing, evaluation, drawing conclusions and making comparisons with other reported studies. There are a multitude of tests which can be used to functionally assess motor function and coordination which can be also incorporated into the testing battery of the likes of rotarod, beam walking, grid walking, staircase test, treadmill test, etc. to name a few, to evaluate motor deficits if any in the animals.

19.9 Conclusion and Future Perspective

To summarize, ethological data is used to improve the standards of animal care and also in drug discovery and basic research. There are over 400 mice and 200 rat strains, and the requirement and behavioural responses differ between many strains, and within each strain, it is important to bear in mind that requirements differ based on the physiological status, sex and age of animals, to state a few. In practice, while

applying a preference-based test to measure feelings, it shall be taken into account that the animals lack long-term preferences [56]. So, seeking an animal's pleasure and designing care programs according to the results using preference tests shall be done with caution. The existence of complexities in preference behavioural studies is another issue to be considered. An example to explain this is that of pigs that exhibit a high preference for straw for foraging. This preference was considered true for every case. But when deeply evaluated, the preference was shown to be dependent upon many other factors. When compounded feed was available, the preference for straw as a foraging option diminished, and as a bedding material on the floor, it was either liked or disliked based upon whether the environment was cool or warm. So, evaluating multiple factors as interlinked components to assess the scenario on a whole is recommended while designing and interpreting preference tests.

Implementing the findings from preferences to suit the purposes is of importance. Intense research housing of pigs on solid floors deprives them of the natural rooting behaviour and social grouping, and a natural environment will be more preferred by them, but aggression, difficulty in health management and setting up of experiments (when animals lodge persistent cannulas, sutures) and data collection can be affected. Design of pens having transparent cage walls with perforations allowing communication using snouts, flooring supplemented with thickly laid straw to encourage rooting behaviour, providing feed pellets sprinkled into the bedding, fruity treats like apples being offered to assist the training of animals as means of positive reinforcement [57], etc. are built in to strike a balance. A similar approach to rabbit housing can also be observed as part of evolving concepts. Wire floors are replaced (some national legislation prevents wire floors for rabbits, e.g. Danish legislation) with solid plastic slatted ones, and providing forage, group housing females and food treats are common features in modern housing.

Hence, problems can be identified and solutions developed, using behavioural studies for better animal care. Furthermore, behavioural studies are instrumental in generating data to aid the research world to find the effects of pharmacologic agents and to understand underlying neuronal mechanisms of complex yet not unravelled diseases like Alzheimer's disease, post-traumatic stress disorder (PTSD) and many more.

References

1. Sørensen D (2007) Animal welfare—an introduction. Springer, Dordrecht, pp 3–14
2. Wahlsten D, Metten P, Crabbe J (2003) A rating scale for wildness and ease of handling laboratory mice: results for 21 inbred strains tested in two laboratories. Genes Brain Behav 2 (2):71–79
3. Brechbühl J et al (2013) Mouse alarm pheromone shares structural similarity with predator scents. Proc Natl Acad Sci U S A 110(12):4762–4767
4. Carew SJ et al (2018) Pheromone-induced odor associative fear learning in rats. Sci Rep 8 (1):1–10
5. Garner JP (2005) Stereotypies and other abnormal repetitive behaviors: potential impact on validity, reliability, and replicability of scientific outcomes. ILAR J 46(2):106–117

6. Harikrishnan V et al (2018) A comparison of various methods of blood sampling in mice and rats: effects on animal welfare. Lab Anim 52(3):253–264
7. Bechard A, Meagher R, Mason G (2011) Environmental enrichment reduces the likelihood of alopecia in adult C57BL/6J mice. J Am Assoc Lab Anim Sci 50(2):171–174
8. van den Broek FA, Omtzigt CM, Beynen AC (1993) Whisker trimming behaviour in A2G mice is not prevented by offering means of withdrawal from it. Lab Anim 27(3):270–272
9. DeLuca AM (1997) Environmental enrichment: does it reduce barbering in mice? Animal Welfare Information Center Newsletter (USA)
10. Kappel S, Hawkins P, Mendl MT (2017) To group or not to group? Good practice for housing male laboratory mice. Animals 7(12):88
11. Gaskill BN et al (2017) The effect of early life experience, environment, and genetic factors on spontaneous home-cage aggression-related wounding in male C57BL/6 mice. Lab Anim 46 (4):176–184
12. Barker TH et al (2017) Assessment of housing density, space allocation and social hierarchy of laboratory rats on behavioural measures of welfare. PLoS One 12(9)
13. Sivian L, White S (1933) On minimum audible sound fields. J Acoust Soc Am 4(4):288–321
14. Kelly JB, Masterton B (1977) Auditory sensitivity of the albino rat. J Comp Physiol Psychol 91 (4):930–936
15. Ehret G (1974) Age-dependent hearing loss in normal hearing mice. Naturwissenschaften 61 (11):506–507
16. Sherwin C, Glen E (2003) Cage colour preferences and effects of home cage colour on anxiety in laboratory mice. Anim Behav 66(6):1085–1092
17. Wren-Dail MA et al (2016) Effects of colored enrichment devices on circadian metabolism and physiology in male Sprague–Dawley rats. Comp Med 66(5):384–391
18. Wren MA et al (2014) Effect of different spectral transmittances through tinted animal cages on circadian metabolism and physiology in Sprague–Dawley rats. J Am Assoc Lab Anim Sci 53 (1):44–51
19. Pérez-Gómez A et al (2014) Signaling mechanisms and behavioral function of the mouse basal vomeronasal neuroepithelium. Front Neuroanat 8:135
20. Melo AI, González-Mariscal G (2010) Communication by olfactory signals in rabbits: its role in reproduction. In: Vitamins & hormones. Elsevier, Amsterdam, pp 351–371
21. Meyer L, Caston J, Mensah-Nyagan AG (2006) Seasonal variation of the impact of a stressful procedure on open field behaviour and blood corticosterone in laboratory mice. Behav Brain Res 167(2):342–348
22. Ammassari-Teule M, Castellano C (2004) Strains of rodents and the pharmacology of learning and memory. Neural Plast 11(3–4):205–216
23. Schwegler H et al (1988) Water-maze learning in the mouse correlates with variation in hippocampal morphology. Behav Genet 18(2):153–165
24. Wehner JM, Sleight S, Upchurch M (1990) Hippocampal protein kinase C activity is reduced in poor spatial learners. Brain Res 523(2):181–187
25. Aggleton JP et al (2010) Lesions of the rat perirhinal cortex spare the acquisition of a complex configural visual discrimination yet impair object recognition. Behav Neurosci 124(1):55
26. Hammond RS, Tull LE, Stackman RW (2004) On the delay-dependent involvement of the hippocampus in object recognition memory. Neurobiol Learn Mem 82(1):26–34
27. Sousa N, Almeida O, Wotjak C (2006) A Hitchhiker's guide to behavioral analysis in laboratory rodents. Genes Brain Behav 5:5–24
28. Antunes M, Biala G (2012) The novel object recognition memory: neurobiology, test procedure, and its modifications. Cogn Process 13(2):93–110
29. Lindner MD et al (1997) Blind rats are not profoundly impaired in the reference memory Morris water maze and cannot be clearly discriminated from rats with cognitive deficits in the cued platform task. Cogn Brain Res 5(4):329–333
30. Gaskill BN et al (2012) Heat or insulation: behavioral titration of mouse preference for warmth or access to a nest. PLoS One 7(3):e32799–e32799

31. Gaskill BN et al (2009) Some like it hot: mouse temperature preferences in laboratory housing. Appl Anim Behav Sci 116(2/4):279–285
32. Bayne KAL et al (2015) In: Fox JG et al (eds) Chapter 38: Laboratory animal behavior, in laboratory animal medicine, 3rd edn. Academic Press, Boston. p, pp 1617–1651
33. Deacon RM (2006) Assessing nest building in mice. Nat Protoc 1(3):1117
34. Pedersen CS et al (2014) PCP-induced deficits in murine nest building activity: employment of an ethological rodent behavior to mimic negative-like symptoms of schizophrenia. Behav Brain Res 273:63–72
35. Frie JA, Khokhar JY (2019) An open source automated two-bottle choice test apparatus for rats. HardwareX 5:e00061
36. Overstreet DH (2012) Modeling depression in animal models. In: Psychiatric disorders. Springer, New York, pp 125–144
37. Dauchy RT et al (2015) The influence of red light exposure at night on circadian metabolism and physiology in Sprague–Dawley rats. J Am Assoc Lab Anim Sci 54(1):40–50
38. Dauchy RT et al (1999) Dim light during darkness stimulates tumor progression by enhancing tumor fatty acid uptake and metabolism. Cancer Lett 144(2):131–136
39. Conour LA, Murray KA, Brown MJ (2006) Preparation of animals for research—issues to consider for rodents and rabbits. ILAR J 47(4):283–293
40. Kapogiannatou A et al (2016) Effect of light colour temperature and intensity on τhε behaviour of male C57CL/6J mice. Appl Anim Behav Sci 184:135–140
41. Nayfield K, Besch E (1981) Comparative responses of rabbits and rats to elevated noise. Lab Anim Sci 31(4):386–390
42. Algers B, Ekesbo I, Strömberg S (1978) The impact of continuous noise on animal health. Acta Vet Scand Suppl (68):1–26
43. Johnson PM (2006) Guidelines for the housing of Guinea pigs in scientific institutions. A.W.B. (ed) ARRP, New South Wales Department of Primary Industries, Orange, NSW, 55p
44. VOIPIO HM (1997) How do rats react to sounds? Scand J Lab Anim Sci 24(Suppl 1):1–80
45. Berezhnoy DS et al (2015) Influence of high-intensity sound vibration on rat behavior. Mosc Univ Biol Sci Bull 70(2):53–57
46. Heinla I et al (2018) Behavioural characterization of C57BL/6N and BALB/c female mice in social home cage–effect of mixed housing in complex environment. Physiol Behav 188:32–41
47. Jørgensen BP et al (2014) A possible link between food and mood: dietary impact on gut microbiota and behavior in BALB/c mice. PLoS One 9(8)
48. Mendelson WB et al (1974) The flower pot technique of rapid eye movement (REM) sleep deprivation. Pharmacol Biochem Behav 2(4):553–556
49. Leach MC et al (2012) The assessment of post-vasectomy pain in mice using behaviour and the Mouse Grimace Scale. PLoS One 7(4)
50. Roughan JV, Flecknell PA (2003) Evaluation of a short duration behaviour-based post-operative pain scoring system in rats. Eur J Pain 7(5):397–406
51. Sotocina SG et al (2011) The Rat Grimace Scale: a partially automated method for quantifying pain in the laboratory rat via facial expressions. Mol Pain 7. 1744-8069-7-55
52. Langford DJ et al (2010) Coding of facial expressions of pain in the laboratory mouse. Nat Methods 7(6):447
53. Sorge RE et al (2014) Olfactory exposure to males, including men, causes stress and related analgesia in rodents. Nat Methods 11(6):629–632
54. Bourin M, Hascoët M (2003) The mouse light/dark box test. Eur J Pharmacol 463(1–3):55–65
55. Grau C et al (2019) Ethanol and a chemical from fox faeces modulate exploratory behaviour in laboratory mice. Appl Anim Behav Sci 213:117–123
56. Sørensen DB et al (2007) The impact of tail tip amputation and ink tattoo on C57BL/6JBomTac mice. Lab Anim 41(1):19–29
57. Sørensen DB (2010) Never wrestle with a pig. Lab Anim 44(2):159–161

Part III

Laboratory Animal Techniques and Practices

Nonsurgical Techniques in Laboratory Animals

20

S. Ramesh and B. Vasanthi

Abstract

Any in vivo experiment involves collection of blood and administration of substances. These procedures involve handling and restraining of animals which often induce stress to the animal and attract scrutiny by ethical agencies. This chapter aims to enlist the common routes of drug administration and the sites of blood collection. Practical information such as volume of solution to be given, anatomical location of sites and size of needles, etc. have been given for the benefit of the reader. This chapter will be useful for an investigator like a practical manual before taking up animal experiments. However, as ethics and law require, it is recommended that any investigator should undergo practical hands-on training before initiating any study. This chapter is better learnt by doing than reading.

Keywords

Laboratory animals · Procedures · Drug administration · Blood collection · Nonsurgical

S. Ramesh (✉)
Department of Veterinary Pharmacology and Toxicology, Madras Veterinary College, Tamil Nadu Veterinary and Animal Sciences University, Chennai, Tamil Nadu, India
e-mail: ramesh.s@tanuvas.ac.in

B. Vasanthi
Poultry Research Station, Centre for Animal Production Studies, Tamil Nadu Veterinary and Animal Sciences University, Chennai, Tamil Nadu, India

© The Author(s), under exclusive license to Springer Nature Singapore Pte Ltd. 2021

P. Nagarajan et al. (eds.), *Essentials of Laboratory Animal Science: Principles and Practices*, https://doi.org/10.1007/978-981-16-0987-9_20

Abbreviations

°C	Degree centigrade
G	Gauge (size of needle)
g	Grams
in.	Inch
kg	Kilogram
max	Maximum
ml	Millilitres
mm	Millimetre
μl	Microlitre

20.1 Introduction

Administrations of drugs and solutions and collection of blood and body fluids are routinely done in biomedical experiments. Toxicological studies involve regular, if not daily, administration of drugs. In vaccine studies, too, vaccines, standards, adjuvants, etc. are administered through various routes. It is important to acquire the skill of restraint and administration before initiating the experiment. Research outcome hinges on the precision of the procedures. Since we deal with exceedingly small quantities of drugs, spillage of even a drop of it outside the desired site may lead to gross errors in experiment. This chapter describes the procedures involved in the restraint, handling, and drug administration in common laboratory animal species.

20.2 Handling and Restraint of Laboratory Animals

The first step in the administration of drugs is the proper handling and restraint of animals. Handling refers to holding the animal in proper position for the purpose of transfer to another cage or surface. Restraint is the art of keeping the animal in fixed position for definite period for the purpose of any manipulation such as blood collection, drug administration, etc. Ethical principles require proper handling of the animal in a manner that they suffer no pain or discomfort. Since rat and mouse are the common species used in most animal experiments, the procedures of handling and restraint of these animals are discussed in detail. However, handling methods for other species like guinea pigs, hamsters, and rabbits are also mentioned.

The question that arises foremost in the mind of a beginner before handling laboratory animals is ‘Will they bite?’. Yes, all animals with teeth tend to bite. The tendency of the animal is to test and taste and bite anything that is presented in the size and shape of a nut/pellet. Animals that are irritated or in pain and discomfort also

tend to bite back. It is therefore important to ensure that the animals are healthy and do not suffer from any pain or discomfort.

In general, animals do not relish being handled and try to evade when a human hand is introduced into the cage. Repeated handling and giving a feeling of comfort will ensure that they eventually take it better. It is therefore important that the animals are repeatedly handled during the acclimatization period. It is a good practice to adopt the five principles while handling laboratory animals to reduce stress to the animal [1]:

1. *Anticipation*: Before handling, anticipate the behaviour of the animal depending upon its physiological status and temperament.
2. *Approach*: The animal should be approached confidently and without alarming the animal. The animal should be made aware of the presence of the handler by calling the animal or allowing the animal to sniff the handler.
3. *Care*: Sufficient care must be taken to handle the animal to avoid struggling of the animal. Special care must be taken while handling sick, injured, or pregnant animals.
4. *Relax*: The handler must relax and comfort the animal by holding it appropriately and train the animal for handling and restraining procedures.
5. *Return*: The handling procedure ends with return of the animal back to its cage. The animal should be placed back carefully into its cage once the intended procedure is complete. The door of the cage must be properly closed to avoid escape of the animal.

Before undertaking any experiment with animals, the investigator must undergo training in the handling of animals, along with knowledge on biology, ethics, and drug administration. National agencies and ethical committees of various institutes have made it mandatory that investigators undergo a basic level training before initiating the in vivo trial. There are many institutes that offer such training programmes.

20.2.1 Rat

Before handling, an investigator must patiently learn to observe the normal behaviour of a rat. A good observation of cage activities will help to identify disease and stress in animals. It is advisable to handle the animals after observing them. A laboratory rat weighing about 100–150 g, suiting the size of a human palm, will be easier to handle for a beginner. On the other hand, mouse is smaller and is also more irritable and 'jumpy' compared to a rat. Therefore, it is good to start with a rat.

In case an animal is non-cooperative and is jumping around, the operator may place the animal in the forearm and folded elbow, giving some leverage to move around, to assuage it, and to reduce the aggression and anxiety of the animal. Then the animal becomes easier to handle.

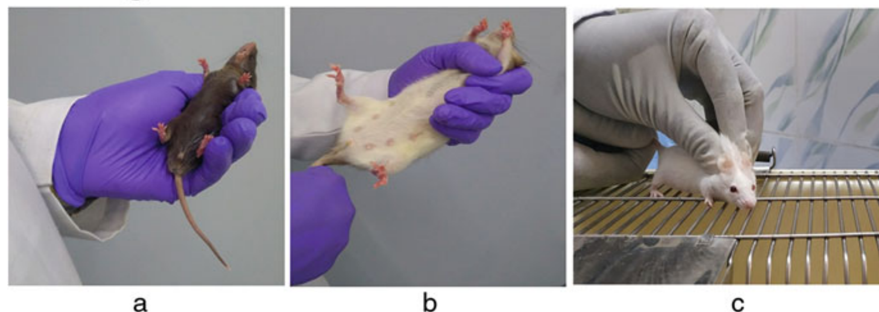


Fig. 20.1 (**a**–**c**) Holding and handling of rat and mouse

For transferring rats from one cage to another, which are kept adjacent to each other, a common practice is to lift the rat by the base of its tail. Care must be taken not to lift animals by the lower parts of the tail as it may cause pain and discomfort. It is stressful to the animal to hold by the tail and must be avoided [2]. Instead, handling tunnels can be used to transfer animals between cages and to habituate animals for handling. The rats that are habituated to handling can normally be picked up easily by grasping them around the shoulders (with the index and middle fingers along the sides of the head and the thumb and remaining fingers under the axilla).

The loose skin in the back of the neck or nape region (called the scruff) can be used to exert a firm grip and handle the rat during drug administration (scruff method) (Fig. 20.1a). However, heavier rats (weighing >250 g) may find it uncomfortable to be handled in this manner. In such cases the animal can be held by its loose skin from the neck to the whole back such that the whole back rests on the palm of the handler (whole body method). This is a suitable method for many routes of drug administration such as oral, intraperitoneal, intramuscular, etc. Sometimes to avoid being bitten by the rat, the neck region of the animal can be held in the gap between index finger and thumb of the handler (Fig. 20.1b). This is good only for a few minutes, and adequate practice is needed to ensure the completion of the process of handling, restraint, and drug administration in a few seconds. For certain routes of drug administration like intravenous and inhalation route, specialized instruments like restrainer and anaesthetic chamber can be used to hold the animal.

While carrying the animal from one room to another, they must be carried in cages and never by hand.

20.2.2 Mouse

Traditionally laboratory mouse was lifted by the base of the tail similar to that of the rat but recent research has shown that this method of handling induces undue stress and anxiety in mouse and that may affect the scientific outcome. For this reason, it is suggested to use either tunnels or cupped hand to pick the animal and then restrain

the animal by the scruff or base of the tail for carrying out various procedures or examination of animals [3]. The mouse being more agile and faster species, caution must be exercised to avoid the animal jumping out of the hands. For holding them, they may be placed on the grids of cage lids to slow down their swift movements (Fig. 20.1c). Holding the mouse loosely with only the tip of the fingers makes the animal uncomfortable and makes it jump or bite.

20.2.3 Rabbit

Rabbit is comparatively easier to handle since it rarely tends to bite. The animal is held by the neck and the ears together. Pulling by holding the ears alone is to be avoided since it can be very painful. It is important to support the lower back of the animal with the other hand, thereby not allowing the heavier lower portion of the animal to hang in the air. While carrying around, the body is supported in the folded elbow and forearm, with the face neatly tucked inside facing the body of the handler. This prevents the animal from facing outside and prevents anxiety. Rabbits may also be restrained by carefully wrapping them in a drape or towel to avoid struggling during injections.

20.2.4 Guinea Pig

Guinea pigs are smaller compared to a rabbit and are more docile. Handling of guinea pigs is easier as there is little fear of being bitten. It is however to be noted that guinea pigs are more sensitive and hence better less handled. The guinea pig is handled with the fingers surrounding the whole upper back. Since they are heavy, the lower part of the body is supported by the other hand and must not be allowed to hang in the air.

20.2.5 Hamster

Hamsters can be restrained by holding them by the scruff between thumb and index finger and placing the back on the palm of the handler. This is an ideal restraining method for intraperitoneal and subcutaneous injections in hamsters.

Points to be borne in mind while handling laboratory animals:

(a) While trying to restrain an animal, trying to grab the animal forcefully or making sudden hand movements must be avoided. The hand may be brought over the cage and allowed to be sniffed by the animal before handling.
(b) The animal is to be held confidently. Nervousness or anxiety of the handler will increase the anxiety levels of the animal too.
(c) The cage must be opened slowly and deliberately to avoid jumping off of the small animals.

(d) When handling, the neck or throat of the animal must not be squeezed. It may be painful, and small mice may die during handling.
(e) Picking a rat or mice by the tip of the tail induces aversion and high anxiety levels in animals which might negatively impact the outcome of research and welfare of the animal.
(f) Mice and young rats when picked by the tail may climb back on its tail and bite the fingers.
(g) Handling the animals daily helps the animals to get used to handling procedures.
(h) When mechanical restrainers are used, it is to be ensured that the diameter of the restrainer is apt so that the animal does not turn around inside the restrainer.

20.3 Routes of Drug Administration in Small Laboratory Rodents

Injection techniques/routes: The common routes of injection are intraperitoneal (IP), intravenous (IV), subcutaneous (SC), intramuscular (IM), and intradermal (ID) routes. The rate of absorption of the injected substance varies with the routes of injection, with IV route being the most rapid. For injections, it is important to use syringes and needles appropriate for the route and species. The recommended needle sizes and injection volumes for different injectable techniques in common laboratory rodents are mentioned in Table 20.1. All equipment used must be sterile and dry. Disposable needles and syringes should be discarded as biohazardous waste. Needles should be cauterized and disposed off in boxes designed to prevent accidental puncture wounds.

20.3.1 Intravenous Injection (IV)

The site for intravenous injection varies with species. Lateral tail vein, ear vein, saphenous vein, jugular vein, and femoral vein are some of the sites used for injecting substances intravenously in common laboratory animals.

20.3.1.1 General Principles of IV Injection

- Prior to the procedure, the site must be cleaned (shaved if necessary) and treated with antiseptic solution. The pressure of application of an antiseptic also helps in easy identification of the blood vessel.
- Slight pressure may be applied in the area proximal to the injection site so that the blood vessel will engorge and be visible.
- Use of appropriate size needles is to be ensured, and the syringes must be kept ready loaded with the substances to be administered before picking the animal.
- The vessel must be punctured at an angle of about 30° to the skin surface. Aspiration of a small amount of blood into the syringe confirms that the needle is in the blood vessel.
- Intravenous administration of all substances should be done slowly.

Table 20.1 Recommended injection sites [4], needle sizes [4, 5], and volumes [6] for different routes of drug administration in laboratory animals

Species / Administration routes		Mouse	Rat	Hamster	Guinea pig	Rabbit
IV	Site	Lateral tail vein	Lateral tail vein	Femoral/ jugular vein	Ear vein/ saphenous vein	Marginal ear vein
	Vol (ml/kg)	5[a] (25[b])	5[a] (20[b])	5[a] (20[b])	1[a] (5[b])	1–5[a] (10[b])
	Size	26–28 G	23 G	25–27 G	25–27 G	23–25 G
IP	Site	Left or right lower quadrant of abdomen	Left or right lower quadrant of abdomen	Left or right lower quadrant of abdomen	Left or right lower quadrant of abdomen	Right lower quadrant of abdomen
	Vol (ml/kg)	5–10	5–10	5–10	10	3–5
	Size	27–25 G	25–23 G	25–23G	25–23 G	23–21 G
IM	Site	[c]Quadriceps/ posterior thigh	Quadriceps/ posterior thigh	Quadriceps/ posterior thigh	Quadriceps/ posterior thigh	Quadriceps/ posterior thigh/lumbar muscle
	Vol (ml/kg)	0.05 ml/site (2 sites/day)	0.1 ml/site (2 sites/day)	0.1 ml/site (2 sites/day)	0.3 ml/site (2 sites/day)	0.25 ml (2 sites/day)
	Size	27 G	25 G	25 G	25 G	25 G
SC	Site	Scruff	Scruff/back	Scruff	Scruff/back	Scruff/flank
	Vol (ml/kg)	5	5	5	5	2.5
	Size	<25 G	<25 G	25 G	23–25 G	21–25 G

IV intravenous route, *IP* intraperitoneal route, *IM* intramuscular, *SC* subcutaneous route of drug administration, *G* gauge

[a]Intravenous single bolus volume (dosed in less than 1 min)

[b]Slow infusion volume (typically dosed over 3–10 min)

[c]Not generally recommended

- After withdrawing the needle at the completion of the injection, pressure must be applied over the injection site for a few seconds to allow blood clotting and prevent hematoma formation.

20.3.1.2 Procedure

In rat and mouse, lateral tail vein is the common site for intravenous injections and is located at 90° on either side of the central tail artery as left and right lateral tail vein (Fig. 20.2a–d). After restraining the animal either by using physical restrainers or anaesthetics, the tail is immersed in warm water (max. 40 °C) for a minute to induce vessel dilation. The tail is restrained, and the needle is introduced at an angle of about 30° to the skin surface with its bevel uppermost, into one of the lateral tail veins, at a distance approximately half way to two thirds from the base of the tail. The vein lies immediately below the skin, and entry of the needle into the vein can be made easier if the tail is bent slightly over the operator's finger and the needle introduced almost parallel to the tail. After penetrating the blood vessel, the syringe plunger can be

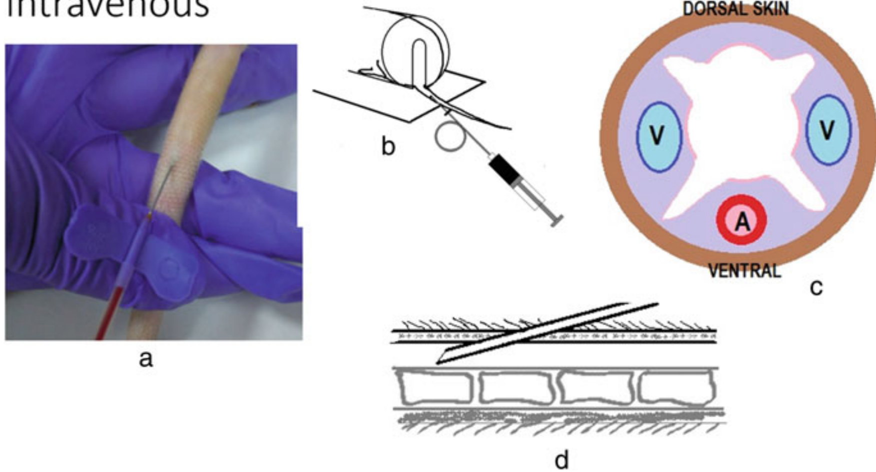


Fig. 20.2 (**a–d**) Intravenous administration: restraint, orientation of vein, administration, and location of needle

pulled back to draw a small amount of blood into the syringe to ensure that the needle is in the vein.

The injection should be made slowly and steadily, taking great care to avoid any changes in the position of the needle and tail, and generally no resistance will be felt during injection procedure if it is properly administered in the vein. After the injection is carried out, the needle may be withdrawn, and gentle pressure may be applied with a sterile swab to seal the vessel and allow clotting to occur. Once bleeding has stopped, the animal may be placed back into its cage and released.

20.3.2 Intraperitoneal Injection (IP)

20.3.2.1 General Principles of IP Injection

- Intraperitoneal injections must be carried out in a sterile manner using sterile needles of appropriate size to avoid post-injection complications like peritonitis.
- Utmost care must be taken to avoid penetrating visceral organs within the abdominal cavity. It is important to draw the needle back after piercing. If a yellowish fluid is withdrawn into the syringe, the needle could be in the bladder. If a greenish brown fluid is withdrawn, the needle could be in the intestine or the caecum. If blood enters the syringe, a blood vessel must have been punctured. In such cases, the syringe must be discarded, and the procedure must be repeated.
- While giving an IP injection, slight resistance will be encountered as the needle passes through the skin and abdominal muscles, but once through, there will be little resistance as the needle enters the abdominal cavity.

Intraperitoneal

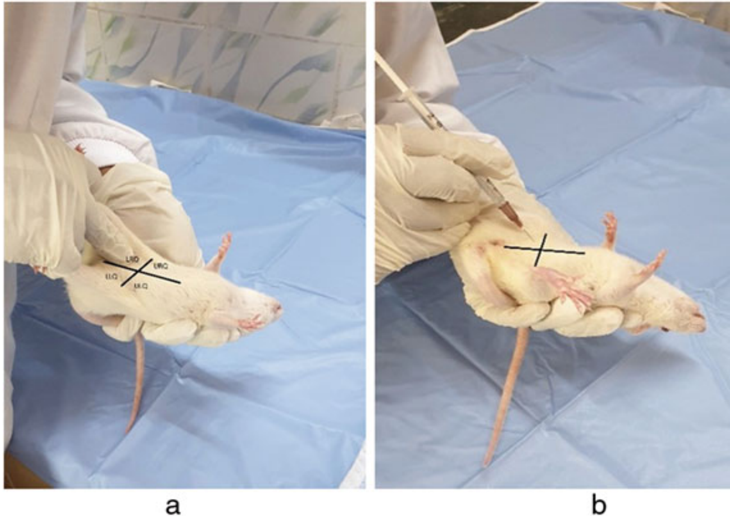


Fig. 20.3 (**a**, **b**) Intraperitoneal administration in rat: location and administration

20.3.2.2 Procedure

To carry out intraperitoneal injection, the animal must be carefully restrained by grasping it around the scruff. Adjust the grip around the animal's scruff, and push the animals' legs forward and tilt the animal head slightly downwards to move the visceral organs cranially. The needle should be angled approximately 45° along the line of the leg, into the lower quadrant (left or right) of the abdomen, lateral to the midline, and injected slowly (Fig. 20.3a, b). This posture will minimize the likelihood of penetration of the abdominal organs. The animal should be closely monitored post-injection for any discomfort after leaving the animal back to its cage.

20.3.3 Intramuscular Injection (IM)

20.3.3.1 General Principles of IM Injection

- Intramuscular injections are generally given in the thigh region of the hind limbs (Fig. 20.4a, b) such as quadriceps muscle area (a large muscle mass over the anterior portion of the femur) and occasionally in the triceps muscles of the forelimb or the lumbar muscles at the back. The muscles posterior to the femur can also be used for injection without damaging sciatic nerve and the femoral vein, artery, and nerve located deep within the tissues near the femur.
- The femoral region is not generally recommended in rats, mice, and hamsters because small muscle mass and larger volumes of material can damage sciatic nerve and cause pain to the animal. When used, only small volumes are injected.

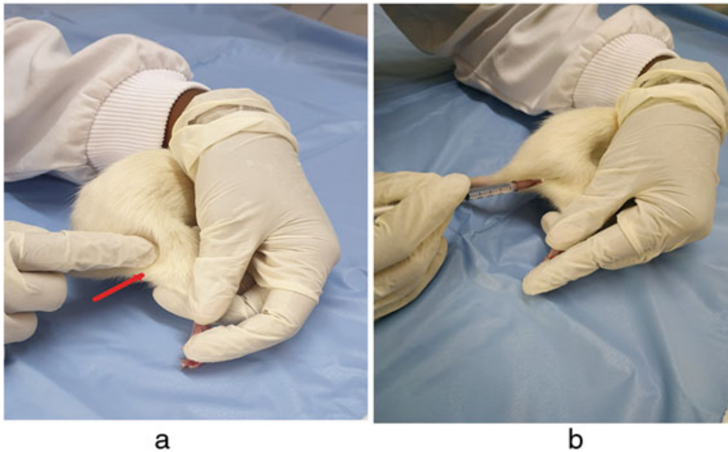


Fig. 20.4 (**a**, **b**) Intramuscular administration in rat

- In rats, up to 0.2 ml substances can be injected per site in the quadricep muscle area using 25 G or smaller size needles to avoid tissue damage. In rabbit, care should be taken not to inject more than 0.5 ml.
- Repeated injections must be avoided as it may result in inflammation and necrosis of the muscle.
- Irritants such as ketamine should not be administered intramuscularly as they may lead to self-mutilation of the affected limb if muscle or nerve damage occurs.

Procedure: After proper restraint, the muscle mass is pinched and raised with one hand. The substance is injected into the elevated tissue with the other hand. The needle is then withdrawn, and the animal is placed back into its cage and released.

20.3.4 Subcutaneous Injection (SC)

Subcutaneous injection is given in the void space between the skin and the underlying tissue when the skin is raised like a tent. The most common site for subcutaneous injection is the loose skin between the shoulder blades or in the flank region, just in front of the hind leg. Relatively large volumes of non-irritating fluids can be injected into these sites.

20.3.4.1 Procedure

For administration of any substance by subcutaneous route, the animal must be restrained and placed on flat surface with a firm grip on the base of its tail. A loose fold of skin is pulled away from the underlying muscles over the shoulders using thumb and index finger to form a tent at the nape (neck region). The animal must be

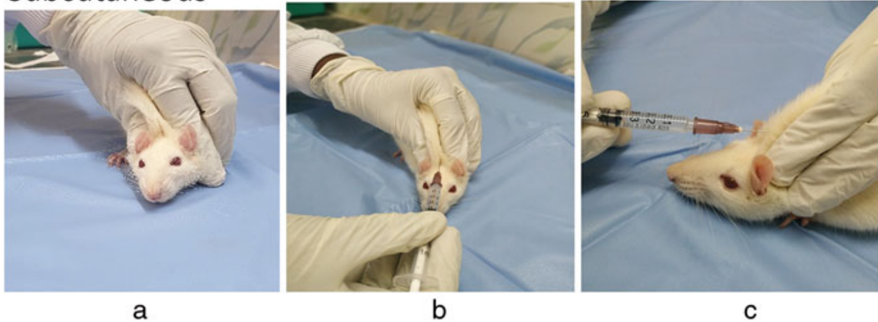


Fig. 20.5 (**a**–**c**) Subcutaneous administration in rat: restraint and administration

held securely to restrict the movement of the head. The needle is then directed into the base of the tented skin (Fig. 20.5a–c), so that inadvertent injury to the fingers with the needle can be avoided. A clear bulge/bleb in the skin will be noted after injecting a large quantity of fluid. After completion of the injection, the animal can be placed back into its cage and gently released. Care must be taken not to enter the underlying muscle or pass the needle through the other side of the tent while injecting.

20.3.5 Intradermal Injection (ID)

Intradermal injection is given into the thick dermal layer of the skin. The needle (usually 25 G or smaller) is directed at an angle of about 30° into the skin that is held taut. The needle may be rotated in the injection site after insertion but prior to withdrawal to minimize leakage of injected fluids. However, in this site only, a small quantity of fluid (0.1–0.2 ml) can be injected, and resistance will be felt to further volumes. A distinct bleb can be noticed when properly injected.

20.3.6 Oral Gavage

Gavaging is the technique used to administer liquid substances orally to the animals. This technique is commonly used in rodents during pharmacological and toxicological studies to deliver a precise amount of liquid preparation into the stomach by using a special gavage needle with a ball at the end attached to a syringe. The smooth rounded surface of the ball prevents injury and entry of the needle into the trachea.

Gavage needles of various ball sizes (ball diameter) and types (curved/straight) are available. It is necessary to ascertain the length of the gavage needle before administering the substance based on the size of the animal. Generally, the distance from the mouth of the animal to its last rib is used for determining the length of the

Table 20.2 Suggested gavage needle sizes for mouse and rat [7]

Mouse				Rat			
Weight (g)	Gauge (G)	Length (in.)	Ball diameter (mm)	Weight (g)	Gauge (G)	Length (in.)	Ball diameter (mm)
Up to 14	24	1	1.25	50–75	20	1	2.25
						1.5	
15–20	22	1	1.25	75–120	18	2	2.25
		1.5					
20–25	20	1	2.25	100–200	18	3	2.25
		3		150–300	16–15	3	3.0
25–30	18	2	2.25	200–350	14–13	3	4.0
30–35		3					

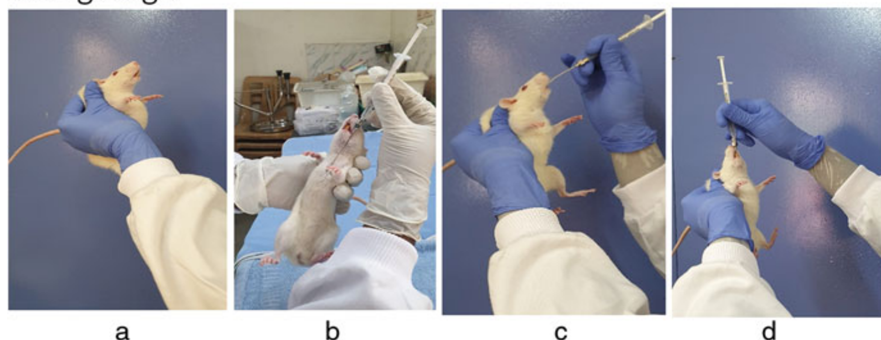


Fig. 20.6 (**a**–**d**) Oral gavaging in rat: restraint, depth of insertion, insertion of gavage, and oral drug administration

gavage needle. The recommended gavage needle sizes for rat and mouse are given in Table 20.2.

20.3.6.1 Procedure

To carry out gavaging, the animal must be restrained properly by holding the animal in a vertical position by its scruff with the head and neck extended. The gavage needle is slowly inserted into the animal's mouth at approximately 45° angle with the horizontal plane, towards left of the oral cavity, by pressing down the tongue of the animal with the ball of the needle. As the needle progresses inside the oral cavity into the oesophagus, raise the animal to a vertical position (Fig. 20.6a–d). The needle should not be forced inside if there is resistance. If the needle does not easily pass into the oesophagus, the needle can be removed and tried again.

When the ball of the needle is in the stomach, the contents of the syringe can be slowly delivered by pushing the plunger of the syringe. After discharging the substance into the stomach, the needle is gently removed, and the animals are placed

back in its cage. Care must be taken not to administer the substance into the trachea of the animal or accidently pierce oesophagus/stomach wall and penetrate thoracic or abdominal cavity. Subcutaneous abscesses may result from infection tracking out from the mediastinum as swellings in the axillary region. If the liquid substance is mistakenly delivered into the lungs of the animal, it is fatal. The maximum recommended volume of substance that can be administered by this route in rat is 10 ml/kg. It should be noted that repeated insertion of the tube for frequent dosing may sometimes cause inflammation and ulceration of the oesophagus.

20.4 Blood Sampling Techniques in Laboratory Rodents

Blood sampling from experimental laboratory animals is one of the critical parts of research as even a small error in the collection procedures may lead to variation in the results. There are a number of efficient and approved methods available for blood sampling in small laboratory rodents, balancing animal welfare, and research needs.

Broadly, the blood sampling techniques in laboratory rodents are classified as:

- Techniques not requiring anaesthesia like collection of blood from saphenous vein, facial vein, dorsal pedal vein, etc.
- Techniques requiring anaesthesia like sampling from tail vein and jugular vein and terminal procedures like cardiac puncture, sampling from orbital sinus, etc.

The choice of the appropriate sampling technique depends on various factors like species, size of the animal, physiological status, quantity of sample required, frequency of sampling, effect of anaesthesia on blood constituents, parameter to be tested, etc. Due considerations are also to be given to the ethical and regulatory principles of different countries while choosing the route of blood collection (Table 20.3).

Table 20.3 Different sites of blood collection in small laboratory animals

	Procedures requiring anaesthesia	
Procedures not requiring anaesthesia	Terminal procedures	Procedures requiring local/ general anaesthetics
1. Saphenous vein 2. Dorsal pedal vein 3. Facial vein	1. Cardiac puncture 2. Retro-orbital sinus 3. Blood collection from posterior vena cava	1. Tarsal vein 2. Jugular vein 3. Tail snipping/transection 4. Cannulation (temporary/ surgical) 5. Marginal ear vein[a] 6. Lateral tail vein[a] 7. Sublingual vein[a]

[a]Can be carried out without anaesthesia by trained persons if animals are properly restrained

20.5 Principles of Blood Collection in Small Laboratory Animals

The important ethical principle of any blood sampling technique is that it should cause minimal stress or pain to the animal. The other basic principles and factors that must be borne in mind during blood sampling in laboratory animals are as follows:

Handling and restraint of animals: The animals should be handled gently and restrained firmly during blood sampling to avoid injury to animal or handler. The animals can be restrained either by using physical restrainers or by anaesthesia (general or local) depending upon the degree of invasiveness and duration of the procedure and the pain inflicted. Blood can be collected from conscious animals that are adequately trained and appropriately restrained. Reward can be given to the animal after each act of bleeding.

Animal handler: The person collecting the blood must be skilled and well trained to recognize the clinical signs of stress and shock in the experimental animals.

Materials used for blood collection: The materials that are required for blood sampling include depilator, antiseptic, gauze, vaseline, collection tubes, local or general anaesthetics (as necessary), capillary tubes, syringes, and sterile needles of appropriate size. The size of the needle (length and diameter) used depends upon the size of the animal and the site of blood collection. It is recommended to use a large bore needle to ensure rapid blood withdrawal without collapsing the vein and avoiding haematoma. The recommended bleeding sites for common laboratory rodents are given in Table 20.4.

20.5.1 Volume and Frequency of Blood Collection

The volume of blood that can be drawn and the frequency of sampling depend on the total blood volume of the animal and the objective of the procedure. As a general principle, the sample volume and frequency should be minimum to equilibrate animal welfare and good research.

The average total circulating blood volume in an animal's body equals approximately 5.5–7% of its body weight, i.e. 55–70 ml/kg body weight [10]. However, the total blood volume is likely to be lower by about 15% in obese and older animals [11]. The normal blood volume and recommended volume of bleeding and recovery period of common laboratory rodents are listed in Tables 20.5 and 20.6.

- When blood samples are collected at frequent intervals, there are guidelines to ensure that the health of the animal is not affected. As a rough guide, in a normal healthy animal, up to 10% of the total blood volume can be taken in a single collection with minimal adverse effects at 2 weeks interval.
- For repeated sampling, a maximum of 1.0% of an animal's total blood volume can be collected every day.
- If a study involves repeated blood sampling, temporary cannulation can be done to reduce pain and stress of repeated sampling.

Table 20.4 Recommended sites of blood collection in common laboratory rodents [8, 9]

Species	Mouse	Rat	Hamsters	Guinea pig	Rabbit
Site of blood collection and permitted conditions	1. Lateral tail vein (incisional method not permitted) 2. Saphenous vein 3. Facial vein 4. Tail prick 5. Distal tail transection 6. Orbital sinus (under general/local anaesthesia) according to stipulations in animal use protocol 7. Sublingual vein 8. Jugular vein 9. Cardiac puncture (terminal only)	Sites mentioned for mouse also holds good for rat except facial vein Other sites include Subclavian and dorsometatarsal vein	1. Jugular vein 2. Tail vein 3. Femoral vein 4. Saphenous vein 5. Gingival vein 6. Sublingual vein 7. Orbital sinus (lateral canthus) 8. Cardiac puncture (terminal procedure)	1. Ear vein 2. Saphenous vein 3. Jugular vein (anaesthetized only) 4. Anterior vena cava/ subclavian vein 5. Metatarsal vein 6. Cardiac puncture (terminal only)	1. Cardiac (terminal in anaesthetized animal) 2. Marginal ear vein (small vein) preferred site 3. Central auricular artery 4. Lateral saphenous vein 5. Cephalic vein 6. Jugular vein

Table 20.5 Total blood volume and recommended maximum sampling for different species [12]

Species	Blood volume Mean (ml/kg)	Blood volume Range (ml/kg)	Blood volume (average)		
			7.5%	10%	15%
Mouse (25 g average weight)	58.6	55–80	110 μl	146 μl	220 μl
Rat (250 g average weight)	64	58–70	1.2 ml	1.6 ml	2.4 ml
Rabbit (4 kg average weight)	56	44–70	17 ml	22 ml	34 ml

Table 20.6 Limit volumes and recovery periods [4]

Blood volume removal (%)	Recovery period
7.5%	1 week
10%	2 weeks
10–15%	4 weeks

- If blood collection volume exceeds 10% of total blood volume, replacement with fluids like ringer lactate solution is recommended.
- The animal may suffer from hypovolaemic shock and anaemia on rapid or frequent blood sampling without fluid replacement.

Monitoring: The health status of the animal should be monitored if they are bled at regular intervals. The parameters like body weight of the animal, packed cell volume (PCV), and haemoglobin levels are better post-bleeding indicators about the recovery rate and health status of the animal. It may take up to 14 days for all the other blood constituents (i.e. cells, proteins) to be replaced and 1 day to recover blood volume in healthy adult animal [12]. In general, it is not safe to draw blood if the haematocrit value is less than 35% or the haemoglobin concentration is less than 10 g/dl [9].

Dilation of blood vessel: In conscious rodents, blood can be more easily collected if the blood collecting site is warmed prior to collection using warm water or gentle massaging, followed by cleaning with alcohol. Topical irritants like xylene must not be used to dilate the blood vessel as they damage blood cells.

Haemostasis: At the end of all non-terminal blood collection procedures, the bleeding from the site of collection must be arrested before replacing the animal back to the cage. Utmost care must be taken to avoid potential adverse effects like hypovolaemic shock, anaemia, stress, haemorrhage, thrombosis, infection, phlebitis, tissue, and nerve damage due to poor bleeding techniques.

All the blood collection methods should be clearly described in the protocol approved by the ethical committee and should be performed as such without any deviation.

20.6 Common Blood Sampling Techniques

20.6.1 Blood Sampling Techniques Without Use of Anaesthesia

20.6.1.1 Saphenous Vein Bleeding Technique

It is one of the least invasive procedures of blood collection and the only visible collection site in hamsters, guinea pigs, and gerbils. The saphenous vein is a tortuous blood vessel located over the lateral aspect of the hock joint, and this site is highly prone for haematoma.

Procedure: The hind leg of the animal is immobilized after manually restraining the animal. The area over the blood vessel is cleaned off hair and wiped with antiseptic until saphenous vein is visible. A light coating of sterile vaseline is applied over the area to facilitate easy blood collection. Slight pressure may be applied gently above the knee joint to visualize the vein. The vein may be punctured using a 22 or 23 G (20–22 G in guinea pig) needle, and blood may be collected with a capillary tube or a syringe with needle (Fig. 20.7a, b). After collection, the punctured site must be compressed with gauze to stop bleeding, and the animal should be returned to the cage only after the bleeding stops at the punctured site.

20.6.1.2 Dorsal Pedal Vein Blood Collection Technique (Rat or Mouse)

Procedure: The animal is restrained and its hind foot around ankle is held exposing the medial dorsal pedal vessel on top of the foot. The foot is cleaned with alcohol, and dorsal pedal vein is punctured with 23 G/27 G needle. Drops of blood are collected in a capillary tube, and pressure is applied to stop bleeding after blood collection.

Facial vein technique: This technique can be applied only in adult mouse. It is a safe and fast technique, and approximately 200 μl of blood can be obtained easily from a healthy adult animal with momentary restraint. In this technique, blood is

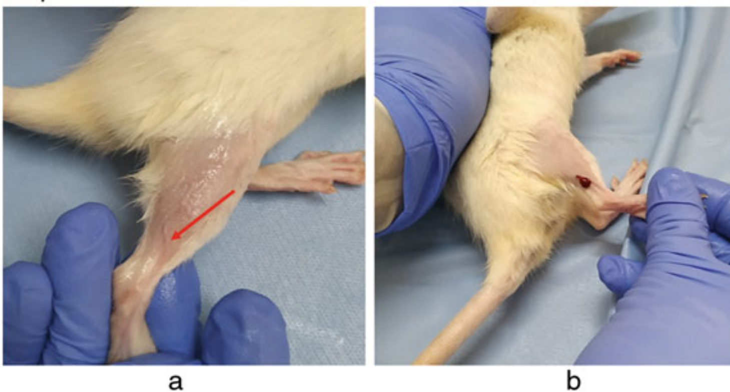


Fig. 20.7 (**a**, **b**) Blood collection: rat saphenous vein—location and collection of blood

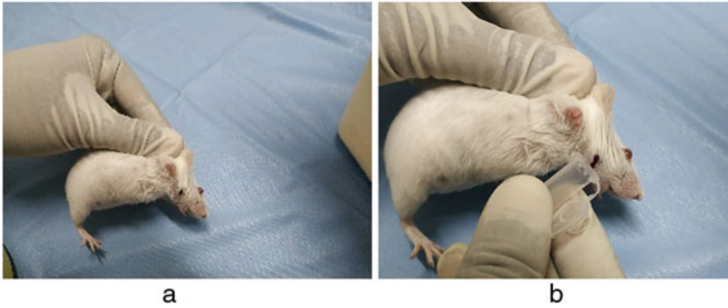


Fig. 20.8 (**a**, **b**) Blood collection: mouse facial vein—location and collection of blood

collected from the submandibular facial vein located below the skin posterior to the facial vibrissae (whiskers) of the jaw.

Procedure: The mouse may be placed on the wire bars of the cage using the dominant hand. The scruff of the mouse is firmly held using thumb and index finger, and the tail is tucked between the last two fingers of the free hand such that the spine of the mouse rests close to the knuckles. The hairless freckle on the side of the jaw is located, and vaseline is applied over the area. The insertion point is just 1 mm behind the freckle on the angle of the jaw and 20–23 G needle is inserted with care at the insertion point. The blood is collected in blood collection tube (Fig. 20.8a, b). The pinch of the skin must be loosened when sample is collected, and the animal is returned to the cage once the bleeding has stopped from the insertion site.

20.6.2 Blood Sampling Techniques Using Local/General Anaesthesia

20.6.2.1 Marginal Ear Vein Blood Collection (Rabbit)

Procedure: The animal should be appropriately restrained. The dorsal surface of the ear is cleaned with alcohol so that the marginal ear vein can be easily located. Local anaesthesia is applied on the collection site before sampling. Using a 26 G needle, blood may be collected away from the base of the ear from its peripheral tip. After collecting blood, slight pressure may be applied on the punctured site to stop bleeding.

20.6.2.1.1 Rodents

Lateral tail vein blood sampling technique: It is a reliable and safe method in rats and mice.

Procedure: The animal is restrained comfortably in an appropriate restrainer, or an anaesthetized animal can be used. The left or the right lateral tail vein is located. The tail is dipped into warm water (40 °C) for 1–2 min and rubbed with alcohol or antiseptic solution for better visibility. After locating the vein, a 23 G (for rat)/25 G

(for mouse) needle is introduced into the blood vessel, and blood is collected. For repeated bleeding, temporary surgical cannulation can be done.

Lateral incision of tail: This technique is followed in rats and mice to collect few drops of blood during serial testing, e.g. measurement of glucose by nicking the lateral tail vein. The animal is immobilized, and a small incision is made in the lateral tail vein. The tail is gently massaged back and forth until blood drops emerge. The blood is collected in collection tube. Slight pressure is applied over the incised area with gauze to stop bleeding or else the presence of blood on the tail can encourage mutilation and further trauma. This technique can be used for repeated small volume blood collections by gently removing the clot/scab. The blood samples may at times be contaminated with tissue and skin products.

Tail snip blood collection: This method is performed only in mice mostly as terminal procedure as it can cause permanent damage to the tail.

Procedure: If not done as terminal procedure, local anaesthesia must be applied on the tail before collecting the blood and incision made 1 mm from the apex of the tail using scalpel blade. After bleeding is arrested, the animals are placed back in the cage.

Jugular vein blood collection: This technique can be applied to collect medium to large volumes of blood, but repeated serial samples cannot be done. Blood can be collected from the jugular veins on either side, but vessels on the right are more accessible.

Procedure: The anesthetized animal is placed on dorsal recumbency, and the hair in the neck region is removed. On extending the neck, the jugular veins can be located on the side to sternoclavicular junction as a blue line. A 25 G needle is inserted, directed from back to front not more than 3–4 mm into the blood vessel, and the blood is withdrawn slowly to avoid collapse of the vein. Gentle pressure may be applied to the bleeding site after blood collection to prevent haematoma. If there is failure in the first attempt, further attempts should not be made for sometime.

Intracardiac puncture: This is a terminal procedure done under general anaesthesia and must be followed by assurance of death or euthanasia.

Procedure: The anesthetized/euthanized animal is placed on dorsal recumbency. The pedal reflex is checked. The needle (20/23 G) is introduced at 20–30° angle just left to the xiphoid process (base of sternum) towards the heart with the bevel facing upwards. After the needle passes the skin, create negative pressure by pulling back the needle and progress the needle to the middle of the chest cavity, and blood will start flowing into the syringe on puncturing the heart.

Retro-orbital plexus blood collection: Retro-orbital sampling is done in rat and mice when serial blood sampling with reasonable sample volumes is required. This technique must be performed only under general anaesthesia. Retro-orbital blood collection as a non-terminal collection is generally discouraged. The drawback with the method is that the blood obtained is likely to be contaminated with local secretions and may not be suitable for assays [13]. If not properly done, there may be tissue damage and periorbital lesions in the animal.

Procedure: The animal is held by the scruff with thumb and index finger of the non-dominant hand with the skin around the eye pulled tight. A capillary tube is

inserted at 30° angle into the medial canthus of the eye with a thumb pressure to pierce the plexus/sinus. The capillary tube is gently removed after collecting blood and the area is wiped with sterile gauze.

Apart from these bleeding sites, gingival vein and sublingual vein can be used as additional bleeding sites in hamsters. These are least invasive and efficient alternative sites for repeated blood sampling in hamsters [14]. During gingival vein puncture, the anaesthetized animal must be placed in dorsal recumbency, and the lower lip of the animal must be pulled down. A 26 G needle with the syringe may be inserted into the mucous membrane below the gingival incisor edge in between lower incisors at 30° to 60° angle in the lower direction for blood sampling.

A comparison of various routes of blood collection techniques in laboratory animals is provided in Table 20.7. A suitable technique appropriate for the species may be chosen for blood collection considering the requirements of the experiment and animal welfare factor.

Table 20.7 Comparison of various routes of blood collection in laboratory animals

Bleeding site	Species applicable [15, 16]	Type of restraint [15]	Volume of blood [15, 17]	Frequency of collection [15, 16]	Animal welfare factor [15, 18]
Saphenous	Mice, rat, guinea pigs, rabbits, ferrets, cats, dogs, small ruminants, larger animals, etc.	MR	Small to medium	Rb	Low tissue damage^{+}
Tail vein	Rats and mice	PR/GA	Small to medium	Rb	Low tissue damage$^{+/++}$
Tail snipping	Mice	GA	Small	Limited Rb	Moderate tissue damage^{+++}
Ear vein	Rabbits, guinea pigs, minipigs, pigs	MR & LA	Small to medium	Rb	Low tissue damage^{+}
Retro-orbital	Mice, rats, hamsters	GA	Medium to large	Limited Rb	Moderate tissue damage$^{+/++*}$
Jugular vein	Mice, rats, guinea pigs, rabbits, ferrets, cat, dog, small ruminants, pigs, etc.	MR	Medium to large	Rb	Low tissue damage, stressful$^{+/++}$
Intracardiac	Mouse, rat, guinea pigs, hamsters, rabbit	GA	Medium to large	Sb (terminal)	Moderate tissue damage^{++*}

Sb single blood collection, *Rb* repeat bleeding, *MR* manual restraining, *PR* physical restrainers, *GA* general anaesthesia, *LA* local anaesthesia, + less stressful, ++ moderately stressful, ++* moderately stressful and stress can be minimized when carried out by following good practices by competent technicians, +++ highly stressful

Note: Scoring is based on the assumption that the procedure is carried out by a well-trained and experienced technician following the principles of handling and restraint of laboratory animals; however, the severity and volume of blood collected depends on the species, skill, and experience of staff and status of the animal

20.7 Conclusion

Experiments with laboratory animals often involve the administration of small quantities of drugs/vaccines/other chemicals. The volumes used are extremely low so that even minor errors in administration can cause variation in results. Thus, training in the areas of handling, restraint to administer drugs and to collect blood from the animals becomes important. Since there are multiple species, it is imperative to have good understanding about individual species beforehand about the techniques used. Before embarking on an in vivo study, researchers must familiarize themselves with the type of needles used, volumes permissible, tools and equipment available, and the precautions to be taken. During the entire procedure, maximum emphasis should be laid on the principles of welfare and ethics since live animals are handled. Of late, regulatory agencies have made it mandatory that any person who wishes to start animal research should have undergone adequate training in handling, administration of drugs collection of blood, etc. These procedures form the basic level of training upon which researchers can build up their skills further. This chapter aims to cover all the key essential aspects of basic procedures to instil confidence in the reader before starting a trial. However, it is reiterated that this chapter is learnt better by doing than reading.

References

1. Handling and Restraint—3Rs resources—National Centre for the Replacement, Refinement and Reduction of Animals in Research. Available from: https://www.nc3rs.org.uk/handling-and-restraint
2. Deacon RMJ (2006) Housing, husbandry and handling of rodents for behavioural experiments. Nat Protoc 1:936–946
3. How to pick up a mouse? Available from: https://www.nc3rs.org.uk/how-to-pick-up-a-mouse
4. Hawk C, Leary S, Morris T (2005) Formulary for laboratory animals, 3rd edn. Blackwell Publishing, Oxford
5. Wolfensohn S, Lloyd M (1998) Handbook of laboratory animal management and welfare, 2nd edn. Oxford University Press, Oxford, p 334
6. Recommended Dose Volume for common laboratory animals IQ 3Rs Leadership Group Contract Research Organization Working group. Available from: https://iqconsortium.org/images/LG3Rs/IQCRO_Recommended_Dose_Volumes_for_Common_Laboratory_Animals_June_2016_%2%282%29.pdf
7. Virginia Tech SOP: oral gavage in the rat (Online), 12 Dec 2017. Available from: https://ouv.vt.edu/content/dam/ouv_vt_edu/sops/small-animal-biomedical/sop-rat-oral-gavage.pdf
8. Blood collection guidelines, research animal resources, University of Minnesota (Online) Available from: https://www.researchservices.umn.edu/services-name/research-animal-resources/research-support/guidelines/blood-collection
9. Guidelines for blood collection: rodents and rabbits, Department of Laboratory Animal Resources, University of Toledo (Online), 25 July 2011. Available from: https://www.utoledo.edu/depts/dlar/docs/Guidelines%20for%20Blood%20Collection_Rodents%20and%20Rabbits.pdf
10. Guidelines for survival bleeding of mice and rats. NIH-ARAC guidelines (Online). 23 Oct 2019. Available from: https://oacu.oir.nih.gov/sites/default/files/uploads/aracguidelines/b2_blood_collection_in_mice_and_rats.pdf

11. Blood sampling (Online). 2009. Available from: https://www.nc3rs.org.uk/blood-sampling-general-principles
12. Guidelines for collection of blood from laboratory animals, Thiel College Institutional Animal Care and Utilization Committee Available from: https://thiel.edu/assets/documents/academics/iacuc/IACUC-guidelines-for-collection-of-blood-from-laboratory-animals.pdf
13. Blood sampling—Rat-Retro orbital (Online). 2009. Available from: https://www.nc3rs.org.uk/rat-retro-orbital-non-surgicalterminal
14. Rodrigues MV, De Casro SO, De Albuquerque CZ, Mattaraia VG, Santoro ML (2017) The gingival vein as a minimally traumatic site for multiple blood sampling in Guinea pigs and hamsters. PLoS One 12:e0177967. https://doi.org/10.1371/journal.pone.0177967
15. Diehl KH (2001) A good practice guide to the administration of substances and removal of blood, including routes and volumes. J Appl Toxicol 21:15–23
16. Blood collection guidelines—research animal resources, University of Minnesota. Available from: https://www.researchservices.umn.edu/services-name/research-animal resources/research-support/guidelines/blood-collection
17. Guidelines for blood collection in laboratory animals, Division of Laboratory Animal Resources, UK Research. Available from: https://www.research.uky.edu/division-laboratory-animal-resources/guidelines-blood-collection-laboratory-animals
18. Tsai PP, Schlichtig A, Ziegler A, Heinrich E, Haberstroh J, Stelzer HD, Hackbarth H (2015) Effects of different blood collection methods on indicators of welfare in mice. Lab Anim 44:301–310

21 Clinical Examination of Laboratory Rodents and Rabbits

Krishnamurthy Venkataraman and B. Sangeetha Raajkamal

Abstract

The clinical examination of laboratory rodents and rabbits is a challenging task since these species instinctively conceal signs of sickness to avoid attracting the attention of its predators, as they would in the wild. However, in biomedical research, the need to understand the clinical signs evinced by these animals under experimentation is of paramount importance as it gives a prelude to the course of the experiment. In laboratory rodents, the clinical signs expressed are the sum of the changes in the normal anatomy and physiology of the animal under experimentation. Therefore, it becomes imperative for the investigator to understand the normal in these animals before classifying a change as abnormal. The various regulatory bodies have developed reommendations/guidelines suggesting ways to understand the clinical signs; however, there is no universal source that can clearly define the clinical signs in these animals because the clinical changes can depend on the strain or stock of the animal being used in the research. The chapter will provide a basic idea to the reader on the means to perform a clinical examination in the laboratory rodents employed in research.

Keywords

Clinical examination · Laboratory rodents · Biomedical research

K. Venkataraman (✉)
Siddha Central Research Institute, Central Council for Research in Siddha, Chennai, Tamil Nadu, India

B. S. Raajkamal
Laboratory Animal Veterinarian, Bangalore, Karnataka, India

© The Author(s), under exclusive license to Springer Nature Singapore Pte Ltd. 2021

P. Nagarajan et al. (eds.), *Essentials of Laboratory Animal Science: Principles and Practices*, https://doi.org/10.1007/978-981-16-0987-9_21

21.1 Introduction

The international and national regulatory bodies mandate the observation of animals for signs of illness, injury, and/or abnormal behaviour by animal care staff. The clinical observation of the rodents has to be performed every day and that the frequency of observation on animals has to be increased on animals that have been subjected to surgery or those animals whose endpoint in the study is imminent or those that have physical deficits. The working group further recommends that the individuals involved in the observation should be trained in recognition of the clinical signs and the individual must exercise professional judgment to decide on the frequency of observation. The purpose of the clinical observations (according to the guide) should be to reduce the risks to the animals involved in the experimentation and to avoid jeopardizing the research work. Since the rodents are known to mask the signs of distress to avoid the unwarranted attention of the predator, an elaborate clinical examination is a requisite to preclude an experiment from further inconveniencing the animal(s) involved.

It is very important to observe and report the clinical signs in laboratory animals. This will help us to assess the animal for its welfare, fix humane endpoints and report to several regulatory bodies. It also enhances the scientific value of any research performed on laboratory animals.

21.2 Role of Veterinarians

The burden of responsible use of animals lies with the veterinarian since the veterinarian is trained in understanding the welfare of the animals. An overzealous and passionate investigator might allow the animal to undergo more distress with the intent to lead the research to its logical conclusion. However, it becomes imperative for the veterinarian to ensure that any animal utilized in the research does not undergo pain and distress that is unwarranted. The laboratory animals instinctively hide the clinical signs of pain and stress to avoid garnering the attention of the predators. The challenge is in understanding the ethology of the laboratory animals that can differ across strains and stocks of the laboratory animals. The laboratory animal veterinarian is therefore required to be in continuous learning as the landscape of biomedical research continues to evolve with the growing demands of the health sector. In the zeal for the advancement of science, one should not ignore the emotional turmoil that the animals undergo while being used in research. Some behavioural changes even warrant euthanasia to prevent further suffering due to the experiment on the animal. It is with this intention that the regulations [1, 2] recommend that the veterinarian should be consulted for advice on designing euthanasia and the procedure is executed under the latter's oversight.

It is well established that no animal can be subjected to experimentation without the approval of the protocol from the regulatory body. The overt clinical signs expressed by laboratory rodents and rabbits are sometimes readily discernible by the users of the vivarium. However, some of the behaviour-based changes can be

Fig. 21.1 (**a**) Alopecia in CD-1 and C57BL/6 mice due to barbering. (**b**) Mouse showing cage fight wounds in the cervical area with swollen ears. (**c** and **h**). Spontaneous skin eruptions in a wistar rat. (**d**) Male B6 mouse showing signs of self-barbering. (**e**) E. Mouse showing neurological deficits like the inability to clasp to the cage grip in the forelimb. (**f**) Hyperkeratosis and erythematous changes on skin in B6 mouse. (**g**) Patchy hair loss due to barbering. (**i**) B6 mouse showing tumour growth of the face with signs of lack of grooming and unkempt hair coat. (**j** and **k**) Incompatibility in a male B6 mouse characterized by hair loss and non-glossy hair coat

mistaken for the normal behaviour of these animals. The changes in the behaviour of these animals can be identified relatively with greater ease by the veterinarian. This signifies the importance of the veterinarian performing the clinical examination to demarcate the signs expressed due to an experimental intervention or simply a sign arising out of non-provision of a welfare need (Figs. 21.1, 21.2, 21.3 and 21.4).

21.3 Observation of the Clinical Signs: Where to Begin?

The observation of clinical signs in the animals begins with the caretakers who feed and water the animals and the technicians who collect data from these animals which include body weight and checking for morbidity/mortality. The information gathered is transmitted to the veterinarian who can initiate a detailed clinical examination. The caretakers involved in feeding and watering the animal can provide useful information such as trivial changes in the behaviour of the animals such as lack of integration of nesting material to construct a nest, building of two nests, microphthalmia in a cage mate, head tilting, bedding material sticking to the perineum of the animal, etc.

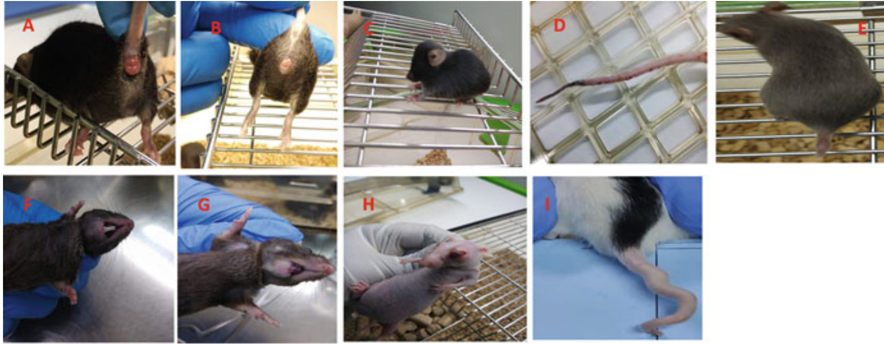


Fig. 21.2 (**a** and **b**) Rectal prolapse in a male C57BL/6 mouse possible due to straining because of low fibre content in the feed or Helicobacter infection. (**c**) Congenital abnormality—Hydrocephalus in a C57BL/6 mouse. (**d**) Tail bite wounds in a mouse with caudal necrosis in the tail. (**e**) Tail stump—congenital abnormality. (**f**) Overgrown incisors in a male C57BL/6 mouse. (**g**) Hair loss due to barbering in a B6 mouse. (**h**) Corneal opacity in a male nude mouse. (**i**) Kinky tail in a male rat

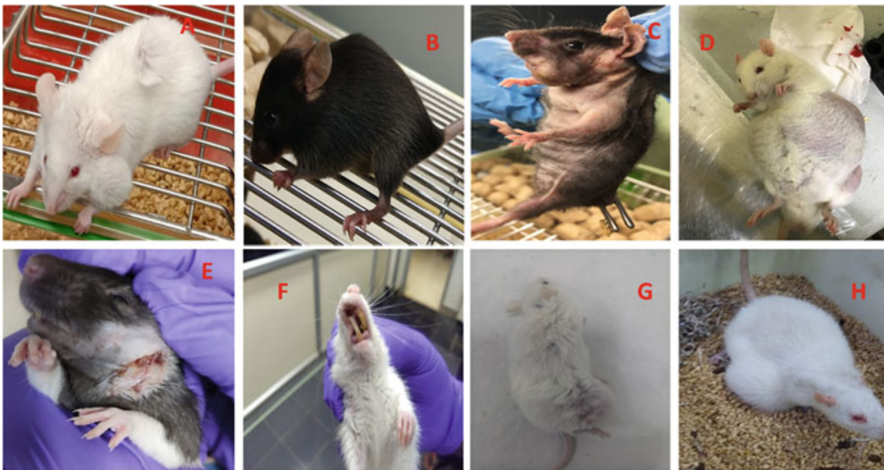


Fig. 21.3 (**a**) Hard mass protruding from the maxillary bone of a male CD-1 mouse. (**b**) C57BL/6 mouse exhibiting dehydration with sunken eyeballs and hunched posture. (**c**) Mandibular tumour in an aged C57BL/6 mouse. (**d**) Ascites in a male Sprague Dawley rat subjected to unilateral nephrectomy. (**e**) Intense pruritis in a male C57BL/6 mouse. (**f**) Malocclusion due to overgrown incisors in a male Sprague Dawley rat. (**g**) Male CD-1 mouse in lateral recumbency showing signs of hypothermia characterized by unkempt hair coat with no signs of grooming. (**h**) Mammary tumour in the caudal mammary glands in a female wistar rat

The clinical observations begin with observing changes in the smell characteristic of the species. The activities such as feeding and watering and cage changing are appropriate times to observe for clinical signs. Since these activities involve the cages to be observed and the animals in it, the abnormal behaviour expressed by the

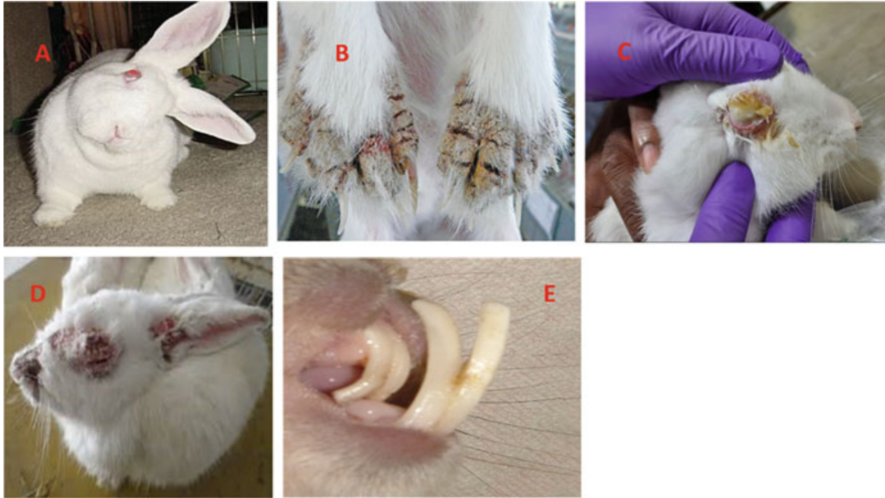


Fig. 21.4 (**a**) Head tilt in a New Zealand White rabbit. Probably middle ear infection caused by Pseudomonas spp. (**b**) Sarcoptic mange infection in a male New Zealand White rabbit characterized by hyperkeratosis and hair loss of the digits. (**c**) Suppurative conjunctivitis due to trauma in the right eye of a female New Zealand White rabbit. (**d**) Mange infection in a non-descript rabbit characterized by erythematous pruritic dermatitis. (**e**) Malocclusion due to overgrown incisors in a male non-descript rabbit

animals within the cage becomes obvious to the eyes of the caretakers. The changes like bite wounds, reduced muscle tone, unkempt coat, non-glossy coat, weak animal in the cohort, progressive deterioration of health like the negative trend in body weight in animals, etc. become evident while performing the routine activities. It is also a practice in many vivarium to allocate scheduled time exclusively for observation of the animals within the cage other than the time when routine activities are performed. Observation of animals, without disturbing the home cage, can provide valuable information like pain after a procedure or incompatible cohort bullying.

The animal caretakers can identify those changes in the cages that can be as trivial as bite wounds on the tail of the animals to as significant as the absence of nest-building behaviour in the cage. They can also identify the animal in the cage that is losing bodyweight. The animal needs to be separated and taken cared of until it exhibits the right amount of growth appropriate for its strain/stock. The latter is an observation that can be easily missed by the veterinarian as routine activities would not involve observation of all the cages every day. The caretakers have to inform the principal investigators or the veterinarian of any significant change. These observations can enable the investigators or the veterinarian to understand the outcome of an experiment or a disease process. This warrants further evaluation of the cage and the corresponding animals to arrive at a tentative diagnosis.

21.4 Role of Cage Environment in Clinical Evaluation

The interaction of the animal with its habitat is the result of the impact of the micro- and the macroenvironment of the animal. The immediate cage environment constitutes the microenvironment, while the room accommodating the cage is the macroenvironment. The microenvironment is also influenced by the changes in the macroenvironment such as temperature and relative humidity. The current school of thought is that the temperature below the thermoneutral zone of rodents causes a negative energy balance, and hence the animal constructs a nest to evade cold to remain euthermic. Under such conditions, it is also not uncommon to sight the animals huddling together be referred to as dogpiling. Besides the temperature and relative humidity, air cycle changes per hour attain significance for the removal of gases like carbon dioxide and ammonia that builds up in the cage. The accumulation of gases can result in respiratory ailments paving opportunities for the commensal to establish an infection. Such information on the environment of the animal can help in narrowing down the aetiology of the observed clinical sign. While looking for the signs, the veterinarian should ensure that noises like welfare compromises, the failure in environmental parameters like temperature, relative humidity, light, noise, vibrations, and ACH (air changes per hour) remain within the range as advised.

21.5 How Should Clinical Sign Monitoring Be Done?

21.5.1 Home Cage Evaluation

Daily inspection of animals helps in many ways to know the health status and clinical signs of the animals. Wide knowledge of the species or strain-specific helps to know what is normal and abnormal. On entering the animal room, the animal technician should observe for any abnormal sounds and smells from the cage. He/she should notice if there are any abnormal cage deposits. The change in water and water consumption should be observed properly and any decrease in feed and water consumption should be reported immediately. When mice are housed in pairs with litter mates, it is important to see if animals remain isolated from the normal groups. If so, the isolated animals must be noticed for their abnormal behaviour including the body conditions, skin coat, and response to stimuli. Ideally, the clinical examination can be initiated by performing careful observation at the cage without retrieving the cage from the rack. The mice are known to construct nests with the enrichment provided in the cages. The ability to construct a nest in the cage is not influenced by gender. However physiological states like pregnancy or the oestrous cycle can determine the outcome of the nest constructing trait of the mice. The building of nests contributes to reduced aggression in the mice. The absence of nest-building activity can be considered to be a deviation from normal. The evidence of the nest building can be used to evaluate the health of the animal after experimentations like administration of test items or post-survival surgeries since

the ability to build a nest would already be known [3, 4]. The inability of the mice to build a nest or to acquire a median score less than or equal to score 3 is indicative of hippocampal damage [5]. If a mouse shows no indication of nest building after being placed in a new cage at the time of its receipt from the vendor's site or during cage changing, it needs immediate veterinary attention [6]. Burrowing and hoarding are the other cage-side observations that can ascertain the health status of the animal.

The interaction between the cage mates and general appearance of the cage mates can provide ample information about the cohort in the cage [6]. The absence of behaviours such as barbering, stereotype, and facial pain expression due to noxious stimuli is indicative of the good health of the cohorts in the colony. These behaviours are also considered to be the welfare indicators of the mice [7]. The ability of a mice cohort in a cage to display the signs of burrowing and hoarding can indicate the positive welfare in that cage [8, 9]. This can be envisaged by the uneven topography of the bedding material in the cage and remnants of feed inside their nests or in the corners of the cage.

21.5.2 Evaluation of the Laboratory Mice by Handling

The laboratory mice should be acclimated to the handler before attempting a detailed physical examination. This would not be possible if the animals are newly received. A trained person can minimize the impact on the new animals. At the time of receipt, the animals that are not in good health can be identified by the absence of glossiness in the coat and obvious secretions around the eyes, nostrils, and urogenital area. Such animals need to be quarantined and screened for infectious diseases. The methods of screening for infectious diseases are discussed elsewhere in this book.

21.5.3 Body Condition Scoring

The scoring of the body condition presents the welfare of the mouse in the cage. Body condition scoring is done by running a finger over the sacroiliac bone on the hip of the mouse. The score ranges between 1 (indicating wasting of the body fat) and 5 (indicating obesity). Score 3 is considered ideal wherein the bones at the sacroiliac region are palpable but not prominent on the inspection with bare eyes [10].

The face of the mouse is to be examined to detect the presence of any obvious changes which can include the overgrowth of the incisors without forcing the scissor bite, pale, or blanched mucosa of the gingiva covering the incisors indicative of anaemia, presence of red pigments (porphyrin) around eyes and nares, loss of whiskers bilaterally (indicative of barbering), and loss of patchy hair over the head (indicative of barbering):

- **Nose:** Opening, colour, moisture, any discharge, any asymmetry, any respiratory sounds.

- **Mouth** (lips, cheek, teeth, gums, tongue, cheek pouches): Position, texture, colour, moisture, any wounds or deformities, abnormal discharge.
- **Eyes:** Position, size of the eyeball, and close fit of the eyelids. Moisture, reflection, transparency of cornea, size and shape of the pupil, any discolorations, or discharge.
- **Eye Abnormality:** Opacity, dilated pupils, constricted pupils, exophthalmia (bulging eye), and enophthalmia (sunken eye) are important if noticed along with other signs (e.g. loss of condition). Conjunctivitis (inflammation of the ocular mucous membranes), abnormal secretions or crusting, or corneal ulcers.

21.5.4 Examination of Limbs

Position, motility, any wounds or deformities, abnormal muscle, tone, or muscle mass, proper use (versus lameness).

The examination of the limbs would include the observation for the weakness which can be ascertained by allowing the mouse onto the grill.

- A weak mouse would not prefer walking until prompted to move.
- Mouse suffering paralysis or paresis or ataxia can also be identified by toe pinch or observing the gait of the moving mouse.
- Aggression which is common in certain strains of the mouse can also be seen if wounds are there in the hind limb paw.
- A neglected mouse can suffer necrosis or gangrene of the affected hind paw which is often missed in cage side evaluation of the animals.

21.5.5 Examination of the Urogenital Area

Observing the urogenital area can allow the examiner to identify rectal trauma, swelling or tumour of the reproductive organ, rectal prolapse, etc. Fight wounds in the scrotum of the male mouse if allowed to persist may worsen as necrotizing dermatitis due to excessive grooming. The prepuce should be checked for the evidence of wounds and the ability to project the glans penis without difficulty. In senile male mice, swelling of the region adjacent to the rectum is an incidental finding [6]. The mammary glands of the aged female mouse can reveal masses indicative of the tumour of the mammary gland.

21.5.6 Neurological Examination in Rodents

Certain compounds can cross the blood-brain barrier and can affect cognition and influence the behaviour in rodents. A series of ten reflex tests can be done to identify the deficit in the specific region of the brain or the spinal cord.

1. Flexion test—tests spinal cord.
2. Grasping reflex—tests cerebral cortex.
3. Righting reflex—tests mesencephalon and pons.
4. Placing reaction—tests cerebral cortex.
5. Equilibrium tests—tests pons and mesencephalon.
6. Corneal reflex—tests pons, medulla, and upper cervical cord.
7. Pupillary reflex—tests pons and mesencephalon.
8. Auditory startle—tests medulla.
9. Toe spreading—tests many levels.
10. Head shaking—tests medulla.

21.5.7 Other Neurological Abnormalities That Are Observed

- **Head:** Head tilt.
- **Tremors:** Involuntary shaking, convulsions, or seizures.
- **Paresis:** Mice unable to walk due to weakness and unable to support its body weight but can move its legs.
- **Paralysis:** There is a loss of voluntary muscle movement in the legs or tail due to an underlying cause.
- **Hyperactivity:** Hyperactivity is a nonspecific clinical sign. Sometimes hyperactivity relates to a nervous system abnormality and seen as a strain-specific phenotype.

21.5.8 Physical Observations

- Thorax: Shape, respiratory movements.
- Abdomen: Shape, tension, superficial structures, (respiratory) movements.
- Pelvis: Shape and symmetry of skeleton and muscles of the pelvis. Structures of the perineum: size, shape, colour, any discharge. Structures of the groin, with special attention to mammary glands in this area, and inguinal lymph nodes.
- Tail: Position, motility, tension (laxness or rigidity), any wounds or deformities.

The abdominal palpation of the mouse cannot be performed elaborately as in the case of the other animals due to its smaller size. Generally, a mouse urinates when handled as a response to external stimuli. In older male mouse, deep palpation of the abdomen can reveal an enlarged size of the kidneys or enlarged male reproductive organs with distended fluids indicative of urinary obstruction. This requires skill and more practice. The abdominal palpation can also inconvenience the animals and can affect the rate of breathing if the liver and spleen are enlarged. The enlarged bladder is palpable during the caudal abdominal palpation. In older females and retired breeders, masses observed on palpation of the abdomen are indicative of uterine tumours.

The clinical examination of the mouse is challenging due to the small size of the animal. The investigator must practice professional judgment in choosing the method of evaluation to identify unthrifty animals from healthy individuals. A glossary of clinical signs can be found in a publication [6].

21.6 Clinical Examination in Laboratory Rabbits

The clinical evaluation can be challenging depending on the housing provided to the rabbits. The floor housing provides the rabbits with opportunities to express their natural traits that are expressed in the wild. However, in research institutions, rabbits are usually housed in cages individually. This can allow the development of stereotypic behaviours that could be misconstrued as clinical signs developed due to experimental intervention.

The abnormal stereotypic behaviour expressed by rabbits housed in cages includes wire gnawing, excessive wall pawing [11–15], somersaulting [16], and overeating [17]. Aggression commonly develops in rabbits that are penned, while rabbits that are housed singly in cages developed stereotype behaviours probably due to the inability to express traits like hopping and socializing with the cage mates.

It should be borne in mind that stereotype behaviours are undesirable findings in singly housed rabbits. Sudden death can happen due to excess grooming by licking of hairs (a sign of stereotype behaviour) resulting in trichobezoars in the intestine causing intestinal stasis and impaction.

The clinical examination of rabbits begins by observing the activities in the room. A noisier room due to the displacement of the enrichment in the cages by the rabbits strongly indicates a lack of welfare in which the animals are raised. With the general condition assessed, attention should be focussed on individual rabbits for other clinical signs.

The examination should begin with the observation of the animal in its cage. The examination should begin from the cranial end and proceed to the caudal end to assuage any fear that the animal may develop with the handler. The lymph nodes of the cervical region, pre-scapular, popliteal, and inguinal, should be palpated for enlargement and uniformity in size to understand the development of infection. Since the rabbits are covered with fur, the examination should involve checking below the level of fur to rule out abscesses. The fur on the underside of the paw should not be trimmed for ease of examination. The urogenital examination is challenging in rabbits as it can make the animals anxious. Care should be taken to avoid any sudden movement, and the anus and urogenital area should be checked for faecal impaction, loose stools stuck in the perineum, and urine scalding. In female rabbits, mammary glands in the abdomen should be checked for swelling as the latter in old rabbits could indicate a tumour that may be malignant. The testes in male animals can be retained in the abdomen during the examination; application of gentle pressure can push the testes in the scrotal bag.

21.6.1 Examination of Individual Rabbit in Its Cage

Checking of the feed trough/water bottle: The rabbit should be ensured if it has fed and taken water from the water bottle. No decrease in the water level in the bottle can indicate blockade or the inability of the animal to consume water with the given bottle. This should prompt the investigator to observe for dehydration and further intervention. Some rabbits can displace feed from the trough which can mean that the animal was not acquainted with the feed or an improperly designed trough that hinders food acquisition by the animal.

21.6.2 Examination of the Face

A closer look at the face can make obvious the presence of snuffles in the rabbits which are characterized by the mucous or mucopurulent discharge from the nares. This could be indicative of a respiratory infection. The examination can also reveal blepharitis which can present with nasolacrimal duct occlusion resulting in the watery discharge from the eyes of the animal. Infection with *Encephalitozoon cuniculi* can cause uveitis in addition to neurological signs. It can cause the animal to lose orientation and often making the animal unable to maintain a steady gait. Corneal opacity can indicate the trauma due to the sharp object in the cage.

21.6.3 Examination of Ear

The ears of the animal can harbour mites giving the ear a non-glossy appearance. The severity of the ectoparasite infestation is proportional to the scales and dryness of the ear. This can result in repeated bouts of scratching. A skin scraping from the ear of the animal observed under the low or high power of the microscope can reveal the presence of mites. The clinical designation of an animal as suffering from mite infestation requires demonstration of the parasite with all its stages of the life cycle along with evidence of crustiness along the margins of the ear pinna and diffuse erythematous zones on the ear. The ear in the rabbits is valuable for the research investigators for accessing its veins and arteries for blood withdrawals and test item administrations. The ear should be arbitrarily divided into the outer segment (flap and ear canal), middle segment (drum and the Eustachian tube), and the inner segment (cochlea and inner canals). The rabbit can be found shaking its head when the observation is made without making the animal aware. The head tilt is because of the accumulation of pus, sequelae of *Pseudomonas* infection. This can often end up affecting the brain.

21.6.4 Examination of Mouth

This should be followed by an examination of the mouth. Since the rabbit's teeth grow ceaselessly, it can result in pathology that is not discernible without detailed examination. A simple overgrown tooth leading to difficulty in feeding might make the animal anorexic, misleading the veterinarian to a systemic infection.

The examination of the mouth cavity is not possible without administering general anaesthesia. The use of cheek dilators or gags is required for the proper visualization of the cheek tooth. Since dental pathologies can affect the other associated structures of the face, a detailed examination of the teeth is warranted. Performing a detailed examination in a live animal requires expertise to avoid fractures of the teeth when instruments are used. Cyndi Brown (2008) has given a detailed insight into the examination of the oral cavity [18]. The examination of the face provides preliminary information to identify if an animal is healthy or sick.

21.6.5 Examination of the Animal by Removal from Its Cage

Once suspected**,** the animal can be removed from the cage for further examination. For this, the handler should have acclimatized the animal to handle. Much more information can be acquired by the veterinarian from the physical examination.

21.6.6 Examination of Skin and Fur

The observer should run the fingers through the fur to check for superficial nits or ticks. The glossiness of the hair can also be ascertained when the fur is examined in detail. The scales on the skin at the base of the hair can be felt. The presence of any self-mutilated wounds can be observed. The loose skin over the dorsum can be pulled to check for tenting which can indicate the level of dehydration. A well-hydrated animal would not present with tenting upon pulling the loose skin.

21.6.7 Examination of Limbs

A rabbit reared in a cage can develop an overgrowth of the nails that would require trimming. The animals with overgrown nails can bleed at nail tips when attempting burrowing on the cage bottom. The medial side of the forepaws should be observed for the evidence of rubbing its nose of any secretions. This can identify an ongoing clinical change in the animal by the change of colour in the medial aspect of the forepaw. The symmetry between the limbs should also be checked to check for any growth or masses.

21.6.8 Abdominal Examination

The organs in the abdomen can be examined by abdominal palpation. The relative sizes of the organs are to be known to make a meaningful conclusion. Since the rabbits held will be of different sizes and weights, the abdominal palpation can be confounding. The presence of any masses can be found by gentle palpation. Further confirmation requires techniques like imaging.

A detailed review of the management practices in rabbit husbandry can yield voluminous information on an observed abnormality in the animals. Rabbits are averse to sudden feed changes when the animals are transferred from the vendor site to the recipient lab. The change in feed can result in animals becoming off-feed or developing loose stools similar to diarrhoea. Faecal examination under a microscope after the floatation technique can throw light on the cause. Oocysts of coccidia can be looked for since a common cause of infectious diarrhoea is hepatic coccidiosis in rabbits. However, diarrhoea due to *Clostridium* species cannot be identified by microscopic evaluation of faeces.

21.7 Some Commonly Observed Clinical Signs in Rodents

Barbering: During grooming, mice tend to pluck the other mice's hair. This is the dominant expression in the mice and may result in alopecia and is more common in C57BL/6 strains. Severe cases may result in ulcerative dermatitis or bite wounds. This can be prevented using an enrichment device.

Fighting: The wounds resulting from fighting are seen in the base and back of the animals.

Ulcerative Dermatitis: Ulcerative dermatitis (UD) is an idiopathic, spontaneous, debilitating syndrome of laboratory mice and is commonly aged and in particular C57BL/6 strain. Pruritus is the characteristic lesions and may lead to severe inflammation. Ulcerative dermatitis is diagnosed by eliminating other causes of dermatitis in laboratory mice.

Malocclusion: Overgrown incisor teeth results in poor weight gain of weaned animals. During malocclusion, the top and bottom teeth are hitting against each other. Usually, the bottom teeth grow long and are easily noticed that interferes in feeding and ultimately leading to poor weight gain.

Rectal Prolapse: Rectal prolapse is commonly found in mouse colonies. It may be due to spontaneous or due to *Helicobacter* and *Citrobacter* species. The animals with rectal prolapse lead to ulceration and necrosis, and secondary bacterial infections invade illness.

