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Experimental Animal Models of Human Diseases

An Effective Therapeutic Strategy

Edited by Ibeh Bartholomew

EXPERIMENTAL ANIMAL MODELS OF HUMAN DISEASES - AN EFFECTIVE THERAPEUTIC STRATEGY

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Meet the editor

Ibeh Bartholomew, PhD, is currently the acting director/head of Medical Biotechnology Department, National Biotechnology Development Agency, Abuja, Nigeria. He holds a doctoral degree in Medical Biochemistry/Immunochemistry. He has obtained several grants and awards. Dr. Ibeh has served as an external examiner and supervised over 30 university students on various HIV research topics. Though his work on HIV serodiscordant infection in blacks is widely acknowledged, his current research interest is on development of efficient immunodeficient mouse models using CRISPR/Cas9-mediated genome engineering technique. He is a member of the International Union of Biochemistry and Molecular Biology and the Nigerian Society of Biochemistry and Molecular Biology.

Contents

Preface

The concept of animal models dealt in this book discusses appropriate mechanistic models for several prevalent human diseases. Animal models are imperative for preclinical trials, disease pathway and pathological elucidation, new drug development, environmental testing, and vaccine construction. Against any odds, the use of animals (from primates to murine), especially rat and mouse, seems indispensable in today's scientific world. The book presents reproducible experimental approach using animal models for the study of human diseases with measurable equivalence to that of humans. It also presents models of high human predictive value. Despite current insights and promising technologies, no scientific method can at this time fully address the limitation(s) of using animal models as complete surrogates for humans. The organization of the book is unique and organic because it brings to the fore the implications, pros and cons, and choice of experimental animals in research design for scientists and clinicians. An important objective of the book is to provide a wider readership of scientist, clinicians, and ethicist. It also provides a multidisciplinary approach to modeling animals for the study of human diseases and on the basics in choosing animal models. The need to qualify and/or standardize animal models is evident at the least to specifically and reproducibly produce a disease process or condition that in multiple important aspects corresponds to the human disease or condition of interest. The question of whether or not there should be a standardized or qualification model is one of the main current controversies in developing animal models for human diseases. This is also addressed in the chapter contributions.

Each chapter of this book further discuses new-generation model of animals for human disease study. Chapter 1 is the introduction describing the major contributions of animal models to modern medicine. The book therefore is divided into three (3) sections: the first section deals with diabetes and obesity in five (5) chapters. Chapters 2 and 3 "Animal Models of Diabetic Retinopathy" x-ray the suitability of available animal models (from chemically induced to transgenic) for therapeutic drug screening and further understanding of the molecular and cellular pathological processes involved in DR. The availability of a holistic animal model that reproduces the pathological progression of human DR is presented. Chapter 4 "Animal Models of Central Diabetes Insipidus: Oxytocin and Low-Sodium Diets as Complementary Treatments" describes the animal model of hereditary human central diabetes insipidus (CDI); the authors asserted that the hydromineral characteristics of these animals do not seem to be fully comparable with an acquired CDI animal model such as transgenic rat TGR(ASrAOGEN)680, TGR(mREN2)27, etc.; additionally, the variations in the neurobiological system evidently predict individualized therapies desirable to improve the quality of life of the patients. Chapter 5 "Animal Models of Obesity: The Potential Role of Nonalcoholic Hepatic Steatosis" describes the mechanisms involved in the progression from NAFLD to

NASH, which are not yet clearly elucidated, as some models have shown unexpected outcomes such as severe malnutrition or the absence of obesity markers and IR after the use of minimal-change disease (MCD) therapies and drugs, respectively. Thus, the importance of evaluating different animal models of obesity is to induce the profile of NAFLD and NASH disease in humans, assessing their mechanisms of action. Chapter 6 "Rodent Models of Metabolic Disorders: Obesity and Diabetes" emphasizes various metabolic disorders that are not only congenital but also can be acquired or coexistent with both types. The chapter describes fully transgenic animal models available, focusing on global emerging pathologies, obesity, diabetes, and metabolic syndrome. The second section with five (5) chapters discuses inflammatory and viral infections. Chapter 7 "Animal Models of Double Incontinence: Fecal and Urinary" considers the use of animals as models to understand pathogenesis, diagnosis, and management of double incontinence (DI), a complex disease of urinary and anal abnormality. The chapter points to animal models as an imperative to train surgeons for perineal tear repair surgery. Chapter 8 "Relevance of the CDE and DDC Mouse Models to Study Ductular Reaction in Chronic Human Liver Diseases" discusses ductal reaction (DR) observed in chronic liver disorders important in hepatic cell restoration, fibrosis, or carcinogenesis. In humans, observational studies are available, but experimental manipulations and lineage tracing are impossible. The authors thus presented and compared two (2) animal models from a practical point of view describing the pathophysiological mechanisms at play in each model and their experimental procedures. Chapter 9 "Experimental Animal Models of HIV/AIDS for Vaccine Trials" details current animal models for invasive investigation of HIV disease mainly in preclinical evaluation of drugs and vaccines and success achieved. A comparison of nonhuman primate and murine model immunogenetics in response to recombinant viruses as options of animal models available for research was tabulated. Chapter 10 "Animal Inhalation Models to Investigate Modulation of Inflammatory Bowel Diseases" examines the suitability of animal inhalation/smoke exposure models for assessing the contrary effects of cigarette smoke (CS) on ulcerative colitis (UC) and Crohn's disease (CD). It crystallizes various technical inhalation approaches, in the context of mouse disease models of IBD. Chapter 11 "The Use of Animal Models in the Study of Colitis" presents the current concept on animal models of inflammatory bowel diseases, describing genetically modified, chemical induction, cell transfer, and spontaneous inflammation models. These models are crucial for the understanding of inflammatory bowel diseases, development of alternative treatments, and more effective therapeutic agents, thus contributing to the control of the disease. The last section with six (6) chapters deals with stress, neurology, and regeneration. Chapter 12 "Evaluation of Animal Models Suitable for Hair Research and Regeneration" evaluates animal models for hair regeneration (alopecia), a frequent dermatological disease with limited treatment options, as well as presents current experimental data from suitable animal model. Chapter 13 "Animal Models of Rheumatoid Arthritis" focuses on the most common in vivo models used for the study of RA, including those related with genetic, immunological, hormonal, and environmental interactions. The chapter considers the CIA model to be the most successful in generating arthritis using type II collagen and adjuvants and evaluating therapeutic compounds both intra-articularly and systemically. Chapter 14 "Animal Models for Chronic Stress-Induced Oxidative Stress in the Spleen: Role of Exercise and Catecholaminergic System" explains an experimental approach including research methodology and evaluated results for combined animal model of chronic social isolation and long-term daily treadmill running in rats and "cross-stressor adaptation hypothesis." The model seems to be a highly predictive animal model for research on the ther-

apeutic role of exercise in human disease caused by chronic stress. Chapter 15 "Studying Side Effects of Tyrosine Kinase Inhibitors in a Juvenile Rat Model with Focus on Skeletal Remodeling" sorts out important and effective treatment for chronic myeloid leukemia (CML) targeting the causative oncogenic TK BCR-ABL1 using tyrosine kinase inhibitor (TKI) imatinib in animal model. As TKI treatment might be necessary for a lifetime, long-term side effects exerted on bone and other developing organs in children are of major concern and are not yet studied systematically; the chapter described a new juvenile rat model to face this challenge and presented extensive experimental evidence. Chapter 16 "Animal Models of Fetal Medicine and Obstetrics" demonstrates the fundamental mechanisms occurring in fetal medicine and obstetric diseases, such as intrauterine growth restriction, preeclampsia, and gestational diabetes. These conditions differ in relation to animal characteristics (size, number of fetuses, placenta barrier type, etc.) and do not exactly mirror the human condition; different pregnant animal models (mice, rats, guinea pigs, chinchillas, rabbits, sheep, and pigs), however, are available as described with respect to advantages and limitations for careful choice of experimental model. Chapter 17 "Animal Model of Parkinson Disease: Neuroinflammation and Apoptosis in the 6-Hydroxydopamine-Induced Model" elucidates the animal-to-human replication of Parkinson disease (PD)-induced 6-hydroxydopamine (6-OHDA) model, which will aid in PD therapeutic design.

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Introduction

Chapter 1

Introductory Chapter: Animal Models for Human Diseases, a Major Contributor to Modern Medicine

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Additional information is available at the end of the chapter

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1. Defining animal models

The use of animals as experimental models for human diseases is currently seen as an imperative in understanding the causes, biology, and prevention of diseases. Animal models over the years have been used extensively in biomedical field since the early 1980s [1]. Current understanding of these models tends to be a specific combination of an animal species, cell, tissue, organ, gene, or a challenge agent, and its directed route of exposure to produce and/or mimic a disease process or pathological condition in multiple important aspects approximating or corresponding to the human disease scenario or condition of interest. An important fact is that the models have to be reproducible.