Vaginal or Uterine Prolapse: The uterus may prolapse through the vagina and vulva after the delivery in some mice due to straining. However, it is important to identify if the prolapsed organ is the rectum or uterus. In rectal prolapse, the prognosis is not favourable.

Anaemia: The footpad of mice is the main indicator for anaemia. The paleness of its footpads is an indicator of an anaemic mouse.

Dehydration: During dehydration, the eyes that appear recessed in their heads, and the facial fur will appear vaguer (due to piloerection). The skin will not return quickly to its original shape when the mouse is picked up. The animals usually remain bunched. In severe conditions, the mice will be cool when touched.

Diarrhoea: The faeces of mice is usually moist but rarely liquid. The mice with the diarrheic condition can be deducted with faeces sticking with bedding materials or on the side of the cage. We can also notice the staining of faeces around the rectum.

Hypothermia: During hypothermia, the body of mice will be cool to touch; the mice will be lethargic, tend to huddle, and lack responsiveness if we try to pick up.

Ruffled Fur: Mice with ungroomed appearance designate a mouse is unwell due to illness.

21.8 Reporting of Clinical Signs

An ideal description of a lesion should include its anatomical location, its symmetry (unilateral/bilateral), nature/extent of spread (diffused or focal), and the use of additional qualifiers like duration (acute, chronic, sudden occurrence) which can provide valuable information of the lesion. The reporting of clinical signs may be improved by using a graphic to depict the location of the lesion and its size for ease of understanding and communication with peers. However, it is ideal to make descriptions with predefined terminologies. It should be understood that the clinical signs observed only inform the changes in the animal, and it cannot enable the observer to offer a diagnosis as the latter requires further investigation by employing other diagnostic methods [19].

21.9 Development of Clinical Sign Scorecard

The investigator is suggested to develop a scorecard for each of the projects that involve experimental animals. At the time of preparing the project, the investigator can envisage the likely development of the clinical signs that can be unique to the experimentation. In case of developing a protocol like experimental autoimmune encephalitis, the mice are expected to develop tail sagging, inability to void urine spontaneously, and inability to maintain a steady gait. These experimental signs develop gradually and consistently over some time as the model establishes in affecting the cerebrospinal cord. The development of a scorecard unique to this experiment based on the above clinical signs (that are anticipated in addition to other signs that can develop in the animals) can inform the investigator of the extent of morbidity in the animal. Upon assigning a score based on the severity of the clinical signs, the investigator or the veterinarian can decide if euthanasia would be warranted in those animals exhibiting the clinical signs.

The scorecard, if it can become a part of the project before initiation, needs to be developed for every animal in the project. The severity of each clinical sign should

have at least three levels of ranking (no clinical sign observed, moderate level of clinical sign, and severe clinical sign) to enable the investigator to decide the welfare of the animals under study. If a severe form of clinical sign(s) is observed to make the animal non-ambulatory, then the decision to euthanize the animal should be taken in consultation with the veterinarian.

At the time of the development of a scorecard, the author should ensure that harmonization is achieved to avoid redundancy/conflicting thoughts in understanding the clinical signs described by an investigator. Harmonization and standardization in reporting the clinical signs can ensure 3Rs in laboratory animal experimentation in biomedical research.

21.10 Conclusion

Understanding the clinical sign in laboratory animals is a daunting and a challenging task. Since the species involved are reluctant to express their signs, mastery of the same can take a long time to achieve. An expert investigation and a sound judgment thereon are defining steps to evaluate the welfare measures and also to relate the trajectory of the research. A veterinarian, that too, a trained one, plays a crucial role. The success of the investigation and the success of the welfare measures hinges on the proper reading of clinical signs. Looking for clinical signs starts the moment one enters the animal room, even before we touch and handle the animals. This chapter has attempted to provide a primer for the clinical signs in a laboratory animal. However, the reader is informed that clinical signs are learned better by observing an animal in a live situation. Though a veterinarian can understand the terms, only hands-on training in an established vivarium under a well-trained veterinarian will make him/her fully competent.

Annexure 1

A list of commonly observed clinical signs is provided below [19]

Sign	Possible causes
Alopecia	Hair loss due to barbering by other animals
Ataxia	Trauma to the caudal spine, post-surgical complication, encephalitis or due to mutation of a certain gene like ATX1,2,3, CACNA1A
Blindness	The drug-related effect, trauma to the eye or retinal generation mutation gene in few inbred mouse strains like C3Hej /J, FVB, CBA, SJL, etc.
Circling	Middle ear infection, the space-occupying lesion in the brain

(continued)

Coprophagia	Physiological behaviour in rodents, high parasite infestation, exocrine pancreatic insufficiency
Dyspnoea	Anaesthetic overdose, drug effects
Dystocia	Strain character, dead pups, non-dilated vaginal canal, or exhaustion of female
Erythema	Local histamine and inflammatory cytokine release due to chemicals
Hyperaesthesia	Post-surgical complication
Pruritus	Ectoparasitic infection
Tremor	Drug treatment-related effects
Vocalization	Aggression in cohort, pain

Annexure 2

Table showing the clinical signs and the possible infectious causes [20]

Clinical signs	Possible causes
Abdominal enlargement	Kilham rat virus Lymphocytic choriomeningitis virus Murine leukaemia viruses
Abortions and stillbirths	*Streptobacillus moniliformis*
Cervical abscesses	*Klebsiella pneumoniae* *Pasteurella pneumotropica* *Streptococcus pyogenes*
Abscess in facial, orbital, and tail	*Staphylococcus aureus*
Preputial gland	*Pasteurella pneumotropica* *Staphylococcus aureus*
Amputations, necrotic, of limbs or tails	*Corynebacterium kutscheri* Ectromelia virus *Mycoplasma arthritidis* Poxvirus(es) in rats
Ringtail	*Streptobacillus moniliformis* Change in humidity
Anorexia	*Corynebacterium kutscheri* Ectromelia virus *Klebsiella pneumoniae* Poxvirus(es) in rats
Birth weight reduced	*Salmonella enteritidis* Sendai virus
Cervical oedema	Sialodacryoadenitis virus
Chattering (mice)	*Chlamydia trachomatis* *Mycoplasma pulmonis* Sendai virus
Circling (or rolling)	Kilham rat virus *Mycoplasma neurolyticum* (experimental) *Pseudomonas aeruginosa*

(continued)

	Streptobacillus moniliformis Theiler's virus
Conjunctivitis	Ectromelia virus *Pasteurella pneumotropica* *Salmonella enteritidis* Sialodacryoadenitis virus *Staphylococcus aureus* *Streptobacillus moniliformis*
Convulsions	Theiler's virus Corneal ulceration Sialodacryoadenitis virus
Cyanosis	*Chlamydia trachomatis* *Haemobartonella muris* *Salmonella enteritidis* *Streptobacillus moniliformis*
Dermatitis and alopecia	Due to infectious agents Dermatophytes (fungi) Ectromelia virus Mouse papule virus *Myobia musculi* *Myocoptes musculinus* *Pasteurella pneumotropica* *Staphylococcus aureus* Due to noninfectious causes Bite (fight wounds) Hair growth arrest
Diarrhoea	*Bacillus piliformis* *Citrobacter freundii* *Giardia muris* Mouse hepatitis virus (infant mice) Mouse rotavirus (infant mice) *Salmonella enteritidis* *Spironucleus muris*
Dyspnoea	*Chlamydia trachomatis* Cilia-associated respiratory bacillus *Corynebacterium kutscheri* *Haemobartonella muris* Murine leukaemia virus *Mycoplasma pulmonis* Sendai virus *Streptococcus pneumoniae*
Emaciation	Lymphocytic choriomeningitis virus *Salmonella enteritidis* *Streptobacillus moniliformis*
Facial abscesses	*Staphylococcus aureus* Facial oedema Ectromelia virus

(continued)

Head tilt	*Mycoplasma pulmonis* *Pseudomonas aeruginosa* Theiler's virus
Ocular discharge	Sialodacryoadenitis virus
Papular Rash	Ectromelia virus Poxvirus(es) in rats
Paralysis of rear legs	Lactic dehydrogenase-elevating virus (in C58 and AKR mice) Polyoma virus [in athymic (nu/nu) mice] *Streptobacillus moniliformis* Theiler's virus
Polypnoea	Cilia-associated respiratory bacillus *Corynebacterium kutscheri* *Mycoplasma pulmonis* *Streptococcus pneumoniae*
Pruritus	Dermatophytes (fungi) *Myobia musculi* *Myocoptes musculinus* *Staphylococcus aureus*
Rectal prolapse	*Citrobacter freundii* (Biotype 4280) *Syphacia* sp.
Respiratory rales	Cilia-associated respiratory bacillus *Corynebacterium kutscheri* *Mycoplasma pulmonis* *Streptococcus pneumoniae*
Skin ulceration	Dermatophytes (fungi) *Myobia musculi* *Myocoptes musculinus* *Staphylococcus aureus*
Snuffling	Cilia-associated respiratory bacillus *Mycoplasma pulmonis* Sendai virus Sialodacryoadenitis virus *Streptococcus pneumoniae*
Swelling (oedema)	Feet and tail Ectromelia virus Poxvirus(es) of rats Ringtail *Streptobacillus moniliformis* Neck Sialodacryoadenitis virus Swollen, reddened joints *Corynebacterium kutscheri* *Mycoplasma arthritidis* *Streptobacillus moniliformis*

References

1. Garber JC, Barbee RW, Bielitzki JT, Clayton LA et al (2011) The guide for the care and use of laboratory animals, 8th edn. The National Academic Press, Washington, DC, pp 113–115
2. Smith JA, van den Broek FA, Martorell JC, Hackbarth H et al (2007) Principles and practice in ethical review of animal experiments across Europe. Lab Anim 41:143–160
3. Hess SE, Rohr S, Dufour BD, Gaskill BN et al (2008) Home improvement: C57BL/6J Mice given more naturalistic nesting materials build better nests. J Am Assoc Lab Anim Sci 47:25–31
4. Jirkof P (2014) Burrowing and nest building behaviour as indicators of well-being in mice. J Neurosci Methods 234:139–146
5. Deacon R (2012) Assessing burrowing, nest construction and hoarding in mice. J Vis Exp 59:2607
6. Burkholder T, Foltz C, Karlsson E et al (2012) Health evaluation of experimental laboratory mice. Curr Protoc Mouse Biol 2:145–165
7. Canadian Council on Animal Care (1968) CCAC guideline:Mice. http://www.https://ccac.ca. Accessed 2 June 2020
8. Deacon R (2006) Burrowing in rodents: A sensitive method for detecting behavioural dysfunction. Nat Protoc 1:118–121
9. Deacon R (2007) Assessing hoarding in mice. Nat Protoc 1:2828–2830
10. Foltz CJ, Ullman-Cuellere M (1999) Guidelines for assessing health condition of mice. Lab Anim 28:28–31
11. Philips C (2007) Animal welfare. In: Kaliste E (ed) The welfare of laboratory animals, vol 2. Springer, Dordrecht, p 211
12. Wiser R (1986) Functional analysis of behavior as a basis for assessing animal justice. An investigation into normal behavior and behavioral disorders in domestic rabbit Zibben. Dissertation, University of Berne
13. Bigler L, Lehmann M (1991) Final report on the examination of the animal justice of a fixed wall cage for domestic rabbit zibben. Report Swiss Federal Veterinary Office, Bern
14. Loeffler K, Drescher B, Schulze G (1991) Influence of different keeping methods on the behavior of test and meat rabbits. TieraerztlUmsch 46:471–478
15. Stauffcher M (1992) Group housing and enrichment cages for breeding, fattening and laboratory rabbits. Anim Welf 1:105–125
16. Podberscek AL, Blackshaw JK, Beattie AW (1991) The behavior of group penned and individually caged laboratory rabbits. Appl Anim Behav Sci 28:353–363
17. Gunn D, Morton DB (1995) Rabbits. In: Environmental enrichment information resources for laboratory animals. AWIC Resource Series
18. Brown C (2008) Intraoral examination in rabbits. Lab Anim 37:405–406
19. Fentener van Vlissingen JM, Borrens M, Girod A, Lelovas P, Morrison F, Saavedra Torres Y (2015) The reporting of clinical signs in laboratory animals. Lab Anim 49:267–283
20. Lindsey JR, Boorman GA, Collins MJ, Hsu CK, Hoosier GLV, Wagner JE (1991) Infectious diseases of mice and rats, National Academy Press, p 260

22 Anaesthesia, Analgesia and Euthanasia of Laboratory Rodents and Rabbits: A Practical Approach

V. S. Harikrishnan

Abstract

Understanding the signs of laboratory animal distress, pain and anxiety and its mitigation is quintessential in obtaining meaningful results from animal studies. Even routine management practices like cage changing, tattooing, transportation, restraint and blood collection can cause stress in animals, and so professional judgement shall be made on the requirement of anaesthesia/analgesia on a case-to-case basis. Various techniques are available to assess the levels of pain in rodents and rabbits and there is a wide spectrum of drugs from which a choice can be made on analgesic/aesthetic administration. Any interaction shall be avoided between anaesthetic/analgesic drugs used and targeted data to maintain the dependability of results, and so the choice of the anaesthetic/analgesic regimen is of utmost importance. Monitoring anaesthesia to ensure a safe recovery and to provide analgesia thereafter requires knowledge and practice. Further, existing practices of euthanasia shall also be judiciously scrutinized by dynamically imbibing the evolving data and recommendations published by scientific groups. This chapter aims to throw light on the basic scientific principles and practices of laboratory animal anaesthesia, analgesia and euthanasia in rodents and rabbits and its welfare components from a practical perspective.

Keywords

Identification of pain · Analgesia · Anaesthesia · Euthanasia · Laboratory animals

V. S. Harikrishnan (✉)
Division of Laboratory Animal Science, BioMedical Technology Wing, Sree Chitra Tirunal Institute for Medical Sciences and Technology, Thiruvananthapuram, Kerala, India
e-mail: harikrishnan@sctimst.ac.in

© The Author(s), under exclusive license to Springer Nature Singapore Pte Ltd. 2021

P. Nagarajan et al. (eds.), *Essentials of Laboratory Animal Science: Principles and Practices*, https://doi.org/10.1007/978-981-16-0987-9_22

22.1 Introduction

Sushruta Samhita that means Suśruta's compendium dating to the sixth century BCE is considered to be the foremost scripture on surgery, and this text scribes on the training of future surgeons. This book recommends the students to be trained using gourds and dead animals in surgical techniques. The use of wine with the incense of cannabis [1] and henbane (*Cannabis indica*) [2] for anaesthesia has been documented in this book. However, the first record of modern anaesthesia dates back to 1846, when doctors and dentists of the likes of Crawford W Long and William TG Morton used sulphuric ether to remove a tumour. The first preclinical test to confirm the efficacy and safety of anaesthetics also dates back to the times of Morton (1840s), when he tested it in his menagerie of pet animals and was satisfied with the performance of sulphuric ether [3]. Contemporarily, nitrous oxide was also used to take the senses off, during dental procedures [3]. Since then, a plethora of pre-anaesthetic and anaesthetic drugs and techniques have been introduced and are proven to be safe and efficient for clinical use after the completion of preclinical trials in laboratory animals. So, a wide spectrum of anesthetics and analgesics with their doses and safety well established and effectiveness proven are readily available to be used in laboratory rodents.

Apart from invasive surgical procedures, even routine management procedures like cage changing, handling and blood collection in laboratory animals can be stressful [4]. Tattooing in laboratory rabbits potentially causes pain and distress, and, in most cases, the management of pain is not addressed [5]. So, in contrast to clinical practice in humans, laboratory animals would require to be anaesthetized, even during some non-surgical procedures, and for those cases in which pain persists, analgesia will have to be provided. Underreporting of anaesthesia and analgesia [6] and lack of attempts to identify and mitigate pain can undermine research. Withholding analgesia cannot be justified in cases where there is a record indicating that the provision of analgesia has no potential impact on the animal model [7]. On the other hand, some disease models, like the model of arthritis, can be adversely affected by the use of NSAIDS [8] since the inflammatory process forms the basis of the generation of the model. In animal models of neuropathic pain and nerve injuries like spinal cord injury, it is debated that neuroinflammatory process and construct validity can be compromised by the usage of several classes of analgesic and anti-inflammatory agents, where a marked concern exists regarding analgesia usage among scientists working in this field [7]. A recent survey on neuropathic pain models which only consisted of spinal cord injury models or spared nerve injury models revealed that the majority of the papers did not mention the use of perioperative analgesia [6]. It is worthwhile to note recent scientific evidence being exposed that postoperative analgesia does not affect neuropathic hypersensitivity outcomes in the spared nerve injury models [9]. Adequate use of analgesia is advocated wherever it favours imparting welfare and promotes closeness in the simulation of the human conditions.

Contemporarily, in developing countries, the lack of competent centres and trainers together with the economic constraints hinder the progress in imparting

awareness and adding competencies in the assessment and mitigation of pain in laboratory animals. Advancement in this field will be hard to percolate to the working levels in such a scenario. Most of the research groups will focus to comply to the national legislation even though it lacks a periodic technical review process and, thus, will miss updated cutting-edge techniques. For instance, for retro-orbital blood collection in rodents, the Danish Animal Experiments Inspectorate insists on anaesthesia as a mandatory requirement, whereas many other national guidelines do not for the same procedure.

Hence, following peer-reviewed publications along with the national/regional statutes to maintain competent techniques in addressing pain and distress is of importance. There are also many instances where data from peer-reviewed publications differ in results leading to uncertainty among researchers to decide upon whether anaesthesia is required or not. As an example, sublingual bleeding in rats had been reported to be performed under general anaesthesia whereas the technique is also done without anaesthesia and could be demonstrated not to involve any distress [10].

Wherever inconclusive and contradictory evidence exists, studies must be conducted to ascertain the underlying facts whether mitigation of pain can affect the outcome in animal models. It is important to probe whether abolishing periprocedural pain can be achieved using any particular class of analgesic without affecting the outcome of the model. It is also necessary to assess the pain levels after an adequately long time in chronic pain models. Anxiety-like behaviour and depression are well reported in rodent models of chronic pain. A comprehensive plan to assess and address these issues shall be part of an animal experimentation proposal.

Conducting surgical procedures in animals without adequate anaesthesia can hinder the observance of the tenets of Halstead leading to improper muscle relaxation. Gentle handling of tissues is not feasible, and underlying structures are hard to be visualized, and in turn, proper haemostasis cannot be achieved leading to compromised outcomes of the model and overwhelming pain can even lead to shock and death. Animals that underwent surgery when left to experience undue pain without postoperative analgesia will have an increased concentration of serum corticosteroids that are anti-inflammatory leading to delayed wound healing and immunocompromise. Altered immune status can render these animals vulnerable to infections. The ability of their immune systems is compromised, disabling them in detecting foreign materials (for instance, the implanted biomaterials or devices) which is the key concept behind biocompatibility tests. Ultimately, the results from such studies lead to retrieving unreliable biocompatibility data. Lack of analgesia also will prolong postoperative recovery time, exerting a negative impact on the study. Those animals not being offered with or being treated with inadequate analgesia tend to move less to drink and eat which could lead to marked postoperative body weight loss and lack of bioavailability of nutrients, thereby prolonging the healing time. Owing to inadequate analgesia, the animals try to restrain their natural depth of breathing since the sutures expand with each inhalation leading to pain. This can result in hypoxia and hypercapnia affecting respiratory homoeostasis and blood gas levels, leading to a delayed recovery process. The decision to provide

anaesthesia for any procedure shall be made considering all these underlying facts, on a case-to-case basis. In this chapter, acceptable practices and practical aspects of the usage of anaesthesia, identification of pain and providing analgesia and the important points to be considered during euthanasia of laboratory rabbits and rodents are being discussed.

22.2 Anaesthesia

22.2.1 Deciding on the Requirement of Anaesthesia

The decision-making step should weigh the stress owing to the injection procedure (in case of injectable anaesthesia) and the lingering stress owing to the anaesthetic drug used to anaesthetize an individual in any particular species. If the procedure for which anaesthesia is planned has to be done multiple times, feasibility, safety and cumulative stress every time the anaesthesia exerts on the animal, in addition to the procedural stress, has to be also assessed. Further, the effect of the anaesthetics on the outcome of the study, in terms of how the anaesthetics can pharmacologically affect the targeted data, shall also be questioned.

22.2.2 Decision-Making Process: A Case Study

22.2.3 Oral Gavage in Rodents

Oral gavage in mice and rats and, in particular, serial oral gavages in animals are a common procedure in toxicological studies. Much data is available to scrutinize the necessity of anaesthesia in this procedure. Oral gavage offers a high-precision technique of enteric dosing of pharmacologic agents for testing and is a straightforward procedure in rodents [11]. However, a series of complications like pneumonia, reduced food intake, weight loss, traumatic oesophagus, pleurisy, stress and mortality are reported owing to the procedure [12, 13]. However, it is also reported that chronic daily oral gavage in awake animals does not affect the welfare of rats acclimatized to handling [14]. In a study in mice, oesophageal damage was inapparent in both awake and anaesthetized mice. However, incomplete retention of administered saline was reported in awake mice in comparison to anaesthetized mice, suggestive of an advantage in procedural success without any added stress in anaesthetized mice [15]. Training of handlers and acclimatizing the rodents to handling are shown to improve the outcome in rodents. Considering all the information available from the previous studies, the decision-making process is difficult, and so the experience of the investigator on pre-existing morbidity/mortality data if any can be used to make the decision. Consideration must be given to the number of animals to be gavaged at the same time and the frequency and duration of gavaging required for the entire study. If any previous experience is not available, and if the investigator is still perplexed, the best way forward is to conduct a pilot study, with

an awake and anaesthetized group and observe the scientific indications before a full-fledged study is executed.

22.2.4 Oral Gavage in Rabbits

Oral administration of substances by intra-gastric gavaging in rabbits is more difficult than in rats and mice [16]. Introducing the nasogastric tube in conscious rabbits requires a topical anaesthetic spray with adequate time for action, after which tubes can be inserted with adequate restraint of the rabbit [17]. Even though many works had been reported indicating chronic gavage of fluids in rabbits, the usage of anaesthesia during oral gavage in rabbits [18, 19] nor the technique in detail is often not reported.

While performing oral gavage in conscious rabbits without the use of any muscle relaxants, a mouth gag to prevent the tube from being damaged by the rabbit's teeth during administration and withdrawal is advised [20]. The mouth gag itself may inflict potential damage to the buccal cavity, teeth and tongue. However, it can prevent fatalities by accidental foreign body insertion of the resected portion of the indwelling ET tube in the trachea owing to chewing. Further, physical restraint itself can be stressful in rabbits that are not as easily trainable as rats. The justification to use an anaesthetic for the procedure gets stronger with all these facts being stated. Xylazine and ketamine were used at the rates of 9 mg/kg and 3.6 mg/kg, respectively, as a single IM injection for gastric intubation in rabbits [16]. Smaller volumes can be syringe-fed (2–3 ml), even though rabbits tend to reject substances that taste unpleasant. In practice, gastric gavaging higher volumes requires care, and the procedure is easier and safer when the rabbits are sedated. Considering all these facts, we arrive at a decision that gastric gavaging anaesthesia in rabbits is beneficial for the precise gastric administration of substances in higher volumes. Anaesthesia is also required topically when nasogastric tube is introduced and must be maintained in situ for longer durations in rabbits.

The algorithm on factors to be considered on decision-making on anaesthetic administration is given in Fig. 22.1.

22.3 Choosing an Anaesthetic Regimen

It is to be borne in mind that there is no rigid rule to choose an anaesthetic regimen to suit all the studies, and a convenient regimen shall be created based on the specific requirement of each study. It is well-known that xylazine and medetomidine are alpha-2 agonists very commonly used in combination with ketamine for a wide range of animal species including rodents and rabbits. However, these drugs elevate blood glucose levels and hence shall not be considered in studies focusing on blood sugar. Most of the anaesthetic drugs alter cardiovascular parameters, especially blood pressure and heart rate, and so for the research aiming to collect data on these values, ethyl carbamate also known as urethane and alpha-chloralose shall be

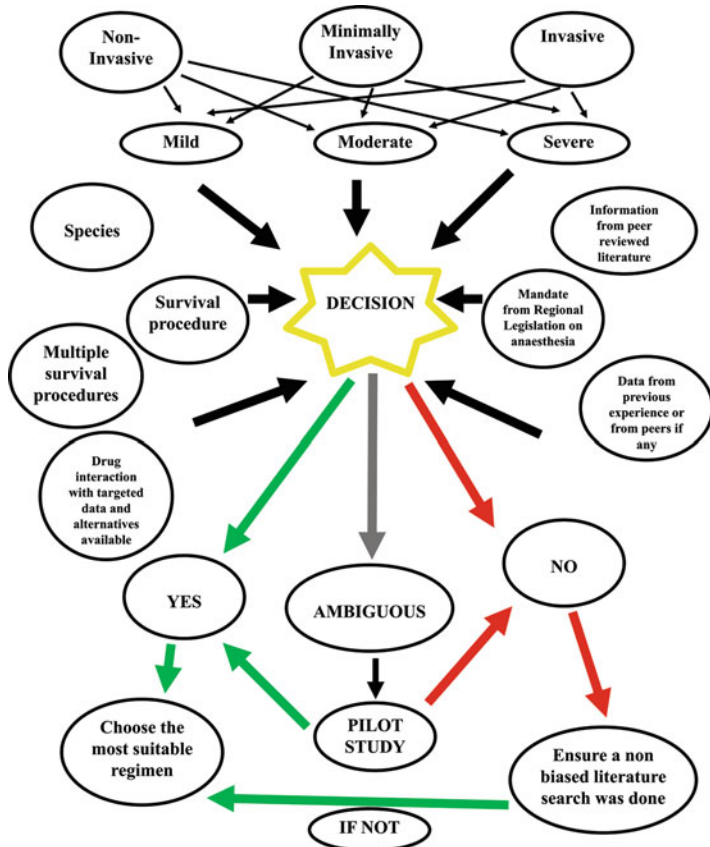


Fig. 22.1 The algorithm on factors to be considered on decision-making of anaesthetic administration

considered as the drugs of choice. While choosing agents and doses, both species and strain-specific contraindications have to be considered. Halothane, an inhalant anaesthetic, is capable of producing a condition known as malignant hyperthermia in certain breeds of pigs. It is also known that the same anaesthetic produces a different level of effects in different strains. Due consideration must be given to the duration of the procedure, depth of anaesthesia required and the number of times anaesthesia is required repeatedly and the interval between them for the procedure in question. For procedures evoking painful responses such as manipulation of the periosteum (involving bone resection or drilling and implantation), induction with injectable anaesthesia and maintenance with inhalant anaesthesia with topical instillation of local anaesthetic at the site of surgery is preferred to using inhalational anaesthesia alone. For transient procedures like retro-orbital bleeding, either isoflurane inhalation or even carbon dioxide as such (under experienced supervision) can be used to

produce momentary anaesthesia, thereby extracting advantage of an instantaneous recovery.

22.4 Various Methods Used to Anaesthetize Animals

22.4.1 Preconditioning of Animals and Considerations on Perioperative Heat Loss

In mice, a physiological condition is known as "torpor" exists, which is characterized by a sharp drop in body temperature as well as metabolism which is in response to lower ambient temperatures and non-availability of food. The expression of torpor in outbred mice differs between individuals of the same litter [21]. It is important to note that a mouse that has not fasted does not express torpor, whereas they get into torpor after 7 h of fasting exhibiting a 15 °C drop in body temperature [22]. So, there is a valid reason to ponder over whether fasting as a part of the surgery or diabetic or toxicological studies in mice is of any value since it introduces a confounding artefact into the research data [23]. Further, the rodents and the rabbits cannot vomit due to a lack of neural components in the brain stem that coordinate different sets of muscles in the gastrointestinal tract to throw up. Hence, the likely complications anticipated in larger laboratory animal species do not exist in rodents and rabbits. The health of rabbits getting deteriorated due to pain during the immediate post-procedural days can be aggravated by preprocedural fasting. Owing to these events, gastric motility decreases, and gut stasis occurs in these monogastric hind-gut fermenters, giving rise to excessive fermentation. The normal proportion of gut microbiota becomes dramatically altered. Yeast and other stray organisms usually present in low numbers (*Escherichia coli* and *Clostridium* species) will start proliferating leading to symptomatic presentation as soft stool or, in severe cases, as true diarrhoea leading to enterotoxaemia progressing to shock, toxaemia and death.

22.4.2 Conservation of Heat

General anaesthesia depresses the central nervous system (CNS) affecting loss of consciousness and reduced muscle tone, and many agents as a side effect will also depress the respiratory and thermoregulatory centres of the brain. The ratio of surface area per gram body weight is relatively higher in small animals, and this makes them remarkably vulnerable to the loss of heat, delaying anaesthetic recovery. Heat loss is aggravated during the surgery when the body cavities are opened. So, it is important to provide warmth using a constant heating pad during surgery for lengthy procedures and a warm and quiet area for postoperative recovery. Hypothermia can be mitigated during the operative and recovery phase by draping the rodents with sterile plastic bubble packs, a cost-effective alternative to heating pads. Windows can be cut in the bubble packs at the required site of surgery as per the

dimensions to suit the purpose of the surgery. The tails being the major organ of thermoregulation in rodents shall also be included to be within the draping, since considerable heat loss can occur from the tails. Applying ophthalmic ointment or saline wetted gauze on the eyes for protection during surgery and recovery underlighting bulbs as a heat source shall be practiced during anaesthesia since dried eyes owing to lack of lachrymation and exposure of the opened eyes to the surgical lamps can both cause damage. Pre-emptive analgesia aims to anticipate and mitigate the pain which will arise owing to the upcoming invasive procedure, which should also be part of a comprehensive pre-anaesthetic regimen. By the time noxious stimuli are being introduced, the plasma concentration of the analgesic would have reached a therapeutic level, mitigating the pain that is arising. Anticipating the perioperative fluid loss and inadequate self-hydration, as a thumb rule, 5 ml and 1 ml of pre-warmed sterile normal saline can be subcutaneously administered using a needle size of 23–25G to adult rats and mice, respectively. In rabbits, using an IV line, fluids can be administered at the rate of 10 ml/kg/h, through the marginal ear vein. Turgor test can be applied during postoperative days, and extra fluid supplementation shall be given if required.

22.4.3 Injectable Anaesthetics

22.4.3.1 Premedication

Premedication or pre-anaesthetic medications are a group of drugs aimed to aid the setting of, compensate inadequacies of and also counterbalancing the side effects of anaesthetics. As an example, the sedative xylazine induces dose-dependent sedation, analgesia and muscle relaxation. Ketamine and xylazine (KX) form a well-working combination and can supplement towards lack of muscle relaxation offered by ketamine. Increased blood pressure is a feature of ketamine, and even though hypotension is produced by xylazine, the popularity of the mixture is owing to the supplemental effects on analgesia, sedation and muscle relaxation. Build-up of bronchial secretions when KX is being used leads to bronchial spasms and airway obstruction in most of the experimental species. In subjects like goats and dogs, xylazine produces vomit, which can lead to aspiration pneumonia and death. Atropine or glycopyrrolate are agents that belong to the class of anticholinergics or parasympatholytics which reduce the secretory effects of KX. However, in rodents and rabbits, owing to higher heart rate and inability to vomit and, in rabbits, a considerable proportion of animals possessing an enzyme known as atropine esterase (atropinase), atropine is not widely used in practice, and KX can directly be given as a mixture in a single syringe with needle size 23–25G. In rabbits, glycopyrrolate can be used as an alternative premedicant.

22.4.3.2 Induction and Maintenance

Induction shall be done with the restraint of minimum duration in a noise-free area, and the animals shall not be disturbed after the injection. The preferred route of KX induction is IP in rodents and IM in rabbits. In the mice owing to their low body

weights, ketamine and xylazine shall be diluted with normal saline to make it up to workable volumes to prevent overdosing and mortalities thereby. Maintenance of KX anaesthesia can be achieved safely and effectively by repeating 50% of the initial dose upon observing the emergence of the animal from anaesthetic plane. However, unacceptable levels of mortality are reported in mice, with this practice and data showing an effective continuous rate infusion through the IP route [24], even though this technique presents many practical difficulties in smaller subjects like mice. Another drug used commonly and effectively in rabbits as a general anaesthetic is propofol. Induction as a bolus can abolish breathing reflexes, and thus an anticipatory readiness to perform endotracheal intubation and mechanical ventilation of the subject is advised. Propofol diluted in normal saline can be used for maintenance as an IV infusion where a mechanical flow controller can be used to adjust the dose if precision pumps are not present. Propofol brings in instantaneous loss of reflexes, and the recovery is just as fast. Sleep time is only 10–15 min in rodents and rabbits for propofol, whereas KX can give up to 100–150 min.

Injectable drugs are popular since they do not require any special equipment. However, due care has to be exercised while calculating the dose as it is not only dependent upon body weight but also on the physiological status (like obese aged females in comparison to young lean males require lesser dosing irrespective of weight) and also on the strain of animals. So, the experience of the anaesthetist plays a major role in the assessment of dosing required. Further, after the induction of anaesthesia, there will be a time lag before the sequential loss of righting reflex, pinna pinch (in rabbits), pedal pinch and tail flinch (in rats and mice) reflexes and comes to the surgical anaesthetic plane. During this time lag, a quiet and dark environment is beneficial. Prolonging the KX anaesthesia can be attempted with half the initial dose of both xylazine and ketamine. It is worthwhile to note that the effects of xylazine can be reversed using atipamezole. Acepromazine if added to KX combination can bring in an additive effect, and the dose of ketamine and xylazine can be brought down to half and one-third, respectively, yet bringing in the surgical plane of anaesthesia in rats.

Barbiturates are generally less popular in rodents and rabbits as an aesthetic agent owing to its potential induction of apnoea and particularly slow recovery, and the doses are excluded from the table (Table 22.1).

22.4.4 Inhalant Anaesthetics

Inhalational anaesthesia is induced by introducing the gas to animals in a chamber and maintenance using a face mask (Fig. 22.2) or an endotracheal (ET) tube. Drugs like isoflurane and sevoflurane have fewer side effects, a larger safety index and lesser occupational hazard in comparison to halothane. The animals need not be weighed because the dose calculation is independent of body weights and restraint of the animals for induction is also not required, thereby avoiding the stress of restraint. Inhalational anaesthesia has a very short induction and recovery time from anaesthesia, and the safety index is substantially high in comparison to injectable

Table 22.1 Commonly used injectable anaesthesia in rodents and rabbits

Drug	Rats	Mouse	Rabbits	Comments
Xylazine+ ketamine	5 mg/kg body weight + 80 mg/ kg body weight IP (preferred) or IM	5 mg/kg body weight + 80 mg/ kg body weight IP	5 mg/kg body weight + 50 mg/ kg body weight IM	In mice dilution to bring up to workable volumes shall be done with sterile saline or water for injection
Xylazine+ ketamine +acepromazine	40–50 mg/kg +2.5 mg/kg + 0.75 mg/kg IM	80–100 mg/kg +10 mg/kg IP + 3 mg/kg IP	–	
Propofol	10 mg/kg IV	10 mg/kg IV	10 mg/kg IV	IV bolus may lead to apnoea and so readiness to intubate and ventilate is required. For very short duration of anaesthesia or to induce anaesthesia, and to be maintained for longer durations with infusions
Diazepam	5 mg/kg IP	5 mg/kg IP	0.5–2.0 mg/kg IV, IM	To be used as sedative or in combination with ketamine for anaesthesia

Adopted from Laboratory Animal Anaesthesia with modifications [25]

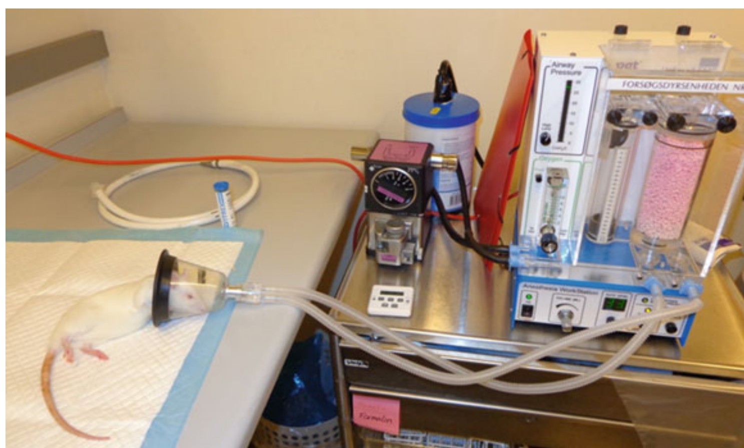


Fig. 22.2 Inhalant anaesthetic system

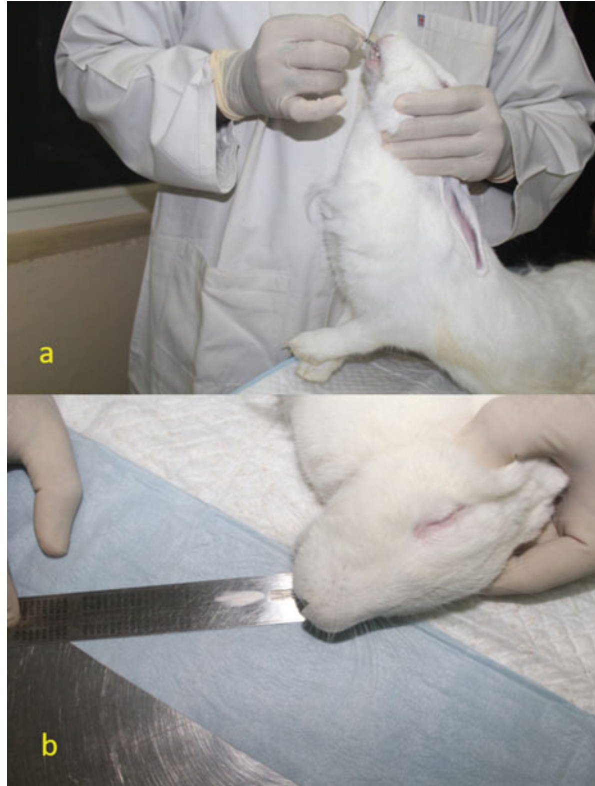


Fig. 22.3 (**a**) An endotracheal intubation in rabbits. (**b**) To verify successful intubation in rabbits

anaesthetics. Their potency is expressed as minimum alveolar concentration (MAC) value and each volatile anaesthetic differs in it. The MAC will be a constant for a given species, for a certain drug. The MAC is defined as the concentration required for any inhalant anaesthetic drug to produce an adequate loss of sensation of a particular painful stimulus in 50% of the subjects. As MAC value goes higher, the potency of the drug will be lesser. Since MAC value represents the concentration to anaesthetize half of the population, in practice, the actual dosing can vary, and it can be expected that 0.5–2 times the MAC value is an effective dose to produce surgical anaesthesia [26]. The gas driven into the lungs achieves tensions in alveoli, blood and tissues, and the tension of the gas in blood enables it to enter the brain where it exerts action.

Endotracheal intubation in rabbits can be done easily by using a blind technique (Fig. 22.3a). The animals after anaesthetic induction have to be held with its forelimbs off the table at a comfortable height of the anaesthesiologist. Using the non-dominant hand with an index finger and the thumb on both the sides of the oral commissures, the mouth shall be opened. Using the dominant hand, a paediatric (without an inflatable cuff) ET tube of the size of 2.5 shall be advanced to the epiglottis. The opening of the epiglottis in the pharyngeal region is indicated by the

condensing of air on the tube. Once this point is reached, the tube can be advanced every time as the animal inspires and successful intubation is indicated by an instantaneous cough reflex and the vapour condensing on the tube or on a steel or mirror surface (Fig. 22.3b). The ET tube shall be fixed in situ after intubation during the entire procedure to prevent accidental dislodging. In rats and mice, specialized equipment like laryngoscope or fibre optic illuminator is required for introducing the ET tube. The rodent can be hung on its incisors in an angle of 45–60° for the anaesthesiologist to view the epiglottis from the top and manoeuvre the cannula into the trachea. Verification can be done by observing the condensation on a dental mirror or a stainless-steel surface.

Open anaesthesia systems using face masks expose the users to the volatile anaesthetics as waste gases are expelled into the operating arena, whereas closed systems work with concentric facemasks or endotracheal tubes with scavenging canisters are safer to work with. Further, it is better to let the exhaust gas tube be driven out of the procedure room using long tubes or working in a table with downward air draft cabinets with adequate exhaust. Fabricating working pods with powerful exhaust fans that can be ducted out of the room shall be considered whenever branded products are not affordable or available.

Any anaesthesia breathing circuit works towards achieving deliverables enlisted as controllable oxygen supply, removal of expired gas (carbon dioxide mixed with expired anaesthetic gas), a supply of anaesthetic gas of choice and optionally integrating provisional positive pressure ventilation or assistance to the subject. The modern rodent anaesthetic machines have the below-mentioned components:

1. Oxygen cylinder with a regulator and a tube that leads to the oxygen flow meter and the flow meter with gradation unit mentioned as litres/minute with a regulator knob.
2. A precision vaporizer of isoflurane (desflurane and sevoflurane are also used, each with dedicated precision vaporizers), which receives a regulated supply from the oxygen flow meter. The flow can be adjusted using controls and is indicated in percentages. Usually, 1.5–2% is used for delivery using masks or endotracheal tubes or 3–4% for induction in a chamber in rats and mice.
3. Connectors from precision vaporizer leading to a T-junction where a valve is present to direct the flow to the induction chamber or to the face mask or endotracheal tube.
4. Outlet to scavenge gases from the chamber as well as from the mask end into a canister with activated charcoal.

If mechanical positive pressure rodent/small animal ventilator is present, the outlet from the precision vaporizer will go into the ventilator. The ventilator will be having a tubing with gases as well as adjusted positive pressure going into the endotracheal tube. In this case, the expired gases will be driven out through another port from the ventilator and will be directed out of the room.

Positive pressure ventilators are largely used in combination with anaesthesia machines and most of the human/large animal machines work using compressed air

to provide pressure for the breathing system, which drives the ventilator. Modern rodent ventilators work with electric power to drive the pistons, which in turn drives in oxygen. Pressure-controlled mode of ventilation will inflate the lungs until the sensors of the ventilator detect that a pre-set pressure is achieved say 20–40 cm^3 of H_2O, whereas the volume-controlled ventilator drives in a pre-set volume to suit the tidal volume of the subject. Tidal volume is calculated at the rate of 10 ml/kg body weight. When a rate of respiration can be set to maintain arterial pH in a range of 7.53–7.45, and where blood gas analysis is not feasible in small subjects where arterial blood collection is not a part of the protocol, the minute volume can be maintained to about 250 ml/min. If an end-tidal carbon dioxide sensor and monitor is present, ventilator settings like minute volume or oxygen concentration can be adjusted to obtain an optimal $ETCO_2$ value of about 40 mmHg.

22.4.5 Local Anaesthesia

Local anaesthesia is important in avoiding pain and stress in many routine procedures carried out in small laboratory animal management. For painful procedures like venepuncture in rabbits and rodents, Eutectic Mixture of Local Anaesthetics (EMLA), a 2.5% mixture of prilocaine and 2.5% lidocaine, is found to be effective. The onset of its action is after about 40 min of application, and covering the area with occlusive dressing is advised during this period. Some procedures potentially produce pain sensation even though the animals are under anaesthetic plane, and for these procedures, local infiltration can be also used in conjunction with general anaesthesia. This technique of combining local infiltration anaesthesia will effectively bring down the total dose of general anaesthesia mitigating the noxious stimuli and effects a much faster recovery. As the recovery is expected to be fast, the anaesthesiologist shall anticipate addressing the issue of pain that will also surface faster. Re-emerging pain can also be anticipated if a reversal of general anaesthesia is done using specific antagonists. For instance, in a short procedure like subcutaneous implantation of material, under a light plane of anaesthesia with local infiltration around the site of skin incision, the procedure can be completed, and the animal can be back to normalcy rapidly. This approach can minimize complications owing to the general anaesthesia and at the same time hasten the recovery process.

22.5 Assessment of the Depth of Anaesthesia and Ensuring the Safety of Subjects (Monitoring)

Specialized equipment is present to indicate heart rate and pulsometer to show oxygen saturation, using custom-made cuffs in rodents and rabbits. However, monitoring devices for mice and rats are costly and not readily available. Even though needle electrodes for ECG monitoring and metallic rigid probes for core body temperature are readily available, affixing these shall be under caution since it

can be a stressful procedure on its own. The most common and practical means of monitoring is still based on the surgeon's and/or the anaesthesiologist's observations. Bleeding tendencies are an indicator of adequate cardiac function. In the animals draped with a transparent cover, abdominal movements can also be noticed when animals are on spontaneous respiration. Further, if the procedure is lengthy, the time to check for pedal pinch or pinna pinch to ensure adequate anaesthetic depth should be predetermined and carried out meticulously. During the procedure, surgeons shall also listen to subtle vocalizations if any and look for slight movements during manipulation of tissues, suggestive of inadequate pain management and anaesthetic depth. In such occurrences, the depth of anaesthesia shall be adequately enhanced. For rabbits, there are small animal monitors that display and also offer auditory cues for vital statistics like beep sounds for heartbeats, and visual indication for body temperature, peripheral capillary oxygen saturation, respiratory rate and if electrodes are affixed—ECG, which eases out the anaesthesiologist's job of monitoring. In rabbits, arterial invasive blood pressure can be obtained as well from auricular artery if cannulated. Anaesthetized animals have a lower heart rate and if a progressive hike in heart rate and blood pressure is observed during anaesthesia, it is an indication that the animal is coming out of anaesthesia, and it is time to supplement adequately the maintenance dose depending upon the stage of the procedure.

22.6 Postoperative Recovery

By the end of the procedure, if the animals are maintained in gaseous anaesthesia, the gases shall be cut off, while continuing with oxygen supply as the animal regains consciousness. If the animals are maintained on mechanical positive pressure ventilation, the rate and volume can be reduced gradually to see whether the animal is returning to spontaneous breathing. As the animal adequately breathes, it shall be weaned off from the ventilator. Before the chewing reflexes strongly return, the endotracheal tube shall be taken out. Failing to do this can result in accidental severing and choking by leaving a segment inside the trachea, which can lead to death. For postoperative recovery, room temperature has to be set to an appropriate level, and the animals shall be observed and monitored closely until consciousness is regained. Feeding and water supply shall be done after complete consciousness is regained. Soft bedding and dim lighting are preferred, and the animals shall be kept in the recovery area until they have completely regained normalcy.

22.7 Analgesia

22.7.1 Quantification of Pain in Laboratory Animals

A decade ago, the trend was to prescribe analgesics for common ailments like dental extraction, as a standard regimen, and the patients were being advised to complete the course as per the prescription. Of late, analgesic prescriptions are being offered only in case of inevitably precipitating pain, indicative of a paradigm shift of concepts in the quantification of pain and its mitigation. In experimental animals, it is possible to assess pain and fix the dosage and duration to optimize offering analgesia to relieve pain and to stop the administration to avoid unwarranted side effects of the drugs. To achieve this, the first step is to assess clinical and behavioural signs of pain (also refer to Chapter on Behaviour). Training of personnel shall be done to identify the relevant and obvious signs of pain. There are several tests like hot plate/cold plate for hyperalgesia, Hargreaves thermal hyperalgesia test, von Frey filaments to assess paw withdrawal threshold to determine mechanical allodynia, tail-flick test against a focused heat source and many more to assess the sensation level of pain in animals, and most of these can be quantified. However, in a clinical setting, measuring pain on a scale or algesiometry tests cannot be used unless being a part of the research protocol because it adds to an unwanted variable in the test protocol. Thus, it is important to detect the clinical and behavioural signs of pain.

22.7.2 Signs of Abdominal Pain in Rats

Twitch: The mostly observed behaviour, which is characterized by a spasmodic rostro-caudally directed jerky movement of skin and underlying tissues on the head or back region, reminding of voluntary localized twitches as seen on cow's skin when being disturbed by biting flies.

Fall and partial fall: The rats lose balance completely or partially and tend to fall to the side while walking and even sitting, or during sitting and self-grooming.

Stagger: Loss of foot stability and imbalance leading to swaying movement or side-to-side movement on ambulation.

Abdominal press: Belly region is pressed to the cage floor.

Hop: Both the rear legs are being lifted and entire hindquarters lifted while being flexed while resting and comes back to resting posture immediately.

Writhe: Catching in of abdominal muscles at the flank region by severe and sudden contraction and holding the contraction for a few seconds until the abdomen relaxes, which is observed during walking or while resting.

Bacharach: The stretching of the back forming an arched back resembling the stretch of cats immediately after waking up by the extension of both hind and forelimbs.

22.7.3 Signs of Abdominal Pain in Mice

Writhe, twitch, stagger, fall, abdominal press, arch, stretch, flinch, raised tail and rear leg lift are enlisted as the specific signs of abdominal pain in mice [27, 28].

Writhe, twitch, stagger, fall, abdominal press and arch are as described in the above section on signs of abdominal pain in rats.

Flinching: A momentary rapid jerky movement of the whole body, as a twitch is observed on the back, but here on the whole body.

Stretch: Often seen in conjunction with the abdominal press in which both hind limbs and forelimbs will be extended out giving an elongated posture.

Raised tail: The tail is held in a lifted position during ambulation.

Rear leg lift or extension: Momentary lifting one of the rear legs and often associated with writhe or abdominal press.

Many of the signs mentioned above come in combinations, and scoring can be done from a 5-min video recording from which a cumulative score can be calculated and adequacy of the given dose of analgesia can be assessed. Rabbits, being a prey species, mask many signs. However, twitches, wince (characterized by a rocking backward movement in conjunction with the closing of eyes and swallowing action), writhe, stagger and abdominal press have been observed as signs of abdominal pain [25]. A general assessment of temperament during handling (increased aggression in comparison to normal individual's temperament) and vocalization in the cage and while handling can be directly associated with pain. The signs mentioned above pertain to abdominal pain, and the pain originating from other body regions needs to be addressed as well.

22.7.4 Assessment of Pain of Non-abdominal Origin

Acute pain of non-abdominal origin can be detected and graded by using grimace scales (Fig. 22.4) in many of the laboratory species (or details, please see Chapter on behaviour). Dark-phase home cage activity, rearing in the cages, weight loss over a period of time, reduced nest-building behaviour in mice, red tears, etc. cannot be considered specific pain-related behaviour, but these can be used as additional parameters to assess the status if the pain is suspected. An important aspect of pain assessment is to select multiple parameters. Building a welfare monitoring protocol with several parameters makes it more likely to detect any signs of altered behaviour.

22.7.5 Choosing an Analgesia Regimen

A wide range of drugs such as non-steroidal anti-inflammatory drugs (NSAIDs), opioids and miscellaneous drugs like tramadol shall be considered as candidates for offering analgesia in laboratory animals. The NSAIDs act by the inhibition of cyclooxygenase (COX), which exists as two isomers—COX-I and COX-II. COX-I protects the gastrointestinal mucosa and activates the platelets, whereas COX-II is

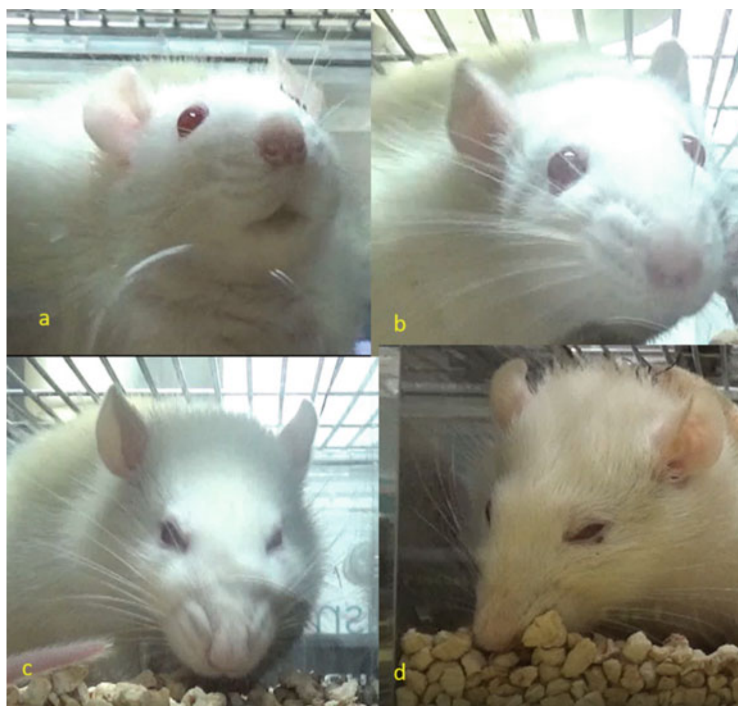


Fig. 22.4 Rat Grimace Scale recordings from a rat in various stages—pre and post-surgery. (**a**) and (**b**) Signs of pain absent. (**c**) Moderate pain and (**d**) obviously under pain

the isomer responsible for pain and inflammation. A third isomer COX-III is also described [29]. The prostanoids, being important mediators of the inflammatory pathway, is being prevented from getting synthesized by prostaglandin H2. Prostaglandin H2 is formed from arachidonic acid by COX which is the first step in the process of synthesizing prostanoids. Important adverse effects of NSAIDs include hepatotoxicity, interfering platelet function, nephrotoxicity, ulceration of the GI tract and GI disturbances, blood dyscrasias and foetal toxicity (as with aspirin). Hence, it should be understood that many of these drugs can potentially interfere with specific research objectives. Newer generation NSAIDs selectively inhibit COX-II and spares COX-I, minimizing the side effects. In optimum doses, for 2 or 5 days, the analgesia can be beneficial, and side-effects are likely to be negligible (although this must be ensured by proper pilot experiments). Prolonging therapy and/or increased dose administration in rodents and rabbits can lead to gastric stasis, ulcer and death. The doses of commonly used NSAIDs in various laboratory animal species are enlisted in Table 22.2.

Opioids exert their action specifically on mu (μ) and kappa (κ) opioid receptors. There are three classes of opioids, and among these, the first category acts as mu agonists (morphine). The second class of opioids are mu antagonists, but they are

Table 22.2 Commonly used analgesics in rodents and rabbits

Drug	Rats	Mice	Rabbits
Meloxicam	5 mg/kg SC	1 mg/kg SC	0.6–1 mg/kg SC
Buprenorphine	0.01–0.05 mg/kg SC or IV, 8–12 h 0.1–0.25 mg/kg PO, 8–12 h	0.05–0.1 mg/kg SC, 12 h	0.01–0.05 mg/kg SC, 12 h
Carprofen	5 mg/kg SC	5 mg/kg SC	4 mg/kg OD
Tramadol	5 mg/kg SC	5 mg/kg SC	–

Adopted from Laboratory Animal Anaesthesia [25]

kappa agonists thereby earning the term mixed agonist/antagonists (pentazocine, nalbuphine). The third class, termed as partial agonists, exhibits agonistic action towards both the mu and kappa receptors (buprenorphine).

Practically, NSAIDs and opioids are often combined to form a therapy, since both the classes act through different pathways termed as "multimodal analgesia". During the first few days of the postoperative period, since the animals will not be feeding and drinking normally, it is advised to provide analgesia through the parenteral route and preferably by subcutaneous route. Moreover, those drugs that don't interfere with the objective of the test but at the same time having a maximum duration of pharmacologic action shall be chosen. For example, among opioids, 12 h is the half-life for buprenorphine which is the longest in the class of opioids, which makes it the drug of choice in most of the studies (4–6 h is the time of pharmacologic analgesic action, and voluntary ingestion of peanut butter or other food treats can be used for a prolonged cover). Among NSAIDs, meloxicam shall be administered once per day and can be injected subcutaneously, both factors making it one of the drugs of choice from an animal welfare point of view and from users' point of view. Some examples of multimodal therapy are meloxicam once-daily with buprenorphine twice-daily injection and carprofen in combination with buprenorphine injections.

22.8 Euthanasia

Euthanasia is the process of inducing death with minimal or no pain or distress in the animal involved. The procedure involves the sudden loss of consciousness followed by irreversible and reliable CNS depression resulting in death. This should be done with minimum restraint to minimize distress. Ascertaining death shall be done by qualified and adequately trained personnel by duly ensuring circulatory and respiratory failure, lowering of body temperature and progression of rigor.

Euthanasia can be broadly classified into chemical (pharmacological) and physical (mechanical) methods. Generally, from an animal welfare viewpoint, chemical methods shall be used whenever possible. Chemical methods are fast and aesthetic and can be performed with minimal restraint/handling and minimally stressful in comparison to mechanical methods. Anaesthetic ether had been in use as an agent both for anaesthesia and euthanasia for long, by the practice of putting a piece of

cotton or gauze dipped in ether to which animals are being made in contact within a jar or cage, with a closed and airtight lid in place. It is important to note that use of ether for any of the aforesaid purposes shall be discouraged for the reason that besides being an irritant to the animals, ether causes potential health hazards to the personnel using it and also is inflammable and an explosive. Irrespective of how refined the technique of euthanasia adopted may be, the surge of pheromones from animals emitted during the euthanasia can also trigger stress among conspecifics. Hence, euthanasia shall be performed at an isolated area within the facility and should not be done within animal rooms. Overcrowding and mixing of several species simultaneously within the carbon dioxide euthanasia chambers shall be avoided. A gradual fill of carbon dioxide is beneficial in bringing the stress to the minimum levels. Studies are showcasing contradictory results on the benefit on euthanasia of rats in their home cage in comparison to euthanasia chamber [30, 31]. Practicalities shall be considered since euthanasia of some animals in a cage is required selectively, and the emerging data in both mice and rats shall be considered while making the choice. Based on periodically evolving scientific data, regulatory bodies shall revise guidelines on euthanasia to stay abreast with technically and ethically sound practices. An example would be the abolition of carbon dioxide euthanasia in rabbits by the EU Directives [32] (Directive 2010/63/EU), while many national guidelines continue to endorse the practice. While using overdosage of anaesthesia as a means of euthanasia, pentothal sodium is the most recommended drug currently, ensuring minimal discomfort and rapid euthanasia by effecting CNS depression followed by cardiac and respiratory arrest. For pentothal sodium, being an irritant during IV or IP injections, the recommended concentration is 18% (200 mg/ml) in a dosage of 200 mg/kg for euthanasia. Instead of parenteral administration involving some amount of restraint leading to stress and pain of injection, oral administration of pentothal in mice is recently experimented in combination with phenytoin mixed in dough and offered as sugar cookies for voluntary ingestion [33]. With agents like thiopentone sodium, caution shall be exercised, since this drug is also an irritant and turns painful characterized by vocalizations if extravasation happens during injections. Lesser reports are available in comparison to pentothal, and so more studies are required to assess effective dilutions to minimize pain during euthanasia. Sedating the animals with xylazine and ketamine is also advisable before the injection of thiopentone. Physical methods, if used, shall be done only after sedation in animals above a certain body weight. The usage of potassium chloride intravenously alone or muscle relaxants like pancuronium bromide or succinylcholine is not recommended unless the animals are anaesthetized, because this can lead to considerable animal sufferings. Neonates are tolerant to hypoxic conditions for long, and it is established that they can survive for longer durations on carbon dioxide exposure. So, carbon dioxide inhalation shall not be used to euthanize neonatal rodents. The carbon dioxide chamber can be a custom-made desiccator, a regulator for the chamber and a leak-free tubing from the cylinder to a plastic desiccator. In case a smaller chamber is required, an acrylic chamber of adequate size can be fabricated, and the end of the tubing from the cylinder shall be connected to the chamber using a valve.

Table 22.3 Methods of euthanasia performed in rodents and rabbits

Technique for euthanasia	Rodents	Rabbits
Anaesthetics overdose	✓	✓
Captive bolt pistol	X	✓
Cervical dislocation	For under 150 g bodyweight. Animals over 150 g shall be sedated	For under 150 g bodyweight. Animals over 150 g shall be sedated
Concussion/percussive blow to the head	Only for animals under 1 kg if no other methods are available	Only for animals under 5 kg if no other methods are available
Decapitation	If other methods are impossible	X
Electric stunning	X	Special equipment required
Inert gases (argon, nitrogen)	✓	X

Adopted with modifications from the 2010/63/EU [32]

The Directive 2010/63/EU [32] is freely downloadable from the website in many languages, and the English version can be found here: {https://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2010:276:0033:0079:EN:PDF}.

This document gives detailed insights on recommendations made on acceptable techniques in all the species of laboratory animals. In short, anaesthetic overdosage is an acceptable technique for all the species and whenever appropriate accompanied with prior sedation. For rabbits, stunning using a captive bolt pistol is recommended. For rabbits and for rodents under 1 kilogram, cervical dislocation can be done, and if they weigh more than 150 grams, the animals shall be sedated before the procedure. Decapitation in rodents shall be considered if and only if other methods are not possible. Euthanasia has to be performed with compassion and obliging to all possible technical requirements, and the personnel who are having any reluctance in carrying out the operations shall be waived from performing the specific task, on moral and humanitarian grounds (Table 22.3).

22.9 Conclusion and Future Directives

Mitigating pain during procedures and afterwards is a strategic issue. Confounding effects of anaesthetic and analgesic drugs selected on research goals and inadequate or avoidance of drugs leading to compromised welfare can seriously hamper the culmination of meaningful research. Much knowledge had been added in recent decades on clinical signs and assessment of signs of pain in most of the species of laboratory animals. It shall be the focus of activities to train more and more personnel who take care of the animals in the assessment of well-being and signs of pain which is specific to the species each one is working with.

References

1. Samhita, S. (1907) English translation by Kaviraj Kunja Lal Bhishagratna, Calcutta, vol. 16, pp. 152–154
2. Singh V (2017) Sushruta: The father of surgery. Natl J Maxillofac Surg 8(1):1–3
3. LeVasseur R, Desai SP (2012) Ebenezer Hopkins Frost (1824–1866) William TG Morton's first identified patient and why he was invited to the ether demonstration of October 16, 1846. Anesthesiology: J Am Soc Anesthesiol 117(2):238–242
4. Balcombe JP, Barnard ND, Sandusky C (2004) Laboratory routines cause animal stress. Contemp Top Lab Anim Sci 43(6):42–51
5. Keating SC et al (2012) Evaluation of EMLA cream for preventing pain during tattooing of rabbits: changes in physiological, behavioural and facial expression responses. PLoS One 7(9)
6. Carbone L, Austin J (2016) Pain and laboratory animals: publication practices for better data reproducibility and better animal welfare. PLoS One 11(5):e0155001
7. Peterson NC, Nunamaker EA, Turner PV (2017) To treat or not to treat: the effects of pain on experimental parameters. Comp Med 67(6):469–482
8. Agha AM, El-Khatib AS, Al-Zuhair H (1999) Modulation of oxidant status by meloxicam in experimentally induced arthritis. Pharmacol Res 40(4):385–392
9. Hestehave S et al (2017) Is there a reasonable excuse for not providing post-operative analgesia when using animal models of peripheral neuropathic pain for research purposes? PLoS One 12 (11):e0188113
10. Harikrishnan V et al (2018) A comparison of various methods of blood sampling in mice and rats: Effects on animal welfare. Lab Anim 52(3):253–264
11. Council, N.R (2010) Guide for the care and use of laboratory animals. National Academies Press
12. Arantes-Rodrigues R et al (2012) The effects of repeated oral gavage on the health of male CD-1 mice. Lab Anim 41(5):129–134
13. de Meijer VE et al (2010) Repetitive orogastric gavage affects the phenotype of diet-induced obese mice. Physiol Behav 100(4):387–393
14. Turner PV et al (2012) Oral gavage in rats: animal welfare evaluation. J Am Assoc Lab Anim Sci 51(1):25–30
15. Jones CP, Boyd KL, Wallace JM (2016) Evaluation of mice undergoing serial oral gavage while awake or anesthetized. J Am Assoc Lab Anim Sci 55(6):805–810
16. Martinic G (2008) A technique for intragastric gavage of radiolabeled liquid cholesterol in rabbits (*Oryctolagus cuniculus*) using a pediatric feeding tube. Lab Anim 37(7):323–328
17. Harcourt-Brown, F. (2002) The rabbit consultation and clinical techniques. Textbook of rabbit medicine, p. 52
18. Mor F, Ozmen O (2010) Effect of vitamin C in reducing the toxicity of endosulfan in liver in rabbits. Exp Toxicol Pathol 62(1):75–80
19. Hakki SS et al (2013) Boron enhances strength and alters mineral composition of bone in rabbits fed a high energy diet. J Trace Elem Med Biol 27(2):148–153
20. Flecknell PA (2000) BSAVA manual of rabbit medicine and surgery. British Small Animal Veterinary Association
21. Kato GA et al (2018) Individual differences in torpor expression in adult mice are related to relative birth mass. J Exp Biol 221(12)
22. Brown JC, Staples JF (2010) Mitochondrial metabolism during fasting-induced daily torpor in mice. Biochim Biophys Acta (BBA)-Bioenerg 1797(4):476–486
23. Jensen TL et al (2013) Fasting of mice: a review. Lab Anim 47(4):225–240
24. Erickson RL et al (2016) Intraperitoneal continuous-rate infusion for the maintenance of anesthesia in laboratory mice (Mus musculus). J Am Assoc Lab Anim Sci 55(5):548–557
25. Flecknell P (2015) Laboratory animal anaesthesia. Academic Press
26. Becker DE, Rosenberg M (2008) Nitrous oxide and the inhalation anesthetics. Anesth Prog 5 (4):124–130. quiz 131-2

27. Leach MC et al (2012) The assessment of post-vasectomy pain in mice using behaviour and the Mouse Grimace Scale. PLoS One 7(4)
28. Miller AL et al (2016) Using the mouse grimace scale and behaviour to assess pain in CBA mice following vasectomy. Appl Anim Behav Sci 181:160–165
29. Chandrasekharan N et al (2002) COX-3, a cyclooxygenase-1 variant inhibited by acetaminophen and other analgesic/antipyretic drugs: cloning, structure, and expression. Proc Natl Acad Sci 99(21):13926–13931
30. Hackbarth H, Küppers N, Bohnet W (2000) Euthanasia of rats with carbon dioxide-animal welfare aspects. Lab Anim 34(1):91–96
31. Hickman DL (2018) Home cage compared with induction chamber for euthanasia of laboratory rats. J Am Assoc Lab Anim Scie 57(6):729–733
32. Parliament, E. (2010). DIRECTIVE 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for scientific purposes. Book DIRECTIVE 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for scientific purposes. City, 2010: p. 33–78
33. Dudley ES, Boivin GP (2018) Evaluation of a commercially available euthanasia solution as a voluntarily ingested Euthanasia agent in laboratory mice. J Am Assoc Lab Anim Sci 57 (1):30–34

Biosafety Consideration in Laboratory Animal Facilities and Working on Infectious Agents

23

Aravindh Babu R. Parthiban, Azhahianambi Palavesam, Karthik Kumaragurubaran, and Dhinakar Raj Gopal

Abstract

Animal experimentation is an integral part of the research and development process especially of those in which vaccines and therapeutics against infectious agents are attempted. The use of infectious agents on live animals exponentially increases the hazards associated with such procedures. The zoonotic potential of several infectious agents also increases the risk to users and the environment. It is therefore imperative to put in place a combination of biological safety measures to minimize such risk. These safety measures are classified as per global standards into biological safety levels which are to be scrupulously followed to prevent the unintentional exposure or release of infectious agents. The chapter aims to provide an overview of the principles of biosafety, hazard characteristics, and considerations such as bio-risk assessment and risk mitigation requirements in laboratory animal facilities. The chapter is intended to be a primer for personnel who work in high containment animal houses. References to global standards and procedural guides are indicated in places for a detailed consultation.

Keywords

Biosafety · Laboratory animal · Risk assessment · Biohazards · Safety requirements

A. B. R. Parthiban (✉) · A. Palavesam · K. Kumaragurubaran · D. R. Gopal
Centre for Animal Health Studies, Tamil Nadu Veterinary and Animal Sciences University, Madhavaram Milk Colony, Chennai, Tamil Nadu, India
e-mail: aravindhbabu.r.p@tanuvas.ac.in; Azhahianambi.p@tanuvas.ac.in; karthik.k@tanuvas.ac.in; dhinakarraj.g@tanuvas.ac.in

© The Author(s), under exclusive license to Springer Nature Singapore Pte Ltd. 2021

P. Nagarajan et al. (eds.), *Essentials of Laboratory Animal Science: Principles and Practices*, https://doi.org/10.1007/978-981-16-0987-9_23

23.1 Introduction

In recent years, a surge of infectious diseases that are transmitted between animals and humans has been recorded. Emerging infectious agents like Hendra virus, Nipah virus, Ebola virus, severe acute respiratory syndrome (SARS) coronavirus, Middle East respiratory syndrome (MERS) coronavirus, and the more recent SARS coronavirus-2 have resulted in a global health catastrophe. Research on infectious agents is on the rise, to combat such persistent threats to human and animal health. Animal models of infectious diseases form a vital aspect of infectious disease research. Studies on animal models involving pathogenic bacteria, viruses, fungi, genetically modified microorganisms, recombinant DNA, recombinant proteins, etc. can pose a direct hazard to the animal house personnel. Therefore, a thorough understanding of biosafety is necessary for personnel involved in such studies. Biosafety of the laboratory or animal facility describes the principles and practices for the prevention of unintentional release or accidental exposure to biological agents and toxins. On the other hand, biosecurity explains physical control of biological agents and toxins in the laboratory to avert their theft, loss, mishandling, unauthorized use, and deliberate release. The chapter aims to provide an overview of the global standards for infectious disease research and consequent biosafety considerations in laboratory animal facilities and such experiments.

23.2 Classification of Microorganisms

The World Health Organization (WHO) categorizes the microorganisms into four risk groups [1]:

Risk Group 1 (no or low individual and community risk)—Microorganism that is unlikely to cause disease in animals or humans is placed in this group.

Risk Group 2 (moderate individual risk, low community risk)—Microorganisms that have the ability to cause human or animal disease but are unlikely to pose a serious hazard are placed in this group. Exposure to these microbes may result in serious disease, but effective prophylaxes are available, and the risk of community spread is limited.

Risk Group 3 (high individual risk, low community risk)—Microorganisms that have the ability to cause serious human or animal disease, but the risk of community spread is limited. Effective prophylaxes are available.

Risk Group 4 (high individual and community risk)—Microorganisms that have the ability to cause serious human or animal disease and that can be readily spread between individuals and a high risk of community spread. Effective prophylaxes are not usually available [2].

Points to Remember

- Each country has its own list of microorganisms that are assigned to specific risk groups.
- Risk assessments should be conducted to those agents that are not listed in the country-specific risk grouping.
- Organisms that are genetically modified (either naturally or artificially) will need a re-evaluation of risk, even if they are already listed under one of the risk groups (e.g. multidrug-resistant *Acinetobacter baumannii*)

23.3 Principles of Working with Infectious Agents

While handling any infectious agent, it is required to consider any microorganism as a potential pathogen (unless otherwise its status is explicitly known) and to handle it with standard microbiological techniques to substantially reduce the risk to laboratory personnel and the environment.

Exposure to the microorganism in laboratory animal facilities occurs more frequently than is generally suspected. The risk of exposure in the animal house is considerably higher than in a typical laboratory set-up. The exposure can occur by the following routes:

1. Inhalation of infectious aerosols (from excretions, etc.)
2. Animal bites and scratches
3. Accidents involving syringe needles or other contaminated sharps
4. Accidental spills
5. Accidental ingestion or exposure to contaminated objects

The likelihood of exposure can increase with the faulty work practices and improper use of personnel protective equipment. Minor spills, aerosols, cuts, and scratches occur more frequently in laboratories, which might not cause a laboratory-acquired infection. However, such insignificant exposures still pose a risk to laboratory personnel.

23.4 Animal Biosafety Laboratory

Laboratory animal facilities are an integral part of research with infectious agents. Therefore, the animal biosafety levels for working with risk group infectious agents are comparable to that of the laboratory standards for the same. For security reasons, the animal house should be isolated from other facilities with strict access restrictions

[1]. The construction design should provide for the decontamination and disinfestation. There are four animal biosafety levels (ABSLs) that are globally recognized. The ABSLs are defined by specific facility requirements, safety equipment, and operating procedures depending on the types of agents being studied.

23.4.1 Animal Biosafety Level 1 (ABSL-1)

ABSL-1 is for experiments that do not involve biological agents or involve microorganisms that are unlikely to cause disease in animals or humans and that pose minimal or no hazard to laboratory personnel and the environment. Animals after quarantine are usually housed in the ABSL-1 facility. ABSL-1 practices, types of equipment, and facility requirements are more often sufficient to maintain laboratory animal breeder houses. However, specific policies such as access control, the health monitoring programme, etc. shall be implemented as required. Access to the ABSL-1 facility should be restricted to authorized persons to minimize contamination. Biomedical waste generated in the ABSL-1 facility should be collected in leak-proof containers and transported to the decontamination plant as per the guidance of the state or country.

23.4.2 Animal Biosafety Level 2 (ABSL-2)

ABSL-2 is for animal work with those microorganisms that have the ability to cause human or animal disease but are unlikely to pose a serious hazard to laboratory personnel. ABSL-2 has specific practices, procedures, containment equipment, and facility requirements and will also include all ABSL-1 requirements. Personnel working in ABSL-2 should be properly trained in handling animals, use of the facility, etc. Biosafety cabinets should be used while handling infectious materials or other operations that can create aerosol. Secondary containment barriers include restricted entry, provision of disposal of biological and personal waste, and unidirectional airflow [3].

23.4.3 Animal Biosafety Level 3 (ABSL-3)

ABSL-3 is for animal work involving indigenous or exotic agents with the potential of aerosol transmission and that have the ability to cause serious human or animal disease but with a limited risk of community spread [4]. ABSL-3 has specific practices, procedures, containment equipment, and facility requirements and will also include all ABSL-2 requirements [5]. A simple schematic of the ABSL-3 laboratory floor plan is shown in Fig. 23.1.

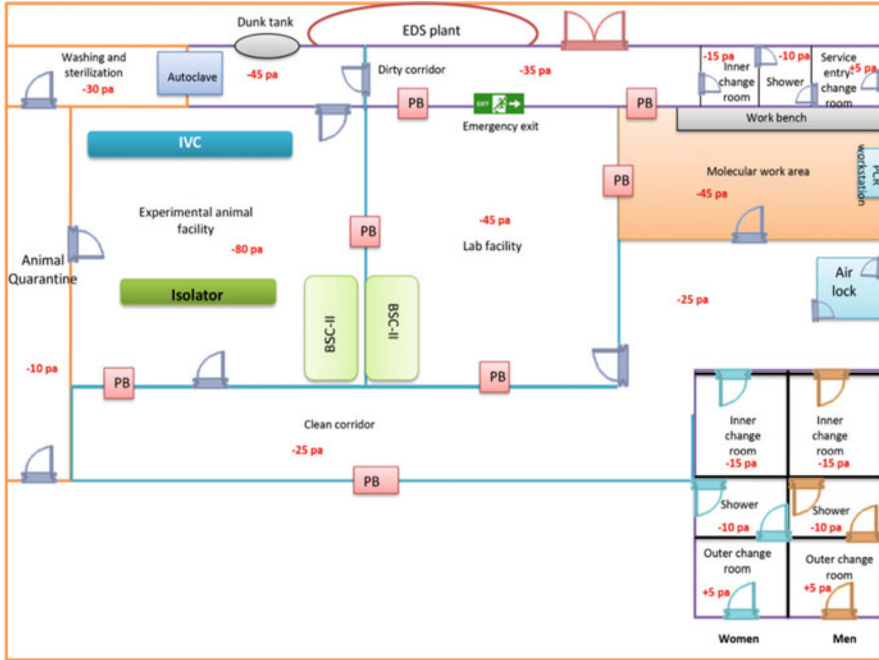


Fig. 23.1 Schematic floor plan for ABSL-3 laboratory including pressure cascade. The pressure gradient in each room is marked in red colour font, and the pressure difference is calculated based on atmospheric pressure

23.4.4 Animal Biosafety Level 4 (ABSL-4)

ABSL-4 is for animal work involving dangerous or exotic agents that pose a high risk of life-threatening disease, aerosol transmission, or related agents with unknown risk of transmission [6].

There are two types of ABSL-4 laboratories

Cabinet type	Suit type
All manipulations like handling infected animals are carried out inside biosafety cabinet class III. The major difference between ABSL-4 cabinet lab and ABSL-3 laboratory is the presence of BSC class III cabinet	All manipulations of handling infected animals are carried out in BSC class II with the personnel wearing a positive pressure suit
BSC class III should be connected through an autoclave, dunk tank, and/or fumigation chamber for decontaminating the material before removal from the cabinet	A positive pressure suit should be decontaminated before removal and exit from the change room. A chemical shower should be used to decontaminate the surface of the suit
The layout of the entry and exit area is similar to ABSL-3 (Fig. 23.2a)	A chemical shower is an additional unit in the layout of the entry and exit area (Fig. 23.2b)

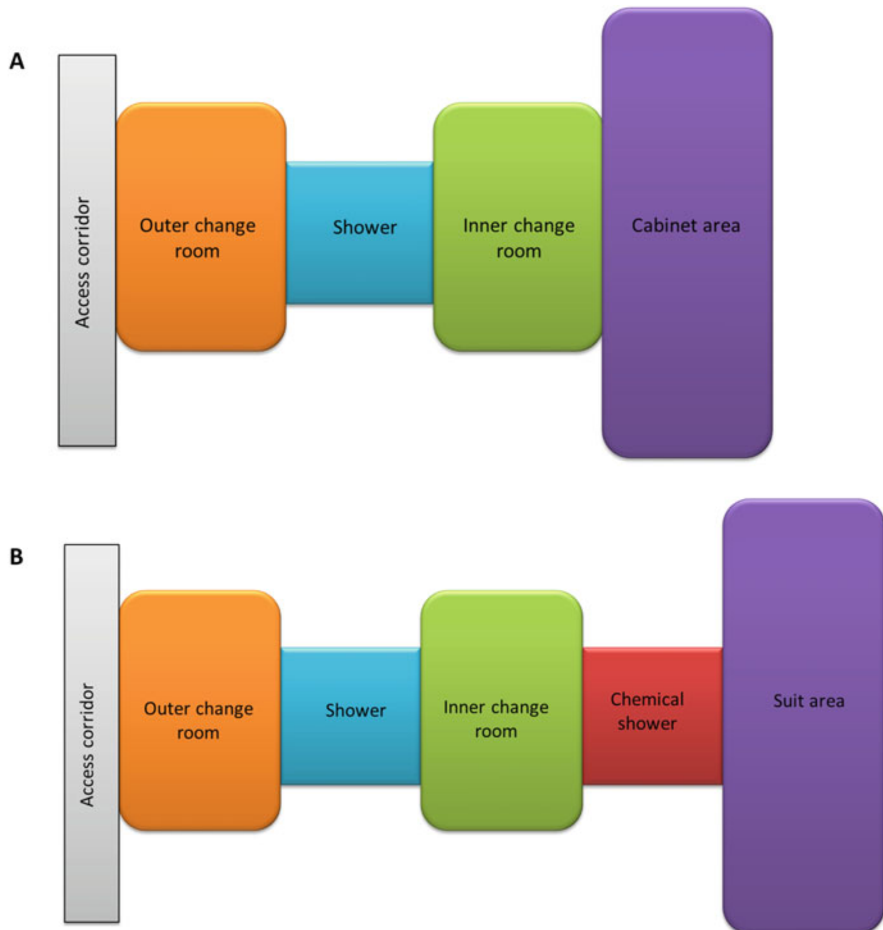


Fig. 23.2 Difference in the entry and exit area of suit and cabinet type ABSL-4 laboratories. Entry and exit of cabinet type lab are similar to BSL-3 laboratories with outer and inner change rooms and a shower (**a**), while the entry and exit of suit lab have an additional chemical shower before the inner change room (**b**)

Agents for which no vaccines or treatments are available should be handled in ABSL-4 laboratory. Novel agents with a high risk of transmission to humans should be handled in ABSL-4 facility until there is clear evidence to reclassify the agent to a lower level of containment. ABSL-4 has specific practices, procedures, containment equipment, and facility requirements and will also include all ABSL-3 requirements [7].

The US Department of Agriculture (USDA) has categorized BSL-3-Agriculture (BSL-3-Ag), a separate work practice for handling pathogens in large animals [8]. The agents that are handled in BSL-3-Ag may pose a serious threat to the

animal, personnel involved, and also the environment. Facility requirements and its operation are close to ABSL-4, and handling infected large animals is a challenge working in these laboratories.

23.5 Individually Ventilated Cages

Small laboratory animals in ABSL-3 may be housed in an individually ventilated caging system (IVC) that protects the animals, handlers/researchers, and also the environment. Inward and outward movement of the air through the cages are filtered through HEPA filters, and negative pressure is maintained inside the cages [9]. The integrity of the sealed cages, alarm system to indicate the breach of airflow, and uninterrupted power supply are the minimum requirements to maintain the biosafety within IVC. Exhaust from the IVC should be rooted through the ABSL-3 exhaust system. IVC cages should only be opened inside the BSC class II cabinet.

23.6 Animal Isolators

Animal isolators are similar to class III BSC where animals can be housed inside a complete barrier. Animal isolators can be maintained in negative pressure for routine testing or positive pressure for maintaining specific pathogen-free animals [9]. Like IVC, the pressure is monitored and alarms to indicate the deviation is available in the isolators. The major drawback of the isolator is the transfer of material inside and outside the chamber, for which bag-in/bag-out system or bio-decontainment hatch or rapid transfer port may be used. A rapid transfer port is a highly useful system for the rapid transfer of materials without contamination [10]. Rapid transfer port operates on the principle of a double door system where one door is present in the port while the other in the isolator (Fig. 23.3a). Once connected, both the exterior surfaces of the doors attach and get sealed thus preventing the exposed surface of the door to the interior of the isolator. Thus, this docking prevents contamination of the isolator (Fig. 23.3b).

Various primary and secondary barrier requirements of ABSL2, 3, and 4 are shown in Table 23.1.

A detailed list of practices and structural requirements for animal biosafety levels are described in the WHO Laboratory Biosafety Manual 3rd Edition [1] and CDC Biosafety in Microbiological and Biomedical Laboratories (BMBL) 5th Edition [11]. Selecting a biosafety level for animal experimentation with a particular agent depends upon several factors such as pathogenicity, stability in environment and aerosols, route of spread and infectivity of the agent, the procedures intended to be used, the quantity and concentration of the agent, the endemicity of the agent in the country, and the availability of prophylactic and/or therapeutic measures.

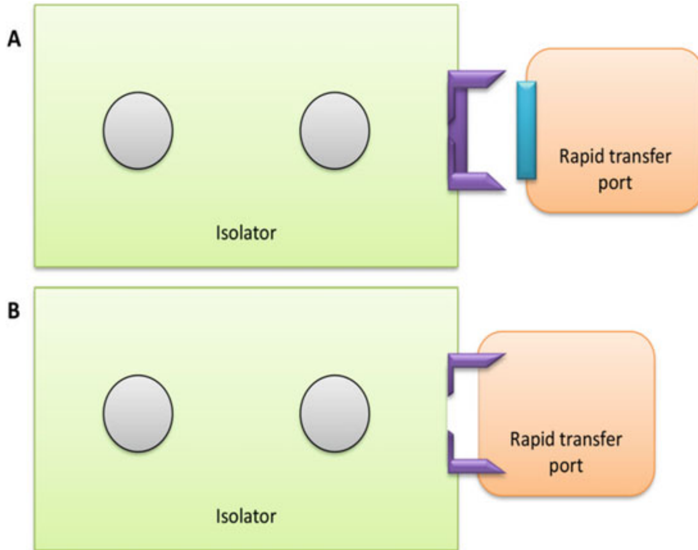


Fig. 23.3 Rapid transfer port system. The system works on the principle of an interlocking system between two doors. One door is present in the transfer port while the other in the isolator (**a**). Upon connection, both doors dock and fall into the interior of the port thereby preventing contamination (**b**)

23.7 General Requirements and Work Practices to be Followed in an ABSL Facility

- The laboratory should be constructed with leak-proof and chemical-resistant walls and floor. Floors should be non-slippery and easy to clean.
- Restricted access to the animal facility and electronic access may be provided for high containment facilities like ABSL-3/4.
- Entry and exit into/from the laboratory should be recorded regularly.
- PPEs like gloves, masks, respirators, coverall, goggles, face shield, head cover, shoe cover, and others as per the requirement of the facility should be worn inside the facility. The street dress is strictly not allowed inside the facility, and laboratory dress should be worn outside the facility [12].
- Eating, drinking, and smoking are strictly prohibited inside the facility.
- Laboratory doors should be closed while working to prevent contamination.
- Mouth pipetting is not allowed.
- Aerosol production should be minimized during centrifugation with the use of a lid.
- A standard operating protocol (SOP) should be available in the facility, and it should be updated yearly. All the personnel working in the laboratory should

Table 23.1 Various primary and secondary barrier requirements of ABSL2, 3, and 4

Barrier	Specifications	ABSL-2	ABSL-3	ABSL-4	
				Cabinet lab	Suit lab
Primary	Biosafety cabinet	Class II	Class II	Class III	Class II
	IVC	No	Yes	Yes	Yes
	PPE	Scrub suit/ coverall, gloves, mask/ respirator, goggles	Scrub suit/ coverall, gloves, mask/ respirator, goggles	Scrub suit/ coverall, gloves, mask/ respirator, goggles	Scrub suit/ coverall, gloves, mask/ respirator, goggles, positive pressure suits
Secondary	Access to the animal facility	Restricted	Restricted	Restricted	Restricted
	Double-door entry	No	Yes	Yes	Yes
	Hands-free handwashing sink at the exit		Yes	Yes	Yes
	The direction of airflow	Yes	Yes. Inward	Yes. Inward	Yes. Inward
	Exhaust air	Not necessarily HEPA filtered	Filtered and exhausted. No recirculation	Filtered and exhausted. No recirculation	Filtered and exhausted. No recirculation
	Autoclave	Yes. Not necessarily double door	Double-door autoclave	Double-door autoclave	Double-door autoclave
	Seamless floor	Yes	Yes	Yes	Yes
	Emergency eyewash	Yes	Yes	Yes	Yes
	Effluent decontamination plant	No	Yes	Yes	Yes

have a clear understanding of the SOP and should have also undergone proper training before working in the laboratory.

- Bio-risk assessment should be carried out, and a clear response plan should be made available.
- Any accident or spill inside the laboratory should be brought to the notice of the officer-in-charge or the biosafety officer.
- An inventory of the available pathogens/infectious agents should be maintained in the laboratory.
- No infectious material or culture should be taken out of the laboratory without prior approval from the biosafety officer.
- Regular auditing of the laboratory should be conducted.

- Animals entering the laboratory should be quarantined before using for an experiment.
- No infected animal should be reused for experimental work.
- All biological wastes generated should be decontaminated before disposal.

23.8 Categories of Biohazards in Animal Biosafety Laboratories

In a laboratory animal facility, wastes like solid (bedding material with faeces), liquid waste (blood, urine, body fluids during necropsy, and wash water), and animal carcasses are generated. Treatment protocol for these wastes may be based on the type of testing and pathogen involved in testing. Waste generated (bedding material, faeces, and dead carcass) from control animals or uninfected animals can be handled as non-hazardous waste and disposed of as per the instructions of the local governing body. On the contrary, waste generated from infected animals should be considered hazardous and should be decontaminated before disposal. Biohazard waste materials should be collected in biohazard bags and packed in leak-proof containers with biohazard labels [13]. Once the materials are decontaminated, it may be disposed of as per the instructions of the governing body.

- Solid waste may be treated by autoclave or chemical inactivation, while liquid waste should be treated by effluent treatment/decontamination plant. Due consideration should be provided when the content of organic particles in the liquid waste is high since the effectiveness of decontamination may be hampered by these organic particles. Under these circumstances, biodegradation using microorganisms, activated sludge reactors, membrane bioreactors, etc. can be used. Ideally, an effluent decontamination plant should have a minimum of two tanks, one to decontaminate and the other to hold.
- Infectious animal carcasses can be collected in biohazard bags, labelled, and stored. A licensed agency can collect these materials for incineration. Autoclaving of the animal carcasses is not recommended as it takes a long time to reach the required temperature in the middle part of the animal carcass. Alkaline hydrolysis can be followed to decontaminate the animal tissues.
- Sharps like needles, broken glass, etc. should be collected separately in puncture-proof containers with closed lids. Biohazard symbol should be labelled over the container. Sharps container should not be allowed to fill till the top of the container, and it should be disposed off once two-third of the container is full. All the collected material may be autoclaved and disposed off as per guidelines.

23.9 Bio-risk in Animal Houses

Bio-risk management in animal houses aims to reduce the risks associated with experimentation on infectious agents and toxins in animal houses. Both biosafety and biosecurity are essential components of risk management. Animal house

biosafety management involves infrastructure controls, standard operating procedures, and personal protective equipment. Animal house biosecurity management involves the security of the facility, personnel, and biological materials. The OIE's Terrestrial Manual, Biosafety, and Biosecurity: Standard for Managing Bio-risks in Veterinary Laboratory and Animal Facilities is the guideline document for developing a risk management program [14].

Biological risk assessment is an expert process that must involve experts with adequate knowledge and experience in biological risk management. However, experts evaluate the risks based on hypothetical scenarios and develop management programs based on insufficient scientific data. Therefore, users must understand the inherent limitations of the assumptions and mitigation strategies. Risk in any process is inevitable due to human involvement; therefore, no process should be considered risk-free during the assessment process.

There are no standard operating procedures for conducting a risk assessment. However, the following risk prioritization method is recommended by the CDC. The CDC method involves the following steps:

1. Identification of potential hazards associated with an infectious agent/toxin or material
2. Identification of procedures and lab activities that could potentially lead to exposure or release of the agent or material
3. Evaluation of the overall competencies and experience of laboratory personnel
4. Evaluation and prioritization of the risks
5. Development, implementation, and evaluation of control methods to minimize the risk of exposure and release

The bio-risk management approach gives the ABSL facility a blueprint to protect the laboratory personnel, experimental animals, general public, and animal population if any unintentional or intentional release of and exposure to biological agents and toxins happens [15, 16]. The bio-risk management should be evidence-based, transparent, affordable, and sustainable. Bio-risk management involves the risks associated with both laboratory biosafety and biosecurity. Biosafety of the laboratory or animal facility describes the principles and practices for the prevention of unintentional release or accidental exposure to biological agents and toxins. On the other hand, biosecurity explains physical control of biological agents and toxins in the laboratory to avert their theft, loss, mishandling, unauthorized use, and deliberate release. The following five components are critical in bio-risk analysis, management, and continual improvement in the biosafety and biosecurity measures:

1. Biohazard identification (What can possibly go wrong?)
2. Bio-risk assessment (What is the likelihood for the occurrence of hazardous events and severity of the harm?)

   Bio-risk assessment is based on the potential animal, human, and environmental exposure of pathogens/toxins and intentional/unintentional release of pathogens and toxins from the animal facility.

3. Bio-risk reduction strategies/bio-risk management (What are the ways to prevent or minimize the occurrence of the hazardous event?)
4. Biological risk communication (How the bio-risks were identified, assessed, and managed?)
5. Monitoring and improvement (How the bio-risk management process is verified, reviewed, and corrected for continual improvement?)

23.9.1 Biohazard Identification

Biohazard identification is the process of detecting, listing, and characterizing the hazards and risk factors that have the potential to cause harm. Categorization and documentation of the potential biohazards of the animal facility/ABSL facility is the first step in the bio-risk analysis process [17]. The biohazard can be the pathogen or the toxin handled in the ABSL, the procedures and protocols followed in the facility, or any situation or act with the potential to cause harm or hazardous event. Broadly, the biohazard of an ABSL facility can be listed under three headings.

23.9.1.1 Biological Agents/Toxins

The biohazardous nature of the biological agent/toxin can be determined by considering the following criteria:

(a) Zoonotic potential
(b) Host range
(c) Infection route
(d) Infectious dose
(e) Latent infection
(f) Shedding of organism
(g) Inactivation methods
(h) Tenacity of the agents outside the host (environmental persistence)
(i) Prevalence and economic burden
(j) Exotic nature of the agents

23.9.1.2 Activities/Procedures Performed using Biological Agents/Toxins and Animals in the Facility

(a) Procedures leading to aerosolization
(b) Containment of animals
(c) Fomites
(d) Inactivation procedure
(e) Transport of biological agents/animals inside the facility

23.9.1.3 The Scale of the Activity and Procedures

(a) The volume of biological agents handled/stored
(b) The concentration of the biological agents handled/stored

23.9.2 The Potential Biohazardous Nodes in an ABSL Facility Are Listed Below

(a) Pathogens/toxins/arthropods handled in the facility
(b) Storage of pathogens/toxins
(c) Disposal of pathogen/toxin materials
(d) Maintenance of experimental animals
(e) Handling of experimental animals
(f) Disposal of experimental animals
(g) Inoculation/collection of biological materials
(h) Storage of samples obtained from animal experiment
(i) Assays using the samples
(j) Disposal of used samples
(k) The traffic of animals/biologicals inside the facility
(l) Personnel access to pathogen/toxin/arthropods inventory

Potential biosafety risks and hazards within an animal containment laboratory (Table 23.2).

The biohazards are very specific to the ABSL/laboratory which emanate on the type of pathogen handled, location of the facility, the structural and procedural biosecurity and biosafety measures, training level of personnel, the policy of the government, etc. Thus, the list of biohazards identified in two different ABSL facilities handling the same pathogen might vary. The primary focus of the biohazard risk is the inventory of biological materials such as pathogens/toxins and arthropods, handling unknown diagnostic specimens, access to the inventory, and disposal of the used pathogen samples.

For example, *the biohazard of the ABSL facility can simply be identified by asking questions such as: Does the pathogen/toxin cause animal or human disease? Does cage changing of infected animals expose the animal handlers to the pathogen? If the answer is YES, the biological material or procedure is considered as a potential biohazard.*

23.10 Bio-risk Assessment

No work with biological agents is devoid of risk.

Bio-risk assessment is a set of analytical procedures designed to identify and characterize biosafety and biosecurity risks in a facility or laboratory. The bio-risk assessment process involves a thorough and robust approach for the *determination of the likelihood of the occurrence of hazard*, *severity of the harm associated with the hazard*, and *detectability of the hazard*. The severity of the harm is estimated based on biological, environmental, and economic impact associated with the release/exposure of the biohazard. A comprehensive bi-risk assessment includes an evaluation of biosafety and biosecurity of the facility. The process of risk assessment should be proactive rather than reactive. The risk of each identified biohazard should

Table 23.2 Potential biosafety risks and hazards within an animal containment laboratory

S. no	Risk	Hazard
Risks and hazards associated with laboratory animals		
1.	Animal cage doors are not properly locked	Escape of infected animals and disease transmission to human and organism spill over to the environment
2.	Animals with symptomatic and asymptomatic zoonotic infection or carrier animals	Disease transmission to laboratory workers and subsequent spill over to the community
3.	Invasive procedures on animals	Splashing of body fluid with zoonotic organisms and disease transmission
4.	Weak sedation of animals while handling	Scratching, biting, and kicking while handling the animals
5.	Handling the large animals in a confined room and breaching the flight distance of the animals	Aggressive behaviour towards animal handlers and physical injury
6.	Improper restring of large animals and no escape route available	Severe physical injury to the animal handlers
Risks and hazards associated with pathogens		
1.	Infectious aerosols from bedding material, animal cage, etc.	Infection to the workers and environment
2.	Improper disposal of contaminated sharp tools used in animal procedures	Injury to the workers and disease transmission
3.	Improper collection and storage of biological fluids and tissues from infected animals	Infection to the workers and environment
4.	Improper disposal of infected animal carcasses/spent medium/bedding material	Infection to the workers and environment
5.	Spill and splash of infectious material	Infection to the workers and environment
6.	Improper transport of infectious material inside the facility and outside the facility	Infection to the workers and environment
7.	Improper transportation of infected animals/arthropods inside the facility	Infection to the workers and environment
Risks and hazards associated with laboratory structure and equipment		
1.	Improper handling of equipment such as X-ray machine, CT scan and ultracentrifuge	Injury to the personnel and building
2.	Improper handling and failure of the HVAC system	Infection to the workers and environment
3.	Use of radiolabeled tracer in animals	The presence of radioisotopes in animal excreta and body fluid expose the workers to radiation injury
Risks and hazards associated with anaesthetic drugs		
1.	Use of gaseous anaesthetics in a confined facility	Health problems in workers exposed to anaesthetics
Risks and hazards associated with inadequate facilities and equipment		
1.	Breakdown or improper maintenance of primary (biosafety cabinet, PPE) and secondary barriers (negative pressure, drainage system, sterilization and disposal system, etc.)	Infection to the workers and environment

be done systematically. Answering the following questions will determine if the identified biohazard is a risk or not a risk:

(a) What is the likelihood of intentional or unintentional release? (negligible/low/moderate/severe)
(b) What is the likelihood of exposure to humans, animals and the environment? (negligible/low/moderate/severe)
(c) What is the likelihood of biological, environmental, and economic consequences?
(negligible/low/moderate/severe)
(d) What would be the severity of the harm to animals and human beings?
(negligible/low/medium/high)
(e) How easily the release or exposure can be detected?
(easily detectable/moderately detectable/tough to detect)
(f) Is there a vaccine available for the pathogen handled in the facility?
Yes/no/available but partially effective

If the answer is negligible, identified biohazard is NOT risk. Biologicals and procedures can be rated as low to very high risk based on the aforementioned systematic risk-assessment protocol. The bio-risk assessment protocol should address all the procedures and protocols associated with the identified biohazard.

Example: Genetically modified Aedes aegypti mosquito infected with dengue virus (Table 23.3)

While conducting a risk assessment, each risk should be assessed individually. The following is a simpler version of the risk assessment sheet (Table 23.4).

23.11 Bio-risk Management

Bio-risk management is the process of selecting suitable containment measures to ensure that biohazards are properly controlled. The selection of appropriate and feasible biosafety and biosecurity measures to prevent the release of and exposure to the biohazard is critical in bio-risk management. Bio-risk management is achieved by one of the following means:

(a) Elimination of identified biohazard (not handling the biological agents)
E.g. risk: *A highly infectious agent with zoonotic potential*
Risk management*: Not to handle and store the infectious agents in the facility*
(b) Substitution (by using alternative technical procedures)
E.g. risk: Transportation of infectious agent culture from culture room to animal facility and risk of spillage
Risk management: Using pass box to move the culture from culture room to animal facility

Table 23.3 An example of bio-risk assessment protocol

Likelihood of release	Likelihood of exposure	The economic consequence in an endemic area	Severity of harm	Detectability	Vaccine availability	Risk status
Moderate	Moderate	Moderate	High	Tough	No	Moderate to Severe

Table 23.4 Simpler version of the risk assessment sheet

Activity	Potential hazard	Likelihood of occurrence	Consequence	Risk rating
Cage changing of infected animals	Infectious aerosol generation	Low/ moderate/high	Exposure of animal attendants	Low/ moderate/ high

(c) Implementing appropriate biosafety and biosecurity measures that include administrative, operational, and structural control and use of personal protective equipment (PPE)

E.g. risk: Cage changing of infected animals and risk of aerosol infection

Risk management: Using PPE such as N95 face mask, face shield, and eye goggles and handling the cage inside the BSC-II cabinet

Organizational policy plays a key role in risk management. Proactive steps including recruitment of qualified and experienced personnel, training and verification of competency of the personnel, approvals from the Institutional Biosafety Committee and Institutional Animal Ethics Committee, biological agent/toxin inventory management, waste management policy, etc. ensure professional risk management.

Operational control includes laying down and scrupulously following standard operating procedures on biosafety and biosecurity. Good laboratory practices, regular disinfection and decontamination procedures, transportation of samples and animals inside the facility, biological agents handling and storage practices, accident reporting, and periodic emergency drills are part of operational control strategies.

Structural control includes the physical features of the facility. Design of work stations, civil structures, equipment, or any other relevant structural aspect of the laboratory that minimizes or prevents exposure to biohazards is considered structural or engineering control to manage the bio-risk.

Example: Risk of unauthorized use of freezer box key which is placed inside an open hook key cabinet

Risk management: Use of biometric devices to access the key cabinet or password-protected key locker

The following is a model of risk management worksheet (Table 23.5).

Table 23.5 Model of risk management worksheet

Activity	Proposed equipment	Proposed work practices	PPE	Risk rating
Cage changing of infected animals	Cage changing station (BSC-II cabinet)	All work should be in BSC-II following BSL-III practices	Overalls with face shields	Low/ moderate/ high

23.12 Risk Communication

Risk communication is designed to inform the stakeholders of the ABSL facility about technical practices and decisions used for handling biohazards and for responding to the release of and exposure to biohazards. Risk communication is an integral part of bio-risk handling and a continuation of biohazard identification, bio-risk assessment, and bio-risk management processes [18]. The bio-risk communication statement should contain the following details:

(a) Details of the biohazard handled by the facility
(b) Benefits of working with the biohazard
(c) Documentation details on the bio-risk analysis performed on the biohazard
(d) Biosafety and biosecurity capabilities of the facility to handle the intentional or unintentional release of the biohazard

The ABSL facility should also prepare a communication document on general preparedness to handle the accidental or intentional release of the biohazard. This document should contain the following details:

(a) Roles and responsibilities of staff involved in the drafting, reviewing, approving, and distributing official communication on preparedness
(b) Contact list (name, contact numbers, email addresses of the national, regional and local disease control agencies/veterinary and public health agencies, security agencies dealing with bio-threat agents such as National Disaster Response Force, physicians and occupational health programme officials, staffs at risk, stakeholders, and affiliates)
(c) "Detailed incident response plan" in the event of unintentional and intentional release of the biohazard

23.13 Monitoring and Improvement

Bio-risk management is an ongoing process where the laboratory biosafety and biosecurity measures are regularly monitored to ensure the effective bio-risk handling system. Periodic internal and external audits on administrative, structural, operational, and PPE should be conducted to identify the areas of noncompliance that need to be documented and corrected and to identify areas for improvement.

Example: Internal audit on bio-risk management (Table 23.6)

Table 23.6 Internal audit on bio-risk management

Internal audit observation/ noncompliance	Perceived risk	Corrective action taken
Freezer box key placed inside an open key cabinet	Theft of biological material	Keys kept inside a password-protected locker/biometry-enabled digi-locker

23.14 Waste Management in Laboratory Animal Houses

The management of wastes from laboratory animal houses in India is governed by the Environment (Protection) Act, 1986 (29 of 1986). In India, a specific framework for this is provided under the Biomedical Waste (Management and Handling) Rules, 1998, which was amended in 2016. The biomedical wastes generated in animal house fall under one of the following categories. A specific colour of the container is also prescribed for segregation of these wastes (Table 23.3).

S. No	Waste type	Colour category	Storage
1	Animal anatomical waste, soiled waste, expired or discarded medicines, chemical waste, chemical liquid waste, discarded contaminated beddings, and microbiology, biotechnology, and other clinical waste	Yellow	Yellow-coloured non-chlorinated plastic bags or containers
2	Contaminated waste which is recyclable	Red	Red-coloured non-chlorinated plastic bags or containers
3	Sharp waste including needles, broken glass, and metals	White	Puncture-proof, Leak-proof, tamper-proof containers
4	Glassware and biomedical implants	Blue	Cardboard boxes with blue-coloured marking

Provision should be made within the premises for a safe, ventilated, and secured location for storage of segregated biomedical waste. Microbiological waste, tissue, and blood waste shall be pre-treated at the premises before disposing off through a biomedical waste disposal service provider [19]. All biomedical waste handling personnel should be given proper training in handling and management of the waste. They should also be routinely immunized against diseases such as hepatitis B, tetanus, etc. These personnel should also be subjected to periodic health check programmes. Appropriate disinfectants should be kept at locations of handling biomedical wastes to deal with accidental spillage or leakage. The commonly used disinfectants are as below (Table 23.7).

Table 23.7 Commonly used disinfectants

S. No	Disinfectant	Intended use	Precautions
1	Sodium hypochlorite	Disinfection of surfaces contaminated with biological wastes	Corrosive; not to be mixed with strong acids
2	Alcohol	Surfaces and table tops	Flammable
3	Chlorhexidine (hand rub/hand wash)	Disinfection of skin and hands	Hand rubs may contain large amounts of alcohol (70%) and inflammable
4	Detergent	Floor cleaning	None

Liquid waste generated at animal houses should be treated and disposed off in accordance with the Water (Prevention and Control of Pollution) Act, 1974 (6 of 1974).

23.15 Regulatory Requirements for Biosafety

In India, the Institutional Biosafety Committee (IBSC) shall be constituted in all animal research organizations handling hazardous microorganisms and/or GE organisms under the provisions of "Rules for the manufacture, use/import/export and storage of hazardous microorganisms/ genetically engineered organisms or cells, 1989" notified by the Ministry of Environment, Forests and Climate Change, Government of India, under the Environment (Protection) Act, 1986 [20]. The terms of reference of this committee involve the supervision and approval of the use of microorganisms in experiments, import and export of biological agents, monitoring the use of genetically engineered biologicals, etc. The committee acts as the nodal point for the implementation of the biosafety guidelines and functions as the first point of contact for biosafety-related decisions within an institution. The composition of the committee includes the Head of the Institution, scientists engaged in the recombinant DNA work, a medical doctor, and a nominee of the Department of Biotechnology. The Institutional Biosafety Committee (IBSC) will also advise on the on-site emergency plan and provides routine updates on the biosafety guidelines of the Review Committee on Genetic Manipulation (RCGM). The committee will also coordinate with the District Level Committee/State Biotechnology Co-ordination Committee and the Genetic Engineering Appraisal Committee.

23.16 Occupational Health and Safety Programme

The occupational health and safety programme (OHSP) in ABSL should aim to be consistent with national and local regulations. While designing an effective OHSP, the institution should consider all aspects of the experimentation including infrastructure, personnel, processes, biohazards, and, more importantly, the animal species. A guideline for designing an OHSP is available in WHO Laboratory Biosafety Manual 3rd Edition [1] and CDC Biosafety in Microbiological and Biomedical Laboratories (BMBL) 5th Edition [11]. A successful OHSP can be implemented only with the active participation of the animal house management, research investigators, animal handlers, and health and safety professionals. The occupational health and safety of animal house personnel in India are not governed by a separate act [21]. However, acts that contain the provisions for the protection of health and safety of workers in India, namely, the Factories Act (1948) and the Mines Act (1952), can be made applicable for such programmes. The Factories Act was

amended in 1987 and stipulates pre-employment examination as a pre-placement procedure, statutory periodic medical examination for a job in hazardous areas.

Additional care and supervision should be implemented in cases where:

1. The experimental risk and hazard are moderate or severe.
2. The human-animal contact period is longer resulting in higher exposure to hazard.
3. Involvement of personnel with a previous history of illness/allergies.

The risk assessment process should consider all personnel who will come into contact with the experimental animal irrelevant of the exposure period. The program shall consider the routine evaluation of exposure risk by personnel interview, periodic diagnostic tests for specific pathogens, vaccinations, and periodic evaluation of respiratory health. Prophylactic immunizations against tetanus, hepatitis B virus, rabies, etc. Pre-exposure immunization (if available) against the pathogens that are proposed to be handled should be offered to personnel at risk of infection. The OHSP is a dynamic program and should be routinely revised based on the assessed risks (Table 23.8).

Table 23.8 Assessment risks

Relevant terminologies	
Biosafety	Laboratory biosafety involves the principles, methods, and practices that are put in place to prevent unintentional exposure to pathogens and toxins or their accidental release. In general, they are the measures to protect the users and environment from the pathogens
Biosecurity	Biosecurity is a multipronged approach to assess and mitigate risks to human, animal, and plant life and health and associated risks for the environment due to the intentional release of the pathogens or toxins. In general, they are measures to protect the pathogens from harmful use
Bio-risk	Bio-risk is the probability of occurrence of accidental infection or unauthorized access or misuse of the pathogen and toxins from the laboratory or animal facility
Bio-risk assessment	This involves processes to identify biosafety and biosecurity risks and their potential consequences
Bio-risk management	This involves the development of procedures and practices to minimize the likelihood of the occurrence of bio-risks. The bio-risk management is a multipronged strategy to identify risks, hypothesize consequences, and develop and validate risk reduction procedures

References

1. Laboratory Biosafety Manual (2004). 3rd edition. World Health Organisation available at https://www.who.int/csr/resources/publications/biosafety/WHO_CDS_CSR_LYO_2004_11/en/
2. Frasier D, Talka J (2005) Facility design considerations for select agent animal research. ILAR J 46(1):23–33
3. Yan LX, Ning XK, Sheng JJ, Cheng LX (2016) The main biological hazards in animal biosafety level 2 facilities and strategies for control. Biomed Environ Sci 29(4):300–304
4. Ming G, Yong W, Jinbiao L, Zhixiang H, Xiangdong L (2019) Biosafety and data quality considerations for animal experiments with highly infectious agents at ABSL-3 facilities. J Biosafety Biosecurity 1(1):50–55
5. Alderman TS, Carpenter CB, McGirr R (2018) Animal research biosafety. Appl. Biosafety: J ABSA Int 23(3):130–142
6. Carpenter CB (2018) Safety considerations for working with animal models involving human health hazards. Anim Model Exp Med 1:91–99
7. Lehner NDM, Crane JT, Mottet MP, Fitzgerald ME (2009) Biohazards: safety practices, operations and containment facilities. Planning and Designing Research Animal Facilities, American College of Laboratory Animal Medicine, pp 347–363
8. USDA (US Department of Agriculture) (2002) Agricultural research service facilities design standards. USDA, ARS, Washington, DC
9. Lipman NS (1999) Isolator rodent caging systems (state of the art): a critical review. Contemp Top Lab Anim Sci 38:9–17
10. Maher JA, Young DO (2013) How to isolate a rodent: biocontainment caging options. Lab Anim 36(2):42–48
11. Biosafety in Microbiological and Biomedical Laboratories (BMBL) (n.d.). 5th ed. Centres for Disease Control and Prevention available at https://www.cdc.gov/labs/BMBL.html
12. Hessler J, Broderson JR, King CS (1999) Rodent quarantine: facility design and equipment for small animal containment facilities. Lab Anim 28:34–40
13. American Committee for Medical Entomology (2001). Arthropod containment guidelines, Version 3.1, December 2001.
14. Biosafety and Biosecurity: Standard for Managing Biological Risk in the Veterinary Laboratory and Animal Facilities in Manual of Diagnostic Tests and Vaccines for Terrestrial Animals (2019). OiE World Organisation for Animal Health. Available at https://www.oie.int/fileadmin/Home/eng/Health_standards/tahm/1.01.04_BIOSAFETY_BIOSECURITY.pdf
15. National Research Council (2011) Guide for the care and use of laboratory animals, 8th edn. National Academies Press, Washington, DC
16. National Research Council (1997) Occupational health and safety in the care and use of research animals. National Academies Press, Washington, DC
17. Swearengen JR, Carpenter CB (2015) Working safely with experimental animals exposed to biohazards. In: Fox JG, Anderson LC, Otto G, Pritchett-corning KR, Whary MT (eds) Laboratory animal medicine, 3rd edn. Elsevier-Academic Press, Cambridge, MA, pp 1297–1312
18. Fontes B (2008) Institutional responsibilities in contamination control for research animals and in occupational health and safety for animal handlers. Inst Lab Anim Res J 49:326–337
19. Pritt S, Hankenson FC, Wagner T, Tate M (2007) The basics of animal biosafety and biocontainment training. Lab Anim 36:31–38
20. Regulations and Guidelines for Recombinant DNA Research and Biocontainment (2017). Department of Biotechnology, Government of India available at http://dbtindia.gov.in/guidelines-biosafety
21. The Draft Labour Code on Occupational Safety, Health and working Conditions (2018) Ministry of Labour and employment, Government of India available at https://labour.gov.in/sites/default/files/Last_Date_Extended_for_OSH_Code_0.pdf

Occupational Health Hazards and Disaster Management in Laboratory Animal Facilities

24

Neeraj Khatri, Priyanka, Ashish Kumar Dubey, Ram Bharti, and Bhupinder Singh Chopra

Abstract

Animal facility offers live biological tools to the scientific community. It is a legal and ethical responsibility of the facility provider/management to protect the employees and researchers from work-related hazards. Animal facility staff and users routinely perform several tasks that expose them to animals, zoonotic agents, infectious materials, and equipment that may impose a possible health risk. Besides, research personnel working with animals are equally prone to occupational health hazards. Unattended hazards may lead to disaster, causing suffering and severe damage to life. A disaster management plan is an absolute requirement for tackling unintended incidents. However, a single plan cannot address all the accidents. Therefore, it is essential to review and upgrade the safety program continuously and regularly. Institutes should devise occupational health and safety management programs to prohibit any harm to individuals and different components of the facility. A successful program needs coordination and joint management by individuals, laboratories/service providers, organizations, and the government.

Keywords

Health hazards · Occupational · Animals · Animal facilities · Disaster management · Zoonosis

N. Khatri (✉) · Priyanka · A. K. Dubey · R. Bharti · B. S. Chopra
IMTECH Centre for Animal Resources & Experimentation (iCARE), CSIR-Institute of Microbial Technology, Sector 39-A, Chandigarh, Punjab, India

Academy of Scientific and Innovative Research (AcSIR), Ghaziabad, Uttar Pradesh, India
e-mail: neeraj@imtech.res.in; priya6192@imtech.res.in; toniahish@imtech.res.in; rambharti@imtech.res.in; bhupindersingh@imtech.res.in

© The Author(s), under exclusive license to Springer Nature Singapore Pte Ltd. 2021

P. Nagarajan et al. (eds.), *Essentials of Laboratory Animal Science: Principles and Practices*, https://doi.org/10.1007/978-981-16-0987-9_24

24.1 Introduction

Occupational health, safety, and emergency plan (OHSP) is an integral part of any biomedical research or academic institution, which adopts ethical practices necessary for the safety of animals and the personnel working with them. Besides breeding and supplying experimental animals to the researchers/scientific community, the facility should focus on the safety and health of animals and personnel, which requires an understanding of the whole scenario of the potential hazards and the management thereof [1]. Enlisting the hazards and potential areas of threats can help to prevent any undesirable incident associated with an animal facility. The tools required to evaluate and mitigate such occupational hazards are called "job hazard analysis" and "job safety analysis" [1]. Disaster leading to fatality and economical wellness is a major concern for humanity. Management of activities to reduce injuries, lethality, and all harmful environments is one of the aspects of disaster management programs. A disaster management plan should be on three levels, i.e., pre-, during, and post-crisis. The pre-existing policies and guidelines substantially facilitate the implementation of standard protocols during an emergency. Having a comprehensive emergency action plan in place considering the routine and essential services, evacuation plan, and other safety guidelines to be followed during an unlikely event of an emergency will help avoid inadvertent procedural delays and achieve the targeted result by the optimal use of available resources.

In addition to the emergency action plan, a medical surveillance program shall be established for the animal caretakers with the maintenance of record of research, personnel involved, and providing a place for animal-related issues as an essential part of the occupational hazards management program [2].

Generally, a disaster management plan is assumed to be prepared for countering a natural disaster such as an earthquake, flood, storm, fire, etc. However, the insufficiency of working personnel and failure of utilities frequently cause an eruption of conditions much like a disaster [3]. The pandemic of COVID-19 compelled the administration world over to impose a complete lockdown, which significantly thinned down the supply of utilities and manpower. This situation had a severe impact on research on animals and led to the complete cessation of new experiments on animals. Therefore, contingent emergency plans for running essential services with limited resources will assist in managing the vivarium in such unprecedented situations.

24.2 Hazard and Safety Measures

Hazards are agents, which cause potential harm to humans, animals, or the environment. Daily exposure of animal care facility workers to animals, zoonotic agents, materials, and equipment required in animal care facilities may involve health risks. Moreover, researchers, who are working on animal experimentation, also have a risk of occupational health hazards. Occupational health hazards in animal care facilities

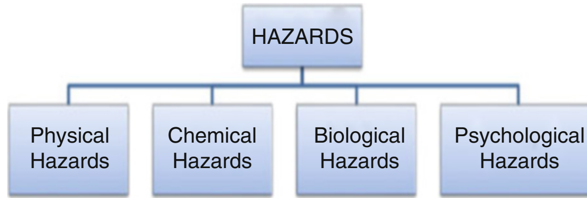


Fig. 24.1 Major classification of hazards

can be classified as physical, chemical, biological, and psychological hazards (Fig. 24.1).

Physical Hazards: The animal care facility itself poses several operational physical health hazards like wounds, injuries, and fractures resulting from slippery floors, lifting heavy materials, etc. Materials such as animal feed if lifted physically can cause muscular, tendon, or ligament injuries. Equipment like animal cages, racks, animal restrain systems, and autoclaves carry the risk of injuries to animal care facility workers. Experiments involving the use of radiation/radionuclides also pose a threat of radiation exposure to the personnel. Besides, an electrical or fire emergency can also erupt in the facility. Therefore, the disaster management plan should include standard operating procedures to tackle these eventualities too.

Chemical Hazards: Animal care facility workers and research scholars (working on animal experimentation) handle carcasses. The carcass itself may carry chemical hazards used while experimentation can be dangerous.

Biological Hazards: This includes zoonoses, animal bites, kicks, and allergies.

24.2.1 Physical Hazards

Physical hazards include all those activities and substances that threaten the physical safety of animals and personnel. It results in injury, illness, and death [4]. Broadly, physical risks (Fig. 24.2) comprise of the following.

24.2.1.1 Ergonomic Threats

It is mainly triggered by the activities which require monotonous movements, such as lifting and moving cages and piles of feeds and fodder, carrying out sterilization methods, and washing. This necessitates the development of a worker-friendly environment to provide a way in which risk factors can be eliminated, for example, cage changing stations for changing cages, pulleys, and trolleys to lift and transport the cages and heavy bags of feed and bedding. The use of service lifts to transport heavy goods within multistoried animal facilities is highly recommended.

24.2.1.2 Sharp Injury Hazards

Sharp injuries may result in workers due to mishandled needles, syringes, glass pipettes, sharp equipment, scalpel blades, damaged cages, and broken glass, which

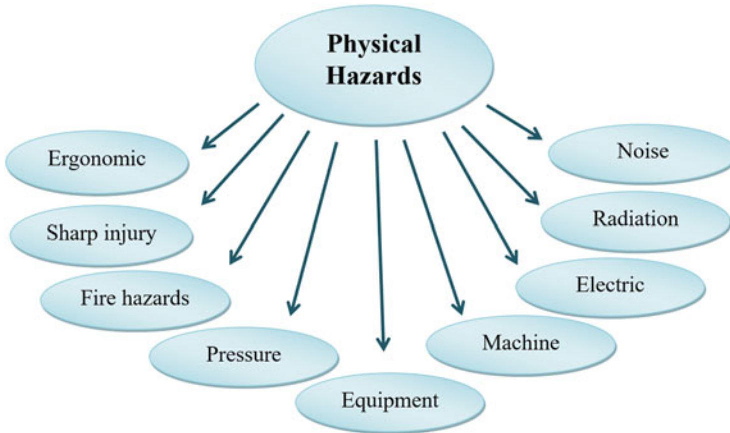


Fig. 24.2 Types of physical hazards

are a source for tissue injuries. For example, while recapping a used needle, it may accidentally puncture the finger of the user; therefore, instead of recapping, it should be immediately disposed of in a needle destroyer and syringe cutter. To minimize this type of hazard, one has to be trained with the disposal of sharp waste. The disposal should be done in a puncture-proof, tamper-proof, and leak-free container after autoclaving by shredding or encapsulating in a metal container or concrete box [5].

24.2.1.3 Fire Hazards

Many laboratories and animal care facilities have incinerators, where flammable liquids are used to burn animal carcasses and involve the use of electrical appliances that may cause an electric fire. The National Fire Protection Association (NFPA), USA, has classified materials A and class B as ordinary combustibles and flammable liquids. Flammable liquids are also used during surgical procedures on animals. These hazards can be prevented by storing flammable liquids at an appropriate temperature and in explosion-proof cabinets.

24.2.1.4 Pressure Vessel Hazards

Pressure-related hazards arise from autoclaves, washing equipment, and gas cylinders. While working with these hazards, one should be careful. Steam is generated under high pressure for running autoclave, if not regulated properly, will cause burst causing severe injury to the operators. Personnel professionally trained in the handling of the equipment must be allowed to work with them, and regular maintenance of the equipment should be done.

24.2.1.5 Electrical Hazards

Electric hazards are classified into four types – fatal electrocutions, electric shock, burns, and falls. Electric outlets and switches in washing areas are at higher risks

from this kind of hazard due to the use of large volumes of water. This hazard can be prevented by using electrical appliances of good quality, waterproof electrical switches, and using insulated goods.

24.2.1.6 Radiation Hazards

While working in the biosafety cabinets, sterilization is done by UV, which is carcinogenic. Areas with the use of UV should be least accessible, and personnel should be aware of the hazard. Proper eye and skin protection are required while working with UV and laser lights. In the areas where lasers or UV light sources are used profoundly, warning signage should be posted outside the room [6]. X-beam imaging is one of the most well-known imaging techniques used for in vitro and in vivo imaging. Instruments include transmission and scanning electron microscope, fluorescent microscope, positron emission tomography scanner, etc. [7]. When handling radioisotopes, personnel need to wear a lead apron, gloves, and radiation badges specific to personnel needs and should be monitored at frequent intervals.

24.2.1.7 Machinery Hazards

Machines with movable parts are the main cause of injury, and all the large equipment can be a source of injury if not handled properly. At purchase, machines and equipment need to be evaluated properly about the functionality and design.

24.2.1.8 Noise Hazards

Animal care facilities have high-clamor territories, for example, a tumult arising from large laboratory animals, washing zones, lodging zones, and mechanical spaces. Exposure to elevated levels of sound can cause lasting hearing loss. Some facilities also have dogs that are highly noisy at times. Personnel working in noise-generating areas like washing area, HVAC machine rooms, and generator rooms must be provided with auditory protection. To protect the damage to ears, earplugs are used to reduce the harmful and annoying effects. Earplugs in both foldable and pre-molded variants are accessible in different sizes. Circumaural defenders (earmuffs), which are plastic vaults that spread the ears and are associated with a spring band that fits on the head or is connected to a hard cap, are also available [8].

24.3 Chemical Hazards

Chemicals are pervasive in research and in laboratory and animal facilities too. They are utilized in disinfection, anesthetization, and taking samples, treatments, etc. Experimental procedures require the administration of lethal substances to animals, and there are chances of their disposal and contamination of the environment and waste torrents [9]. Chemical hazards include several chemicals as mentioned in Fig. 24.3.

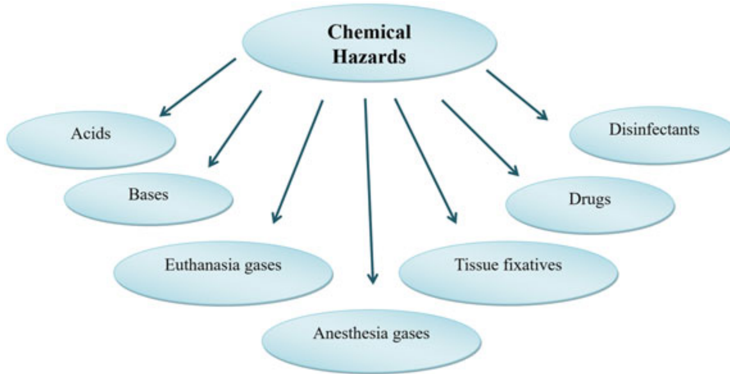


Fig. 24.3 Types of chemical hazards

24.3.1 Disinfectants

Disinfectants are used in the facility to clean the premises and surfaces to destroy pathogenic microbes [10]. While using any disinfectant, we must be aware of the procedures for handling the material. Glutaraldehyde, crystalline phenol, and many other chemicals are generally used for disinfecting the materials, and their exposure may lead to skin irritation. Glutaraldehyde is also used as a tissue fixative for histopathological examination [11]. Inhalation of glutaraldehyde will cause lung problems of both chronic and acute nature. Asthma, irritation in the lungs and nose, sneezing, wheezing, and bleeding nose may develop. Skin contact will result in irritation to the eyes, skin rashes, etc. [9]. It is advised, therefore, that proper precautions should be taken while using different types of disinfectants and chemicals. Alcoholic disinfectants (spirit, etc.) are also used by veterinarians and technicians particularly when preparing for any surgical procedure on animals. While doing surgery, spirit swabs are wiped over the skin, and if electrocautery (electrosurgical unit) is used immediately, it catches fire. Potential items of threat include electrosurgical unit and motorized drilling units (dental burrs, motorized bone drills, etc.). These items are widely used in animal surgical facilities, and if proper procedures are not followed and if any untrained person is doing the procedure, it can catch fire rapidly and burn the skin.

24.3.2 Acids and Bases

Any substance, which causes a visible injury to the tissue by chemical action, is corrosive and is classified as solids, liquids, and gases. Corrosive compounds include sulfuric acid, phosphoric acid, and hydrogen peroxide, which irritate the skin and eyes and affect the breathing system [6]. These compounds need to be stored in shelves or cabinets that are lower or at floor height in secondary

containment to avoid spillage. These chemicals should be kept in a well-ventilated environment or near the ventilated system.

24.3.3 Euthanizing Agents

Euthanizing agents in an animal facility are used for humane termination of life. It includes isoflurane, halothane, nitrous oxide, and carbon dioxide which when inhaled can cause hypoxemia. This causes inflammation of inhalation and should be used by trained personnel with respiratory protection.

24.3.4 Anesthetizing Agents

Anesthetic agents are used while performing surgery and during animal experiments. Inhaling anesthetic gases above the minimum value can be hazardous to both the animal and the personnel. The waste anesthetic gases circulating in the procedure room after the experiment cause nausea, headaches, fatigue, and sometimes sterility. It can be prevented by routine monitoring for leakage of gases. Fume hoods should be used for the storage of gaseous anesthetic agents like isoflurane and halothane. Ketamine, a commonly used anesthetic agent, can cause an assortment of neurological, cardiovascular, mental, urogenital, and stomach manifestations [12].

24.3.5 Tissue Fixatives

In histopathology labs, formaldehyde and glutaraldehyde are utilized for fixing the tissues. Formaldehyde has been reported as a probable human carcinogen [13] but can even cause corneal damage if spatters into the eyes. Besides, this can irritate the ear, nose, and throat pathway upon inhalation. A procedure room and biosafety cabinet should be used along with PPE (personal protective equipment) while doing histopathological studies.

24.3.6 Hazardous Drugs

Hazardous drugs include all those pharmaceutical drugs, which have been regarded as toxic by the American Society of Hospital Pharmacists. They may potentially induce carcinogenicity, gene toxicity, infertility, etc. While using these drugs, a proper protocol has to be prepared before experimenting. Streptozotocin, a diabetogenic chemical, has been reported to induce tumor and liver toxicity [14]. In India, many of the anesthetics and sedatives such as ketamine, pentobarbital, tramadol, etc. used in animal facilities fall under Schedule X drugs. Schedule X of Drugs and Cosmetics Rules, 1945, covers habit-forming and narcotic drugs that are required to

be kept under lock and key in a specified area accessible only to authorized personnel.

24.4 Biological Hazards

Biological hazards related to animal care facilities are also termed as animal-related hazards (Fig. 24.4). In general, these are the secondary effect of the physical hazards that results from direct human and animal contact during the cage transfer process, animal restraining, or any other procedures.

24.4.1 Bites, Scratches, and Kicks

These are considered as physical hazards caused by animals while handling them during restraining or during the procedure of changing the cages. Personnel and researchers need proper training for animal handling, restraint techniques, and awareness of all the species-specific behavior of animals. A first aid kit should be kept in the facility with all the primary components, viz., cotton, disinfectants, bandages, and antiseptic ointments. First aid procedures should also be made familiar to the staff. For example, in the case of a rodent bite or scratch, the wound should be cleaned immediately with soap and water, and an antiseptic and bandage should be applied. Further medication may be taken as per the prescription of a physician.

24.4.2 Allergens

Allergens are those substances (part of the animal body) that result in immune response in the body and cause hypersensitivity. It is a common hazard that develops

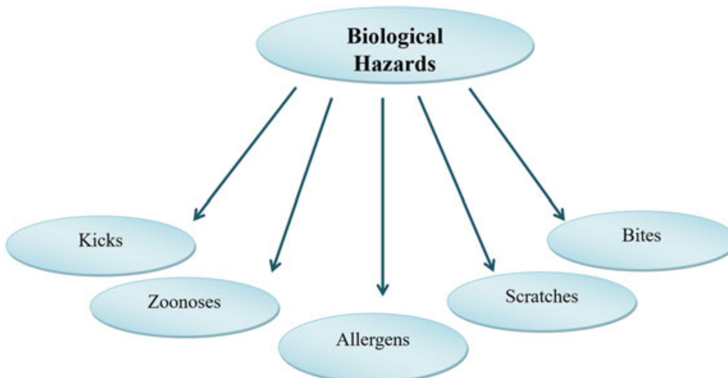


Fig. 24.4 Types of biological hazards

Table 24.1 Allergies associated with animal facilities and their symptoms

Allergy	Symptoms
Urticaria	Skin redness, itchiness, welts or "hives"
Conjunctivitis	Sneezing, eye itchiness, clear nasal drainage, or nasal congestion
Rhinitis	Sneezing, nose itchiness, clear nasal drainage, nasal congestion
Asthma	Cough, wheezing, chest tightness, or shortness of breath
Anaphylaxis	Itching, hives, throat tightness, eye or lip swelling, difficulty swallowing, hoarseness, shortness of breath, dizziness, fainting, nausea, vomiting, abdominal cramps, diarrhea
Atopy	Genetic tendency to inherit IgE-mediated reaction due to animal products

in personnel working with animals. Animal hair, furs, skin, urine, saliva, etc. can act as allergens. Individuals with prior respiratory conditions are at higher risk of developing allergies. Allergies can be species-specific and are induced by proteins. These proteins vary from species to species, and a person can be allergic to a single species or multiple species.

Some common allergies with their symptoms are given below (Table 24.1).

Allergies can be avoided by segregating the vivarium from other facilities and by using PPEs such as gloves, masks, hair and shoe covers, and safety glasses. Allergic aerosols can be reduced by increasing the frequency of air changes in the animal rooms. Small animals can be housed in individually ventilated cages (IVCs) that should be opened only in a laminar hood or cage change station for performing husbandry practices or experimental interventions. The use of bedding disposal stations for discarding used bedding prevents allergies due to dust. Care must be taken while doing procedures on animals, and hands should be washed with soap after completion of work. In case of any kind of allergic reaction or symptoms, a physician should be consulted.

24.4.3 Zoonoses

Zoonoses are infectious diseases whose primary reservoir is animals and the secondary reservoir is human. They are transmitted from non-human to human in zoos, farms, animal facilities, etc. There are lesser chances of zoonoses infection in labs as the animals are bred and managed within the labs, with no exposure to wild animals. Still, fatal zoonotic diseases may occur if proper procedures are not followed while working with infectious agents. It is essential to know zoonotic agents for safety as it will reduce the chances of infection and spread of the disease if one gets infected. Individuals with poor health status are at higher risk of getting an infection. Zoonotic agents reach the body through inhalation, ingestion, mucous membrane, and wounds. Some of the biological agents along with the diseases are listed below (Table 24.2).

Table 24.2 Zoonotic agents related to occupational hazards while working in animal facilities

Biological agent	Zoonotic disease
Viruses	Lymphocytic choriomeningitis virus (LCMV), hantavirus, herpes B, herpes simplex, measles, poxviruses, rabies, Lassa fever (Lassa virus)
Bacteria	*Salmonella*, *Shigella*, *Campylobacter* (gastrointestinal diseases); *Chlamydia psittaci* (psittacosis; a bird disease); *Mycobacterium tuberculosis*; *Brucella*; *Coxiella burnetii* (Q fever); *Streptobacillus moniliformis* (rat bite fever); *Rochalimaea henselae* (cat scratch disease); *Clostridium tetani* (tetanus); *Mycobacterium marinum* (an aquatic infection); *Erysipelothrix rhusiopathiae* (a marine animal infection), *L. interrogans* (leptospirosis), *Yersinia enterocolitica* (yersiniosis)
Fungi	Dermatophytes (ringworm)
Protozoans	*Giardia*, *Cryptosporidium*, *Entamoeba* (gastrointestinal diseases); *Toxoplasma gondii* (toxoplasmosis)
Worms	*Hymenolepis* spp. (tapeworm)

24.5 Occupational Health and Risk Management

Prevention of illness, injury, or any kind of damage to the environment is the primary purpose of occupational health and risk management. Provisions should be made to reduce the menace and potential hazards. To curtail the occupational hazards to healthcare professionals, risk management programs should be established in the facilities for safe and smooth functioning without any occupational health threats at four main levels, i.e., personnel, animals, environment, and the nation.

24.5.1 Basic Concepts

For effective working of overall activities, safe practices are essential. Different guidelines and SOPs are available that can vary among organizational bodies about hazards, instrumentations, and record maintenance to ensure the safety programs. However, the following basic practices are essential for safety and occupational health purposes.

24.5.2 Risk Assessment

Risk assessment is a continuous program involving trained personnel to evaluate threat-related risk management programs. It relies on the type and amount of hazards released depending on the laboratory. A brief medical examination of the working person should be done to assess the pre-existing medical condition. Such an analysis would help in avoiding risk-prone/susceptible individuals from potentially hazardous activities.

24.5.3 Minimizing Exposure

The best way to deal with hazards is avoidance maximally. The basic protection requirement is a PPE kit (Fig. 24.5) which includes coverall, gloves, face mask, hair cover, shoe cover, safety glasses, first aid kit, etc. Various activities and work areas

Fig. 24.5 Illustration of personnel protective equipment (PPEs)

Fig. 24.6 Cage change station

can be categorized according to the risk involved, such as low-, medium-, or high-risk areas, and only sufficiently trained personnel should be allowed to carry the respective activities.

Further, procedures involving animals and their waste products can be carried in biosafety stations specifically designed to trap aerosols and allergens generated during these operations (Figs. 24.6 and 24.7).

24.5.4 Training of Users

Training is mandatory for any new employee about safety measures, standard operating procedures, duties, hazards, disposal of hazards, etc. under the supervision of an experienced person. The worker must be periodically monitored and assessed for following the protocols.

Fig. 24.7 Waste disposal station

24.5.5 Guidelines and SOPs

Guidelines are essential for an organization to conduct proper work and prevention of occupational hazards of the personnel involved in working with animals. It ensures the safety of the personnel, animals, and equipment. Standard operating procedures must be followed by each employee of the laboratory. Any deviation in the fulfillment of these rules will lead to perturbation of the program.

24.5.6 Record Maintenance

All the records regarding animals, use of infectious agents in animal experiments, use of schedule X drugs and health records of staff and their vaccination schedule, if any, etc. should be maintained in an easily accessible manner.

24.5.7 Responsibility and Coordination

Responsibility is the most crucial for any type of laboratory/organization. The responsibility is also categorized at different levels, be at the institute, manager, and/or implementer level. There must be coordination among all the stakeholders for the accomplishment of the desired goals of safety programs. Failure from any of the stakeholders may lead to the failure of these plans.

24.5.8 Diversity

It is mandatory to set down the levels of risk for the individuals coming in direct/indirect contact with animals that are taxing to assign for every person as each person has a different grade of infections. In an animal facility, there will be principal investigators, veterinarians, scientists, laboratory technicians, students, security staff, and workers, coordinating the wellness of all the activities. Training on safety and hazards must be provided to different employees irrespective of education and cadre.

24.5.9 Institutional Bodies

It is a legal and ethical responsibility of the institution (including the management) that houses the animal facility to protect all the employees and researchers working therein from the work-related hazards. The institute may divide the responsibilities among different institutional bodies looking after specific objectives. The biosafety committee may identify and regulate the use of hazardous substances in the institute and can facilitate the animal ethics committee in mitigating potential biohazard-associated **risks** (Fig. 24.8).

24.6 Disaster Management

Disaster can be defined as any accident or negligence that can cause severe damage and suffering to human life leading to fatality, property damage, or deterioration of the environment to a level that requires remedial intervention from outside the

Fig. 24.8 Role of institutional management

affected community [15]. Allergens, bio-aerosols, epizootic and zoonotic diseases, and other experiment-associated infections are potential biological hazards residing in an animal facility along with laboratory animals that can turn a small incident into disaster [16].

Disaster management consists of plans or guidelines for organizing resources and key responsibilities, which can be adopted from disaster management agencies. Most people presume it to be the operations carried out after a disaster has happened. It also includes the set of processes planned to prevent and tackle a disaster, carried at the time or after an emergency condition to limit the ill effects to minimize the loss [17]. The World Organisation for Animal Health (OiE) has established strategies for disaster management that concern animal and veterinary public health. Guidelines of OiE use an integrated methodology for managing natural, man-made, and technical adversities, their interventions adopted by various stakeholders [18]. The Federal Emergency Management Agency (FEMA) is an important resource for emergency management information. It serves with a motto to decrease the loss of life, property, and safeguard the nation's infrastructure by following a comprehensive, risk-based, emergency management program [19].

Disaster plans for an animal facility could vary from place to place depending upon the nature of the facility and the volume and types of animals, staff requirements, weather, and regional conditions, i.e., located in the natural disaster-prone area, etc. Although it is quite challenging to develop a perfect disaster management plan that combats all kinds of emergencies and applicable to all the animal facilities, the disaster management agencies world over trail the following phases of disaster management to combat the adverse situation.

24.7 Different Phases of Disaster Management

(a) Prevention and mitigation } Pre-disaster preparation
(b) Preparedness }

(c) Response } Post-disaster action
(d) Recovery }

24.7.1 Prevention and Mitigation

Prevention includes activities that potentially prevent an emergency. However, not all emergencies and disasters can be prevented, but the associated risks can always be mitigated. Mitigation has been defined as "the lessening or minimizing of the adverse impacts of hazardous events" by the United Nations Office for Disaster Risk Reduction (UNDRR). According to the decision (EU), 2019/420, of the European Parliament and the Council, prevention is an action taken for decreasing the hazards

or mitigating significance of an anticipated disaster for people, animals, the environment, and property [20]. To prevent an adverse condition, an animal facility should address the following preventive measures.

24.7.1.1 Risk Assessment

Health and safety specialists with knowledge and training in relevant disciplines should be part of hazard assessment and the development of protocols to avoid these risks. The institutional occupational health and safety program (OHSP) should identify potential risks in the work environment and conduct a critical assessment of the associated risks. Plenty of information can be had from already published literature about the hazardous substance being worked upon. Professionals in the domain concerned may be consulted to analyze the hazardous risks. An effective OHSP ensures that the risks associated with the use of experimental animals are recognized and abridged to admissible levels [21].

Animal care facilities have biological, physical, and chemical hazards generated daily and in large quantities. Hazards should be classified based on the harm level on every prospective using scientific methods/facts.

24.7.2 Preparedness

European Parliament and of the Council (Decision (EU) 2019/420) defines preparedness as a state of commitment and capabilities of human and material means, procedures, societies, and establishments that empowers for taking advance actions to ensure effective, safe, rapid retort to adversity [20].

24.7.2.1 Categorizing Emergencies

Emergency management organizations like FEMA Define Emergencies is a recording method to help classify and list emergencies. Such classification will help in designing guidelines and policies for the effective management of the disaster. FEMA generally divides emergencies into three distinct groups [19]:

I. **Natural emergency.**
Generally, the most frequently occurring disasters are natural emergencies that include weather, seismic, and ocean-related events, e.g., earthquakes, floods, tornadoes, hurricanes, etc.

II. **Technical emergency.**
Mechanical and human errors result in technical emergencies and include HVAC problems, computer system errors, chemical spillages, and operational disaster fall under technical emergencies.

III. **Civil emergency.**
Events like terrorist attacks, vandalism and worker strikes, purposive human-oriented issues come under civil emergencies.

24.7.2.2 Developing Policies and Protocols

The policies, guidelines, and protocols are the central elements of a disaster management plan that is put into action during an emergency. These policies, guidelines, and protocols provide a general figure for steps to be taken in response to an emergency. Animal facility disaster management plan includes the development of standard operating procedures (SOPs) for different activities performed in facilities starting from entry-exit procedures during an emergency. It includes a floor plan of the facility, qualification, and validation of equipment. These SOPs are updated to their latest versions based on the documented operational data from a particular time. The development of such protocols assists personnel to identify available resources.

24.7.2.3 Displaying Contacts and Resources

A list of available contacts and resources should be included in the disaster plan that can be used during emergency events. Besides, a telephone and accident alert system should be placed in strategic locations such as a hallway or access corridors to enable communication by/with personnel trapped in the building for passing emergency alerts. A list of information about the suppliers of important services should also be on display who can be contacted during an emergency, e.g., suppliers for periodic refueling of backup generator fuel and criteria for contacting specific emergency personnel notification lists should be up to date. Appropriate items such as floor plan, names, and numbers of fire station, Red Cross, hospital, police station, etc. and weather condition information can also be displayed in this section.

The facility building should be equipped with fire alarm and firefighting equipment, a water sprinkler system, covering the entire work, and an animal housing area (no water sprinkler inside the animal room). The working staff should also be trained in using these systems. Also, the entry/exit passages and stairs should be equipped with a secured emergency power supply and fluorescent indicator tapes to aid emergency exit.

24.7.2.4 Building Up an Emergency Response Team

The greatest disaster plan is achieved by a team of personnel or team that would counter an emergency. This team should include individuals, experts from different expertise, including animal facility staff and researchers, and nominees from regulating bodies. The initial action of the response team should be to define its mission, goals, and modus operandi. The members of this group are required to have a provision of support from the administration for the strict execution of set action strategies and SOPs. Eventually, the facility disaster plans should be integrated with site-wise or local disaster plans. The creation of an emergency managing group is helpful and crucial for a rapid response. This team may have representation from personnel from the animal facility security, laboratory animal resources, the IAEC management or research administration, and governmental agencies.

24.7.2.5 Identifying Critical Systems and Obligations

A disaster management plan should direct plans to sustain and bear up the cost due to the loss of critically important things and systems and obligations in the facility (Fig. 24.9). To mitigate these losses, it is critical to meticulously specify all important animal facility-specific systems and obligations [22].

There are two general categories of critical systems and obligations:

1. Mechanical systems.
2. Personnel obligations.

24.7.2.6 Biocontainment of Potential Hazards

Biocontainment is the management of bio-risk associated with animal facilities. Facilities that offer animals and space to study (animal biosafety laboratories) hazardous microorganisms must ensure that the infectious agents do not spread among animals and must not escape the facility. Infected animals should be housed individually along with the safety consideration of the associated humans [23]. In an unlikely event, the housing and facility should be able to shield the outer world from potential hazards. All work involving infectious agents has to be done in designated biosafety levels (Class I–IV) which have been commissioned and validated by regulatory agencies [24]. The biomedical waste generated by such biosafety labs should be disposed of as per the guidelines on biomedical waste management [25, 26].

24.7.2.7 Training Staff and Testing Emergency Equipment

The disaster management exercise aims to increase the efficiency of staff and personnel of the facilities to improve preparedness and response at all levels beforehand, during, and post-disasters. The focus of disaster management training is normally on refining the practical skills of the staff and also on team management so that all components work together in a coordinated manner during an emergency. It is aimed to encourage the exchange of knowledge, experience, and development of networks amongst the disaster managing agencies. Training is provided to the personnel on fire drills and to the personnel involved in regular emergency evacuation testing of building facilities. These mock drills mimic real scenarios; it provides practical training and assesses our risk management plans and procedures. Likewise, equipment/instruments to be used in a critical emergency such as firefighting equipment should be examined and kept operational. The disaster plan should be made readily available to all personnel and members of the facility.

24.7.3 Response

United Nations Office for Disaster Risk Reduction (UNISDR) defines response as a mean of emergency duties and community support at the time or soon after a disaster to prevent loss of human and animal lives, health effects, ensure civic care, and meet the primary comestible requirements of the suffering humankind and animals [27].

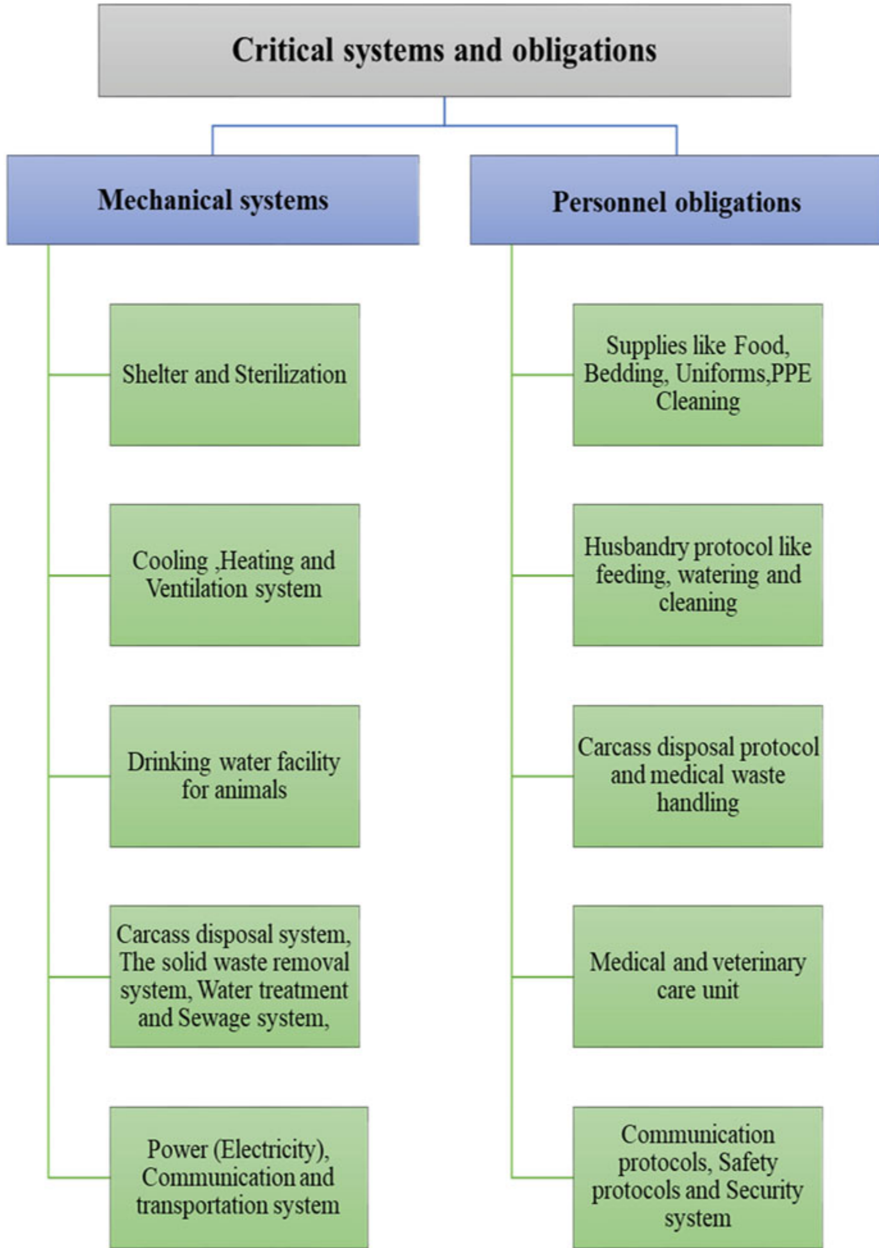


Fig. 24.9 Critical systems and obligations

Whatever the emergency or disaster condition may be, human life is the foremost priority. Situations may arise when not all the animals could be saved, and conditions may arise that necessitates euthanasia based on:

- Shortage of safe shelter, feed, water, and other resources.
- Injured or sick animals with bleak chances of recovery.
- Animals in an experiment that may not be carried forward.

24.7.4 Recovery

United Nations Office for Disaster Risk Reduction (UNISDR) defines recovery as the restoration and improvement of facilities, livelihoods, and living conditions of disaster-affected communities. It includes efforts made to reduce disaster risk factors and actions taken to return the situation to normal and safer than before. In the context of a vivarium, earliest attempts should focus on the rehabilitation of rescued animals. Injured animals must be given first aid to minimize pain and suffering. In case the housing is destroyed, the first and foremost necessity is to move the animals to safer places, with arrangements of food and water. It is quite possible, especially in large animals, that the symptoms of post-disaster trauma can cause distress and fear. Providing the animals certain environmental enrichment efforts could help in overcoming the stress and attaining normal behavior. Subsequently, breeding and research programs may be restored after going through the requirements of different stakeholders.

24.8 Summary

Occupational safety and health hazards and disaster management plans are an important aspect of any animal facility. The facility is an integral part of any research institute and is responsible for breeding, supply, and management of various species of animals. Trained technical staff proficient in animal handling practices, instrumentation, and housekeeping are essential for running these facilities. Working with animals has potential occupational health hazards in terms of work-related injuries, bites, infections, etc. Risk management should be the primary focus of the facility to protect the personnel and surroundings from these hazards. Potential hazards can be minimized by developing SOPs and following guidelines provided regarding the use of instruments, chemicals, and biomedical waste disposal. Guidelines should be followed strictly. Failure to do so could lead to disaster, affecting human lives and animal resources. The responsibility for occupational safety and health hazards and disaster management program lies with the head of the animal facility, and management of the facility must constitute a designated team of personnel from the animal facility, researchers, and institutional administration. The team should be mandated to develop standard operating procedures, to be followed for the safety of the staff

and the users of the facility. These SOPs must be monitored and reviewed at regular intervals.

References

1. Schmitt JM, Wilson DE, Raber JM (2018) Occupational safety and health. In: Weichbrod RH, Thompson GAH, Norton JN (eds) Management of animal care and use programs in research, education, and testing. CRC Press/Taylor & Francis, Boca Raton, FL, pp 279–318. https://doi.org/10.1201/9781315152189-14
2. Sharpe D (2009) Implementing a medical surveillance program for animal care staff. Lab Anim 38(8):275–278. https://doi.org/10.1038/laban0809-275
3. Huerkamp MJ, Mallon D, Percifield G (2018) Facility design, planning, and renovation, management of animal care and use programs in research, education, and testing. CRC Press/Taylor & Francis, Boca Raton, FL. https://doi.org/10.1201/9781315152189-18
4. Physical Hazards/Canadian Centre for Occupational Health and Safety (2020). Available at: https://www.ccohs.ca/topics/hazards/physical/#ctgt_1-3 (Accessed: 12 June 2020)
5. Datta P, Mohi GK, Chander J (2018) Biomedical waste management in India: critical appraisal. J Lab Phys 10(1):6–14
6. National Research Council (2011) Prudent practices in the laboratory, Prudent practices in the laboratory. National Academies Press
7. Ahn S, Jung SY, Lee SJ (2013) Gold nanoparticle contrast agents in advanced X-ray imaging technologies. Molecules 18(5):5858–5890. https://doi.org/10.3390/molecules18055858
8. National Research Council (1997) Occupational health and safety in the care and use of research animals. National Academies Press, Washington, DC
9. Thomann WR (2003) Chemical safety in animal care, use, and research. ILAR J 44(1):13–19
10. Alaimo RJ (2001) Handbook of chemical health and safety, ACS Handbooks. Oxford University Press, Oxford, New York
11. CDC (2001) Glutaraldehyde: occupational hazards in hospitals. https://doi.org/10.26616/NIOSHPUB2001115
12. Orhurhu VJ et al (2020) Ketamine toxicity. In: StatPearls. StatPearls Publishing, Treasure Island (FL). Available at: http://www.ncbi.nlm.nih.gov/books/NBK541087/
13. Zhang L et al (2009) Formaldehyde exposure and leukemia: a new meta-analysis and potential mechanisms. Mutat Res/Rev Mutat Res Elsevier 681(2–3):150–168
14. Eleazu CO et al (2013) Review of the mechanism of cell death resulting from streptozotocin challenge in experimental animals, its practical use and potential risk to humans. J Diab Metab Disord 12:60
15. The Disaster Management Act 2005, c. I (2005). India. Available at: https://ndma.gov.in/en/disaster.html.
16. Li XY et al (2016) The main biological hazards in animal biosafety level 2 facilities and strategies for control. Biomed Environ Sci Elsevier Ltd 29(4):300–304
17. Nikbakhsh E, Zanjirani Farahani R (2011) Humanitarian logistics planning in disaster relief operations. In: Logistics operations and management. Elsevier, Waltham, MA, pp 291–332
18. OiE (2016) Guidelines for National Veterinary Services. Available at: https://www.oie.int/doc/ged/D8801.PDF.
19. FEMA (2004) An in-depth guide to citizen preparedness are you ready? Available at: www.fema.gov (Accessed: 29 June 2020).
20. Official Journal of the European Union (2019) Decision (EU) 2019/420 of the European Parliament and of the Council of 13 March 2019 amending Decision no 1313/2013/EU on a Union Civil Protection Mechanism. Off J Eur Union L77:I/1–I/14
21. Goodly LJ et al (2016) Developing a comprehensive animal care occupational health and safety program at a land-grant institution. J Am Assoc Lab Anim Sci 55(1):50–57

22. Suckow MA, Lamberti GA (2017) Institutional animal care and use committee, principles of animal research for graduate and undergraduate students. Elsevier. https://doi.org/10.1016/B978-0-12-802151-4.00004-9.
23. Bayne K, Turner PV (2013) Laboratory animal welfare, laboratory animal welfare. Academic Press. https://doi.org/10.1016/C2010-0-66027-5.
24. Frieden, T. R. et al. (2011) Morbidity and Mortality Weekly Report Guidelines for Biosafety Laboratory Competency CDC and the Association of Public Health Laboratories Centers for Disease Control and Prevention MMWR Editorial and Production Staff MMWR Editorial Board
25. The Bio-Medical Waste Management (Amendment) Rules-2018 (2018). India. Available at: https://cpcb.nic.in/rules-3/.
26. The Bio-Medical Waste Management Rules-2016, s. 8 (2016). India. Available at: https://cpcb.nic.in/rules-3/.
27. UNISDR (2007) I S D R International Strategy for Disaster Reduction National Platforms for Disaster Risk Reduction. Available at: www.unisdr.org

Preclinical Imaging for Laboratory Rodents 25

Pradip Chaudhari

Abstract

Laboratory animals play an important role in biomedical research and act as a backbone of new drug research and development. Biomedical research is primarily dependent on laboratory rodents. There is a steady shift of therapeutic strategies for diseases from physiological to cellular and presently molecular targets. There is a huge advancement in personalized medicine and ways of drug research in the recent past that is primarily due to the implementation of 3Rs in animal study design and looking into molecular insights of the disease processes with the help of precise tools such as noninvasive imaging. Imaging technologies proved extremely important in diagnosis and evaluating human diseases in clinics. Besides, it has an important role in biomedical research in oncology and other research areas including stem cell research, bone metabolism, endocrinology, neurology, cardiology, and nanotechnology. The complex pathways involved in the initiation and progression of the disease can be visualized and quantitatively monitored using noninvasive imaging technologies. The strong preclinical data is the backbone of biomedical research, and new drug development and imaging technologies provide relevant accurate information. This chapter is focused on the dedicated noninvasive preclinical imaging modalities for their present role in laboratory animal science. This chapter also deals with protocols for positron emission tomography (PET), single-photon emission computed tomography (SPECT), and X-ray computed tomography (CT) for rodent imaging.

P. Chaudhari (✉)
Small Animal Imaging Facility, Animal Oncology Gr., Advanced Centre for Treatment, Research and Education in Cancer (ACTREC), Tata Memorial Centre, Navi Mumbai, Maharashtra, India

Homi Bhabha National Institute, Anushaktinagar, Mumbai, Maharashtra, India
e-mail: pchaudhari@actrec.gov.in

© The Author(s), under exclusive license to Springer Nature Singapore Pte Ltd. 2021

P. Nagarajan et al. (eds.), *Essentials of Laboratory Animal Science: Principles and Practices*, https://doi.org/10.1007/978-981-16-0987-9_25

Keywords

Preclinical · Noninvasive imaging · Laboratory rodents · Animal model

25.1 Introduction

Preclinical noninvasive imaging is looking into internal organ structures, their functions, and molecular events happening at the cellular level in live laboratory animals for biomedical research purposes. The milestones in the history of biomedical science go hand-in-hand with advances in technologies to perform research [1]. Preclinical imaging has appeared as an exceptional tool in the drug discovery process [2]. The discovery of X-rays by German physicist Wilhelm Rontgen in 1895 introduced imaging in human health, and this event has paved the way to the world of noninvasive imaging. In years to come immediately after the discovery of X-ray, it became a primary investigation in disease diagnosis and treatment monitoring, and to date, it is still an important primary screening tool [3, 4].

The drug discovery process for the treatment of various human and animal diseases furthers their way to a commercial utility which includes multiple milestones such as drug target identification and its validation and optimization and then its assessment for therapeutic efficacy in the most appropriate animal model system.

The promising molecules need to be evaluated on a fast track as in current high-throughput assays, this process of novel drug discovery is expedited with quantitative and accurate tools of translation. In the recent past, molecular imaging of rodents has been incorporated as the most accurate tool to lessen the time of the new drug discovery process. In general, preclinical imaging systems for rodents can be classified primarily as anatomical, physiological, and molecular modalities (Fig. 25.1).

The basic understanding of cancer initiation, progression, and metastasis has played an important role in deciphering several molecular and pathophysiological processes and designing potential targets for cancer diagnosis and treatment. The visualization of intricate pathways plays a significant role in identifying the key target molecules to develop therapeutic strategies.

Drug discovery is a long multistep process to transform a new potential candidate molecule into a final drug for human clinical application (Fig. 25.2).

This process involves multiple disciplines such as in silico target evaluation for affinity and toxicity, biochemical evaluations, pharmacological testing for kinetics and dynamic characterization, and after in vitro assays detailed study for efficacy and in vivo target affinity. The in vitro studies with cancer cell lines are the basis for target validation and lead optimization, but these studies have limitations as they do not mimic the complex biological environment in living mammals [5, 6]. Furthermore, the drug distribution and metabolism cannot be evaluated in isolated cell line studies, which are important parameters to take the molecules ahead for the clinical application stage [7]. Hence incorporation of an appropriate biological system/model

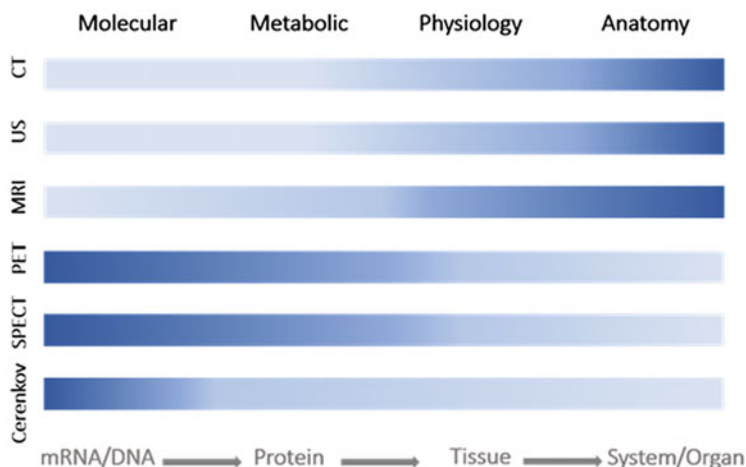


Fig. 25.1 Preclinical imaging modalities with categorization on the basis of abilities to visualize from structural to functional and molecular processes. These modalities are described in the text (*CT* computed tomography, *US* ultrasound, *MRI* magnetic resonance imaging, *PET* positron emission tomography, *SPECT* single-photon emission computed tomography; Cerenkov; *OI* optical imaging consisting bioluminescence and fluorescence imaging)

is the way forward to prevent drug failures in later stages. The shortcoming of in vitro evaluation is answered using a suitable animal model system. The tools for accurate visualization and quantitation of the disease processes have been considered to bridge the gap between in vitro studies and clinical trials.

The ability of noninvasive preclinical imaging in deciphering molecular information in cancer initiation, progression, and assessment of response to the treatment of candidate drugs has made this technology one of the primary tools in drug discovery research [8]. This concept of preclinical imaging and its technology has grown to higher levels in a short period because of its ability in translating ideas, ligands, and small molecules to the bedside.

25.2 Preclinical Imaging Techniques

Preclinical noninvasive diagnostic imaging includes several modalities that are important in visualization and monitoring/assessment of molecular mechanisms in disease processes. The most commonly used modalities are X-ray computed tomography (CT), positron emission tomography (PET), single-photon emission computed tomography (SPECT), etc. There are several applications of these technologies in biomedical research involving laboratory animals and human disease diagnosis [9–12]. Bioluminescence and fluorescence imaging are currently confined to animal subjects and being tested for their clinical utility [13, 14]. The information received by imaging procedures are supportive to each other and improve target/lesion

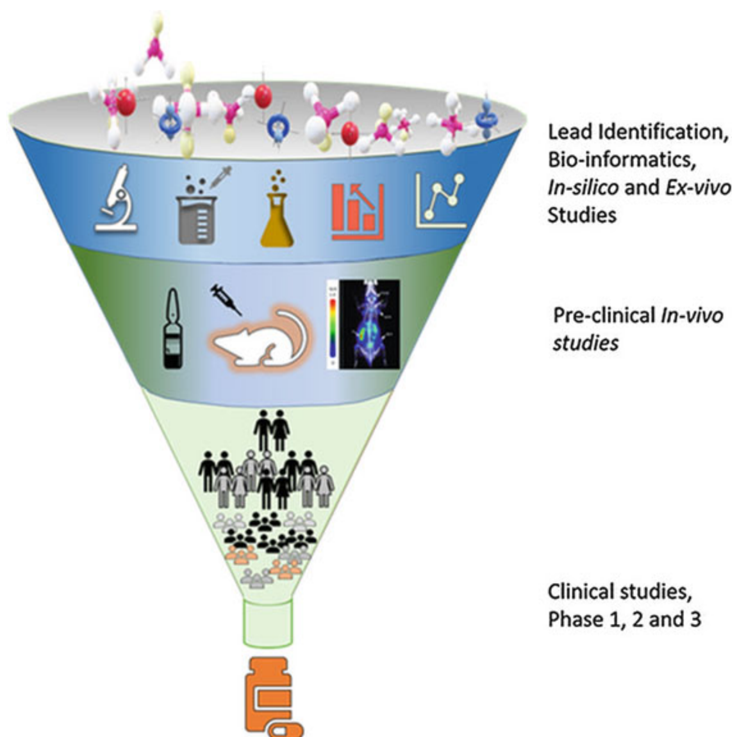


Fig. 25.2 Drug discovery process depicting steps such as lead identification, bioinformatics studies, in vitro assays, in vivo evaluation in laboratory animals and clinical trials in human subjects. This representation indicates that a number of potential leads enter at the beginning and few get into the clinical practice indicating resource involvement in the process

localization and allow morphological measurement and assessment of intricate biochemical, physiological, and molecular processes.

In this era of imaging, multiple modalities are used either stand-alone or in combination as per research requirements. Hybrid or fusion imaging is preferred over a single modality due to the advantage of combining multiple aspects of the disease processes in a single framework. Hybrid imaging makes it easy to visualize molecular/biochemical/functional processes over a morphological platform.

The hybrid imaging technique requires precise seamless co-registration of imaging data and compatibility of these technologies to fuse the acquired data. The common hybrid imaging combinations are microPET-CT and microSPECT-CT; this enhances understanding of obtained molecular/functional/biochemical data as it is read on the morphological background. This significantly improves information of the underlying pathophysiological and molecular processes [15]. A large amount of data is obtained noninvasively without any pharmacological response or discomfort to the animals under investigation. The radiotracer, radiopharmaceutical, or contrast agents are administered in very small quantities which do not cause any

pharmacologic signals impacting organ physiology or adverse drug reaction. These procedures are painless for animals and performed under general anesthesia to immobilize the animal during the image acquisition process, which reduces artifacts created by movements often called motion artifacts and gives high-resolution data with high reproducibility. These procedures are performed under general anesthesia which helps to reduce handling stress and pain in disease models. The images are obtained with the resolution to the submillimeter scale.

The new generation preclinical scanners make use of high sensitivity detector system and gives output with very high-image-resolution data. This detector assembly has efficient signal amplification and its further transmission without losses to the output devices. The dedicated algorithm processes and analyzes these signals to form high-resolution images.

The anticancer drug discovery has incorporated noninvasive preclinical imaging due to its ability to quantitatively validate cancer models and its drug test efficacy in the same animal over a prescribed time period. The preclinical in vivo imaging studies obtain longitudinal data of the animal model, hence reduces the number of control animals in the study design. This factor also decreases variability in the study and increases precision of the outcome. The most commonly used tracer/radiopharmaceuticals used in PET, SPECT, and CT are listed in Table 25.1. The detailed characteristics of major imaging technologies are listed in Table 25.2.

The choice of an imaging procedure, radiopharmaceuticals, radiotracer, and contrast agent depends upon the organ or target system/cellular mechanism to be monitored. The imaging procedures and radiopharmaceuticals/contrast agents/tracers used in preclinical setup are similar to clinical scenarios; hence, there is a smooth, accurate, and reliable transition of preclinical information to the human application. The present generation rodent scanners use similar image acquisition protocols as clinical ones. These protocols are routinely used in the validation of animal models of human diseases. That's the reason there is increasing interest seen in preclinical imaging to make the drug discovery process faster and dependable. The drug discovery process is resource-intensive, time-consuming, costly, and not certain, particularly in late clinical evaluation phases. Hence to bring down the attrition rate of new potential molecules during later stages of human clinical trials, advanced translational preclinical imaging technologies are incorporated during the process of preclinical evaluation. These translational technologies also assist in the prediction and understanding of the advantages/drawbacks while utilizing animal models in drug discovery. The common translational imaging modalities are described below.

25.3 Positron Emission Tomography (PET)

Positron emission tomography is a nuclear molecular imaging procedure that allows evaluating the distribution and target affinity of the molecules/probes labeled with positron-emitting radioisotopes. It provides noninvasively spatial and temporal distribution in an animal model system. PET has become a popular and essential

Table 25.1 The most commonly used tracer/radioisotope/contrast material in major imaging technologies

Imaging modality	Tracer/radioisotope/contrast material	References
PET radioisotopes	Fluorine-18	[18]
	Carbon-11	[55]
	Gallium-68	[56]
	Iodine-124	[20]
	Rubidium-82	[57]
	Copper-64/62	[21]
	Nitrogen-13	[22]
	Oxygen-15	[58]
	Carbon-11	[59]
SPECT radioisotopes	Technetium-99 m	[60]
	Thallium-8	[61]
	Iodine-131/125	[62]
		[63]
Contrast agents	Iodinated compounds	[64]
	Nanoparticles	[65]
		[66]
Bioluminescent markers/reporters	Luciferins	[67]
	Pro-luciferins	[68]
		[69]
		[70]
		[71, 72]

tool for preclinical assessment in the last three decades because of its precision and sensitivity. PET has immense ability to obtain molecular information of the disease process, which is important for target identification and designing strategies for specific targeting, which directly enhances translation of the basic biological information. The usefulness of PET has increased to understand underlying cellular and molecular events inducing disease processes and its progression [10]. PET has higher sensitivity in comparison with other nuclear imaging technologies, besides it quantitates precisely biological and biochemical processes in vivo [12, 16]. PET technology has greatly influenced human clinical practice, since its introduction in the mid-1970s. Considering the impact of this technology on human disease management, efforts have started to explore this technology in drug discovery. This has resulted in developing preclinical scanners to image a mouse like a man. Over two decades there is a huge advancement in technology used in animal imaging scanners. The new generation scanners are moved from analog to a completely digital interface with either single or multimodality hybrid version equipped with solid-state advanced detector materials [17].

Table 25.2 Overview of the characteristics of imaging techniques

Imaging modality	Resolution (spatial)	Basis	Advantage	Disadvantage
Computed tomography	15–100μm (preclinical) 5–7 mm (clinical)	Ionizing radiation	Accurate visualization of structural differences Possibility of whole-body imaging	Limited ability to visualize soft tissue lesions High radiation exposure
Single-photon emission tomography (SPECT)	0.5–2 mm (preclinical) 8–10 mm (clinical)	Ionizing radiation	Functional/ physiological measurement Possibility of whole-body imaging	Availability of radioisotopes, low spatial resolution, and radiation exposure
Positron emission tomography (PET)	1–2 mm (preclinical) 5–7 mm (clinical)	Ionizing radiation	Metabolic and molecular processes measurement Possibility of whole-body imaging	Availability of radioisotopes, radiation exposure
Magnetic resonance imaging	25–100μm (preclinical) 2–5 mm (clinical)	Magnetism	Accurate imaging of soft tissue	Expensive Suitable for only soft tissue No whole-body imaging
Optical imaging	2–3 mm (preclinical)	Light	Measurement of activity with time	Limited applicability No whole-body imaging
Ultrasound imaging	~1 mm (preclinical)	Sound waves	Soft tissue and blood flow assessments	Limited applicability No whole-body imaging
Cerenkov imaging	0.5–0.7 mm (preclinical)	Cerenkov light	Molecular processes measurement	Limited applicability Still in research and development state

25.3.1 Biomedical Research Applications

Biomedical research is based on animal model systems for various human diseases. The radiopharmaceuticals targeted for the specific disease process, physiology, or organ system are often used to validate the mouse models of human diseases. Similarly, in drug discovery research, evaluation of the biodistribution pattern of radiolabeled drug or complex is used to know and understand the target affinity and pharmacokinetics of a new drug entity.

Imaging and biodistribution are two major applications used in in vivo studies to evaluate the distribution pattern of radiolabeled drugs/molecules in biomedical research. During these studies, tracking the path of radiotracer is very crucial as it provides the opportunity to study the animal as a whole from the perspective of

tracer uptake and in vivo dynamics such as metabolism and excretion in a complex multi-organ scenario. The radiolabeled complexes are evaluated in vivo in living animals after intravenous administration. To check the distribution of the tracer elements conventionally, these animals are euthanized and measured real-time uptake in the organs of choice. On the contrary, imaging utilizes the same animals throughout the experimental period without any need to sacrifice them which improves research data quality and animal ethics and welfare.

^{18}F is the most popular PET tracer due to its relatively long half-life for synthesis reaction and imaging procedures; hence, ^{18}F-based tracers have become practically relevant [16, 18]. Other long-lived PET radioisotopes are ^{124}I and ^{64}Cu with a half-life of 4.2 days and 12.7 h, respectively [19–21]. The feasibility of shipping these radionuclides from the production site to preclinical labs is easy. These radionuclides are suitable for metabolic and physiological clearance and organ or lesion uptake studies, where tracers are tracked over an extended period. In addition, generators consisting of long-lived parent radionuclide (^{62}Zn/^{62}Cu and ^{68}Ge/^{68}Ga generator) and short-lived daughter radionuclides (^{62}Cu, $T1/2 = 9.7$ min; ^{68}Ga, $T1/2 = 68$ min) are more convenient for preclinical facilities as one can procure generators as per research requirement of the laboratory [22]. Hence the cost-effective option of the generator system boosts preclinical research than on-site preclinical research cyclotrons. The amount of a radiotracer injected to get interpretable image data depends on the specific activity of the radionuclide. The specific activity refers to the amount of radioactivity per unit mass of the radionuclide. Typically, a dose of tracer injection for laboratory rodents is in the range of 1.85–37 MBq. The consideration of radiation exposure to vital organs is an important criteria while selecting the dosage in humans; however, in animals, obtaining quality image data is of primary importance [23]. In rodents, the volume of administration is important considering the total blood volume. Extensive literature is already existing on the use of PET tracers in various laboratory animals from rodents up to primates. The primary research fields with the applicability of PET are oncology, neurology, cardiology, regenerative medicine, etc. The commonly used tracers in preclinical labs and human bioimaging labs are listed in Table 25.3.

25.3.2 PET Instrumentation

The basis of PET technology is the unique decay characteristics of positron-emitting radioisotopes. During emission of a positron (the antiparticle to the electron with the same mass but with an opposite electric charge) from the nucleus occurs, it travels a short distance (typically a few tenths of a millimeter), and then it undergoes an annihilation producing two gamma photons of 511 keV in equal and opposite directions as depicted in Fig. 25.3. Present advanced microPET scanner has one-to-one coupled Phoswich-Avalanche Photodiode (APD) detector, which is optically sealed with high-sensitivity scintillation crystals having nanosecond decay time. This detector assembly gives high spatial resolution, and the energy resolution is enhanced with advanced APD coupling to the scintillator. This scintillation detector

Table 25.3 PET tracers for molecular imaging in oncology in the preclinical and clinical setup

PET radionuclide	Half-life	PET radiotracer (molecule)	Molecular mechanism of tumor uptake	Application
F[18]	110 m	F[18]-fluorodeoxyglucose	FDG is a glucose analogue transported from the blood into cells by glucose transporters (predominantly GLUT1)	Tumor glucose metabolism imaging
		F[18]-fluorothymidine (FLT)	Fluorothymidine is an analogue of the nucleoside thymidine (deoxythymidine) and substrate for thymidine kinase I (TK1), which is transported from the blood into cells by active transport	Tumor cell proliferation imaging
		F[18]-Fluoromisonidazole (FMISO)	[F18] FMISO is reduced to RNO2 radicals and binds selectively to macromolecules within hypoxic cells	Tumor hypoxia imaging
		F[18]-Fluoroazomycin arabinoside	Nitroimidazoles are reduced and bind selectively to macromolecules within hypoxic cells	Tumor hypoxia imaging
		F[18]-Fluoroethyl-l-tyrosine (FET)	Amino acid transport system	Tumor proliferation and protein synthesis
		F[18]-Fluoroestradiol	Binding with overexpressed hormone receptors	Breast tumor imaging
		F[18]-Sodium fluoride (NaF)	Incorporation of fluoride ions in the bone matrix to form crystals of fluorapatite	Bone metastasis imaging
C[11]	20.4 m	C[11]-Methionine (MET)	Amino acid transport system	Tumor protein synthesis
		C[11]-Choline	Cell membrane synthesis targeting related to upregulation of choline kinase associated with cancer	Prostate cancer and brain tumor imaging
Ga[68]	68.1 h	Ga[68]-DOTATATE	Binding to somatostatin receptors (SSTRs)	Neuroendocrine tumor imaging
		Ga[68]-Prostate-specific membrane antigen (PSMA)	Binding to PSMA, which is membrane glycoprotein overexpressed in prostate cancers, disease recurrence	Prostate cancer imaging
Cu[64]	12.7 h	Cu[64]-Diacetyl-bis (N-methylthiosemicarbazone) [ATSM]	Non-nitroimidazole compound binding to hypoxic cells	Tumor hypoxia imaging

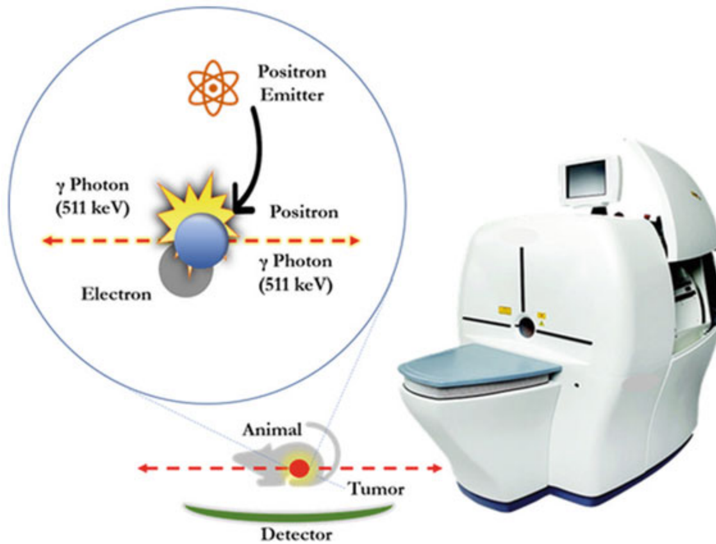


Fig. 25.3 Schematic of the preclinical PET/SPECT-CT trimodality scanner along with depiction of an annihilation reaction occurring as a resultant of decay process of positron emitting radionuclide. The emitted positrons interact with electrons in the surrounding tissues and give rise two photons of 511 keV in equal and opposite direction

assembly efficiently attenuates 511 keV gamma-ray photons with minimum signal losses. The challenge in designing preclinical experiments is mainly due to the short half-life of PET radioisotopes, and hence animal imaging time point's selection needs to be optimized as per physical and biological decay of the radiotracer.

PET provides information on both spatial and temporal distribution of the radiotracer in a single study [24]. PET data can be further analyzed by applying tracer kinetic models to get the time-activity curves [25]. In routine study proposals, image-based percentage injected dose data is required, but few studies need tracer kinetic modeling to get dynamic information about the administered tracer [26]. The dedicated scanners for rodent imaging have gone through vast development during the last two decades with several advances in detector technology and simplifying the complex scanner designs. The first dedicated animal PET scanner was designed for brain imaging in nonhuman primates [27, 28]. This was the start of the exciting journey of a dedicated animal scanner, which now reached a stage of multimodality scanners exclusive for laboratory rodents [29].

25.3.3 Selected PET Protocol

25.3.3.1 Protocol: I (Fig. 25.4)

- *Aim:* To study the biodistribution pattern and uptake of F [18] FDG distribution in normal and tumor-bearing mice [30].

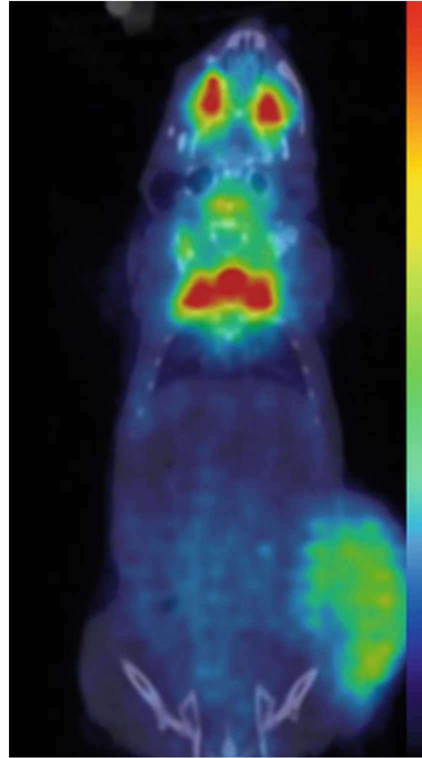


Fig. 25.4 NOD SCID mouse bearing MCF-7 xenograft administered with 18F-FDG (18.5 MBq, intravenously 1.5 h prior to scan) showing uptake in tumor (arrow)

- *Materials:* Male/female NOD SCID/nude mice aged between 6 and 8 weeks subcutaneous xenograft tumor-bearing are required for the study.
- *Radiopharmaceutical:* F [18] Fluorodeoxyglucose [FDG]—of concentration 18.5 MBq/0.5 ml.
- Anesthesia: Mice need to be immobilized before the administration of the radiotracer. The whole procedure is performed under inhalation anesthesia using isoflurane and oxygen in anesthesia induction chamber.
- *Procedure:* PET imaging and data analysis steps.