It is obvious that laboratory animals play a crucial role in scientific research, discovery, and technological advances and in a substantive manner improve the lives of people and other useful animals. It may suffice to say that animals are used as models to study human biology and diseases and as test subjects for the development and testing of drugs, vaccines, and other biologicals (i.e., antibodies, hormones, etc.) to enhance and promote human health. This book, therefore, was written for medical practitioners, drug/therapeutic agent developers, biomedical scientists/bioengineers research students, bioethicists, behavioral scientists, and the general public who aspire to enrich their understanding of human diseases and development of effective therapeutics using animal models as clearly defined herein. Over the last century, almost all medical knowledge, treatment regimes, and medical device development have involved research using animals. Disease experimentation using animal models may be a deliberate design or an inevitable choice which possible due to the common descent of all organisms which even in the face of evolution many of them conserve their metabolic, developmental, and genetic material.

2. Trendy outlook

Animals were used to study human physiology and anatomy in the second century AD as documented by a Greek physician and philosopher, Galen, using mainly apes and pigs [2]. Galen applied his findings directly to humans without considering taxonomic relatedness. It was until the late sixteenth century that this error began to be recognized. Previously in 1865, a French physiologist by name Claude Bernard published the first book, *An Introduction to the Study of Experimental Medicine* [3], advocating the use of chemical and physical induction of disease in animals for biomedical research. Around that same time, Louis Pasteur in France and Robert Koch in Germany introduced the concept of specificity into medicine and the "germ theory of disease."

It is noted that from 1901, two-thirds of the Nobel Prizes in medicine have relied majorly on animal models for their research, more recently seven (7) of the last ten (10) were animal model-based breakthroughs (**Table 1**) [4]. Researchers now rely heavily on development of animal models to explore all areas of medical science specifically in the assessment of pathogenic mechanisms, diagnostic and therapeutic procedures, vaccine development, nutrition, metabolic diseases, and the efficacy of novel drug development as captured in this book.

A typical instance in the trending use of animals as disease model is the transition from nonhuman primates such as chimpanzee to mouse/rat models in diabetic retinopathy (Chapter 2/3) and in HIV research (chapter 9) [5]. Larger animals are deemed relatively closest to humans (e.g., chimpanzee). However, these animals have become increasingly difficult to maintain and to handle; besides their costly nature. A more disturbing fact is that most human diseases could not be replicated in them, and the causative human agent hardly infects these nonhuman primates as well as difficulty in development of human symptoms and therapeutic responses. Scientists, therefore, resulted to started developing simpler and effective models most especially transgenic (humanized) mouse models [6] that mimic human responses to study and understand various aspects of infectious agents, pathogenesis, disease progression, nature of protective immunity and vaccine development. An ideal animal model for human disease research should possess certain characteristics as a prerequisite for a standard model. The chapters presented in this book elucidate the following notable characteristics of a chosen animal model:

(i) A close relative or closely associated with the host tissue distribution, disease progression, and similar route of infection, if not identical.

(ii) Disease course should be relatively shorter in the animal model, to allow for completion of the efficacy test in reasonable time, permitting rapid transition to human clinical testing.

(iii) Despite the differences in genetic makeup of humans and animals, there should be sufficient disease correlation and pathological equivalence.

(iv) The model should be easy to maintain, work with, easily available in adequate number, relatively inexpensive, and free of regulatory constraints.

Year	Nobel Laureate	Animal model	Contribution to modern medicine
2015	William C. Campbell and Satoshi Ōmura and Youyou Tu	Mice, dogs, sheep, cattle, chickens, monkeys	Campell and Omura for discoveries concerning a novel therapy against infections caused by roundworm parasites and Youyou Tu for her discoveries concerning a novel therapy against malaria
2014	John O'Keefe and May-Britt and Edvard I. Moser	Rats	Discoveries of cells that constitute a positioning system in the brain (an inner GPS)
2013	James E. Rothman	Hamsters	Discoveries of machinery regulating vesicle traffic, a major transport system in our cells
2013	Thomas C. Südhof	Mice	Discoveries of machinery regulating vesicle traffic, a major transport system in our cells
2012	Sir John B. Gurdon	Frogs, mice	For the discovery that mature cells can be reprogrammed to become pluripotent
2012	Shinya Yamanaka	Frogs, mice	For the discovery that mature cells can be reprogrammed to become pluripotent
2011	Bruce A. Beutler	Mice	Discoveries concerning the activation of innate immunity
2011	Jules A. Hoffmann	Flies	Discoveries concerning the activation of innate immunity
2011	Ralph M. Steinman	Mice	For his discovery of the dendritic cell and its role in adaptive immunity
2010	Robert G. Edwards	Rabbits	The development of in vitro fertilization
2009	Carol W. Greider	Protozoan, mouse, frog	Discovery of how chromosomes are protected by telomeres and the enzyme telomerase
2009	Elizabeth H. Blackburn	Protozoan, mouse	Discovery of how chromosomes are protected by telomeres and the enzyme telomerase
2009	Jack W. Szostak	Protozoan	Discovery of how chromosomes are protected by telomeres and the enzyme telomerase
2008	Harald zur Hausen	Hamster, mouse, cow	Discovery of human papilloma viruses causing cervical cancer
2008	Françoise Barré-Sinoussi	Monkey, chimpanzee, mouse	Discovery of human immunodeficiency virus
2008	Luc Montagnier	Monkey, chimpanzee, mouse	Discovery of human immunodeficiency virus
2007	Mario R. Capecchi	Mouse	Discoveries of principles for introducing specific gene modifications in mice by the use of embryonic stem cells
2007	Sir Martin J. Evans	Mouse, chick	Discoveries of principles for introducing specific gene modifications in mice by the use of embryonic stem cells
2007	Oliver Smithies	Mouse	Discoveries of principles for introducing specific gene modifications in mice by the use of embryonic stem cells

Year	Nobel Laureate	Animal model	Contribution to modern medicine
2006	Andrew Z. Fire	Nematode roundworm	Discovery of RNA interference—gene silencing by double-stranded RNA
2006	Craig C. Mello	Nematode roundworm	Discovery of RNA interference—gene silencing by double-stranded RNA
2005	Barry J. Marshall	Piglet	Discovery of the bacterium *Helicobacter pylori* and its role in gastritis and peptic ulcer disease
2004	Richard Axel	Mouse, *Drosophila* (fruit flies)	Discoveries of odorant receptors and the organization of the olfactory system
2004	Linda B. Buck	Mouse	Discoveries of odorant receptors and the organization of the olfactory system"
2003	Paul C. Lauterbur	Clam, mouse, dog, rat, chimpanzee, pig, rabbit, frog	Discoveries concerning magnetic resonance imaging (MRI)

Table 1. Contributions of lab animals to biomedical research (adapted from Foundation for Biomedical Research [4]).

3. Expert view vs. common sense

Many scientific articles and books written in recent times have attempted to bridge the gap between effective animal model and the equivalent human pathological replication. It may seem controvertible on the acceptance of animal models as equivalent to human testing. As this may not apply in all cases, however, there are notifiable instances where animal models may substantially suffix. This is exemplified by the US FDA Animal Efficacy Rule (also known as Animal Rule) which applies to development and testing of drugs and biologicals in animal models to reduce or prevent serious/life-threatening conditions caused by exposure to lethal or permanently disabling toxic agents (chemical, biological, radiological, or nuclear substances) and in instances where human efficacy trials are not feasible or ethical [7].

In this book, animal models of global disease of interest were extensively discussed. The seventeen (17) chapters presented by experienced experts in the field detailed the practical and theoritical steps in animal model development and various approaches to achieve and/or develop specific models X-raying their limitations, interspecies variations, and comparison of different models (chemically induced, biological, xenograft, syngeneic, and genetically modified) which best suited for good experimental results. The book is designed to assist researchers make a beneficial choice of experimental animal relevant to their research design, hypothesis, and expected results. The chapters as much as intriguing presents scientific bases for choice of experimental animals on notable and widely researched global disease of interest ranging from central diabetes insipidus, diabetic retinopathy, hair research and regeneration, skeletal remodeling, ductular reaction in chronic human liver diseases, induced oxidative stress, inflammatory bowel diseases, and double incontinence HIV/AIDS to neuroinflammatory disease.

One of the factors impeding the translation of knowledge from preclinical to clinical studies has been the limitations of in vivo disease models in which specific animal models discussed

in the chapters tend to address. Regulatory authorities, however, require vaccine candidates to undergo preclinical evaluation in animal models before they enter the clinical trials in humans [8]. The overarching goal of a new vaccine is to stimulate the immune system to elicit an effective response against the pathogen it is being designed for; currently, experts have noted that no alternatives to the use of live animals exist for evaluation of the vaccine response despite advances in computational sciences for the search of an in silico model. One of the issues bordering scientific expediency in the development and use of animal models is on bioethics and animal rights. Thus, qualification and ethical consideration need appropriate clarification.