  Overnight fasting of the animals is required before the PET scan for optimum image acquisition.

  A caliper is used to measure the tumor volume of the tumors if it is palpable before the scanning for data interpretation and further correlation.

  The F [18] FDG dose administered is in the range of 4–18.5 MBq and is injected through the tail vein of the animal. The whole procedure is performed under general anesthesia.

  Pre- and post-injection radioactivity in the syringe needs to be measured in a dose calibrator to estimate the exact dose administered.

A heating pad is used to revive the animals before transferring to the IVC cage. The animals are to be taken for imaging. In the case of FDG imaging, scanning is performed 1.5 h post-injection.

- Further, the raw images are required to be reconstructed using a dedicated software running the MLEM-2D reconstruction algorithm. Reconstructed images are required to be visualized and analyzed in dedicated software for quantitation.

25.4 Single-Photon Emission Computed Tomography (SPECT)

microSPECT is relatively an older technique compared to PET, which underwent significant advancement in the past few years. The most important advantage of this technology over PET is the easy availability of a wide range of radiopharmaceuticals targeting various organ systems. The radiopharmaceuticals used in human nuclear medicine centers are useful for rodent imaging. SPECT utilizes gamma emitters, ^{99m}Tc (technetium), which is the most common radionuclide. Technetium-99m is readily available on tap due to efficient, inexpensive ^{99}Mo (molybdenum)–^{99m}Tc (technetium) generator system. ^{99m}Tc can be repeatedly eluted from the generator system. Technetium-99 m has 6 h short half-life with versatile chemistry useful for radiolabeling which is an added advantage for SPECT applications in biomedical research. The present advanced detector technologies allow the use of multi-isotope imaging probes concurrently giving insights into disease processes in a single study [11]. The other SPECT radioisotopes of preclinical research potential are thallium-201 (201Tl), indium-111 (^{111}In), gallium-68 (^{68}Ga), iodine-123/125 ($^{123/125}$I), tin-117 m (^{117m}Sn), lutetium-117 (^{117}Lu), etc. [31–37].

SPECT is a three-dimensional imaging that gives functional qualitative and quantitative data. There is a wide range of SPECT isotope-labeled complexes suitable for specific targeting of physiological and molecular processes observed in the initiation, progression, and response monitoring. The quantitative kinetic information concerning time can be obtained using these tracers, which is valuable in terms of organ function. The commonly used technetium-99 m radiopharmaceuticals in research and clinical practice are listed in Table 25.4.

25.4.1 SPECT Instrumentation

The main detector system of SPECT scanner is gamma-ray detection assembly which provides a distribution pattern of radiotracer for the position, intensity, and time, noninvasively. This detector registers signals based on radionuclide energy; the signal is amplified and converted to digital signals. There are X, Y, and Z signals representing the point of origin of the signal (X and Y coordinates), and Z represents its intensity. The dedicated data acquisition, processing, and analysis algorithms enable visualization and quantitation of acquired data. SPECT preclinical instrumentation has improved significantly in terms of sensitivity, resolution, and analytical abilities. The first-generation gamma scanners were having a small FOV (field of

Table 25.4 Common technetium-99 m radiopharmaceuticals for diagnostic imaging studies

Radiopharmaceutical	Mechanism of localization	Application
^{99m}Tc-MDP (methylene diphosphonate)	Physical adsorption on hydroxyapatite matrix	Bone imaging
^{99m}Tc-DIPA (diethylenetriaminepentaacetic acid)	Renal glomerular filtration	Renal functional imaging
^{99m}Tc- MAG3 (mercaptoacetyltriglycine)	Tubular secretion	Renal functional imaging
^{99m}Tc(III)-DMSA (dimercaptosuccinic acid)	Binding to renal parenchyma	Renal scan
^{99m}Tc(V)-DMSA (dimercaptosuccinic acid)	Tumor cell uptake	Medullary carcinoma of thyroid
^{99m}Tc-Mebrofenin	Hepatobiliary excretion	Hepatobiliary imaging
^{99m}Tc-Sulfur colloid	Phagocytosis	Liver, spleen, and bone marrow imaging
^{99m}Tc-MAA (macro-aggregated albumin)	Capillary blockade	Lung perfusion
^{99m}Tc-DTPA (diethylenetriaminepentaacetic acid) aerosol	Sedimentation in bronchioles	Lung ventilation
^{99m}Tc-Pertechnetate	Thyroid function (trapping)	Thyroid imaging

view), low sensitivity, and low resolution as compared to currently available scanners. The presently available scanners have a solid-state CZT (cadmium zinc telluride) detector having improved energy resolution. The detector is placed on a common gantry, for SPECT and CT in dual-modality systems. Recent scanners have dockable SPECT and CT assembly for optimal output of single modality independently. Present systems have universal animal imaging beds allowing its use with multiple scanners of different modalities. In the case of SPECT technology, collimator is required to collimate gamma rays for improvement of image resolution. The major collimators available for SPECT scanners are high-resolution parallel hole (HRPH) and single- and multi-pinhole low-energy collimator, which are useful for imaging animals administered with different radioisotopes. Currently available preclinical scanners have a resolution to the level of 0.5 mm after data reconstruction.

25.4.2 Selected SPECT Protocols

25.4.2.1 Protocol: I (Fig. 25.5)

- *Aim:* To study the functional assessment of the renal system [38].
- *Materials:* Mouse (Swiss albino) male/female, aged 6–8 weeks, and weigh around 20–25 gm.
- *Radiopharmaceutical:* ^{99m}Tc-DTPA (^{99m}Tc-diethylenetriaminepentaacetic acid).
- *Method:*

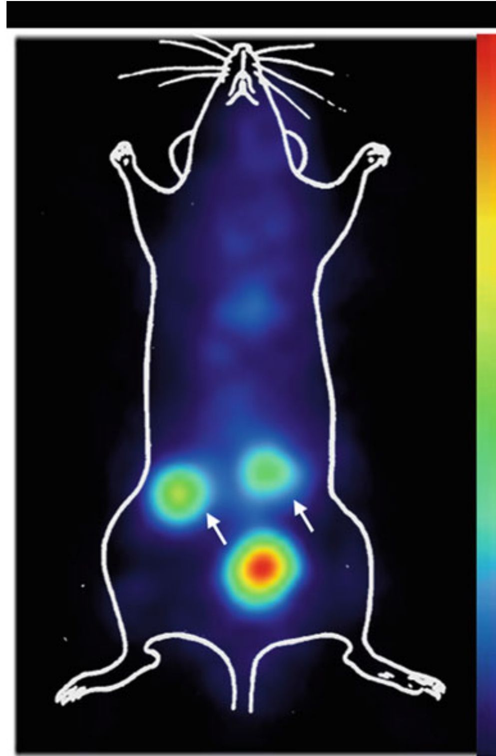


Fig. 25.5 99mTc-DTPA SPECT dynamic scan and clearance pattern in kidneys (arrow). The 5.5 MBq was injected intravenously via tail vein using P10 catheter. Dynamic images were acquired at the rate of one frame per 6 s for 30 min

Preparation of the injection:

All the steps are to be performed strictly under aseptic conditions.

Initially, an amount of 5 ml of the sodium pertechnetate (^{99m}Tc) solution of radioactivity ~200 MBq is added to the cold kit vial content to obtain a final concentration of radioactivity.

The sterile eluate from the technetium (^{99m}Tc) generator, if necessary, is diluted with sodium chloride sterile isotonic solution (0.9% saline).

The vial content shall be mixed thoroughly and incubated for 15 min at room temperature.

The ^{99m}Tc-DTPA must then be ready to be administered within 5 h from the time of preparation.

- *Anesthesia:* The animals need to be immobilized before the administration of the radiotracer. The whole procedure is performed under inhalation anesthesia using isoflurane and oxygen in anesthesia induction chamber.
- *Procedure:* SPECT imaging and data analysis steps.
- An intravenous injection dosed with 5–7.5 MBq of activity of ^{99m}Tc-DTPA through the tail vein to immobilize mice under isoflurane anesthesia.
- Pre- and post-injection radioactivity of the syringe was measured to calculate the dose.

- The animals can be taken for imaging immediately after the injection for 30 min. Images acquired at the rate of one frame per 6 s.
- The animal is positioned in a way so that the SPECT can acquire a whole-body scan.
- The acquired imaging data will be reconstructed using the standard filter back-projection reconstruction method. Reconstructed images will be visualized in dedicated software for image analysis and quantitation.

25.4.2.2 Protocol: II (Fig. 25.6)

- *Aim:* To study the functional assessment of the skeletal system [39].
- Materials: Mouse (Swiss albino) male/female, aged 6–8 weeks, and weigh around 20–25 g.
- *Radiopharmaceutical:* ^{99m}Tc-MDP (methylene diphosphonate).
- *Method:*

  Preparation of the injection:

  All the steps are to be performed strictly under aseptic conditions.

  Initially, an amount of 5 mL of sodium pertechnetate (^{99m}Tc) solution with a maximum activity of ~200 MBq is added to the content of vial content.

  The eluants from the technetium (^{99m}Tc) generator, if necessary, is diluted with sodium chloride sterile isotonic solution (0.9% saline) to achieve target activity for further use.

  The pH value of the solution needs to be in the range between 5.0 and 7.0.

  The vial content should be mixed thoroughly and incubate for 10 min at room temperature.

  The ^{99m}Tc-MDP must then be ready to be administered within 6 h from the time of preparation.

  The (^{99m}Tc)-MDP complex formation crucially relies on the presence of Sn^{2+} ions. Therefore, the sodium pertechnetate solution should be free from any oxidizing agents.
- *Anesthesia:* The animals need to be immobilized before the administration of the radiotracer. The whole procedure is performed under inhalation anesthesia using isoflurane and oxygen in anesthesia induction chamber.
- *Procedure:* SPECT imaging and data analysis steps.
- An injection dosed with 5–7.5 MBq of activity of ^{99m}Tc-MDP is administered through the tail vein to immobilize mice under isoflurane anesthesia.

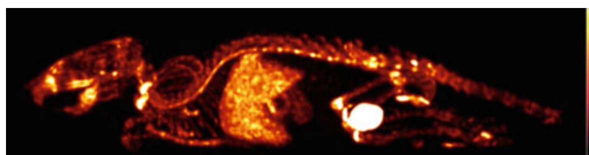


Fig. 25.6 99mTc HDP whole-body bone scan in mouse (94.35 MBq administered intravenously 5 h prior scan). (Acknowledgment: Molecubes, Belgium)

- Pre- and post-injection radioactivity of the syringe was measured to calculate the dose.
- The animals can be taken for imaging immediately after the injection for 30 min.
- The animal is positioned in a way so that the SPECT can acquire a whole-body scan.
- The acquired imaging data will be reconstructed using the standard filter back-projection reconstruction method. Reconstructed images will be visualized in dedicated software for image analysis and quantitation.

25.4.2.3 Protocol: III (Fig. 25.7)

- *Aim:* To study the functional assessment of the hepatobiliary system [38, 40].
- *Materials:* Mouse (Swiss albino) male/female, aged 6–8 weeks, and weigh around 20–25 g.
- *Radiopharmaceutical:* ^{99m}Tc-mebrofenin.

  Method:

    Preparation of the injection:

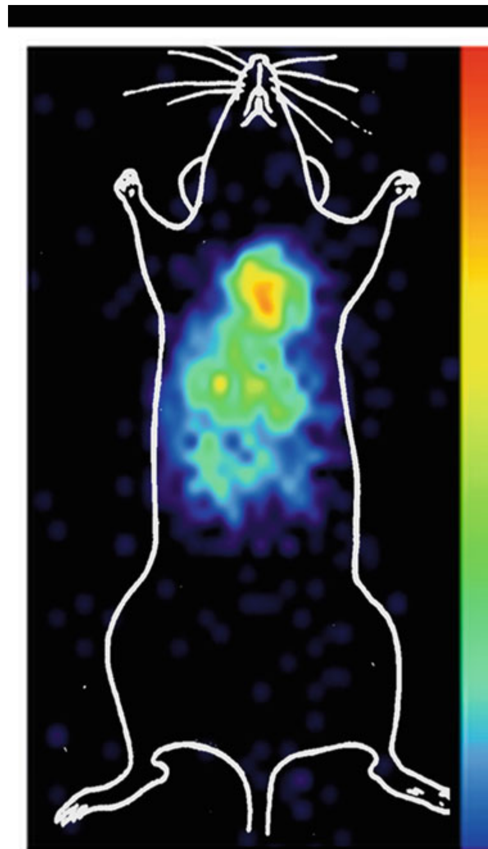


Fig. 25.7 99mTc-Mebrofenin SPECT dynamic scan and clearance pattern in the liver. The 5.5 MBq was injected intravenously via tail vein using P10 catheter. Dynamic images were acquired at the rate of one frame per 6 s for 30 min

- All the steps are to be performed strictly under aseptic conditions.
- Initially, an amount of 5 ml of the sodium pertechnetate (^{99m}Tc) solution of radioactivity ~200 MBq is added to the cold kit vial content to obtain the final target radioactivity.
- The sterile eluate from the technetium (^{99m}Tc) generator, if necessary, is diluted with sodium chloride sterile isotonic solution (0.9% saline).
- The vial content shall be mixed thoroughly and incubated for 15 min at room temperature.
- The ^{99m}Tc-mebrofenin then is ready to be administered within 5 h from the time of preparation.
- *Anesthesia:* The animals need to be immobilized before the administration of the radiotracer. The whole procedure is performed under inhalation anesthesia using isoflurane and oxygen in the anesthesia induction chamber.
- *Procedure:* SPECT imaging and data analysis steps.
- An injection dosed with 5–7.5 MBq of activity of ^{99m}Tc-mebrofenin is administered through the tail vein to the immobilized mice under general anesthesia.
- Pre- and post-injection radioactivity of the syringe was measured to calculate the dose.
- The animals can be taken for imaging immediately after the injection for 30 min, and dynamic images were acquired at the frame rate of one frame per 6 s.
- The animal is positioned in a way so that the SPECT can acquire a whole-body scan.
- The acquired imaging data will be reconstructed using the standard filter back-projection reconstruction method. Reconstructed images will be visualized in dedicated software for image analysis and quantitation.

25.5 Computed Tomography (CT)

The microCT is the most popular high-resolution imaging modality to image animals and objects. Its usefulness in the localization of lesions in hybrid scanners is indispensable. There is significant technological advancements in system resolution which gives precise morphological details [9, 41]. It is most useful for imaging high-density structures like bones in in vivo and ex vivo settings. Imaging of soft tissue structures and vasculature is feasible due to the availability of organ-specific contrast agents for rodents. Lesion localization in PET, SPECT, MRI, and optical image data is precisely achieved using CT image overlay techniques. Besides, it can be used very well for mapping lesion vascularity, bone trabecular structures, and pulmonary metastasis in cancer models [42–44]. The scanning procedures are very fast and of high quality (~50 µm resolution). High-resolution (HIRES) scanning acquisition mode is used for specimen imaging, which has a resolution of 5–15µm.

25.5.1 CT Instrumentation

There is an external source of radiation in CT modality as opposed PET and SPECT, where the animal itself acts as a radiation source after administration of radiotracers. The animal is placed under the useful field of view of the scanner, and the X-ray beam from the source rotates around the animal [45]. Current (mA) and voltage (kV) are important parameters to determine the resolution and image acquisition time. An increase in the number of X-rays are used for enhancement of image contrast, which is achieved by increasing current to the system. The strength of the X-ray varies as per the tissue density. Tissue density influences the absorption of X-rays resulting in image quality. X-ray source rotates around the subject, and the detector placed on the opposite side acquires a set of two-dimensional images, which are processed using dedicated data processing algorithms to form three-dimensional views.

25.6 Magnetic Resonance Imaging (MRI)

MRI is known for its accuracy to scan anatomically and morphologically soft tissues such as muscles, ligaments, brain and spinal cord, etc. Animal or preclinical MRI is commonly used in the research field of neurology and oncology. MRI imaging is commonly used in human clinical practice; hence, to tap the potential of this modality for biomedical research, microMRI has evolved to scan the brain and other soft tissue organs of laboratory animals. Nuclear magnetic resonance (NMR) spectroscopy principle is also the basis of animal scanner. Human and animal MRI scanners differ in the strength of the magnet used for the generation of the magnetic field. The magnetic field strength is three- to fourfold higher in animal scanners due to the tiny size of the rodents as compared to human adults. It gives excellent spatial and contrast resolutions, but the primary constraint for microMRI is the requirement of technical expertise and expenses involved in scanner and infrastructure development. In earlier generation scanners, the study acquisition time was considerably long to the tune of hours and which was a major drawback for animal studies. This has been resolved to some extent in advanced scanners making it possible to visualize real-time processes in mice and rats. The functional studies in fully awake rodents have been reported, which are necessary for some behavioral studies to circumvent pharmacologic signals initiated due to anesthetic agents [46].

25.7 Optical Imaging

Bioluminescence and fluorescence is the basis of optical imaging [47, 48] in which bioluminescence utilizes enzymatic reactions based on chemiluminescence and fluorophores (green and yellow fluorescent proteins) are required for fluorescence imaging. There is an issue of autofluorescence causing interference in image data below 700 nm, which has been addressed by the use of near infrared probes. The

image acquisition is simple and quick, and technology is highly sensitive; however, the shorter depth of penetration makes this modality limited for deep-seated lesion visualization. The applications of this technology in residual disease diagnosis and examination of surgical margins have a promising future [49–51].

25.8 Cerenkov Luminescence Imaging (CLI)

Russian physicists Pavel Cherenkov, Ilya Frank, and Igor Tamm received the Nobel Prize in Physics (1958) for the discovery of Cherenkov radiation made in 1934. These radiations have several applications in diagnostic imaging and therapy. The Cerenkov luminescence was described in 1934, but only in the recent past it has emerged in biomedical research as an imaging modality. These radiations are produced while advancing charged particles with a speed greater than light in a dielectric medium. The major advantage of this modality is the utilization of commonly used radioisotopes in nuclear medicine [13]. This imaging technology couples luminescence and annihilation coincidence detection and can carry out imaging on multiple animals with high sensitivity. Hence, it's an upcoming promising technology for screening multiple probes and animals. However, several technical challenges are needed to be solved to make it a routine preclinical and clinical modality [52].

25.9 Animal Handling Protocol for Imaging

Mouse tumor models play a substantial role in cancer research for understating the cause, initiation, and progression of the disease and developing potential anticancer therapies, which has led to the development of dedicated preclinical imaging modalities for mouse imaging. The general protocol applicable for imaging studies with a mouse is described here. The schematic steps of the workflow are illustrated in Fig. 25.4.

25.9.1 Animal Immobilization

It is important for the animal to be immobilized for imaging studies during the entire process; therefore, suitable anesthetic agent administration is required. However, during the animal imaging procedure, the body of the mouse is maintained through the image at physiological temperature for obtaining normal scans. The heated beds are used for this purpose during scanning of the animal, and after scanning, heated pads and lamps are used for an uneventful recovery. The immobilization is also necessary to avoid contamination of the imaging bed and surrounding animal preparation area as these animals are administered with radioactive substances.

25.9.2 General Anesthesia

Isoflurane (2-chloro-2-(difluoromethoxy)-1, 1, 1-trifluoro-ethane) is a safe and common inhalation anesthetic agent for imaging procedures. Initially, the animal is placed gently in the induction chamber containing 2–5% isoflurane. Later anesthesia is maintained with 1–2% of isoflurane throughout the imaging procedure. Nose cones are used for the delivery of a gaseous anesthetic agent to the mouse during the scanning procedure. Intramuscular (IM) or intraperitoneally (IP) ketamine (100–200 mg/kg body weight)/xylazine (5–16 mg per kg body weight) combination is recommended in case of unavailability of inhalation anesthesia [53]. The vital parameters of the mouse such as respiratory rate and electrocardiogram (ECG) are monitored using inbuilt or external monitoring devices.

25.9.3 μPET, μSPECT, and μCT Imaging

After the induction of general anesthesia, PET or SPECT radiotracer or CT contrast material is administered. Administration of the optimal amount of radioactivity is important to obtain quality images. Fasting for 4–5 h prior imaging is recommended and required for uniform tracer distribution. The radiotracer is injected directly into the tail vein through P10 customized catheter. To increase the visibility of the veins, the tail is immersed in warm water for a minute, or wiping with absolute alcohol before administration of the radiotracer is recommended. To minimize occupational radiation exposure while injecting animals, a syringe shield is used.

The entire procedure of the radiotracer uptake and completion of the imaging experiment takes approximately 5–30 min, and repetitive imaging at various time points is carried out in anticancer drug efficacy experiments. Before study, the animal is placed in a separate individual cage on an absorbent bedding material to contain radioactive excretion through urine and feces. The anatomical fiducials are used in some studies for a better understanding of the anatomical sites; these fiducials are point sources created with a small amount of radioactivity. They are placed close to the proximity of the anatomic site of interest while acquiring PET/SPECT and CT data.

25.9.4 μMRI and Optical Imaging

In general, the procedure for MRI and optical imaging is similar to μPET, μSPECT, and μCT procedure as described above. The specific requirements for μMRI and optical imaging are mentioned here. As there is a high magnetic field in MRI, safety while working is entirely different and needs compatible accessories/tools in the imaging core. A special orientation program is necessary for users of the facility.

Optical imaging doesn't need stringent safety regulations, but while preparing animals, the hair coat is removed to receive quality signals; hence, nude mice-based models are highly recommended for such studies. In the case of optical imaging

procedures, anesthetized animals are placed in a light-tight box and imaged with a CCD camera for the time required for the experiment (typically for a few minutes). The recently advanced scanners are well equipped with microCT modality for better localization of the lesions.

25.10 Post-Imaging Care and Record-Keeping

Animals should be transferred to the designated cages and racks in vivarium post-imaging studies as these animals are required for longitudinal imaging experiments at various time points. The cage environment should be fresh and clean. The animal cages should be labeled properly with the mention of administered radioactive tracers, time of injection, and quantity of the tracer. These details are important for cage changing and disposal of bedding material containing radioactivity. The cages should be monitored routinely with the help of a survey meter.

25.11 Future of Preclinical Imaging

The translational preclinical research has been revolutionized due to the introduction of preclinical imaging in the recent past. Hybrid imaging has transformed this field from planar mode to exciting visualization capabilities and accurate quantitative abilities. 3D projections of organs, vasculature, and molecular insights provide remarkable information about target and target affinities. This will change the patient care approach and facilitate the entry of personalized medicine to clinics. In coming years, modalities will play an incredible role in the drug discovery process [54].

References

1. Rogers J, Malliaras G, Someya T (2018) Biomedical devices go wild. Sci Adv 4(9):eaav1889
2. Hargreaves RJ (2008) The role of molecular imaging in drug discovery and development. Clin Pharmacol Ther 83(2):349–353
3. Röntgen WK (1896) A New form of radiation. Science 3(72):726–729
4. Wang L, Lin ZQ, Wong A (2020) COVID-Net: a tailored deep convolutional neural network design for detection of COVID-19 cases from chest X-ray images. Sci Rep 10(1):19549
5. Call JA, Eckhardt SG, Camidge DR (2008) Targeted manipulation of apoptosis in cancer treatment. Lancet Oncol 9(10):1002–1011
6. Loisel S et al (2007) Relevance, advantages and limitations of animal models used in the development of monoclonal antibodies for cancer treatment. Crit Rev Oncol Hematol 62 (1):34–42
7. Hartung T (2008) Thoughts on limitations of animal models. Parkinsonism Relat Disord 14 (Suppl. 2):S81–S83
8. De Saint-Hubert M et al (2011) Preclinical imaging of therapy response using metabolic and apoptosis molecular imaging. Mol Imaging Biol 13(5):995–1002
9. Schambach SJ et al (2010) Application of micro-CT in small animal imaging. Methods 50 (1):2–13

10. Buscombe JR, Wong B (2013) PET a tool for assessing the in vivo tumour cell and its microenvironment? Br Med Bull 105:157–167
11. Jang BS (2013) MicroSPECT and MicroPET Imaging of small animals for drug development. Toxicol Res 29(1):1–6
12. de Kemp RA et al (2010) Small-animal molecular imaging methods. J Nucl Med 51(Suppl 1):18S–32S
13. Thorek D et al (2012) Cerenkov imaging - a new modality for molecular imaging. Am J Nucl Med Mol Imaging 2(2):163–173
14. Galban CJ et al (2010) Applications of molecular imaging. Prog Mol Biol Transl Sci 95:237–298
15. Lee JS, Kim JH (2014) Recent advances in hybrid molecular imaging systems. Semin Musculoskelet Radiol 18(2):103–122
16. Dandekar M, Tseng JR, Gambhir SS (2007) Reproducibility of 18F-FDG microPET studies in mouse tumor xenografts. J Nucl Med 48(4):602–607
17. Herschman HR (2003) Micro-PET imaging and small animal models of disease. Curr Opin Immunol 15(4):378–384
18. Maddahi J et al (2020) Phase-III Clinical Trial of Fluorine-18 Flurpiridaz positron emission tomography for evaluation of coronary artery disease. J Am Coll Cardiol 76(4):391–401
19. Haubner R et al (2010) Positron emission tomography tracers for imaging angiogenesis. Eur J Nucl Med Mol Imaging 37(Suppl. 1):S86–S103
20. Pham TT et al (2020) Iodine-124 based dual positron emission tomography and fluorescent labeling reagents for in vivo cell tracking. Bioconjug Chem 31(4):1107–1116
21. Shi S et al (2019) Copper-64 labeled PEGylated exosomes for in vivo positron emission tomography and enhanced tumor retention. Bioconjug Chem 30(10):2675–2683
22. Ma Z et al (2019) Photonuclear production of medical isotopes 62,64Cu using intense laser-plasma electron source. Matter Radiat Extremes 4(6):064401
23. Kung MP, Kung HF (2005) Mass effect of injected dose in small rodent imaging by SPECT and PET. Nucl Med Biol 32(7):673–678
24. Cherry SR, Gambhir SS (2001) Use of positron emission tomography in animal research. ILAR J 42(3):219–232
25. Ferl GZ et al (2007) Estimation of the 18F-FDG input function in mice by use of dynamic small-animal PET and minimal blood sample data. J Nucl Med 48(12):2037–2045
26. Bullich S et al (2018) Validation of noninvasive tracer kinetic analysis of (18)F-Florbetaben PET using a dual-time-window acquisition protocol. J Nucl Med 59(7):1104–1110
27. Cutler PD et al (1992) Design features and performance of a PET system for animal research. J Nucl Med 33(4):595–604
28. Watanabe M et al (1992) A high resolution PET for animal studies. IEEE Trans Med Imaging 11(4):577–580
29. Chaudhari P (2015) Preclinical animal model and non-invasive imaging in apoptosis. In: Proteases in apoptosis: pathways, protocols and translational advances, Proteases in apoptosis: pathways, protocols and translational advances, 1st edn. Springer International Publishing. XV, Switzerland, p 245
30. Chandrani P et al (2017) Drug-sensitive FGFR3 mutations in lung adenocarcinoma. Ann Oncol 28(3):597–603
31. Valdes Olmos RA et al (1997) Thallium-201 SPECT in the diagnosis of head and neck cancer. J Nucl Med 38(6):873–879
32. Su N et al (2015) Iodine-125-labeled cRGD-gold nanoparticles as tumor-targeted radiosensitizer and imaging agent. Nanoscale Res Lett 10:160
33. Notni J et al (2017) In vivo pet imaging of the cancer integrin alphavbeta6 using (68)Ga-labeled cyclic RGD nonapeptides. J Nucl Med 58(4):671–677
34. Chakraborty S et al (2008) 177Lu-EDTMP: a viable bone pain palliative in skeletal metastasis. Cancer Biother Radiopharm 23(2):202–213

35. Ishihara M et al (2018) Optimal thallium-201 dose in cadmium-zinc-telluride SPECT myocardial perfusion imaging. J Nucl Cardiol 25(3):947–954
36. Wendt RE 3rd et al (2020) Radiation safety considerations in the treatment of canine skeletal conditions using 153Sm, 90Y, and 117mSn. Health Phys 118(6):702–710
37. Tang Y et al (2019) Indium-111 labeled bleomycin for targeting diagnosis and therapy of liver tumor: optimized preparation, biodistribution and SPECT imaging with xenograft models. J Radioanal Nucl Chem 322(2):545–551
38. Chavare A et al (2017) Safety and bioactivity studies of Jasad Bhasma and its in-process intermediate in Swiss mice. J Ethnopharmacol 197:73–86
39. Gaut C et al (2020) SPECT Imaging of muscle Injury with [(99m)Tc]MDP in a mouse model of muscular dystrophy. Mol Imaging Biol 22(3):562–568
40. Malhi H et al (2002) 99mTc-mebrofenin scintigraphy for evaluating liver disease in a rat model of Wilson's disease. J Nucl Med 43(2):246–252
41. Liu CN et al (2019) Nanoparticle contrast-enhanced micro-CT: a preclinical tool for the 3D imaging of liver and spleen in longitudinal mouse studies. J Pharmacol Toxicol Methods 96:67–77
42. Badea CT et al (2019) Functional imaging of tumor vasculature using iodine and gadolinium-based nanoparticle contrast agents: a comparison of spectral micro-CT using energy integrating and photon counting detectors. Phys Med Biol 64(6):065007
43. Kerckhofs G et al (2018) Simultaneous three-dimensional visualization of mineralized and soft skeletal tissues by a novel microCT contrast agent with polyoxometalate structure. Biomaterials 159:1–12
44. Linxweiler J et al (2017) Experimental imaging in orthotopic renal cell carcinoma xenograft models: comparative evaluation of high-resolution 3D ultrasonography, in-vivo micro-CT and 9.4T MRI. Sci Rep 7(1):14249
45. Willmann JK et al (2008) Molecular imaging in drug development. Nat Rev Drug Discov 7 (7):591–607
46. Harris AP et al (2015) Imaging learned fear circuitry in awake mice using fMRI. Eur J Neurosci 42(5):2125–2134
47. Sato A, Klaunberg B, Tolwani R (2004) In vivo bioluminescence imaging. Comp Med 54 (6):631–634
48. Andersson-Engels S et al (1991) Fluorescence imaging and point measurements of tissue: applications to the demarcation of malignant tumors and atherosclerotic lesions from normal tissue. Photochem Photobiol 53(6):807–814
49. Blanco VM et al (2016) Optical and nuclear imaging of glioblastoma with phosphatidylserine-targeted nanovesicles. Oncotarget 7(22):32866–32875
50. Patrick PS et al (2014) Dual-modality gene reporter for in vivo imaging. Proc Natl Acad Sci U S A 111(1):415–420
51. Chibhabha F et al (2020) Non-invasive optical imaging of retinal Abeta plaques using curcumin loaded polymeric micelles in APPswe/PS1DeltaE9 transgenic mice for the diagnosis of Alzheimer's disease. J Mater Chem B 8(33):7438–7452
52. Zhang Z et al (2019) Endoscopic Cerenkov luminescence imaging and image-guided tumor resection on hepatocellular carcinoma-bearing mouse models. Nanomedicine 17:62–70
53. Stokes EL, Flecknell PA, Richardson CA (2009) Reported analgesic and anaesthetic administration to rodents undergoing experimental surgical procedures. Lab Anim 43(2):149–154
54. Barbet J et al (2016) Editorial: innovative radiopharmaceuticals in oncology and neurology. Front Med (Lausanne) 3:74
55. Miyakita Y et al (2020) Usefulness of carbon-11-labeled methionine positron-emission tomography for assessing the treatment response of primary central nervous system lymphoma. Jpn J Clin Oncol 50(5):512–518
56. Segard T et al (2019) Comparison between gallium-68 citrate positron emission tomography-computed tomography and gallium-67 citrate scintigraphy for infection imaging. Intern Med J 49(8):1016–1022

57. Feher A et al (2020) Serial assessment of coronary flow reserve by Rubidium-82 positron emission tomography predicts mortality in heart transplant recipients. JACC Cardiovasc Imaging 13(1 Pt 1):109–120
58. Nakao R et al (2020) Papillary muscle ischemia on high-resolution cine imaging of nitrogen-13 ammonia positron emission tomography: association with myocardial flow reserve and prognosis in coronary artery disease. J Nucl Cardiol. https://doi.org/10.1007/s12350-020-02231-z
59. Gómez-Vallejo V et al (2019) The radiopharmaceutical chemistry of Nitrogen-13 and Oxygen-15. Radiopharm Chem:237–254
60. Ramos-Membrive R et al (2020) In vivo SPECT-CT imaging and characterization of technetium-99m-labeled bevacizumab-loaded human serum albumin pegylated nanoparticles. J Drug Del Sci Technol:101809
61. Liang J et al (2019) SPECT Imaging of treatment-related tumor necrosis using Technetium-99m-labeled rhein. Mol Imaging Biol 21(4):660–668
62. Spanu A et al (2020) Neck lymph node metastasis detection in patients with differentiated thyroid carcinoma (DTC) in long-term follow-up: a 131I-SPECT/CT study. BMC Cancer 20 (1):239
63. Babin V et al (2020) Design of iodinated radioligands for SPECT imaging of central human 5-HT4R using a ligand lipophilicity efficiency approach. Bioorg Chem 96:103582
64. Hsu JC et al (2018) An all-in-one nanoparticle (AION) contrast agent for breast cancer screening with DEM-CT-MRI-NIRF imaging. Nanoscale 10(36):17236–17248
65. Kim J et al (2018) Assessment of candidate elements for development of spectral photon-counting CT specific contrast agents. Sci Rep 8(1):12119
66. Kale T et al (2019) Albumin based iohexol nanoparticles for computed tomography: an in vivo study. J Biomed Nanotechnol 15(2):236–247
67. Hall MP et al (2018) Click beetle luciferase mutant and near infrared naphthyl-luciferins for improved bioluminescence imaging. Nat Commun 9(1):132
68. Vandergaast R et al (2020) Enhanced noninvasive imaging of oncology models using the NIS reporter gene and bioluminescence imaging. Cancer Gene Ther 27(3):179–188
69. Yao Z et al (2020) Multicomponent bioluminescence imaging with a π-extended luciferin. J Am Chem Soc 142(33):14080–14089
70. Cheng G et al (2020) Increased formation of reactive oxygen species during tumor growth: ex vivo low-temperature EPR and in vivo bioluminescence analyses. Free Radic Biol Med 147:167–174
71. Coutant EP et al (2019) Gram-scale synthesis of luciferins derived from coelenterazine and original insights into their bioluminescence properties. Org Biomol Chem 17(15):3709–3713
72. Ortega Ugalde S et al (2019) Evaluation of luminogenic substrates as probe substrates for bacterial cytochrome P450 enzymes: application to *Mycobacterium tuberculosis*. SLAS Discov 24(7):745–754

Part IV

Specialty Areas of Laboratory Animal Science

Management of Specific Pathogen-Free (SPF) Mice and Rats

26

G. H. Mohan, R. V. Shwetha Reddy, and C. Yogesh

Abstract

Specific pathogen free (SPF) status is a relative term that varies from one animal facility to another depending on the number of pathogens in their exclusion list to be tested and determined free. The two main critical aspects of SPF rodent facilities management include an engrained health surveillance program and an exclusion list or SPF list. The veterinarian in charge of the animal facility in consultation with institutional management and users of the animal facility will decide on the exclusion list of pathogens. There are several factors like personal safety, animal welfare, and research quality which will influence the preparation of the exclusion list. Animals for modern biomedical research need to have defined levels of pathogens and other infectious agents that may change the results of research. It is a well-known fact that the microbiological quality of experimental animals has an influence on animal welfare and can affect the validity and reproducibility of research data. This chapter attempts to describe the various aspects of the management of SPF mice and rat facilities.

Keywords

SPF · Sentinel · HVAC · Immunodeficient

G. H. Mohan (✉) · R. V. Shwetha Reddy · C. Yogesh
Animal Care and Resource Centre (ACRC), National Centre for Biological Sciences, Bengaluru, Karnataka, India
e-mail: mohan@ncbs.res.in; shwetharvr@ncbs.res.in; yogeshc@ncbs.res.in

© The Author(s), under exclusive license to Springer Nature Singapore Pte Ltd. 2021

P. Nagarajan et al. (eds.), *Essentials of Laboratory Animal Science: Principles and Practices*, https://doi.org/10.1007/978-981-16-0987-9_26

Abbreviations

FDA Food and Drug Administration
IVC Individual ventilated caging system
PCR polymerase chain reaction
PPE Personal protective equipment

26.1 Introduction

Specific pathogen free (SPF) is a term used for laboratory animals that are free from a predefined group of pathogens. SPF animals are different from germ-free animals (which are free of all microorganisms including gut microbiota) or gnotobiotic animals (germ-free animals with only certain known strains of bacteria and other known microorganisms are present). A specific pathogen-free (SPF) animal should not contain any members of a defined list of pathogenic microorganisms and parasites [1].

Specific pathogen free status is a relative term that varies from one animal facility to the other depending on the number and nature of pathogens in their exclusion list to be tested and determined free. In any rodent facility of SPF status, it is very important to establish a well-defined health monitoring program and a list of organisms to be excluded from the facility [2]. An attending veterinarian in consultation with the institutional management and users of the animal facility will decide on the exclusion list of pathogens. In the preparation of the exclusion list, the first and foremost consideration is the personal safety of individuals (animal facility staff and users) who are in contact with animals. The laboratory animals must be tested and determined free of species-specific pathogens of zoonotic importance. The second consideration is animal welfare, meaning that the exclusion list must include species-specific pathogens that are highly virulent and can cause high mortality in the animal colony. The maintenance of SPF status for such pathogens can help prevent the disease outbreaks that can result in the total eradication and loss of the animal colony. The SPF status of the animals can also help in optimizing the breeding and fecundity of laboratory animals. The immunocompromised animals survive better in SPF than non-SPF conditions. The third consideration is the quality of research wherein the results obtained using experimental animals must be reproducible and universally acceptable. It is important to maintain infection-free animals as the presence of even asymptomatic pathogens could interfere with research outcomes [3–5]. Precise knowledge of the health status of your tool (laboratory animal) helps in better standardization of your experiments (and therefore use fewer animals). The SPF animals are maintained free of defined pathogens and other infectious agents that may interfere with the results. Animal welfare and research data reproducibility are greatly influenced by the environment where animals are maintained, microbiological quality, and genetic quality of the experimental animals

used in research. SPF animals play a vital role in the generation of novel genetically modified models. There are also regulatory requirements like the quality assurance of laboratory animals as required for the Organisation for Economic Co-operation and Development (OECD), International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH), Food and Drug Administration (FDA), etc. Considering the above criteria, most laboratory animal facilities implement the FELASA [6] (Federation of European Laboratory Animal Science Associations) recommendations on health monitoring policy.

26.2 The Physical Design of SPF Animal Facility

The designing of SPF animal facilities is combined with animal housing and management which serves as an essential requirement for the well-being and quality of animals used for research. Appropriate animal husbandry programs should be implemented to provide a clean environment and housing to animals considering their physical, physiological, and behavioral needs so that they grow, mature, and breed normally. The personnel, animals, and equipment traffic pattern/movement in an animal facility has a major impact on the microbiological/health status of the animals. Facility design features and operational procedures that support circulation patterns aim to separate clean and dirty items. This separation minimizes contamination by directing facility traffic from areas of lesser contamination to those of greater contamination. Several types of corridor floor plans may be used in an SPF animal facility.

26.2.1 Single Corridor Floor Plan

This is commonly used in conventional facilities. This plan consists of a central corridor with no separation of traffic for clean and dirty operations. To avoid cross-contamination, the clean and dirty items/activities should be separately timed. Both dirty and clean cages are often shrouded to avoid aerosol contamination

26.2.2 Two-Corridor Floor Plan

In this design, each animal room has two doors. One door is for the entrance of clean items from a clean corridor, and the second door is for the removal of dirty items into the dirty corridor so that it becomes possible to carry out clean and dirty operations in designated corridors which reduce the possibility of contaminating the clean equipment, materials, and animals. Pass boxes can be used to transfer containers of supplies into and out of the facility. The airflow in these facilities is controlled to promote the direction of airflow from the clean hallway to the animal room and then to the dirty hallway to maintain the sterility of the facility.

26.2.3 Three-Corridor Floor Plan

This design effectively maintains a separation of clean and dirty items. A single clean corridor located in the center of the facility serves rooms on both sides. Each room exit door opens into a dirty corridor that wraps around the periphery of the floor plan.

The single corridor floor plan has the highest risk for cross-contamination among animal rooms. However, a dual-corridor floor plan will reduce the potential for cross-contamination with a one-way circulation from clean to dirty areas. The same applies to the three-corridor floor plan, which is a variation on the dual-corridor floor plan and has the same benefits [7] (Fig. 26.1a, b).

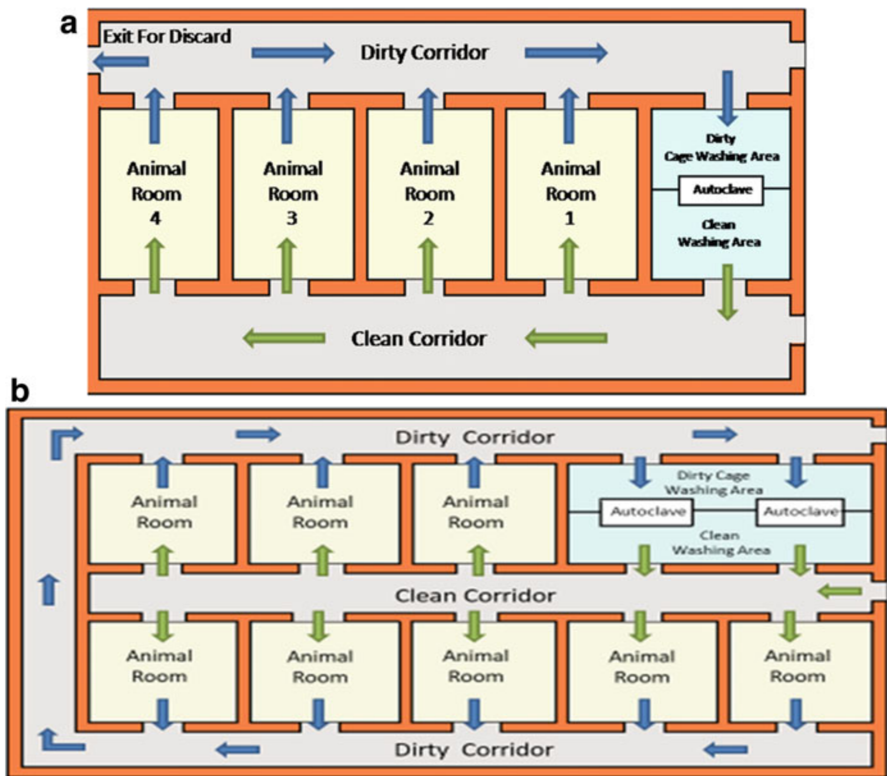


Fig. 26.1 (**a** and **b**) Two-way and three-way corridor system

26.3 Barrier Facilities

Barrier facilities can be designated for bioexclusion (protecting the animals) or biocontainment to prevent pathogens from leaving the room. The barrier system is the facility where the entry air and exhaust air are filtered. A barrier system that combines constructive features, equipment, and operating methods is mandatory in SPF facility housing. It helps to stabilize the enclosed environment and also to minimize the probability of pathogens and other undesirable organisms infecting the enclosed animal population [2].

Barriers should be made at the personnel level (wearing sterilized protective apparel like the gown, mask, head cover, autoclaved scrubs or lab coats, shoe covers, and gloves), facility-level (room, rack, and/or cage levels individual ventilated caging system (IVCs)). Depending on the species housed, animal health, and microbiological status, the type of personal protective equipment may change.

All the materials that go inside the barrier animal room must first be autoclaved and then pass through a portal from the clean corridor, and waste materials are passed out of the facility via a separate route. Air pressure is maintained positive in the surrounding areas to avoid airborne contamination. Usually, the biosafety personnel determine the containment controls and worker protections and through a risk assessment of the biohazardous agents used in the research programs. In contrast, the air pressure in a biocontainment room is negative relative to the surrounding areas to help contain airborne contaminants.

26.3.1 Containment Levels of SPF Facility

Containment defines the facilities, procedures, and processes that could prevent people and the environment from exposure to microorganisms. This can be done using good microbiological practices (e.g., aseptic technique) to prevent cross-contamination and the spread of contamination [8]. Based on the risk proportions, the Centers for Disease Control (CDC) have established animal biosafety facility levels for animal facilities as ABSL1, ABSL2, ABSL3, and ABSL4 [9]. ABSL-1 facility involves works on agents on laboratory animals that don't cause human infection; the ABSL-2 facility involves works on agents on laboratory animals that cause moderate risk to humans. ABSL-3 contains agents that cause serious and potentially lethal infections and have known potential for aerosol transmission; ABSL-4 contains nonindigenous (exotic) agents that pose a high individual risk of life-threatening disease and for which there is no available vaccine or treatment [10].

26.3.2 Primary Barriers

Primary barriers are an immediate line of defense that prevents hazardous biological agents from being exposed to animals, humans, and the environment. They are designed to contain the agent and prevent exposures from infectious splashes or

Fig. 26.2 Personal protective equipment for SPF room, biological safety cabinet

aerosols when animals are kept in an open cage or in rooms within which personnel, equipment, and animals move freely:

1. Personal protective equipment (PPE) (Fig. 26.2a)
2. Staff and animal care personnel generally wear dedicated clothing before entry
3. Face mask (special mask based on need—N95 or PAPR)
4. Hair cap
5. A lab coat or coverall/jumpsuits (reusable or disposable based on the type of work)
6. Gloves (latex or nitrile etc.)
7. Closed-toe shoes and shoe covers

26.4 Barrier Systems

Laminar airflow cabinets. Laminar airflow is a feature of cage changing cabinets, biosafety cabinets, and animal holding cubicles that provide a continuous flow of HEPA-filtered air in the unidirectional stream. This reduces or eliminates air turbulence inside the cabinet which could cause contamination of the items inside the cabinet. All the activities such as handling, cage changing, etc. should be done in a laminar airflow cabinet (Fig. 26.2b).

26.4.1 Biological Safety Cabinets (BSCs)

For working on SPF animals Class I, biosafety cabinets are not recommended. Usually, Class II cabinets are used. Class II cabinets provide complete protection

to the animals and the personnel. There are four subtypes of Class II cabinets, viz., A1, A2, B1, and B2. These types are based on the amount of air recycled and exhausted. These can be used for handling SPF animals. There are cage changing/transfer stations that belong to the Class II type used routinely for cage changing procedures.

SPF animals can also be housed in a micro-isolator. It resembles and works as an animal-sized Petri dish. The advantage is that the microbiological barrier at the cage level can be maintained. The micro-isolator cages contain a plastic, filter-top lid that fits over the wire food hopper and can be used with or without a ventilated rack system. Micro-isolator cages must only be opened only inside a biosafety cabinet or a cage changing station.

When used in a HEPA-filtered individually ventilated caging (IVC) system, each cage is docked on a ventilated rack that provides HEPA-filtered air at the cage level. The IVC system provides a barrier type of protection for animals and prevents airborne particulates and infections and protects both animals and personnel. Each cage is individually supplied with filtered air under positive pressure. Cage exhaust air is then transferred/captured separately as it exits above each cage filter top, assuring barrier protection for personnel.

There are many advantages of micro-isolator/IVC such as avoidance of cross-contamination from animals of different species/strains in the same room, prevention of infection to, and the possibility of maintaining different species in the same room. It is not recommended to maintain the different species in the same room, but sometimes it may also be acceptable to house different species in the same room—for example, two species that have a similar pathogen status and are behaviorally compatible as long as animal handling devices remain separate between systems [10]. The IVCs play a very important role in maintaining the health status of the animals in the SPF facility. Hence the IVC system will be an effective housing system for SPF animals.

26.4.2 Secondary Barriers

1. Engineering controls
2. HVAC (heating, ventilation, and air conditioning)
3. HEPA filters in HVAC
4. Airlocks
5. Differential airflow – positive and negative pressure

26.4.2.1 HVAC: Heating, Ventilation, and Air Conditioning

Maintaining constant humidity and temperature within the normal range in SPF animal facility helps minimize the stress and physiological alteration in SPF animals. So, these parameters should be constantly monitored to provide an optimum environment for animal well-being. The temperature and humidity of the environment are affected by housing design, cage type, number and size of animals, ventilation,

and type and frequency of bedding change. Animal holding room temperatures are monitored by a digital or dry and wet bulb thermometer.

Many pathogens of animals can be airborne or travel on fomites. An HVAC system prevents airborne and fomite infection and can avoid the risk of cross-contamination in the SPF facility [10].

HVAC systems shall be designed in such a way to meet the demands of SPF housing that includes maintaining optimal temperature and humidity. The HVAC systems must ensure adequate ventilation capacity to control fumes, odors, airborne contaminants, and relative pressure differentials between spaces to prevent cross-contamination. HVAC systems should be independent of HVAC systems of other buildings. Usually for an SPF animal facility, N+1 redundancy (standby) is needed to prevent failure of HVAC.

26.4.2.2 HEPA Filters in HVAC (Fig. 26.3)

HEPA filters should have 99.99% efficiency and be capable of removing particulates of 0.3 microns for an SPF facility. These filters are capable of removing airborne contaminants in the supply and the exhaust of the HVAC system. HEPA filters must be tested every 6 months and to be monitored (with manometers or other pressure indicating devices) regularly and replaced following the manufacturer's instructions.

26.4.2.3 Airlocks

Airlocks should (Fig. 26.3) be installed for a transition area before SPF entry for personnel (e.g., a change room for gowning, de-gowning), equipment, and materials. A cascade airlock system having high pressure on one side and lower pressure on the other side can prevent cross-contamination inside SPF.

Fig. 26.3 Airlock and HVAC for SPF rooms entry

26.4.2.4 Differential Airflow: Positive and Negative Pressure

Positive pressure helps to prevent containments from entering the room. Animal holding rooms, surgical suites, and barrier rooms are kept under positive pressure ventilation. Negative pressure helps to keep containments from leaving the room. Animal quarantine rooms and rooms where biohazards or infectious agents are handled should have negative pressure ventilation.

Besides, dedicated clean anterooms should be present off the corridor, where personnel wears PPE before entering the animal room. This anteroom helps segregate infectious items to minimize contaminations. A dirty anteroom is also needed to remove dirty materials from the room without cross-contamination. Besides, a handwash sink needs to be provided at the anteroom (Fig. 26.4).

26.5 Decontamination in SPF Room

Decontamination is important for the elimination of pathogens in animal facilities, thereby preventing contamination within barrier systems. Any contamination from animal rooms or outside sources can be transmitted to other areas that potentially affect animal health status, thus compromising research outcomes. A wide range of agents including quaternary ammonium compounds, phenol-based products, and alcohol are commonly applied for decontamination of animal rooms by manual wiping, fogging, or vapor and gas exposure. H_2O_2 is commonly used as a biocide agent and found safe to use for effective biologic inactivation. VHP (vaporized hydrogen peroxide)-based decontamination methods are widely used as alternatives to formaldehyde because of their ease of use, higher levels of sterility assurance, and overall cost savings [11].

Fig. 26.4 Clean and dirty corridor for SPF rooms

26.5.1 Feed and Water for SPF Animals

Feed and water must be sterilized before they come in contact with the SPF animals. So while receiving on the clean side of the facility, these materials should be completely sterile from microbial contaminants. The feed should be either gamma-irradiated or autoclaved. The sterile feed is available in commercial markets. The clean side of the SPF facility should be provided with clean water (reverse osmosis water) or autoclaved water. The water should be again treated with acid or chlorination to minimize/eliminate the contamination. It is to be ensured that treatment procedure should not have any effect on the physiology of the animal, water consumption, gut microbiota, and experimental results. The frequency of water bottle change will depend on the type of treatment used for sterilizing the water. The water bottles should be changed completely with new bottles periodically rather than refilling them. It is necessary to conduct periodic microbial monitoring of water samples in the SPF facility, to keep track of the sterility level of drinking water.

26.5.2 Microenvironmental Factors

The microenvironment of the cage influences animal well-being and experimental data. Bedding and the nesting materials used should have good moisture absorption capacity to minimize the growth of microorganisms and limit the animals coming in contact with excreta. Besides, nesting material should also help in thermoregulation. Considering all the above factors, the appropriate selection should be made from the different types of bedding material that are available. While sourcing bedding material, the vendors' manufacturing process, storage, and transportation methods should be taken into consideration. During transportation, the bedding may be contaminated with toxins and other microbial contaminants. Bedding should be placed off the floor either on pallets or racks to avoid contamination and to maintain sterility of the bedding. During autoclaving appropriate drying time should be provided for complete drying of bedding, and failing to do so results in loss of absorbance and growth of microbes due to moisture present in the bedding that in turn contaminate the SPF animals. The soiled or dirty cage bedding should be removed and replaced with a fresh clean cage periodically. The frequency of cage bedding change depends on multiple factors such as the number of animals, size of the animals, housing, and type of bedding material used.

26.5.3 Disinfection, Sterilization, and Environmental Microbiological Monitoring

In SPF rodent facility, it is very important to implement the cleaning and disinfection of all materials that have direct or indirect contact with animals. Sanitation of cages and equipment in cage and rack washer with hot water and detergents or disinfectants can be effective but requires considerable attention to detail. The

surfaces of the cages are rinsed free of residual chemicals, and the personnel operating the machine/equipment should take all the safety measures to protect themselves from exposure to hot water or chemical agents used in the process. Cleaning and disinfection are necessary to sterilize caging and associated equipment to ensure that pathogenic or opportunistic microorganisms are not presented into specific pathogen-free or immune-compromised animals [10].

Cages and other associated equipment must be regularly sterilized with disinfectants or with an automated cage and rack washer and further autoclaved before housing the animals. The effectiveness of cage washers/tunnel washers can be monitored by RODAC (Replicate Organism Detection and Counting) to evaluate the machine's effectiveness. Proper evaluation of sterilization using an indicator tape for autoclaving along with biological indicators needs to be done quarterly to ensure proper sterilization of autoclave.

In addition to the routine sterilization procedures in the facility, regular environmental microbial monitoring of the SPF facility is very important. This can be done either by swabbing and culturing using test strips that assess for the presence of NAD (nicotinamide adenine dinucleotide) or ATP (adenosine triphosphate) as the bioindicator to evaluate the efficiency of sanitization, decontamination, air quality, and hygienic practices [11, 12].

26.5.4 Pest Control Program/Management

Regular pest control programs are essential to prevent, control, or eliminate the infestation by pests in an animal facility. The program is essential that helps prevent the entry of vermin into the facility. Frequently scheduled and documented program of control and monitoring should be implemented in the SPF animal facility [13]. The pest control program should comply with federal, state, or local regulations. The best time to seal off possible pest entry areas is during facility construction. Walls and floors must be free of cracks and crevices. Pipelines drain, and air filters should be well sealed and inspected often. Pest control programs include using either physical and chemical means laboratories to control pest infestation. Traps, sticky boards, boric acid or silica powder, and housekeeping which are noninvasive will also aid in the control of pests. Most importantly there should be a proven method for eliminating feral or wild rodents in the laboratory by using traps in areas outside having improperly sealed doorways or walls.

26.5.5 Animals

It is important to procure animals from known sources with a known microbial status of the animals. A suitable quarantine and biosecurity protocols should be followed during receiving animals from other sources. Also, a risk analysis of each shipment from other sources should be made and evaluated by the veterinarian. Recent health status reports should be reviewed. At the end of the quarantine period [14], the health

monitoring of animals should be carried out in the recipient institution. Animals that do not meet institutional health requirements should be discarded or re-derived. During quarantine of these animals, a proper standard operating procedure should be placed on housing, handling of animals, and equipment to prevent cross-contamination. It is necessary to establish a specific traffic pattern in the rooms and aseptic technique to prevent cross-contamination. As a general rule husbandry procedures are performed starting from lower risk animals to the highest risk animals. Strict biosecurity protocols of changing gloves and cleaning workspace in between the different rooms should be employed [14].

26.5.6 Personnel

Animal users, facility visitors, and staff pose an important risk in introducing and/or spreading infection. Immunocompromised animals are often susceptible to otherwise innocuous organisms, e.g., *Staphylococcus aureus* and *Klebsiella pneumonia*. Personal protective equipment (PPE) should be worn when personnel are working with the animals. A proper sticky mat or foot dip can be placed before the entry of the facility. Besides, a comprehensive standard operating procedure (SOP) should be in the place that includes various procedures carried out in an SPF animal facility.

26.6 Risk Factors for Transmission of Agents in SPF Facility

26.6.1 High Risk: As Suggested by FELASA (Ref. [6])

1. Frequent introduction of lab animals and different species/strains.
2. Units of varying microbiological status near.
3. Introduction of animals from different breeding colonies.
4. Movement of animals out of the room for various procedures and in return.
5. Access of pests or wild rodents to animal rooms and storage areas.
6. Multipurpose facilities with various kinds of experiments.
7. Frequent turnover of animal care personnel.
8. Shared equipment that cannot be easily disinfected (e.g., imaging, behavioral equipment).
9. The frequent entry of research personnel apart from animal care staff.
10. Transplantable tumors, hybridomas, cell lines, blood products, and other biologic materials can be sources of both murine and human viruses that can contaminate rodents or pose risks to laboratory personnel.

26.6.2 Lower Risk

1. Closed breeding colonies
2. Occasional introduction of animals with strict quarantine
3. One or a few types of experiments

26.7 Experimental Procedures

The entry of animals into the facility; the use of biomaterials such as cells, parasites, viral stocks, purified proteins, or antibodies; cleaning, disinfection, and sterilization processes; the housing; husbandry; and the personnel must be carefully controlled to reduce the risk [2]. The materials like cell lines, tissue fluids, etc. should be tested (MAP and RAP test) for contamination before they are used on animals. Rodent viruses or mycoplasma contaminate cell lines. Common procedure rooms and surgical or imaging facilities are usually shared facilities and may present a risk of cross-contamination; therefore, disinfection between the procedures helps to reduce the risk. The biosecurity measures have to be monitored strictly to avoid the risk of eliminating the potential of introducing an adventitious agent into the facility. It is important to follow standard husbandry practices like regular health monitoring of animals, environmental microbial quality in the animal facility, proper sterilization, disinfection procedures, and assessing the quality of materials used in the facility. So, these considerations are important in maintaining the specific pathogen-free (SPF) laboratory mice and rats.

26.8 Health Monitoring and Surveillance

Health monitoring programs is the term applied to find out the subclinical infections in rodents that cause detrimental effects on the health of the animals as well as the outcome of the research. These programs are also termed as sentinel monitoring programs or rodent health surveillance. The health status of resident colonies of laboratory animals is detected by implementing these programs that support the production of healthy animals and also assuring that the results of the experiment are unaffected by undesirable organisms. The demand for health monitoring increases especially in the case of rodents when their transferring are there between the institutions.

The periodic health surveillance of high-density mice colonies can be accomplished through their effective health management and care. The most crucial part of these programs is the evaluation and documentation of the microbial status of the colony which will be evaluated based on the requirement and goal of the institution. For efficient implementation of these, a program has to expect a culture of coordination between all stakeholders involved in animal care and their use that promote the appropriate health screening and interpretation of result accompanied by relevant action.

A high level of health monitoring comprised of reliable and sensitive diagnostic methods is particularly required for specific pathogen-free facilities. SPF animals are bred continuously in controlled environmental conditions to remain free from specific pathogens and need to be tested regularly for an extended period. These animals are not innately resistant to the specified pathogens or infections, but they can be produced as specific pathogen-resistant (SPR) species. Even the removal of stock animals from the designated facility besides if they are not showing any disease symptoms or not infected is also lost their SPF status. Usually, the SPF animals may be designated as "high health" stock even if they are transferred to other well-established units having a history of the severe disease surveillance program.

Health surveillance programs involve the myriads of batteries or panels of tests which are directed at specific frequencies to evaluate the microbial or health status of animals housed in such kind of facility. Usually, the samples are taken from the animal which is part of the colony or sentinels who are purposely exposed to the colony for the specific purpose of health monitoring. Mostly, the colony-raised animals are preferred as compared to sentinel because these animals have a huge chance of introducing an unwanted agent into the colony. The most advisable thing that selects the sentinel is only those mouse strain that is especially susceptible to specific pathogen during the health evaluation of colony. Further, SPF status is generally monitored by exposing sentinel animals to dirty bedding of colony animals and testing the sentinel animals. Moreover, the information on the health and welfare of animals can be achieved by careful observation in their home cage also [2]. Overall, it is most useful to screen the colonies for a defined list of pathogens only, and the results are shared with the users or public. SPF facility should provide a rigorous health monitoring program regularly for effective tracking of the microbial status of animals.

26.8.1 Sentinel Program

The exposure to dirty bedding, direct contact, and air exhaust are the most current sentinel program. The first two methods are commonly used for this program, however, recently the air exhaust also gained importance after the introduction of the IVC system for the housing of rodent colonies. The population surveyed for health monitoring has to be placed with sentinel animals that are indirectly exposed to the infectious agents by using soiled bedding. The selection of strain, stock, age, and sex is an important factor counted during the choosing of sentinel animals. Mostly the outbred stocks having a robust immune response to a larger pool of antigens and also having high vigor and available at a relatively low cost are used as the sentinels for health monitoring programs. The heterozygous mice may be used as sentinels for those facilities housing the immune-compromised animals like nude or SCID mice [15]. In inbreeding facilities, retired breeders may also require health screening as usually they have mostly been exposed to infectious agents for an extended period and have more chance of seroconversion during this long period.

26.8.2 Types of Sentinel System

1. Dirty bedding sentinels—by exposure of sentinels to the dirty bedding from the used cages. The transfer of contamination is primarily by fecal-oral contamination.
2. Direct contact sentinels—by directly placing sentinels in the same cage with normal animals. The transfer of contamination is by direct contact, aerosol, urine, and the fecal-oral route. The infection may spread to uninfected cages.
3. Air-exhaust exposure—this is effective in detecting aerosolized pathogens in the IVC system.

Dirty bedding sentinels are considered the most effective method for the detection of pathogens transmitted by the fecal-oral route including the mouse hepatitis virus (MHV), mouse parvovirus (MPV), Theiler's murine encephalomyelitis virus (TMEV), and *Helicobacter* spp. However, all agents could not easily spread by this method. The transmission of viruses including the Minute virus of the mouse (MVM), *Clostridium piliforme*, Murine Norovirus (MNV), pinworms, *Pneumocystis murina*, sialodacryoadenitis virus (SDAV), and endoparasites (excluding the pinworms) is achieved only partially by this method, whereas the transmission of the mouse rotavirus (EDIM), *Pseudomonas aeruginosa*, and cilia-associated respiratory (CAR) bacillus is highly effective by applying the soiled bedding method [16].

26.8.3 Sentinel Animal Selection

Importantly the sentinel animals must be obtained from specified vendors or breeding colonies and should be free from specifically undesired organisms. Further, the sentinel animals should provide every opportunity to become infected if the rodent colony has to screen for the pathogens. For capturing the high exposure to airborne pathogens, the sentinel cages should be placed near the outflow of air in the room. Moreover, the sentinel cages should be contaminated with dirty bedding material obtained from the colony animals. It benefits in capturing pathogens spread by the fecal-oral route.

Genetically modified animals should not be employed as sentinels because they may not produce a measurable antibody response.

26.8.4 Sample Size

To perceive at least one positive animal, a minimum of 10 animals should be monitored if the suspected prevalence rate of infection is 30% at the confidence level of 95%. But if the prevalence of infection is less than 30%, then the infection may not be detected with a 95% confidence level. However, the rate of infection detection depends on the test method used. The sero-monitoring methods usually are

Table 26.1 Calculation of the number of animals to be monitored

Suspected prevalence rate (%)	Sample sizes (N) at different confidence levels		
	95%	99%	99.9%
10	29	44	66
20	14	21	31
30	10	13	20
40	6	10	14
50	5	7	10

Ref. [6]

sensitive and measure higher prevalence as connect to direct methods which directly detected the presence of the microorganism. By using SEO monitoring, the level of confidence may be increased by the screening of the same number of animals (Table 26.1). If diseases having an infection rate of 50% or more like (high infection rate) like in the case of Sendai and MHV, reduce the number of animals to detect the presence of these pathogens. Conversely, the low infection rates required a larger sample size [17].

The sample size is calculated from the following formula:

Assumptions:
1. Both sexes are infected at the same rate
2. Population size > 100 animals.
3. Random sampling.
4. Random distribution of infection.

$$\log\ 0.05/\log\ N = \text{Sample Size}$$

N = % of noninfected animals
0.05 = 95% confidence level relation of sample size to prevalence rate

26.8.5 Test Frequency

The frequency of health monitoring usually depends upon the prevalence of infectious agents and their biological characteristics. Frequent testing is recommended for highly infectious agents. The health monitoring frequency may also indicate the potential impact of an agent on a research program underway. The current FELASA recommended the minimum test frequencies [15, 17]. The sampling frequency highly depends upon many biological considerations. A minimum of 14 to 21 days from the time of infection requires serological estimation. This minimum duration is mostly needed to get the antibody titer in the serum of the infected animal. However, depending upon the rate and method of transmission, the

additional time is also required for the animal to show a detectable antibody titer and to reach maximum morbidity. In conclusion, at least a 3–6 weeks period of time may be required from the introduction of the agent and to produce sufficient serologic evidence of the organism in animals.

26.9 Diagnostic Methods

The serological test is mainly used for the detection of microbial infections in animal populations including flow cytometry immunoassay and immunofluorescent assay. Other methods such as DNA analysis using polymerase chain reaction (PCR), clinical chemistry, microbial culture, histopathology, and immunohistochemistry are also applied to confirm the diagnosis [18].

26.9.1 MFIA

Multiplex diagnostic system that uses fluorescent microbeads coated with purified antigens is helpful for the simultaneous serodetection of various infectious agents [18].

26.9.2 ELISA

The most common method used to assess the presence of viral agents in serum for the presence of specific antibodies. The viral antibodies are mostly detected by using the ELISA method. The ELISA and IFA are sensitive methods and primarily employ for most of the viral agents and mycoplasma.

26.9.3 IFA

It is often used as a confirmatory rather than the primary assay. The main strength of the IFA test is the evaluation of fluorescence morphology and location in the interpretation of reactions. The IFA detects cross-reacting antibodies or rodent parvoviruses (MVM, KRV, and H-1) in a better way as compared to standard ELISA. The reaction produces strong nuclear fluorescence which is very characteristic [19].

26.9.4 PCR

The PCR technique is the most reliable method for the detection of *Helicobacter* spp. [20, 21], CAR bacillus, and *Pneumocystis* spp. in laboratory animals.

26.9.5 Necropsy and Histopathology

After euthanizing the animals, the necropsy usually performs for the systematic examination of the carcass for abnormalities and the examination of organs for the appearance of any gross abnormalities. However, in routine, if the organs look normal grossly, the histopathology is not necessarily required. The additional appropriate methods should be further employed to know the etiology of gross changes. The infection of extraintestinal bacteria such as *Klebsiella* spp. or uncultivable bacteria *Clostridium piliforme* can be checked by histopathology. The presence of lesions may support positive serology results or it may also reveal about the organisms not included in routine screening programs. This is especially important in the case of immunocompromised animals which have a chance of opportunistic infections by a variety of organisms. The presence of ectoparasites should be checked by a careful examination of the skin and fur of animals. However, endoparasites can be diagnosed by a variety of techniques like PCR, direct visual examination, or smears of intestinal contents by employing floatation and microscopy of feces. The adhesive tapes can be used for sampling around the anus.

The description of parasites should be likely according to the species name. For the detection of most bacterial and fungal agents, cultural techniques should be employed. The samples are generally collected from the genital mucosa, the large intestines, nasopharynx, and trachea; however other appropriate sites may be used for sampling depending upon the requirement. If the lesions are presumed to be of bacterial origin, it should be cultured [9, 22].

26.10 Interpretation of Health Monitoring (HM) Test Report

It is highly expected to verify any positive results by using other methods at least as specific as the first one. The results ought to be confirmed by performing repeated sampling and testing of sentinels or resident animals. It is advisable that if there are positive results, then these results should also be confirmed by another laboratory. Many findings revealed that the results obtained from different diagnostic laboratories may differ. The differing or borderline results need to be further investigated before concluding on the agent status. A health management action plan should be put in place while waiting for confirmatory results to avoid the potential spread of contaminants. Confirmation processes in progress should be mentioned as additional information in the HM report.

Each assay has the probability of getting false-positive and false-negative results. In such condition, the prevalence data may support the estimation of the predictive value of test results. The rare agents are less anticipated to be observed in a population and therefore less likely to produce true positives. During the serological testing of several sera. Generally, the presence of antibodies in animal serum implies a current or previous infection, but there is a chance that it may also be due to vaccination, maternal antibodies, and cross-reaction. Indeed, the uncertainty of false-

positive results may also exist with PCR, for example, due to sample contamination or nonspecific amplification of DNA [6].

26.11 Prevention of Disease Outbreaks: Do's and Don'ts

1. Do quarantine animals received from outside, and observe to reduce the direct spread of an agent.
2. Do have containment barriers at the room or facility level.
3. Do wear proper personal protective equipment like gloves, masks, caps, gowns, shoe covers, etc.
4. Do use IVC or micro-isolator cage systems to limit airborne transmission.
5. Do use animal cage changing stations for the animal procedure which helps to prevent airborne particles.
6. Do thorough cleaning and disinfection of equipment used and areas after each use, e.g., procedure tables and anesthetic machines.
7. Do change PPE between different species of animals or groups of animals as required.
8. Do screening of agents before quarantine release of animals into the animal holding rooms of the facility.
9. Do strictly follow facility SOPs/guidelines.
10. Do replace the old water feeder/bottle with a new one during cage changing.
11. Do not lift/open IVC outside the cage changing station/laminar airflow.
12. Do not refill water bottles.
13. Do not transfer back the rodents that have jumped out onto the floor back into their cages.
14. Do not allow movement from contaminated areas of the facility to uncontaminated areas.
15. Do not use the same instrument for surgery on two different animals without sterilizing them in between.
16. Do not transfer leftover feed from one animal cage to the other cage.
17. Do not swap enrichment devices between cages without sterilizing them.

26.12 Production Methodology of SPF Laboratory Animals

The animals free of contamination or having a minimum contaminant are the main objective behind the production of SPF animals. The production of these animals is achieved by cesarean section. The placenta serves as a very efficient barrier and checks the fetus from becoming infected with most bacteria, viruses, and parasites. Therefore, the most satisfactory and recommended system for getting the SPF animals is first raising them by germ-free methods and maintaining them under pathogen-free conditions.

There are various constraints in managing these SPF animals, especially in the pretext of nutrition and fertility. The germ-free conditions have to be strictly maintained by establishing controlled contamination. Therefore, the animals are required to be housed in IVC system that helps in maintaining a super clean status and then transferred to a minimal disease breeding area from which they will supply for the experimental purpose. The weaned animals not used for the breeding replacements are not kept in the breeding unit, while they are transferred to the stock room in the experimental area. The utmost care has been taken during the transfer of these animals to avoid contamination. The movement of animals should be unidirectional as it moves from the highest clean environment to minimal pathogen status unit. Reverse movement of animals is strictly prohibited to prevent cross-contamination [13].

Decontamination from specific pathogens of the low-barrier colonies of laboratory rodents is achieved by the employment of the rederivation method. This process finally helps in converting these colonies to the SPF state. The animals with these specific pathogens should be housed in a low-barrier facility with all strict biosecurity measures. Subsequently, an optimized rederivation model of laboratory animals comprised of a series of embryo-technological methods can be employed. The sequelae include the freezing and cryopreservation of embryos; their decontamination by washing in sterile media; cultivation for 48 h; then finally, transfer to recipients with the SPF state. After obtaining these animals, strict health monitoring surveillance should be carried to check the pathogen status and then transferred to a high-barrier facility if it meets the pathogen status of the high-barrier facility.

26.13 Conclusion

The SPF animal facilities are constructed with design features that enhance the welfare of the animals, support research, facilitate the management of operations, and provide biocontainment capabilities. There should be regular monitoring of environmental parameters within the facility that can affect the animals and impact experimental data. Humidity, temperature, air, light, and noise must be monitored and controlled. Maintaining an ideal environment for the animals also requires attention to their enrichment and social interactions. Sanitization, disinfection, sterilization procedures, and equipment must be appropriate for the animal colonies as well as research needs. The systems needed to manage animal quality in support of the research are complex and require highly trained, motivated, and competent laboratory animal technicians.

References

1. Laaldin N, Baloch SR, Noor A, Aziz A, Gul A, Rajput TA, Babar MM (2020) Animal models: bridging cross-species variation through animal biotechnology. In: Genomics and biotechnological advances in veterinary, poultry, and fisheries. Academic Press, Cambridge, pp 183–207

2. Schlapp G, Fernandez-Grana G, Arevalo AP, Crispo M (2018) Establishment of an environmental microbiological monitoring program in a mice barrier facility. Anais da Academia Brasileira de Ciências 90(3):3155–3164
3. Sztein JM, Kastenmayer RJ, Perdue KA (2011) Pathogen-free mouse rederivation by IVF, natural mating and hysterectomy. In: Advanced protocols for animal transgenesis. Springer, Berlin, Heidelberg, pp 615–642
4. Burkholder T, Foltz C, Karlsson E, Linton CG, Smith JM (2012) Health evaluation of experimental laboratory mice. Curr Protoc Mouse Biol 2(2):145–165
5. Shek WR (2008) Role of housing modalities on management and surveillance strategies for adventitious agents of rodents. ILAR J 49(3):316–325
6. Mähler M, Berard M, Feinstein R, Gallagher A, Illgen-Wilcke B, FELASA Working Group on Revision of Guidelines for Health Monitoring of Rodents and Rabbits et al (2014) FELASA recommendations for the health monitoring of mouse, rat, hamster, guinea pig and rabbit colonies in breeding and experimental units. Lab Anim 48(3):178–192
7. National Institute of Health Office of Research Facilities. (2003) NIH Design Policy and Guidelines, Animal Research Facilities.
8. Health and Safety Services. Biological and Genetic Modification Safety Policy. Health and Safety Services. [Online] http://www.bris.ac.uk/safety/biosafety/#policy
9. Chosewood LC, Wilson DE (2009) Biosafety in microbiological and biomedical laboratories. In: US Department of Health and Human Services, Public Health Service, Centers for Disease Control and Prevention. National Institutes of Health
10. National Research Council (2010) Guide for the care and use of laboratory animals. National Academies Press
11. Kimura T (2012) Effective decontamination of laboratory animal rooms with vapour-phase (‘vaporized’) hydrogen peroxide and peracetic acid. Scand J Lab Anim Sci 39:17–23
12. Parker A, Wilfred AG, Hidell TB (2003) Environmental monitoring: the key to effective sanitation. Lab Anim 32:1–9
13. Weichbrod RH, Thompson GAH, Norton JN (eds) (2017) Management of animal care and use programs in research, education, and testing. CRC Press
14. Leszczynski JK, Tackett J, Wallace-Fields M (2018) Basic animal facility management. In: Management of animal care and use programs in research, education, and testing. CRC Press/ Taylor & Francis, Boca Raton, FL
15. Shakthi Devan RK, Parthiban N, Samarendra N, Suresh P (2011) Sentinel monitoring program: quality assurance in laboratory animal facility. J Lab Anim Sci 1(1):68–76
16. de Bruin WCC, van de Ven EME, Hooijmans CR (2016) Efficacy of soiled bedding transfer for transmission of mouse and rat infections to sentinels: a systematic review. PLoS One 11(8): e0158410
17. Nicklas W (2008) International harmonization of health monitoring. ILAR J 49(3):338–346
18. Burkholder T, Foltz C, Karlsson E, Linton CG, Smith JM (2012) Health evaluation of experimental laboratory. Curr Protoc Mouse Biol 2:145–165
19. Khan IH, Kendall LV, Ziman M, Wong S, Mendoza S, Fahey J, Griffey SM, Barthold SW, Luciw PA (2005) Simultaneous serodetection of 10 highly prevalent mouse infectious pathogens in a single reaction by multiplex analysis. Clin Diagn Lab Immunol 12:513–519
20. Baker DG (1998b) Natural pathogens of laboratory mice, rats, and rabbits and their effects on research. Clin Microbiol Rev (2):11, 231–266. Baker DG (2003)
21. Fox JG, Dewhirst FE, Tully JG, Paster BJ, Yan L, Taylor NS, Collins MJ, Gorelick PL, Ward JM (1994) Helicobacter hepaticus sp. nov., a microaerophilic bacterium isolated from livers and intestinal mucosal scrapings from mice. J Clin Microbiol 32:1238–1245
22. Shek WR, Smith AL, Pritchett-Corning KR (2015) Microbiological quality control for laboratory rodents and lagomorphs. In: Laboratory animal medicine. Academic Press, Boston, MA, pp 463–510

Good Laboratory Practice (GLP) Requirements for Preclinical Animal Studies 27

K. Srinivasan, K. Tikoo, and G. B. Jena

Abstract

There are persistent efforts to discover newer drugs to address huge unmet needs in the treatment of various disease conditions. The process of drug discovery and development involves both the preclinical and clinical studies which are very challenging and have huge commercial interests. Following the scientific malpractice in the pharmaceutical industries and contract research laboratories in the USA, the "good laboratory practice (GLP)" regulations were enforced in the arena of preclinical drug testing in the late 1970s. In 1981, the Organization for Economic Cooperation and Development (OECD) also developed its GLP principles which are internationally accepted. Given the public health and environmental safety concerns, various regulations and guidelines emphasize the compliance for GLP in the preclinical or nonclinical health and environmental safety studies. GLP assures the regulatory authority that the nonclinical safety data they receive from the sponsors are of high quality and accuracy that can be relied on in the risk assessment process for the product registration. The accreditation and compliance for GLP are voluntary for the test facilities and are required in the case of the regulatory submission. The present chapter covers the overview of the principles of GLP with the primary focus on requirements related to the *test system* in preclinical animal studies during the new drug development.

K. Srinivasan (✉) · K. Tikoo · G. B. Jena
Department of Pharmacology and Toxicology, National Institute of Pharmaceutical Education and Research (NIPER) (Autonomous Body Under Ministry of Chemicals and Fertilizers, Government of India), Mohali, Punjab, India
e-mail: ksrinivasan@niper.ac.in; tikoo@niper.ac.in; gbjena@niper.ac.in

© The Author(s), under exclusive license to Springer Nature Singapore Pte Ltd. 2021

P. Nagarajan et al. (eds.), *Essentials of Laboratory Animal Science: Principles and Practices*, https://doi.org/10.1007/978-981-16-0987-9_27

Keywords

Good laboratory practice (GLP) · Safety · Preclinical animal studies · Drug development

27.1 Introduction

Despite the great advancement in present healthcare systems, there persist unmet medical needs across the wide array of disease conditions. The leading pharmaceutical companies continue to make efforts to discover and launch new drugs into the market through the "drug discovery and development" process [1]. Unfortunately, there have been issues of serious harm owing to the drugs containing toxic impurities, adulterated, fake/counterfeit drugs, or drugs with unknown serious adverse reactions. Industrialization and urbanization over centuries coupled with uncontrolled use of industrial chemicals have also caused the frequent exposure of people which have, at times, led to the episodes of accidental mass poisoning [2]. Many countries have laid down laws and regulations to control and oversee the development and distribution of pharmaceuticals, cosmetics, and feed additives, on the one hand, and industrial chemicals, pesticides, and other similar products, on the other hand. Regulations require that every manufacturer or sponsor must establish data through a battery of scientific studies to ascertain that the products are efficacious and do not pose any hazard to humans and the environment. It is known since ancient times that there is a significant relationship between therapeutic and toxicological effects of a chemical that forms the basis of the famous adage of Paracelsus (1493–1541) *sola dosis facit venenum* (Latin) which means "the dose alone makes the poison." It states that "all the substances are poisonous and it is the right dose that differentiates a poison and a drug" [3]. Hence, the utility of any new chemical or product is subject to benefit-risk assessment which is a cornerstone for the drug approval process. The preclinical animal studies play a significant role in drug development, and outcomes from these studies greatly help in defining safe human doses for clinical trial and disease management [4–7]. The safety studies also provide a scientific rationale for public health guidance that defines maximum allowable concentrations of diverse contaminants in drinking water, food, and the environment. Deeply concerned with the quality of safety data owing to the public health and environment protection, various regulations and guidelines mandate that the preclinical or nonclinical health and environmental safety studies of chemicals intended for the regulatory submission should be carried out under the robust quality system known as "good laboratory practice" (GLP) [8–14]. The present chapter covers the overview of the principles of GLP with the main focus on test system requirements in preclinical animal studies during new drug development.

27.2 Drug Discovery and Development

The discovery and development of a new drug are exhaustive, time-consuming, and risky business as the entire process from the discovery to licensing of the drug into the market can take 10 to 15 years costing pharmaceutical companies billions. Approximately, for every 9000 to 10,000 molecules synthesized or isolated as potential drug candidates, only 1 (on average) would eventually qualify as a drug reaching the market. Though there are several reasons for the failure and attrition of the success rate of molecules during drug development, a significant proportion of them fails due to safety reasons [15, 16]. Drug discovery involves many steps such as target identification and validation, hit identification, and lead identification and optimization for identifying potential drug candidates for further development. In the drug discovery program, the pharmaceutical industries generally follow either the phenotype- or the target-based approach [17]. Both these approaches differ in the way that leads to therapeutic target and lead identification. In the phenotype screening approach (forward or classical pharmacology), the scientists screen the chemical libraries of compounds using validated bioassays (intact cells or whole organism models) to identify molecules that produce a desirable response in the phenotype. In this approach, the compounds are first discovered, and then an effort is made to identify the biological target of the compounds. But in the case of the target-based approach (reverse pharmacology or reverse chemical biology), the high-throughput screening of the compounds is carried out to find potential drug candidates that bind with the new or specific established target which is mostly proteins (membrane or nuclear receptors, enzymes, transporters, ionic channels, etc.) linked to the human disease [18]. Target identification and validation constitute the early steps of the target-based approach which became so popular after the human genome sequencing and the advent of the "omics" technologies like genomics, proteomics, and metabolomics. Apart from in silico and in vitro methods, the use of genetically modified whole animal models (knockouts and transgenics) allows for the studies on the phenotypic changes following the genetic manipulation and validation of the target [19].

Drug development encompasses both preclinical and clinical investigations that transform a drug candidate from the discovery phase to a product licensed for marketing by the appropriate regulatory authorities. The preclinical evaluation of a drug candidate consists of in vitro, ex vivo, and in vivo pharmacological and toxicological assays. Importantly, the preclinical animal toxicology/safety studies that can be performed throughout all stages of drug development help in the assessment of the various toxicological properties of the compound that include single-dose acute and repeated dose toxicity studies, safety pharmacology, genotoxicity, reproductive and developmental toxicity, carcinogenicity, and other safety studies as depicted in Fig. 27.1. It also helps in the identification of a safe dose level for human exposure by the suitable extrapolation of animal dose for initiating the clinical trials [20, 21]. Toxicology studies should be conducted following national or international regulatory guidelines under the norms of GLP for enabling

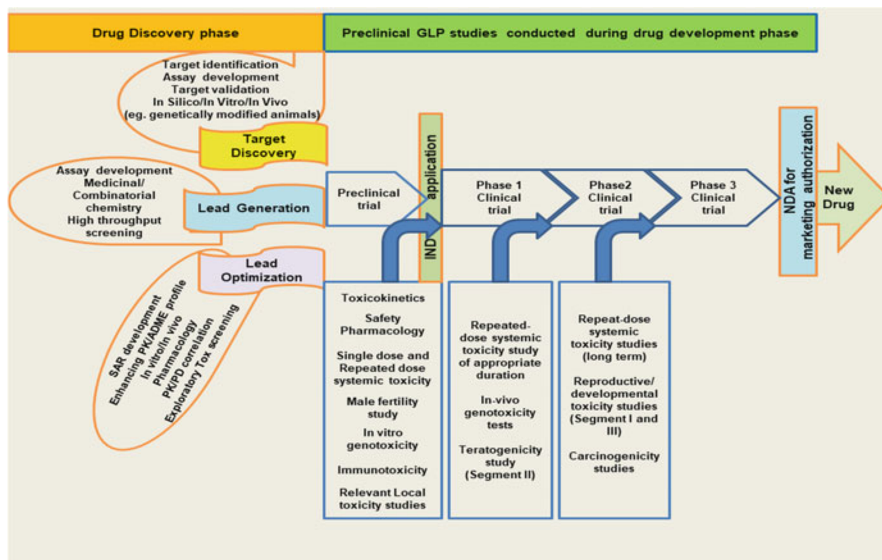


Fig. 27.1 Overview of the regulatory requirement of GLP preclinical animal studies for undertaking different phases of clinical trials and marketing of a new drug during drug development

the submission of the investigational new drug (IND) and new drug applications (NDA) to the regulatory authority.

27.3 Brief History of Drug Regulations and Good Laboratory Practice

The federal laws, regulations, and standards set for the drug approval process in the United States are considered to be more stringent and demanding as compared to that of the rest of the world. In 1820, the pioneering efforts in drug regulations led to the establishment of US Pharmacopeia, the first compendium of standard drugs for the United States. In 1906, the Pure Food and Drugs Act was promulgated that required every drug must satisfy the official standards of composition, strength, and purity. The use of animals in the testing of human pharmaceuticals dates back to 1937 triggered by the toxicity of sulfanilamide elixir (a liquid formulation containing sulfonamide antibiotic dissolved with toxic solvent 73% diethylene glycol) causing deaths of hundreds of adults and children. This prompted the passage of the 1938 US Federal Food, Drug, and Cosmetic Act (which superseded the Pure Food and Drugs Act, 1906) requiring some animal testing and premarketing evidence of safety for new drugs. Yet another tragedy that shocked the whole world was the multiple incidences of the rare birth defect, phocomelia in babies (majorly in Europe and also in other countries like South America, Canada, and Australia) linked with thalidomide, a sedative-hypnotic that was then prescribed for treating morning sickness in

pregnant women. Subsequently, the landmark "Kefauver-Harris Amendment Act" in 1962 demanded the substantial proof of safety and also the efficacy of drugs before granting NDA that marked the start of the FDA approval process in its modern form [1, 22, 23]. Thalidomide disaster demonstrated for the first time the interspecies variability in drug response as mice, conventionally used for screening, are found less sensitive to thalidomide toxicity as compared to other species such as rabbits and nonhuman primates. This had necessitated the conduct of toxicity studies on fetal development in two species, a rodent and non-rodent (mostly the rabbit due to its response to thalidomide) to cover the likelihood of either one of these species might be predictive of response in humans [24, 25]. After the "thalidomide disaster," the regulatory requirements for safety testing of pharmaceuticals became stringent all over the world. In the late 1970s, enhanced public concerns and the complaints surrounding the safety of the chemical products prompted FDA to make series of investigations in leading pharmaceutical companies and testing laboratories that uncovered multiple incidents of scientific misconduct and data fraud. The most prominent case was that of Industrial Bio-Test (IBT) Laboratories, the largest contract research organization (CRO) in the world at that time, conducting approximately 35–40% of all safety studies in the United States. More than 22,000 toxicology studies had been conducted during its existence, mainly supporting the assessment of pesticides and other chemicals, pharmaceuticals, and cosmetics [26]. FDA investigation at IBT in April 1976 had exposed serious violations of sound scientific conduct that included horrible animal husbandry practices, including constant cage flooding and drowning of animals by automatic watering systems and frequent escape of animals. Further, there were poor record-keeping, poorly trained study personnel, poor study plan design, inadequate characterization of the test item and test system, poor health of animals, improper calibration of equipment, replacement of dead animals with naive animals with no documentation of replacement, test animals receiving incorrect doses or wrong test article, the inclusion of data of urine and blood samples without actually collecting them, laboratory animals first reported as dead and later shown as alive, blatant data fabrication, fraud, and even the destruction of records. Approximately 75% of the over 900 studies audited at IBT were invalidated. Marketed products from invalidated studies had to be suspended until new studies were conducted to ascertain the safety of products. The horrifying experience from this investigation led to the establishment of a rigorous quality system, the so-called "good laboratory practice (GLP)." The GLP regulations for FDA under the provision of the Federal Food, Drug, and Cosmetic Act was proposed and enacted by US Congress on November 19, 1976, and published in the Federal Register as a final rule on December 22, 1978, as 21 CFR Part 58 good laboratory practices for the nonclinical studies. The US environmental protection agency (EPA) also faced similar problems with the nonclinical safety data it received for the permission of products like pesticides and industrial chemicals. EPA also adopted GLP regulations and published the final rules in 1983 in two parts, 40 CFR 160 and 40 CFR 792 which describe GLP for performing studies on health and environmental hazards, pesticide residues in food and water, and chemical fate testing. Since then, it has become mandatory for every manufacturer or sponsor to

apply GLP in studies required for the regulatory submission and product registration in the United States [9, 11, 27–30].