4. Need for standardization of model

There is a need to qualify and/or standardize animal models. Qualification of an animal model implies that a specific animal species given a specific challenge agent by a specific route produces a disease process or condition that in multiple important aspects corresponds to the human disease or condition of interest [9]. The experts' discussion (chapters) presents the need for standardization or qualification of models. The question of whether or not there should be a standardized or qualified model is the basis of one of the main current controversies in developing animal models for human diseases. Having a standardized animal model relates to the appropriate research use and may be regarded as a complete and precise description of intended use and application of the qualified animal model in drug development and regulatory processes. The process must specify the details necessary to replicate the model. Other criteria may be summarized as follows:

(a) Known and identified animal thus proposed for use

(b) Known and characterized challenge agent

(c) Procedural information for the challenge agent exposure

(d) Identification of the primary and secondary endpoints

(e) Potential triggers for intervention

5. Next-generation models

An interesting aspect of the book is the respective discussion in each chapter of next-generation models and how perceived limitations of current animal models could be obviated. Recent animal model research has focused on the (i) refinement of existing models and the development of new ones, (ii) use of these models to research key questions about the disease pathology, and (iii) key findings with these models testing therapeutic and vaccine concepts [10]. Margaret Hamburg wrote "We must bring 21st century approaches to 21st century products and problems" [11]. This scientific era entails rapid and unprecedented development of enabling biotechnologies with great promise for the future.

6. What this book argues

As implied above, the concept of animal models dealt with in this book discusses appropriate mechanistic models for selected prevalent human diseases. An animal model is imperative for preclinical trials, disease pathway and pathological elucidation, new drug development, and vaccine construction. Against any odd, the use of animals especially rat and mouse seems indispensable in today's scientific world. The book presents reproducible experimental approach using animal models for human diseases with measurable equivalence to that of humans. It also presents models of high human predictive value. Despite the current insights and promising technologies, no scientific method can at this time fully address the limitation(s) of using animal models as complete surrogates for humans.

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Section 2

Diabetics and Obesity

Chapter 2

Animal Models of Diabetic Retinopathy (Part 1)

Larissa H.C. Tang, Ian Y.H. Wong and Amy C.Y. Lo

Additional information is available at the end of the chapter

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Abstract

Diabetic retinopathy (DR) is one of the leading causes of preventable vision impairment and blindness in the working-age population worldwide. Numerous animal models have been developed for therapeutic drug screening and to further our understanding of the molecular and cellular pathological processes involved in DR. In this book chapter, we describe the cellular, molecular and morphological features of mouse models of DR as well as their respective advantages and limitations. To date, no animal model can holistically reproduce the pathological progression of human DR; most only display early or advanced lesions of DR. However, a thorough understanding of genotypic and phenotypic expressions of existing models will facilitate researchers' selection of the appropriate model to simulate their desired clinical scenarios.

Keywords: animals, blood glucose, blindness, diabetic complications, diabetes mellitus/pathology/physiopathology, neovascularization, proliferative, retinal vessels

1. Introduction

Diabetes mellitus is a growing epidemic and a major contributor to the global burden of disease [1]. Insulin deficiency leading to hyperglycemia occurs in type 1 diabetes (T1D or insulin-dependent diabetes mellitus) as a result of autoimmune destruction of pancreatic beta islet cells. Type 2 diabetes (T2D or non-insulin-dependent diabetes mellitus) is characterized by insulin resistance, often due to physical inactivity and obesity, and may progress to impaired insulin production. T1D is unpreventable as of current understanding, while T2D, the more common type of the two, is preventable.

Diabetic retinopathy (DR) is one of the most common microvascular complications of diabetes and one of the leading causes of preventable vision impairment and blindness in the

working-age population worldwide. It can be broadly classified as non-proliferative diabetic retinopathy (NPDR) or proliferative diabetic retinopathy (PDR). According to the AAO International Clinic DR Disease Severity Scale, NPDR is further subdivided into mild, moderate or severe NPDR, depending on the extent of microaneurysm, intraretinal hemorrhage, venous beading and intraretinal microvascular abnormality (IRMA) formation [2]. With worsening retinal ischemia and increasing microvascular damage, NPDR may progress to PDR, which is characterized by the presence of neovascularization and/or vitreous or preretinal hemorrhage [2]. Severe cases of PDR may result in retinal edema, tractional retinal detachment and neovascular glaucoma. Diabetic maculopathy or macular edema, the most common cause of vision loss, may also arise at any stage of DR [3].

DR-associated visual impairment results in large socioeconomic costs for both the society and individuals. This calls for effective screening methods and increased efforts to understand the pathophysiological progression and to look for effective treatment strategies using both experimental animal models and clinical trials.

2. Pathological features of human diabetic retinopathy

Although DR has long been considered as a hyperglycemia-mediated microangiopathy, it has been recognized as a neurodegenerative process in view of the presence of neurodegenerative abnormalities preceding clinically apparent microvascular changes. Numerous cellular and molecular changes reflective of the DR pathogenesis have been identified, though the multifactorial nature of DR makes it challenging to clearly identify clinically relevant pathogenic pathways implicated in each stage of retinopathy. The common clinical, cellular, molecular features and functional changes of human DR are summarized in **Table 1**.

2.1. Cellular and molecular features

The DR hallmark lesions of capillary basement membrane (BM) thickening [4, 5] and pericyte loss [6] or apoptosis [7, 8] have been well described in human patients. Other microvascular changes include blood-retinal barrier (BRB) disruption (as evidenced by fluorescein leakage) [9] and the presence of acellular capillaries [6]. In regards to hemodynamics, it has typically been reported that retinal blood flow is increased in NPDR [10–12]. Conversely, in PDR, the nature of retinal blood flow changes appears to be dependent on the degree of non-perfusion and the pathological features present, with no marked increases in blood flow in cases with arterial narrowing [9, 10, 13]. As persistent inflammation is also implicated in DR, studies have demonstrated increased leukostasis (increased leukocyte entrapment and leukocyte endothelial cell adhesion) in diabetic retinae, perhaps resulting from increased expression of adhesion molecules (e.g. ICAM-1) in human DR [14].

Histologically, retinal thinning, particularly thinning of the pericentral total retinal thickness and the retinal nerve fiber layer (RNFL), is present in both T1D and T2D patients with no DR, NPDR or pre-proliferative DR [15–19]. Studies analyzing individual intraretinal layer thicknesses showed thinning of the ganglion cell layer (GCL), RNFL, inner plexiform layer (IPL)

Features	Non-proliferative diabetic retinopathy (NPDR)	Proliferative diabetic retinopathy (PDR) (in addition to features of NPDR)
Clinical features [2]	• Intraretinal hemorrhages • Microaneurysms • Cotton wool spots • Venous beading • IRMAs (e.g. vessel tortuosity, venous loops, vessel dilatation)	• Neovascularization • Retinal or vitreous hemorrhage • Tractional retinal detachment (advanced) • Neovascular glaucoma (advanced) • Retinal edema (can occur at any stage of DR)
Cellular and molecular features	• RGC loss [20] • Reactive gliosis (overexpression of GFAP expression in Müller cells) [21] • Activated microglia [22] • Decrease in retinal thickness (total, RNFL, GCL, INL, IPL) [15–19] • Pericyte loss [6] or apoptosis [8] • Leukostasis [14] • Capillary BM thickening [4, 5] • Acellular capillaries (associated with microaneurysms) [6] • BRB breakdown [9] • Capillary non-perfusion and obliteration • Increased retinal blood flow [10–12] • Decreased arteriole-to-venule ratio (decreasing with increasing DR severity) [29]	• Retinal blood flow may be increased [11] or equivalent to that of normal patients [9, 10, 13] • Infiltration of activated microglia into subretinal space (diabetic maculopathy) [22]
Functional changes (ERG)	• Increased OP peak latencies [25] • Reduced OP amplitudes [23, 25] • Delayed OP implicit times [23–25] • Increased b-wave implicit time [26] • (Reduced b-wave amplitude) [30]	• Reduced b-wave amplitude [25, 27, 28]

Table 1. Overview of common clinical, cellular, molecular features and functional changes of human DR.

and inner nuclear layer (INL) in patients with minimal DR as compared with controls, while such a difference was not observed in diabetic patients without DR [16, 19]. Numerous studies have also documented evidence suggestive of increased retinal ganglion cell (RGC) loss in DR [20].

In addition to neural apoptosis, reactive gliosis is another prominent feature of DR. Expression of glial fibrillary acidic protein (GFAP), an intermediate filament protein expressed by astrocytes, is normally confined to the proximal retina in non-diabetic retinae. In DR, there is aberrant overexpression of GFAP by Müller cells spanning across the entirety of Müller cell processes [21]. Microglial cells are also activated in NPDR [22]. In PDR, the microglia surrounds the neovascularization area in the vitreous, with subsequent infiltration and migration of activated microglia into the subretinal space in cases with diabetic macular degeneration [22].

2.2. Electrophysiological alterations

Electroretinographic (ERG) alterations have long been documented in diabetic patients prior to the development of visible lesions of retinopathy. Delay in implicit times of oscillatory

potential (OPs), particularly OP1, precede retinopathy development [23, 24]. The OPs are generated by inner retinal neurons and are often considered to be reflections of feedback circuits between amacrine and bipolar cells and/or circuits between amacrine and ganglion cells. Eyes with NPDR display a reduction in OP amplitudes [24, 25] and an increase in OP peak latencies [25]. There is some discrepancy regarding the onset of changes in b-wave responses, which are largely generated by depolarizing bipolar cells with some contribution from Müller cells. B-wave implicit times appear to be increased even in early stages of DR [26] while reductions in b-wave amplitudes have been suggested to be predominantly found in eyes with PDR [25, 27, 28]. Changes in OP amplitude and implicit times have also been suggested to be a reflection of the severity and prospective progression of DR [24, 25, 27].

3. Models of diabetic retinopathy

Animal models of DR can be broadly classified into (1) diabetic models by pharmacological induction, diet induction or genetic manipulation and (2) non-diabetic models of proliferative retinopathy and angiogenesis. To date, no diabetic models fully develop end-stage retinopathy, arguably due to the short lifespan of animals and differing anatomical structure from humans. Non-diabetic models are thus used to mimic the pathophysiology of end-stage DR, specifically the proliferative pathogenesis and neovascularization in the retinal vasculature. These models, however, are not DR-specific, and display phenotypes common to other conditions with retinal neovascularization. While animal models are useful for drug testing and furthering our understanding of the molecular and cellular pathological processes involved in DR, no single model can holistically reproduce the pathological features of human DR. BRB breakdown, for example, is exhibited in numerous animal models. Yet macular edema resulting from the increase in permeability of retinal capillaries is seldom observed. Judicious evaluation and selection of models according to research objectives is critical to avoid inappropriate translation of experimental findings to the clinical situation. An overview of existing models used to study DR is summarized in **Table 2**. The cellular, molecular and morphological features of existing animal models of DR are described in Section 4 of this chapter and Section 1 of the following chapter (Animal Models of Diabetic Retinopathy Part 2).

3.1. Diabetic models

3.1.1. Pharmacological induction of diabetes

Pharmacological induction of diabetes is most commonly performed using streptozotocin (STZ), a naturally occurring antibiotic in *Streptomyces acromogenes*, or alloxan, a pyrimidine derivative. Both chemicals destroy the β-cells of the pancreatic islets. STZ is preferentially used over alloxan due to its greater stability and more preferable chemical properties [31]. T1D or T2D can be induced by varying the dosage and/or number of doses administered, or by combination administration with other treatments (e.g. STZ injection with nicotinamide administration or high fat diet feeding). The use of this model to induce T1D is more common due to the inability of the two chemicals to directly induce insulin resistance. Low doses of

Model			Diabetes	Advantages	Limitations
Diabetic models	Pharmacological induction	• STZ-induced • Alloxan-induced	Type 1 (or 2)	• Quick induction • Lower cost	• Individual animals may demonstrate resistance to STZ-hyperglycemia induction • Requires exogenous injections • Short lifespan of animals • Toxicity of drugs
	Genetically diabetic	• Mice - T1D: $Ins2^{Akita}$ mouse, NOD mouse - T2D: db/db mouse, KKA^{y} mouse • Rats: - T1D: Biobreeding (BB) rat - T2D: Wistar Bonn/Kobori (WBN/Kob) rat, Zucker diabetic fatty (ZDF) rat, Otsuka Long-Evans Tokushima fatty (OLETF) rat, non-obese Goto-Kakizaki (GK) rat, spontaneously diabetic Torii (SDT) rat, TetO rat	Type 1 or 2	• Consistent phenotype • High success rate of hyperglycemia induction • No further manipulation required	• Higher cost • Breeding time required
	Diet-induced	Galactose-feeding	Type 2	• Longer lifespan of animals • Allows for analysis of retinal features in animals beyond 1 year of age • Isolated elevation of hexose levels without metabolic abnormalities of diabetes	• Longer time required to develop DR features

Model			Diabetes	Advantages	Limitations
Non-diabetic models	Oxygen-induced retinopathy (OIR)	• Continuous hyperoxia → normoxia • Alternating cycles of hyperoxia and hypoxia → normoxia	/	• Consistent and reproducible neovascularization	• Phenotype not specific to DR • Mostly for small rodents (mice, rats) • Only applicable to newborn rodents • Neovascularization in undifferentiated retina • Varying ocular angiogenesis responses in differing strains of rats • Spontaneous regression of neovascularization features within 1 week of neovascularization development
	Retinal occlusion	• Retinal vein occlusion	/	• Neovascularization in fully differentiated retinae • Quick induction of neovascularization response	• Phenotype not specific to DR • Acute ischemia
	Intraocular injection	• Vascular endothelial growth factor (VEGF) • (Fibroblast)	/	• Displays NPDR and PDR features (VEGF injection)	• Phenotype not specific to DR • Mainly applicable to large animals (e.g. rabbits) • Long duration of exogenous injection of pro-angiogenic molecules required • Mimics proliferative vitreoretinopathy more than ischemic retinopathy (fibroblast injection)
	Transgenic mice	• Mice: Kimba mice, Akimba mice, TgIGF-I mice	(Akimba: type 1)	• Exhibits reproducible neovascularization	• Cost • Some strains not commercially available • Phenotypes may not be specific to DR • Changes do not necessarily occur due to prolonged hyperglycemia

Table 2. Overview of existing models used to study DR.

insulin are required for maintenance of STZ or alloxan-induced diabetic animals. It is important to note that failure of hyperglycemia induction may occur in individual animals due to STZ resistance. Blood glucose monitoring is hence essential for confirmation of hyperglycemia development. A review by Lai and Lo [32] comprehensively details existing regimens for induction of diabetes using STZ.

3.1.2. Genetically diabetic animals

Spontaneous hyperglycemia can occur in animals carrying endogenous mutations. Inbreeding of mutated animals with wild-type animals generates reliable hyperglycemic models with consistent phenotype expression. However, the establishment of large colonies may be time-consuming. The target genes for genetic manipulation in specific animal models (e.g. insulin 2 gene mutation in the *Ins2*^{Akita} mouse; leptin receptor gene mutation in the *db*/*db* mouse) are detailed in Section 4 of this chapter and Section 1 of the following chapter.

3.1.3. Diet induced

Experimental galactosemia via feeding with 30–50% galactose can also be used to induce diabetic retinopathy. Galactose feeding causes the isolated elevation of blood aldohexose levels. Other metabolic abnormalities (e.g. alterations in insulin, glucose, fatty acids, amino acid levels) characteristic of diabetes are absent in this model [33]. Despite the long feeding time required for the onset of DR-like lesions, these animals have a longer lifespan than other diabetic models. The model may hence be able to reflect the retinal complications arising from a prolonged period of isolated elevated hexose levels.

3.2. Angiogenesis models

3.2.1. Oxygen-induced retinopathy (OIR) model

Originally developed as a model for retinopathy of prematurity, the oxygen-induced retinopathy (OIR) model has also been used to investigate angiogenesis in other retinal diseases, including proliferative DR. The OIR model is mostly used in small rodents such as mice and rats. In brief, neonatal rodents are exposed to hyperoxia to induce vaso-obliteration. Upon removal from hyperoxia, hypoxia develops in the retina. This triggers a compensatory revascularization response, resulting in neovascularization [34]. This model differs from DR in that OIR-induced neovascularization occurs in incompletely differentiated retinae, while neovascularization in DR results from progressive retinal ischemia and capillary obliteration in fully differentiated retinae.

3.2.1.1. OIR mouse model

The OIR mouse model involves exposing postnatal 7-day-old (P7) mice to 75% oxygen for 5 days before placing them back in normoxia at P12. Upon return to room air, vessel regrowth occurs at P12–P17, with neovascularization beginning at P14. Neovascularization peaks at P17 and complete spontaneous resolution is subsequently achieved by P25 [35, 36].

3.2.1.2. OIR rat model

The OIR rat model involves either continuous hyperoxia or alternating cycles of hyperoxia and hypoxia. In general, the continuous hyperoxia model involves placing rats under 80% oxygen conditions for 22 hours per day until P11. Rats are then transferred to room air for 7 days (P11–P18). In the alternating hyperoxia model, newborn rat pups are exposed to sustained cycles of hyperoxia (50–80%)/hypoxia (SHH) for 14 days and subsequently returned to room air [37, 38]. OIR methods involving the use of varying oxygen concentrations have been described.