27.4 International Recognition of GLP

Consequent to US FDA GLP regulations, many countries have realized the need for GLP, and the international harmonization of these standards became the topic of debate and need of the hour to avoid nontariff trade barriers for the chemicals between countries. The Organization for Economic Co-operation and Development (OECD), an intergovernmental organization consisting of representatives of 37 industrialized countries (from Europe, South and North America, the Asia and Pacific region, and the European Commission) which regularly meet to discuss the issues of mutual concern, coordinates and harmonizes policies and work together to respond to international problems. GLP was one of such international issues discussed by OECD, culminating in the establishment of "The OECD Principles of Good Laboratory Practice." These principles were recommended for the use in their member countries in 1981 as per the OECD council decision on Mutual Acceptance of Data (MAD). It stated that "data generated in the testing of chemicals in one OECD member country following OECD test guidelines and OECD principles of GLP shall be accepted in other member countries for purposes of assessment and other uses relating to the protection of man and the environment" [C(81)30(Final)]. Recently, the OECD revised the principles of GLP in 1997 which superseded the original principles adopted in 1981 [8, 31]. Besides the GLP principles, the OECD has published the various guidance documents under "the OECD series on principles of GLP and compliance monitoring" for the test facilities as well as monitoring authorities either as consensus documents (e.g., study director responsibilities, quality assurance unit), advisory documents (e.g., sponsor responsibilities, test item characterization), and other GLP-related issues [32]. Further, the OECD has published test guidelines for harmonizing the scientific conduct of various studies which are split into five series, namely, physical-chemical properties, effects on biotic systems, environmental fate and behavior, health effects, and other test guidelines. The OECD test guidelines are globally considered as the standard reference tool for chemical testing. Section 4 of health effects mainly covers the guidelines related to different preclinical safety studies. Importantly, some of the revised OECD guidelines have revolutionized animal testing by promoting animal welfare and three Rs (replacement, reduction, and refinement) principles. For example, the much-criticized test guideline 401 (LD50 acute toxicity testing) being superseded with test guidelines such as 420, 423, and 425 that require only a very small number of animals, replacement of the conventional animal skin, and eye irritation/corrosion studies with some non-animal alternative test methods and also the release of the guidance document towards the refinement of safety testing with the use of clinical signs as humane endpoints in the experimental animals [33].

Because of the growing internationalization of research and testing, an important OECD council decision in 1997 had extended and set out a stepwise procedure for non-OECD countries/economies to take part as full adherent members in this MAD system [34]. In India, National GLP Compliance Monitoring Authority (NGCMA) was established by the Department of Science and Technology, consequent to the approval of the Union Cabinet on April 24, 2002. India, after being in the observer/provisional status, became full adherent to the MAD system in the OECD's working group on GLP since March 3, 2011 [35]. It was a major boost to Indian industries since data generated henceforth by Indian GLP-certified test facilities (by NGCMA) shall be acceptable for the assessment in all OECD members and other full adherent countries. The main driving force for every GLP compliance monitoring authority is to assure the receiving or regulatory authorities (e.g., US FDA and EPA in the United States, EMA and ECHA in EU, PMDA in Japan, and CDSCO and CIB in India) that the data of safety studies from the GLP-certified test facilities can be relied upon during the assessment of risks or hazards to humans, animals, and/or the environment [29, 36–39].

MAD system and GLP helps (a) ensure high quality, integrity, and validity of safety test data submitted in support of new drug or product registration; (b) assure data accuracy, reproducibility, and consistency; (c) reduce the chances of unreliability and fraud and provide confidence regarding the authenticity of preclinical safety data in hazard and risk assessment to regulatory authorities; (d) allow for the auditability and reconstruction of the entire study even years after the study is completed; (e) ensure comparability of results and promote mutual recognition of results and global acceptability; (f) limit wastage of resources and reduce time and costs for industry, government, and other stakeholders; (g) prevent duplication of studies, reduce the use of animals, and promote animal welfare considerations, (h) facilitate the exchange of information and remove nontariff trade barriers; and (i) promote safety towards the protection of human health and environment [30, 40].

27.5 Overview of GLP and General Requirements

GLP is defined as a quality system concerned with the organizational process and the conditions, under which nonclinical health and environmental safety studies are planned, performed, monitored, recorded, archived, and reported [8]. It is an overall quality management system rather than the scientific framework concerning only the organizational process and the conditions, but the basic concept of GLP however, can complement and lead to better science through a more organized way of study performance. The ultimate purpose of GLP is to promote and maintain the quality of safety test data through meticulous documentation, encompassing the study process step by step and its environment. These principles of GLP apply to all nonclinical health and environmental safety studies conducted in the laboratory, field, and greenhouses to meet the regulatory requirements in the assessment for the registration of pharmaceuticals, cosmetic products, food/feed additives, veterinary drugs, medical device and for the regulation of pesticides, industrial chemicals, and similar

products. Often, the test item is of synthetic origin but may also be of natural or biological sources including living organisms. GLP is, however not a regulatory requirement for basic research, exploratory drug discovery studies, pharmacological screening/efficacy and bioequivalence, pharmacokinetic/absorption, distribution, metabolism, and excretion (ADME) studies where there is no assessment for the safety of the drug. GLP also does not apply to pharmaceutical manufacturing and human clinical trials where yet another quality systems of good manufacturing practice (GMP) and good clinical practice (GCP), respectively, are to be used [20, 28, 41, 42]. The following are the GLP principles as specified in document No. 1 (ENV/MC/CHEM (98)17) of OECD which provides an internationally accepted view of GLP [8]:

- Test facility organization and personnel including the responsibilities of test facility management, sponsor, study director, principal investigator, and study personnel.
- Quality assurance program including the duties of quality assurance personnel.
- Facilities in general and for test systems and test and reference items.
- Equipment, reagents, and materials.
- Test systems (physical-chemical and/or biological).
- Test and reference Items.
- Standard operating procedures for different activities of the test facility.
- Performance of study which includes the content of the study plan and conduct of the study.
- Reporting of study results.
- Storage and retention of records and materials.

At the outset, the primary requirement is of the "test facility" which is defined as "the persons, premises and operational unit(s) that are necessary for conducting the nonclinical health and environmental safety study." The test facility is required to meet the GLP principles before they can be considered as GLP compliant by the compliance monitoring authorities. There is no such standard design or size for the construction of the facility as it depends on the scope of institutional activities, type, and specific requirements of studies. The test facility should be suitably constructed and established on a location that minimizes the interference to the test system and environment affecting the validity of the study. The test facility may be designed simply for testing the physical-chemical properties of the test items involving only analytical instruments or complex nonclinical safety studies involving animals as test systems. In general, the test facility should have a sufficient number of rooms and areas for allowing an adequate degree of separation of the various operations to assure the proper conduct of each study. The suitable rooms and areas may include for housing test systems, receipt of materials, storage for bedding, diet and other supplies, test item receiving and preparation room, laboratories for various analysis/testing, necropsy, the archive for dry and wet materials, washing, utilities, shower room, sterilization/autoclaving, waste disposal, clean and dirty corridors, etc.

Test facility management (TFM) has the formal authority and responsibility for the organization and functioning of the test facility in compliance with GLP

principles. TFM should ensure that the studies are conducted free from influences of any external source that could affect the compliance and conclusions of the studies and/or the test facility [43]. TFM ensures the availability of an adequate number of qualified and trained personnel, appropriate facilities, equipment, and other materials for the proper and timely conduct of the study. For each study from the sponsor, an individual with the appropriate qualifications, experience, and training from the pool of study personnel is appointed by the TFM as the study director. The study should be performed following the approved study plan and standard operating procedures (SOPs). The study plan is a document containing the objectives and experimental design for the conduct of the study whereas SOPs represent the documented procedures that describe how to perform tests or activities normally not specified in detail in the study plan or test guideline. For example, the receipt, health examination, handling, identification, necropsy procedure of the test system (animal), analytical methods, equipment operations, test item characterization, and many other functioning related to the TFM, quality assurance, and archiving are not usually incorporated in detail in the study plan but are prepared as SOPs. The relevant SOPs for the study and test facilities should be written by the concerned individual(s)/study personnel that shall be approved by TFM before its implementation. Although GLP does not specifically point out the content and quantum of the details to be incorporated in the SOPs, it is the responsibility of TFM to ensure that SOPs adequately cover all the appropriate and technically valid procedures as there is a likelihood that the study may be rejected by the regulatory authority based on the technical ground (e.g., the application of insensitive and obsolete techniques or methods in the study). The study plan is approved by the study director with the dated signature, and it should also be approved by the TFM and the sponsor if required by national legislation in the country where the study is being performed. Regardless of national regulations, it is a good practice to get the study plan also signed by the TFM and sponsor to ensure acceptance by all the concerned stakeholders before the study initiation. The revised OECD GLP principles require that the study plan should indicate the test method (OECD or any other test guidelines or method) adopted and state whether the scientific conduct of the study complies with GLP or not. Once the study plan is signed, the study should be entered on the master schedule sheet in a stipulated format. The master schedule is nothing but the compilation of the salient information of all the completed and ongoing studies being updated over a specified period that helps TFM in the tracking of study and further assessment of the current workload at the test facility.

The study director is the single point control with ultimate accountability to ensure that the scientific, regulatory, and administrative aspects of the study are controlled. His or her responsibility cannot be shared with anyone as there is no such role or provision for deputy study director in OECD principles of GLP. However, it allows for the replacement of the study director as and when necessary by TFM to be done and documented as per the established procedures. In case of a multisite study or when the phase(s) of the study is delegated to an outside location(s) (“test site”), the study director of the test facility cannot go directly often to monitor, and the person called the “principal investigator” may be designated by TFM (on the behalf of study director) with the defined responsibilities for delegated phases of the study at

the test site in consultation with the sponsor, study director and test site management. Again, the responsibility of the study director for the overall conduct of the study cannot be delegated to the principal investigator(s) that includes the approval of the study plan, amendments, and the final report. Whether the study is conducted in single or multisite, it should be ensured that there is one study director, one study plan, and one study report for any given study. The study director is eventually responsible for the scientific validity of data and the GLP compliance of the study report confirming with his or her signed and dated statement on the extent of GLP compliance [30, 44, 45]. There should be separate facilities and documented procedures for the receipt, handling, storage, labeling, and sampling of the test item under the study. Test item identification and characterization are important for GLP compliance and should be properly documented in the study plan and study report [46]. It also requires the determination of the stability, homogeneity, and concentration of the test item in the vehicle if it is administered in the vehicle as formulation. Likewise, the chemicals, solutions, and reagents used in the study should be appropriately labeled with the details of identity, source, expiry or retest date, date of preparation, storage condition, etc. GLP requires that the appropriate validation, calibration, and periodic maintenance of equipment including the computerized systems are mandatorily done in the process of the generation, storage, and retrieval of data and for controlling environmental factors relevant to the study as per the SOPs.

The study personnel are responsible for the quality of the study data collected and for recording them promptly and accurately. If any change or correction is to be made on raw data, it can be done by the individual with his or her dated initial without obscuring the previous entry (just cutting across the wrong entry in such a way that it is still readable) by citing the reason therein for the change. In case of any deviation (unintended changes) from the study plan or SOP during the conduct of the study, the impact of this deviation on the quality and integrity of study outcome should be assessed and recorded by the study director, and appropriate corrective action needs to be taken accordingly. In case of any amendment to the study plan (intended changes), it should be justified and primarily approved by the study director and readily communicated to concerned study personnel, quality assurance unit, and stakeholders concerned as per SOP. If the amendment involves additional animals or different procedures, the necessary approval of the animal care and ethics committee should also be obtained. The approved amendment copy may be annexed to the relevant study plan and/or SOP preferably on the front side so that it may be easily seen and followed by all the personnel. It is of paramount importance for the study personnel to take care of all the safety and health precautions to minimize the risk to themselves and to maintain the integrity of the study. In the event of any relevant health complaint, it should be reported to the appropriate person as per the SOP so that the concerned study personnel can be excluded from the operations that may affect the study validity.

GLP requires an internal quality assurance (QA) program to inspect and audit studies in compliance with GLP [47]. The QA program is undertaken by a single or group of few individuals as designated by TFM. They directly report to and are

responsible for assuring the TFM of GLP compliance of the studies being performed. GLP has not specified any qualifications or experience for QA persons but stated that these individual(s) need to be familiar with the test procedures and should not be involved in the conduct of the study being assured. Although it is not mandatory as per GLP, TFM may assign QA personnel to review SOPs written by the different facility personnel for their GLP compliance before being approved by TFM. However, QA personnel are responsible for reviewing and verifying the study plan to ensure that it covered all the necessary information required for GLP compliance before being approved by the study director. QA persons generally conduct different types of inspections (study-based, facility-based, and process-based) as per their written SOP. They audit the final report to verify that the materials, methods, and observations are properly described and the results reported there entirely and accurately reflect the raw data of the studies. Besides the study director's statement on GLP compliance, it is also important to include the signed statement from the QA person in the final report to confirm that the study is the reflection of raw data, specifying the types and their dates of inspections, etc. Altogether, the GLP principles require that all the information and the data sought by the study plan should be incorporated in the study report. After the study is completed, the copies of the final report are distributed to the concerned stakeholders including the original copy to the sponsor. It is the responsibility of the study director that all the study-related documents and materials like the draft and final study plan/report, logbooks, test items, specimens, etc. are archived promptly [48–50].

The archive facility is again a unique structure of GLP unlike the non-GLP environment and is meant for providing the safe, secure storage, and retrieval of different dry and wet materials related to the study conducted in the test facility. Once the study materials are archived, it becomes the responsibility of TFM, and no one can access the archived materials including the study director or inspectors without the necessary permission from TFM. Only the authorized person(s) is permitted to enter and access this area, and the movement register of the material in and out of the archives should be properly maintained. As all the study-related materials are safely stored under the custody of a designated person of TFM (archivist), the study reconstruction (a unique feature and advantage of the GLP system) is possible reasonably at any time even years after the completion of the study whenever required. The archival period of various documents, raw data, test items, specimens, etc. varies and needs to be followed as per SOPs or as defined by the regulations of the respective country. It is beyond the scope of this chapter to describe every GLP principle that may be referred to elsewhere [8]. It primarily focuses on the GLP requirements related to the "test system" and associated test system facilities in the conduct of pre−/nonclinical health and safety studies.

27.6 Test System and Its Facilities

The test system means any biological, chemical, or physical system or a combination thereof used in a study. Biological test systems are often the animals, but they can also be bacteria, cells, organs, and plants. There are different types of animal test

systems including the vertebrate and invertebrate species employed in the nonclinical health and environmental safety studies such as rodents (e.g., rats, mice, hamsters, guinea pigs), non-rodents (rabbit, dog, monkey), birds, zebrafish, earthworm, higher aquatic plants, etc. However, the following section specifically describes the GLP requirements where the test system is an animal used in preclinical safety testing during new drug development. Majorly, the conventional models of the rodent species (rats and mice) and non-rodents (rabbit, dogs, and nonhuman primates) are used as test systems while performing various preclinical safety evaluations as in Fig. 27.1. The specific details over the usage of different test systems (rodent and/or non-rodent species) and regulatory test guidelines/ methodologies for executing each of the aforesaid nonclinical safety investigations are beyond the purview of this chapter and may be referred elsewhere [10, 12, 33]. Besides, transgenic animal models are also used in chemical safety determination for its mutagenic and carcinogenic potential and while unraveling its underlying mechanisms [51, 52]. As it involves the use of animals, GLP requirements should also take into consideration the respective national animal care and welfare regulations/guidelines of the country where the experiments are being conducted [53–56].

27.6.1 Isolation of Test Systems and Studies

GLP requires that the test system facility should have a sufficient number of rooms or areas to allow for the physical separation or isolation of different test systems (animal species) to prevent disease transmission and to minimize the anxiety associated with interspecies conflict. Animals of the same species supplied by one supplier might likely be different from another supplier in terms of harboring microbial agents, and hence intraspecies separation is also warranted if procured from multiple sources having different microbiological status [54]. Besides the isolation of different test systems, isolation of individual projects by separately housing the animals of different studies is required to reduce the accidental administration of or exposure to the test item(s) which is not the subject of the study and to avoid the mix-up of animals that could compromise the quality and validity of either of the study outcome. It has been observed that some test substances or preparations are volatile or gaseous or known to form aerosols that led to cross-contamination of the test systems as well as the control samples from the naive animals when the different studies performed concurrently in the same room [57]. Isolation of the test system study-wise becomes extremely necessary while studying biohazardous agents as a test or reference item which may be of infectious organisms, carcinogenic or radioactive substances, etc. In such a case, there must be a special set up in the study room for handling the test system, and necessary approval as per the national regulatory requirements should be obtained, given the public health hazard and environmental protection. The best principle is always to have only one species from a single vendor housed in any one room and only one study conducted per room. If mixed housing of different animal test systems or studies in the same room

is necessary, the facility must provide adequate differentiation by space and special containment in the room (e.g., laminar flow cabinets or filtered or micro isolation cages or isolators or individually ventilated caging system and cage changing station) and unique identification for the suitable isolation to minimize the event of animal mix-ups, disease transmission, and cross-contamination.

Irrespective of the separation of test systems, all the animals newly procured from the outside sources for the study should be kept under isolation until their health status has been evaluated in a designated room, namely, “quarantine room” as per the established veterinary clinical procedure. Upon the arrival of the animals, it must be counted for the number of the animals and physically checked for the sex and weight of the animals and also verify the species, strain/breed, age of the animals with the supplier’s delivery document to ensure if it satisfies the specification of the animals as ordered and defined in the study plan. The records on the source, date of receipt, and the condition of test systems upon arrival should be maintained. The documents like animal order/issue forms, health certificates (freedom from pathogens and parasites if any), transport, and vendors’ invoices should be stored as raw data. A proper quarantining of these animals effectively decreases the likelihood of introducing pathogenic organisms into an established colony or the animals of ongoing studies at the test facility and also helps in limiting the transmission of zoonotic infections to humans if any. The accreditation status of the animal supplier if any along with its quality control measures and knowledge of the background history of the animals may limit the quarantine period of the animals necessary for the inspection upon arrival. However, the duration of quarantine varies from a week to 1 month for small animals and up to 6 weeks for large animals (dog, cat, monkey, etc.) which can be extended based on the type of infection or suspected infection in the experimental animals to allow them sufficient time for the expression of clinical signs of disease. In case of any unusual mortality or morbidity occurs, the particular lot of the animals should not be used in studies and, when appropriate, should be humanely destroyed as per the appropriate euthanasia procedure. Since any overt or subclinical infection in animals might complicate the outcome and interpretation of experimental results, the ways for their suitable detection and elimination should be done to ensure the optimal health and wellbeing of the animals during the study. For example, rodents may harbor internal parasites or some other pathogenic agents subclinically which may aggravate and produce clinical manifestations due to stress associated with test item effects or/and experimental procedure during the study. Hence, at the experimental starting date of a study, it should be ensured that test systems are free of any disease or condition that is likely to interfere with the goal or conduct of the study. Besides a health certificate from the veterinarian, it is always a good practice to set certain criteria (as user qualification) by the study director that the test system (animal) should qualify to justify for its health status and acceptance before being placed in for the proposed study. The parameters not limited to the following may be considered for the above criteria; animal weight, nonpregnancy (check abdomen), circling, nasal discharge, lacrimation, piloerection, genital organs, forelimbs, hind limbs for the abnormalities, animal locomotion, and ectoparasites if any (good and suitable for experiments or not) [30].

27.6.2 Acclimatization

Regardless of the duration of quarantine, biological test systems, in general, should be acclimatized to the test or laboratory environment for a sufficient period before the first administration or application of the test or reference item. It supports the animals to recover from the shipment or transport-induced stress and also allows the animals for their stabilization for physiological, psychological, and nutritional changes in their new environment. The length of the stabilization depends on the type and duration of animal transportation, the species involved, and the intended use of the animals. Some test guidelines generally recommend that the rodents like rats are allowed an acclimatization period of at least 5 days before the dosing. During the acclimatization, the health status of the animals should be ascertained daily, and unsuitable animal(s) are identified and removed before randomization and grouping of the animals as per SOP and study plan requirement. Any disqualification of animals during the acclimatization period should be justified and documented. For instance, OECD guidelines of toxicity studies recommend that the variation of weight among animals should be minimal not exceeding ±20% of the mean weight of each sex and those animals shall not be incorporated into the study and appropriately disposed of or rehabilitated as per SOP or animal ethics committee approval [33, 58].

27.6.3 Isolation of Sick Animals, Disease Diagnosis, Control, and Treatment

The health of all the animals should be monitored before and after treatment with a test item as per the study plan or test guideline. Animals that exhibit clinical signs of disease conditions or that become injured during the study should be isolated from other animals by housing them in isolation cages/units either in the same room or separate room (meant for sick animals) as appropriate for the containment of the agents of concern. The appropriate diagnostic laboratory services (microbiology, serology, clinical pathology, and parasitology) should be available within the test facility or maybe outsourced to an accredited laboratory. In case of any positive infection in animals, the source of possible infection or contamination of the test system through facility personnel, feed, water, bedding, and pest (wild rodents and insects) must be given due consideration in infection control measures [59]. The GLP does allow for the treatment or control of disease conditions that develop during the study to ensure that there is no unacceptable degree of deterioration of test systems and if necessary, to maintain the integrity of the study. Any diagnosis and treatment of disease should be recorded. The veterinarian is responsible for instituting the treatment or control measures, including euthanasia following the diagnosis of an animal disease or injury. There should be a full-time veterinarian to provide 24 h of veterinary care to the experimental animals including during the weekend and emergency. The veterinarian has to discuss the situation with the study director to decide the suitable course of action consistent with the objectives of the

study plan. The study director has to analyze and record the influence of drug treatment, if any, on the outcome of the study. In any case, the veterinarian has the ultimate authority to decide and act to protect the health and wellbeing of the animals and personnel. Proper personal hygiene, wearing of personnel protective equipment, and further restriction of entry of personnel inside the animal test system facility should always be practiced to reduce the chance of contamination and disease transmission.

27.6.4 Animal Care, Housing, Handling, and Environment

Unlike physical test system (e.g., equipment), biological test systems (animals) are very complex and react very sensitively and differently to disturbances in their environment and handling. Therefore, the quality and reproducibility of the test data derived from these test systems can be ensured only through the maintenance of proper conditions required for different species of laboratory animals. The person should be well trained in the proper and humane method of handling as improper handling can cause undue stress and compromise the animal health, behavior, and validity of study results. The criteria for ensuring the GLP compliance of the biological test system are more extensive than the one for the physical-chemical systems which include the practices and observations on animal care, husbandry (feeding, watering, bedding, etc.), environmental conditions (temperature, humidity, ventilation, lighting/light cycle and noise), and other animal housing method (single or group-housed/conventional or barrier maintained including caging arrangement on the rack). They must follow the respective national animal welfare legislation and guidelines for meeting species-specific requirements. The GLP principles are not certainly intended to change, supersede, or replace the respective national regulations. For instance, the SOPs should cover all technically valid practices to ensure that test systems under the study are subject to optimum care, housing, and environmental conditions as any abnormal deviation can complicate the test item treatment-related toxic effects and scientific validity of the results [33, 54, 58].

27.6.5 Animal Identification

An important aspect of the use of a biological test system is its proper identification. Since the individual test systems are to be removed from their home cages or containers for any reason (e.g., cage cleaning, dosing, treatment, veterinary examinations, etc.) during the conduct of the study, GLP requires that it should bear appropriate identification, wherever possible. For example, individual identification may not be feasible or appropriate in sucking rodents and fishes. Many test facilities initially use a temporary identification method by temporary marking on the animals and/or a simple cage card system during animal quarantine/ acclimatization to ensure animal accountability. It is followed by a permanent identification method after the randomization and grouping of the animals before

the start of the dosing period as per the study plan. There are different methods of permanent animal identification such as tattooing, ear tags and collars, permanent tail marking using a marker pen, color coding with suitable agents, and occasionally ear punching, notching, and toe clipping. Further, all the relevant information required to properly identify the test systems should appear in their cages or containers *w*hich may include different color coding cage card systems for the vehicle control and different doses of treatment groups [60]. Each identification method has its advantages and disadvantages. The shortcomings of the same have to be addressed while evolving the SOP for animal identification based on the test system selected, housing (single or group), and type of study. For instance, tail marking and color coding in rodents may fade over the period and should be redone periodically whereas ear tags or collars may fall off requiring replacement, and some metal ear tags and dyes in the tattoo may be a cause of sensitization response affecting the outcome especially during the Guinea pig sensitization study. The identification number of the animal should be at least unique, preventing the mix-up of animals of various groups within and also between the studies (if performed in the same room concurrently) which may jeopardize the validity of the study. Cage cards generally do not require to be preserved as raw data if the identification information is properly followed and transferred in the study report. In case animals are randomized for the grouping, the copy of the randomization or statistical tables needs to be preserved as raw data.

27.6.6 Cleaning, Sanitation, and Quality Analysis

During use, the cages or containers, racks, and related equipment and areas of animal housing should be cleaned and sanitized periodically as per standard guidelines [53]. The efficacy of disinfection or sterilization procedures should be ensured through periodic microbiological analysis with swab samples taken from the sanitized surfaces of the animal supplies or floors as per SOP. A chemical indicator is used for ensuring successful autoclaving or sterilization cycle of supply materials for the batchwise assessment while a biological indicator may be used for periodical assessment. Deodorants should not be used inside the animal facilities as a substitute for good sanitation practices (like bedding change, cage cleaning, and good ventilation) and for masking the animal odors as it may expose the animals to volatile chemicals leading to changes in animal behavior and metabolic process. Any material that (principally includes feed, water, and bedding) comes into contact with the test system should be free of contaminants at levels that would interfere with the study. It is always advisable to use quality, certified feed from a reputed supplier/vendor who can provide appropriate documentary evidence for ensuring its nutritional composition (proximate principles, minerals, vitamins, etc.) and contaminants (e.g., heavy metals, pesticides, microbes, aflatoxin) within the permissible limits as defined in the SOPs/study plan [61]. Apart from the supplier's report, the test facility may get feed analysis done from an accredited laboratory for some critical nutrients and contaminants to cross-check and verify the feed quality

periodically as per SOP as well. The selection of feed (chow or purified diet or irradiated/fortified autoclavable diet) is the scientific discretion of the study director based on the type of test system involved (conventional or genetically modified animals) and the availability of historical control data generated with the particular diet appropriate for the duration of the study. Likewise, water and bedding should also be analyzed to ensure that they are free from contaminants known to interfere with the results of a study. Documentation of such analyses shall be maintained as raw data. Bedding should be changed as required by the sound husbandry practice as often as necessary to keep the animals comfortably clean and dry. Importantly, the bedding should be of good quality having better absorbent/binding property, nonstaining to the animal fur and skin, inedible for animals to eat and free from toxic contaminants that could cause problems to the animals and personnel. For example, cedar and pine bedding materials contain aromatic hydrocarbons that can induce hepatic microsomal enzymes and hence not appropriate and recommended for use in nonclinical laboratory studies. Although GLP principles do not specify, the beddings like quality rice husk, corn cob, and aspen wood bedding may be used and justified. At times, it is advisable to visit the supplier's unit by the QA personnel for the visual inspection of their quality assurance and biosecurity measures adopted there to minimize the risk of contamination of these products. It is also advisable to have at least two qualified suppliers for any animal critical item like feed and bedding to avoid problems faced by unforeseen disruption of supply by the primary supplier.

27.6.7 Storage Rooms

Apart from the room for the test system, the test system facility should have storage rooms or areas as needed for animal supplies and equipment. As the adequate supply of feed and bedding should be available all time during the study, there should be adequate storage rooms or areas away from the test system. It is inadvisable and even opposed to good animal husbandry practices to store animal supplies (e.g., feed and bedding) in animal housing rooms. The bags of animal feed and bedding are kept off the floor for easy cleaning. It should also provide adequate protection against infestation, contamination, and/or deterioration. The areas meant for the storage of feed and other perishable supplies should be in a temperature-controlled environment to minimize their deterioration. For instance, the Guinea pig feed contains a high content of vitamin C which is heat-labile and hence needs to be kept under cool storage conditions to retain and maintain its nutritive value until the expiry period. The first line of vermin control should be at the perimeter level aimed at controlling or preventing the entry of vermin into a facility. The facilities can be designed and constructed to allow for the perimeter control of vermin or rodents with the elevated basement off the ground. It should be ensured that there is no damage in the wall and floor which might favor harboring of pests, and hence impervious flooring is mandatory for pest control. The use of chemicals if any in pest control should be documented. Care should be taken to protect the feed and bedding from

contamination by toxic chemicals. Besides, there should be facilities for the handling, collection, segregation, and storage of different biomedical wastes including animal carcasses, contaminated/soiled wastes, and other hazardous wastes for the disposal that should be carried out in a manner not jeopardizing the validity of the study. There should be safe storage of wastes (e.g., cold storage for dead animals) before removal from the testing facility. Waste disposal should be carried out safely and may be outsourced to an authorized party as per SOP following respective national regulations (e.g., Biomedical waste management rules, 2016 in the case of India [28, 53].

27.6.8 Test System Characterization and Justification

The revised principles of GLP require that the characterization of test systems (e.g., animals, in vitro) should be described in the study plan as per written SOP. Test systems have to be properly characterized to ensure their suitability for the respective studies utilizing them. This is the GLP requirement that can be directly accomplished by the information from the supplier. The suppliers in some countries might be registered with the national regulatory or voluntary accreditation schemes that assure users with additional documentary evidence that the test systems supplied are of defined quality for the compliance of GLP [61, 62]. The quality of the animals should not be compromised at any cost as the cost of the animals is not significant compared to the expenses incurred for entire preclinical testing. It should be noted that enormous efforts and expenditure spent on infrastructural facilities and environmental control cannot overturn the impact of poor-quality animals on a study. Hence, it is always prudent to procure and use the animals of defined quality in such a way that no variables (e.g., pathogens, colony renewal, genetic and pesticide contamination, veterinary treatments, transport problems, etc.) compromise the quality and validity of the study. As per revised GLP principles, the characterization of a particular animal test system can usually be accomplished by documenting the animal species, strain, substrain, source of supply, number, body weight range, sex, age and other pertinent information in the study plan. Though there is no clarity about what is other "pertinent information," the statement of defined quality [phenotype/genotype (if applicable) and specific pathogen-free status if any] obtained from the supplier may be mentioned in the study plan. In addition to the characterization, the study plan also requires the justification for the selection of the test system. Even though scientific test guidelines recommend the use of a particular test system, it is still mandatory to mention in the study plan the reasons for choosing behind the particular test system. It is mostly based on its relevance of biological process, the predictive validity of the animal model in eliciting toxic response compared to the human system and often on the availability of historical background data. Besides, GLP requires careful adherence to some other technical details and issues about the animal test system that should be addressed in the study plan by the study director for the GLP compliance of the study (according to the type of study and test guideline). At the end of the study, the study director prepares a study report

which may generally include the information and description on the experimental design, randomization, and grouping, all materials and methods/procedures, conditions, type and frequency of analysis, dosing techniques, and justification for the route of administration, measurements, observations, and examinations (e.g., toxic/clinical signs, mortality, veterinary physical examination, ophthalmoscopy, hematology, clinical blood chemistry, urinalysis, necropsy, gross, and histopathology) and other details as sought by the study plan/test guideline. The histopathological evaluation of tissue samples as one of the primary endpoints of the toxicity study is usually carried out by the slide-reading expert toxicopathologist. Considering its critical role in risk assessment, many of the receiving authorities expect some level of peer review by another study pathologist for assuring the concrete interpretation of histopathological findings and for arriving at a definite conclusion [63–66].

27.7 Conclusion

GLP is a quality management system intended to assure the data quality and integrity from the pre- or nonclinical health and environmental safety studies meant for regulatory oversight. GLP compliance reduces the chance of scientific malpractice by the responsible way of study conduct and accurate documentation being monitored under the established internal quality assurance program as well as external monitoring authorities. GLP is significantly distinct from other quality systems of laboratory accreditation like ISO/IEC, and therefore the data generated under the quality system other than GLP cannot be considered as an alternative for regulatory compliance. There are multiple requirements to meet the GLP principles which are very diverse and flexible that should be convincingly complied and demonstrated by the test facility through its established procedures and practices in such a way that support but do not interfere with the quality and the scientific validity of the study. IND and NDA enabling preclinical studies are required to comply with GLP principles including on the test system during new drug development. Generally, healthy conventional animal models (rodents and non-rodents) are utilized as a test system in the nonclinical assessment of safety risks of test items. The GLP requirements for the animal test system involve ethical considerations and compliance with the national animal welfare laws/guidelines. The requirement of animals of defined quality and test system characterization is mandatory and challenging as there are only a few accredited laboratory animal suppliers able to provide documentary evidence for the GLP compliance. Unlike the physical test system, animals are very sensitive to the changes in the laboratory environment and also susceptible to contamination, morbidity, and mortality at any time during the study which may jeopardize the entire study and thus must be given utmost care and consideration. GLP is a dynamic system and amenable to the emerging developments in the global regulatory arena which include the advent and addition of newer test systems (e.g., genetically modified animals and non-animal alternatives), test guidelines, and technologies in safety testing. The OECD principle of GLP is an internationally accepted system of GLP, and the accreditation for GLP allows for the international

acceptability of safety data for the risk assessment under the MAD system. The MAD system also avoids repetitive testing, saving plenty of personnel and financial resources besides sparing a huge number of animals being tested. As there is scope for the studies for the regulatory submission (for marketing authorization) not only in the country of origin but also to receiving authorities of another country, the efforts are being made to harmonize the technical requirements and data reporting (e.g., OECD harmonized template for various studies/test guidelines) for efficient risk assessment, electronic data storage and transfer. Beyond regulatory compliance, the scope of GLP is expanding and being explored for its applicability in other basic scientific research and testing as well. Overall, the compliance with GLP is obligatory for preclinical studies required for product safety evaluation and regulatory approval that contribute to promoting good science and improving the safety of public health and the environment [67–71].

References

1. Sinha S, Vohora D (2018) Drug discovery and development: an overview. In: Pharmaceutical medicine and translational clinical Research. Academic Press, Boston, pp 19–32
2. Gad SC (ed) (2001) Regulatory toxicology. CRC Press, Boca Raton, FL
3. Kille JW (2017) Regulatory toxicology. In: A comprehensive guide to toxicology in nonclinical drug development. Academic Press, Cambridge, pp 499–539
4. Gad SC (ed) (2006) Animal models in toxicology. CRC Press, Boca Raton
5. Saganuwan SA (2017) Toxicity studies of drugs and chemicals in animals: an overview. Bulg J Vet Med 20(4):291–318
6. Colerangle JB (2017) Preclinical development of non oncogenic drugs (small and large molecules). In: A comprehensive guide to toxicology in nonclinical drug development. Academic Press, Cambridge, pp 659–683
7. Dorato MA, Buckley LA (2007) Toxicology testing in drug discovery and development. Curr Protoc Toxicol 31(1):19–11
8. OECD (1998) OECD Principles of good laboratory practice (as revised in 1997)
9. Bode G (2018) Regulatory guidance: ICH, EMA, FDA. In book: Drug discovery and evaluation: methods in clinical pharmacology:1–54
10. New Drugs and Clinical Trials Rules (2019). https://cdsco.gov.in/opencms/export/sites/CDSCO_WEB/Pdf-documents/NewDrugs_CTRules_2019.pdf
11. Food and Drug Administration (2004). Comparison chart of FDA and EPA Good Laboratory Practice (GLP) regulations and the OECD principles of GLP. https://www.fda.gov/inspections-compliance-enforcement-and-criminal-investigations/fda-bioresearch-monitoring-information/comparison-chart-fda-and-epa-good-laboratory-practice-glp-regulations-and-oecd-principles-glp
12. ICH M3(R2) (2009) Guidance on nonclinical safety studies for the conduct of human clinical trials and marketing authorization for pharmaceuticals M3 (R2). In: International conference on harmonisation of technical requirements for registration of pharmaceuticals for human use
13. Cwiertniewicz J (2005) Introduction to the good laboratory practice regulations. Lab Anim 34 (3):29–32
14. ECHA. REACH tests need to comply with good laboratory practice. https://echa.europa.eu/-/reach-tests-need-to-comply-with-good-laboratory-practice
15. Gad SC (2016) Drug safety evaluation. Wiley, New York, NY
16. Chorghade MS (ed) (2007) Drug discovery and development, Volume 2: Drug development, Wiley

17. Takenaka T (2001) Classical vs reverse pharmacology in drug discovery. BJU Int 88:7–10
18. Hughes JP, Rees S, Kalindjian SB, Philpott KL (2011) Principles of early drug discovery. Br J Pharmacol 162(6):1239–1249
19. Russell C, Rahman A, Mohammed AR (2013) Application of genomics, proteomics and metabolomics in drug discovery, development and clinic. Ther Deliv 4(3):395–413
20. Andrade EL, Bento AF, Cavalli J, Oliveira SK, Schwanke RC, Siqueira JM et al (2016) Non-clinical studies in the process of new drug development-Part II: Good laboratory practice, metabolism, pharmacokinetics, safety and dose translation to clinical studies. Braz J Med Biol Res 49(12):e5646
21. Sahota PS, Popp JA, Hardisty JF, Gopinath C (eds) (2013) Toxicologic pathology: nonclinical safety assessment. CRC Press, Boca Raton, FL
22. Food and Drug Administration. A history of the FDA and drug regulation in the United States. https://www.fda.gov/media/73549/download
23. Rahalkar H (2012) Historical overview of pharmaceutical industry and drug regulatory affairs. Pharmaceut Reg Aff S11:002
24. Vargesson N (2015) Thalidomide-induced teratogenesis: History and mechanisms. Birth Defects Res C Embryo Today 105(2):140–156
25. Kelsey FO (1988) Thalidomide update: regulatory aspects. Teratology 38(3):221–226
26. Schneider K (1983) Faking it. The Case against Industrial Bio-Test Laboratories. Amic J 4:14–26
27. Baldeshwiler AM (2003) History of FDA good laboratory practices. Qual Assur J 7(3):157–161
28. Weinberg S (ed) (2007) Good laboratory practice regulations. CRC Press
29. FDA. Good laboratory practices for nonclinical laboratory studies. http://www.accessdata.fda.gov/scripts/cdrh/cfdocs/cfcfr/CFRSearch.cfm?CFRPart=58&showFR=1
30. Seiler JP (2005) Good laboratory practice: the why and the how. Springer Science & Business Media
31. OECD (1981) Decision of the Council concerning the Mutual Acceptance of Data in the Assessment of Chemicals. [C(81)30]
32. OECD (1997) OECD Series on Principles of Good Laboratory Practice and Compliance Monitoring. https://www.oecd-ilibrary.org/environment/oecd-series-on-principles-of-good-laboratory-practice-and-compliance-monitoring_2077785x
33. OECD (1994) OECD Guidelines for the Testing of Chemicals. https://www.oecd.org/chemicalsafety/testing/oecdguidelinesforthetestingofchemicals.htm
34. OECD (1997) Decision of Council Concerning the Adherence of non-Member countries to the Council Acts related to the Mutual Acceptance of Data in the Assessment of Chemicals. [C(97)114]
35. NGCMA. https://dst.gov.in/ngcma
36. EMA (European Medicines Agency) Good laboratory practice compliance. https://www.ema.europa.eu/en/human-regulatory/research-development/compliance/good-laboratory-practice-compliance
37. Jawahar N, Lakshmi VT (2017) Regulatory requirements for the drug approval process in US, Europe and India. J Pharm Sci Res 9(10):1943–1952
38. Guidance document on toxicology for registration of Chemical pesticides in India, September 2017., http://ppqs.gov.in/sites/default/files/toxguidancedocsept2017.pdf
39. PMDA (Pharmaceuticals and Medical Devices Agency) Ministerial ordinance on Good Laboratory Practice for nonclinical safety studies of drugs. https://www.pmda.go.jp/files/000153713.pdf
40. Turnheim D (1993) Benefits of good laboratory practice as a tool to improve testing. Hum Exp Toxicol 12(6):528–532
41. Hickman S, Ogilvie B, Patterson T (2018) To GLP or not to GLP? Drug Discov 19:61
42. Carson, P. A., Dent, N. J. (Eds.). (2007) Good clinical, laboratory and manufacturing practices: techniques for the QA professional. Royal Society of Chemistry

43. OECD Document No. 21 (2020) OECD Position Paper Regarding Possible Influence of Sponsors on Conclusions of GLP Studies
44. OECD Document No. 8 (1999) The Role and Responsibilities of the Study Director in GLP Studies
45. OECD Document No. 13 (2002) The Application of the OECD Principles of GLP to the Organisation and Management of Multi-Site Studies
46. OECD.Document No. 19 (2018) Advisory document of the working group on good laboratory practice on the management, Characterisation and use of test items
47. OECD Document No. 4 (1999) Quality Assurance and GLP
48. World Health Organization (2010) Handbook: good laboratory practice (GLP): quality practices for regulated non-clinical research and development. World Health Organization
49. Stiles T (1997) The revised OECD principles of good laboratory practice. a reflection upon the impact of the proposed changes on pre-clinical safety testing: Part 1: Scope, definition of terms, responsibilities. Qual Assurance J Qual Assurance J Pharmaceut Health Environ Prof 2 (1):13–18
50. Heinz-Taheny K (2013) Pathology and GLPs, quality control and quality assurance. In: Haschek and Rousseaux's Handbook of toxicologic pathology. Academic Press, pp 519–536
51. Boverhof DR, Chamberlain MP, Elcombe CR, Gonzalez FJ, Heflich RH, Hernandez LG et al (2011) Transgenic animal models in toxicology: historical perspectives and future outlook. Toxicol Sci 121(2):207–233
52. Nohmi T, Masumura K, Toyoda-Hokaiwado N (2017) Transgenic rat models for mutagenesis and carcinogenesis. Genes Environ 39(1):1–32
53. CPCSEA. CPCSEA guidelines for laboratory animal facility, India http://cpcsea.nic.in/WriteReadData/userfiles/file/SOP_CPCSEA_inner_page.pdf
54. National Research Council (US) (2011) Guide for the care and use of laboratory animals, 8th edn. National Academies Press (US), Washington, DC
55. Canadian Council on Animal Care (2003) Guidelines on: laboratory animal facilities — characteristics, design and development. (as revised 2020) https://www.ccac.ca/Documents/Standards/Guidelines/Facilities.pdf,
56. Deshmukh P (2018) Animal research facility for conducting GLP study. MOJ Toxicol 4 (3):205–207
57. European Commission Guidance Document for GLP inspectors and GLP Test Facilities (2004) Cross-contamination of control samples with test item in animal studies
58. OECD (2008) Repeated Dose 28-day Oral Toxicity Study in Rodents. OECD Guidelines for the Testing of Chemicals. Section 4: Health Effects
59. Prichett-Corning, K. R., Shek, W. R., Henderson, K. S., Clifford, C. B. (2009) Companion guide to rodent health surveillance for research facilities (No. 636.02 COM)
60. World Health Organization (2010) Good laboratory practice training manual: for the trainee: a tool for training and promoting Good Laboratory Practice (GLP) Concepts in Disease Endemic Countries. World Health Organization
61. OECD Document No. 5 (1999) Compliance of Laboratory Suppliers with GLP Principles
62. OECD Document No. 14 (2004) The Application of the Principles of GLP to in vitro studies
63. Borgert CJ, Becker RA, Carlton BD, Hanson M, Kwiatkowski PL, Sue Marty M, Witorsch RJ (2016) Does GLP enhance the quality of toxicological evidence for regulatory decisions? Toxicol Sci 151(2):206–213
64. Wallace JM, Trundy RL (2018) Animal research pathology: regulatory and safety considerations. ILAR J 59(1):111–118
65. OECD Document No 16 (2014) Guidance on the GLP Requirements for Peer Review of Histopathology
66. Seaton M (2014) The study pathologist's role in GLP studies: a regulator's perspective. Toxicol Pathol 42(1):285–285
67. Becker RA, Janus ER, White RD, Kruszewski FH, Brackett RE (2009) Good laboratory practices and safety assessments. Environ Health Perspect 117(11):A482–A483

68. OECD document No. 18 (2016) OECD Position Paper Regarding the Relationship between the OECD Principles of GLP and ISO/IEC 17025
69. OECD Harmonised Templates for reporting chemical test summaries. http://www.oecd.org/ehs/templates/
70. Bolon B, Baze W, Shilling CJ, Keatley KL, Patrick DJ, Schafer KA (2018) Good laboratory practice in the academic setting: fundamental principles for nonclinical safety assessment and GLP-compliant pathology support when developing innovative biomedical products. ILAR J 59(1):18–28
71. Jena GB, Chavan S (2017) Implementation of Good Laboratory Practices (GLP) in basic scientific research: translating the concept beyond regulatory compliance. Regul Toxicol Pharmacol 89:20–25

Assisted Reproductive Technologies (ARTs) in the Laboratory Mouse

28

Rahul Thorat

Abstract

Biomedical research has been hugely enriched by the advent of genetically engineered mouse models (GEMM). The latest genome editing technologies have enabled the development of numerous targeted genome-edited animals. GEMMs are valuable assets to the researchers for studying the pathophysiology of several human diseases. For the development of such animal models, assisted reproductive technologies (ARTs) in the laboratories play a very crucial role. ART helps in the preservation of valuable strains and safeguards them from infectious disease outbreaks and natural calamities. This also helps in the global distribution of strains to fulfill the research demands. ARTs involve superovulation, in vitro fertilization, cryopreservation of germplasms, and embryo transfer. The principles and practical aspects of ARTs are described.

Keywords

Superovulation · Embryo cryopreservation · Mouse sperms · In vitro fertilization · Embryo transfer

Abbreviations

ARTs	Assisted reproductive technologies
GEMM	Genetically engineered mouse models
IVF	In vitro fertilization

R. Thorat (✉)
Advanced Centre for Treatment, Research and Education in Cancer (ACTREC), Tata Memorial Centre, Navi Mumbai, Maharashtra, India
e-mail: rthorat@actrec.gov.in

© The Author(s), under exclusive license to Springer Nature Singapore Pte Ltd. 2021

P. Nagarajan et al. (eds.), *Essentials of Laboratory Animal Science: Principles and Practices*, https://doi.org/10.1007/978-981-16-0987-9_28

28.1 Introduction

The use of laboratory animals in biomedical research is inevitable. The mouse is the most commonly used species due to its definite genetic background and the ease of carrying out any genetic modifications [1]. With the advancements in gene-editing technologies, thousands of new strains are being quickly produced. Their numbers have dramatically increased in recent years. The maintenance of a colony of genetically engineered mouse models (GEMM) requires lots of efforts and attention. Therefore, it is of utmost importance to safeguard these strains from loss by any means like a breeding failure, catastrophe, infectious disease outbreaks, or genetic drift [2–4].

For the protection of such strains, assisted reproductive technologies (ARTs) offer a viable solution. The ARTs help reduce manpower, save space on the shelves, enable global distribution of strains in the cryopreserved form, and very importantly cater to the principles of animal welfare and 3Rs.

The first successful freezing of mouse embryos was done at Whittingham Laboratory, Biology Division, Oak Ridge National Laboratory, USA, in 1972 using a controlled slow-freezing method [5]. This was the major breakthrough in the use of ARTs. In subsequent years, several laboratories across the globe modified the protocols and developed simpler, reproducible, and more efficient methodologies. Currently, researchers engaged in the production of GEMM routinely adopt ART. Superovulation, in vitro fertilization; cryopreservation of germplasms like sperms, ovaries, or embryos; and embryo transfer are the most commonly used ARTs.

This chapter describes various commonly used ARTs in laboratory animals.

28.2 Superovulation

Superovulation has been used in the production of genetically modified mice since the late 1980s. As far as laboratory animals are a concern, the collection of embryos is possible from naturally mated and superovulated females. However, it would require sacrificing large numbers of naturally mated donor females in comparison with the superovulated females. Considering the animal welfare and ethical concerns [6], superovulation offers a better option than natural mating. Due to superovulation, there is a significant decrease in the number of donor animals by increasing the number of ovulated oocytes obtained from a single animal [7]. Superovulation efficiently synchronizes the estrus in females to maximize the use of each female being sacrificed.

28.2.1 Hormones

Superovulation can be achieved by artificial induction of follicle maturation using exogenous hormone, pregnant mare serum gonadotropin (PMSG) which acts by

stimulating follicle-stimulating hormone (FSH), and ovulation effect using human chorionic gonadotropin (hCG) by stimulating luteinizing hormone (LH).

28.2.2 Preparation of Hormone Injections

PMSG (Prospec Bio, cat. no. HOR- 272, 1000 IU): Reconstitute the lyophilized powder (1000 IU/vial) by addition of 5 ml of embryo-tested water (Sigma, cat. no. W1503). Make aliquots of 250 μl into 1.5 ml tubes, and store at -80 °C deep freezer. Dilute the aliquot by 750 μl of embryo-tested water, and inject 100 μl per mouse by intraperitoneal (IP) route. The final dose would be 5 IU per mouse.

hCG (Sigma, cat. no. CG 10-10VL): Reconstitute the lyophilized powder (2500 IU/ vial) by addition of 5 ml of embryo-tested water. Make aliquots of 100 μl into labeled 1.5 ml tubes and store at -80 °C. Dilute the aliquot by 900 μl of embryo-tested water, and inject 100 μl per mouse by IP route. The final dose would be 5 IU per mouse.

28.2.3 Factors Affecting the Superovulation Response

Genetic background, age, weight, time of injection, dose of hormones, and reproductive performance of stud males can affect the successful induction of superovulation. The incidence of interstrain variability is reported due to exogenous hormones. The response of superovulation varies from poor that is less than 5 oocytes per mouse (A/J mouse) to more than 40 oocytes per mouse (129S1 mouse) [8]. Three to four major genetic factors are attributed to the differences among the strains in the efficient induction of superovulation [8–12].

Apart from genetic differences, superovulation is sensitive to the weight and age of the female donor mice [13, 14]. The best age for superovulation varies between 3 and 6 weeks. The body weight of mice and dose of exogenous hormones are key factors in maximizing superovulation rates, provided other variables like a light-dark cycle, housing conditions, noise, and vibration levels are controlled [15].

28.2.4 Dose and Time of Injections

The optimum dose of hormones is 5 IU PMSG and 5 IU hCG for 3–4 weeks old age/or 10–15 g body weight of mouse [7–10, 12, 14, 16]. The dose has to be increased with an increase in the body weight. The time between PMSG and hCG injection is highly correlated to each other. On day 1, inject PMSG between 1:00 and 2:00 pm. Inject hCG, on day 3, 47–48 h post-PMSG injection. The ovulation takes place 12–13 h post-hCG injection. Allow donor mice for mating with the stud males in a 1:1 ratio. The age of stud males should be 8–10 weeks of age. Do not put females for mating with stud males, in case if you wish to collect the oocytes. Hormone injections can be scheduled on a weekly basis as per the desired stage of

preimplantation embryos. There are different stages of preimplantation embryos, namely, one-cell (0.5 day post coitus), two-cell (1.5 dpc), eight-cell to morula- (2.5 dpc), and blastocyst stage (3.5 dpc).

28.2.5 Checking for Vaginal Plugs (VP)

Place the female and male for cohabitation; check the females early on the next day for vaginal plugs. The plug is formed due to deposition of secretions by male accessory sex glands which indicate that mating has occurred. However, there is no guarantee that all VP-positive females will become pregnant. To observe the plug, lift the female by holding the tail, and observe the vaginal orifice for white color mass. The day of the vaginal plug is considered as 0.5 dpc timed pregnant female. Figure 28.1 shows the differentiation between vaginal plug positive female with negative.

28.3 Cryopreservation

Cryopreservation is the method of cooling of organelles, cells, tissues, or any other biological material to extremely low temperatures for an infinite period of time. Cryopreservation of germplasms like embryo, sperms, and ovaries is possible with varied success rates without causing any adverse effects.

28.3.1 Embryo Cryopreservation

Controlled rate freezing, vitrification, and rapid freezing are the three techniques being widely used for embryo cryopreservation [17].

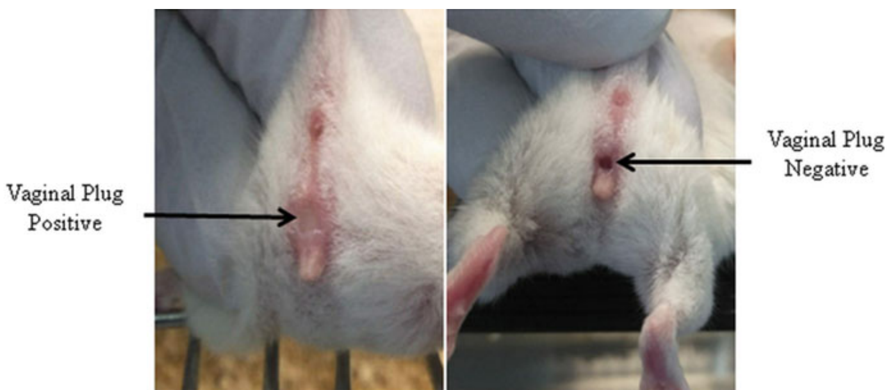


Fig. 28.1 Detection of vaginal plug

In this section, two methods of embryo cryopreservation adopted at ACTREC animal facility are described.

28.3.2 Controlled Rate Freezing

Controlled rate freezing or slow rate freezing is based upon the principle of dehydration. The cooling rate is controlled to remove water from the embryo, preventing cryoinjury occurring due to the formation of ice crystals [3, 18, 19]. Embryos are equilibrated in a 1–2 M solution of permeable cryoprotective solutions. During the process, intracellular water is replaced with the permeable cryoprotective solution. Controlled rate freezing prevents intracellular crystallization by osmotic dehydration of intracellular water. The degree of dehydration depends upon the rate of the cooling, duration of cooling, and the final temperature at which freezing process is terminated. The success of controlled rate freezing depends upon several key factors such as the stage of embryo, concentration of cryoprotectant, rate of cooling, seeding temperature, freezing/thawing procedure, embryo culture system, and surgical transfer of embryos.

Scientists have proved competence of freezing and thawing of all the preimplantation stage of embryo with varied success rates [5, 20, 21]. ACTREC animal facility prefers eight-cell to morula-stage (2.5 dpc) of embryos for cryopreservation.

28.3.3 Materials and Methods

28.3.3.1 Cryopreservation Media

1. Embryo washing: M2 medium, Sigma, cat. no. M-7167.
2. Embryo culture: M16 medium, Sigma, cat. no. M-7292 and potassium-supplemented simplex optimized medium (KSOM).
3. Nakagata laboratory, Division of Reproductive Engineering, Centre for Animal Resources and Development (CARD), Kumamoto University, Japan, has developed CARD media. The online manual for CARD mouse reproductive technology can be accessed at http://card.medic.kumamoto-u.ac.jp/card/english/sigen/manual/onlinemanual.html
4. COOK Medical, Australia, provides a medium for embryo washing, freezing, thawing, etc.

However, many of these cryopreservation solutions have a relatively short shelf life so in some cases where import logistics issues may take time or for facilities that perform these procedures on a regular basis, it might be worth considering making these media in-house. All these media can be made in-house quite easily, but they will need to be batch tested before experimental use.

We use media from Cook Medical, Australia. The composition of cryopreservation and culture media can be viewed at http://www.cookmedical.com. For details of the cryopreservation kit, see Table 28.1.

Table 28.1 Cryopreservation kit, Cook Medical, Australia

Sydney IVF cryopreservation kit (K-SICS-5000)	Sydney IVF thawing kit (K-SITS-5000)	Sydney IVF blastocyst medium (K-SIBM-50)
1. Vial F1—Sydney IVF cryopreservation buffer 2. Vial F2—Sydney IVF cryopreservation buffer with 1.5 M propanediol 3. Vial F3—Sydney IVF cryopreservation buffer with 1.5 M propanediol and 0.1 m sucrose	1. **Vial T1**—Sydney IVF cryopreservation buffer with 1.0 M propanediol and 0.2 m sucrose 2. **Vial T2**—Sydney IVF cryopreservation buffer with 0.5 M propanediol and 0.2 m sucrose 3. **Vial T3**—Sydney IVF cryopreservation buffer with 0.2 M sucrose. 4. **Vial T4**—Sydney IVF cryopreservation buffer	A bicarbonate-buffered medium that is used to promote the development of early embryos to blastocysts. The Blastocyst Medium is a complex medium that contains essential and non-essential amino acids, glucose, and human serum albumin

28.3.4 Collection of Eight-Cell to Morula-Stage Embryos at 2.5 dpc

1. For superovulation and vaginal plug identification, refer to previously mentioned protocols.
2. Euthanize 2.5 dpc VP-positive mouse by cervical dislocation. Cervical dislocation is the preferred method of euthanasia to obtain a good-quality embryos over gaseous isoflurane euthanasia [22].
3. Place the mouse in the supine position. Give abdominal "V" type cut to the skin and muscle. Carefully remove both the oviducts along with a 1–2 mm part of the utero-tubular junction. There is a chance that embryos hide at the utero-tubular junction.
4. Place the individual animal oviducts in a drop of 100 μl pre-warmed F1 (K-SICS-5000) solution in a 60 mm sterile plastic tissue culture dish. One dish is sufficient for four females. Do not mix the drops which will make it easy to calculate the embryo yield per female. Warm F1 solution at 37 °C in a 5% CO_2 for 10–20 min.
5. Slowly flush the oviducts through the infundibulum with 100–200 μl of F1 medium using a 30G needle. Embryos that have an intact zona pellucida, round in shape, normal size, usual cytoplasmic granulation, free of adherent debris, and without any damaged blastomeres should be selected [4]. With the help of a glass pipette attached to mouth pipette assembly (Sigma, cat. no. A5177-5EA), transfer all the embryos into a fresh drop of F1 medium. Give three to four sequential washings to get rid of the debris. Alternatively, an M2 medium can be used for embryo washing.
6. Move embryos from F1 drop into F2 and F3 (K-SICS-5000) medium for 10 min of equilibration in each drop.

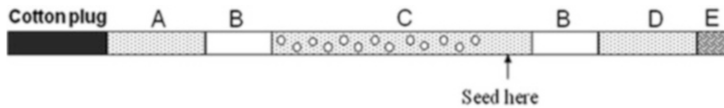


Fig. 28.2 Schematic representation of 0.25-ml-capacity mini straw: (**a** and **d**) F3 medium; (**b**) air bubble; (**c**) embryos in F3 medium; (**e**) sealed end

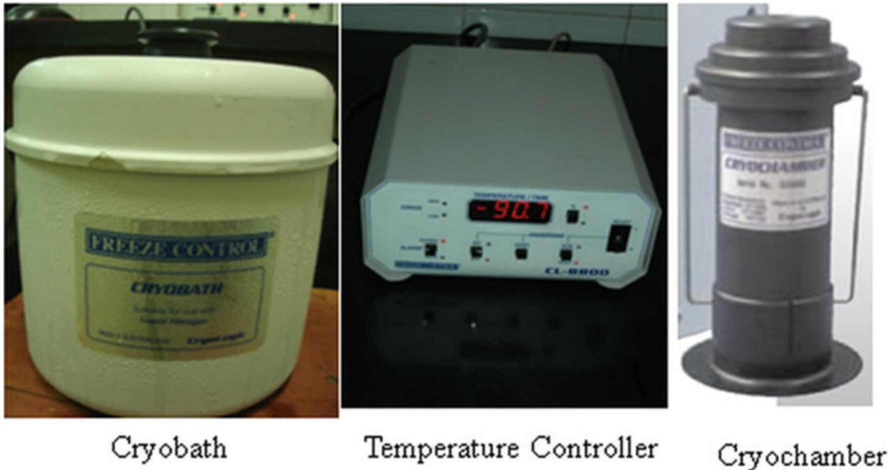


Fig. 28.3 Freeze control system. CryoLogic Pty. Ltd., CL-8800

28.3.5 Loading of Embryos into Straws

1. A 0.25-ml (133 mm)-capacity sterile flexible mini straw (IMV Technologies, cat. no. 006430) can be used. One straw is sufficient for 20–25 embryos. Label the straws by giving a unique identification number or strain code.
2. Load the straw with three columns of F3 medium separated by air bubbles from a central column containing the embryos (see Fig. 28.2).
3. Seal the open ends of the straws with the help of a plastic sealing machine.

28.3.6 Freezing

1. At our laboratory, freezing is performed using an apparatus, Freeze Control System (CryoLogic Pty. Ltd., Australia model CL-8800) (see Fig. 28.3).
2. Stand the cryochamber into a cryobath containing liquid nitrogen (LN2). Place the straws in a cryochamber in the vertical position, keeping the cotton plug at the upper side. Connect the cryochamber securely to the temperature controller which is further connected to a personal computer.
3. Set the cooling conditions are as follows:
   - From room temperature (24 °C) to −7 °C at 2 °C/min, give initial holding time at −7 °C for 5 min.

- Manual seeding is performed at −7 °C. Seeding is performed by cotton bud soaked in LN2. Touch a cotton bud at the upper-end meniscus of the central column of the media containing embryos (see Fig. 28.2).
- From −7 to −30 °C at 0.5 °C/min.
- From −30 to −120 °C at 1 °C/min.
- Hold straw at −120 °C for 5 min. Plunge the straws into liquid nitrogen for long-term storage for an indefinite period.

We refer to protocols developed by Pomeroy KO, Uechi et al. [3, 23], and the Jackson Laboratory (Bar Harbor, ME, USA).

28.3.7 Thawing

1. Transfer the straw into the Dewar flask containing LN2.
2. With the help of pre-cooled forceps, hold the straw horizontally at room temperature (24 °C) for 40 s, and transfer straw at 37 °C water bath for another 30 s. Do not agitate the straw in the air or water bath.
3. Wipe the straw with sterile tissue paper and cut both ends with the scissor. Slowly expel embryos in a drop of T1 medium. Do sequential transfer from T1 to T4 (K-SITS-5000) medium to remove the cryoprotectant, and sucrose with 5 min of equilibration in each drop.
4. Assess the morphological appearance of thawed embryos under a stereoscopic microscope (Nikon, SMZ 1500). Discard fragmented or partially damaged blastomeres or fractured zona pellucida embryos.

We refer to the thawing protocol developed by Macas et al. [24].

28.3.8 Embryo Culture

1. Warm 75 μl drop of the blastocyst medium (K-SIBM-50) covered with mineral oil (Sigma, cat. no. 5301) in a 35 mm sterile plastic tissue culture petri dish at 37 °C, 5% CO_2 for 1–2 h.
2. Transfer morphologically assessed thawed embryos in pre-warmed blastocyst medium. Incubate the plate in a CO_2 incubator at 37 °C, 5% CO_2 for 24 h.
3. On the following day, the viability of the eight-cell to morula-stage embryos can be assessed by the ability to develop into early or fully expanded blastocysts.
4. Do uterine transfers of well-developed blastocysts in 2.5 dpc pseudopregnant female mice.

28.4 Vitrification

After the first report of mammalian cryopreservation, more technological advancements have been made. Researchers emphasized to make the procedure simple and reproducible, reduce cost/time, and increase the overall success rate. Rall and Fahy in 1985 developed the novel technology, "vitrification" [25].

Vitrification is a simple method of directly plunging embryos into LN2 after a short exposure to cryoprotectant solutions of high concentrations. Vitrification is beneficial over the slow-freezing method in two ways. First, no cryoinjury caused due to intra- and extracellular ice crystal formation. Second, the vitrification method reduces the time required and no costly equipment is required [17]. Vitrification is possible from the one-cell stage to the blastocyst stage of embryos. The success of vitrification is depending upon the strain of animals, stage of embryo, type/concentration of cryoprotectant, time of exposure to cryoprotectant solution, warming procedure, and embryo transfer.

ACTREC laboratory animal facility has adopted protocol originally developed by Mochida et al. [26].

28.4.1 Materials and Methods

28.4.1.1 Vitrification Media

1. Dulbecco's phosphate-buffered saline (DPBS) (Sigma, cat. no. D-4031).
2. Ficoll-sucrose (FS) solution (Table 28.2).
3. Equilibration solution (EFS20) and vitrification solution (EFS40): (see Table 28.3).

Use a 0.45 μm filter for sterilization of the solutions. Make aliquots and store at 4 °C for up to 6 months.

28.4.2 Carrier System

Different carrier systems are being widely used, namely, open pulled straws (OPS), electron microscope grids, CryoLoops, thin plastic strips, and cryovials [26–30]. We do use round bottom cryovials (Corning Life Sciences, cat. no. CLS430488-500EA).

Table 28.2 Composition to make Ficoll-sucrose (FS) solution

Reagent	Per 10 ml DPBS
Ficoll PM 70 (Sigma, cat. no. F-2878)	4.27 g
Sucrose (Sigma, cat. no. S-7903)	2.44 g
Bovine serum albumin (BSA) (Sigma, cat no. A-8806)	30 mg (3 mg/ml)

Data were taken from Mochida et al. [26]

Table 28.3 Composition to make equilibration (EFS20) and vitrification (EFS40) solutions

Reagent	EFS20 20% (v/v) ethylene glycol (EG), 24% (w/v) Ficoll, and 0.4 mol/L sucrose in DPBS with BSA	EFS40 solution 40% (v/v) ethylene glycol (EG), 18% (w/v) Ficoll, and 0.3 mol/L sucrose in DPBS with BSA
Ethylene Glycol, (Sigma, cat. no. 324558)	1 ml	2 ml
Ficoll-sucrose (FS) solution (see Table 2)	4 ml	3 ml

Data were taken from Mochida et al. [26]

28.4.3 Vitrification

1. For superovulation and collection of embryos, refer to previously mentioned protocols.
2. Aliquot 50 µl drop of vitrification (EFS40) solution into a labeled cryovial.
3. Transfer up to 25–30 embryos into 50 µl drop of equilibration solution (EFS20) in a 60 mm sterile plastic tissue culture petri dish for 2 min. Check the morphology of embryos under a stereomicroscope. Typically, dehydrated embryos show a shrunken morphology. If they are not dehydrated properly, wait for another 1 or 2 min.
4. After 1 and a half minutes, pick up the embryos from EFS20 with a minimum amount of medium, and transfer them into cryovial, containing EFS40 solution. Wait for 1 min. For better results, adjust the timing of picking up embryos so that all embryos can be transferred into EFS40 within 2 min. Avoid longer exposure which causes toxic effects on embryos.
5. Plunge the cryovial into LN2.

28.4.4 Warming (Thawing)

Materials and Methods

Thawing solutions (TS1) and (TS2) with two different concentrations of sucrose are required (see Table 28.4).

Table 28.4 Composition of warming solutions, TS1 and TS2

Reagents	(TS1) 0.75 M sucrose
Sucrose	Dissolve 2.56 g in DPBS* and bring the total volume to 10 ml
BSA	Add 30 mg of BSA onto the surface of the solution. Put the tubes at 4 °C until BSA is completely dissolved
	(TS2) 0.25 M sucrose: Dilute 10 ml TS1 (0.75 M) with 20 ml DPBS*

Data were taken from Mochida et al. [26]

Use a 0.45 μm filter for sterilization. Make aliquots and store at 4 °C up to 1 month. *, DPBS solution can be replaced with M2 medium (Sigma, cat. no. M-7167):

1. Warm 75 μl drop of the blastocyst (K-SIBM-50) or M16 (Sigma, cat. no. M-7292) or KSOM medium covered with mineral oil (Sigma, cat. no. 5310) in a 35 mm plastic tissue culture petri dish at 37 °C in 5% CO_2 for 1–2 h. Warm TS1 solution tube at 37 °C in a water bath.
2. Transfer the cryovial into a Styrofoam box or Dewar flask containing LN2. Take out the vial and quickly remove the cap. Discard LN2 from cryovial, and wait for 30 s at room temperature (24 °C).
3. Add 850 μl of pre-warmed TS1 solution into the cryovial. Mix the solution by gentle pipetting without forming bubbles. Transfer the complete volume into a 60 mm sterile plastic tissue culture petri dish. Wait for 3 min. Slowly rotate the plate after 2 min.
4. Make a 60 mm sterile petri dish containing three drops of 50 μl of TS2 solution. After 3 min in TS1, pick up morphologically good-quality embryos, and transfer into the first drop of TS2. Wait for 3 min. Do sequential transfer of embryos from the second drop to the third drop of TS2 solution.
5. Transfer the embryos into embryo culture medium (K-SIBM 50) or M16 or KSOM. Observe the embryos after 24 h of incubation for blastocyst development.
6. Do uterine transfers of well-developed blastocysts in 2.5 dpc pseudopregnant female mice.

28.5 Cryopreservation of Mouse Spermatozoa

For many decades, cryopreservation of embryos is a preferred method to safeguard the mouse strain. The disadvantage of embryo cryopreservation is that several hundreds of preimplantation embryos are needed to be cryopreserved for which a large number of donor mice are required to be sacrificed [31]. Many genetically modified mice strains are poor responders for superovulation. Therefore, a relatively large breeding colony needs to be established, which leads to an additional burden on space, cost, manpower, etc. Hence, embryo cryopreservation is not a good option with the large number of mouse lines that are being generated each year.

To overcome these issues, sperm cryopreservation and in vitro fertilization (IVF) were attempted and standardized. The first attempt of cryopreservation of spermatozoa was carried out by C. Polge [32]. Afterward, efforts were made to increase the efficiency, rapidity of procedures, and overall success rate of sperm cryopreservation. Finally, in 1990, the first successful attempt of cryopreservation and retrieval of mouse spermatozoa was published [33]. Since then several laboratories have successfully standardized sperm cryopreservation by suitably modifying the protocols [34–42].

There are several advantages of sperm cryopreservation. The main advantage is that a single desired male can yield 10,000,000 to 30,000,000 spermatozoa. If all frozen-thawed spermatozoa from a single male are used for in vitro fertilization, at least 500 oocytes can be fertilized [43]. Sperm cryopreservation saves lots of space in the animal facility, reduces the financial burden in the upkeep of live mouse line colony, reduces the risk of genetic drift, and avoids the need for transportation of mice between research facilities. Therefore, sperm cryopreservation may provide the best and economical alternative to embryo cryopreservation [2, 44, 45].

During the process of standardization, the use of 18% raffinose and 3% skim milk as a cryoprotective agent (CPA) was a significant breakthrough [43, 46–48]. N. Nakagata and T. Takeshima reported that the success rate of sperm cryopreservation is strain-dependent [47]. Unfortunately, cryoprotectant efficiency even after the use of 18% raffinose and 3% skim milk was unsatisfactory for many important inbred strains, including the most popular mouse strain, C57BL/6 [47]. The sperms of C57BL/6 mouse are highly sensitive to freezing and thawing procedures [49]. The scientist has made attempts to improve the success rate by several ways. The addition of amino acid, especially L-glutamine, improved the fertilization rates of IVF of frozen-thawed sperm by boosting the cryoprotective effect of 18% raffinose pentahydrate 3% skim milk solution [50]. L-glutamine increases the post-thaw sperm motility and stabilizes the plasma membrane in mammalian sperm, which further decreases the damage associated with freeze-thaw procedures [51].

The addition of methyl β-cyclodextrin (MBCD) in sperm preincubation medium increased the fertilization ability of the sperms [52]. MBCD promotes cholesterol efflux much faster and efficiently from the plasma membrane of sperm, inducing sperm capacitation [53].

The treatment of oocytes with reduced glutathione (GSH) helps to cleave disulfide bonds and induce morphological changes in the zona pellucida (ZP). This provides the sperms to have easy penetration through the ZP which increases the fertilization efficiency. Thus, sperm cryopreservation and IVF protocol were optimized which provided a high recovery rate of frozen-thawed C57BL/6 mouse sperm [52, 54].

Our facility has adopted the above modifications originally developed at Dr. Naomi Nakagata laboratory at the Center for Animal Resources and Development (CARD), Kumamoto University, Japan.

28.5.1 Materials and Methods

28.5.2 Sperm Freezing

28.5.2.1 Cryoprotective Agent (gCPA) Containing 100 mM L-Glutamine (See Table 28.5)

1. Add 584 mg L-glutamine to 40 ml of embryo-tested water in a 50 ml disposable tube. Vortex the tube for 3 min.

Table 28.5 Composition cryoprotective agent (gCPA)

Reagents	Catalog no.	mg/40 ml
Raffinose pentahydrate	Sigma, R-7630	7200
Skimmed milk	Himedia, GRM-1254	1200
L-glutamine	Sigma, G8540	584
Embryo-tested water	Sigma, W1503	–

Data taken from Nakagata, N [55]

Table 28.6 Composition for making mHTF

Reagent name	Catalog number	mg/100 ml
Sodium chloride (Nacl)	Sigma, S5886	593.8
Potassium chloride (Kcl)	Sigma, P5405	35.0
Magnesium sulfate heptahydrate ($MgSO_4 \cdot 7H_2O$)	Sigma, M7774	4.9
Potassium phosphate monobasic (KH_2PO_4)	Sigma, P5655	5.4
Calcium chloride dihydrate ($CaCl_2 \cdot 2H_2O$)	Sigma, C7902	75.5
D-(+)-Glucose	Sigma, G6152	50.0
Sodium DL-lactate solution (ml)[a]	Sigma, L7900	0.34
Sodium pyruvate Na-pyruvate	Sigma, P4562	3.7
Penicillin G	Sigma, P4687	7.5
Streptomycin	Sigma, S1277	5.0
Phenol red solution 0.5% (ml)[a]	Sigma, P0290	0.04
Sodium bicarbonate ($NaHCO_3$)	Sigma, S5761	210.0
BSA (albumin bovine serum, fraction V, fatty acid-free)	Sigma, A-8806	400.0

[a]Indicates the volume of a reagent. Data were taken from Kito, S and Ohta, Y [56]

2. Add 7200 mg raffinose pentahydrate and 1200 mg skimmed milk powder into the tube. Vortex for 3 min.
3. Incubate the solution at 60 °C in a water bath for 90 min. Vortex for 3 min after every 30 min of incubation.
4. Divide the solution into 1 ml aliquots into 1.5 ml centrifuge tubes. Centrifuge the samples at 10,000 RPM for 60 min.
5. Carefully collect 0.7–0.8 ml supernatant of each sample from the central region of the tube, and discard the pellets.
6. Filter the supernatant using a 0.22 μm syringe filter. Aliquots and store at room temperature. Use within 3 months.

28.5.2.2 Modified High Calcium HTF Medium (See Table 28.6)

Filter through a 0.22 μm filter and store in aliquots at 4 °C for up to 3 months.

- Mineral oil.
- 70% ethanol.

Animals: Male mice over 12 weeks of age**.** Proven males also can be used. However, 1-week mandatory rest to a donor male mouse should be given.

Equipment

- Semen straw (see Fig. 28.4).
- Floating sperm-freezing apparatus (see Fig. 28.5b).
- LN2 container (see Fig. 28.5a).
- Temperature-controlled water bath.
- Analytical weighing balance.
- Laboratory use osmometer.
- Pipettes: 1000 μl, 100 μl, 20 μl, 10 μl capacity.
- Syringe filters: 0.22 μm and 0.45 μm size.
- Microscopes: dissecting and stereoscopic microscope.
- Surgical instruments: iris forceps, scissors,
- Stopwatch.
- Kimwipes, tissue towels.
- 50-ml-capacity disposable tubes
- Petri dish: 60 mm and 35 mm size sterile plastic tissue culture petri dish.
- CO_2 incubator.
- Laminar hood or biosafety hood.
- Centrifuge machine.
- Microfuge tubes: 1.5 ml capacity.
- Hot plate: temperature controlled.
- Glass thermometer.
- Refrigerator, −20 °C or − 80 °C deep freezer.
- Sealing machine.
- Marker pen.
- Dewar flask.
- Styrofoam box.
- Syringe 1 ml capacity.
- Safety goggles, cryo gloves.
- Straw storage Daisy Goblets with various color visitubes.
- Long stainless-steel forceps.

28.5.3 Preparation of Straws

Prepare 10 numbers of flexible mini straws per mouse (see Fig. 28.4).

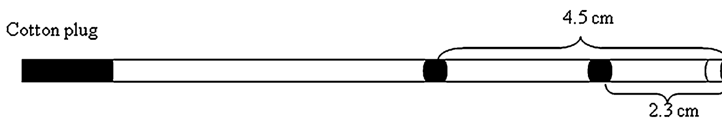


Fig. 28.4 A 0.25-ml (133 mm)-capacity flexible mini straw. Mark straw at distance 4.5 cm and 2.3 cm from the open end. Label the straws near the cotton plug. (Adopted from MRC Harwell institute, UK)

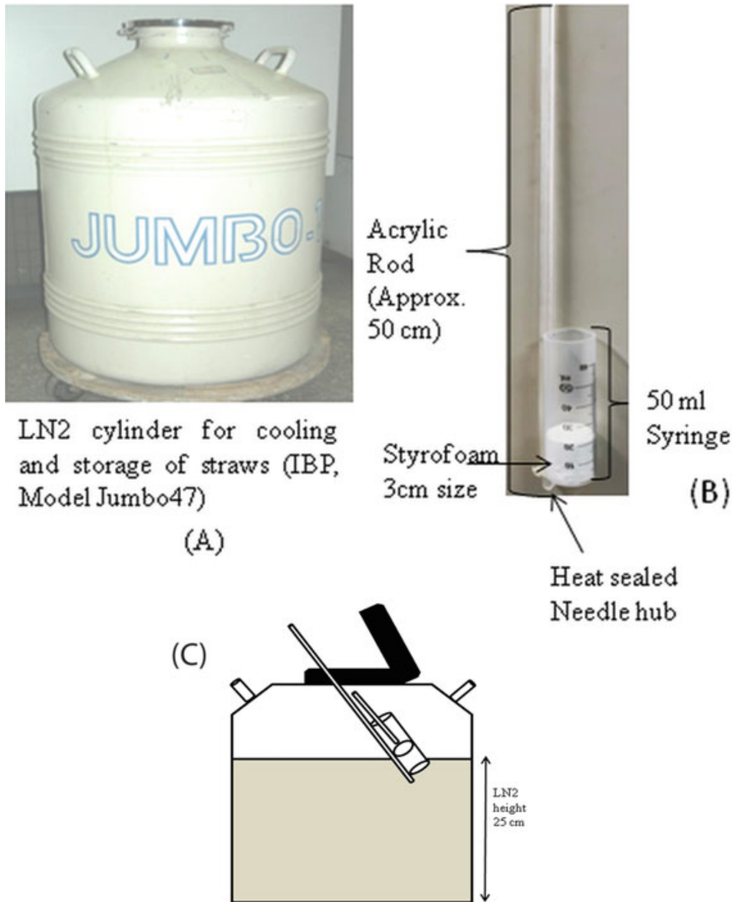


Fig. 28.5 Liquid nitrogen (LN2) container for storage (**a**), floating sperm-freezing device (**b**). Made from a 50 ml syringe attached to an approximately 2 feet acrylic rod. Heat seal the needle hub. Fix approx. 30 mm size Styrofoam at the bottom of syringe. (Originally prepared at Nakagata Laboratory, CARD, Japan) (**c**). Cooling chamber. The depth of LN2 = 25 cm

28.5.4 Cryoprotectant Plate

1. Put the first drop of 60 μl sperm cryoprotective agent (gCPA) in the center of the 35 mm sterile plastic tissue culture dish. Cover the drop with mineral oil (see Fig. 28.6a).
2. Add another drop of 60 μl of gCPA into the first drop so that the final volume would be 120 μl. This makes a drop tall and semispherical (see Fig. 28.6b).

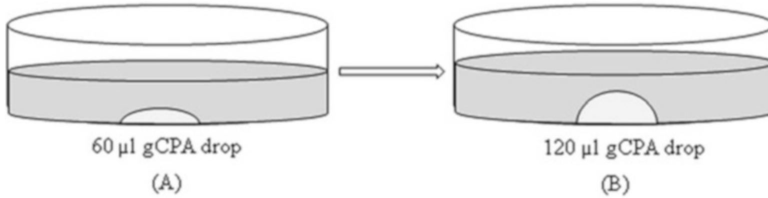


Fig. 28.6 (**a**) A 35 mm tissue culture grade plastic petri dish containing 60 µl and (**b**) 120 µl cryoprotectant agent (gCPA) covered with mineral oil

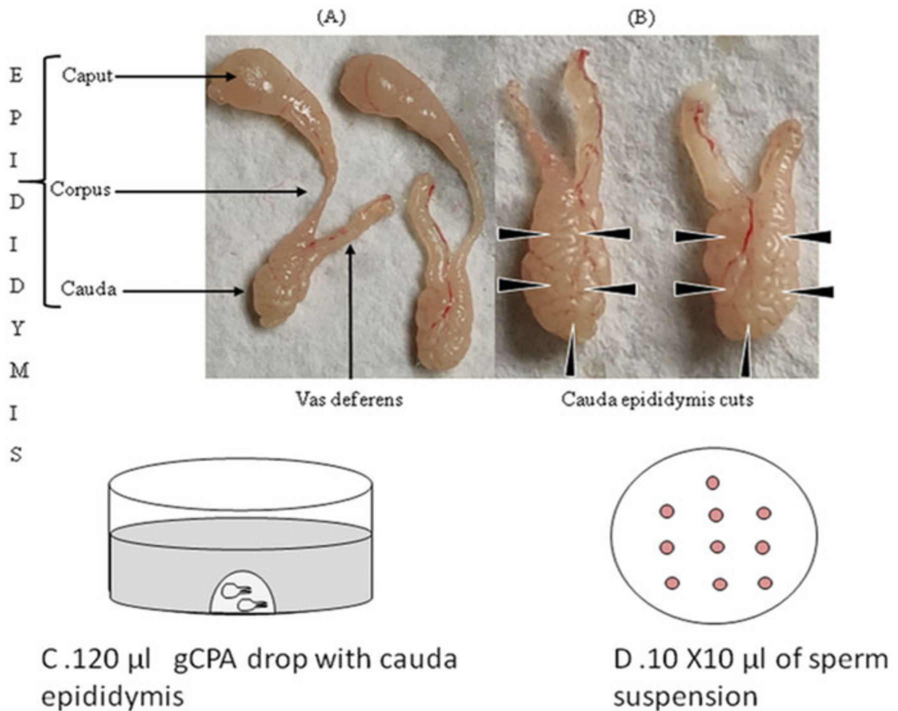


Fig. 28.7 (**a**) Entire epididymis separated from the testis. (**b**) Cauda epididymis, cut at various places. (**c**) A 120 µl gCPA drop with cauda epididymis. (**d**) 10 × 10 µl aliquots of sperm suspension

28.5.5 Collection and Freezing of Spermatozoa

1. Sacrifice mouse by cervical dislocation, and under aseptic conditions, collect both the cauda epididymis along with 2–3 mm vas deferens. Place the cauda on sterile tissue paper, and gently clean the adipose tissue, and squeeze the blood vessels to drain out blood (see Fig. 28.7a, b).

2. Transfer both the cauda into a 120 μl drop of gCPA covered with mineral oil in a 35 mm sterile plastic tissue culture petri dish (see Fig. 28.7c). For one cauda, use 60 μl drop of gCPA.
3. Under the dissecting microscope, give five to six cuts over cauda epididymis using fine iris scissors or a sharp surgical blade (see Fig. 28.7b). Place the dish on a warmer plate at 37 °C temperature for 3 min. Gently swirl the dish clock and anticlockwise after each minute for 3 min. Make 10 drops of 10 μl of sperm suspension on a 60 mm sterile petri dish (see Fig. 28.7d).
4. Connect 1 ml syringe at the cotton plug end, and slowly aspirate pre-warmed mHTF medium up to the level reaches 4.5 cm mark, aspirate air up to the 2.3 cm mark (see Fig. 28.4), aspirate one drop of sperm suspension, and continue to aspirate air until the mHTF medium reaches half way in the cotton plug. Seal both ends of the straw using a heat-sealing machine.
5. Transfer sealed straws into floating sperm-freezing apparatus (see Fig. 28.5b) keeping cotton plug end at the upper side. Place the freezing apparatus into the LN2 cylinder (see Fig. 28.5c) for 10 min. The depth of LN2 must be 20–25 cm. After holding for 10 min, plunge the straws directly into the liquid nitrogen.

28.5.6 Sperm Thawing

Thawing protocol is adopted from work published by Takeo (2011) [54].

28.5.6.1 Media and Solutions

Composition of Sperm Preincubation Medium (TYH + 0.75 mM MBCD) (See Table 28.7)

Filter the solution through a 0.22 μm filter and store 1 ml aliquots at 4 °C for up to 3 months:

Table 28.7 Composition of reagents for making TYH and MBCD

Reagent name	Cat. number	mg/100 ml
Sodium chloride (NaCl)	Sigma, S5886	697.6
Potassium chloride (KCl)	Sigma, P5405	35.6
Magnesium sulfate heptahydrate ($MgSO_4 \cdot 7H_2O$)	Sigma, M7774	29.3
Potassium phosphate monobasic (KH_2PO_4)	Sigma, P5655	16.2
Sodium pyruvate Na-pyruvate	Sigma, P4562	5.5
D-(+)-Glucose	Sigma, G6152	100.0
Calcium chloride dihydrate ($CaCl_2 \cdot 2H_2O$)	Sigma, C7902	25.1
Methyl-β-cyclodextrin	Sigma, C4555	98.3
Penicillin G	Sigma, P4687	7.5
Streptomycin	Sigma, S1277	5.0
Sodium bicarbonate ($NaHCO_3$)	Sigma, S5761	210.6
Poly vinyl alcohol	Sigma, P8136	100.0

Data taken from Nakagata N [57]

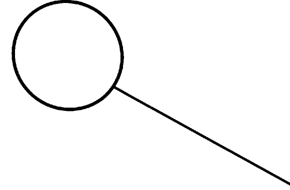


Fig. 28.8 Cotton plug pusher

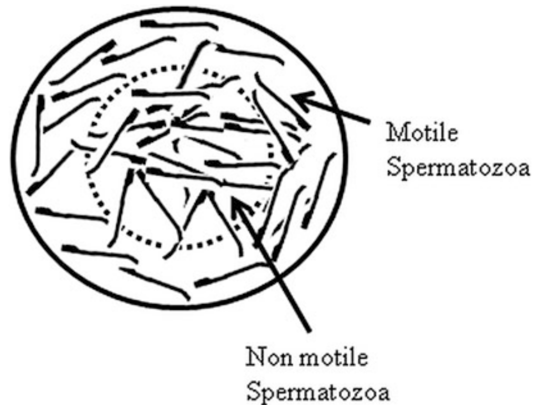


Fig. 28.9 Motile and nonmotile spermatozoa after thawing

1. Put a drop of 90 μl sperm preincubation medium into the center of a 35 mm petri dish, and overlay with mineral oil. Keep the plate for equilibration at 37 °C, 5% CO_2 for 20–30 min.
2. With the help of forceps, remove the straw from the storage tank, and transfer it into a 37 °C water bath for 10 min. Straw floats into the water bath so make sure that straw should completely immerse in the bath. Do not agitate the straw in water.
3. After 10 min, remove the straw, and hold it horizontally without shaking. Gently wipe the straw with sterile tissue paper. Cut the heat-sealed end of the straw, and push the cotton plug with the help of a cotton plug pusher (see Fig. 28.8) to expel 10 μl sperm suspension into the center of the drop of 90 μl sperm preincubation medium without expelling mHTF medium. Do not shake the plate to compromise the viability and motility of spermatozoa.
4. Incubate the plate for 30 min at 37 °C, 5% CO_2 in the incubator. Check the motility of sperms at the periphery of the preincubation drop while dead and nonmotile sperms at the center (see Fig. 28.9).

28.6 In Vitro Fertilization (IVF) Using Frozen Spermatozoa

Recovery of frozen mouse strains can be accomplished by in vitro fertilization (IVF). IVF is nothing but fertilization of mature oocytes and sperms (frozen-thawed or fresh sperms) in a tissue culture dish under controlled conditions. IVF can generate large

Table 28.8 Composition of fertilization medium

GSH stock	GSH working solution
Add 30.7 mg reduced glutathione (GSH) Sigma, cat. no. G4251, in 1 ml mHTF medium	(For frozen-thawed sperm samples) Add 50 μl of the GSH stock into a 5 ml mHTF medium. Mix gently. The final concentration is 1 mM GSH. Filter the solution through a 0.22 μm filter, and store at 4 °C

numbers of embryos which facilitates colony expansion [58]. However, IVF is influenced by the genetic background of strain, quality of oocytes, quality of sperms, media concentration, and environmental conditions.

28.6.1 Materials and Methods

28.6.1.1 Fertilization Media Containing Reduced Glutathione (GSH) (See Table 28.8)

In Vitro Fertilization

1. Refer to previously mentioned protocol for superovulation in donor mice.
2. Prepare three 60 mm sterile plastic tissue culture petri dishes, first dish, sperm preincubation dish (TYH + 0.75 mM MBCD); second dish, fertilization dish with reduced glutathione; and third dish containing mHTF medium. Equilibrate the sperm preincubation and fertilization dish for 15–20 min and mHTF dish for 4–5 h at 37 °C, 5% CO_2.
3. At the beginning, thaw the spermatozoa as per the previously mentioned sperm thawing protocol. Incubate the thawed spermatozoa in a CO_2 incubator for a minimum of 30 min.
4. After 30 min, sacrifice and collect the oviducts from 4 to 5 superovulated females (see Fig. 28.14c). Transfer the oviducts into mineral oil covered on the fertilization drop. Under a stereomicroscope, hold each oviduct down with forceps, and gently tear the swollen ampulla with a needle to release the cumulus-oocyte complex (COC). Using a needle, drag the COC through mineral oil into the fertilization drop. Repeat the procedure for all the oviducts (see Fig. 28.10a).
5. Take 10 μl of the motile sperm suspension from the peripheral part of the sperm preincubation drop by using a 10-μl-capacity pipette tip (see Fig. 28.10b). Incubate the dish at 37 °C, in 5% CO_2 for approximately 3–4 hrs.

28.6.2 Washing and Culture

1. After incubation in the fertilization drop, sequentially transfer all the oocytes from drop number one to three of mHTF medium. Transfer good-quality probable zygotes in drop number four of 150 μl mHTF medium for overnight culture at

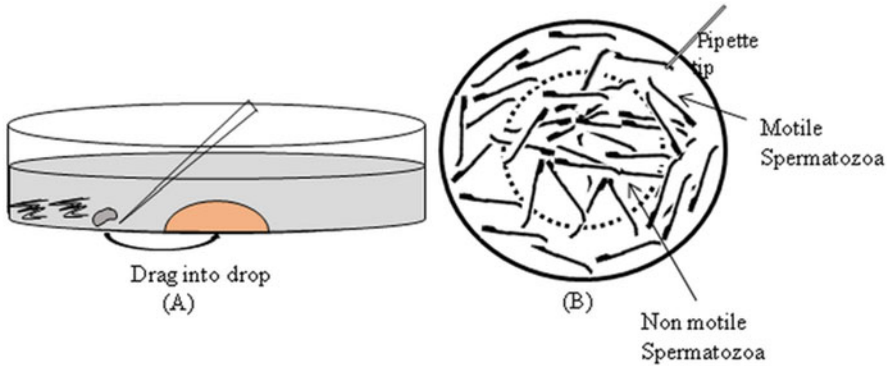


Fig. 28.10 (**a**) A 90 μl fertilization drop containing oviducts and COC (**b**) sperm preincubation drop containing motile and nonmotile spermatozoa. Pick up motile sperms with the pipette tip

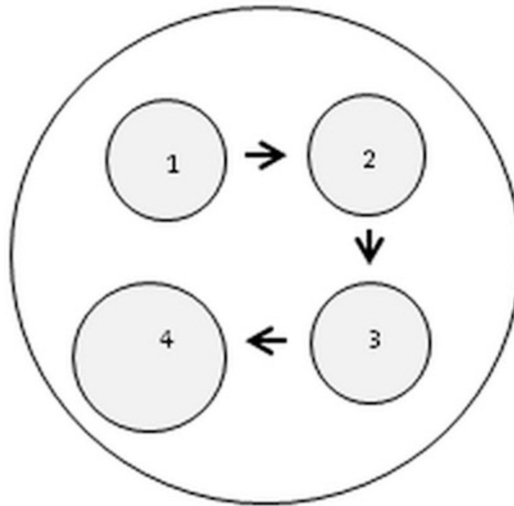


Fig. 28.11 A 60 mm culture dish, number 1–3 is 100 μl drops of mHTF medium and number 4 is 150 μl drop of mHTF medium for overnight culture

37 °C, 5% CO_2 (see Fig. 28.11). In the next day, observe and count the cleaved two-cell embryos.

2. Surgically transfer the two-cell embryos into the oviduct of 0.5-day-old pseudopregnant foster mothers, or cryopreserve the embryos for future use or culture them in blastocyst media (K-SIBM-50) or KSOM +AA media.

Embryo Culture medium (See Table 28.9).

Table 28.9 Composition of potassium simplex optimized medium (KSOM) supplemented with amino acids

Reagent name	Catalog number	mg/100 ml
NaCl	Sigma, S 5886	555
KCl	Sigma, P 5405	18.5
KH2PO4	Sigma, P 5655	4.75
MgSO4 7H2O	Sigma, M 7774	4.95
CaCl2 2H2O	Sigma, C 7902	25
NaHCO3	Sigma, S 5761	210
Glucose	Sigma, G 6152	3.6
Na-Pyruvate	Sigma, P 4562	2.2
DL-lactic Acid, sodium salt	Sigma, L 1375	0.174 ml
10 mM EDTA	Sigma, E-6635	100 μl
Streptomycin	Sigma, S 9137	5
Penicillin	Sigma, P 7794	6.3
0.5% phenol red	Sigma, P 0290	0.1 ml
L-Glutamine	Sigma, G 8540	14.6
MEM essential amino acids	Gibco, 11,130–051	1 ml
MEM non-essential AA	Sigma, M 7145	0.5 ml
BSA	Sigma, A-4378	100

Data were taken from Lawitts J.A. and Biggers J.D [59]

28.7 Embryo Transfer

Surgical embryo transfer is possible from the one-cell stage to the blastocyst stage. Depending upon its stage, embryos can be transferred into an oviduct of 0.5 dpc and uterine horn of 2.5 dpc pseudopregnant female mice. For blastocyst transfer, 2.5 dpc pseudopregnant female is more suitable than 3.5 dpc because embryos get sufficient time for implantation. This is called an asynchronous embryo transfer. The success of implantation is higher in asynchronous transfer than in synchronous transfer [60].

28.7.1 Pseudopregnant Female

The pseudopregnant recipient females used for embryo transfer are obtained by natural mating with vasectomized males [7]. The choice for pseudopregnant females is outbred strains like CD-1 or Swiss Webster or F1 hybrid like B6D2F1 (cross between C57BL/6 female and DBA/2 male) or B6CBAF1 (cross between C57BL/6 females and CBA male) due to their good maternal behavior. However, CD-1 and Swiss Webster mice get fatty too early which makes the surgical procedure difficult. On the contrary, F1 hybrids do not get fatty and can be kept for a longer time. The coat color of the pseudopregnant females should be considered for embryo transfer.

28.8 Surgical Procedures

In this section, various surgical procedures like vasectomy, uterine, and oviduct transfer of embryos are described in detail.

28.8.1 Vasectomy

Vasectomized males (sterile males) are bred with females to obtain pseudopregnant mice for oviduct or uterine transfer.

28.8.1.1 Materials and Methods

1. The choice of strain is Swiss Webster or CD1 mice (8–10 weeks) having proven breeding performance.
2. All the surgical instruments should be cleaned in a 0.1% sodium hypochlorite solution and sterilized by autoclaving. Preferably, carry the surgical procedures under laminar airflow to avoid pathogenic cross-contamination.
3. Anesthesia is performed by IP injection of a combination of Inj. Xylazine and Inj. Ketamine at the rate of 10 mg/kg and 90 mg/kg body weight, respectively. Alternatively, inhalation anesthesia like isoflurane can be used. The induction by isoflurane gas in oxygen can be achieved by induction and maintenance anesthesia (for induction, 5% isoflurane, and 2 L/min oxygen following maintenance anesthesia at 2.5% isoflurane at 1 L/min oxygen). Check the pedal reflexes after 10–15 min to confirm the plane of anesthesia.
4. Apply the eye ointment or put a drop of normal saline on both eyes to avoid corneal dehydration. To maintain the fluid balance, inject the warm 0.9% normal saline at the rate of 10 ml/kg body weight subcutaneously.
5. Place the mouse in the supine position, and remove the fur from the midline area of size 3 $\times$ 3 cm (see Fig. 28.12a). Sterile the area by alternate wiping with 10% povidone-iodine and 70% ethanol for three times.

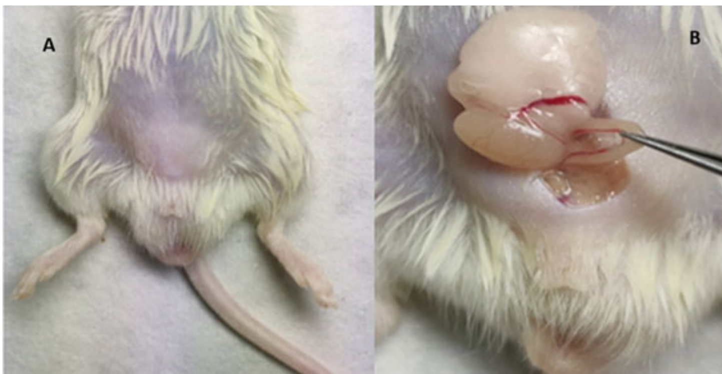


Fig. 29.12 (**a**) Incision site for vasectomy. (**b**) A tubular structured vas deferens

6. Hold the skin with sterile fine forceps and give a 1–1.5 cm midline incision. Expose the abdominal cavity, hold the supra-testicular fat pad, and slowly pull out the testis along with the epididymis and vas deferens. The vas deferens can be recognized by its tubular structure and blood vessel running along one side (see Fig. 28.12b).
7. Slowly separate the vas deference from the blood vessel. Ligate the vas deferens at two sites by keeping approx. 1 cm distance. Cut the middle portion of the ligation. Keep the cut piece of vas deferens on paper, and slowly move back testis into the abdominal cavity. Follow the same procedure for another testis. Confirm both the pieces of the vas deferens on paper before closing the surgical wound.
8. Suture the muscle with the help of an absorbable suture of size 5.0. Close the surgical wound by using tissue adhesive or wound clips or by suturing with nylon thread.
9. Place the mouse on a thermal plate at 38 °C. Inject the analgesics like Inj. Buprenorphine at 0.05 to 0.1 mg/kg body weight subcutaneously every after 12 hrs for 3 days. After 10 days, remove the surgical thread or wound clips.
10. After sufficient rest of 2 weeks, test the vasectomized males by allowing them to mate with sexually mature females. Observe the females for pregnancy.

28.8.2 Uterine Transfer

The technique of uterine transfer was developed by McLaren and Michie. This was the first report of successful live birth was obtained from in vitro cultured mouse embryos [61]. Later this technique is extended by Rafferty [62] and Hogan et al. [63]

28.8.2.1 Materials and Methods

1. For uterine transfer, use a 2.5-day-old pseudopregnant female mouse. The choice of strains used is mentioned earlier. The body weight of the mouse should be ranging between 25 and 30 g. Anaesthetize the animals as per protocol mentioned at 6.1.
2. Remove the patch of fur at the paralumbar fossa region. Sterile the skin by using sequential wiping with a swab of 10% povidone-iodine and 70% alcohol alternatively for three times (see Fig 28.14a).
3. Load the embryo transfer pipette in a sequence. First, aspirate a small amount of F1 medium, followed by a 0.5–1 cm air bubble, then F1 medium, second air bubble, and finally draw blastocysts with a minimal volume of the medium. Alternatively, M2 media can be used.
4. Make 0.5 to 1 cm incision 5 mm away from the midline. Slide the skin to the left and right until the ovary or fat pad is visible through the abdominal wall. Hold the abdominal wall with forceps, and give 0.5 cm of the incision with a fine iris scissor.
5. Pick up the fat pad associated with the ovary and clamp with Bulldog clamp. To avoid dryness, apply sterile normal saline on the ovary and uterine horn. Gently

hold the tip of the uterine horn using blunt forceps and with the help of a 26-gauge needle perform a hole at 2–3 millimeters down from the utero-tubular junction (see Fig. 28.14b). Pull out the needle and insert the embryo transfer pipette through the hole into the uterine lumen. Gently blow the blastocysts along with the second air bubble and medium.
6. Unclip the Bulldog clamp and push the fat pad, ovary, and uterus back into the body cavity. Suture the muscle with 5.0 absorbable sutures, and close the skin wound by tissue adhesive (3 M Vet Bond) or wound clips. Place the mouse on a warm plate maintaining a temperature of 38 °C for quicker recovery.
7. Transfer the female to the original cage, and provide cage enrichment and nesting material to avoid cannibalism of pups.

28.8.3 Oviduct Transfer

The first oviduct transfer of mouse embryo was done by Tarkowski [64]. Infundibulum or oviduct transfer is being carried out to rederive mouse strains by specific pathogen-free (SPF) method or transfer of 1 or two-cell stage embryos after microinjection/ or IVF.

28.8.4 Materials and Methods

28.8.4.1 Infundibulum Embryo Glass Transfer Pipette

Embryos are handled using pulled borosilicate glass pipettes. The length and outer and inner diameters of the glass pipette are a very critical part while pulling the glass pipette. The length gives the maximum control while aspirating and releasing the embryos. The OD and ID should be precise to avoid less friction, easy penetration, and release of embryos into the infundibulum. Approximate dimensions are the outer diameter is around 200–220 μm, inner diameter is around 150–180 μm, and the tapered length of the pipette is approximate 3–5 cm.

28.8.4.2 Loading of Embryos in a Pipette

- Load the transfer pipette with pre-warmed M2 medium approximately 1–1.5 cm to allow maximum and precise control of airflow during aspiration and release of the embryos.
- Then aspirate sequentially, a small air bubble around 0.5 to 1 cm, then embryos with a minimum volume of M2 medium. For an inexperienced person, take one more additional air bubble at the tip to prevent embryo loss while transferring (see Fig. 28.13).

28.8.4.3 Transfer

1. Use 0.5 dpc pseudopregnant female mice for ovarian transfer.
2. Using sharp iris scissors and forceps, make a straight 10 mm skin incision approximately 5 mm away from the midline. Separate the skin from the body

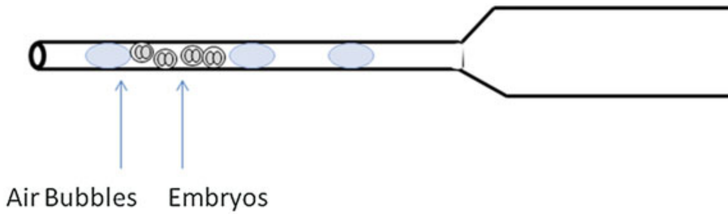


Fig. 28.13 Loading of glass pipette with embryos

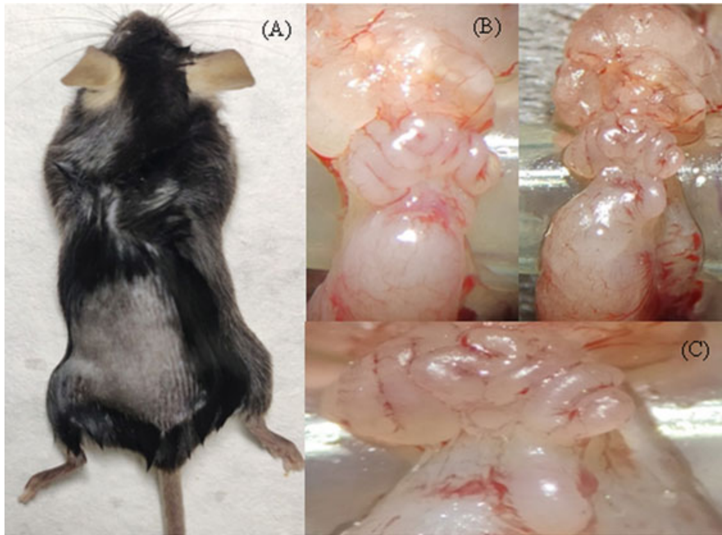


Fig. 28.14 (**a**) Surgical incision site for embryo transfer. (**b**) Ovary, oviduct, utero-tubular junction, and uterine horn. (**c**) Oviduct

wall by blunt dissection using scissors tips. Give the incision to the body wall until the white fat pad associated with the ovaries is visible. Grasp the fat pad with blunt forceps and pull it through the incision. Clip the ovarian fat pad with a bulldog clamp (see Fig 28.14c).

3. Gently tear the bursa over the ovary with two pairs of iris forceps to locate the opening of the infundibulum. If bleeding occurs, blot the blood with the help of a sterile cotton swab or Kimwipes.
4. Insert the tip of the loaded glass pipette through the infundibulum, and blow until the air bubble is visible within the ampulla. Observation of air bubbles is an indication of the successful transfer of embryos into the infundibulum.
5. Unclip the Bulldog clamp and push back the ovary and uterus into the abdominal cavity. Close the muscle incision by absorbable catgut number 5.0 and skin by interrupted nylon sutures or tissue adhesive like 3 M Vet Bond. Embryo transfer can be done unilaterally or bilaterally.

6. Transfer the animal to a fresh cage and provide enrichment into the cage.
7. Observe the abdominal distension after 10 days and full-term delivery by 20 days after surgery.

Note: To gain experience of infundibulum transfers, initially do practice to locate infundibulum; do the transfer using dye or Affi-Gel beads. The challenges during infundibulum transfer are small reproductive tract of the mouse, bleeding which may interfere to see the opening of the infundibulum, peristaltic movement of the abdomen leads to movement of the oviduct, the expulsion of embryos due to positive pressure inside the oviduct, clogging of embryo transfer tip due to blood.

28.9 Conclusion

With the advancement of genetic engineering techniques in the recent past, the number of genetically modified mouse strains has exploded. Assisted reproductive technologies (ARTs) help researchers to safeguard these valuable strains against loss due to various catastrophes. The value of these techniques to maintain genetic quality, slowing genetic drift, discusses the possibility to avoid transporting live animals and helping reduce the pathogen transfer into facilities. The laboratories engaged in the development of genetically modified strains should develop ART. Researchers can use these protocols for implementing the effective use of ART in the facility.

References

1. Beck JA, Lloyd S, Hafezparast M et al (2000) Genealogies of mouse inbred strains. Nat Genet 24:23–25
2. Landel P (2005) Archiving mouse strain by cryopreservation. Lab Anim 34(4):50–57
3. Pomeroy KO (1991) Cryopreservation of transgenic mice. Genet Anal Tech Appl 8(3):95–101
4. Wayss KW, Michaela K, Johannes S (2005) Cryopreservation of transgenic mouse embryos- an eight year's experience. J Exp Anim Sci 43:69–85
5. Whittingham D, Leibo S, Mazur P (1972) Survival of mouse embryos frozen to −196° and −269°. Curr Sci 178:411–414
6. Russell WMS (2005) The three Rs: past, present and future. Anim Welf 14:279–286
7. Nagy A, Gertsenstein M, Vintersten K et al (2003) Manipulating the mouse embryo: a laboratory manual, 3rd edn. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, pp 148–149
8. Byers SL, Payson SJ, Taft RA (2006) Performance of 10 inbred mouse strains following assisted reproductive technologies (ARTS). Theriogenology 65:1716–1726
9. Suzuki O, Asano T, Yamamoto Y et al (1996) Development in vitro of preimplantation embryos from 55 mouse strains. Reprod Fertil Dev 8:975–980
10. Vergara GJ, Irwin MH, Moffatt RJ et al (1997) In vitro fertilization in mice: strain differences in response to superovulation protocols and effect of cumulus cell removal. Theriogenology 47:1245–1252
11. Spearow JL (1988) Major genes control hormone-induced ovulation rate in mice. J Reprod Fertil 82:787–797

12. Spearow JL, Barkley M (1999) Genetic control of hormone-induced ovulation rate in mice. Biol Reprod 61:851–856
13. Gates AH (1956) Viability and developmental capacity of eggs from immature mice treated with gonadotrophins. Nature 177:754–755
14. Gates AH (1971) Maximizing yield and developmental uniformity of eggs. In: Daniel JC (ed) Methods in mammalian embryology. WH Freeman, San Francisco, CA, pp 64–75
15. Luo C, Zuñiga J, Edison E et al (2011) Superovulation strategies for 6 commonly used mouse strains. J Am Assoc Lab Anim Sci 50(4):471–478
16. Auerbach AB, Norinsky R, Ho W, Losos K, Guo Q, Chatterjee S, Joyner AL (2003) Strain-dependent differences in the efficiency of transgenic mouse production. Transgenic Res 12:59–69
17. Mello MRB, Queiroz VS, Lima AS et al (2001) Cryopreservation of mouse morulae through different methods: slow-freezing, vitrification and quick-freezing. Braz J Vet Res Anim Sci 38 (4):160–164
18. Bryant G (1995) DSC measurement of cell suspensions during successive freezing runs: implications for the mechanism of intracellular ice formation. Cryobiology 32:114–128
19. Campos-Chillon LF, Walker DJ, Torre-Sanchez JF et al (2006) In vitro assessment of a direct transfer vitrification procedure for bovine embryos. Theriogenology 65:1200–1214
20. Whittingham D (1974a) Embryo banks in the future of developmental genetics. Genetics 78:395–402
21. Whittingham D (1974b) The viability of frozen-thawed mouse blastocysts. J Reprod Fertil 37:159–162
22. Audrey Roustan, Jeanne Perrin, Anaı¨s Berthelot-Ricou, Erica Lopez, Alain Botta, Blandine Courbiere (n.d.) Evaluating methods of mouse euthanasia on the oocyte quality: cervical dislocation versus isoflurane inhalation
23. Uechi H, Tsutsumi O, Morita Y et al (1999) Comparison of the effects of controlled rate cryopreservation and vitrification on 2-cell mouse embryos and their subsequent development. Hum Reprod 14:2827–2832
24. Macas E, Min X, Paul JK et al (1991) Developmental capacities of two-cell mouse embryos frozen by three methods. J In Vitro Ferti Embryo Transfer 8(4):208–212
25. Rall WF, Fahy GM (1985) Ice-free cryopreservation of mouse embryos at −196 °C by vitrification. Nature 313(6003):573–575
26. Mochida K, Hasegawa A, Taguma K et al (2011) Cryopreservation of mouse embryos by ethylene glycol-based vitrification. J Vis Exp e3155:57
27. Yan CL, Yang QE, Zhou GB et al (2008) Open-pulled straw (OPS) vitrification of in vitro fertilised mouse embryos at various stages. Acta Vet Hung 56:245–253
28. Son WY, Yoon SH, Yoon HJ et al (2003) Pregnancy outcome following transfer of human blastocysts vitrified on electron microscopy grids after induced collapse of the blastocoele. Hum Reprod 18:137–139
29. Saki G, Dezfuly FG (2005) Vitrification of human oocyte using cryoloop. Iran J Reprod Med 3:19–24
30. Ryu EK, Hur YS, Ann JY et al (2012) Vitrification of mouse embryos using the thin plastic strip method. Clin Exp Reprod Med 39(4):153–116
31. Thorat RA, Ingle AD (2012) An attempt of cryopreservation of mouse embryos at the ACTREC laboratory animal. Facility India Exp Anim 61(2):139–145
32. Polge C, Smith AU, Parkes AS (1949) Revival of spermatozoa after vitrification and dehydration at low temperatures. Nature 164:666–668
33. Tada N, Sato M, Yamanoi J et al (1990) Cryopreservation of mouse spermatozoa in the presence of raffinose and glycerol. J Reprod Fertil 89:511–516
34. Songsasen N, Leibo S (1997) Cryopreservation of mouse spermatozoa. Cryobiology 35:255–269

35. Tao J, Du J, Kleinhans FW et al (1995) The effect of collection temperature, cooling rate and warming rate on chilling injury and cryopreservation of mouse spermatozoa. J Reprod Fertil 104:231–236
36. Penfold LM, Moore HD (1993) A new method for cryopreservation of mouse spermatozoa. J Reprod Fertil 99:131–134
37. Sztein JM, Farley JS, Young AF et al (1997) Motility of cryopreserved mouse spermatozoa affected by temperature of collection and rate of thawing. Cryobiology 35(1):46–52
38. Okuyama M, Isogai S, Saga M et al (1990) In vitro fertilization (IVF) and artificial insemination (AI) by cryopreserved spermatozoa in mouse. J Fert Implant 7:116–119
39. Yokoyama M, Akiba H, Katsuki M et al (1990) Production of normal young following transfer of mouse embryos obtained by in vitro fertilization using cryopreserved spermatozoa. Exp Anim 39:125–128
40. Takeshima T, Nakagata N, Ogawa S et al (1991) Cryopreservation of mouse spermatozoa. Exp Anim 40(4):493–497
41. Nakagata N, Takshima T (1992) High fertilizing ability of mouse spermatozoa diluted slowly after cryopreservation. Theriogenology 37:1283–1291
42. Tada N (1993) Effect of pre-freezing equilibration and post-thawing centrifugation on the fertilizing capacity of frozen mouse epididymal spermatozoa. Cryo-Lett 14:195–206
43. Nakagata N (1996) Use of cryopreservation techniques of embryos and spermatozoa for production of transgenic (Tg) mice and for maintenance of Tg mouse lines. Lab Anim Sci 46 (2):236–238
44. Marschall S (1999) Hrabe de, Angelis M. Cryopreservation of mouse spermatozoa: double your mouse space. Trends Genet 15:128–131
45. Critser JK, Mobraaten LE (2000) Cryopreservation of murine spermatozoa. ILAR J 41:197–206
46. Nakagata N (1993) Production of normal young following transfer of mouse embryos obtained by *in vitro* fertilization between cryopreserved gametes. J Reprod Fert 99:77–80
47. Nakagata N, Takeshima T (1993) Cryopreservation of mouse spermatozoa from inbred and F_1 hybrid strains. Exp Anim 42(3):317–320
48. Nakagata N, Ueda S, Yamanouchi K et al (1995) Cryopreservation of wild mouse spermatozoa. Theriogenology 43:635–643
49. Sztein JM, Farley JS, Mobraaten LE (2000) In vitro fertilization with cryopreserved inbred mouse sperm. Biol Reprod 63(6):1774–1780
50. Takeo T, Nakagata N (2010) Combination medium of cryoprotective agents containing L-glutamine and methyl-{beta}-cyclodextrin in a preincubation medium yields a high fertilization rate for cryopreserved C57BL/6J mouse sperm. Lab Anim 44(2):132–137
51. Renard P, Grizard G, Griveau JF et al (1996) Improvement of motility and fertilization potential of postthaw human sperm using glutamine. Cryobiology 33:311–319
52. Takeo T, Hoshii T, Kondo Y et al (2008) Methyl-beta-cyclodextrin improves fertilizing ability of C57BL/6 mouse sperm after freezing and thawing by facilitating cholesterol efflux from the cells. Biol Reprod 78:546–551
53. Choi YH, Toyoda Y (1998) Cyclodextrin removes cholesterol from mouse sperm and induces capacitation in a protein-free medium. Biol Reprod 59:1328–1333
54. Takeo T, Nakagata N (2011) Reduced glutathione enhances fertility of frozen/thawed C57BL/6 mouse sperm after exposure to methyl-Beta-cyclodextrin. Biol Reprod 85(5):1066–1072
55. Nakagata N (2000) Cryopreservation of mouse spermatozoa. Mamm Genome 11(7):572–576
56. Kito S, Ohta Y (2005) Medium effects on capacitation and sperm penetration through the zona pellucida in inbred BALB/c spermatozoa. Zygote 13(2):145–152
57. Nakagata N. (2011) Cryopreservation of mouse spermatozoa and in vitro fertilization. In: Hofker M., van Deursen J. (eds) Transgenic mouse methods and protocols. Methods in molecular biology (methods and protocols). vol. 693. Humana Press
58. Thornton CE, Brown SD, Glenister PH (1999) Large numbers of mice established by in vitro fertilization with cryopreserved spermatozoa: implications and applications for genetic resource banks, mutagenesis screens, and mouse backcrosses. Mamm Genome 10:987–992

59. Lawitts JA, Biggers JD (1993) Culture of preimplantation embryos. Methods Enzymol 225:153–164
60. López-Cardona AP, Fernández-González R, Pérez-Crespo M et al (2016) Effects of synchronous and asynchronous embryo transfer on postnatal development, adult health, and behavior in mice (published correction appears). Biol Reprod 94(1):20
61. Mc Laren A, Michie D (1956) Studies on the transfer of fertilized mouse eggs to uterine foster-mothers. I. Factors affecting the implantation and survival of native and transferred eggs. J Exp Biol 33:397–416
62. Rafferty RA (1970) Methods in experimental embryology of the mouse. Johns Hopkins Press, Baltimore
63. Hogan B, Beddington R, Costantini F et al (1994) Manipulating the mouse embryo, 2nd edn. Cold Spring Harbor Laboratory Press, New York
64. Tarkowski AK (1959) Experimental studies on regulation in the development of isolated blastomeres of mouse eggs. Acta Theriol 3:191–287

Generating Transgenic Animal Models: Recent Technological Advancements

29

Neerja Wadhwa, Nirmalya Ganguli, and Subeer S. Majumdar

Abstract

Since the generation of the first transgenic animal by Gordan and Ruddle, several methods and procedures have been developed for the manipulation of the genome. This chapter highlights several technical advancements in animal transgenesis during the last few decades that enabled us to annotate every gene with its specific function. The various transgenic techniques such as pronuclear microinjection, embryonic stem cell-mediated gene targeting, somatic cell nuclear transfer, retrovirus gene transfer, sperm- and testicular-mediated gene transfer, Cre-lox mediated gene targeting, RNAi-mediated gene targeting, and CRISPR-Cas9-mediated gene editing along with their refinements, improvements, limitations, and applications for biomedical research are described. This chapter outlines the state-of-the-art animal transgenesis, thereby offering various technological opportunities in the field of biomedical research.

Keywords

Animal transgenesis · Transgenic techniques · Transgenic methods · Transgenic animals · Pronuclear microinjection · Embryonic stem cell-mediated gene

N. Wadhwa
Embryo Biotechnology Laboratory, National Institute of Immunology, New Delhi, India

N. Ganguli
Gene and Protein Engineering Laboratory, National Institute of Animal Biotechnology, Hyderabad, Telangana, India

S. S. Majumdar (✉)
Embryo Biotechnology Laboratory, National Institute of Immunology, New Delhi, India

Gene and Protein Engineering Laboratory, National Institute of Animal Biotechnology, Hyderabad, Telangana, India
e-mail: subeer@niab.org.in

© The Author(s), under exclusive license to Springer Nature Singapore Pte Ltd. 2021

P. Nagarajan et al. (eds.), *Essentials of Laboratory Animal Science: Principles and Practices*, https://doi.org/10.1007/978-981-16-0987-9_29

targeting · Somatic cell nuclear transfer · Testicular transgenesis · Cre-lox · RNAi · CRISPR-Cas9

29.1 Introduction

The fundamental goal of many studies in genomics is to determine the physiological function of an identified gene. In this context, transgenic animal technology has completely revolutionized the biological world by becoming a privileged way of analyzing gene function. Transgenic animals are genetically altered animals generated by a method known as "transgenesis" in which any foreign gene "transgene" is introduced into an animal so that the gene is expressed and inherited by the offspring. Several transgenic techniques have been developed over the past few decades for the production of transgenic animals. Each transgenic technique has its efficiency and expression levels and has advantages and disadvantages. Transgenic technologies have enabled researchers to generate transgenic animal models to study human development, diseases, and disorders. This chapter summarizes the state-of-the-art animal transgenesis, thereby providing varied technological opportunities within the field of biomedical research.

29.2 Historical Background

The ability to introduce foreign genes into the animals provides a really powerful tool for dissecting various biological processes and systems. The first successful embryo transfer was done by Walter Heape in Angora Rabbits in 1891 [1]. Later, when the culture system was developed in 1940, oocytes could sustain themselves through several cleavage divisions, thereby providing possibilities for variable means of embryo manipulation [2].

The term "transgene" first coined by Gordan and Ruddle 1981 refers to any foreign gene that gets integrated into the host genome [3, 4]. The animal whose genetic composition gets altered by the insertion of foreign genes is referred to as a "transgenic animal." The whole procedure is called "transgenesis" [4, 5].

Gordon et al. [4] illustrated the introduction of a foreign gene into the mice by embryo pronuclear microinjection, a methodological gene transfer approach being widely employed for studying molecular and cellular functions of numerous novel identified genes. Since then, many transgenic approaches like embryonic stem cell-mediated gene transfer, retrovirus-mediated gene transfer, somatic cell nuclear transfer, sperm- and testicular-mediated gene transfer, Cre-lox, RNAi, and CRISPR-Cas9 technology have been effectively used to generate transgenic mice and many other animal species such as rats, rabbits, chicken, dogs, pigs, fishes, cattle, sheep, and primates. As new technologies are emerging, techniques continue to evolve to widen the relevance of transgenic animals for exploring the genetic function, for genetic improvement, and for their use as bioreactors, animal disease models, and organ transplantation.

29.3 Techniques for the Generation of Transgenic Animals

There are various techniques for the incorporation of a foreign gene to generate transgenic animals. The gene transfer methods include pronuclear microinjection, embryonic stem cell-mediated gene transfer, retrovirus-mediated gene transfer, somatic cell nuclear transfer, sperm- and testicular-mediated gene transfer, Cre-lox, RNAi-mediated gene targeting, and CRISPR-Cas9-mediated gene editing.

29.4 Pronuclear Microinjection

The most common and well-characterized procedure for generating transgenic mice is direct pronuclear microinjection of one-cell fertilized embryos. The method was successfully used by Gordon and colleagues [4, 5]. The method involves the injection of DNA carrying the gene of interest into the male pronucleus of fertilized embryos [6]. Fertilized embryos are obtained from superovulated female donor mice mated with stud mice. One-cell fertilized embryos are microinjected with an ultrathin glass capillary pipette filled with a DNA of interest. Approximately 1–2 picolitres of DNA is injected into the male (larger) pronucleus until a visible increase in the pronuclear perimeter is observed (Fig. 29.1). The microinjected embryos are surgically transferred and implanted into the oviducts/uteri of pseudopregnant females (female mice mated with vasectomized male mice) that are physiologically receptive to the embryos for implantation. After 19 days of the gestation period, the recipient surrogate mice give birth to offspring which can be screened for transgene

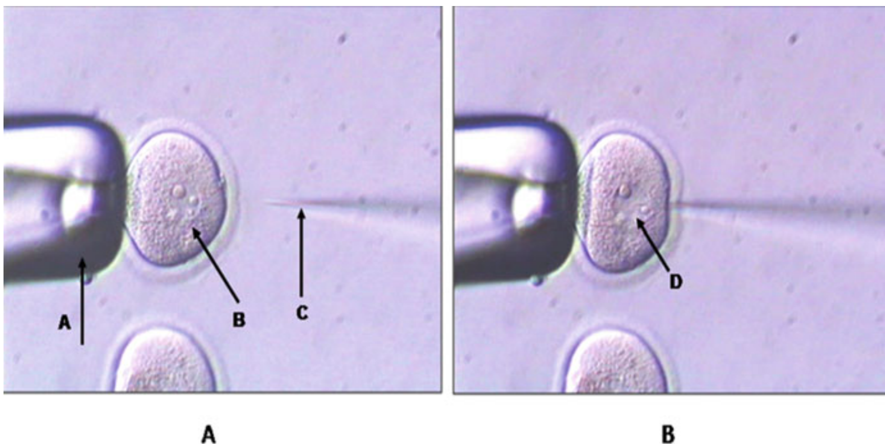


Fig. 29.1 Pronuclear microinjection of DNA into mouse fertilized oocytes. (**a**) Holding pipette is used to hold an oocyte. (**b**) Male pronuclei being bigger is focused to make it visible for microinjecting DNA. (**c**) The injection pipette is loaded with DNA of interest. (**d**) Injection pipette is inserted into male pronucleus to inject suspended DNA. A sudden increase in the perimeter of the pronucleus confirms its microinjection

integration by PCR, slot blot, and Southern hybridization. Transgenic mice can then be bred to create various transgenic lines. The work of researchers in the field of transgenesis has revealed several prominent features of pronuclear microinjection [7–12]. The efficiency varies with 10–40% of mice containing the transgene [13]. The integration process of the transgene is essentially random having no control over the site where the gene gets integrated nor on the copy number of the transgene which gets integrated [13, 14]. The DNA normally integrates at a single site in the genome with a multicopy insertion in a head-to-tail fashion [15, 16]. Integration mostly occurs at the one-cell stage, thereby passing the transgene on to subsequent generations. Genomic integration is an additive process as the host gains new information. The genome of the host remains unaffected except at the locus of integration, where deletions may take place rarely, thereby disrupting endogenous genes.

The major advantage of microinjection is its technical reliability in terms of gene expression and its utilization in a wide variety of species. There is no limitation on the length of the foreign gene to be microinjected. It has been shown that long DNA fragments can successfully be inserted to generate transgenic animals. As the technique involves microinjection at the one-cell zygotic stage, all subsequent cells derived from it are transgenic. The technique is highly stable in terms of gene expression being transmitted from one generation to the next generation.

The major disadvantages of the pronuclear technique are the micromanipulator equipment is highly sophisticated and expensive, the operating techniques require skilled personnel, the process is time-consuming, and the embryo survival rate is quite low. The method also has a major disadvantage that random incorporation of the transgene into the host genome might lead to consequences of inactivation of any of the host genes or activation of an oncogene leading to tumor formation in the host.

29.5 Embryonic Stem Cell-Mediated Gene Transfer

The next popular method for creating transgenic animals is the embryonic stem (ES) cell-mediated gene transfer which is a complicated but dominant approach for germline gene insertion [17]. ES cells are pluripotent stem cells that are obtained from the inner cell mass of the blastocyst and are used for generating transgenic mice. ES cells have the exclusive property of pluripotency to develop into any other type of cell or tissue in an adult organism. The ES cells are retrieved using a micropipette and cultured. Typically, ES cells are altered by the process of homologous recombination using a mutated targeting construct. The transgene is inserted into the ES cells utilizing electrical current (electroporation) [18], chemiporation, or with the help of viruses [19]. The positive transgenic ES cells are then selected from among non-transgenic cells and are furthered cultured to create transgenic ES cell colonies. The transgenic ES cells are then injected into a fresh blastocyst stage embryo where they are placed near the cells of the inner cell mass (Fig. 29.2). The blastocyst is then implanted into the uterus of a pseudopregnant female [20].

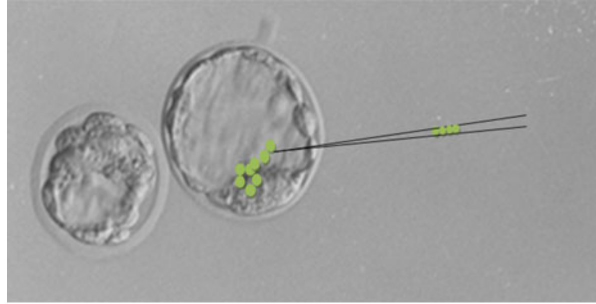


Fig. 29.2 Embryonic stem cell-mediated gene transfer. The embryonic stem cells with a specific gene of interest (represented by green color circles) are microinjected into the mouse blastocyst by injection pipette

The chimeric pups generated are screened for germline transmission and are bred further to generate pure transgenic animals. The level of chimerism can also be judged by observing the coat color of mice after birth. This technique has enabled variable transgenic approaches in the mouse including targeted gene knockout, targeted gene repair/replacement, conditional gene targeting, and gene trap reporter systems [21].

An advantage of using ES cells over microinjection is the prior selection of transgenic ES cells using selectable markers before implanting them into a blastocyst, increasing the efficiency of the process. Secondly, they allow targeted incorporation of the transgene through homologous recombination avoiding random integration as found in microinjection [22].

The obstacles for the generation of ES cell-derived transgenic animals is the complexity associated with the generation, characterization, and maintenance of pluripotent ES cell lines. ES cell-mediated gene transfer is presently not much applied to variable species other than mice because it has not been possible to develop such complex ES cell lines in other animals [23, 24].

29.6 Somatic Cell Nuclear Transfer

Somatic cell nuclear transfer (SCNT) also known as "somatic cloning" is another method to generate transgenic animals. In this technique, the nucleus from any somatic cell is removed and injected into an enucleated oocyte, thereby reprogramming the donor nuclei into totipotency (Figs. 29.3 and 29.4). Reconstructed oocytes are surgically transferred to a pseudopregnant surrogate mother. The offsprings born are genetically identical to the one from where the somatic nucleus is taken [25].

This method has been widely used for animal cloning and the first mammal to be cloned was "Dolly" – the sheep [26]. Later successful cloning of other species such as cattle, goats, and pigs [27, 28] was also done. Transgenes can also be inserted into the donor somatic cells in vitro, for the production of genetically modified animals by inserting transgenic nucleus into the enucleated oocytes [29].

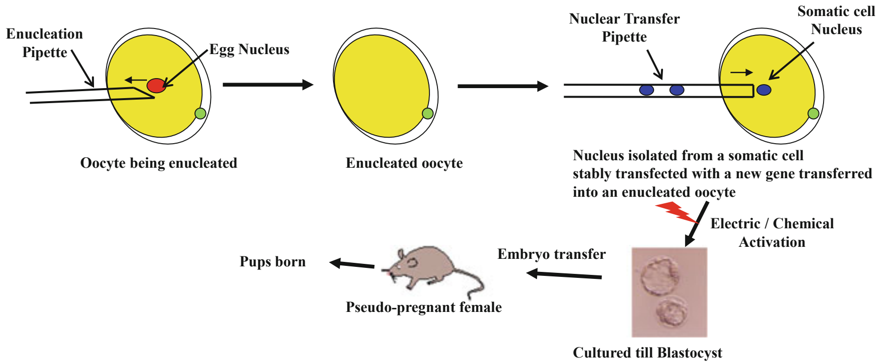


Fig. 29.3 A flowchart of the various steps involved in the generation of a transgenic mouse by somatic cell nuclear transfer

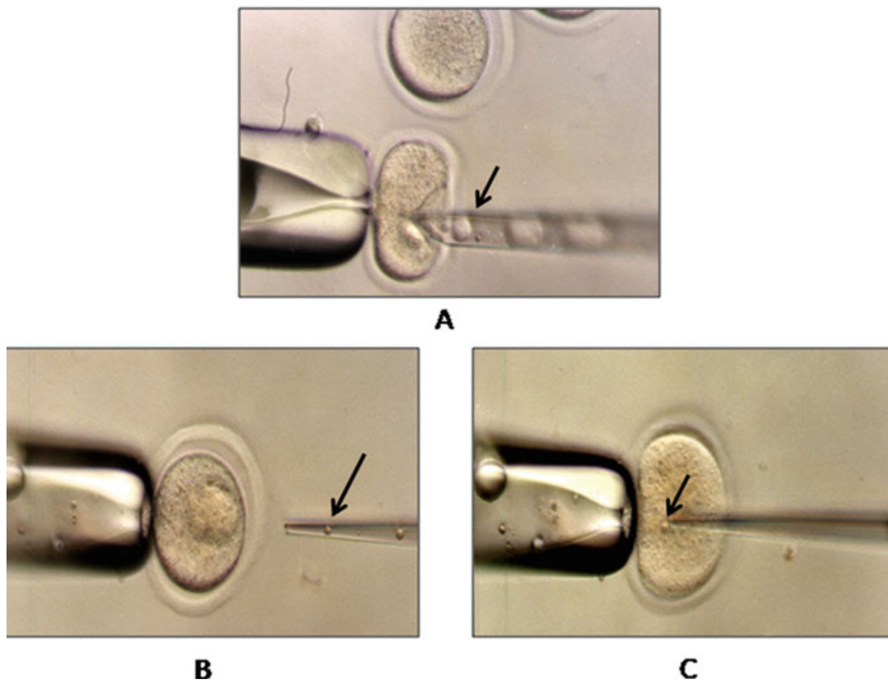


Fig. 29.4 Cloning by somatic cell nuclear transfer. (**a**) Enucleation of mouse oocyte, pulled chromatin material can be seen in an enucleation pipette represented by an arrow. (**b**) Mouse somatic cumulus cell nucleus are taken in the transfer pipette for injection into the enucleated oocyte, represented by an arrow. (**c**) The nucleus of the cumulus cells is injected into the enucleated oocyte of the mouse. A nucleus can be seen inside the cytoplasm marked with an arrow

One of the significant advantages of using SCNT is that the offspring produced are truly transgenic. Also, SCNT allows predetermination of the sex and phenotype of the transgenic animals to be produced. For instance, for the mass production of recombinant proteins into milk, transgenic animals can be chosen to be females at the embryonic stage before embryo transfer. However, the disadvantage of the technique is that it is relatively inefficient (success rate 1–3%) in comparison to ES cell transgenesis. Only a small percentage of reconstructed embryos remain viable to form live animals. It also suffers from high mortality rates of fetuses and embryos [30].

29.7 Virus-Mediated Gene Transfer

In 1974, Jaenisch and Mintz [19] reported the first gene transfer by microinjection of viral DNA into the blastocysts. Retroviruses are usually used as vectors to transmit genetic material into the cell as they can infect host cells. A retrovirus carries its genetic material in the form of RNA. Offspring derived from this method are chimeric as retrovirus doesn't integrate into all of the germ cells. The success rate with this technique in terms of transgenic animals is extremely low [31]. However, expression levels of the transgene can be overcome by the use of lentiviruses. Microinjection of lentiviruses in zygotes has led to the establishment of stable transgenic lines. Lentiviral gene transfer has led to greater efficiency in the transgenic animal production in livestock [32, 33].

29.8 Sperm-Mediated Transgenesis

The millions of spermatozoa produced by a male in a day, if manipulated, can be used as a carrier of the transgene for delivery into an oocyte leading to the generation of transgenic animals. The manipulated spermatozoa are utilized for generating transgenic animals with the help of various assisted reproductive techniques such as in vitro fertilization, artificial insemination, intracytoplasmic sperm injection, etc.

The first report about such manipulation of sperm as a carrier of transgene through internalization/absorption of foreign DNA in the sperm came in 1971 [34]. The DNA marked with tritiated thymidine was incubated with rabbit spermatozoa, and it got internalized inside the head of the sperm. Later, the method was used for many other species also [35, 36].

Later, researchers tried to deliver transgene in the sperm cells by electroporation [37]. But this method was not very efficient. The incorporation of a transgene in the sperm cells was also performed by the restriction digestion method. In this method, limited restriction enzyme digestion of the sperm of *Xenopus* sp. was performed to decondense the genomic DNA located in the sperm head followed by integration of the linearized plasmid DNA into the nuclei [38]. This method was found to be superior to simple incubation mediated incorporation of transgene.

Though the sperm-mediated transgenesis method was simple, often the delivered transgene remains episomal with a loss of expression of phenotype with the growing age of the animal. The treatment given to the sperm made the sperm nonmotile or with aberrant phenotype which makes these techniques difficult to use.

29.9 Testicular Transgenesis

As the attempts of transgenesis through sperm were partially successful, germ cells located inside the testis were identified as targets for gene delivery. Initially, injection of a liposome-reporter-plasmid complex into the busulfan pre-treated seminiferous tubules of mice was attempted [39, 40]. The transgene was expressed in few tubules. Mating of such transgenic males with wild-type females generated transgenic offspring, but in most of them, the transgene remained episomal [41].

The spermatogonial stem cell (SSC)-related studies arose interest when it was shown that spermatogenesis can be restored by injection of either total or partial germinal cell population into the seminiferous tubules of a recipient testis devoid of endogenous germ cells [42, 43]. A retrovirus was used to transfect SSC in vitro before transplanting these germ cells into the seminiferous tubules [44]. This generated transgenic mice that expressed transgene integrated permanently in the genome [45–49]. Although this method was successful certain drawbacks like the silencing of the integrated transgene [50, 51], the low fertility rate of the recipient animals following spermatogonial transplantation [42] and reduced fertility of recipient animals were observed [46, 52, 53]. In a different approach, Yamazaki et al. (1998) injected DNA into the testicular seminiferous tubules followed by electroporation [54]. Long-lasting expression of the transgene in such DNA-injected and electroporated testes was evident, but they failed to generate any transgenic offspring [55].

Recently another efficient and less time-consuming method of generating transgenic animals via permanent integration of genes into SSC has been established. The method involves surgical exposure of the testis under the stereo zoom microscope in a sterile environment followed by testicular injection of the linearized DNA directly given in the testis and subsequent electroporation using square wave pulses [56]. Despite its higher efficiency, this method is not widely used since surgical steps are involved in this procedure, thereby increasing the chances of postsurgical infection and impotency of male mice. Another superior method was developed which involved the delivery of transgene DNA into the testis nonsurgically followed by electroporation using a tweezer electrode [57] (Fig. 29.5). It was found to be a simple method with the generation of transgenic mice in a short time [58–60]. It was robust, did not involve any surgical procedure, and was ethically better. Recently transgenesis through the delivery of transgene in the germ cells by hypotonic shock has also been reported [61]. This is much simpler and involves the delivery of transgene suspended in hypotonic solution directly into the testis, externally. The DNA is delivered in the germ cells due to induced hypotonicity.

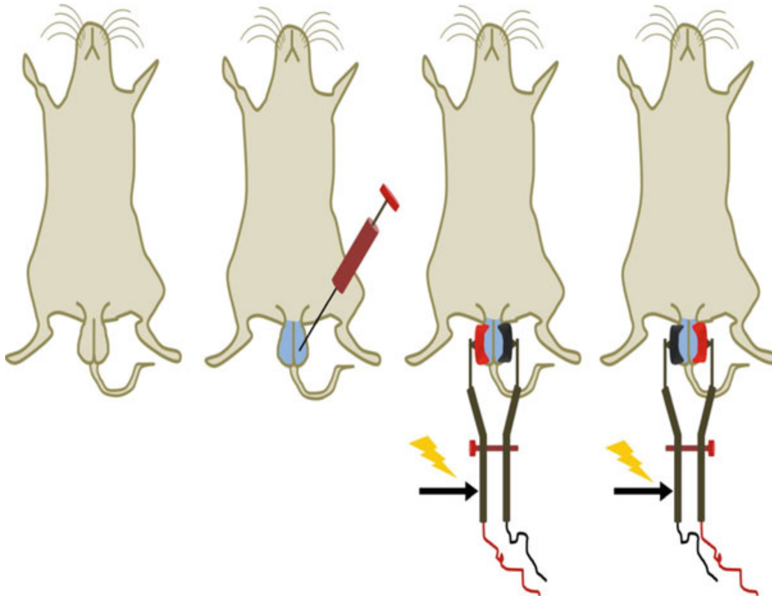


Fig. 29.5 Schematic representation of the nonsurgical external in vivo testicular electroporation procedure. (i) The anaesthetized mouse; (ii) DNA injection in both testes; (iii) electroporation in both the testes, held together, using tweezer type electrode; (iv) electroporation by changing the poles (red and black) of the tweezer type electrode after four pulses

Other models of gene overexpression and gene downregulation in mice have been developed using the external testicular electroporation-based methods.

29.10 Cre-lox-Mediated Gene Targeting

Manipulation of host genome by conventional transgenic and ES cell methods, though, had significantly assisted studies of gene function, often resulting in embryonic lethality with no viable mouse. The random integration of the inserted gene was found to have its effect in multiple cells and tissue types making it difficult to distinguish the specific function of the gene in a particular cell and tissue.

Conditional gene targeting was a strategy used to resolve this specificity problem. This technique is based on the Cre recombinase/locus of the X-over P1 system (Cre/LoxP) to attain tissue-specific gene targeting.

Two independent genetically engineered mouse lines Cre recombinase and LoxP are generated to achieve a tissue-specific gene deletion. These two mice are then mated to generate offspring where both the Cre gene and the LoxP-flanked gene are expressed. In tissues with the No Cre gene, the target gene is present and functions normally. In those tissues, where Cre is expressed, the target gene of interest would be deleted. Cre recombinase recognizes the LoxP site and replaces the DNA segment

between the two LoxP sites generating transgenic mice by gene targeting. When the floxed mouse and the Cre-expressing mouse are bred and progeny inherit both of them, in the tissue where the Cre recombinase is expressed, the DNA segment flanked by the LoxP sites will be excised and inactivated, thereby generating tissue-specific regulation of the gene [62, 63]. The Cre/Lox system is a precious tool to understand molecular biology with the advantage of functioning in almost any type of cell. This versatility has allowed researchers to start and stop the expression of the gene at any time.

This method also suffers from various drawbacks. In the case of double or triple knockout in the same animal, the number of transgenes and the number of breedings becomes laborious and time-consuming. It is not possible to alter single or few nucleotides (e.g., point mutations) utilizing this technique. Another issue reported with Cre mouse lines is the allele parental inheritance [64]. Besides, it may sometimes cause unintended gene expression in different cell types. It is to be noted that the generation of two different transgenic lines (Cre and LoxP lines) requires a considerable amount of time and money.

29.11 RNAi-Mediated Gene Silencing

Gene silencing mediated by RNA interference (RNAi) refers to a process of degrading messenger RNAs (mRNAs) by complementary RNA-RNA binding. This phenomenon occurs naturally inside the cell, as it is a crucial process for posttranscriptional regulation. Utilizing this phenomenon, a double-stranded RNA (dsRNA) is used to target an mRNA of interest and inserted into a DNA vector [65]. On transcription, the resultant dsRNA activates the endogenous RNAi mechanism, binding to and finally degrading the target mRNA resulting in the inhibition of gene expression. The DNA vector may have a sequence allowing inducible expression of the dsRNA by the addition of the antibiotics. When drinking water of an animal containing this type of sequence is supplemented with an antibiotic, RNAi proceeds. Removal of the antibiotic exerts a reverse effect [66]. This allows for the system to be inducible and reversible. RNAi-induced gene knockdowns are generated through the incorporation of small hairpin RNA (shRNA) expressing constructs as transgenes via conventional pronuclear microinjection. The knockdown effect gets passed onto many generations in transgenic animals. In this technique, the gene of interest is not completely removed, so it is used for the generation of knockdown mouse models instead of knockouts. Though it is a recent discovery, it has become a significant tool in drug discovery, new biological and therapeutic strategies. This innovative technique when used in association with classical transgenic methods offers the advantage of quick and specific genetic manipulation in several species and better regulation of transgene expression. RNAi delivers a key advantage of inducible and reversible gene silencing not offered by traditional genetic approaches. The major disadvantages of this method are the off-target effects and incomplete or partial knockdowns of the target gene [67].

29.12 CRISPR/Cas9 System for Editing Mouse Genome

Genome editing has rationalized biological sciences by its ability to precisely edit simple and complex genomes. It includes a choice of various tools, such as zinc-finger nucleases (ZFNs), transcriptional activator-like effector nucleases (TALENs), and the most recent technique of using clustered regularly interspaced short palindromic repeat (CRISPR)/CRISPR-associated nuclease 9 (Cas9) system. ZFNs are targetable DNA cleavage proteins for cutting target sequences at any site [68]. TALENs stimulate double-stranded breaks in target sequences [69]. Although ZFNs and TALENs have been broadly used for genome editing, they suffer from limitations such as nonspecific mutations and the time- and labor-consuming process of vector design [70]. Therefore, recently, interest has been diverted towards the use of CRISPR/Cas9. CRISPR/Cas9 is an RNA-guided nuclease specifically targeting DNA sequences via nucleotide base pairing. The introduction of the CRISPR/Cas9 system was adapted from the bacteria and archaea wherein they constitute a part of their viral defense system [71, 72]. Out of three types of CRISPR systems, the type II system is used for gene editing. The CRISPR/Cas9 mediates genome editing by complementary base pairing. A single guide RNA (gRNA) recognizes any target DNA sequence and guides the endonuclease component Cas9 to cut the DNA by creating double-strand breaks. Once the DNA is excised, the cell's DNA repair machinery is used to add/delete/edit pieces of genetic material by replacing them with a customized DNA sequence [73] (Fig. 29.6). To improve further specificity of Cas9 nuclease, it has been modified and engineered to edit target DNA at the desired loci in eukaryotic cells [74]. It is a simple, efficient, and inexpensive tool that can be easily tailored to target specific sequences by modification of few nucleotides in the guide RNA. Secondly, it is considerably efficient at cleaving targeted genomic sequences [75]. The CRISPR/Cas9 tool is easy to design as it involves only gRNA and Cas9 nuclease. This system has been successfully applied to edit genes in mammalian embryos such as mice [76], rats [77], monkeys [78], and pigs [79].

29.13 Problems, Prospects, and Ethics

Since the generation of the first transgenic animals in 1980, remarkable achievements have been made in the field of transgenic technology. The low efficiency of the transgenic techniques, uncontrolled gene expression, low and unstable integration, and animals born with abnormalities have been the major limiting factors of the conventional transgenic techniques. These merits and demerits are continuously being resolved. Several novel and innovative techniques have also emerged which have increased the accuracy and efficiency of transgenesis. With the advent of new gene-editing technologies, the ability to manipulate genomes has become amenable to almost all species as it provides a more precise, targeted, and practical approach for generating new animal models for biomedical research, which in turn expands its applicability. Transgenic animals, primarily mice, have revolutionized knowledge of mammalian developmental genetics. It has made

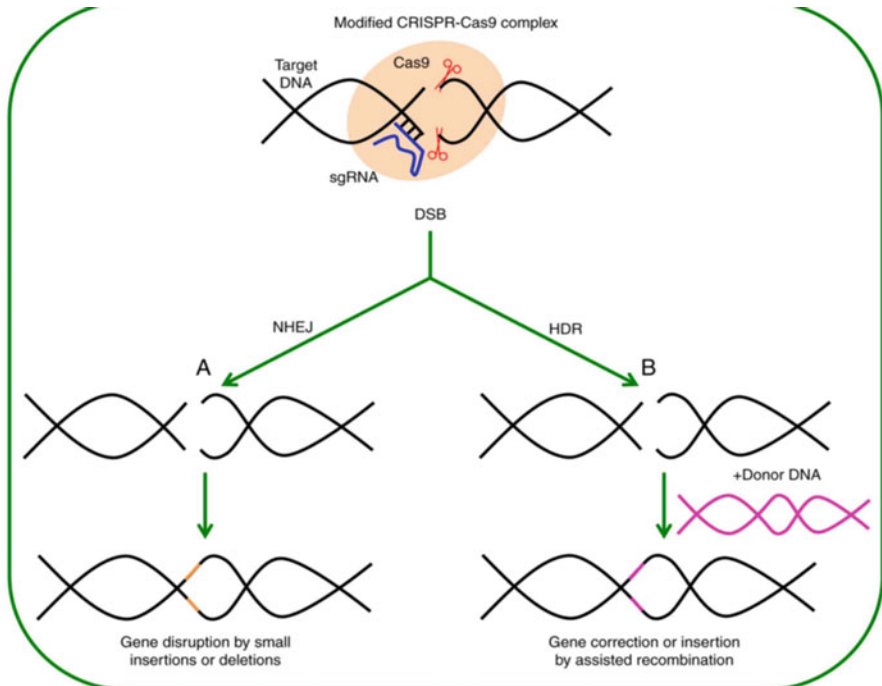


Fig. 29.6 CRISPR/Cas9 system. Cas9 and gRNA complex binds with the DNA close to the PAM site. Double-strand break is created at the target site which can be repaired via NHEJ (nonhomologous end joining) resulting in insertion/deletion or homology-directed specific repair can occur where specific DNA can be introduced to modify the gene. (Source: Rodríguez et al. 2019; 43(4))

considerable contributions to human health. Transgenic animals have widely been used for the discovery of gene function, animal disease models, organ transplantation, genetic improvement of animals, and production of pharmaceutically important proteins using animal bioreactor, etc.

New innovative techniques would continue to develop and expand further, but researchers must be aware of both expected and unexpected consequences of the technique chosen for gene manipulation. Ethics on animal welfare and human health and environmental issues must be considered. The three Rs principles must be kept in mind before the application of any innovative technique on the generation of transgenic animals. It should be equipped with a high level of ethical values and animal welfare. There are safety concerns in the generation of transgenic animals as it may lead to problems of allergies, toxicities, tumourogenesis, etc. There may be an effect on the host, and it may disturb the ecological balance and species diversity. Therefore, considerations of ethical and moral issues, animal welfare issues, economic and commercial issues, regulatory issues, etc. all need to be discussed widely when these new emerging technologies of transgenesis are to be used for mankind. It is advisable to involve all scientific, nonscientific stakeholders in discussions about the applications of any innovative transgenic technology.

Acknowledgments The authors would like to acknowledge the National Institute of Immunology, New Delhi; National Institute of Animal Biotechnology, Hyderabad; and the Department of Biotechnology for all their support.

References

1. Moore SG, Hasler JF (2017) A 100-Year Review: Reproductive technologies in dairy science. J Dairy Sci 100:10314–10331. https://doi.org/10.3168/jds.2017-13138
2. Pinkert CA (2014) Introduction to transgenic animal technology. In: Transgenic animal technology: a laboratory handbook, 3rd ed. In: pp 3–13
3. Ferkany JW (1996) Molecular biology and biotechnology. In: Robert A. Meyers (ed.) A comprehensive desk reference. VCH Publishers, New York. 1995. xxxviii + 1034 pp. 21.5 × 27.5 cm. ISBN 1-56081-925-1. $59.95. J Med Chem 39:1565–1566. https://doi.org/10.1021/jm960027m
4. Gordon JW, Ruddle FH (1981) Integration and stable germ line transmission of genes injected into mouse pronuclei. Science (80) 214:1244–1246. https://doi.org/10.1126/science.6272397
5. Singhal M, Kansara N (2010) Transgenic animals: production and application. Int J Pharm Sci Res 1:12–22
6. Ittner LM, Götz J (2007) Pronuclear injection for the production of transgenic mice. Nat Protoc 2:1206–1215. https://doi.org/10.1038/nprot.2007.145
7. Kaur G, Gulati N, Mandal A et al (1998) Expression and stable germline transmission of neomycin-resistance gene in transgenic mice. Indian J Exp Biol 36:1209–1215
8. Baskaran S, Datta S, Mandal A et al (2002) Instability of CGG repeats in transgenic mice. Genomics 80:151–157. https://doi.org/10.1006/geno.2002.6813
9. Kaul-Ghanekar R, Majumdar S, Jalota A et al (2005) Abnormal V(D)J recombination of T cell receptor β locus in SMAR1 transgenic mice. J Biol Chem 280:9450–9459. https://doi.org/10.1074/jbc.M412206200
10. Mehta AK, Majumdar SS, Alam P et al (2009) Epigenetic regulation of cytomegalovirus major immediate-early promoter activity in transgenic mice. Gene 428:20–24. https://doi.org/10.1016/j.gene.2008.09.033
11. Alam MP, Datta S, Majumdar S et al (2010) Comparative analysis of DNA methylation in transgenic mice with unstable CGG repeats from FMR1 gene. Epigenetics 5:241–248. https://doi.org/10.4161/epi.5.3.11417
12. Datta S, Alam MP, Majumdar SS et al (2011) Nucleosomal occupancy and CGG repeat expansion: A comparative analysis of triplet repeat region from mouse and human fragile X mental retardation gene 1. Chromosom Res 19:445–455. https://doi.org/10.1007/s10577-011-9206-7
13. Brinster RL, Palmiter RD (1984) Introduction of genes into the germ line of animals. Harvey Lect 80:1–38
14. Palmiter RD, Brinster RL (1986) Germ-line transformation of mice. Annu Rev Genet 20:465–499. https://doi.org/10.1146/annurev.ge.20.120186.002341
15. Smirnov A, Fishman V, Yunusova A et al (2020) DNA barcoding reveals that injected transgenes are predominantly processed by homologous recombination in mouse zygote. Nucleic Acids Res 48:719–735. https://doi.org/10.1093/nar/gkz1085
16. Naoto H, Cho A, B Kulkarni A (2010) Engineering transgenic constructs and mice. Curr Protoc Cell Bio:1–12. https://doi.org/10.1002/0471143030.cb1910s42.Overview
17. Bronson SK, Smithies O (1994) Altering mice by homologous recombination using embryonic stem cells. J Biol Chem 269:27155–27158
18. Xia X, Zhang SC (2007) Genetic modification of human embryonic stem cells. Biotechnol Genet Eng Rev 24:297–310. https://doi.org/10.1080/02648725.2007.10648105

19. Jaenisch R, Mintz B (1974) Simian virus 40 DNA sequences in DNA of healthy adult mice derived from preimplantation blastocysts injected with viral DNA. Proc Natl Acad Sci U S A 71:1250–1254. https://doi.org/10.1073/pnas.71.4.1250
20. Longenecker G, Kulkarni AB (2009) Generation of gene knockout mice by ES cell microinjection. Curr Protoc Cell Biol 44. https://doi.org/10.1002/0471143030.cb1914s44
21. Ledermann B (2000) Embryonic stem cells and gene targeting. Exp Physiol 85:603–613
22. Capecchi MR (1989) Altering the genome by homologous recombination. Science (80) 244:1288–1292. https://doi.org/10.1126/science.2660260
23. Thomson JA, Kalishman J, Golos TG et al (1995) Isolation of a primate embryonic stem cell line. Proc Natl Acad Sci U S A 92:7844–7848. https://doi.org/10.1073/pnas.92.17.7844
24. Notarianni E, Galli C, Laurie S et al (1991) Derivation of pluripotent, embryonic cell lines from the pig and sheep. J Reprod Fertil Suppl 43:255–260. https://doi.org/10.1530/biosciprocs.2.020
25. Markoulaki S, Meissner A, Jaenisch R (2008) Somatic cell nuclear transfer and derivation of embryonic stem cells in the mouse. Methods 45:101–114. https://doi.org/10.1016/j.ymeth.2008.04.002
26. Wilmut I, Schnieke AE, McWhir J et al (1997) Viable offspring derived from fetal and adult mammalian cells. Nature 385:810–813. https://doi.org/10.1038/385810a0
27. Robinson AW, Do VH (2014) Somatic cell nuclear transfer in mammals: reprogramming mechanism and factors affecting success. Cloning Transgenes 03:129. https://doi.org/10.4172/2168-9849.1000129
28. Wadhwa N, Kunj N, Tiwari S et al (2009) Optimization of embryo culture conditions for increasing efficiency of cloning in buffalo (Bubalus bubalis) and generation of transgenic embryos via cloning. Cloning Stem Cells 11:387–395
29. Schníeke AE, Kind AJ, Ritchie WA et al (1997) Human factor IX transgenic sheep produced by transfer of nuclei from transfected fetal fibroblasts. Science (80) 278:2130–2133. https://doi.org/10.1126/science.278.5346.2130
30. Solter D (2000) Mammalian cloning: advances and limitations. Nat Rev Genet 1:199–207
31. Bihon Asfaw A, Assefa A (2019) Animal transgenesis technology: a review. Cogent Food Agric 5. https://doi.org/10.1080/23311932.2019.1686802
32. Shinohara T, Kanatsu-Shinohara M (2020) Transgenesis and genome editing of mouse spermatogonial stem cells by lentivirus pseudotyped with Sendai virus F protein. Stem Cell Rep 14:447–461. https://doi.org/10.1016/j.stemcr.2020.02.001
33. Pfeifer A (2004) Lentiviral transgenesis. Transgenic Res 13:513–522
34. Brackett BG, Baranska W, Sawicki W, Koprowski H (1971) Uptake of heterologous genome by mammalian spermatozoa and its transfer to ova through fertilization
35. Lavitrano M, Camaioni A, Fazio VM et al (1989) Sperm cells as vectors for introducing foreign DNA into eggs: genetic transformation of mice. Cell 57:717–723. https://doi.org/10.1016/0092-8674(89)90787-3
36. Camaioni A, Russo MA, Odorisio T et al (1992) Uptake of exogenous DNA by mammalian spermatozoa: specific localization of DNA on sperm heads. J Reprod Fertil 96:203–212. https://doi.org/10.1530/jrf.0.0960203
37. Horan R, Powell R, Bird JM et al (1992) Effects of electropermeabilization on the association of foreign DNA with pig sperm. Syst Biol Reprod Med 28:105–114. https://doi.org/10.3109/01485019208987687
38. Kroll KL, Amaya E (1996) Transgenic Xenopus embryos from sperm nuclear transplantations reveal FGF signaling requirements during gastrulation. Development 122:3173–3183
39. Bucci LR, Meistrich ML (1987) Effects of busulfan on murine spermatogenesis: cytotoxicity, sterility, sperm abnormalities, and dominant lethal mutations. Mutat Res Fundam Mol Mech Mutagen 176:259–268. https://doi.org/10.1016/0027-5107(87)90057-1
40. Kim J-H, Jung-Ha H-S, Lee H-T, Chung K-S (1997) Development of a positive method for male stem cell-mediated gene transfer in mouse and pig. Mol Reprod Dev 46:515–526. https://doi.org/10.1002/(SICI)1098-2795(199704)46:4<515::AID-MRD10>3.0.CO;2-V

41. Celebi C, Auvray P, Benvegnu T et al (2002) Transient transmission of a transgene in mouse offspring following in vivo transfection of male germ cells. Mol Reprod Dev 62:477–482. https://doi.org/10.1002/mrd.10143
42. Brinster RL, Zimmermann JW (1994) Spermatogenesis following male germ-cell transplantation. Proc Natl Acad Sci U S A 91:11298–11302. https://doi.org/10.1073/pnas.91.24.11298
43. Brinster RL, Avarbock MR (1994) Germline transmission of donor haplotype following spermatogonial transplantation. Proc Natl Acad Sci U S A 91:11303–11307. https://doi.org/10.1073/pnas.91.24.11303
44. Nagano M, Shinohara T, Avarbock MR, Brinster RL (2000) Retrovirus-mediated gene delivery into male germ line stem cells. FEBS Lett 475:7–10. https://doi.org/10.1016/S0014-5793(00)01606-9
45. Nagano M, Brinster CJ, Orwig KE et al (2001) Transgenic mice produced by retroviral transduction of male germ-line stem cells. Proc Natl Acad Sci U S A 98:13090–13095. https://doi.org/10.1073/pnas.231473498
46. Kanatsu-Shinohara M, Ogonuki N, Inoue K et al (2003) Long-term proliferation in culture and germline transmission of mouse male germline stem cells. Biol Reprod 69:612–616. https://doi.org/10.1095/biolreprod.103.017012
47. Kanatsu-Shinohara M, Toyokuni S, Shinohara T (2005) Genetic selection of mouse male germline stem cells in vitro: offspring from single stem cells1. Biol Reprod 72:236–240. https://doi.org/10.1095/biolreprod.104.035659
48. Hamra FK, Gatlin J, Chapman KM et al (2002) Production of transgenic rats by lentiviral transduction of male germ-line stem cells. Proc Natl Acad Sci U S A 99:14931–14936. https://doi.org/10.1073/pnas.222561399
49. Ryu BY, Orwig KE, Oatley JM et al (2007) Efficient generation of transgenic rats through the male germline using lentiviral transduction and transplantation of spermatogonial stem cells. J Androl 28:353–360. https://doi.org/10.2164/jandrol.106.001511
50. Cherry SR, Biniszkiewicz D, van Parijs L et al (2000) Retroviral expression in embryonic stem cells and hematopoietic stem cells. Mol Cell Biol 20:7419–7426. https://doi.org/10.1128/mcb.20.20.7419-7426.2000
51. Klug CA, Cheshier S, Weissman IL (2000) Inactivation of a GFP retrovirus occurs at multiple levels in long-term repopulating stem cells and their differentiated progeny. Blood 96:894–901. https://doi.org/10.1182/blood.v96.3.894
52. Ogawa T, Dobrinski I, Brinster RL (1999) Recipient preparation is critical for spermatogonial transplantation in the rat. Tissue Cell 31:461–472. https://doi.org/10.1054/tice.1999.0060
53. Brinster CJ, Ryu B-Y, Avarbock MR et al (2003) Restoration of fertility by germ cell transplantation requires effective recipient preparation. Biol Reprod 69:412–420. https://doi.org/10.1095/biolreprod.103.016519
54. Yamazaki Y, Fujimoto H, Ando H et al (1998) In vivo gene transfer to mouse spermatogenic cells by deoxyribonucleic acid injection into seminiferous tubules and subsequent electroporation. Biol Reprod 59:1439–1444. https://doi.org/10.1095/biolreprod59.6.1439
55. Yamazaki Y, Yagi T, Ozaki T, Imoto K (2000) In vivo gene transfer to mouse spermatogenic cells using green fluorescent protein as a marker. J Exp Zool 286(2):212–218. https://doi.org/10.1002/(SICI)1097-010X(20000201)286:2<212::AID-JEZ13>3.0.CO;2-C
56. Dhup S, Majumdar SS (2008) Transgenesis via permanent integration of genes in repopulating spermatogonial cells in vivo. Nat Methods 5:601–603. https://doi.org/10.1038/nmeth.1225
57. Usmani A, Ganguli N, Sarkar H et al (2013) A non-surgical approach for male germ cell mediated gene transmission through transgenesis. Sci Rep 3:3430. https://doi.org/10.1038/srep03430
58. Ganguli N, Ganguli N, Usmani A, Majumdar SS (2015) Isolation and functional characterization of buffalo (Bubalus bubalis) ??-casein promoter for driving mammary epithelial cell-specific gene expression. J Biotechnol 198:53–59. https://doi.org/10.1016/j.jbiotec.2015.02.001

59. Basu S, Arya SP, Usmani A et al (2017) Defective Wnt3 expression by testicular Sertoli cells compromise male fertility. Cell Tissue Res. https://doi.org/10.1007/s00441-017-2698-5
60. Shukla M, Ganguli N, Sen Sharma S, Majumdar SS (2018) Sertoli cell specific decline in NOR-1 leads to germ cell apoptosis and reduced fertility. J Cell Biochem 119:6514–6526. https://doi.org/10.1002/jcb.26698
61. Usmani A, Ganguli N, Jain SK et al (2016) Robust generation of transgenic mice by simple hypotonic solution mediated delivery of transgene in testicular germ cells. Mol Ther Methods Clin Dev 3. https://doi.org/10.1038/mtm.2016.76
62. Sosa MAG, De Gasperi R, Elder GA (2010) Animal transgenesis: an overview. Brain Struct Funct 214:91–109
63. Burgio G (2018) Redefining mouse transgenesis with CRISPR/Cas9 genome editing technology. Genome Biol 19. https://doi.org/10.1186/s13059-018-1409-1
64. Rocha-Martins M, Cavalheiro GR, Matos-Rodrigues GE, Martins RAP (2015) From gene targeting to genome editing: transgenic animals applications and beyond. An Acad Bras Cienc 87:1323–1348. https://doi.org/10.1590/0001-3765201520140710
65. Fire A, Xu S, Montgomery MK et al (1998) Potent and specific genetic interference by double-stranded RNA in caenorhabditis elegans. Nature 391:806–811. https://doi.org/10.1038/35888
66. Zhang J, Wang C, Ke N et al (2007) A more efficient RNAi inducible system for tight regulation of gene expression in mammalian cells and xenograft animals. RNA 13:1375–1383. https://doi.org/10.1261/rna.520707
67. Boettcher M, McManus MT (2015) Choosing the right tool for the job: RNAi, TALEN. or CRISPR Mol Cell 58:575–585
68. Palpant NJ, Dudzinski D (2013) Zinc finger nucleases: looking toward translation. Gene Ther 20:121–127
69. Ma ACH, Chen Y, Blackburn PR, Ekker SC (2016) TALEN-Mediated mutagenesis and genome editing. Methods Mol Biol:17–30
70. Ebina H, Kanemura Y, Misawa N et al (2015) A high excision potential of TALENs for integrated DNA of HIV-based lentiviral vector. PLoS One 10. https://doi.org/10.1371/journal.pone.0120047
71. Doudna JA, Charpentier E (2014) The new frontier of genome engineering with CRISPR-Cas9. Science 80:346
72. Sternberg SH, Richter H, Charpentier E, Qimron U (2016) Adaptation in CRISPR-Cas Systems. Mol Cell 61:797–808
73. Wu X, Kriz AJ, Sharp PA (2014) Target specificity of the CRISPR-Cas9 system. Quant Biol 2:59–70
74. Cong L, Ran FA, Cox D et al (2013) Multiplex genome engineering using CRISPR/Cas systems. Science (80) 339:819–823. https://doi.org/10.1126/science.1231143
75. Jinek M, Chylinski K, Fonfara I et al (2012) A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity.(Research Article)(Author abstract)(Report). Science (80) 337:816–821
76. Zhu X, Xu Y, Yu S et al (2014) An efficient genotyping method for genome-modified animals and human cells generated with CRISPR/Cas9 system. Sci Rep 4. https://doi.org/10.1038/srep06420
77. Alcantar TM, Rairdan XY (2019) Microinjection and oviduct transfer procedures for rat model generation with CRISPR-Cas9 technology. Methods Mol Biol:273–294
78. Kang Y, Chu C, Wang F, Niu Y (2019) CRISPR/Cas9-mediated genome editing in nonhuman primates. DMM Dis Model Mech 12. https://doi.org/10.1242/dmm.039982
79. Whitworth KM, Lee K, Benne JA et al (2014) Use of the CRISPR/Cas9 system to produce genetically engineered pigs from in vitro-derived oocytes and embryos. Biol Reprod 91. https://doi.org/10.1095/biolreprod.114.121723

30 Mouse Models of the Humanized Immune System

Kshama Jain, Prakriti Sinha, K. Varsha Mohan, and
Pramod Kumar Upadhyay

Abstract

The development of humanized mice by engraftment of functional human cells and tissues in immunodeficient mice strains has helped in bridging the gap between human and mice immune systems. Owing to their close similarity with the human immune system, humanized mice have been widely used as a valuable preclinical tool in the understanding of the pathophysiology of various human diseases and evaluation of drugs as well as cell-based therapeutics. The current chapter discusses the development of various humanized mouse models and their application in the understanding of human disease biology and therapeutic discovery.

Keywords

Humanized immune system · Hu-PBL mice · Hu-SRC mice · Hu-BLT mice · Immunodeficient mice · Infectious diseases · Autoimmune disorders · Transplantation studies · Cancer · Drug discovery

Abbreviations

GvHD	Graft versus host disease
HIS	Humanized immune system

K. Jain · P. Sinha · K. V. Mohan (✉)
Product Development Lab, National Institute of Immunology, New Delhi, India
e-mail: jainkshama@nii.ac.in; prakriti@nii.ac.in; kvarshamohan@nii.ac.in

P. K. Upadhyay (✉)
Laboratory National Institute of Immunology, New Delhi, India
e-mail: pkumar@nii.ac.in

© The Author(s), under exclusive license to Springer Nature Singapore Pte Ltd. 2021
P. Nagarajan et al. (eds.), *Essentials of Laboratory Animal Science: Principles and Practices*, https://doi.org/10.1007/978-981-16-0987-9_30

Hu-BLT mice	Bone marrow, liver, thymus
hu-PBL	Human peripheral blood lymphocytes
Hu-SRC	Human SCID repopulating cell
HvG	Host versus graft response
NSG-NOD	SCID gamma

30.1 Introduction

The fundamental understanding of human biology is largely based on the in vivo studies conducted on various animal models. The animal models used in biological studies are usually rodents and nonhuman primates (NHPs) [1, 2]. However, the biological differences in these animal models and humans are the main technical challenges in the translation of animal studies to clinical trials [3–5].

To overcome the difficulties in translating animal results to clinical applications, researchers have engineered an innovative platform called "humanized mice" which essentially mirrors the human biological system at cellular as well as molecular levels [6, 7]. In simpler words, a humanized mouse represents a mouse engrafted with functional human cells or tissue or gene of interest. One of the well-studied humanized animal models is the HIS (human immune system) mouse model which simulates the functional human immune system [8]. The functional human immune system comprises a vast network of cells and tissues spread all over the body. The human immune system protects the body by innate and adaptive immune mechanisms. The key components of the innate immune system are the granulocytes, macrophages, dendritic cells, NK cells, and mast cells. The adaptive immune response is primarily mediated by B and T cells. Thus the generation of a complete and functional human immune system in a mouse model provides a unique platform for investigating the pathogenesis as well as treatment therapies of various human diseases including infectious diseases [9–11], autoimmune disorders [12], graft rejections [13], and cancer [14].

30.2 Mouse Model with Humanized Immune System (HIS)

30.2.1 Mice Strains for HIS Generation

The key requisite for the generation of the humanized mouse model is the selection of an immunocompromised or immunodeficient mice strain which ensures the successful engraftment of human cells and tissues in recipient mice by reducing the risk of graft rejection. For generating immunocompromised animals, mutations are introduced in different genes responsible for immune cell development and differentiation. An initial study for the generation of humanized mice was conducted in SCID (severe combined immunodeficiency) mouse models which are known to

Table 30.1 Details of various immunodeficient mice strains used for generating humanized mice

Name	Genetic background	Other details	References
NSG (NOD-SCID. gamma)	NOD.Cg-$Prkdc^{Scid}Il2rg^{tm1Wjl}$/SzJ	• IL-2rg null • Support reconstitution of human immune system	[16]
NOG (NOD. Gamma)	NOD.Shi.$CgPrkdc^{Scid}$ $Il2rg^{tm1Sug}$	• Mutated IL-2rg which do not bind cytokines • Supports reconstitution of human immune	[18]
NRG (NOD.Rag. Gamma)	NOD-$Rag1^{null}$ $IL2rgamma^{null}$	• IL-2rg null • Support reconstitution of human immune system.	[21]
BRG (BALB/c. Rag.Gamma)	BALB/cA-$Rag2^{null}Il2rg^{null}$	• Support reconstitution of human immune system	[90]
SRG-15	$S^{h/m}Rag2^{-/-}/IL2rg^{-/-}$-$15^{h/m}$	• Support survival of transplanted NK cells and myeloid cells	[1]
MISTRG	M-$CSF^{h/h}$ IL-3/GM-$CSF^{h/h}$$SIRPA^{h/m}TPO^{h/h}$ $Rag2^{-/-}$ $Il2rg^{-/-}$	• Support survival of transplanted NK cells	[8]
HUMAMICE	HLA-$A2^{b/b}$/$DR1^{b/b}$/H-2-$b2m^{-/-}$/$IAb^{-/-}$/$Rag2^{-/-}$/$IL2rg^{-/-}$/$Perf^{-/-}$	• Supports reconstitution of human immune system in HLA restricted manner • Delayed GVHD	[91]

have mutations in Prkdc gene. Later, various immunodeficient mouse models were developed by manipulating the Prkdc gene and the recombination-activating gene (RAG) or by crossing immunodeficient SCID and RAG mice with nonobese diabetic (NOD) mice [15]. However, the most significant advancement in humanized mice generation occurred by the development of IL-2Rγ knockout mouse models lacking functional NK cells. Various commonly used immunodeficient models based on IL-2Rγ knockout mice are NSG (NOD-SCID gamma mouse) [16, 17], NOG (NOD/Shi-scid/IL-$2R\gamma^{null}$) [18, 19] NRG(NOD/RAG1/$2^{-/-}$IL$2R\gamma^{-/-}$) [20, 21], and BRG (BALB/cA-$Rag2^{null}Il2r\gamma^{null}$) [22].

NSG mice are highly immunodeficient and thus are widely used for generating humanized mice. The high immunodeficiency of NSG mice is caused by two genetic mutations; mutation in Prkdc and null mutation in IL2 receptor common gamma chain. The mutation in PKRDC leads to nonfunctional B and T cells leading to the dysfunctional adaptive immune system of animals. The mutation in IL2 receptor hampers the cytokine signaling through multiple receptors, making animals deficient in functional NK cells. The details of various other immunodeficient strains used for humanized mice studies along with their genetic background and acronyms are summarized in Table 30.1.

30.2.2 Approaches for Establishing the Human Immune System (HIS)

In addition to the immunodeficiency, creating a supportive microenvironment further enhances the engraftment and development of human cells in mice. Thus preconditioning of animals before cell infusion is a critical step for mice humanization. The partial or absolute suppression of the mouse hematopoiesis generates a conducive environment for human HSC engraftment and development. It helps in the homing of human HSC in mice BM niches and their development by inducing a feedback hematopoietic regeneration process. The most common preconditioning regimen for suppressing mouse hematopoiesis is sublethal irradiation with gamma or x-rays. An alternative way of preconditioning is by using chemical agents such as busulfan and 5-fluorouracil. These chemotherapeutic agents exert similar hematopoietic effects and are easier to use [23].

Thus the successful establishment of the human immune system in mice is achieved by transplanting the cells or tissues of interest in the preconditioned immunocompromised mouse models. The three most commonly used transplantation approaches for generating HIS mice are discussed below.

30.2.2.1 Transplantation of Human Peripheral Blood Lymphocytes (Hu-PBL mice)

This approach of establishing HIS mice is the simplest one, which is based on the transplantation of human peripheral blood-derived mononuclear cells in immunodeficient mouse models [24]. The model is popularly known as Hu-PBL-SCID model. This model enables the successful engraftment of human $CD3^+$ T cells along with a few B cells and myeloid cells [25, 26]. This model provides a good platform for the study of T-cell activation and effector functions as well as analysis of immunosuppressive drugs.

This HIS model is extensively used for T-cell-mediated immune responses such as GvHD allograft rejection [27]. However, the rapid engraftment and expansion of T cells in the immunodeficient recipient mice increase the risk of lethal xenogeneic graft-versus-host disease (GvHD) [25]. As the engrafted T cells show aggravated response against the mice MHC molecules, various genetic strains of immunodeficient mice lacking MHC-I and MHC- II have been developed [28, 29].

30.2.2.2 Transplantation of Human Stem Cells (Hu-SRC mice)

Hu-SRC model has been developed by transplanting human $CD34^+$ hematopoietic stem cells (HSCs) in various strains of immunodeficient mice. The very first Hu-SRC model was developed in CB17.SCID mice; however, the most commonly used strains these days are $IL2r\gamma^{null}$ immunodeficient mice [30]. The procurement of $CD34^+$ HSCs can be done from multiple sources such as fetal liver, umbilical cord blood, adult bone marrow, or G-CSF-mobilized blood [22]. However, the engraftment efficiency varies according to the source of cells with fetal liver or cord blood-derived $CD34^+$cells having the highest engraftment [22]. As this model is developed by HSC engraftment, it develops human immune cells of all the lineages including B

cells, T cells, innate immune cells, and precursors for erythrocytes, megakaryocytes, and granulocytes.

A major limitation with Hu-SRC model is the lack of cytokines essential for the development and growth of various lineages from HSCs [22, 27, 31]. This issue can be addressed by delivering human cytokines into humanized mice by various modes such as transgenic cDNA expression, knock-in technology, viral expression vectors, and plasmid delivery [32].

Another major limitation with this model is that the human HSC-derived T cells when educated on the murine MHC become H2-restricted [33] which, in turn, reduces interactions of T cells with HLA-expressing antigen-presenting cells (APCs). This limitation, too, has been overcome by the delivery of vector-mediated HLA by HLA-expressing IL2rγ^{null} immunodeficient strains of AAV in NSG mice [32, 34–38].

30.2.2.3 Transplantation of Bone Marrow, Liver, Thymus (Hu-BLT) Mice

The human bone marrow-liver-thymus model popularly known as the Hu-BLT mouse model is the most robust HIS model. BLT model is generated by implanting fragments of the human fetal liver and thymus along with the intravenous injection of CD34$^+$ HSCs from the same donor [39, 40]. The development of the BLT model is most vigorous on the NSG platform [41, 42]. The major limitations associated with the BLT model are the limited availability of human fetal tissues, technical surgical skills required, and ethical constraints associated with the use of human fetal tissue.

The development of various HIS models has significantly contributed to the research advancements related to various human-specific disease conditions such as infections, autoimmune conditions, graft vs. host disease (GvHD), and cancer (Fig. 30.1). The application of various HIS models in each of these conditions is discussed in following sections.

30.3 Application of HIS Model in Infectious Diseases

30.3.1 HIS Model in Viral Infection Studies

Viruses infections are often target specific making it difficult to replicate the infections and pathogenesis of human-specific viruses in mouse models. Despite some similarities between mouse and human biology, several differences still exist between the human and mouse immune systems. Hence a more accurate reflection of the host-virus interaction can be achieved by “humanizing” the mouse immune system and virus-target cells. However, the kind of immune reconstitution would depend on the kind of virus and its pathogenesis [43].

Humanizing Immune System

For viruses with tropism specific to blood cells or more conserved target receptors or cell type, humanizing the immune system is sufficient to establish an infection and

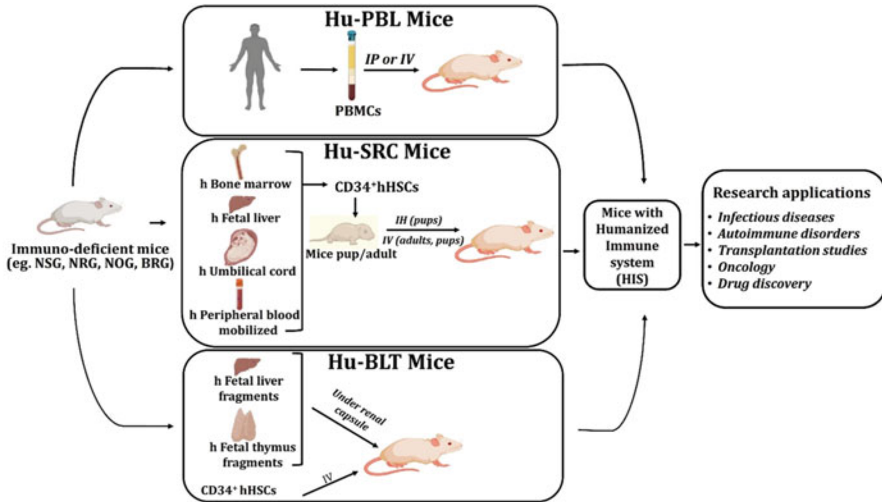


Fig. 30.1 Research applications of humanized immune system (HIS) mice

subsequent studies, e.g., lymphotropic viruses like HIV, EBV, and flaviviruses like DENV and Zika virus.

Humanizing target organ and immune system: When the virus has specific species and tissue tropism, the target tissue/organ has to be reconstituted along with the immune system for the establishment of a successful infection, e.g., the hepatotropic viruses like HAV, HBV, HCV, HDV, and HEV.