3.2.2. Retinal occlusion

Retinal vein occlusion via laser photocoagulation or photodynamic therapy has been used to induce neovascularization in fully differentiated retinae of mice, rats, pigs and monkeys [39–43]. This model induces a near immediate neovascular response with development of retinal edema within hours and the development of intravitreal vessels within days. As DR is predominantly a chronic ischemic disorder, the use of these retinal occlusion models involving periods of reperfusion following acute ischemia induction is less suitable.

3.2.3. Intraocular injection of vascular endothelial growth factor (VEGF)

In view that pro-angiogenic molecules are strongly implicated in retinal neovascularization, researchers have injected VEGF and cultured fibroblasts into monkeys and rabbits, respectively. Intravitreal injection of VEGF in monkeys successfully induced the development of many NPDR and PDR features [44]. However, the rabbit model involving intravitreal injection of fibroblasts mimicked proliferative vitreoretinopathy more than ischemic retinopathy, as the elicited neovascular response was more traumatic and inflammatory than ischemic [45, 46].

3.2.4. Transgenic models

Transgenic mouse models of neovascularization include the Kimba mouse, Akimba mouse and transgenic mouse overexpressing insulin growth factor I, as detailed in the following section.

4. DR features of animal models

Among all of the existing animal models of DR, mice and rats are most commonly used, possibly due to their small size, availability, genetic tractability and relatively faster development of DR lesions as compared with larger animals. **Table 3** summarizes the cellular, molecular and morphological features of mouse models of DR. Features of rat and non-rodent models are detailed in the next chapter (Animal Models of Diabetic Retinopathy Part 2).

Mouse model	Type of diabetes	Hyperglycemia onset	Cellular, morphological and vascular features of human DR displayed in mouse models (Age at which correlates are first reported unless otherwise specified) (+Time post treatment: diabetes, galactosemia, or induction of VEGF overexpression)		
			NPDR features	**PDR features**	**Functional changes (ERG)**
STZ injection	1 (or 2)	Within 1 week (wk)	• *7 days*$^{+}$: Müller cell gliosis* [63] RGC loss* [63] • *8 days*$^{+}$: increased vascular permeability [109] (*2 mo*$^{+}$) [52] • *2 wks*$^{+}$: increased RGC apoptosis [50] • *3–4 wks*$^{+}$: decreased total, GCL, IPL, OPL thickness [51] • *4 wks*$^{+}$: decreased arteriolar velocity [58, 59] Decreased venular velocity [58] Decreased arteriolar and venular shear rates [58] Decreased arteriolar and venular blood flow rate [58] Decreased arteriolar and venular diameter (not observed at 8 wks post diabetes induction) [59] • *21 days*$^{+}$: increased acellular capillaries* [63] (*6 mo*$^{+}$) [53] (*9 mo*$^{+}$) [51, 54] IRMAs* [63] Possible venous dilation or beading* [63] Preretinal neovascular tufts* [63] • *5 wks*$^{+}$: reactive gliosis and increased number of astrocytes [47] • *6 wks*$^{+}$: reduced number of RGCs [48] (*7 wks*) [49] (*10 wks*) [50] • *2 mo*$^{+}$: pericyte loss [52] (*6 mo*$^{+}$) [53] (*9 mo*$^{+}$) [54] Leukostasis [56, 57] (*3 mo*$^{+}$) [51, 54] • *10 wks*$^{+}$: decreased total, INL and ONL thickness [50] • *3 mo*$^{+}$: increased number of leukocytes [54] • *17 wks*$^{+}$: capillary basal lamina thickening [55] • *6 mo*$^{+}$: capillary apoptosis [53]	• *21 days*$^{+}$: neovascularization* [63] • *17 wks*$^{+}$: increased density of capillaries suggestive of neovascularization [110] *in a novel FOB_FT strain of mice	• *4 wks*$^{+}$: decreased OP3 and total OP amplitude [60, 61] Prolonged OP2, 3 implicit time [61] • *6 mo*$^{+}$: decreased a-wave and b-wave amplitudes [51]
Alloxan injection	1 (or 2)	1–4 days [64, 65]	• *7 days*$^{+}$: disorganized capillaries* [63] • *21 days*$^{+}$: microaneurysm* [63] IRMA-like lesions* [63] Capillary dilatation with preretinal neovascular lesions* [63] • *3 mo*$^{+}$: shortened dendrites in microglia [64] *in a novel FOB_FT strain of mice		• *3 wks*$^{+}$: decreased b-wave amplitude [65, 66] • *3 mo*$^{+}$: decreased b/a-wave ratio [64] Delayed OPs [64]

Mouse model	Type of diabetes	Hyperglycemia onset	Cellular, morphological and vascular features of human DR displayed in mouse models (Age at which correlates are first reported unless otherwise specified) (⁺Time post treatment: diabetes, galactosemia, or induction of VEGF overexpression)		
			NPDR features	**PDR features**	**Functional changes (ERG)**
Galactose-fed	/	/	• *11 mo*⁺: reduced number of endothelial cells [67] (*22 mo*) [69] Pericyte loss [67] (*22 mo*) [69] (*26 mo*) [33] • *13 mo*⁺: acellular capillaries [68] (*15 mo**) [33] (*20 mo*) [67] (*21 mo*) [33, 69] • *21 mo*⁺: saccular microaneurysms [33] Increased capillary BM thickness [33] *50% galactose diet (remaining = 30% galactose diet)		
Ins2^{Akita}	1	4 wks of age (male mice)	• *8 wks*: retinal apoptosis [71–74] Increased leukocytes adherent to vessels [71] Activated microglia [71] (*21 wks* [76]) • *12 wks*: increased vascular permeability* [71] (*9 mo* [73]) Reduced total retinal and outer retina thickness (*in vivo*) [77] • *22 wks*: reduced INL and IPL thickness (*ex vivo*)^* [71] Reduced number of RGCs* [71, 72, 75] (*9 mo* [77]) • *6 mo:* reduced inner retinal thickness (INL-NFL)*(in vivo)* [77] • *25 wks*: increased GFAP expression in Müller cells* [76] Microgliosis [76] • *7 mo:* amacrine cell apoptosis [74] *31–36 wks*: increased number of acellular capillaries [71] • *6–9 mo*: microaneurysm formation [73] • *9 mo:* increased capillary BM thickness [73] *Conflicting results from alternate studies ^In vivo imaging techniques failed to reveal inner retinal thinning [76, 78]	• *6–9 mo*: retinal neovascularization [73] • *7 mo*: decreased retinal blood flow rates [79]	• *3 mo*: reduced b-wave amplitude *9 mo:* • Reduced scotopic b-waves [73] • Reduced a, b-wave amplitude [77] • Increased a, b-wave implicit time [77] • Reduced OP amplitude [77] • Increased OP implicit time [77] • Reduced b/a-wave ratio [77]

Mouse model	Type of diabetes	Hyperglycemia onset	Cellular, morphological and vascular features of human DR displayed in mouse models (Age at which correlates are first reported unless otherwise specified) (+Time post treatment: diabetes, galactosemia, or induction of VEGF overexpression)		
			NPDR features	**PDR features**	**Functional changes (ERG)**
NOD	1	Female mice: initial onset at 12–14 wks of age [81]; 80% reaching hyperglycemia at 30 wks	• *3 wks*+: arteriolar vasoconstriction (in close proximity to venules) [83] • *4 wks*+#: ganglion cell, pericyte, endothelial cell apoptosis [84] Retinal capillary BM thickening [84] Perivascular edema [84] • *6 mo:* retinal microvessel loss [85] Major vessel vasoconstriction or degeneration [85] #changes became more obvious after 12 weeks of hyperglycemia	• *6 mo*: disordered focal proliferation of new vessels [85]	
db/db	2	4–8 wks of age [86]	• *8 wks*: reduced number of RGCs [88] Increased apoptotic cells in GCL [88] (15 mo) [87], INL [89] and GCL [89] Glial activation (increased GFAP expression in Müller cells) [88, 89] (15 mo) [87] ONL thinning [88] DNA fragmentation in photoreceptors [88] Increased glutamate levels and reduced GLAST content [88] BRB disruption [89] (*19 weeks*) [111] (*15 mo*) [87] • *16 wks*: reduced central and peripheral total retinal thickness [88] • *18 wks*: increased RBC velocity [91] • *18–20 wks:* increased VEGF and decreased PEDF in vitreous [94] • *22 wks:* retinal capillary BM thickening [93] • *26 wks*: pericyte loss [92] (*15 mo*) [87] • *31 wks*: increased endothelial cell/pericyte ratio [112] Acellular capillaries [112] (*34 wks*) [92]	• *15 mo*: retinal capillary proliferation [87]	• *8 wks:* • Progressive reduced c-wave amplitude [90] • Reduction in fast oscillation amplitude [90] • *12 wks* • Reduction in off response amplitude [90] • *16 and 24 wks* • Increased b-wave implicit time [88] • Reduced b-wave amplitude [88, 90] • Increased oscillatory potential (OP) implicit time (scotopic conditions) [88] • Reduced OP amplitude (scotopic conditions) [88] • *24 wks* • Reduced a-wave amplitude [90]