30.3.1.1 Blood Trophic Viruses

30.3.1.1.1 Human Immunodeficiency Virus (HIV)

HIV is an RNA virus that has tropism to immune cells like $CD4^+$ T cells, macrophages, and dendritic cells, causing $CD4^+$T-cell depletion and host immunodeficiency [44]. Infection models commonly used for HIV studies are NHP-based and mouse-based, with the latter ones being more popular. While the mouse is impervious to infection by HIV, all three humanized mouse models are susceptible to HIV infection [45]. The SCID model was the earliest model to study the efficacy of potential HIV vaccines [46]. This model has also been used to study HIV-associated pathology to the CNS and strategies to mitigate the pathology [47] while newer reports have used the NSG, BLT, and myeloid-only or T-cell-only models to further these studies [48]. The NSG-based HIS models have been used to evaluate the efficacy of CCR5 knocked-out HSCs in resisting HIV infection [49]. The BLT model offers the most comprehensive depiction of the HIV pathogenesis by allowing mucosal route of transmission, i.e., intravaginal and intrarectal [50], and also allows assessment of antiretroviral therapy in a triple knockout BLT model ($Rag2^{-/-}$ $Il2R^{-/-}$ $Cd47^{-/-}$) [51].

30.3.1.1.2 Epstein Barr Virus (EBV)

EBV is a DNA virus that primarily infects B cells and causes B-cell lymphoma. Much of the pathogenesis of EBV is yet to be understood. The development of HIS mice has provided a suitable platform for studying EBV biology and developing therapeutic strategies [52]. The significance of EBV protein, BPLF1, in the viral infectivity was ascertained in γ-irradiated and HSC reconstituted mice [53]. The NSG mouse model has been instrumental in ascertaining T-cell response against EBV-infected B cells [54] as well as in assessing the effectiveness of PD-1/CTLA-4 blockade as a therapeutic strategy in T-cell-mediated control of EBV-induced lymphomas. The NK cells were also found to be important innate immune responders for tumorigenesis control in EBV infection, as seen in the NSG model [55]. This model also enabled the study of coinfection of EBV and Kaposi sarcoma-associated herpesvirus (KSHV) and revealed enhanced tumorigenesis and lytic EBV replication on coinfection.

30.3.1.1.3 Dengue Virus (DENV) and Zika Virus

The DENV is an RNA virus and is majorly transmitted via mosquitoes, causing symptoms ranging from mild flulike symptoms to hemorrhagic fever and hypovolemic shock. The immune system, liver, and endothelial cell lining of blood vessels are believed to be the major target tissues, and further studies are required to fully elucidate the pathogenesis [56]. Wild-type mice are resistant to infection with DENV; however successful infection with relevant immune-pathogenesis was obtained via mosquito bite initially in NSG mice [57]. Subsequently, BLT-NSG mice were found to develop neutralizing antibodies against DENV and held the potential for vaccine studies, therapeutic antibody [58], and antiviral drug testing [9]. HIS mice have also been instrumental in delineating aspects of dengue pathogenesis. Human fetal liver transplanted-NSG mice were receptive to laboratory-adapted and clinical strains of DENV. It also helped identify that dengue-induced thrombocytopenia was due to the reduction of human megakaryocytes and their progenitors in bone marrow [59]. Similarly, the role of NK cells in the DENV pathogenesis was reaffirmed in the NSG model, thus showing the importance of IFN-γ and the interaction of NK cells and dendritic cells in the inhibition of the DENV replication and further infection [60].

Zika virus is another RNA *Flavivirus*, transmitted via mosquitoes and has similar but milder symptoms as DENV infection. The BLT model is permissive to Zika virus infection along with the generation of neutralizing antibodies [61].

30.3.1.2 Hepatotropic Viruses

Hepatotropic viruses like hepatitis viruses cause liver inflammation and damage. While hepatitis A and E cause acute infection, which can be self-cleared, hepatitis B and C can cause chronic infections which may lead to cirrhosis, cancer, and liver failure. These hepatotropic viruses have specific tropism to human hepatocytes and hence require double humanization for their infection and subsequent

immunological studies. The double humanization of animals in such viral studies is done by simultaneous humanization of mice liver with mature human hepatocytes and of mice blood with human hematopoietic cells. Humanization of the mouse liver generally involves damage to the indigenous mouse hepatocytes followed by engraftment of healthy human hepatocytes [62]. A combination of HIS system in the humanized liver mice allows studying the entire pathophysiology of these viruses. Although several mouse models were developed to study the hepatitis viruses, the HIS models have been used more extensively with the HBV and HCV viruses.

30.3.1.2.1 Hepatitis B Virus (HBV)

A double humanized system for studying the complete pathogenesis of the liver-tropic pathogens, like a HIS-HUHEP model, was created in BALB/c Rag2$^{-/-}$ IL-2Rγc$^{-/-}$ NOD.*sirpa*uPA$^{tg/tg}$ mice. These mice were injected intrahepatically with human HSCs and intrasplenically with human hepatocytes. They showed 20–50% liver chimerism and stable development of the myeloid and lymphoid lineages [63]. This HIS-HUHEP was then infected with HBV intraperitoneally resulting in a high viral load with the complete HBV lifecycle. The HBV pathogenesis was also recapitulated in a double humanized model using NSG mice with human HLA-A2 transgene (A2/NSG) transplanted with human HSCs and liver progenitor cells (A2/NSG-huHSC/Hep mice). This model supported HBV infection for up to 4 months as well as the development of immune response and liver pathology which could be alleviated with the administration of neutralizing antibodies [64]. HBV-infected uPA/SCID mice transplanted with primary human hepatocytes were used to assess the efficacy of non-lytic human T cells electroporated with HBV- or HCV-specific TCRs in suppressing HBV or HCV replication, respectively. As desired, these cells did not cause lysis of the infected cells (which is the major cause of liver damage), rather secretion of the antiviral protein, APOBEC3, reduced the viral replication levels [65]. Hence these mouse models serve not only to study the life cycle and pathogenesis of the pathogens but also to assess new therapeutic strategies.

30.3.1.2.2 Hepatitis C Virus (HCV)

A humanized liver and immune system model were created by expressing FK506 binding protein and caspase 8 under albumin promoter (AFC8) to induce liver cell death, in BALB/c Rag2$^{-/-}$ γ C-null immunodeficient mice. This was engrafted with human HSCs and hepatocyte progenitor cells (AFC8-hu HSC/Hep) and then infected with HCV. T-cell response as well as the development of liver inflammation and fibrosis was observed. This enabled studying the finer mechanisms and therapeutics for the disease. Another model utilized a single precursor, i.e., CD34^{+} human fetal liver cells transplanted by intrahepatic injection in NSG mice which further differentiated into HSCs and liver progenitor cells [43]. These mice when challenged with intravenous injection of HCV not only supported the HCV lifecycle but depicted the development of hepatitis and immune response [11]. This model was

used to further study the pathogenic effects of HCV infection like the development of fibrosis, adenoma, and carcinoma within 28 weeks postinfection [66].

30.3.2 HIS Model in Bacterial Infection Studies

Bacterial infections are generally less host-specific than viral infections. However, the HIS mouse model is of value for getting a clearer understanding of the pathogenesis in the human context or the pathogens with a human-specific tropism.

Salmonella enterica, serovar Typhi, is a bacterial pathogen transmitted by infected food and causing typhoid fever in humans. *S. typhi* infection has been established in several HIS mouse models by using RAG-$2^{-/-}$/$\gamma c^{-/-}$ mice and engrafting human fetal liver hematopoietic stem cells as well as human leucocytes [67]. The model exhibited disease symptoms as well as the immune response against the pathogen [67]. Another model employed NOD-SCID mice engrafted with human HSCs recapitulating much of the pathology of human typhoid infection [68].

Staphylococcus aureus is an opportunistic pathogen causing skin and soft tissue infections in humans. Although several animal models have increased our understanding of its pathogenesis, however, the HIS mouse model reveals more human-specific insights. Intraperitoneal infection of *S. aureus* in HSC-containing NSG mice resulted in a more aggressive infection compared to wild-type mice [69]. This model displayed typical cutaneous lesions and highlighted the importance of neutrophils as well as *S. aureus* virulence protein PVL in the disease phenotype.

Bacterial sepsis is another severe inflammatory condition and the most common cause of death in intensive care units. The differences in mouse and human system warranted the need for a HIS mouse model of sepsis. The NSG mice with human HSCs were used to create a sepsis model, by cecal ligation and puncture (CLP) method. As seen in sepsis patients, these mice had a significant elevation in pro- and anti-inflammatory cytokines as well as apoptosis of T and B cells [54]. In a similar study using the BLT mice, it was shown that the presence of human immune cells caused a cytokine storm and ultimately death of the CLP-induced septic animals. The siRNA-mediated inhibition of HMGB1 resulted in reduced cytokine storm and rescued the animals from death [70]. A neonatal model of sepsis using group B streptococcus in NSG mice and human HSCs was created and was further used to test the efficacy of treatment with antibacterial therapy [71].

30.4 HIS Model in Autoimmune Conditions

Autoimmune diseases are the result of complex interactions between the immune system and other body tissues. They have multiple trigger sources and mechanisms. However, the exact mechanisms are yet to be fully elucidated. The HIS mouse model allows the study of immunological aspects of autoimmune disorders in a small animal system.

One critical autoimmune disorder is systemic lupus erythematosus (SLE) which leads to multiple organ damage and mortality. SLE has been modeled in HSC-containing NSG mice platform by injecting pristine, a synthetic mineral oil that elicits autoimmunity. This recapitulated the key features of SLE such as the production of antinuclear antibodies, nephritis, and pulmonary serositis offering a validated system to study the disease mechanism and evaluate therapies [12].

Type I diabetes, characterized by autoimmunity against insulin-producing cells, has been difficult to study in mice. Early work using NOD-SCID mice involved injecting human autoreactive T cells and APCs in streptozotocin-treated animals. Most of the transplanted T cells could not migrate into the islets of Langerhans and cause diabetes, emphasizing the need for additional factors for the generation of the phenotype [72]. In a novel approach, HLA-DQ8-transgenic NSG mice were generated by crossing HLA-DQ8-Tg NOD-SCID mice with NSG mice followed by engraftment of human fetal thymus and HSCs [73]. Therapeutic strategies for type I diabetes have also been assessed using an autoantigen vaccine candidate to induce T regs in human HSC engrafted NSG-HLA-DQ8 transgenic mice [74].

Rheumatoid arthritis is another example of an autoimmune condition affecting the joints and synovial membrane in elderly patients. A HIS model to study the same was constituted by using NSG mice with human HSCs in which arthritis was induced by injecting complete Freund's adjuvant into the joints. There was a production of human IgG in response to the immunization along with inflammation and migration of immune cells to the injected site, which was alleviated when treated with respective drugs [75].

Inflammatory bowel disease (IBD) is a chronic inflammation in the GI tract and may be caused by gut microbiota, allergens, genetic factors, and immune dysregulations. The exact mechanisms are still under investigation [76]. A simple mouse model to study IBD was developed by injecting allergic donor PBMCs into NOD-SCID mice, along with the allergen, which led to strong inflammation of the colon and IgE response against the allergen. In another similar approach, NSG mice were engrafted with PBMCs from ulcerative colitis (UC) as well as healthy donors and challenged with oxazolone in ethanol solvent, a UC inducer. It was observed that mice with PBMCs from UC patients had more pronounced symptoms of inflammation like edema, fibrosis, and infiltration of T cells, reflecting the predisposition of the donor towards the disease and thus offering a platform for further studies [77].

30.5 HIS Mice in Transplantation Studies

30.5.1 In Graft vs. Host Response

Graft-versus-host disease (GvHD) remains a severe consequence of transplantation leading to morbidities and in extreme cases can cause mortality. It is seen usually in allogeneic lymphocyte or bone marrow transplantations in immune-suppressed or immunodeficient individuals. The condition can be acute or chronic based on the kinetics of the GvHD. NOG, NOD-Scid, NOD-Scid-B2m, NOD-ScidIL2rg$^{-/-}$, and

$Rag2^{-/-}\gamma c^{-/-}$ mice strains have been extensively used for humanized models in GvHD studies [78]. These models can help not only distinguish between host and donor GvHD responses due to different species-specific cells and cytokines but also distinguish the trajectories of acute (aGvHD) or chronic responses (cGvHD) wherein the morphological changes and symptoms equivalent to human kinetics distinguish the type of responses. An aGvHD response in mouse models is predominated by Th1 response along with TGF α and IL1 cytokine secretion [31]. On the other hand, cGvHD develops systemic fibrosis along with autoantibodies and Th2 responses in mouse models. The studies in humanized models can be further conditioned by the influence of material transfer as the choice can attune the development of GvHD responses. Literature indicates that the material transfer of human peripheral blood lymphocytes can elicit an acute reaction, while organ transplants of bone marrow, liver, and thymus develop a chronic GvHD reaction [78]. Depending on the material transfer (cells or organ), the model can further modulate the kinetics of the reactions. The severity of GvHD and the rate of response through engraftment level modulations can aid in modeling the intensity of clinical symptoms. Injections of T-cell-depleted HSCs and SCID or NOD-Scid mice display low engraftment and less development of the response. But the injection of human peripheral blood lymphocytes in $IL2rg^{-/-}$ mice develops a stable model, and macrophage depletion in any model creates an acute system [7]. Thus, the study of immune infiltration and cytokines in humanized mouse models can be of consequence in GvHD research.

30.5.2 In Host vs. Graft Response

Allogeneic organ transplant often leads to rejection of the solid organ due to immune response by the host. Through the aid of the immunosuppressive drugs in engraftment, the graft survival for a prolonged time is restricted by host vs. graft response (HvG). Humanized mice with close resemblance to host immune responses hold the interest of current research as humanized mice mimic host systems while non-humanized can render limited or alternative insight into the mechanism. The difference is seen in vascular allograft wherein humanized mice mimicked human mononuclear response while non-humanized mice displayed smooth muscle response [78]. Thus immune-deficient strains with human immune cell engraftment can tolerate allogeneic/xenogeneic grafts while eliciting human systemic immune responses. A successful posttransplantation arteriosclerosis model has been generated for transplantation studies, wherein Scid/bg or $Rag^{-/-}\gamma c$ mice with pig epicardial or human arteries were used in HIS mouse models. The $Rag^{-/-}\gamma c$ transfer established a stable model to study post graft arteriosclerosis [78]. However, the low material transfer of human B cells in the model was a limitation. Models for macrophage-mediated allograft rejection of the skin and artery have been introduced by selective enrichment of $CD34^{+}$ human peripheral blood HSCs and subsequent material transfer to $NOD\text{-}ScidIL2rg^{-/-}$ or SCID/bg [1]. Solid-organ transplant studies have also been made possible with disease conditions. In the models, human hepatocytes were transferred in Ncr nude mice and NOG mice along with

hepatitis B and C viruses for paired influence studies [79]. Similarly, human fetal tracheal transplants in SCID or SCID/bg flank allowed cystic. Further, the model was also used for tracheal transplantation studies. Thus, humanized mice allow for wider variation within the purview of human response limitations in small animals.

30.6 HIS Mice in Cancer Studies

The use of humanized mice in oncological research has propelled the understanding and evaluation of immunotherapy in recent studies. Several models have been established using patient-derived xenografts in immune-deficient strains with the material transfer of immune cells [80]. These models are especially advantageous in understanding antibody and T-cell priming efficacy against cancer cells [81]. A recent study with renal cell carcinoma (RCC) model with PBMCs was used to indicate the function of prime T-cell and B-cell response against carbonic anhydrase IX in RCC90. These models also helped to understand the complex stromal interaction with tumor physiology. Studies showed that tumor-engrafted humanized mice displayed antitumor activity when lymphocytes were accumulated in the cancerous tissue [81]. Further antigen-specific T-cell response has been evaluated by the adoptive transfer of T cells from humanized mice into melanoma models [82]. Humanized mice can further advance interaction studies between tumor development and oncolytic viruses. Tsoneva et al. demonstrated a vaccinia virus in the humanized lung carcinoma model and analyzed the efficacy of CTL4 therapy [83]. Thus, the combinatorial effects of complex models are made possible. Etiological studies wherein the gut microbiota are inoculated in humanized mice have been used to understand the host response against cancer in the presence of microbes [84]. This has been done using fecal transplantation in germ-free humanized mouse models. While the humanization of mice is advantageous in cancer research, the development of a stable model has several limitations. In the most nascent form, NSG mice have undergone material transfer of $CD34^{+}$ HSCs sourced from the fetal liver, thymus, umbilical cord, or bone marrow [79]. A 50–250 cGY whole-body-irradiated NSG mice of 2–3 weeks were humanized by adoptive transfer of close to 0.1 million HSCs. The model is said to be complete since 25% CD45 human cells could be detected in mice peripheral blood 4 weeks post transfer [80]. Alternatively, PDX models or patient-derived xenograft models have made complex interactions possible wherein tumor fragments are engrafted in the aforementioned humanized models. Several selective models for specific cancers have also been evaluated for their stability. Some of the models included NSG-SGM3 expressing GM-MCSF and stem cell factors allowing enhanced engraftment of HSCs [85]. NBSGW mice allow the development of a stable model without irradiation. The SRG-15 mice have displayed better intraepithelial lymphocyte maturation along with innate cell and NK development. These models with PDX have allowed complex interactions and therapeutic research with an ambient variation.

The humanized mouse models have proven to be useful in precision medicine. However, there are several limitations especially in oncological research that needs

to be addressed. Several tumor models such as prostate cancer are difficult to establish in PDX models due to the additional conditions and factors required for tumor development. These models also require a long duration of time to establish. Patient-derived xenografted humanized mouse typically takes 6 months to generate a stable model. The timeline may vary depending upon the type of cancer. Further, genetic heterogeneity in tumors as a consequence of continuous serial cycle passage cannot be fairly represented in dissected tumor engrafted in models [80].

30.7 HIS Mice in Drug Discovery

Drug discovery has brought about a paradigm shift in aiming at the specific targets with increasingly fewer side effects. The accuracy is progressively building towards precision medicine wherein the chronic toxicity and off-target action can be mitigated. The enhancement has been possible due to the development of better small animal models which preclude the need for multiple preclinical drug studies. This enables translation to clinical conditions with a shorter timeline and unburdens the economic stake during drug development. The preclinical development is enabled by representing the biological complexities of the human system in small animals. The humanized mice with human immune response and tissue engraftment can qualify the study for multiparametric evaluation. The toxicity, immune response, and molecule cross-reactivity can be studied in the same setup which would otherwise require multiple preclinical setups in increasingly complex mammals.

There are several humanized mouse models wherein the immune-deficient strains such as NOG, SCID, NOD, and NOD-SCID are subjected to human PBMC or HSC transplantation. Further conditioning is done by engraftment of human xenogeneic grafts such as the liver in the same model to study the detoxification of the molecules. The selection of the appropriate model enables the fitting parameters to function. For instance, NOG-EXL host supports better engraftment of $CD34^{+}$ cells due to the cytokine repertoire (hGM-CSF, IL3), while other strains carrying certain mutations improve its application in the study [86]. However, a humanized model with a broader immune response is favored with xenogeneic engraftment of the liver.

Humanized mice have been particularly favored for drug toxicity studies as several in vivo models have failed to mimic the clinical responses. TGN1412 has been known to cause cytokine release storm (CRS) in clinical studies. However, the drug has had a limited response in non-primate studies at saturating dose. But the humanized models administered with relevant doses displayed appropriate CRS responses [87]. The manifestation limited the adverse outcomes. Further another NSG strain called NSG-SGM3 mouse model reproduced the clinical CAR T treatment side effects including delayed onset of neurotoxicity [88]. This clinical phenotypic response allowed the identification of macrophage IL6 as a pathology inducer and further permitted targeted drug development to prevent neurotoxicity. Various models with induced drug targets in disease have also been developed. Models for colitis [89], rheumatoid arthritis, HIV, and tissue-specific diseases have been

established by human tissue engraftment or induction of infection using virus post-engraftment of receptive cells in the mice host.

30.8 Conclusion

As our understanding of human biology continues to increase, humanized mouse models are going to play a significant role in analyzing the human immune response in experimental setups. Humanized mouse models are also critical in the development of disease-specific immunotherapies and evaluation of their efficacies. With the advent of novel immunodeficient strains, these models are poised to have extensive scope especially in conditions where no other relevant preclinical models are available. However, one must tread with caution since humanized mice are models of human disease and as true for all the model systems, they have various merits and demerits associated. None of the existing humanized mouse models fully reproduce the absolute functionality of the human immune system. However, with an in-depth understanding of their strengths and weaknesses, these models can be used in preclinical studies to address specific questions guiding the precision medicine alternatives in the clinical scenario. In all, HIS mouse models represent an exciting platform in the field of immunotherapy and can be a great future tool to advance our knowledge.

References

1. Herndler-Brandstetter D et al (2017) Humanized mouse model supports development, function, and tissue residency of human natural killer cells. Proc Natl Acad Sci U S A 114(45):E9626–E9634
2. Phillips KA et al (2014) Why primate models matter. Am J Primatol 76(9):801–827
3. Greek R, Rice MJ (2012) Animal models and conserved processes. Theor Biol Med Model 9 (1):40
4. Mestas J, Hughes CCW (2004) Of mice and not men: differences between mouse and human immunology. J Immunol 172(5):2731–2738
5. van der Worp HB et al (2010) Can animal models of disease reliably inform human studies? PLoS Med 7(3):e1000245
6. Bosma GC, Custer RP, Bosma MJ (1983) A severe combined immunodeficiency mutation in the mouse. Nature 301(5900):527–530
7. Pearson T, Greiner DL, Shultz LD (2008) Creation of "Humanized" mice to study human immunity. Curr Protoc Immunol 81(1). https://doi.org/10.1002/0471142735.im1521s81
8. Rongvaux A et al (2014) Development and function of human innate immune cells in a humanized mouse model. Nat Biotechnol 32(4):364–372
9. Frias-Staheli N et al (2013) Utility of humanized BLT mice for analysis of dengue virus infection and antiviral drug testing. J Virol 88(4):2205–2218
10. Marinho CRF et al (2015) De Novo generated human red blood cells in humanized mice support *Plasmodium falciparum* infection. PLoS One 10(6):e0129825
11. Keng CT et al (2016) Characterisation of liver pathogenesis, human immune responses and drug testing in a humanised mouse model of HCV infection. Gut 65(10):1744–1753
12. Gunawan M et al (2017) A novel human systemic lupus erythematosus model in humanised mice. Sci Rep 7(1):16642

13. Tobin LM et al (2013) Human mesenchymal stem cells suppress donor CD4(+) T cell proliferation and reduce pathology in a humanized mouse model of acute graft-versus-host disease. Clin Exp Immunol 172(2):333–348
14. Pan C-x et al (2017) Humanized mice (humice) carrying patient-derived xenograft (PDX) as a platform to develop immunotherapy in bladder cancer (BCa). J Clin Oncol 35(Suppl. 6):381–381
15. Shultz LD, Ishikawa F, Greiner DL (2007) Humanized mice in translational biomedical research. Nat Rev Immunol 7(2):118–130
16. Shultz LD et al (2005) Human lymphoid and myeloid cell development in NOD/LtSz-scid IL2R gamma null mice engrafted with mobilized human hemopoietic stem cells. J Immunol 174 (10):6477–6489
17. Ishikawa F et al (2005) Development of functional human blood and immune systems in NOD/SCID/IL2 receptor {gamma} chain(null) mice. Blood 106(5):1565–1573
18. Ito M et al (2002) NOD/SCID/gamma(c)(null) mouse: an excellent recipient mouse model for engraftment of human cells. Blood 100(9):3175–3182
19. Suzuki M et al (2012) Induction of human humoral immune responses in a novel HLA-DR-expressing transgenic NOD/Shi-scid/cnull mouse. Int Immunol 24(4):243–252
20. Danner R et al (2011) Expression of HLA class II molecules in humanized NOD.Rag1KO. IL2RgcKO mice is critical for development and function of human T and B cells. PLoS One 6 (5):e19826
21. Pearson T et al (2008) Non-obese diabetic-recombination activating gene-1 (NOD-Rag1 null) interleukin (IL)-2 receptor common gamma chain (IL2r gamma null) null mice: a radioresistant model for human lymphohaematopoietic engraftment. Clin Exp Immunol 154(2):270–284
22. Shultz LD et al (2012) Humanized mice for immune system investigation: progress, promise and challenges. Nat Rev Immunol 12(11):786–798
23. Robert-Richard E et al (2006) Human cell engraftment after busulfan or irradiation conditioning of NOD/SCID mice. Haematologica 91(10):1384
24. Mosier DE et al (1988) Transfer of a functional human immune system to mice with severe combined immunodeficiency. Nature 335(6187):256–259
25. Ito R et al (2009) Highly sensitive model for xenogenic GVHD using severe immunodeficient NOG mice. Transplantation 87(11):1654–1658
26. King MA et al (2009) Human peripheral blood leucocyte non-obese diabetic-severe combined immunodeficiency interleukin-2 receptor gamma chain gene mouse model of xenogeneic graft-versus-host-like disease and the role of host major histocompatibility complex. Clin Exp Immunol 157(1):104–118
27. Kenney LL et al (2016) Humanized mouse models for transplant immunology. Am J Transplant 16(2):389–397
28. Yaguchi T et al (2017) Human PBMC-transferred murine MHC class I/II-deficient NOG mice enable long-term evaluation of human immune responses. Cell Mol Immunol 15(11):953–962
29. Brehm MA et al (2018) Lack of acute xenogeneic graft-versus-host disease, but retention of T-cell function following engraftment of human peripheral blood mononuclear cells in NSG mice deficient in MHC class I and II expression. FASEB J 33(3):3137–3151
30. Kamel-Reid S, Dick JE (1988) Engraftment of immune-deficient mice with human hematopoietic stem cells. Science 242(4886):1706–1709
31. Theocharides APA et al (2015) Humanized hemato-lymphoid system mice. Haematologica 101 (1):5–19
32. Akkina R et al (2016) Improvements and limitations of humanized mouse models for HIV research: NIH/NIAID “Meet the Experts” 2015 workshop summary. AIDS Res Hum Retrovir 32(2):109–119
33. Watanabe Y et al (2009) The analysis of the functions of human B and T cells in humanized NOD/shi-scid/γcnull (NOG) mice (hu-HSC NOG mice). Int Immunol 21(7):843–858
34. Brehm MA et al (2013) Humanized mice for the study of infectious diseases. Curr Opin Immunol 25(4):428–435

35. Brehm MA et al (2014) Generation of improved humanized mouse models for human infectious diseases. J Immunol Methods 410:3–17
36. Legrand N et al (2009) Humanized mice for modeling human infectious disease: challenges, progress, and outlook. Cell Host Microbe 6(1):5–9
37. Walsh NC et al (2017) Humanized mouse models of clinical disease. Ann Rev Pathol: Mech Dis 12(1):187–215
38. Sharma A et al (2016) Respiratory Syncytial Virus (RSV) pulmonary infection in humanized mice induces human anti-RSV immune responses and pathology. J Virol 90(10):5068–5074
39. Lan P et al (2006) Reconstitution of a functional human immune system in immunodeficient mice through combined human fetal thymus/liver and CD34+ cell transplantation. Blood 108 (2):487–492
40. Melkus MW et al (2006) Humanized mice mount specific adaptive and innate immune responses to EBV and TSST-1. Nat Med 12(11):1316–1322
41. Denton PW et al (2012) IL-2 receptor γ-chain molecule is critical for intestinal T-cell reconstitution in humanized mice. Mucosal Immunol 5(5):555–566
42. Kalscheuer H et al (2012) A model for personalized in vivo analysis of human immune responsiveness. Sci Transl Med 4(125):125ra30
43. Lai F, Chen Q (2018) Humanized mouse models for the study of infection and pathogenesis of human viruses. Viruses 10(11):643
44. Maartens G, Celum C, Lewin SR (2014) HIV infection: epidemiology, pathogenesis, treatment, and prevention. Lancet 384(9939):258–271
45. Policicchio BB, Pandrea I, Apetrei C (2016) Animal models for HIV cure research. Front Immunol 7:12
46. Mosier DE et al (1993) Resistance to human immunodeficiency virus 1 infection of SCID mice reconstituted with peripheral blood leukocytes from donors vaccinated with vaccinia gp160 and recombinant gp160. Proc Natl Acad Sci 90(6):2443–2447
47. Koneru R, Olive MF, Tyor WR (2014) Combined antiretroviral therapy reduces brain viral load and pathological features of HIV encephalitis in a mouse model. J Neurovirol 20(1):9–17
48. Honeycutt JB, Garcia JV (2017) Humanized mice: models for evaluating NeuroHIV and cure strategies. J Neurovirol 24(2):185–191
49. Myburgh R et al (2015) Lentivector knockdown of CCR5 in hematopoietic stem and progenitor cells confers functional and persistent HIV-1 resistance in humanized mice. J Virol 89 (13):6761–6772
50. Victor Garcia J (2016) Humanized mice for HIV and AIDS research. Curr Opin Virol 19:56–64
51. Lavender KJ et al (2018) An advanced BLT-humanized mouse model for extended HIV-1 cure studies. AIDS 32(1):1–10
52. Münz C (2017) Humanized mouse models for Epstein Barr virus infection. Curr Opin Virol 25:113–118
53. Whitehurst CB et al (2015) Knockout of Epstein-Barr virus BPLF1 retards B-Cell transformation and lymphoma formation in humanized mice. MBio 6(5):e01574–e01615
54. Unsinger J et al (2009) Sepsis-induced human lymphocyte apoptosis and cytokine production in "humanized" mice. J Leukoc Biol 86(2):219–227
55. Chijioke O et al (2013) Human natural killer cells prevent infectious mononucleosis features by Targeting Lytic Epstein-Barr Virus infection. Cell Rep 5(6):1489–1498
56. Martina BEE, Koraka P, Osterhaus ADME (2009) Dengue virus pathogenesis: an integrated view. Clin Microbiol Rev 22(4):564–581
57. Cox J et al (2012) Mosquito bite delivery of dengue virus enhances immunogenicity and pathogenesis in humanized mice. J Virol 86(14):7637–7649
58. Robinson LN et al (2015) Structure-guided design of an anti-dengue antibody directed to a non-immunodominant epitope. Cell 162(3):493–504
59. Sridharan A et al (2013) Inhibition of megakaryocyte development in the bone marrow underlies dengue virus-induced thrombocytopenia in humanized mice. J Virol 87 (21):11648–11658

60. Costa VV et al (2017) Dengue virus-infected dendritic cells, but not monocytes, activate natural killer cells through a contact-dependent mechanism involving adhesion molecules. mBio 8(4): e00741–e00817
61. Schmitt K et al (2018) Zika viral infection and neutralizing human antibody response in a BLT humanized mouse model. Virology 515:235–242
62. Iannacone M, Guidotti LG (2015) Mouse models of Hepatitis B Virus pathogenesis. Cold Spring Harb Perspect Med 5(11):a021477
63. Langsley G et al (2015) A novel mouse model for stable engraftment of a human immune system and human hepatocytes. PLoS One 10(3):e0119820
64. Walker CM et al (2014) Hepatitis B virus infection and immunopathogenesis in a humanized mouse model: induction of human-specific liver fibrosis and M2-like macrophages. PLoS Pathogens 10(3):e1004032
65. Koh S et al (2018) Nonlytic Lymphocytes engineered to express virus-specific T-cell receptors limit HBV infection by activating APOBEC3. Gastroenterology 155(1):180–193. e6
66. Ray RB et al (2017) Hepatitis C virus mediated chronic inflammation and tumorigenesis in the humanised immune system and liver mouse model. PLoS One 12(9):e0184127
67. Firoz Mian M et al (2010) Humanized mice are susceptible to Salmonella typhi infection. Cell Mol Immunol 8(1):83–87
68. Libby SJ et al (2010) Humanized nonobese diabetic-scid IL2r null mice are susceptible to lethal Salmonella Typhi infection. Proc Natl Acad Sci 107(35):15589–15594
69. Knop J et al (2015) *Staphylococcus aureus* infection in humanized mice: a new model to study pathogenicity associated with human immune response. J Infect Dis 212(3):435–444
70. Ye C et al (2012) Human macrophage and dendritic cell-specific silencing of high-mobility group protein B1 ameliorates sepsis in a humanized mouse model. Proc Natl Acad Sci 109 (51):21052–21057
71. Ernst W et al (2013) Humanized mice, a new model to study the influence of drug treatment on neonatal sepsis. Infect Immun 81(5):1520–1531
72. van Halteren AGS et al (2004) Homing of human autoreactive T cells into pancreatic tissue of NOD-scid mice. Diabetologia 48(1):75–82
73. Tan S et al (2017) Type 1 diabetes induction in humanized mice. Proc Natl Acad Sci 114 (41):10954–10959
74. Serr I et al (2016) Type 1 diabetes vaccine candidates promote human Foxp3+Treg induction in humanized mice. Nat Commun 7(1):10991
75. Misharin AV et al (2012) Development of a new humanized mouse model to study acute inflammatory arthritis. J Transl Med 10(1):190
76. Lee SH, Kwon Je, Cho M-L (2018) Immunological pathogenesis of inflammatory bowel disease. Intestinal Res 16(1):26
77. Nolte T et al (2013) Oxazolone and ethanol induce colitis in non-obese diabetic-severe combined immunodeficiency interleukin-2Rγnullmice engrafted with human peripheral blood mononuclear cells. Clin Exp Immunol 172(2):349–362
78. Hogenes M et al (2014) Humanized mouse models in transplantation research. Transplant Rev (Orlando) 28(3):103–110
79. Morton JJ et al (2016) XactMice: humanizing mouse bone marrow enables microenvironment reconstitution in a patient-derived xenograft model of head and neck cancer. Oncogene 35 (3):290–300
80. Choi Y et al (2018) Studying cancer immunotherapy using patient-derived xenografts (PDXs) in humanized mice. Exp Mol Med 50(8):99
81. Jespersen H et al (2017) Clinical responses to adoptive T-cell transfer can be modeled in an autologous immune-humanized mouse model. Nat Commun 8(1):707
82. Hu Z et al (2016) Human melanoma immunotherapy using tumor antigen-specific T cells generated in humanized mice. Oncotarget 7(6):6448–6459
83. Tsoneva D et al (2017) Humanized mice with subcutaneous human solid tumors for immune response analysis of vaccinia virus-mediated oncolysis. Mol Ther Oncol 5:41–61

84. Staley C et al (2017) Stable engraftment of human microbiota into mice with a single oral gavage following antibiotic conditioning. Microbiome 5(1):87
85. Gu Z et al (2017) Evaluation of the correlations between patient-derived xenograft (PDX) model-based mouse trials and cancer patient-based clinical trials. J Clin Oncol 35(Suppl. 15): e23140
86. Magnotti E, Marasco WA (2018) The latest animal models of ovarian cancer for novel drug discovery. Expert Opin Drug Discov 13(3):249–257
87. Stoddart CA et al (2016) TGN1412 Induces lymphopenia and human cytokine release in a humanized mouse model. PLoS One 11(3):e0149093
88. Norelli M et al (2018) Monocyte-derived IL-1 and IL-6 are differentially required for cytokine-release syndrome and neurotoxicity due to CAR T cells. Nat Med 24(6):739–748
89. Hooper LV, Littman DR, Macpherson AJ (2012) Interactions between the microbiota and the immune system. Science 336(6086):1268–1273
90. Traggiai E et al (2004) Development of a human adaptive immune system in cord blood cell-transplanted mice. Science 304(5667):104–107
91. Ashkar AA et al (2017) Creation of an immunodeficient HLA-transgenic mouse (HUMAMICE) and functional validation of human immunity after transfer of HLA-matched human cells. PLoS One 12(4):e0173754

31 Necropsy Procedures for Laboratory Animals

Yogeshkumar V. Murkunde

Abstract

Necropsy is the ultimate diagnostic destiny of most of the experimental animals. Necropsy is the systematic dissection of an animal after death, to establish the cause of disease by macroscopic and microscopic examination of tissues. Necropsy facilitates the conduct of appropriate serologic and microbiologic examinations and harvest of tissues and organs for histological evaluations. The necropsy and histology data are pivotal in laboratory animal research, toxicology, and pathology.

Keywords

Necropsy · Autopsy · Dissection

Abbreviations

b. w.	Body weight
DF	Davidson's fluid
GI	Gastrointestinal
IPA	Isopropyl alcohol
MDF	Modified Davidson's fluid
NAD	No abnormality detected
NBF	Neutral buffer formalin
PBS	Phosphate-buffered saline

Y. V. Murkunde (✉)
Centre for Toxicology and Developmental Research, Sri Ramachandra Institute of Higher Education and Research (Deemed to be University), Porur, Chennai, Tamil Nadu, India
e-mail: yogesh.tfm@sriramachandra.edu.in

© The Author(s), under exclusive license to Springer Nature Singapore Pte Ltd. 2021

P. Nagarajan et al. (eds.), *Essentials of Laboratory Animal Science: Principles and Practices*, https://doi.org/10.1007/978-981-16-0987-9_31

PM Postmortem
SOP Standard operating procedure

31.1 Introduction

Necropsy is the ultimate diagnostic destiny of most of the experimental animals. Laboratory animals death may occur spontaneously or due to certain disease conditions in a colony. However, a death of an animal, if properly investigated, will provide an opportunity to gain significant insight on the husbandry practices and diseases (bacterial, fungal, parasitic, viral) and will help prevent further losses within the colony. Further, laboratory animals are sacrificed during routine health surveillance and during or after experiments in toxicology and efficacy studies [1].

Necropsy is the systematic dissection of an animal after death, necessary to establish the cause of disease by macroscopic and microscopic examination of tissues. Although "autopsy" and "necropsy" are synonymous, "necropsy" is the conventional term for postmortem examination of nonhuman species, and an "autopsy" is the term used for humans. Necropsy includes not only the dissection of the carcass and gross observation of all tissues but also the collection of appropriate tissue specimens and measurements of the body, internal organs, and body fluids, i.e., weight, size, length, volume [2] for histological evaluations. Necropsy also facilitates the collection of samples for appropriate parasitic, serologic, and microbiologic examinations. Necropsy and histology data are pivotal in laboratory animal research, toxicology, and pathology processes therein [3]. The adage "once lost is lost forever" fits aptly for necropsy as information which could have been significant cannot be retrieved once the tissue is discarded or lost during necropsy.

The study pathologist/veterinarian, prosector, and assistant weighing the organ are responsible for collecting necropsy data. Identification and interpretation of the abnormal findings during necropsy is a complex process. The pathologist is consulted at the beginning for assistance in designing the study, for performing postmortem examinations, and for the evaluation of observed lesions [2]. Many academic/small animal laboratories may not afford a veterinary pathologist in the necropsy team. In such cases, diagnostic necropsies shall be conducted by specially trained staff. Because, without knowledge of lesions, causative factors, and disease mechanisms, the investigation could be flawed [4]. The involvement of trained veterinarians is necessary for training and competency assessment of researchers for common procedures, monitoring of procedures, and preventing the unacceptable level of suffering that may influence animal welfare and disease diagnosis, etc., e.g., anesthesia, euthanasia, blood collection, and dissection [5].

For planning the necropsy procedure, several factors should be considered inline with the objectives of the study. While designing the experimental protocol, the methods for the euthanasia and necropsy, tissues to be collected, and types of fixatives must be thoroughly deliberated. If the study plan/protocol is not included

with such details, a separate necropsy protocol (quick guide) should be followed. Deviation if any, must be recorded [1, 6, 7].

There is no single best method for necropsy; procedures differ between laboratories, pathologists, and the type of study involved. It also differs according to the objectives of the experiment and established procedures, etc. This chapter does not discuss the superiority of one method over others. However, a general description given here can be used on any laboratory animal species and will certainly add value in producing a better result.

The appropriate techniques including gross observation and histology techniques form the basis for accurate pathological interpretations during laboratory animal research. Gross pathology findings observed at necropsy should be documented promptly in a descriptive written report accompanied by good-quality photographs. All the organs shall be collected properly [8]. Histological techniques follow the necropsy that involves appropriate preservation/fixation of tissues, processing, embedding in appropriate medium, microtomy sections, staining, and mounting a coverslip over a stained section.

Although this chapter details about postmortem procedures in rodents and rabbits in toxicology/experimental research perspectives, the general principles are relevant to all laboratory animals including organ-specific efficacy studies. The necropsy procedures involved in reproduction and developmental toxicity studies are not discussed here.

The chapter describes the following techniques involved in necropsy:

1. Laboratory design and safety concerns.
2. Prerequisites and preliminary arrangements.
3. Euthanasia.
4. Necropsy general.
5. Dissection procedure and sequence.
6. Weighing of tissues.
7. Retention of tissues/samples.
8. Dispatch of tissues/samples to laboratories, e.g., histology.
9. Post-necropsy procedures.

31.2 Elements of Good Practices

31.2.1 Necropsy Room Design and Safety Concerns

- Special attention is required towards the design, availability of instruments, and overall setup of the necropsy room.
- Flooring, partitions, postmortem table, laminar hoods, and safety cabinets must be simple in design to clean and sterilize or recurrent washing.
- Considering the exposure to the heavy amount of toxic substances such as anesthetics, fixatives, solvents, and allergens [9, 10] and infectious agents, the necropsy room should have adequate ventilation and exhaust system. Downdraft

systems which will exhaust the noxious fumes from the base of workstations are the new norms for necropsy laboratories [11].

- Additional containment systems such as biological safety cabinets and laminar flow hoods should be considered.
- Additional safety measures such as closely fitting personal protective equipment with a ventilated helmet provided with high-efficiency filters are highly recommended for the necropsy of animals suspected to harbor organisms of zoonotic or hazardous nature.
- Appropriate containment and stringent observance of personal hygiene are essential due to the risk of microbial contamination.
- While designing, space for the anesthetic instrument, gas cylinders, pipings, and an outlet for waste gases including charcoal canister is to be planned to facilitate the use of inhalational anesthetics.
- Position and intensity of light, temperature, ventilation airflow, air pressure, and water/drainage lines are also important aspects while planning necropsy room design [10].
- Electronic machines, e.g., weighing balances and computers as well as electrical sockets and wiring, shall be protected from liquids.
- During the postmortem, the personnel involved must wear closed-front protective clothing, hand gloves, head cap, and facemask including protective goggles.
- It is recommended to remove ornaments like rings, bracelets, or wristwatches during the conduct of necropsy. Eating, drinking, smoking, applying cosmetics, mouth pipetting, touching an open body part, or using contact lenses without protective glasses are prohibited in the necropsy room.
- The plastic bags, containers, tubes, etc. should be kept as accessible but not near the carcass and surfaces of such, and other items like telephones and doorknobs shall be protected from spillages and contamination.
- Standard operating procedures (SOPs) describing the details of decontamination methods, sanitation practices, and hygienics shall be available in the necropsy room for ready reference.

31.2.2 Prerequisites of Necropsy

- As necropsy is a crucial act in which errors are seldom repairable, well-trained, conscientious personnel with sufficient knowledge of anatomy and dissection are indispensable for successful postmortem examination [12].
- The junior staff has to undergo training by working under an experienced pathologist to ensure continuity of procedure. Such training must underline the value of scrupulousness and consistency joined with proficiency [13].
- To avoid overlap between studies, a necropsy schedule must be maintained. The study director/investigator must share the study schedule so that the necropsy staff will prepare the place for the proposed necropsy. It is recommended to have a schedule, describing the experimental dates, proposed necropsy dates, type of study, animal species, total animals, and study requirements.

- A copy of the final study plan/protocol and relevant amendment(s), if any, shall be distributed to the pathologist and necropsy section for the pre arrangements like preparation of specimen containers with labels.
- It is critical to use all accessible data of the test item and study data in organizing and making initial decisions about necropsy and histopathology-related procedures in toxicity studies. This will help the study pathologist to take critical decisions regarding the collection of all expected organs having an impact on test item administration and collection of appropriate samples.
- Knowledge of physical/chemical characteristics of the test item and study data like clinical signs, body weight changes, clinical pathology, and any other procedures done during the exposure or recovery phase of the study is valuable for the pathologist during interpretation [3].

31.2.3 Preliminary Arrangements for Scheduled Sacrifices

- It is to be ensured that trained staff including a prosector, a euthanasia assistant, a person for organ weighing, and tissue transfer are present on the day of necropsy. The alternative yet the best option is an individual prosector station is provided with a weighing balance, documentation system, and tissue collection jar, which makes individual prosector responsible for a complete necropsy of an animal.
- Before proceeding with the necropsy, it is necessary to ascertain that relevant documents have been received and are available in the necropsy area.
- It should be ensured that all necessary equipment such as sharp scissors, slides, pots, and trays are available, and the instruments such as balances, pipettes, and centrifuges are available and calibrated.
- Any variations from “normal necropsy routine” must be clearly explained to the prosector before commencing necropsy.

31.2.4 Documentation

- The concerned study director/investigator shall give the requisition for necropsy along with details of animal history. It is recommended to have appropriate formats for each activity to record the information properly.
- The tissue collection list shall be displayed at every necropsy station with a sign of the in-charge/study pathologist.
- Alternatively, this documentation process may be appropriately computerized.

31.2.5 Materials and Equipment Required for the Conduct of Necropsy

Surgical types of equipment are suitable for most postmortem procedures:

1. Euthanasia/anesthesia equipment and instruments.
2. Down drafted necropsy table/ventilated work station.
3. Necropsy board, pins, forceps.
4. Operating and dissecting scissors.
5. Blade handle, scalpel blades.
6. Knives and bone cutters.
7. Needles and syringes (3, 5, and 10 ml capacity).
8. Squeeze bottles and swabs.
9. Thread, labels, and paper towels.
10. Vials for liquid specimens.
11. Tissue collection tray, cups/bowls, or petri plates.
12. Containers/jar—small and large, tissue cassettes, vials to retain tissues for histology.
13. Sterilized tools for sampling for microbiological specimens.
14. Leak-proof containers and plastic bags of various sizes (bacteriology, mycology, parasitology, virology, molecular, chemistry samples).
15. Plastic screw-cap containers are preferred over glass containers.
16. A calibrated weighing balance with different weight ranges to weigh organs.
17. A dissecting scope/magnoscope or stereo-microscope will be an invaluable aid.
18. PPE including apron gloves, facemask, head cap, goggles, etc.

The staff shall take and unpack only what is needed, so that unused equipment/items shall not be contaminated.

31.2.6 Labeling

Labeling of the specimen is an important but often neglected step. It must be ensured that samples are collected in a properly labeled container respective to that animal.

Main tissue retention pots are labeled with the study number, group, sex, animal number, and necropsy date as a minimum. Labels may be written with indelible ink or preferably preprinted. Adhesive tape entirely over the label will avoid the dislodging of a label by moisture.

31.2.7 Fixatives/Reagents

- Adequate fixation is the most crucial successful histopathology slide preparation. For routine toxicologic and diagnostic purposes, 10% neutral buffered formalin (NBF) is the preferred fixative. However, there are other fixatives to fix tissues with each having its advantages and limitations.
- Before commencing necropsy, the necropsy in-charge shall ascertain that the required fixatives are available in sufficient quantity for the number of animals to be necropsied.

- Solutions and fixatives like 0.9% normal saline, 70% isopropyl alcohol, 10% neutral buffered formalin, Davidson's fluid, modified Davidson's, etc. shall be kept ready for use at the time of necropsy, as per requirement.
- Specimen containers filled with fixatives shall be kept ready with the label of necropsy number and date.
- A disinfectant solution shall be kept ready for use for cleaning, after completion of necropsy. It is also recommended to fumigate the necropsy room after completion of necropsy procedures.

31.2.8 Composition of Commonly Employed Fixatives

31.2.8.1 Neutral Buffered Formalin 10% (1 L)

37—40% Formaldehyde	–	100 ml
Distilled water	–	900 ml
Sodium phosphate monobasic, monohydrate	–	4 g
Sodium phosphate dibasic, anhydrous	–	6.5 g

31.2.8.2 Davidson's Fluid (1 L)

37—40% Formaldehyde	–	20 ml
Ethanol	–	350 ml
Glacial aacetic acid	–	100 ml
Distilled water	–	530 ml

31.2.8.3 Modified Davidson's Fluid (1 L)

37—40% Formaldehyde	–	300 ml
Ethanol	–	150 ml
Glacial acetic acid	–	50 ml
Distilled water	–	500 ml

31.3 Basic Anatomical Terms Related to Topography, Planes, and Regions

31.3.1 Topography of Animals (Fig. 31.1)

The following basic terms are useful during identification, description, and documenting of necropsy findings (Table 31.1).

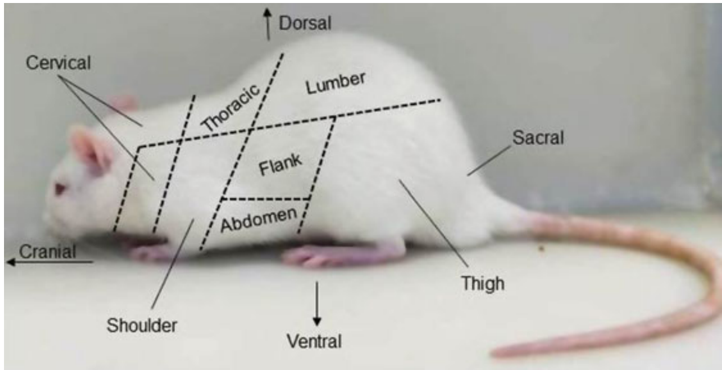


Fig. 31.1 Regions of body

Table 31.1 Topographical term with explanations

Term	Explanation
Anterior	Directed towards the front
Bilateral	On two sides
Caudal	Directed towards the tail
Cranial	Directed towards the head
Distal	In the direction of the edge of the structure
Dorsal	In the direction of the back
Lateral	Directed towards either side
Medial	Directed towards the middle line (median)
Posterior	Directed towards behind
Proximal	Directed towards the beginning/base of the structure
Radial	With equal parts arranged around a central body
Rostral	Directed towards the nose or the mouth
Superior	Directed upwards
Unilateral	On one side
Ventral	Directed towards the abdomen (belly)

31.3.2 The Major Planes of Sections (Table 31.2)

Table 31.2 Terms related to planes of the body with explanation

Plane	Explanation
Median	Longitudinally passing through the middle of the body from front to back, dividing it into left and right halves
Frontal (coronal)	Transversally passing through the body from side to side, perpendicular to the median plane, dividing the body into front and back parts
Sagittal	Vertically passing through the body parallel to the median plane dividing the body into left and right portions
Transverse (dorsoventral),	Vertically passing through the body, perpendicular to the sagittal planes, and dividing the body into front and back portions
Horizontal	Passing through the body, perpendicular to both the frontal and median planes, dividing the body into upper and lower parts
Vertical	Perpendicular to a horizontal plane, dividing the body into left and right or front and back portion

31.4 Euthanasia

- The term euthanasia is typically used to describe the process of ending the life which minimizes or eliminates pain and distress to the animal while necropsy [14].
- Euthanasia is discussed in detail in another chapter of this book; however, the following are some of the accepted brief methods used for euthanasia of laboratory animals.

31.4.1 Euthanasia by Carbon Dioxide

- CO_2 asphyxiation is the most commonly employed, inexpensive method. It shall be avoided in the case of inhalation studies. Co-administration of O_2 with CO_2 is no more recommended since it has little or no advantage [14, 15].
- Whenever gross pathological observation is part of an experiment, animals are subjected to CO_2 gas asphyxiation up to full sedation level, the spinal cord is severed dorsally at the atlas region by a surgical blade to confirm the painless death, and then exsanguination is done by severing the major blood vessels (e.g., jugular/cephalic) without damaging the key organs in the region.

31.4.2 Euthanasia by an Overdose of Anesthetics Followed by Rapid Exsanguination

- The method of an overdose of anesthetics is routinely used for inhalation studies. However, the anesthetics used should not be given by the inhalation route.
- While the animal is still under deep anesthesia, exsanguinations should be done by severing the major blood vessels (jugular/cephalic) without damaging the key organs in the region. This will help in draining all the blood and facilitate accurate gross examination of organs.

31.4.3 Euthanasia by Cervical Dislocation

- This method is preferred when any other physical or chemical method of euthanasia is expected to interfere with the observations or may interact with the test items administered. It is employed for animals with less than 200 gm body weight.
- If adequate dislocation is not achieved, decapitation or exposure to a high level of CO_2 should be used immediately as a backup method [15]. Alternatively, the spinal cord may be severed as explained above in Sect. 31.4.1.

31.5 Necropsy

Upon receipt, an animal shall be checked for its identity along with group and cage identity as mentioned on the animal cage card and matched with necropsy requisition for animal identification number/mark, pathology report form. Any errors in animal identity are immediately brought to the notice of the study director/investigator and the in-charge Necropsy and any corrective action have to be done at once.

31.5.1 Clinical History

The external features of the animal are scrutinized and compared to any relevant comments recorded in vivo as mentioned below.

31.5.2 External Examination (Fig. 31.2)

- Thorough external examination of an animal and its documentation is a crucial aspect of gross pathological evaluations.
- General body condition, external surfaces of the body, and all orifices are observed for abnormality and recorded.
- The general condition includes state of nutrition and muscle mass, skin/fur alterations, and superficial lesions/parasites if any.

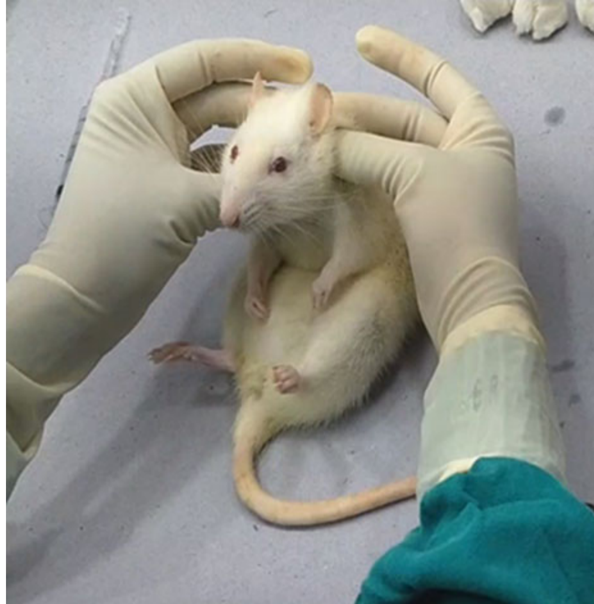


Fig. 31.2 External examination

- While carefully examining the external surfaces of the body, the presence of wounds, infectious processes, and palpable masses will be obvious.
- Swollen, smooth, and glossy skin would indicate edema/anasarca.
- The change in color of eyes and hair coat shall be recorded.
- The discolored, rough, crusty, dry, and hirsute appearing fur indicates health concerns.
- Observations of the natural orifices, like the mouth, the nose, and the anus, are very important to complete the external examination.
- Petechiae, superficial erosions of the mucosa, ulcers, or vesicles may be recorded. Exudates or hemorrhages may be visible at nasal openings, whereas the anal opening would reveal feces or bloody discharge.
- Excessive growth or wear can be looked for at the teeth and nails.
- Palpable masses shall be confirmed and described.
- The masses described in the document have to be checked and verified in the carcass.
- Palpable masses may be found at necropsy, which has not previously been described in vivo. Such new mass shall be given the next available mass number, and the description shall include the mass location, e.g., upper left thorax with a comment, "mass found at necropsy." A rough diagram about the location of the mass will help later during interpretation.
- Where superficial examination at the start of a necropsy fails to identify a palpable mass, which is indicated as still being present, the prosectors shall inform the in-charge, necropsy, or study pathologist who proceeds to identify the mass before the necropsy proceeds further.

- When the in-charge, necropsy, and/or study pathologist confirm a mass is not present, skin with subcutis at the site of the mass shall be retained and the same is documented with the sign and date.

31.5.3 General Considerations

- After exsanguination as explained above, the carcass shall be flushed with freshwater or wiped with cotton so that organs retain the original color.
- Gross pathological examination of all body cavities and internal organs shall be done to check any abnormalities in situ as well as after harvesting/removal of organs. Any abnormality shall be recorded in a specific pathology report form [16]. Wherever possible photographs are taken for comparison with that of controls.
- During the removal of organs, due care shall be taken that they are not compressed/damaged with fingers/forceps.
- To avoid drying of tissues, tissues have to be submerged in normal saline.
- While collecting the lung tissues, using forceps to handle, placing the lungs on bloating paper may damage the lung parenchyma.
- Before weighing and transferring the tissues for fixation, the fascia/connective tissue surrounding the tissues should be removed e.g., capsule covering the kidney.
- All tissues sampled shall be retained in a suitable fixative as soon as possible.
- For identification of preserved tissues, the ear tag, used as animal marking, should be retained in the fixative jar.
- On completion of the necropsy, the remains of the carcass, together with any organs and tissues not required, are not discarded until jar/tray checks have been completed.
- Carcass remains shall be placed in an opaque bag with the label “carcass for disposal.” For carcinogencity study, it is recommended to retain carcass remains along with protocol tissues in the wet archive.
- Any deviation and subsequent corrective action during the necropsy are notified immediately to the study director/investigator who has to document this in the study file.

31.5.4 Recording of Gross Pathology Findings

- Correct description of the gross observations is the most crucial part of pathological evaluations of a study/experiment.
- Detailed recording of gross pathology observations helps the veterinary pathologist during slide examination for histopathology.
- The use of general terms that are used to refer to food or household objects must be avoided as it could be misleading [16, 17].

- Subjective descriptions also should be avoided. For example, a "large" for one observer might be normal for another. The word "common" is not a synonym for "normal." Weighing or measuring objects will be more insightful.
- While recording any gross abnormality/finding, the specific criteria such as mentioning the anatomic location of lesion on organ, number, distribution, color, size, shape, consistency, and texture shall be used wherever applicable. Severity grade (minimal, mild, moderate, marked, and severe) and spread (focal/multifocal) of particular lesion shall be mentioned. A lexicon provided in appendix may be used as a guide.
- **Location:** Describe the anatomic site and its relationship to other organs and tissues, e.g., dorsal, ventral, caudal, cranial, the right side of the stomach, left kidney, etc.
- **Color:** Description of color should be by primary colors. A combination of colors, shades, and degrees may also be used to describe in between changes like pale-yellow, mottled liver, stippled, the streak etc. However, colors referring to objects or fruits, etc. shall be avoided, For example, all oranges are not orange in color.
- **Size and number:** Size should be measured in a metric unit (mm or cm) and three dimensions. The use of names of fruits and vegetables to indicate size should be avoided. Masses or nodules can be counted one to five and later "more than five" [18].
- **Shape and symmetry:** Some of the words to describe the shape and symmetry include discoid, oval, flat, round, conical, nodular, lobular, bulbous, tortuous, punctate, fusiform, laminated, clustered, lace-like, wedge-shaped, straight-edged, etc.
- **Consistency and texture:** Expression of consistency and texture of the organs after palpation of the organs and cutting them open includes terms such as hard, firm, or soft, as well as inspissated, turgid, gelatinous, mucoid, fluctuant, crepitant, brittle, gritty, granular, friable, pliable, viscous, stringy, adhesive, dry, caseous, homogenous, gas-filled, etc.

Physiological changes such as gastrointestinal congestion or postmortem hypostasis in the lung and liver may be recorded as antemortem changes. Lesions may also be caused at death, such as those due to CO_2 exposure, damage due to scissors, etc. It is more often the case that these observations may be artifacts or agonal changes (i.e., caused at death, either physically or chemically). Such changes should also be recorded with a proper description of the artifact. The photograph of the finding with a label and metric scale shall be taken at the discretion of the in-charge, necropsy, and/or study investigator for future reference. Wherever possible, photographs shall be taken in comparison with that of controls and using a scale.

31.6 Dissection Procedure for Rodents/Lagomorphs/Guinea Pigs/Hamsters

- The necropsy routine is not a precise guide to the dissection sequence. The precise order of the necropsy may vary due to study-related requirements or sometimes due to individual variation.
- Autolysis begins as soon as cadaveric rigidity is over; hence the necropsy must be carried out as soon as possible after death/euthanasia.
- It is preferred that a uniform system of dissection shall be followed, either starting from abdominal organs towards the brain or vice versa.
- Once the animal is sedated/euthanized as described earlier, ensure the cessation of respiration and heartbeat before cutting open. Place the animal dorsally on the necropsy table, so that the ventral surface of the animal is facing the dissector.
- Lift the skin at the pelvic region, give a small cut, and take a midline incision from the pelvic region to the approximate start of the first rib. Follow the cut patterns as in Figs. 31.3 and 31.4. Alternatively, one may initiate the cut at thigh region, severe the blood vessels carefully without damaging muscles and nerves (Fig. 31.5) and then proceed with midline incision as explained earlier.
- The ventral skin shall be reflected to allow observations of the subcutaneous tissues and related structures. Any abnormalities found are recorded (Fig. 31.6).

31.6.1 Examination of Glands (Salivary, Lacrimal, Clitoral, or Preputial) and SubMandibular Lymph Node

- After severing the spinal cord, the skin from the ventral neck is removed quickly to expose the major blood vessels and salivary glands with a submandibular lymph node. These glands and lymph nodes should be removed, before severing blood vessles for exsanguination.
- Salivary glands—mandibular, sublingual, and parotid—are paired, found tightly adhered to the muscles, and have a tough covering (Fig. 31.7).
- With the help of forceps, gently grasp the tip of the salivary gland closest to the thorax, cut the surrounding tissues with scissors, and slowly pull outward.
- Alternatively, these glands can be removed by blunt dissection. The closed tips of scissors are inserted into the tissue and then opened to split tissue. Once

Fig. 31.3 Initial incision

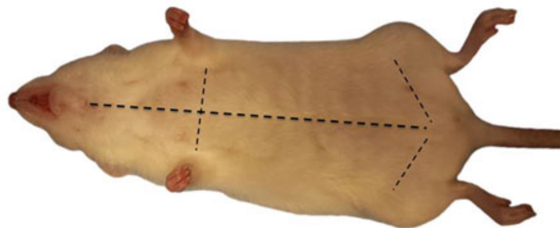


Fig. 31.4 Ventral midline incision

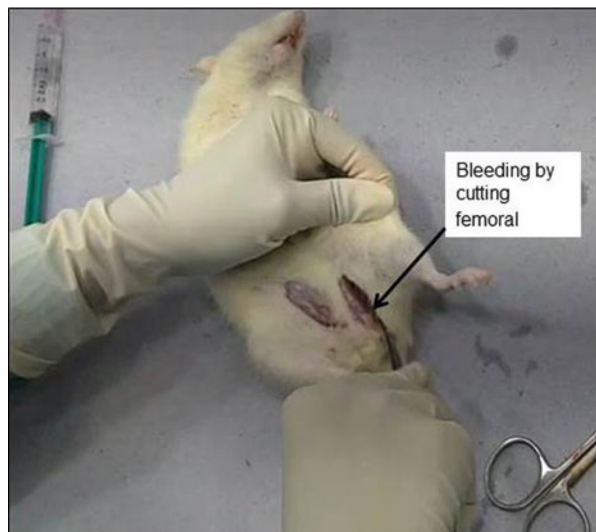


Fig. 31.5 Exsanguination by severing femoral/Saphenous vessels

underlying muscles are cleared, severe residual attachments are severed and lifted. At least one nick is given to the glands to allow penetration of the fixative.

- Extraorbital lacrimal glands are flat, brown-gray, placed next to the parotids, on the ventrolateral side of the head. Extraorbital glands are removed from either side by grasping the tip of the gland with forceps, the gland is isolated from the eye socket using scissors, and both glands are immersed in fixative.

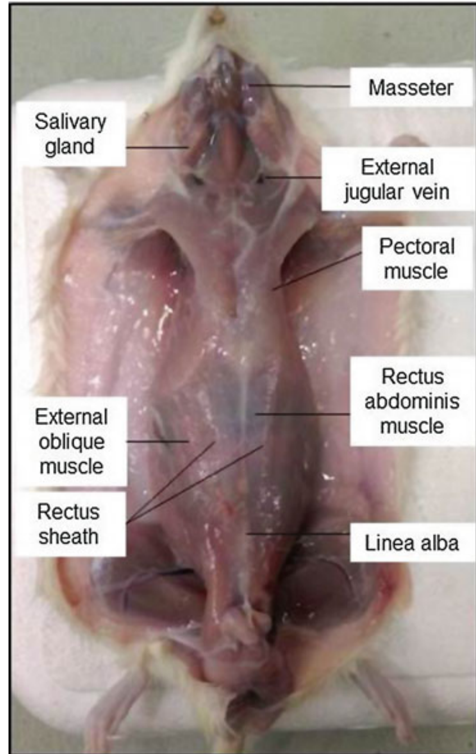


Fig. 31.6 Subcutaneous observation

- The preputial gland located bilaterally cranial to the penis is a dark gray colored, leaf-shaped specialized sebaceous glands in males. The clitoral gland is the females' equivalent of preputial situated lateral to the clitoris near the inguinal mammary glands. The glands are isolated from the surrounding tissue by grasping the tip gently with forceps.
- The mandibular lymph nodes are grasped carefully and dissected (Fig. 31.7).

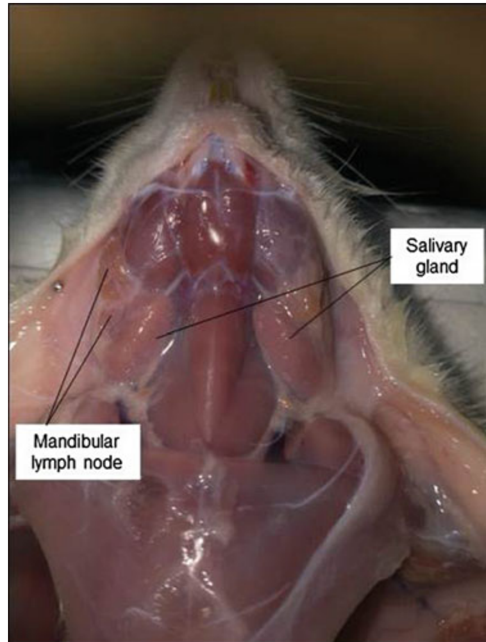
31.6.1.1 Superficial Lymph Nodes

- Superficial lymph nodes, namely, popliteal, superficial cervical, brachial, axillary, and inguinal, are bilateral, grayish, and found subcutaneously.
- The mandibular, axillary, and/or popliteal lymph nodes are the most often examined peripheral lymph nodes.
- Nodes are grasped gently at the base and dissected out.

31.6.1.2 Head

- The brain autolyzes quickly after death and shall be taken out as early as possible. To remove the eyes, the skin from the nape to the snout is cut through a median longitudinal line. The edges of the skin are pulled outward so that the eyes

Fig. 31.7 Salivary glands and lymph



protrude from the orbit. The attachments are cut off to isolate the globes and harderian glands.

- The optic nerves are removed along with the eye. After removing muscles and periosteum from the occipital region, one blade of the scissor is introduced into the foramen magnum. Keeping scissor tips pointed upwards, the skull is through the midline. The roof of the skull is grabbed and flipped with forceps.
- While dissecting, the integrity of the dura is critical to protect the brain from injury. The cranial cap is lifted, exposing the brain. The skull is inverted in a nose-up direction, and the forceps are inserted carefully starting below the olfactory lobes moving beneath the cerebrum till the cerebellum.
- The cranial nerves and any connective tissue that prevents the brain from falling are gently cut from the skull.
- The brain will fall from the skull by gravity after carefully inverting. The pituitary gland shall be fixed and retained in situ with the skull bone unless required for weighing.
- If the weight of the pituitary gland is required, partial fixation is preferred before removing the gland.
- Where a pituitary mass is present, an attempt to detach may result in rupture of the capsule and consequent disintegration. Hence, the pituitary gland shall be fixed in situ.
- After removing these organs, the skull with or without pituitary shall be retained in situ for nasal tissue.

- If the nasal cavity is removed at this point, a lower jaw along with the tongue is cut from the head and the head portion is cut from the carcass.
- Fixative is flushed through the nasal cavity till it comes out from nasopharynx nares.

31.6.1.3 Hind Limbs

- To collect the femur, a cut is made through the skin of the hind limb, the muscles are cleared as much as possible and then cut below the knee joint and above the hip.
- The femur should be transferred to a pre-labeled container containing phosphate buffer saline. If the impression smears of bone marrow are required, a dedicated staff shall critically take quick impression smears from one femur as bone marrow cell morphology deteriorates rapidly. If a femur is being collected for genetic toxicity study, containers with femurs shall be handed over to the responsible person.
- Sciatic nerve shall be collected along with the skeletal muscle dissected from one hind limb; the other hind limb shall be retained whole.
- The skeletal muscle sample shall be taken from the quadriceps group, but for the hind limb intramuscular injection studies, the muscle sample shall be taken from the triceps muscle.
- The femur with joints and tibia intact from one hind limb is removed and retained.

31.6.1.4 Mammary Glands and Skin

31.6.1.4.1 Rodents

- The rats have a total of six pairs of mammary glands (thoracic and abdominal three each) while the mouse has five pairs (three thoracic and two abdominal) (Fig. 31.8).

  The caudal mammary gland with skin is sampled from the inguinal region.
- Representative samples of rodent mammary glands are sampled from the caudal/inguinal region where the mammary tissue is in ample amounts.
- A transverse section of approx. 1 × 3 cm including the nipple is cut with the help of scissors.
- The section is placed in a fixative along with a section of the skin.

31.6.1.5 Parenteral Sites

- In dermal studies, the test items are applied topically onto the skin surface. The test site shall be retained flat, keeping the hair side up.
- In intramuscular studies, a representative sample of injection sites including adjacent skin shall be retained.
- In subcutaneous studies, the test items are injected under the skin between subcutis and muscle. A representative sample of parenteral site skin and underlying musculature shall be retained flat, keeping the hair side down.
- In intravenous studies, the test items are generally injected via the lateral tail veins in rodents. The tail shall be slit at 1 cm intervals to allow macroscopic

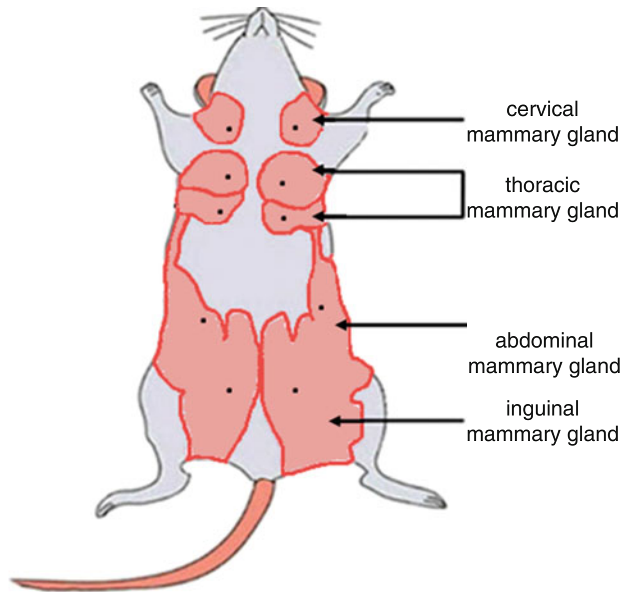


Fig. 31.8 Mouse mammary gland

examination and fixative penetration and retained in three roughly equal segments.
- In the case of rabbits, the test items are generally injected via the marginal ear veins. Hence, the pinnae should be retained whole and together to aid left and right identification.

31.6.1.6 Abdominal Cavity (Figs. 31.9 and 31.10)

- The tips of the scissors are held pointed upwards.
- Using forceps, the abdominal skin is lifted at the inguinal region and cut through it using scissors without damaging underlying organs.
- A small cut is made near the pubis and slit in a cephalic direction, and the skin is loosened from the underlying connective tissue.
- The skin is dissected along the median line longitudinally, from the pubic to the lower jaw.
- The flaps are pinned to the tray/necropsy board if needed.
- The abdominal wall is gently lifted with forceps at the pubis and cut through the pubic to the bottom of the rib cage, avoiding damage to the underlying organs. The abdominal viscera are exposed and observed in situ.
- The diaphragm, which is a thin sheet of muscle that separates the thoracic and abdominal cavity is visible.
- The soft, saclike stomach can be located beneath the liver.
- With scissors, a cut is made at each sphincter to remove the stomach.

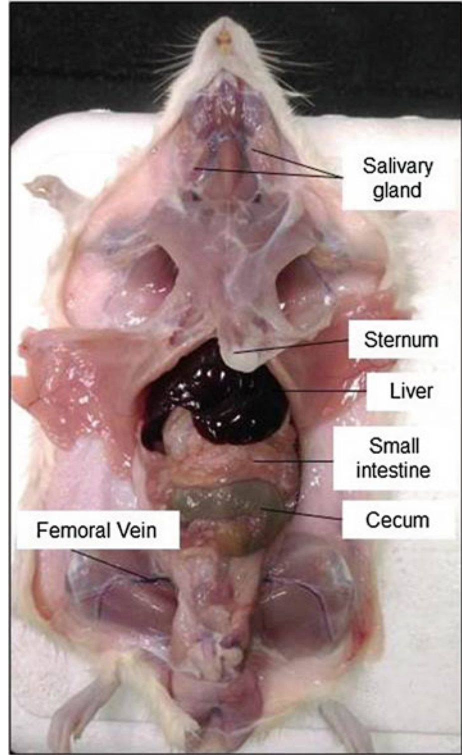


Fig. 31.9 Abdominal cavity

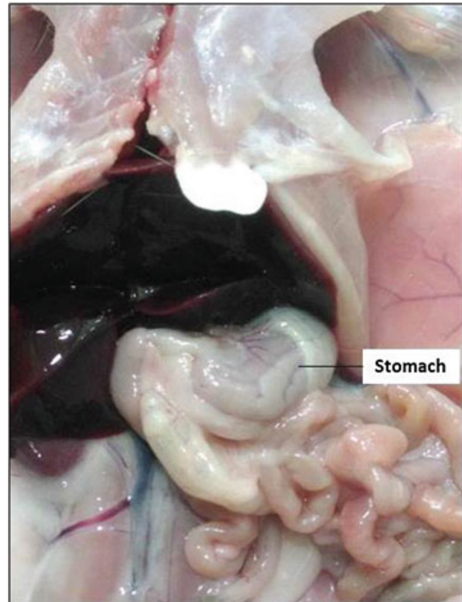


Fig. 31.10 Stomach

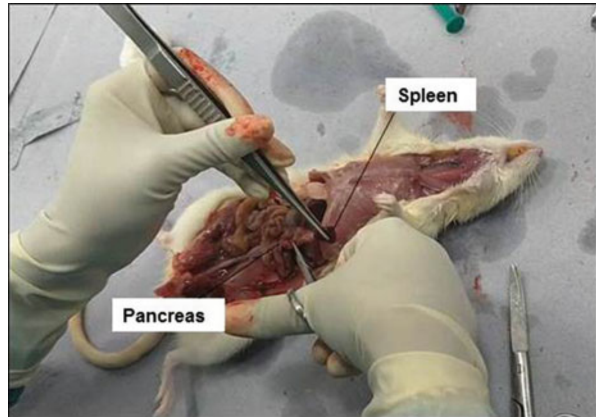


Fig. 31.11 Spleen and pancreas

- Using scissors, a cut is made through greater curvature to open the stomach. The contents are flushed out, and the texture of its inner walls is noted.
- The small intestine is observed. The U-shaped duodenum is the first part of the small intestine, starting from the lower end of the stomach. The small intestine is held in place by the mesentery.
- The mesentery is cut through to release the small intestine. The intestines are handled gently to prevent the destruction of the villi. If necessary, the small intestine is cut open, the contents are gently flushed, and the mucosal surface is examined using a magnoscope to examine the villi.
- The small intestine opens in the large intestine which wider and leads into the rectum. The large intestine is carefully maneuvered to uncoil it (Fig. 31.12).
- Alternatively, all digestive systems can be removed and placed on the table to observe all the parts.
- The reddish to brownish-white or tan-colored pancreas is a flattened gland visible beneath the stomach and between the duodenal loops (Fig. 31.11). Unlike most other mammals, the rat has no gall bladder.
- The spleen can be found wrapped around the stomach is elongated, oval, slightly curved, and dark red in color. It is soft in consistency and covered with a thin transparent capsule (Fig. 31.11).
- The mesenteric lymph nodes are located in the mesentery alongside the colon, immediately adjacent to the cecum. They are yellow and ovoid to spherical nodules with a slightly thick and firm texture compare to the surrounding fat and mesentery.
- The mesenteric lymph nodes are removed first, followed by the gastrointestinal tract.
- Samples of the gastrointestinal tract are rinsed with 0.9% saline to remove contents before immersion in fixative. Wherever necessary, the fecal matter present inside shall be gently squeezed with the flattened surface of the scissors and removed. Alternatively, fixative may be injected in multiple sites of intestine which may preserve the integrity of mucosal surface much better (Fig. 31.12).

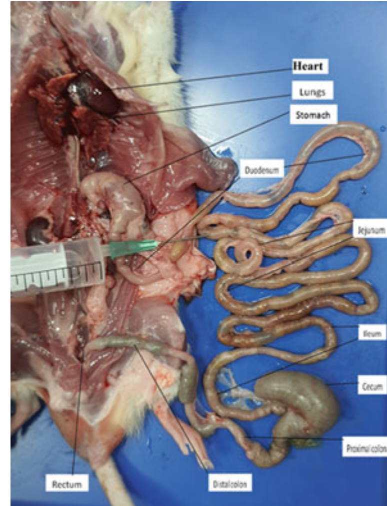


Fig. 31.12 Uncoiling of intestine and fixative infusion

- The stomach is detached together with the esophagus and duodenum and opened along its greater curvature, rinsed in normal saline to remove contents. The mucosal surface of both glandular and non glandular portion is examined.
- The stomach is carefully stapled flat onto suitable material (filter paper, premoistened with formalin) with a portion of the duodenum and esophagus on either side.
- The cecum is opened two-thirds along its greater curvature from the ampulla and sampled with the portion of the ileum and colon.
- A midsection of the jejunum is removed, and a sample of the rectum is taken along with the anus.
- Peyer's patches which are the organized gut-associated lymphoid tissues (GALT) are present as small nodules on the non-mesenteric surface of the jejunum and ileum in the rat [3].
- To collect the spleen, the connective tissue is held and cut through the gastrosplenic ligament and hilum. While removing the spleen, the pancreas that is attached is also removed easily.
- To collect the liver, the diaphragm is gently grasped along with the falciform and coronary ligaments and pulled away from the liver, with the help of small cuts.
- The liver is lifted with index and middle fingers, and all its attachments to the intestines and stomach are cut off. All the lobes of the liver are removed as a whole. The excess blood is removed by blotting.
- In rats, the caudate and posterior-lateral lobes are retained whole, and transverse sections from the median, right anterior, and left lobe are taken, after weighing while fixation.
- In mice, all the liver lobes are retained, and the median lobe is retained with the gall bladder in situ.
- In rabbits, liver sections approximately 10 mm from the left and median lobes are retained.

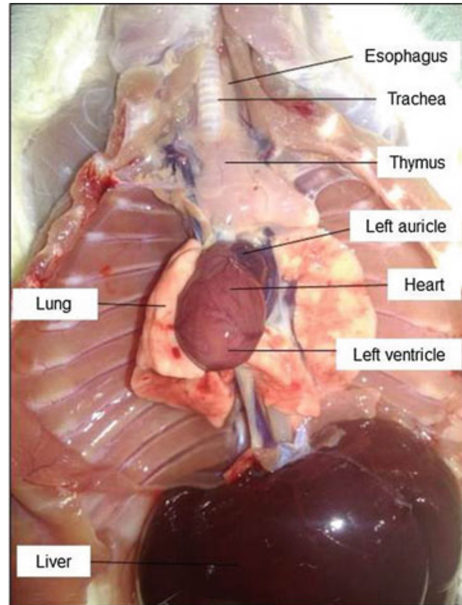


Fig. 31.13 Neck and thoracic cavity

31.6.1.7 Neck and Thoracic Cavity

- The structures of the neck and thoracic cavity are exposed by removing the skin and muscle overlying the ventral thoracic and cervical regions (Fig. 31.13).
- The xiphoid cartilage is grasped and lifted, and two lateral cuts are made on each side of the ribcage and one cut across near the clavicle to take out the sternum. The thoracic cavity is opened wide enough to expose the heart and lungs.
- The muscles at the ventral neck including those overlying the trachea are removed.
- Parathyroids embedded in thyroids is located caudal to the larynx. Parathyroids are not visible grossly.
- Thyroids with parathyroids are to be removed during necropsy only if needed for weighing.
- The thymus, a bilobed whitish translucent organ, lies above the heart, shrunken in aged animals compared to younger ones. The thymus is lifted with forceps and cut carefully without damaging any blood vessels below.
- The forceps are placed perpendicular to the trachea, and the trachea is grasped near the jaw with forceps and cut completely through the trachea with scissors.
- The esophagus and trachea are severed by this cut.
- Without loosening the grip on the trachea, it is lifted and snipped off attachments if any. Alternatively, the trachea is pulled upwards, snipping underlying tissue with scissors until the entire set, i.e., trachea (with larynx, esophagus, aorta, and thyroids), lungs, and heart (“pluck”) have been removed.
- The esophagus is retained along with the trachea/lungs.

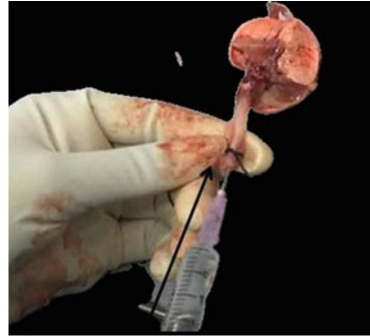


Fig. 31.14 Fixative infusion in lung

- The heart is removed. The heart shall be emptied of blood, appropriately.
- The lungs are placed flat in the tray.
- A piece of suture/thread material is tied loosely around the trachea.
- Approximately 3 or 5 ml syringe is filled with a fixative.
- A blunt needle (26/18-gauge needle) is inserted into the opening of the trachea.
- The fixative is infused slowly inside the lungs until it fully inflates. Underinflation/overinflation is to be avoided (Fig. 31.14).
- The amount of fixative needed to fully inflate the lungs varies with age, strain, and health of the animal.
- Seeping of fluid or foaming will indicate overinflation.
- Flat appearance of lungs or found unfilled in areas will indicate underinflation.
- Due care shall be taken not to puncture the lung with a needle.
- The thread is tightened around the trachea to prevent the backflow of fixative from the lungs. If the lungs are to be weighed, the esophagus shall be removed before weighing. The lungs are weighed, lightly inflated with fixative as above.

31.6.1.8 UroGenital System

- Once the GI tract is removed, the bean-shaped kidneys can be located lying against the dorsal wall of the abdomen.
- The kidneys are removed using forceps by isolating and cutting between the kidney and the ureter. For studies requiring individual identification of the left and right kidney, a transverse section of the right kidney shall be made unilaterally (Fig. 31.15).
- The adrenal glands are small and light-pink nodules at the anterior of each kidney and can be removed by grasping the adnexa beneath (Fig. 31.16).
- The ureters are very small tubes visible connecting the kidneys to the urinary bladder.
- If the ureters are prescribed organ for collection, they are collected before the collection of kidneys by grasping the inferior end of the ureter with forceps and lifting upwards.

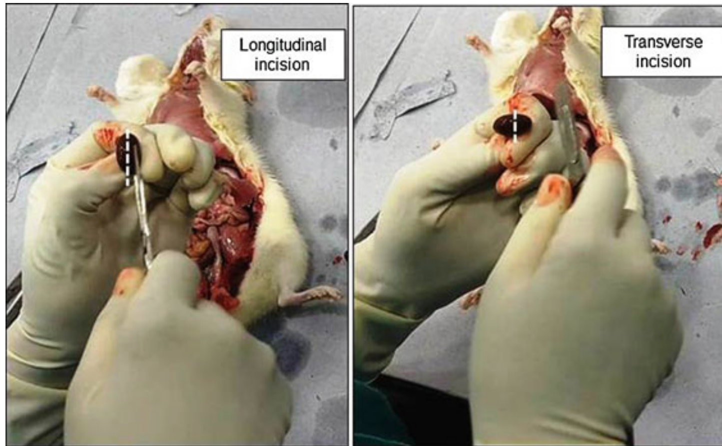


Fig. 31.15 Incision of kidney

- The urinary bladder is examined and inflated with a small amount of fixatives. It is removed by grasping with forceps at the highest point of the bladder and cut at the level of the neck of the bladder (Fig. 31.17).

31.6.1.9 Female Reproductive Organs (Fig. 31.18)

- The uterus is a Y-shaped, pale pink bicornuate organ seen just below the bladder. It has two cornua (horns) on the left and right side, each having its cervix and uniting caudally.
- The uterine horns run cranially and terminate at the oviducts.
- Horns run caudally through the cervix into the vagina, a muscular tube that extends till the vulva which forms an external opening of the body.

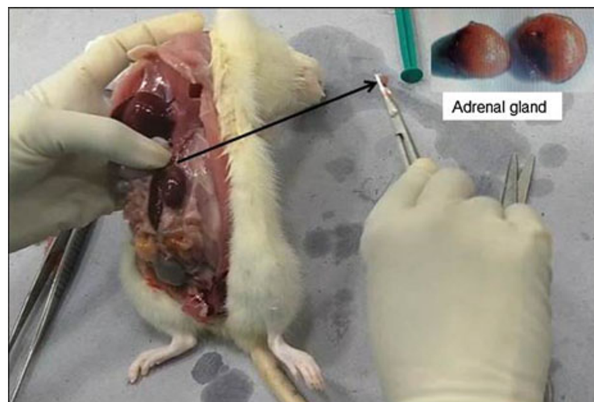


Fig. 31.16 Kidney and adrenals

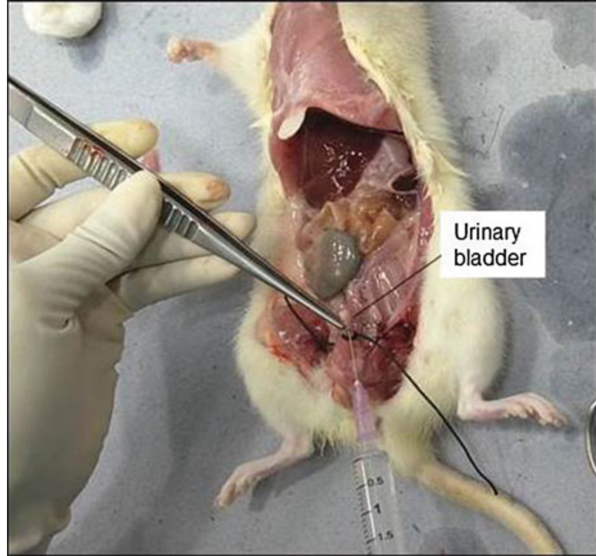


Fig. 31.17 Fixative infusion in urinary blader

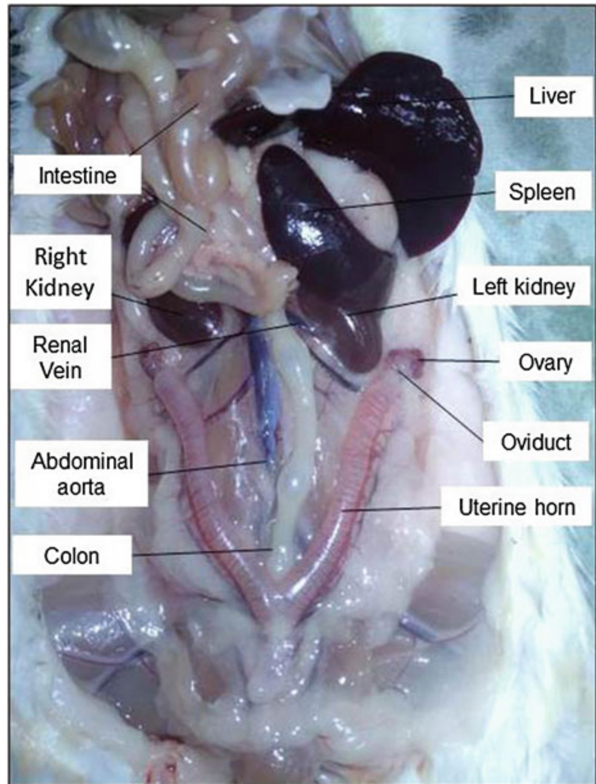


Fig. 31.18 Female urogenital organs

- The ovaries are oval, grape cluster-like structures at the distal end of the uterine horn after the oviduct.
- The vagina and vulva are grasped and lifted upwards and the attachments with skin are removed. While cutting traction is applied and pulled upward and removed along with the uterus and ovary.
- If a uterus is not to be weighed, then the vagina shall be collected with the uterus and cervix. The collection of the urinary bladder is not to be missed.

31.6.1.9.1 Male Reproductive Organ (Fig. 31.19)

- Testes are oval-shaped paired organs with a smooth surface and placed in the scrotal sacs. The scrotal sac is incised and opened. Otherwise, the testes are pulled out from the scrotum by grasping and pulling the fat pads from the lower abdomen with forceps. For removing the testes, the vas deferens are cut followed by the removal of tissue connections from the scrotum.
- The epididymis is found attached to the testis dorsally along the lower margin. The epididymis is found as three distinct parts, i.e., caput (head) at top of the testis, corpus (body) running along with the testis, and cauda (tail) at bottom of the testis.