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Mouse model	Type of diabetes	Hyperglycemia onset	Cellular, morphological and vascular features of human DR displayed in mouse models (Age at which correlates are first reported unless otherwise specified) (+Time post treatment: diabetes, galactosemia, or induction of VEGF overexpression)		
			NPDR features	**PDR features**	**Functional changes (ERG)**
KKA^{y}	2	5 wks of age [96]	• *3 mo* • Retinal neuronal cell apoptosis in GCL and medial INL [98] • Increased capillary BM thickness [98]		
OIR	/	/	• *P18* [99] Reduced IPL and total retinal thickness Decreased outer segment length Müller cell gliosis (increased GFAP expression) Activated microglia	• *P18 (postnatal day 18)* [99] • Intravitreal neovascularization across all retinal eccentricities • Decreased vessel profiles in deep plexus • Absence of vessels in the inner retinal plexus	• *p18* [99] • Reduced a-wave, b-wave amplitude • Increased b-wave implicit time • Reduced OP3, OP4 amplitude
Kimba (trVEGF-029)	/	/	• *P7* reduced total, INL, ONL thickness [101] • *P28* reduced IPL and outer segment thickness [101] Microaneurysms [101, 113] (*10 wks*) [100] Vascular leakage [101] (moderate phenotypes displaying decline in leakage at 9 weeks and cessation of leakage at 19 wks (mild and moderate phenotypes)) [102] Tortuous vessels [101] (*9–19 wks*) [102], capillary dropout [101] • *6 wks*: increased leucocyte adhesion and leucostasis [102] • *9 wks*: pericyte loss* [102] Acellular capillaries* [102] Reduced vessel length* [102] Reduced area coverage by vessels* [102] Reduced number of crossing points* [102] • *10 wks*: capillary non-perfusion [100] *for Kimba mice displaying moderate signs of retinopathy; the observed changes were observed at 24 weeks of age for those with a mild phenotype	• *P28* • Neovascularization [100]	

Mouse model	Type of diabetes	Hyperglycemia onset	Cellular, morphological and vascular features of human DR displayed in mouse models (Age at which correlates are first reported unless otherwise specified) (*Time post treatment: diabetes, galactosemia, or induction of VEGF overexpression)		
			NPDR features	**PDR features**	**Functional changes (ERG)**
Akimba	/	/	• *8 wks*: uneven retinal thickness on OCT [78] Pericyte loss [103] Microaneurysms [78] Hemorrhage [78] Vascular leakage (cessation at 20 weeks) [78] Reduced endothelial junction protein levels [103] Vessel tortuosity, dilatation, constriction, beading; venous loops [78] Capillary dropout and capillary non-perfusion [78] Retinal edema [78] • *24 wks:* RGC loss [78] Neural retina thinning [78]	• *8 wks:* • Retinal detachment [78] • Neovascularization [78]	
TgIGF-I	/	/	• *2 mo:* pericyte loss [107] Retinal capillary BM thickening [107] Acellular capillaries [107] • *3 mo:* Increased GFAP expression in Müller cells and astrocytes [107] Increased VEGF [107] • ≥*6 mo*: venule dilatation [107] IRMAs [107] BRB disruption *7.6 mo:* reduced ONL and INL thickness [114]	• ≥*6 mo* • Retina and vitreous neovascularization [107] • Retinal detachment [107] • Neovascular glaucoma [107]	• *7.5 mo* • Reduced scotopic b-wave amplitude and oscillatory potential amplitude [114]
Intraocular VEGF injection [108]	/	/	• *2–4 wks**: venous dilatation Microaneurysm • *8 wks**: vascular leakage *post VEGF injection	• *12 wks** • Increase in number of retinal blood vessels in INL	

Table 3. Summary of the cellular, molecular and morphological features displayed in mouse models of DR. This table has been modified from a review by Lai and Lo [32].

4.1. Mouse models

4.1.1. Pharmacological

4.1.1.1. STZ induced

STZ-induced mice are one of the most commonly used DR models for DR characterization and therapeutic drug studies. The mice develop hyperglycemia within 1 week after being injected with a dose of STZ.

STZ-induced mice have been reported to exhibit various NPDR features. Signs of neuronal degeneration, including a decrease in RGC number and reactive gliosis, were observed as early as at 5–6 weeks post-hyperglycemia induction [47–50]. Thinning of the GCL, IPL, OPL and total retinal thickness occurred at 3–4 weeks of hyperglycemia [51], with INL and outer nuclear layer (ONL) thinning by 10 weeks [50]. Microvascular changes included increased vascular permeability within 8 weeks of hyperglycemia, pericyte loss as early as at 2 months [52–54], capillary basal lamina thickening at 17 weeks [55], capillary apoptosis [53] and increased acellular capillaries by 6–9 months [51, 53, 54]. Persistent inflammation resulted in leukostasis at 2–3 months of hyperglycemia [51, 54, 56, 57] with an increased number of leukocytes in the microvasculature at 3 months [54]. Hemodynamic changes have also been documented. There was a decrease in arteriolar and venular velocity, shear rates, blood flow rates and diameter at 4 weeks of hyperglycemia [58, 59]. However, the changes in the arteriolar and venular diameters were no longer apparent at 8 weeks of hyperglycemia and hence may not be a reproducible feature of the model. ERG demonstrated decreased total OP and OP3 amplitudes with prolonged OP2-3 implicit times at 4 weeks of hyperglycemia [60, 61]. One study also noted decreased a- and b-wave amplitudes, though this was not evident in the majority of reports [51].

Evidence regarding diabetes-induced RGC apoptosis and loss remain controversial. Some studies reported increased RGC apoptosis within 2 weeks of diabetes induction [50] and decreased RGC numbers by 6–10 weeks of diabetes [48, 50]. Others found no evidence of RGC apoptosis or GCL cell loss after up to 10 months of hyperglycemia [51, 56, 62]. The transient increase in neural apoptosis and astrocyte activation that regressed after a longer duration of diabetes in one study suggested that such changes may have been induced by STZ toxicity [53]. Variations in the onset of DR features may be attributable to the use of different strains of mice (despite most using C57BL/6 mice) or differing STZ-injection protocols.

More recently, in a study of various inbred strains of mice selected using "The Collaborative Cross" mouse resource, the FOT_FB strain was identified to exhibit a wide range of NPDR and PDR lesions within a significantly shorter duration of hyperglycemia induction. Classical features of neurodegeneration including Müller cell gliosis and RGC loss were displayed 7 days after diabetes induction. Other lesions included IRMAs, dilated vessels resembling venous dilatation and venous beading, increased acellular capillaries, and signs of vessel invasion into the avascular vitreous cavity [63]. The presence of PDR features absent in conventional strains of mice with STZ-induced diabetes may be attributable to the expression of genes implicated in DR in the FOB_FT strain [63]. Though further characterization studies on

this model may be needed, the FOT_FT mouse may represent a novel resource for the study of DR related genes and for testing of therapeutic interventions targeting vascular, neural and inflammation-mediated damage in DR.

4.1.1.2. Alloxan induced

Few studies have examined neuronal and vascular DR features of alloxan-induced diabetic C57BL/6 or albino mice, perhaps due to the absence of demonstrable lesions. About 3 months of alloxan-induced diabetes in C57BL/6 mice failed to induce neuronal apoptosis, glial activation, and microaneurysm and hemorrhage formation [64]. Only functional changes on ERG were observed, with decreased b-wave amplitudes at 3 weeks in albino mice [65, 66] and decreased b/a-wave amplitude ratio and increased OP latency at 3 months of hyperglycemia in C57BL/6 mice [64]. Morphologically, shortened dendrites and thickened proximal processes of microglia suggested the activation of microglia after 3 months of diabetes [64]. In the less conventionally used FOT_FB mouse strain, the study reported disorganized capillaries within 7 days of diabetes induction [63]. By 21 days of diabetes, microaneurysms, IRMAs and capillary dilatation with preretinal neovascular lesions were found in the mice retinae [63].

4.1.2. Diet induced

Mice fed with a 30% galactose diet were found to have reduced endothelial cells and pericyte loss beginning as early as at 11 months of hypergalactosemia [67]. With prolonged hypergalactosemia, the number of acellular capillaries increased [33, 67–69]. By 21–22 months, microvascular changes, including saccular microaneurysms and capillary BM thickening, were present [33]. Variations in age of reported features exist depending on the strain of mice used and the percentage of galactose incorporated into the mice's diet. The majority of reports used mice on a 30% galactose-fed diet.

4.1.3. Transgenic diabetic mice

4.1.3.1. Ins2^{Akita} mouse

The *Ins2*^{Akita} mouse is a T1D mouse model carrying an endogenous point mutation in the *Mody4* locus (i.e. Insulin 2 gene) with an autosomal dominant mode of inheritance. The mutation results in misfolding of the insulin protein, leading to beta-cell death and decreased insulin secretion, with subsequent development of hypoinsulinemia and hyperglycemia at around 4 weeks of age in male mice. Female mice are less commonly used for DR studies due to their remission to a mild to moderate hyperglycemic state after sexual maturation following transient hyperglycemia during puberty [70]. Males, on the other hand, develop progressive hyperglycemia, resulting in a shortened average survival time of 305 days [70].

Early subclinical DR features in heterozygous *Ins2*^{Akita} mice retinae have been consistently reported by numerous studies. Cellular changes observed in humans, including increased retinal apoptosis [71–74] and activated microglia, have been documented in mice as early as at 8weeks of age. RGC loss by 22 weeks has also been evidenced by several groups [71, 72, 75].

Morphologically, there was abnormal swelling in RGC somas, axons and dendrites, with increased dendritic length in ON-type RGCs in three-month old mice [75]. One study revealed increased GFAP expression in Müller cells in 25-week-old mice [76], yet another only found increased GFAP immunoreactivity in astrocytes [71].

Retinal microvascular changes consistent with clinical NPDR have been documented in *Ins2*^{Akita} mice. It is important to note that advanced DR clinical correlates of proliferative DR, such as preretinal neovascularization, have not yet been detected in this model. Studies have reported increased leucocyte adhesion to retinal vessels in eight-week-old mice [71] with increased retinal vascular permeability [71, 73] and presence of acellular capillaries [71] in older mice. *Ex vivo* and *in vivo* histological analyses demonstrated inner retinal thinning at 22 weeks [71, 74] and 6 months, respectively [77], conceivably due to dopaminergic and cholinergic amacrine cell loss or dendritic atrophy [74]. Total and outer retinal thinning had been evidenced earlier on at 3 months of age [77]. By 9 months, there was increased capillary BM thickness, with evidence of neovascularization and worsening microaneurysm formation [73]. The use of *in vivo* imaging techniques (OCT) in other studies, however, failed to show evidence of retinal thinning [76, 78] and neovascularization (both by histology and *in vivo* imaging techniques) in 25-week-old mice [76]. Vascular function assessments revealed significantly reduced retinal blood flow rates, blood cell velocity and vascular wall shear rates without signs of increased hypoxia in mice after 26 weeks of hyperglycemia [79]. Corresponding functional deficits, as documented by significantly reduced scotopic a-wave, b-wave and OP amplitudes, increased a-wave, b-wave and OP implicit times, and reduced b/a-wave ratio have also been found in mice 9 months of age [73, 77]. It has been suggested that differences in reported DR morphological features may be due to the potential presence of *rd8* mutations in the *Crb1* gene in C57BL/6 N mice used for the generation of *Ins2*^{Akita} mice. Affected mice have been described to display signs of retinal degeneration and ocular lesions due to the presence of *rd8* unrelated to the mutated genes of transgenic mice [80].

Despite its short average lifespan [70], the *Ins2*^{Akita} mouse is a well-characterized model of T1D exhibiting changes associated with early DR. It's stable insulin-deficient diabetic state that does not require exogenous administration of insulin and lack of systemic immunologic modifications makes it ideal for DR therapy testing. However, it still fails to display preretinal neovascularization and other features of advanced-stage DR.

4.1.3.2. Non-obese diabetic (NOD) mouse

The Non-obese diabetic (NOD) mouse spontaneously develops T1D beginning from 12 to 30 weeks of age. An autoimmune process involving $CD4^+$ and $CD8^+$ cells triggers insulitis and subsequent overt T1D in 80% of female and 20% of male mice by the age of 30 weeks [81, 82].

After 3 weeks of hyperglycemia, constriction of retinal arterioles in close proximity to venules was observed in NOD mice [83]. There was evident degeneration of RGCs, endothelial cell and pericyte apoptosis, retinal capillary BM thickening, perivascular edema and microvascular occlusion by 12 weeks of hyperglycemia (pathological changes initially arose after 4 weeks of hyperglycemia) [84]. Six-month-old mice exhibited further vascular changes, including retinal microvessel loss, vasoconstriction or degeneration of major vessels and focal proliferation of new vessels [85].

Only female mice were used in the studies due to the inconsistent and low rates of hyperglycemic induction in males. However, estrogen is speculated to play a protective role in DR. This may arguably affect the interpretation of potential therapeutic drug studies [32]. Although the NOD mouse represents an autoimmune diabetic model similar to the pathogenesis of human T1D, the onset of hyperglycemia is highly variable, making it a less reliable model for DR studies.

4.1.3.3. Db/db mouse

The C57BL/KsJ-*db*/*db* or *Lepr*$^{db/db}$ (db/db) mouse is a T2D model carrying a mutation of recessive inheritance in the leptin receptor gene. Homozygotes develop obesity at 3–4 weeks of age, and hyperglycemia at 4–8 weeks [86].

The mice exhibited progressive neuronal cell loss [87], glial activation [87], neuroretinal thinning, BRB disruption and accumulating glutamate concentrations accompanied with downregulation of the glutamate/aspartate transporter (GLAST) as early as at 8 weeks of age [88, 89]. Progressively worsening retinal function and retinal pigment epithelium dysfunction with persistent hyperglycemia have been evidenced by ERG changes (a-wave, b-wave, c-wave and oscillatory potential changes) beginning at 8 or 16 weeks of age [88, 90]. Sustained hyperglycemia is also suggested to be associated with increased RBC velocity in these mice at the age of 18 weeks [91], though the nature of microcirculatory hemodynamic changes in diabetes remains controversial. Upon lowering of blood glucose levels by dietary restriction, many of the observed neurodegeneration abnormalities regressed or were arrested [88]. Such findings suggest that the observed neurodegeneration features are attributable to the effect of diabetes as opposed to genetic factors.

Microvascular complications, such as pericyte loss [87, 92], presence of acellular capillaries [92] and thickening of the capillary BM [93], were also displayed in this model. Retinal angiogenesis dysregulation in these mice is further supported by corresponding associated biochemical changes in the vitreous and retina associated with DR pathogenesis (increased VEGF) and decreased pigment epithelium-derived factor (PEDF)) [94, 95]. The presence of more advanced features of DR, however, is limited to the proliferation of retinal capillaries at 15 months of age [87].

While the model confers signs of retinal neurodegeneration, the mice have a shortened life span and do not breed well [86]. Homozygote females are infertile and homozygote males have low fecundity. Despite such limitations, with numerous reports characterizing structural abnormalities and increasing studies examining its functional deficits in recent years, the db/db mouse remains an extensively used model for therapeutic drug research.

4.1.3.4. KKA^{y} *mouse*

The KKA^{y} mouse (or Yellow KK mouse) is a congenic strain of the KK mouse. It was created through the transfer of the yellow obese gene (A^{y}) into KK mice, on the basis that diabetic traits were inherited by polygenes [96]. The mice develop hyperglycemia, hyperinsulinemia and obesity beginning at around 5 weeks of age and display marked hyperglycemia by 16 weeks of age [96]. At the age of 40 weeks, the mouse reverts back to normal [97]. Only one

study to date has documented retinal changes in the KKA^{y} mouse. The study reported retinal neuronal cell apoptosis in the GCL and inner INL [98] with capillary BM thickening [32] after 3 months of hyperglycemia.

4.1.4. Angiogenesis models

4.1.4.1. Oxygen-induced retinopathy (OIR)

Characterization of retinal features exhibited by mouse models of OIR has been performed on postnatal day 18-old (P18) mice [99]. Documented cellular features included reduced IPL, outer segment (central and mid-peripheral) and total (central) retinal thickness, and increased gliotic Müller cells and reactived microglia predominantly in areas where deep plexus vascularization was absent. Substantial intravitreal angiogenesis was present in all retinal eccentricities. The number of vessels was reduced in the inner and deep vascular plexues (central and mid-peripheral), with the central retina remaining fairly avascular. Corresponding functional changes on the ERG were also observed. A-wave, b-wave, OP3 and OP4 amplitudes were reduced and the b-wave implicit time was increased. The OIR model is not widely utilized for therapeutic drug studies for DR, owing to the spontaneous regression of neovascularization within a week of its development.

4.1.4.2. Kimba mouse

The Kimba trVEGF029 mouse (Kimba) is a neovascularization model whereby photoreceptor-specific human $VEGF_{165}$ overexpression is induced using a truncated rhodopsin promoter [100]. The Kimba mouse line displays features most similar to NPDR or early PDR out of the four hVEGF-overexpressing transgenic mouse lines generated, while displaying stable mild to moderate retinopathy for at least 3 months [100]. The phenotypic observations discussed below correspond to the Kimba trVEGF029 individuals displaying mild or moderate retinopathy.