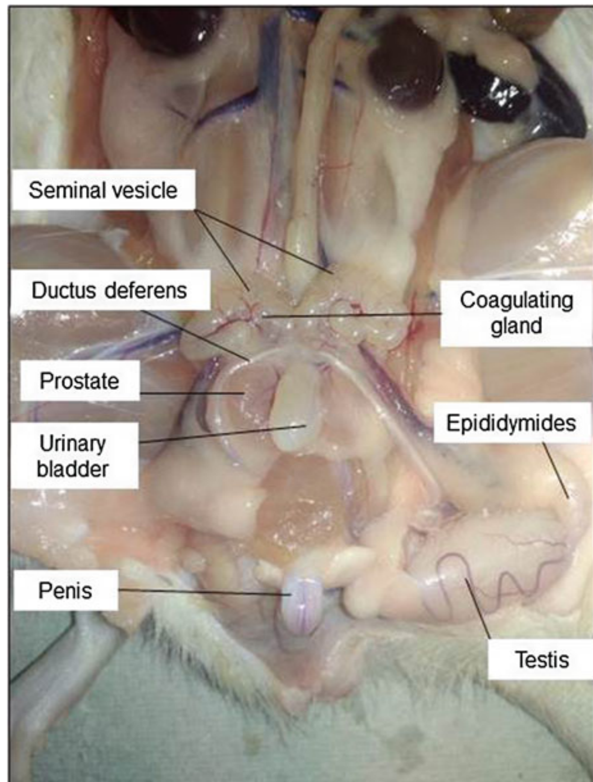


Fig. 31.19 Male urogenital organs

- The seminal vesicles along with coagulating glands are paired glands, whitish to creamy colored, positioned anteriorly to the urinary bladder and attached to the prostate gland at the midline.
- The prostate is the largest accessory gland is light tan-colored and located below the urinary bladder. It is composed of four lobes. The dorsal and ventral lobes are covered by seminal vesicles.
- To remove the prostate and seminal vesicles, the base of the urinary bladder is lifted and cut beneath the prostate without pricking any gland.
- The adventitious tissues attached to the testes, epididymis, prostate, and seminal vesicles are removed before weighing.

31.6.1.10 Spinal Column

- The remnants of the ribs are cut parallel to and close to the spinal column on both sides.
- Muscle, fat, and other soft tissues are cut from the spinal column using curved scissors.
- The spinal column is separated from the carcass by making a transverse cut at the level of the femurs.
- Once cleared of soft tissues, the column can be cut into three pieces, i.e., the cervical, thoracic, and lumbar regions.
- The aorta can be collected along with the spinal cord.

The necropsy procedure for the moribund animal shall be the same as described above..

The necropsy procedure for the dead animal shall be the same as described above except that the organs should be collected and preserved depending upon the suitability.

31.7 Weighing of Tissue

- Prescribed organs shall be weighed using an appropriate calibrated weighing balance.
- It is recommended that each necropsy station/prosector is given individual balance so that the prosector weighs the tissues immediately after harvest and transfers the tissues in the fixative jar.
- Such stations can also be supported by online data capturing systems.
- In routine setup, tissue e-tray shall be transferred immediately to the person weighing the organs.
- Organ weights shall not be recorded for animals killed or dying before terminal necropsy unless otherwise specified in the study plan/protocol. Organs that are found grossly abnormal shall not be weighed or if weighed, and appropriate comment shall be made.
- Each organ shall be “damp-dried” by placing on wet cotton, weighed, and recorded in the pathology report form or using a computer system.

- Weights of paired organs should be entered combined unless otherwise specified in the study plan/protocol.
- Prosectors should ensure that the fat or fascia attached to the organ is removed properly before weighing. The organ weight crossing the lower or higher limit shall be weighed twice to counter check for any error.
- After completion of the weighing of all organs of an animal, the pathology report form is signed by the person weighing the organ and then by the pathologist.

31.8 Organ-Specific Procedure

- Thyroids shall be partially fixed by moistening or immersion in NBF.
- Pituitary weights may also be recorded after partial fixation similarly to the thyroid.
- Prostates shall be weighed attached to the urethra trimmed to the limit of the glands.
- The liver is weighed after removal of the gall bladder in mice and rabbits.
- Lung weights may include approximately six tracheal rings above the bifurcation.
- Occasionally due to abnormality, individual organ weight may be deemed unsuitable for use in subsequent calculations, e.g., mass/node/abscess/bigger cyst in the liver or kidney. Therefore, the nature and incidence of gross findings shall be considered in conjunction with requests for subsequent statistical comparisons and the interpretation of such comparisons.
- Immediately after completion of organ weighing, the logbook of the balance should be closed with a statement providing a summary of the day's proceedings on organ weights and duly signed and dated.

31.9 Retention of Tissues/Sample

- At the end of the animal study after successful necropsy, what remains are the accumulated records and specimens.
- The results from these animal studies and diagnostic necropsies depend on the observations and the specimens collected. The precise sampling procedure is an essential aspect of effective necropsy [19].
- As mentioned earlier, during necropsy samples are collected not only for histology but also for electron microscopy, microbiology, molecular biology, and chemical studies.
- Literature should be referred or laboratories should be consulted for particular techniques for the collection and transport of such specimens.

31.10 Sample for Histological Examinations

31.10.1 Fixation

- After harvesting all required tissues and after weighing as applicable, organs should be transferred immediately into a pre-labeled container containing 10% neutral buffered formalin as a fixative (except the eyes and testes).
- While tissues are transferred in fixative jars, a necropsy introduction sheet should be filled indicating the tissues that are collected.
- The eyes of rodents and lagomorphs are initially fixed for 18–24 h and 36–48 h, respectively. Post-fixation eyes should be washed for 5 min in tap water and transferred into 10% NBF.
- Testes shall be retained in tissue cassettes with necropsy number and transferred to a pre-labeled common jar containing MDF for 48 h, then washed for 5 min in tap water, and transferred to 10% NBF. Where appropriate, the testes and epididymis of rodent and lagomorphs' studies can be retained in Bouin's solution for 12–36 h and 36–48 h, respectively, then washed for 5 min in tap water and transferred to 10% NBF.
- After completing fixation for the eyes, testes, and epididymis as applicable the relevant section on the necropsy introduction form shall be signed off.
- Other fixatives and methods of fixation shall be used as mentioned in the study plan/protocol and or in any amendments. A minimum of 48 h of fixation is recommended for tissues fixed in 10% NBF.

31.10.2 Distribution of Tissues in Histology Pots/Cassette/ Containers and Labeling

- Pots/cassette/containers shall be of sufficient size to allow adequate fixation of the tissues and organs placed in them. It is recommended that the volume ratio of fixative should be at least 10 times greater than the tissue.
- The number and size of pots/cassette/containers used shall depend upon the size, type, and the number of tissues to be retained (Fig. 31.20).
- To improve the identification of small organs and abnormalities and to allow the use of different fixatives and to obviate loss, some organs are fixed in appropriately sized containers/vials/cassettes and they are then retained in the main pot(s). For example, small vials/tubes/tissue cassettes are used to retain small organs, viz., adrenals, eyes, Harderian glands, lachrymal glands, ovaries, sciatic nerves (on absorbent paper), and if separate, mandibular L/N, optic nerves, pituitary, thyroids and parathyroids, and urinary bladder. All these small vials/capsules shall be transferred to the same container containing other organs of the same animal.
- Small palpable masses (roughly $\leq$2–3 cm dia.) shall be removed completely and labeled with a piece of a card stating mass identification number/letter. Large masses (roughly $\geq$3 cm dia.) shall be sliced, and one or more samples of tissue

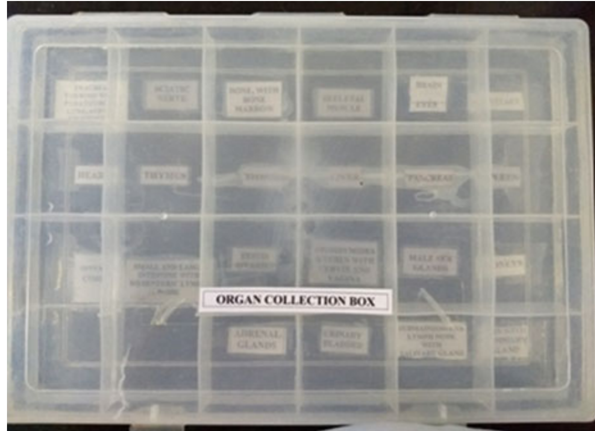


Fig. 31.20 Organ collection box

including skin as applicable may be sampled and labeled with an identification number.

- Small or soft masses unsuitable for labeling with a piece of the card shall be fixed in appropriately sized and labeled containers. Labels shall include the mass number/letter.
- Samples of rodent caudal mammary tissue and skin are placed on absorbent paper. If cranial mammary samples are retained, they shall be placed onto the labeled card.
- Rodent thyroids attached to a section of the trachea are retained with the tongue and larynx if the thyroid is not being weighed. Rodent thyroids if weighed are retained in an appropriately sized tube.
- Rodent peripheral nerves, if removed, are placed onto absorbent paper and retained in a tube. Peripheral nerves from all other animals are stapled onto the card and fixed in the main pot.
- Turbinate is required for retention on inhalation and oncology studies. The turbinate is back-flushed with NBF before immersion in NBF. The turbinate should be inverted and NBF gently infused through the nasopharyngeal opening in the roof of the mouth. NBF is passed through the turbinates and taken out through the nares when the procedure is performed correctly.
- Other abnormalities, in addition to those noted in organs and tissues already collected and retained, are also retained in separate tubes with an appropriate label.
- To assist in the identification of specimens, the tattoo mark, ear tag, or identity disk are retained in fixative with the tissues. All remaining tissue for mice and the remainder of the gastrointestinal tract for rats and hamsters shall be retained in the main jar.
- The jar/tray check procedure is a very important quality check and must be performed carefully, usually by a second person. The check procedures ensure correct labeling on all tubes and main retention jar(s). During or at the end of the necropsy, each tissue should be checked for correct identification. Each tissue is

removed from the tray and transferred to the main pot (or appropriate tube within the main pot).

- Possibly macroscopic comments shall also be ensured at the pot check. Errors if any shall be corrected immediately.
- A ball of cotton wool shall be placed on top of the fixative to retain the floating organs like lungs, skin, etc. immersed in fixative, and the container shall be closed tightly with its cap.

31.10.3 Sample Collection for Parasitological Examination

Due to specific predilection sites, sampling for ectoparasiticides should be done from different areas including eyelids, ears, and nasal areas [20].

- For identification of endoparasites, larvae, and eggs, samples from intestinal contents or feces should be collected during the necropsy and examined as wet preparation.
- Fresh samples stored in clean, leak-proof containers are always preferable.
- Samples for the examination of protozoan oocysts, helminth eggs, and adult helminths can be refrigerated [20–23].

31.10.4 Sample Collection for Microbiology

An active phase of infection may be observed by careful examination of animal specimens during routine health monitoring studies. Bacterial culture is the most important tool in the diagnosis of bacterial infections. Ample literature is available that describes general techniques and sampling procedures for bacteriological cultures from rodents [24–28]. However, a few salient features must be kept in mind by prosectors:

- To avoid accidental contamination, samples for bacterial, fungal, and viral culture and serology are collected before the organs are handled during post mortem.
- An inappropriate sampling procedure may result in the overgrowth of the causative agent.
- Sampling shall be done as early as possible after the death of animals because of the decrease in the viability of pathogenic bacteria and mycoplasma in tissues.
- Sterile instruments or swabs should be used in an aseptic environment to collect tissue specimens for bacterial culture. Swabs should remain in the transport medium until they reach the microbiology laboratory. The samples from the gut or intestine should be collected last.
- The surface of the thick capsuled organs as well as of abscesses and pustules with a sterile scalpel should be disinfected before the content is swabbed from the inside of the capsule.
- Body fluids, such as urine or blood, are sampled either by swabbing or aspiration with a sterile needle and syringe.

31.10.5 Sampling for Serology, Molecular Techniques, etc.

Serology is a commonly employed tool for diagnosis and in health monitoring programs [29, 30].

- Serological blood samples can be aspirated from live animals or immediately after sacrifice with a syringe or a Pasteur pipette from the heart or by severing the blood vessels from the thorax or the axillary/inguinal areas [31].
- Cross-contamination of specimens with nucleic acid during sampling and specimen preparation will lead to serious errors and must be avoided.
- Before sampling, the instruments are sterilized by heat or disinfectant to destroy nucleic acid remnants. Sterile tubes or containers with specimens are stored immediately at −80 °C [32].

31.11 Dispatch of Samples to Histology Laboratory

- The number of pots of tissue is counted before dispatch to histology.
- Pots are transported in secured boxes whenever the containers are leaving the site.
- An accidental drop of a single jar not only will cause spillage of noxious formalin but possibly loss of small tissues.
- The pots of wet tissues are dispatched to histology along with a copy of the requisition for pathology form and a completely filled-in necropsy introduction form. The individual pathology report should be sent within a week of necropsy.
- The necropsy introduction form(s) is signed by the member of staff after the transfer of tissues and or changing fixative(s) and for dispatch of tissues to histology.

31.12 Post-Necropsy

31.12.1 Personal Hygiene

- After the necropsy, the hands are washed thoroughly.
- The protective clothes and gloves are taken off before leaving the necropsy laboratory. If possible, taking a shower/bath before leaving lab premices is highly recommended.
- Contaminated disposable items, such as gloves, etc., should be transported in leak-proof containers for sterilization before destruction.

31.12.2 Necropsy Tools

- After use, necropsy tools shall be rinsed in hot water thoroughly.
- Tools are to be sterilized using disinfectant [phenolic/water 1:10, (v/v)].

- After sterilization, the tools are cleaned with a dry clean cotton cloth and allowed to air dry.
- Used scalpel blades and needles, etc. should be kept in appropriate “sharps” containers for decontamination before destruction as per local biosafety regulations [9, 33].

31.12.3 Necropsy Room

- After completion of necropsy, the necropsy station should be cleaned with tap water.
- The room floor is to be cleaned with water and then with a soap solution and finally mopped with an approved disinfectant solution.

31.12.4 Disposal of Carcass Waste

- After completion of necropsy, carcass waste along with other disposal material if any shall be handed over for disposal as per local statutory laws.
- For disposal procedures and labeling of containers of biological waste, local regulations should be consulted.
- Remnants of carcasses and tissues may be autoclaved or incinerated as permissible.

31.13 Conclusion

A simple yet efficient necropsy technique will facilitate a meaningful evaluation of all organs and findings. Though the detailed SOP and study plan are the critical document, a quick guide with checklists helps to avoid errors. Proper planning, skills, training, and good documentation practice are key to good necropsy. Conducting necropsy is a highly skilled job and is very important that one undergoes training under an experienced pathologist. Even though dead animals are handled, delicate handling of tissues during dissection and sampling is an important aspect of necropsy. Although gross examinations, collection of lesions, and specified tissues are critical goals regardless of study design, the quality of necropsy is dependent on every phase of procedures. The quality of a study can be no better than the quality of necropsy. The outcome also depends on excellent coordination between the entire necropsy team—prosectors, technicians, investigators, and pathologists. Hence successful necropsy is a team effort. The success of the whole study hinges heavily on the gross examination, sample collections, and the subsequent results taken from these samples.

Acknowledgement The pathology team of CEFTE, SRIHER, and IIBAT are duly acknowledged for the assistance. I also acknowledge my mentors Dr. Monique Wells, Dr. Robert Maronpot, Dr. Ronald Herbert, and Ms. Natasha Clayton for honing me as a relevant pathology professional. Ms. S. Shanmugapriya and my daughter, Devanshi, are acknowledged for their support.

Appendix 1: Necropsy Lexicon

Localization		Findings		Distribution	Color/ appearance	Consistency	Size/number	Shape
Left lateral lobe	Entire body surface	Abdominal size	Failed to collapse	Anterior	Black-brown	Soft	Enlarged	Round
Right lateral lobe	Fur	Abnormal shape	Focus(i)	Posterior	Clay-colored	Firm	Small	Rounded edges
Median lobe	Skin	Abnormal content	Gas-filled	Dorsal	Gray-white	Hard (reserved for mineral and bone)	Pinpoint	Elongated
Caudate lobe	Head region	Abnormal consistency	Foreign body/ material	Ventral	Green	Flaky	Number (give number up to 5)	Oval
Right cranial lobe	Sub cutis	Abrasion(s)	Fracture/ broken	Internal	Liver-colored	Friable	if more than 5 use >5	Linear
Right middle lobe	Panniculus muscle	Absence of hair	Hair loss	External	Tan	Pasty	<1 mm	Tortuous
Right caudal lobe	Eye region	Abscess(es)	Hernia	Cephalic	Red	Clumped/ caseous	1—3 mm	Lobulated
Right accessory lobe	Eyelid	Accentuated lobular pattern	Hyperinflation	Cranial	Yellow	Gelatinous	3—5 mm	Pedunculated
Left lobe	Nose region	Adhesion(s)	Ingesta-filled	Caudal	Orange	Grainy/granular	0.5—1 cm	Irregular
Left cranial lobe	Nostril	Autolysis	Intussusception	Well demarcated	Blue	Gritty	1—3 cm	Nodular
Left middle lobe	Cheek region	Calculus(i)	Lactating	Poorly demarcated	Purple	Foamy	3—5 cm	Cavernous

(continued)

Localization		Findings		Distribution	Color/ appearance	Consistency	Size/number	Shape
Left caudal lobe	Flank	Callus	Mass(es)	Focal	White	Thick	>5 cm	
All lobes	Abdominal region	Cannibalized	Nodule(s)	Multifocal	Pale	Watery	<5 cm	
Visceral surface	Inguinal region	Concretions	Not present	Diffuse	Opaque	Brittle		
Parietal surface	Anal region	Contracted	Opacity	Center/ central	Cloudy	Clotted		
Phrenic surface (diaphragmatic)	Perianal region	Crust	Organ obscured by lesion	Periphery/ peripheral	Translucent	Crepitant		
Margin	Scrotum	Cyst	Parasite		Clear	Dry		
Entire surface	Vulva	Deformity	Perforation		Mottled	Moist		
Papillary process	Forelimb	Depressed area	Prolapse		Multi-colored	Smooth		
Cranial pole	Hind limb	Displacement	Raised area/ plaque		Dark	Rough		
Caudal pole	Tip of tail	Dilatation	Rupture			Mucous/mucoid		
Greater curvature	Base of tail	Discoloration	Scab			Viscous		
Lesser curvature	Entire tail	Discharge	Scar			Stringy		
Capsule	Mandibular region	Distended	Soiled			Rubbery		
Cortex	Ear region	Diverticulum	Sore			Shiny		
Medulla	External ear canal	Erosion	Torsion			Dull		
Papilla	Auricle	Emaciation	Thickened					

Cortico-medullary junction	Atrial appendage	Feces-filled	Undescended testis/testes					
Pelvis	Ventricle	Fissure(s)	Urine-filled					
Renal adipose tissue	Mucosa	Fistula	Vascularization					
Hilus	Serosa	Fluid						
	Lumen	Fluid-filled						

References

1. Everitt JI, Gross EA (2006) Euthanasia and necropsy. In: Suckow MA, Weisbroth SH, Franklin CL (eds) The laboratory rat, 2nd edn. Elsevier Academic Press, Burlington, MA. (Chap. 20)
2. Mihai G, Craig S (2012) The laboratory rabbit, Guinea Pig, Hamster, and other rodents. American College of Laboratory Animal Medicine. Academic Press, Boston, pp 117–139
3. Fiette L, Slaoui M (2011) Necropsy and sampling procedures in rodents. Drug Safety Evaluation: Methods and Protocols 691:39–67
4. Feinstein RE, Waggie KS (2010) In: Jann Hau, Steven J. (eds) Schapiro—Postmortem procedures in handbook of laboratory animal science. Essential principles and practices, vol. I. CRC Press, Boca Raton
5. Guidelines for the Veterinary Care of Laboratory Animals, Report of the FELASA/ECLAM/ESLAV Joint Working Group on Veterinary Care (2008) Lab Anim 42(1):1–11. https://doi.org/10.1258/la.2007.007027
6. Mann PC, Hardisty JF, Parker MD (2002) Managing pitfalls in toxicologic pathology. In: Haschek WM, Rousseaux CG, Wallig MA (eds) Handbook of toxicologic pathology (Chap. 9), 2nd edn. Academic Press
7. Bucci TJ (2002) The practice of toxicologic pathology: basic techniques. In: W. M. Haschek, C. G. Rousseaux, M. A. Wallig (eds.) Handbook of toxicologic pathology, vol 1, pp. 681–784
8. Relyea MJ, Miller J et al (2000) Necropsy methods for laboratory mice: Biological characterization of a new mutation. In: Sundberg JP, Boggess D (eds) Systematic approach to evaluation of mouse mutations. CRC Press, Boca Raton, FL
9. USDHHS (2007) Biosafety in microbiological and biomedical laboratories, 5th edn. ASM Press, Washington, DC. http://www.unh.edu/ehs/pdf/CDC-NIH-BMBL-5.pdf
10. WHO Laboratory Biosafety Manual (2004) 3rd ed. Geneva. This publication may also be at http://www.who.int/csr/resources/publications/biosafety/WHO_CDS_CSR_LYO_2004_11/en/print.html
11. Citino S (2000) Design considerations for necropsy facilities: a clinician's perspective. https://www.vin.com/apputil/content/defaultadv1.aspx?id=3980804&pid=11125&print=1
12. Bucci TJ (1991) Evaluation of altered morphology. In: Haschek WM, Rousseaux CG (eds) Handbook of toxicologic pathology, 1st edn. Academic Press, San Diego, CA, pp 23–36
13. OECD Series on Testing and Assessment No. 125, (2010) Guidance document on histopathology for inhalation studies. ENV/JM/MONO 16, http://www.oecd.org/officialdocuments/publicdisplaydocumentpdf/?cote=env/jm/mono(2010)16&doclanguage=en
14. Leary S, Underwood W et al (2020) AVMA guidelines for the euthanasia of animals. 2020th ed.. American Veterinary Medical Association, Schaumburg
15. Canadian Council on Animal Care (2020) CCAC guidelines on: euthanasia of animals used in science. Canadian Council on Animal Care, Ottawa. www.ccac.ca/Documents/Standards/Guidelines/Euthanasia.pdf
16. Parkinson CM, O'Brien A et al (2011) Diagnostic necropsy and selected tissue and sample collection in rats and mice. J Vis Exp 54:e2966. 1–7
17. King JM, Roth-Johnson L et al (2005) The necropsy book, 4th edn. Charles Louis Davis, D.V.M., Foundation, Gurnee, IL
18. Feldman DB, Seely JC (1988) Necropsy guide: rodents & the rabbit. CRC, Boca Raton
19. Noel RB (1982) Toxicity testing, hazard assessment, and data quality assurance in respect to use of laboratory animals. In: Bartosek I, Guaitani A, Pacei E (eds) Animals in toxicological research. Raven Press, New York
20. Flynn RJ (1973) Parasites of laboratory animals. Iowa State Univ. Press, Ames, Iowa
21. Lassen ED, Weiser G (2004) Laboratory technology for veterinary medicine. In: Thrall MA et al (eds) Veterinary hematology and clinical chemistry (Chap. 1). Wiley-Blackwell, Ames
22. Garcia LS, Arrowood M et al (2017) Laboratory diagnosis of parasites from the gastrointestinal tract. Clin Microbiol Rev 31(1):e00025–e00017. https://doi.org/10.1128/CMR.00025-1

23. Smith PH, Seklau EW et al (2007) Collection, preservation, and diagnostic methods. In: Baker DG (ed) Flynn's parasites of laboratory animals, 2nd edn. Blackwell Publishing, Ames, Iowa
24. Weisbroth SH (1979) Bacterial and mycotic diseases, vol 1. Academic Press, New York, pp 193–241. (The Laboratory Rat: Biology and Diseases)
25. Cassell GH, Davidson MK et al (1983) Recovery and identification of murine mycoplasmas. In: Tully JG, Razin S (eds) Methods in mycoplasmology. Diagnostic mycoplasmology, vol 2. Academic Press, New York, p 129
26. Kornerup HA (2000) Handbook of laboratory animal bacteriology (Chap. 2). CRC Press, Boca Raton, FL
27. Shek WR, Gaertner DJ (2002) Microbiological quality control for laboratory rodents and lagomorphs. Lab Anim Med:365–393. https://doi.org/10.1016/B978-012263951-7/50013-2
28. Songer JG, Post KW (2008) General principles of bacterial disease diagnosis. In: Songer JG, Post KW (eds) Veterinary microbiology: bacterial and fungal agents of animal disease. W.B. Saunders, St Louis, pp 10–20
29. National Research Council (US), Committee on Infectious Diseases of Mice and Rats (1991) Infectious diseases of mice and rats. National Academies Press (US), Washington, DC, p 4. Health Surveillance Programs. Available from: https://www.ncbi.nlm.nih.gov/books/NBK235145/
30. Nicklas W, Baneux P et al (2002) Recommendations for the health monitoring of rodent and rabbit colonies in breeding and experimental units. Lab Anim 36:20. http://www.felasa.eu
31. Hrapkiewicz K, Medina L (2007) Clinical laboratory animal medicine. An introduction, 3rd edn. Blackwell Publishing, Oxford, England
32. Compton SR, Riley LK (2001) Detection of infectious agents in laboratory rodents: traditional and molecular techniques. Comp Med 51:113
33. Members Of The Joint Working Group On Veterinary Care (2008) Guidelines for the Veterinary Care of Laboratory Animals: Report of the FELASA/ECLAM/ESLAV Joint Working Group on Veterinary Care. Lab Anim 42(1):1–11. https://doi.org/10.1258/la.2007.007027

Surgical Models of Laboratory Animals

32

R. K. Shakthi Devan

Abstract

Animal models play a pivotal role in biomedical research. Surgical rodent models are widely used in the preclinical drug discovery process. A well-planned surgery with aseptic techniques including intraoperative as well as postoperative care is necessitated for the successful surgical outcomes and survivability of animals. Consideration should be given for the pre-emptive and postoperative analgesia of choice with appropriate dosage regimen to prevent pain and distress in the animals. Furthermore, good management practices, minimal handling, socialization, and environmental enrichment provide comfort by reducing the stress in the animals that underwent surgery.

Keywords

Animal models · Aseptic techniques · Survival surgery · Laparotomy · Anesthesia · Analgesia · Postoperative care

Abbreviations

BUN	Blood urea nitrogen
CCA	Common carotid artery
CCI	Chronic constriction injury
ECA	External carotid artery
ICA	Internal carotid artery
PDS	Polydioxanone

R. K. S. Devan (✉)
Syngene International Limited, Bangalore, Karnataka, India

© The Author(s), under exclusive license to Springer Nature Singapore Pte Ltd. 2021

P. Nagarajan et al. (eds.), *Essentials of Laboratory Animal Science: Principles and Practices*, https://doi.org/10.1007/978-981-16-0987-9_32

PE	Polyethylene
PTFE	Polytetrafluoroethylene
PVDF	Polyvinylidene fluoride
VCC	Vena cava caudalis

32.1 Introduction

Laboratory animal models play a pivotal role in preclinical research to understand the pathophysiology and provides fundamental information on various therapeutic strategies [1]. Surgery is commonly performed in laboratory animals used in research, teaching, and experiments. Several aspects are to be considered before surgery in laboratory animals. Factors such as animal size, metabolic rate, and occurrence of hypothermia are crucial to the success of the procedure. The selection of suitable anesthesia, method of administration, and continuous monitoring until the animal is ambulatory is important. The surgical procedure is itself a technical skill, and the personnel should have adequate training in the procedures and species involved [2]. Maintenance of asepsis is very important to consider for successful outcomes. Intraoperative monitoring of animals such as the exposed tissues and mucous membranes will be an indication of tissue oxygenation. Generally, hypothermia is intensified while using biosafety cabinets, downdraft tables, or fume hoods [3]. In cases of longer duration of surgery where the procedure involves more than 30 min may be monitored using pulse-oximetry, electrocardiogram, and capnography to record real-time parameters of heart rate, pulse rate, and levels of oxygenation for an uneventful and smooth recovery. Fluid therapy is administered through subcutaneous or intraperitoneal route in split doses as necessary until the animal is able to consume feed and the water of its own. Record keeping is necessary with details including animal particulars; type of surgery; postoperative care including wound dehiscence or conditions, suture, or staple removal; and daily checks of the individual who performs the tasks. The pain scoring indicators are often used to determine the wellbeing of animals based on species-specific behaviors. Enrichments should be provided as per the need depending upon the type of materials and their suitability. This chapter describes some of the surgical animal models commonly used in biomedical research and experiments.

32.2 General Considerations

The functional facility should dedicate areas where the surgical procedure is conducted either a separate operating room or specific location within the suite by separation from other activities [4]. Personnel performing surgical procedures must have been trained appropriately to ensure that good surgical technique is followed particularly asepsis, gentle handling of tissues, appropriate use of instruments,

minimal dissection of tissue, effective hemostasis, and correct use of suture materials and suture patterns [4–7]. However, training in the species concerned is important due to the variations in anatomy and physiology including the requirement of anesthetic and analgesic drugs for the surgery.

Survival surgery in rodents should be performed using anesthesia. Analgesia must be given pre-emptively as well as during the postoperative period [8, 9]. Based on the study requirements and outcomes, the provision for analgesia is planned unless contraindicated and justified with scientific reasons in the animal ethics committee.

Aseptic technique is important while performing surgery to maintain the sterility in the surgical field and prevent the infections at later stage during the postoperative care. The aseptic surgery is ensured by using sterile instruments, preoperative preparation of sites, sterile gloves, and adhering aseptic principles during the intraoperative period. In some cases, a sterile field is not possible and required to perform the procedure with the "tips only" technique to minimize the level of contamination. However, the use of antibiotics should not be considered as an alternative to the aseptic procedure [4].

Pre-emptive use of analgesia is valuable since it helps to reduce the anesthetic doses required. Evidences point to greater pain relief by this than when administered postoperatively [10]. Analgesic drugs are important to reduce pain induced during the intraoperative period [11]. Moreover, it improves the intraoperative stability of animals and optimizes the postoperative care and eventually the wellbeing of animals [12]. In addition, an ophthalmic lubricating ointment is applied to prevent the dryness or damage of the cornea of the eyes during surgery. The anesthetic and analgesic protocol before, during, and after surgical procedures should be approved by the institutional animal ethics committee.

The catheters to be used for various procedures are chosen depending on the need as various catheters of different characteristics such as silicone, polyethylene, polyurethane, Teflon, and polytetrafluoroethylene (PTFE) are available. Besides, tethers or hollow metal tubing with flexible devices to protect the catheters are fixed and are externalized from the animal for easy access for administration of test compounds and multiple blood collection. In cases where inhalational anesthesia is used in animals and remaining waste gas anesthesia can be scavenged appropriately with a charcoal canister, other engineering controls such as spot extractor or local exhaust ventilation to minimize the possible occupational hazard of operators can be used. Batch surgery is commonly performed in rodents to conduct the surgeries in single settings. Hence, sufficient surgical instruments should be sterilized so that each set may be used for 4–5 animals and required to be sterilized in between the animals using a hot bead sterilizer (250 °C) for up to 60 s. It is to be noted that the instruments be allowed to cool before use.

The choice of suture materials should depend on minimal tissue reactivity, comfort while handling, ease of sterilization, and being non-allergenic. Suture size, tensile strength, flexibility, surface texture, and good knot security are other important factors to consider in the selection of materials. The degree of tissue reaction largely depends on the physical and chemical characteristics of the suture

materials. The surgeon should apply an appropriate suturing pattern based on the surgical sites that aids in preventing wound dehiscence and favors the healing process. Of late wound clips that provide quick closure of the skin closures are being used. But wound clips can be removed by the animals leading to tissue reactions higher than conventional suturing techniques. Skin glue (e.g., Vetbond, Dermabond) is available which is easy to fix but requires closer monitoring of the sutured sites to avoid any wound dehiscence during the postoperative period.

The catheterization technique is widely used in laboratories for confirmed intravenous delivery of test substances and collection of blood at predetermined time points from the animals; thereafter, the terminal end of the cannula is closed with a stopper. Moreover, the tether setup is used to extend the catheters in such a way that repeat sampling can be performed in conscious, unrestrained animals. In general, up to 10% of the circulating blood volume may be collected in cases of multiple collections, and an equal amount of heparin saline is infused to maintain the blood volume of animals.

32.3 Type of Surgery and Procedure

32.3.1 Major Survival Surgery

Major survival surgery involves the penetration and exposes of the body cavity, thereby impairment of physical or physiological functions of the animals or involvement of extensive tissue dissection or transection (e.g., thoracotomy, laparotomy, craniotomy).

32.3.2 Minor Survival Surgery

Minor survival surgery does not expose the body cavity and causes little impairment of the body functions. Generally, animals do not show signs of postoperative pain when recovering from these minor surgical procedures and return to normal function within a short time of at least 48–72 h (e.g., cannulation of peripheral blood vessels, subcutaneous implantation of medical devices, wound suturing, and percutaneous biopsy).

32.3.3 Non-survival Surgery

It refers to surgeries to obtain the intended parameters as a part of the study design and euthanized before recovery from anesthesia. Non-survival surgery may be performed in clean conditions rather than a complete aseptic manner since it is a terminal procedure.

32.3.4 Microsurgery

It refers to the specialized surgical procedure performed under an operating microscope with precision tools and various operating techniques in animals. These procedures are employed to the anastomosis of blood vessels and nerve tissues including organ transplantation in the experimental setup.

32.3.5 Aseptic Technique

It is a process or procedure used to minimize microbial contamination at the lowest possible and prevent the transfer of potentially pathogenic organisms to a surface that results in the development of infection.

32.3.6 Sterilization

It refers to any process that destroys all forms of microorganisms including spores on surfaces, and these can be achieved by using ethylene oxide gas, chemical, and autoclaving. This is used for the instruments, devices, and other materials (Table 32.1).

Table 32.1 Sterilization process used for surgical instruments, devices, and materials

Agent/ method	Process/ examples	Comments
Steam sterilization	Autoclave	Sterilization process at 121 °C for 15 min or flash sterilization at 131 °C for 3 min
Gas Sterilization	Ethylene oxide	It requires 30% relative humidity or greater to ensure effectiveness against spores. It is irritant and required adequate aeration after the sterilization process
Chlorine	Chlorine dioxide Sodium hypochlorite	Generally, corrosive depending upon the concentration and surgical items or devices should be rinsed with sterile saline before use
Hydrogen Peroxide	Hydrogen peroxide	Sterilization requires several hours, Corrosive and irritating. Vapor-based technology is also available
Aldehydes	Glutaraldehyde	It has bactericidal and sporicidal properties. Several hours are required for sterilization, corrosive, and irritation properties
Alcohol	Ethanol, Isopropanol	Alcohol is neither sterilant nor high-level disinfectant. It can be used for some procedures but prolonged contact time is necessary
Dry heat	Hot bead sterilizer Dry heat chamber	Flash sterilization within minutes and allowed to cool before contacting tissues. The irradiation process may also be used
Other chemicals	Beta propiolactone	It is used as a sterilant for tissue grafts, blood plasma products, and surgical instruments

32.3.7 Disinfection

It is a chemical or physical process used for the destruction of pathogenic organisms that includes all vegetative forms of microbes but not necessarily the spores.

32.3.8 Antiseptic

It refers to antimicrobial substances that are applied over the tissue, skin, or other living surfaces to reduce the possibility of infection or sepsis. Antiseptics are used at surgical sites after the surgery or until the suture removal because it is less harmful to the skin (e.g., povidone-iodine, chlorhexidine, chlorxylenol).

32.3.9 Equipment and Materials Used in Surgery

The following items are listed, and some of them are required based on the type of surgery and duration of the procedure involved:

- Catheters for cannulation (silicone, polyethylene (PE), polytetrafluoroethylene (PTFE), silastic, and Tygon) with a range of sizes and syringes.
- Thermo-controlled surgical platform/thermal blanket, infusion pump, anesthetic apparatus, and monitoring devices such as temperature probe, pulseoxymetry, capnography, and other special devices.
- Surgical instruments including scalpel, iris scissors, spatula, scissors, scalpel, bulldog clamp, hemostats, forceps, staples, tethers, and hot bead sterilizers.
- Anesthetics, analgesics, 70% isopropyl alcohol, povidone-iodine, chlorhexidine 2%, isotonic saline solution, ophthalmic lubricating gel, heparinized saline, surgical gloves, aprons, sterile gauze, and surgical drapes.
- Suture materials and structure: absorbable monofilament (natural, chromic catgut; synthetic, polydioxanone (PDS), polycaprone glycolide, polyglyconate); absorbable braided (synthetic, polyglactin, lactomer); nonabsorbable monofilament (natural, ethilon; synthetic, polypropylene); nonabsorbable braided (natural, silk; synthetic, braided polyester). Alternatively, wound clips and tissue adhesives are used making the incised wounds relatively easy to close.

32.3.10 Preoperative Procedure

All survival surgery should be performed by adhering to aseptic techniques by using appropriate scrubbing, sterile gloves, masks, hair cap, apron, and sterile instruments chosen for the type of surgery. The animal hair and dander from the surgical sites should be shaved using an electric shaver. Alternatively, hairs can be removed by applying depilatory cream just before the surgery. The surgical site should be scrubbed with appropriate disinfectant solution (chlorhexidine or povidone-iodine

and 70% isopropyl alcohol) at least 3 times from center to periphery of the surgical site. The skin surface should be free of cut wounds or injury. Sterile surgical drapes are applied to cover the body surface except for the surgical site. Lubricant ointments are applied to the eyes to prevent dryness during the surgery. Anesthesia should be selected based on the type and duration of surgery and administered as recommended as a loading dose followed by a maintenance dose until the completion of the surgical procedure.

32.3.11 Intraoperative Procedure

It involves minimal handling of tissues and aseptic techniques including monitoring of animals until the compilation of surgery. Anesthetic depth should be ensured by checking the vital signs (palpebral reflex, toe pinch, heart rate, and respiration). Assessment of dehydration has to be monitored, and then fluid therapy should be initiated as necessary. Suture patterns and closing patterns are important to prevent wound dehiscence, and consideration should be given for the type of suture materials such as absorbable (catgut, Polyglycolic acid-PGA) and nonabsorbable (silk, Prolene, etc.) Alternatively, wound clips or surgical glue may be used based on the nature of the surgery. Proper apposition at the suture site is ensured to avoid dead space. The body temperature should not be allowed to drop less than normal by using a source of warmth such as through thermo-controlled blankets or thermo-controlled surgical platforms. The animal should be monitored carefully till recovery and unconscious animals should not be left unattended for any reason.

32.3.12 Postoperative Care

The animal is transferred to a dedicated room or separate recovery area and exposed to a heat source to prevent hypothermia until the recovery from the anesthesia. Soon after the recovery, the animal should be maintained in cages with soft bedding materials, and sterile cotton may be also placed along with bedding materials. Analgesia is administered as per the recommended dose levels (e.g., buprenorphine, meloxicam, ketoprofen, carprofen, or tramadol) for at least 48–72 h or until the recovery period depending upon the surgical procedure involved. Managing postoperative pain management in the rodent is important by identifying an optimal analgesic plan for surgery that can be a critical aspect of animal welfare [13].

In cases, where the analgesia is known to interfere with research outcomes, the analgesic may be avoided during the postoperative period, based on prior clearance from the ethics committee. Antibiotics may not be necessary to administer if the surgical procedures are performed aseptically and competently even if batch surgeries are conducted where several animals underwent the same surgical procedure in a single session. However, fluid replacement should be considered based on the type of surgery. Warm saline, ringer lactate solution can be given subcutaneously after assessing conditions of dehydration.

32.4 Common Surgical Techniques Used in Laboratory Animals

32.4.1 Jugular Vein Cannulation

The jugular vein cannulation enables the collection of multiple blood samples and administration of formulations/drugs through the intravenous route. The surgery is usually performed in rats and guinea pigs under anesthesia (inhalational/injectable). The dorsal median and ventrolateral region of the neck is shaved and then swabbed with povidone-iodine. The animal is placed over the thermo-controlled surgical platform, and a small incision is made over the ventrolateral neck region to locate the jugular vein. The vein is isolated by clearing the adhering tissues, and a supporting plate is placed underneath the vein for better visualization. The cranial side of the vein is tied to block the blood flow, and the caudal end is prepared for cannulation by making a small nick over the vein using iris scissors. A silicone or polyethylene catheter of suitable size (PE-50 approximately 2–3 cm) is inserted carefully by inserting towards the heart. The patency of the vein is verified by aspirating blood and then flushing with heparinized saline to avoid block in the catheter. Thereafter, the catheter is tied with a surgical knot using silk, and the other end of the catheter is subcutaneously tunneled to the dorsal neck region by using a trocar and exteriorized through a small incision. The catheter is secured properly at the dorsal neck region to avoid any displacement of the cannula. The incisions are closed appropriately using either silk, wound clips, or tissue adhesive, and povidone-iodine is applied over the surgical sites and the animal is allowed to recover. In general, single and/or dual cannulation is performed based on the study requirement. It is important to maintain the patency which can be checked by flushing with heparinized saline at periodic intervals. Polyurethane catheters can be used with antithrombotic coatings that are shown to reduce thrombosis of the catheter [14] (Figs. 32.1, 32.2 and 32.3).

32.4.2 Carotid Artery Cannulation

The carotid artery cannulation is performed to collect multiple blood samples, administration of test formulations, brain perfusion, and hemodynamic parameters in rats and guinea pigs. The animal is anesthetized using either an inhalational or injectable agent. The surgical plane of anesthesia is ensured by checking toe pinch, palpebral, or pedal reflexes. The dorsal and ventral neck areas are shaved and swabbed with povidone-iodine thereafter, and the animal is placed on a thermo-controlled surgical table. A small incision is made on the dorsomedial, as well as ventral-lateral neck region. Muscle layers are cleared to access the carotid artery, and a loose knot is placed towards the heart, and the other ends should be tied with a knot using silk. The bulldog clamp is used to block the blood flow to the cranial end. A silicone or polythene catheter (PE-50) is inserted through the incision and secured with a knot. The catheter is tunneled subcutaneously to the dorsal neck region using a trocar and exteriorized through a small incision. The incision is sutured closed with

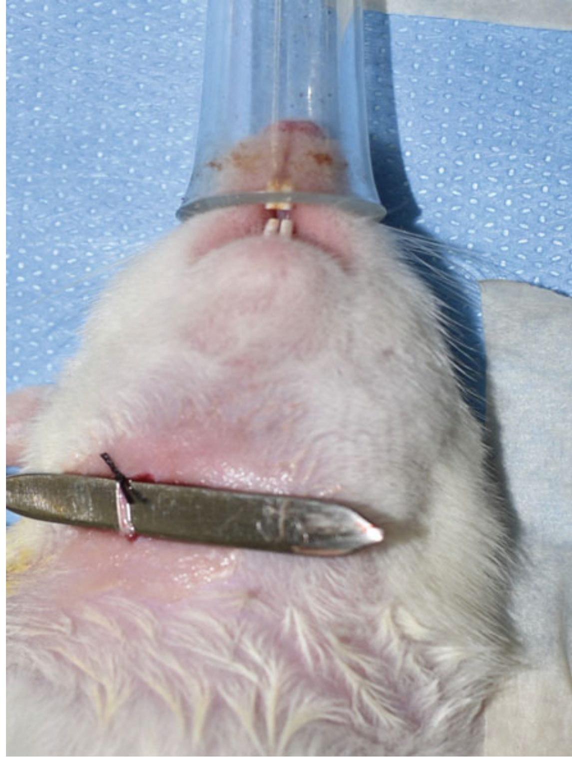


Fig. 32.1 Jugular vein identification for canulation

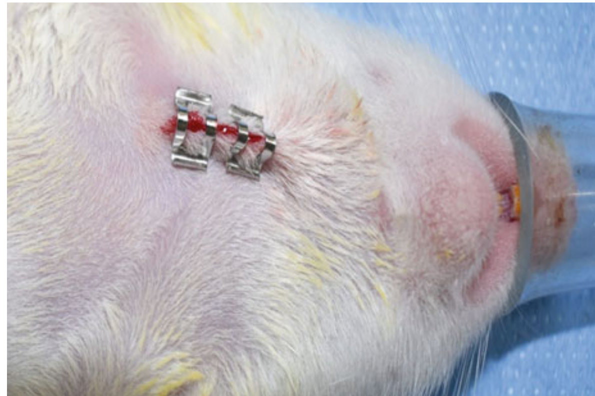


Fig. 32.2 Jugular area suture closure by using wound staples

silk, and povidone-iodine is applied over the surgical sites. The animal can recover from anesthesia, and patency of the catheter is ensured by withdrawing blood and flushing heparin saline at periodic intervals. In the case of brain perfusion, the common carotid artery is ligated, and a catheter containing heparin saline is infused

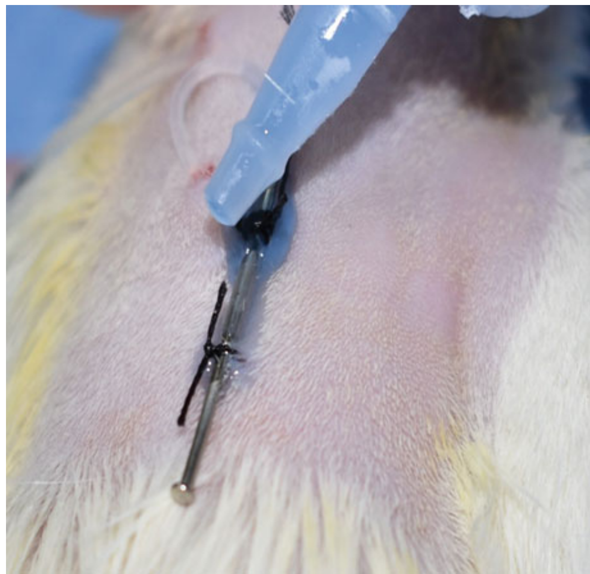


Fig. 32.3 Dorsal neck incision closed with silk and application of tissue adhesive

to perfuse the brain by ligating corresponding external carotid arteries to avoid the perfusion to extracerebral tissues [15, 16].

32.4.3 Bile Duct Cannulation

Bile duct cannulation is generally performed in rats and guinea pigs to understand the biliary metabolism and elimination pathways of test compounds or drugs. The animal is anesthetized using an injectable or inhalational agent. The abdominal as well as dorsal neck region is shaved and wiped with povidone-iodine to prepare the surgical sites. The surgical plane of anesthesia is ensured by toe pinch, palpebral, or pedal reflexes, and the animal is placed over the thermo-controlled surgical platforms. Sterile gauze soaked in warm saline is placed over the abdomen. A midline incision is made in the linea alba of the abdomen. The muscle layers and peritoneum are exposed. The bile duct is exposed, and a supporting plate is placed underneath for better visualization. The caudal side of the bile duct is tied to block the bile flow. A polyethylene catheter (PE-10) of suitable size is inserted into the common bile duct. A silk thread loop is tied on the cranial side to secure the cannula. The duodenum is located and a loose knot is placed over it. A small cut is made in the duodenum, and a polyethylene cannula (PE-50) is inserted and secured properly using silk thread knots. The muscle incision is closed with a simple interrupted suture, and the skin layers are closed using wound clips or suture knots. Povidone-iodine is applied over the surgical sites [17].

32.4.4 Bile Duct Ligation

The procedure of bile duct ligation aids in understanding the alterations in the levels of endogenous compounds, physiological changes, and pharmacokinetic profiling of test compounds in a condition like cholestasis and fibrosis. Generally, rats or mice are used for the bile duct ligation model. The animal is anesthetized using an injectable or inhalational agent. The surgical plane is ensured by toe pinching or pedal reflex. A midline laparotomy is made at the linea alba of the abdomen. The muscle layers are separated using blunt forceps or retractors, and the bile duct is exposed. A metal plate is inserted underneath for better visualization. The bile duct may be ligated partially or completely based on the study requirements and onset of disease progression. A double knot is applied over the bile ducts securely. After this, the peritoneum, as well as muscle layers, is closed by continuous suturing with absorbable materials, and the skin is closed by simple interrupted suture or wound clips. As a consequence, bile stasis occurs in the system, and liver fibrosis develops within a few days after surgery [18–20].

32.4.5 Duodenum and Bile Duct Cannulation

The rat is commonly used for this procedure for absorption of sites in pharmacokinetic studies as well as to understand the elimination pathways of test compounds via bile. Rat is anesthetized with an injectable or inhalational agent, and the surgical plane is ensured by toe pinching and pedal reflexes. The abdominal and dorsal back region is shaved and swabbed with povidone-iodine. The abdominal cavity is opened, and an indwelling polyethylene catheter (PE-10) is inserted into the bile duct (PE-50). Another catheter is inserted into the duodenum at the level of the common bile duct for the recirculation/infusion of bile. Both cannulas are passed through the abdominal musculature and then rooted subcutaneously to the dorsal neck area and exteriorized through a small incision. The control bile may be collected from the next day of surgery. Bile or bile salt solution may be infused through the duodenal cannula throughout the experiment. The flushing of the catheter is not necessary because the bile flow is continuous [21].

32.4.6 Portal Vein Cannulation

The portal vein cannulation aids in the collection of multiple collections of portal blood and is used for intraportal administration of drugs to understand the hepatic first-pass effect of test compounds or drugs. The rat is anesthetized using injectable or inhalational anesthesia. Once the animal reaches the proper surgical plane, the abdominal area is shaved and then swabbed with povidone-iodine, and the animal should be covered with sterile drapes. A midline incision at the linea alba of the abdominal region is made. The portal vein is located under the liver. A supportive plate is placed below the portal vein and cleaned with a moist sterile cotton swab to

remove the adhering tissues, and a loose knot is made around the portal vein without blocking blood flow. A polyethylene catheter (PE-10 tip with PE-50 body) is inserted into the junction of two ileal veins such that the catheter enters into the portal vein up to the first bead (0.8 mm) towards the liver. The ends of the loose knot are tied around the bead to secure properly, and tissue glue also is used at the junction of the catheter and vein. The supportive plate is removed carefully; thereafter the peritoneum and abdominal muscles are closed by a continuous suture pattern. The skin wound is closed by a simple interrupted suture. The animal is allowed to recover from anesthesia. Patency of the catheter is checked and then exteriorized to the dorsal neck region subcutaneously through a trocar and sutured closed [22, 23].

32.4.7 Intestinal Perfusion Technique (Open and Closed Methods)

The surgical technique involves jejunum and mesentery cannulation to investigate the rate of absorption and/or its underlying metabolism of compounds under in situ experimental conditions. Rat is anesthetized using injectable or inhalational anesthesia. The surgical plane of anesthesia is ensured by toe pinch or pedal reflexes. The abdominal and the dorsal neck regions are shaved. A midline incision is made at the linea alba of the abdomen, and muscle layers are separated using retractors. The intestinal segment is pulled out of the abdomen and placed over a moist cotton pad. The intestinal contents are squeezed, and a suitable segment of approximately 10 cm is selected for cannulation, and the remaining tissues are returned into the abdominal cavity. For the open-loop method, a small cut is made at proximal and distal ends of the intestinal segment in which two 4.5-cm-long cannulas, e.g., Tygon Microbore tubing, is inserted and secured with a ligature. The isolated segment draining the blood of the mesenteric vein is identified, and loops of thread are placed at the cranial and caudal ends of the vein. The polyethylene (PE-50) is inserted towards the caudal end of the vein, and the cannula is secured at both ends of the loops with tied knots. The test compound or drug is perfused through the Tygon tubing from the proximal end of the intestinal segment, and blood is collected at predetermined time points from the distal end. The isolated intestinal segment is covered with cotton gauze and periodically moistened with warm and sterile saline. In the end, a systemic sample may be taken from a cardiac puncture.

In the closed-loop method, the distal end of the intestinal segment at approximately 10 cm is tied securely, and then the test compound is dosed directly into the lumen from the proximal end using Tygon tubing of around 4 cm. The proximal end is also closed by tying securely by suture to form a closed intestinal loop. The predetermined blood collection is the same for open and closed methods. This procedure is terminal, and the animal is euthanized at the end of the experiment [24–26].

32.4.8 Surgical Implantation of Osmotic Pump

The surgical implantation of an osmotic pump either subcutaneous or intraperitoneal site facilitates sustained, controlled release of test compounds or drugs in unrestrained animals. This procedure is used especially for compounds with a short half-life. The rat will be anesthetized preferably with inhalational anesthesia since it is a minor procedure, and the dorsal thoracic area skin is shaved and then swabbed with povidone-iodine. A small incision adjacent to the site chosen for pump placement is made, and the subcutaneous pocket is large enough to allow free movement of the pump depending upon the site (e.g., Alzet pump 100 μL to 2000 μL; micro-osmotic pump) selected for implantation. An osmotic pump is inserted into pocket, and the incision is sutured closed with silk or wound clips. In the case of intraperitoneal implantation, laparotomy is performed by preparing the site, and the incision is made at the linea alba of the abdomen and muscle layers separated, and then an osmotic pump of the chosen size is carefully inserted into the abdominal cavity. The peritoneum and muscle layers should be closed by a simple continuous absorbable material, and the skin is sutured by simple interrupted using silk or wound clips or tissue adhesive. The animal is allowed to recover and monitored for any signs [27–30].

32.4.9 Spinal Cord Ligation

The spinal cord ligations are a suitable model for analgesic screening against neuropathic pain and evaluation of the peripheral nerve injury upon treatment of test formulation or drug in rats. Anesthesia is used either injectable or inhalational, and the dorsal surgical site is shaved, aseptically prepared by povidone-iodine. A longitudinal incision is made approximately from the lumbar (L3) to the sacrum (S2) and connective tissues, and paraspinal muscles are carefully separated without damaging the underlying nerves. Using sharp and blunt dissection, the left L6 and S1 posterior interarticular processes are exposed to allow visualization of the L6 transverse process, which is gently removed. The underlying fascia is carefully teased to expose the left L4 and L5 spinal nerves distal to their emergence from the intervertebral foramina. The nerves are gently separated, and the L5 nerve is tightly ligated with 6–0 silk suture. The left L6 spinal nerve is then located just caudal and medial to the sacroiliac junction and tightly ligated with 6–0 silk suture [31, 32].

32.4.10 Chronic Constriction Injury

The chronic constriction injury (CCI) model is used to induce chronic neuropathic pain and is assessed by using test compounds or drugs in rats. The manifestation of abnormal pain sensory such as allodynia and hyperalgesia resembles the symptom produced in humans. The anesthetized animal is ensured for the surgical plane and

placed over a thermo-controlled surgical platform, and the mid-thigh area is aseptically prepared. A small incision is made at the skin parallel to the lateral femoral region of the left hind leg, and muscles surrounding the incision by blunt cutting through the connective tissues in between the gluteus superficialis and biceps femoris muscles are separated. The sciatic nerve is exposed beneath these muscles allowing clear visualization and gently freeing up approximately 10 mm from the surroundings proximal to the trifurcation of the sciatic nerve. At least four ligatures at 1 mm spacing should be made by loosely tying around the sciatic nerve using silk or chromic catgut. Loose tying ensures that epineural circulation is preserved. After 4 days of recovery, the hind paw responses to tactile, mechanical, or thermal, and cold stimuli may be evaluated. The CCI model reliably induces thermal and mechanical hypersensitivity, and spontaneous pain eventually leads to the animal lifting the paw and licking the affected paw [33–35].

32.4.11 Femoral Vein/Artery Catheterization

The femoral vein catheterization is used to collect blood at multiple time points and continuous infusion or injection of test compound or drug in the unrestrained, conscious animals. Similarly, femoral artery catheterization is used for arterial blood pressure measurement and multiple sampling with higher volumes. The rat is anesthetized using an injectable or inhalational agent, and the surgical plane is ensured by checking the toe pinch or pedal reflexes. A small incision is made at the medial femoral region, and connective tissue is separated until the femoral vein and artery are distinguished by their colors. The vein is dark red in color, and the artery is brighter than the vein and is pulsating. The vein, artery, and nerve can be separated by placing a spatula underneath the above structures. Sterile cotton buds or forceps are used to segregate the vein from the artery and nerve sheath. A loop of thread is used to block the blood flow to the distal end, and a bulldog clamp is applied at the caudal site. Thereafter, a small cut is made using an iris scissor, and then a silicone or polyethylene catheter (PE-50) is inserted into the lumen of the vein and securely tied with the cannula. It is required to prefill the catheter with heparinized saline by blocking the other end of the catheter so that blood loss can be controlled while inserting the cannula into the lumen of the vein or artery. The surgical site is sutured closed with silk, and povidone-iodine is applied; thereafter patency is checked at a periodic interval [36, 37] (Figs. 32.4 and 32.5).

32.4.12 Nephrectomy

The rodents are commonly used as a model for nephrectomy to be used in metabolism and renal clearance studies [38, 39]. The surgical removal of the kidneys is performed as a bilateral nephrectomy for acute renal failure [40] or unilateral and partial nephrectomies by dissecting 5/6 nephrectomies for a chronic renal failure model [41]. The animal is anesthetized using an injectable or inhalational agent, and

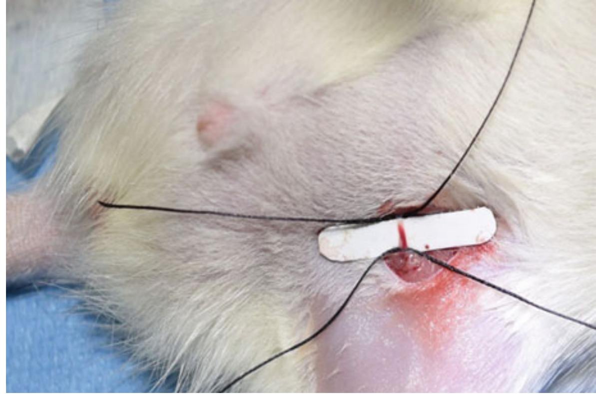


Fig. 32.4 Femoral vein exposed for cannulation

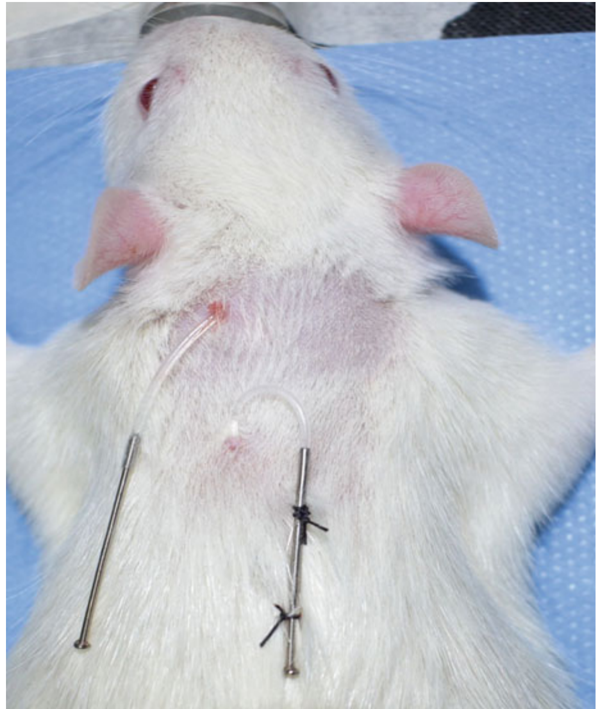


Fig. 32.5 Dual cannula exteriorized through neck region

the surgical plane is ensured by toe pinching or pedal reflexes. The abdominal area is shaved and swabbed with povidone-iodine, and a midline incision is made at the linea alba of the abdomen. The muscle layers are separated and the kidneys are exposed. Careful attention should be paid to ligate the structures such as a renal artery, renal vein, and ureter without any damage to adrenal vessels that are connected directly to the kidneys and resect the ligated vessels of all three structures

one by one. The peritoneum and muscle layers are closed by simple continuous and skin layer by a simple interrupted suture. As a consequence of surgery serum BUN and creatinine will be elevated in the system, and these diagnostic biomarkers are indicators of renal function. Body temperature as an important parameter is also to be monitored since the procedure alters metabolic turnover and those animals with a lower metabolic rate are hypothermic.

32.4.13 Bladder Cannulation

Bladder cannulation is commonly performed in female rodents to administer test materials or cell lines to develop tumor implantation model in rodents. The animal will be anesthetized using inhalational or injectable agents, and the abdomen area is aseptically prepared. A sterile endovenous catheter (20–22 G) is inserted into the bladder through the urethra, and phosphate buffered solution (PBS) is administered to identify the location of the bladder. A midline incision is made at the linea alba of the lower abdomen and muscle, and the peritoneal layer is separated to expose the bladder. The surrounding intestinal and accessory organs are cleared and wetted with moist gauze. The bladder is cannulated by creating a hole using a needle (20G), and the catheter tip is inserted slowly by holding the bladder with blunt forceps. The catheter is secured with purse-string suture by firmly tightening, and tissue adhesives are also applied to prevent leaks from the bladder. The catheter is exteriorized by subcutaneous tunneling out at the back of the neck region for administration of test substance or removing fluids from the bladder. The peritoneum and muscle layers are closed by a simple continuous pattern and skin suturing by silk [42–44].

32.4.14 Hepatectomy

This procedure is performed to understand the pharmacokinetics, liver mass regeneration, analysis of signaling pathways, and hepatocarcinogenesis. Hepatectomy is the surgical removal of the liver either partial or total hepatectomy that is critical due to the anatomical structures [45–47]. The animal is anesthetized with an injectable or inhalational agent, and the surgical plane is ensured by pinching the toes or pedal reflexes. A midline laparotomy incision is made by exposing the xiphoid process and hepatic lobes. The main aspect to be considered is the need for portosystemic shunting that the intestinal blood supply drains into the portal vein which receives 10–25% of cardiac output in most species. The diversion of blood or occlusion of the portal vein is essential while performing the procedure to prevent hemorrhage. However, clamping the portal vein alone and without portosystemic shunting will lead to splanchnic congestion which causes ischemic injury in splanchnic organs [48, 49]. The next aspect is the connection between *vena cava caudalis* and the liver. It is necessary to reestablish the flow in the *vena cava caudalis (VCC)* by creating anastomosis [50]. The hepatectomy is carried out by devascularization technique by following a V-shaped prosthesis, Y-shaped graft, mesentericocaval side-to-side

shunt, a silicone catheter, or splanchnic clamping of the portal vein. The procedures of Y-shaped or V-shaped prosthesis using polyethylene aid in the removal of intrahepatic VCC because the prosthesis is used to replace the intrahepatic section [51, 52]. Alternatively, the combination of side-to-side mesentericocaval shunts with vascular graft for the intrahepatic part can be used [53]. The hepatic artery ligation along with the silicone catheter can block the hepatic blood flow and is useful for devascularization [54]. The portal vein clamping is applied for hepatic devascularization, and the intrahepatic part of VCC is kept intact, but the clamping leads to splanchnic congestion, and this approach is used for short experiments with limited time in animals [55]. In all procedures of hepatectomy, the animal is monitored carefully since the procedures are long and the blood flow of major organs involved. Any remnant parts are cleaned up before suturing the peritoneum and abdominal muscles followed by the skin with a simple interrupted suture. The surgical site is wiped, and povidone-iodine is applied until the postoperative period.

32.4.15 Ovariectomy

This procedure is commonly performed to develop osteoporosis model, endocrine physiology, and superovulation in rodents. The animal is anesthetized using either an injectable or inhalational agent, and the surgical plane of anesthesia is ensured by checking the pedal reflexes and toe pinch. A midline dorsal longitudinal incision (5–10 mm) is made from the second to fifth vertebrae, and the skin is retracted using bulldog clamps, and the ovary is exposed by retraction of the peritoneum. The base of the ovary is tied with silk and surgically excised to remove the ovary. The peritoneum and muscle layers are sutured closed, and skin layers are apposed with interrupted suture or tissue glue. Similarly, the other side of the ovary is also removed in the same manner [56].

32.4.16 Adrenalectomy

The procedure is commonly performed in research involving steroidal hormone metabolism and obesity in rodents. The animal should be anesthetized, and the surgical plane of anesthesia is ensured by toe pinch or pedal reflexes. A midline incision is made at the level of the first to third lumbar vertebrae, and the incision is directed laterally to expose the paracostal musculature. Further, the incision is made at the caudal most rib, and adrenal is identified by retraction of the spleen at the left lateral side or cranial retraction of the liver at the right. Hemostats are applied to the vessels supplying blood to the adrenals, and thread knots are applied to block the blood flow of the vessel adjacent to the gland. The adrenal gland is surgically excised, and the other side of the adrenal also made a cut to remove the gland. The bilateral adrenalectomized animal should be provided normal saline for water consumption [57]. Similarly, glucose (15 g/L) is administered to prevent

hypoglycemia. Furthermore, the paralumbar approach of the adrenalectomy procedure has been well described in the rat [58].

32.4.17 Craniotomy

Craniotomy is performed in rodents for research involving intrahippocampal injection, microinjection of cell lines, behavioral experiments, focal ischemia, and wired and wireless recordings of the brain. This procedure requires a specialized stereotaxic apparatus and skills to perform the surgery with extreme care and caution. The animal is administered with injectable or inhalational anesthesia; the head region is shaved and aseptically prepared using povidone-iodine. The surgical plane of anesthesia is ensured by toe pinch or pedal reflex, and lidocaine drop is applied on the skull. The provision of thermo-controlled blanket is used to maintain the body temperature, and the rectal probe is also used to monitor the temperature since the procedure is likely to last long. The animal is mounted on a stereotaxic frame with ear bars into the ear canal. The mouth is secured with the anterior part of the device, and position the notch with the mouth bar. The nose guard is placed over the animal's nose to the attached bite plate of the device, and upper incisors are positioned over the bite plate after moving the tongue to the side in such a way to prevent the movement of the head which is secured firmly with ear bars. A midline incision of the skin is made from the frontal cranial bones to the back of the parietal cranial bones of the skull (20 mm long for rats, 10 mm long for mice). The periosteum is retracted carefully to the edge of the skull, and the musculature of the back of the neck is also retracted using bulldog clamps to keep the incision open by pinching off the skin. The skull surface is scraped gently, and the surface is made dry to aid better adherence of the glue after the surgery. After ensuring that the head level is with a ruler of the device, and checking for a 90° angle between the ruler and middle of the animal scalp, the guide cannula is positioned onto its mount as the first step to find the lambda located just in between the eyes of the animal by touching the anterior skull, and recording the dorsoventral coordinate. Next step, by placing the cannula right at the bregma of the posterior skull, record the anterior-posterior and lateral coordinates by adding or subtracting from bregma with the aid of stereotaxic atlas [59]. The identified area is marked by a sterile pencil, and a battery-operated driller is used to create a hole, and the located site is reached by pushing gently, and the spongy structure of the bone is lifted away from the skull with forceps. Adding a drop of saline aids in lifting the skull and also prevents bleeding of the dura. The gel foam soaked in saline is applied on the dura matter to stop the bleeding, if any. Adequate care is taken to avoid penetration of drill bit through meningeal membranes (dura, arachnoid, and pia matters) or blood vessels, and the dura mater is dried, and a glass cover of 5 mm is laid over the dura mater. A drop of cyanoacrylate-based glue is added to the opposite hemisphere over the skull and gently spread all around with the help of a needle. The glue is spread over the entire skull surface and allowed to dry. A mix of dental acrylic is applied on top of the entire skull surface and secured with a small rim of the coverslip, and acrylic is

allowed to harden, and suture is made with polyvinylidene fluoride (PVDF). The animal will be monitored carefully to recover from the anesthesia, and povidone-iodine is applied on the surgical site as well as analgesic regimen during postoperative care [60–63]. Depending upon the target area and location, the skull is drilled, and holes are made including the removal of the regions of interest as part of the procedure.

32.4.18 Occlusion of Middle Cerebral Artery

Rodents are ideal models for cerebral ischemia/stroke models of the brain due to the close resemblance to humans. The animal is anesthetized with injectable anesthesia for induction and inhalational anesthesia (isoflurane) for maintenance. This has been shown to be effective due to better control during the procedure and lower mortality at post-ischemia after 7 days of the procedure [64]. The animal is placed on a supine position over thermo-controlled surgical table, and the limbs are fixed with adhesive tape. A midline incision is made on the neck, and gently split the left and right sternohyoid muscles by blunt dissection, and retract the common carotid artery (CCA) situated underneath the right sternohyoid muscle laterally to the trachea. The artery on either the left or the right side should be isolated without damaging the vagus nerve and ligated. The CCA is bifurcated into two different arteries such as the internal carotid artery (ICA) that supplies blood to the head region which further separates into two branches as middle cerebral artery (MCA) and pterygopalatine artery. The first bifurcation of CCA is identified, and the surrounding tissues of the external carotid artery (ECA) are cleared after exposing and tied with two distant knots at the distal part to prevent the backflow. The ECA is cut in between the knots, and the tied section attached proximally to the CCA junction should be straightened further to allow the filament to enter the ICA. Subsequently, the second bifurcation is also cleared for better visualization of MCA. The microvascular clip is placed temporarily at the proximal ICA junction, and the tied section of ECA is incised to insert the monofilament and required to ensure the monofilament reaches the CCA junction. A knot is made just below the arteriotomized section of the ECA, and then the microvascular clip is removed permanently to enable the insertion of the filament. The filament should be facilitated by straightening the MCA at 11 mm and 20 mm for mice and rats, respectively. The intensity of the infarction is typically related to the MCA occlusion period. A period of 60–90 min of occlusion is seen to obtain a reproducible infarct volume [65, 66]. The filament is then removed, and the knot is tightened at the ECA, and the blood flow is restored to anterograde movement. Alternatively, the thromboembolic stroke model also developed using fibrin-rich blood clots, and lodgment of the embolus is attained by advancing the catheter (PE-50) rostrally from the ECA arteriotomy site into the ICA to reach the tip of the catheter to the origin of MCA. The catheter is advanced in such a way to feel the resistance that indicates the catheter reaches the origin of the MCA due to the anatomic narrowing, and slowly inject the emboli to form a ball to block the MCA, and then retract the catheter 5 min after the clot delivery [67]. The knot is

secured by tightening (4–0 silk) around the ECA trunk to ligate the arteriotomy, and remove the aneurism clip. The areas should be swabbed with soaked drapes to clean up the micro clots if any, and then the skin layers are sutured with a simple interrupted pattern or wound clips applied; thereafter, povidone-iodine is applied over the surgical sites. The infarction can be assessed by Doppler flowmetry to ensure the occlusion of MCA in rodents. Furthermore, the infarct volume can be evaluated by histological analysis [68–72].

32.4.19 Tracheotomy

The surgical procedure is used in rodents, guinea pigs, and rabbits mainly for intubation of cannula and artificial respiration into the trachea for the respiratory parameter measurements. The animal is anesthetized using injectable inhalational anesthetic agents, and the surgical plane of anesthesia is ensured by toe pinching or pedal reflexes. The surgical area is shaved and aseptically prepared by swabbing with povidone-iodine. A ventral midline cervical incision is from the manubrium sterni, and up to the level of the hyoid bone, blunt dissection is made to expose the sternocleidomastoid as well as sternohyoid muscles. The tracheal ring structures visualized after retracting the muscles and recurrent laryngeal nerve including inferior thyroid artery are carefully separated. A small incision is made on the trachea using an iris scissor, and a suitable size cannula is inserted by securing through knots. The other end is connected with a ventilator for artificial respiration to the animals for measuring the lung function parameters, and these procedures are usually terminal, and animals will be euthanized. The neck region covered with moist gauze and temperature is monitored during the experiment [73–75].

32.4.20 Vasectomy

The surgical procedure in rodents is commonly performed for transgenic animal production and studies related to reproduction. The surgery is performed either abdominal or scrotal sac approach using inhalational or injectable anesthesia. The surgical site is prepared aseptically by swabbing with povidone-iodine. For abdominal approach, a midline incision is made, and then abdominal muscles are separated and located the bilateral vas deferens by gently pulling out the abdominal cavity. The vas deferens is serially ligated at a 3 mm distance using 4–0 silk and transected between the ligatures, and the same procedure is performed on the contralateral testis. The testes and remaining structures are returned into the abdominal cavity, and the peritoneum, muscle layers, and skin are sutured using silk. Similarly, the scrotal sac method is also preferred to avoid the manipulation of abdominal organs. The surgical incision (0.5 cm) is made on the scrotal sac, and remove the vas deferens of both the testes by gently exposing them from the scrotal sac. The vas deferens is serially ligated and transected, and then the same procedure is performed on the other side of the testis. The scrotal sac is either sutured closed, or surgical glue is also used,

and the surgical site is applied with povidone-iodine, and then allow the animal to recover from the anesthesia [76, 77].

References

1. Garguilo S, Greco A, Gramanzini M, Esposio S, Affuso A, Brunetti A, Vesce G (2012) Mice anesthesia, analgesia, and care, Part I: Anesthetic considerations in preclinical research. ILAR J 53(1):E55–E69
2. Pritchett-Corning KR, Mulder GB, Luo Y, White WJ (2011) Principles of rodent surgery for the new surgeon. J Vis Exp 47:e2586. 1–4. PMID: 21248700
3. Hoogstraten-Miller SL, Brown PA (2008) Techniques in aseptic rodent surgery. Curr Protoc Immunol. Chapter 1, Unit-1.1.14. https://doi.org/10.1002/0471142735.im0112s82
4. Guide for the Care and Use of Laboratory Animals. (2011). The National Academies Press, Washington, DC
5. ACLAM Position Statement on Rodent Surgery (2016) J Am Assoc Lab Anim Science 55 (6):822–823
6. Brown MJ, Pearson PT, Tomson FN (1993) Guidelines for animal surgery in research and teaching. Am J Vet Res 54:1544–1559
7. Heon H, Rousseau N, Montgomery J, Beauregard G, Choiniere M (2006) Establishment of an operating room committee and a training program to improve aseptic techniques for rodent and large animal surgery. J Am Assoc Lab Anim Sci 45:58–62
8. Flecknell P (2018) Rodent analgesia: assessment and therapeutics 2018. Vet J 232:70–77. PMID: 29428096
9. Flecknell PA, Thomas AA (2015) Comparative anesthesia and analgesia of laboratory animals. In: Chapter 39, Veterinary anesthesia and analgesia: the fifth edition of lumb and jones, pp 754–763. https://doi.org/10.1002/9781119421375.ch39
10. Fish RE, Brown MJ, Danneman PJ, Kras AZ (2008) Anesthesia and analgesia in laboratory animals, 2nd edn. Elsevier, USA
11. Elizabeth A, Nunamaker EA, Julia L, Goldman JL, Adams CR, Fortman JD (2018) Evaluation of analgesic efficacy of meloxicam and 2 formulations of buprenorphine after laparotomy in female Sprague-Dawley rats. J Am Assoc Lab Anim Med 57(5):498–507. PMID: 30092855
12. Hedenqvist P, Roughan JV, Flecknell PA (2000) Effects of repeated anaesthesia with ketamine/ medetomidine and of pre-anaesthetic administration of buprenorphine in rats. Lab Anim 34:207–211
13. Nunamaker EA, Goldman JL, Adams CR, Fortman JD (2018) Evaluation of analgesic efficacy of meloxicam and 2 formulations of buprenorphine after laparotomy in female Sprague-Dawley Rats. J Am Assoc Lab Anim Sci 57(5):498–507
14. Foley PL, Barthel CH, Brausa HR (2002) Effect of covalently bound heparin coating on patency and biocompatibility of long term indwelling catheters in the rat jugular vein. Comp Med 52:243–248
15. Feng J, Fitz YV, Li Y, Fernandez M, Puch IC, Wang D, Pazniokas S, Bucher B, Cui X, Solomon SB (2015) Catheterization of the carotid artery and jugular vein to perform hemodynamic measures, infusions and blood sampling in a conscious rat model. J Vis Exp 95:e51881. 1–6. PMID: 25741606
16. Tsui BCH, Mosher SJ, Yeung PKF (1991) A reliable technique for chronic carotid arterial catheterization in the rat. J Pharmacol Methods 25(4):343–352
17. Rath L, Hutchison M (1989) A new method of bile duct cannulation allowing bile collection and re-infusion in the conscious rat. Lab Anim 23:163–168
18. Kirkland JG, Godfrey GB, Garreet R, Kakkar S, Yeh BM, Corvera CU (2010) Reversible surgical model of biliary inflammation and obstructive jaundice in mice. J Surg Res 164 (2):221–227. PMID: 19932898

19. Georgiev P, Jochum W, Heinrich S, Jang JH, Nocito A, Dahm F, Clavien PA (2008) Characterization of time-related changes after experimental bile duct ligation. Br J Surg 95 (5):646–656. PMID: 18196571; 19932898
20. Tannuri AA, Coelho MM, Goncalves JD, Santos MM, Ferraz dS LF, Bendit I, Tannuri U (2012) Effects of selective bile duct ligation on liver parenchyma in young animals: histologic and molecular evaluations. J Paediatr Surg 43(93):513–522. PMID: 22424347
21. Wang YM, Reuning RH (1994) A comparison of two surgical techniques for preparation of rats with chronic bile duct cannulae for the investigation of enterohepatic circulation. Lab Animl Sci 44(5):479–485. PMID: 7844957
22. Strubbe JH, Bruggink JE, Steffens AB (1998) Hepatic Portal vein cannulation for infusion and blood sampling in freely moving rats. Physiol Behav 65(4–5):885–887. PMID: 10073496
23. Matsuda Y, Konno Y, Satsukawa M, Kobayashi T, Takimoto Y, Morisaki K, Yamashita S (2012) Assessment of intestinal availability of various drugs in the oral absorption process using portal vein cannulated rats. Drug Metab Dispos 40(12):2231–2238. PMID: 22930277
24. Mudra DR, Borchardt RT (2009) Absorption barriers in the rat intestinal mucosa: 1. Application of an in situ perfusion model to simultaneously assess drug permeation and metabolism. J Pharm Sci 99(2):982–998. https://doi.org/10.1002/jps.21912
25. Shimomura M, Masuda S, Saito H, Sakamoto S, Uemoto S, Tanaka K, Inui K (2002) Roles of the Jejunum and ileum in the first pass effect as absorptive barriers for orally administered tacrolimus. J Surg Res 103(2):215–222. PMID: 11922737
26. Cao X, Gibbs ST, Fang L, Miller HA, Landowski CP, Shin H, Lennermas H, Zhong Y, Amidon GL, Yu LX, Sun D (2006) Why is it challenging to predict intestinal drug absorption and oral bioavailability in human using rat model. Pharma Res 23(8):1675–1686. PMID: 16841194
27. Tsai YC, Sung YH, Chang PJ, Kang FC, Chu KS (2000) Tramadol relieves thermal hyperalgesia in rats with chronic constriction injury of the sciatic nerve. Fund Clin Pharmacol 14:335–340
28. Liang M, Ly J, Zou L, Yang W, Xiong Y, Chen X, Guan M, He R, Zou H (2015) A modified murine model of systemic sclerosis: bleomycin given by pump infusion induced skin and pulmonary inflammation and fibrosis. Lab Investig 95:342–350
29. Lee R, Reese C, Bonner M, Tourkina E, Hajdu Z, Riemer EC, Silver RM, Visconti RP, Hoffman S (2014) Bleomycin delivery by osmotic minipump: similarity to human scleroderma interstitial lung disease. Am J Physiol-Lung Cell Mol Physiol 306:L736–L748. PMID: 24583879
30. Tan T, Watts SW, Davis RP (2011) Drug delivery: enabling technology for drug discovery and development, iPRECIO micro infusion pump: programmabe, refillable and implantable. Front Pharmacol 44(2):1–13
31. Kim SH, Chung JM (1992) An experimental model for peripheral neuropathy produced by segmental spinal nerve ligation in the rat. Pain 50(3):355–363
32. Hogan Q, Sapunar D, Modric-Jednacak K, McCallum B (2004) Detection of neuropathic pain in a rat model of peripheral nerve injury. Anesthesiology 101(2):476–487
33. Austin PJ, Wu A, Moalem-Taylor G (2012) Chronic Constriction of the Sciatic Nerve and Pain Hypersensitivity Testing in Rats. J Vis Exp (61):e3393. 1–6
34. Attal N, Jazat F, Kayser V, Guilbaud G (1990) Further evidence for 'pain-related' behaviours in a model of unilateral peripheral mononeuropathy. Pain 41(2):235–251. PMID: 2164179
35. Tanck EN, Kroin JS, McCarthy RJ, Penn RD, Ivankovich AD (1992) Effects of age and size on development of allodynia in a chronic pain model produced by sciatic nerve ligation in rats. Pain 51:313–316
36. Rigali A, Di Loreto VE (2009) Experimental surgical models in the laboratory rat. CRC Press, Boca Raton, FL
37. Jespersen B, Knupp L, Northcott CA (2012) Femoral arterial and venous catheterization for blood sampling, drug administration and conscious blood pressure and heart rate measurements. J Vis Exp 59(e3496):1–8

38. Qi Z, Whitt I, Mehta A, Jin J, Zhao M, Harris RC, Fogo AB, Breyer MD (2004) Serial determination of glomerular filtration rate in conscious mice using FITC-inulin clearance. Am J Physiol Renal Physiol 286:F590–F596
39. Moravek J, Schuck O, Hatala M, Priborsky J (1987) Preclinical modeling of changes in drug kinetics caused by acute renal failure in rats. J Pharmacokinet Biopharm 15:15–23
40. Hoke TS, Douglas IS, Klein CL, He Z, Fang W, Thurman JM, Tao Y, Dursun B, Voelkel NF, Edelstein CL, Faubel S (2007) Acute renal failure after bilateral nephrectomy is associated with cytokine-mediated pulmonary injury. J Am Soc Nephrol 18:155–164. PMID: 17167117
41. Naud J, Michaud J, Beauchemin S, Hebert MJ, Roger M, Lefrancois S (2011) Effects of chronic renal failure on kidney drug transporters and cytochrome P450 in rats. Drug Metab Dispos 39:1363–1369
42. Reis LO, Sopena JM, Favaro WJ, Martin MC, Simao AF, Reis RB, Andrade MF, Domenech JD, Cardo CC (2011) Anatomical features of the urethra and urinary bladder catheterization in female mice and rats. An essential translational tool. Acta Cir Bras 26(2):106–110
43. Oliveira PA, Pires MJ, Nobrega C, Arantes-Rodrigues R, Calado AM, Carrola J, M Ginja M, Colaco A (2009) Technical report: technique of bladder catheterization in female mice and rats for intravesical instillation in models of bladder cancer. scand. J Lab Anim Sci 36(1):5–9
44. St Clair MB, Sowers AL, Davis LA, Rhodes LL (1999) Urinary bladder catheterization of female mice and Rats. Contemp Top Lab Anim Sci 38(3):78–79
45. Vestergaard B, Agerso H, Lykkesfeldt J (2013) Nephrectomized and hepatectomized animal models as tools in preclinical pharmacokinetics. Basic Clin Pharmacol Toxicol 113:75–86
46. Nevzorova YA, Tolba R, Trautwein C, Liedtke C (2015) Partial hepatectomy in mice. Lab Anim 49(S1):81–88
47. Andersen KJ, Knudsen AR, Kannerup A, Sasanuma H, Nyengaard JR, Hamilton-Dutoit S, Erlandsen EJ, Bo Jorgensene B, Mortensen FV (2013) The natural history of liver regeneration in rats: description of an animal model for liver regeneration studies. Int J Surg 11(9):903–908
48. Bedirli A, Sakrak O, Soyuer I, Muhtaroglu S (2004) Portosystemic shunt prevents apoptosis in rat intestinal mucosa caused by total hepatic ischemia. Eur Surg Res 36:293–299
49. Kogure K, Suzuki M (1992) Effects of hepatic inflow occlusion on changes in plasma potassium, histamine, and norepinephrine in rats. Circ Shock 36:290–298
50. Aller MA, Lorente L, Prieto I, Moquillaza LM, Arias J (2009) Hepatectomies in the rat: a look at the caudate process through microsurgery. Dig Liver Dis 41:695–699
51. Azoulay D, Astarcioglu I, Astarcioglu H, Lemoine A, Majno P, Bismuth H (1997) A new technique of one-stage total hepatectomy in the rat. Surgery 121:219–222
52. Yamaguchi Y, Bollinger RR, DeFaria E, Landis B, Quarfordt S (1989) A simplified single stage total hepatectomy in the rat with maintenance of gastrointestinal absorptive function. Hepatology 9:69–74
53. Engelbrecht GH, Hickman R, Kahn D (1999) One-stage total hepatectomy in the rat using microvascular anastomoses. Microsurgery 19:95–97. PMID: 10188833
54. Ping H, Zhen-Fu C, Shao-Qing X, Ming L, Jian W, Guo-Qing Z (2001) An in vivo rat model for assessment of extrahepatic metabolism. J Pharmacol Toxicol Methods 45:181–185
55. Gu J, Lu K, Xia P, Tang M, Dai Q, Ma D (2009) Pharmacokinetics of propofol and extrahepatic UGT1A6 gene expression in anhepatic rats. Pharmacology 84:219–226
56. Souza VR, Mendes E, Casaro M, Tada A, Antirio AF, Oliveira FA, Ferreira CM (2019) Description of ovariectomy protocol in mice. Methods Mol Biol 1916:303–309. PMID: 30535707
57. Hayward AM, Lemke LB, Bridgeford EC, Theve EJ, Jackson CJ, Cunliffe-Beamer TL (2006) Biomethodology and surgical techniques. The mouse in biomedical research. Academic Press, Elsevier, Boston, MA
58. Waynforth HB, Flecknell PA (1992) Experimental and surgical techniques in the rat, 2nd edn. Academic Press, New York
59. Paxinos G, Watson C (2009) The rat brain in stereotaxic coordinates, compact, 6th edn. Elsevier, Academic Press, Amsterdam

60. Mostany R, Portera-Cailliau C (2008) A craniotomy surgery procedure for chronic brain imaging. J Vis Exp. https://doi.org/10.3791/680
61. Fornari RV, Wichmann R, Atsak P, Atucha E, Barsegyan A, Beldjoud H, Messanvi FC, Thuring CM, Roozendaal BR (2012) Rodent stereotaxic surgery and animal welfare outcome improvements for behavioral neuroscience. J Vis Exp 59(e3528):1–4
62. Geiger BM, Frank LE, Caldera-Siu AD, Pothos EN (2008) Survivable stereotaxic surgery in rodents. J Vis Exp 20(e880):1–3
63. Poole EI, McGavin JJ, Cochkanooff N, Corssby KM (2019) Stereotaxic surgery for implantation of guidecannulas for microinjection into the dorsomedialhypothalamus in young rats. MethodsX 6(2019):1652–1659
64. Zausinger S, Baethmann A, Schmid-Elsaesser R (2002) Anesthetic methods in rats determine outcome after experimental focal cerebral ischemia: mechanical ventilation is required to obtain controlled experimental conditions. Brain Res Brain Res Protoc 9:112–121. [PubMed: 12034330]
65. Durukan A, Tatlisumak T (2007) Acute ischemic stroke: overview of major experimental rodent models, pathophysiology, and therapy of focal cerebral ischemia. Pharmacol Biochem Behav 87:179–197. [PubMed: 17521716]
66. Thomas CS (2005) Rodent models of focal stroke: size, mechanism and purpose. NeuroRx 2:396–409. [PubMed: 16389304]
67. Krueger K, Busch E (2002) Protocol of a thromboembolic stroke model in the rat: review of the experimental procedure and comparison of models. Investig Radiol 37(11):600–608. PMID: 12393972
68. Park S, Shin J, Hong Y, Kim S, Lee S, Park K, Lkhagvasuren T, Lee SR, Chang KT, Hong Y (2012) Forced exercise enhances functional recovery after focal cerebral ischemia in spontaneously hypertensive rats. Brain Sci 2:483–503. PubMed: 24961257
69. Rupadevi M, Parasuraman S, Raveendran R (2011) Protocol for middle cerebral artery occlusion by an intraluminal suture method. J Pharmacol Pharmacother 2(1):36–39. https://doi.org/10.4103/0976-500X.77113:10.4103/0976-500X.77113. PMID: 21701645
70. Lee S, Lee M, Yunyung H, Won J, Lee Y, Kang S, Chang K, Hong Y (2014) 25206884 Middle cerebral artery occlusion methods in rat versus mouse models of transient focal cerebral ischemic stroke. Neural Regen Res 9(7):757–758. PMID: 25206884
71. Zhang L, Zhang RL, Jiang Q, Ding G, Chopp M, Zhang ZG (2015) Focal embolic cerebral ischemia in the rat. Nat Protoc 10(4):539–547. PMID: 25741989
72. Sommer CJ (2017) Ischemic stroke: experimental models and reality. Acta Neuropathol 133:245–262. https://doi.org/10.1007/s00401-017-1667-0
73. Zaki Ghali MG (2018) Microsurgical technique for tracheostomy in the rat. Methods X 5:61–67. PMID: 29511643
74. Irazuzta J, Hopkins P, Gunnoe P, Brittain E (1997) Simple method of multipurpose airway access through percutaneous tracheostomy in rabbits (Oryctolagus cuniculus). Lab Anim Sci 47 (4):411–413. PMID: 9306316
75. Boon MS, Daniero JJ, , Saxena S, Balceniuk M.(2015). Tubeless tracheotomy for survival airway surgery in the leporine model. Laryngoscope 125(3):680–684
76. Miller AL, Wright-Williams SL, Flecknell PA, Roughan JV (2012) A comparison of abdominal and scrotal approach methods of vasectomy and the influence of analgesic treatment in laboratory mice. Lab Anim 46:304–310. https://doi.org/10.1258/la.2012.012078
77. Lavers AE, Swanlund DJ, Hunter BA, Tran ML, Pryor JL, Roberts KP (2006) Acute effect of vasectomy on the function of the rat epididymal epithelium and vas deferens. J Androl 27 (6):826–836. https://doi.org/10.2164/jandrol.106.000745