Vascular changes in this model have been documented as early as at postnatal day 7 (P7), with INL, ONL and total retinal thinning as one of the first features displayed. P28 mice exhibited classical features of NPDR (tortuous vessels, microaneurysms, vascular leakage and capillary hemorrhages) that progressed with increasing age [100–102]. The development of such retinal vascular abnormalities was accompanied by increasing adherent leucocyte numbers corresponding to the severity of the abnormality observed [102]. Counter intuitively, vascular leakage began to cease at 9 weeks among moderate phenotypes, but this is most likely due to the significant reduction in $hVEGF_{165}$ expression. Mild neovascularization and altered retinal vasculature demonstrating reduced vessel length, coverage area and crossing points have also been reported in mice 9 weeks of age [102]. However, the observed neovascular changes in such VEGF models occur in the outer retina, as opposed to the inner retina as seen in DR. While new vessels typically grow into the vitreous in DR, vessel growth in this model occurs in the opposite direction, from the capillary bed to the ONL. There has not been widespread use of the Kimba mouse in DR studies perhaps as a result of the commercial unavailability of the mouse.

4.1.4.3. Akimba mouse

To create a hyperglycemic model displaying signs of PDR, the *Ins2*^{Akita} mouse was crossbred with the Kimba mouse to generate the Akimba mouse (*Ins2*^{Akita}*VEGF*$^{+/-}$). With the inheritance of diabetic and retinal neovascular phenotypes from parental strains, the Akimba mouse exhibits most characteristic features of NDPR and PDR, with the exception of preretinal neovascularization. By 8 weeks of age, the Akimba mouse had developed major retinal microvascular abnormalities including vessel tortuosity, venous loups, vessel beading, vascular dilatation, microaneurysms and non-perfused capillaries [78]. Increased vascular leakage was accompanied with lowered levels of endothelial junction proteins [103]. Significant capillary drop out resulted in leakage cessation at 20 weeks of age [78]. Neural retinal thickness decreased with age [78]. Severe loss of ganglion cells and complete photoreceptor loss occurred in 24-week-old mice [78]. Retinal edema, neovascularization and retinal detachment were also present in the mice at an early age. The vascular changes observed here were more severe than those of Kimba mice [78], suggestive of the dual (and possibly synergistic) effects of simultaneous hyperglycemia and VEGF upregulation, and the potential use of this model to study the interaction of these two factors in DR. However, the vascular abnormalities may have developed predominantly due to VEGF upregulation rather than longstanding hyperglycemia as seen in human DR, making the model unsuitable for etiological studies. In spite of such dissimilarities in the sequential pathogenic processes, the Akimba mouse is a unique model simulating an advanced human DR retinal environment.

4.1.4.4. TgIGF-I mouse

Insulin-like growth factor I (IGF-I) is a VEGF inducer that has been associated with the pathogenesis of DR. Clinically, increased levels of IGF-I have been found in the vitreous of DR patients [104, 105]. To create a model of neovascularization via increased VEGF expression, the RIP/IGF-I chimeric gene was first introduced into mice with a C57BL/6-SJL background, and these mice were subsequently backcrossed to CD-1 mice to create transgenic mice overexpressing insulin-like growth factor I (TgIGF-I) [106]. The mice were reported to exhibit NPDR-like features at the age of 2 months, including pericyte loss, retinal capillary BM thickening and presence of acellular capillaries [107]. With increasing age, there was progressive development of venule dilatation, IRMAs, retinal and vitreous neovascularization, and subsequent retinal detachment [107]. The model has also been found to induce rubeosis iridis, neovascular glaucoma and cataract under normoglycemic and normoinsulinemic conditions [107].

4.1.4.5. Intraocular injection of VEGF

As intraocular injections of VEGF are less feasible in rodent models, subretinal injection of a binary recombinant adeno-associated virus construct producing green fluorescent protein (GFP) and VEGF was used in one study. VEGF overexpression resulted in microaneurysm formation, venous dilatation and vascular leakage [108]. However, the model failed to induce the pronounced neovascularization seen in transgenic animals and was only able to manifest

some features of NPDR. Significant new vessel formation was restricted to the INL of VEGF expression site. Only one mouse displayed signs of retinal degeneration with blood vessel growth into the subretinal space.

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Chapter 3

Animal Models of Diabetic Retinopathy (Part 2)

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Additional information is available at the end of the chapter

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Abstract

Diabetic retinopathy (DR) is one of the leading causes of preventable vision impairment and blindness in the working-age population worldwide. Numerous animal models have been developed for therapeutic drug screening and to further increase our understanding of the molecular and cellular pathological processes involved in DR. Following our discussion of mouse models in "Animal Models of Diabetic Retinopathy Part 1," we describe the cellular, molecular, and morphological features of both rodent and nonrodent models of DR and their respective advantages and limitations in this chapter. To date, no animal model can holistically reproduce the pathological progression of human DR; most only display early or advanced lesions of DR. However, a thorough understanding of genotypic and phenotypic expressions of existing models will facilitate researchers' selection of the appropriate model to simulate their desired clinical scenarios.

Keywords: animals, blood glucose, blindness, diabetic complications, diabetes mellitus/pathology/physiopathology, neovascularization, proliferative, retinal vessels

1. DR features of animal models

Among all the existing animal models of diabetic retinopathy (DR), mice and rats are most commonly used, possibly due to their small size, availability, genetic tractability, and relatively faster development of DR lesions as compared with larger animals. **Tables 1–3** summarize the cellular, molecular, and morphological features of rat and nonrodent models of DR. Features of mouse models are detailed in the previous chapter (Animal Models of Diabetic Retinopathy Part 1).

Rat model	Type of diabetes	Hyperglycemia onset	Cellular, morphological, and vascular features of human DR displayed in rat models (Age at which correlates are first reported unless otherwise specified) (+Time post treatment: diabetes, galactosemia, or induction of VEGF overexpression)		
			NPDR features	**PDR features**	**Functional changes (ERG)**
STZ injection	1	Within one wk	• *1 wk*+: reduced total retinal thickness [93] • *2 wks*+: increased retinal apoptosis [94] BRB breakdown [5, 93] (*16 wks*+ [6]) • *4 wks*+: Müller cell gliosis [2, 3] (*6 wks*+ [4]) (*12 wks*+ [5]) Reduced NeuN-positive cells in GCL [3] (*4 mo* reduced number of cells in GCL [1]) Reduced astrocyte number [5] (*6 wks*+ [2]) Increased number of microglia [5] (*4 mo*+ [3]) Reduced number of cells in ONL [93] • *6 wks*+: reduced astrocyte processes [2] • *8 wks*+: increased adherent leukocytes [7] • *16 wks*+: increased acellular capillaries [6] (*8 mo*+ [1]) Decreased number of pericytes [6] • *4 mo*+: reduced NeuN-positive cells in INL [3] • *8 mo*+: pericyte ghosts [1] • *12 mo*+: capillary BM thickening [8]		• *2 wks*+: decreased b-wave amplitude [4] (*11 wks*+ [9]) (*16 wks*+ [6]) Decreased OP amplitude [4] (*16 wks*+ [6]) • *8 wks*+: decreased OPs (not observed at 11 wks) [95] • *11 wks*+: increased a-wave implicit time [9] Decreased a-wave amplitude [9] (*10 wks*+ [4]) Delayed OPs [9]
Alloxan injection	1		• *6 wks*+: BRB breakdown [96] • *5 mo*+: capillary occlusion with endothelial cell swelling [11] • *8 mo*+: retinal microvascular cell death [10] • *9 mo*+: IRMAs in lower capillary layer [11] • *12 mo*+: pericyte ghosts [12] Increased no. of acellular capillaries [12] (*18 mo*+ [10]) Capillary BM thickening [12]	• *2 mo*+: neovascularization in mid-periphery region [11] • *5 mo*+: neovascularization in center and mid-periphery regions [11] • *9 mo*+: neovascularization in all regions [11]	

Rat model	Type of diabetes	Hyperglycemia onset	Cellular, morphological, and vascular features of human DR displayed in rat models (Age at which correlates are first reported unless otherwise specified) (⁺Time post treatment: diabetes, galactosemia, or induction of VEGF overexpression)		
			NPDR features	**PDR features**	**Functional changes (ERG)**
Galactose-fed	/	/	• *4 mo*⁺: increased retinal microvascular cell apoptosis^ [10] • *12 mo*⁺: pericyte ghosts [12] (*23 mo*⁺)*^ [13] Increased no. of acellular capillaries [12] (*23 mo*⁺)*^ [13] Capillary BM thickening [12] (*23 mo*⁺)*^ [13] (*28 mo*⁺)^ [14] • *24 mo*⁺: dilated, hypercellular vessels*^ [13] IRMAs* [13] • *28 mo*⁺: disrupted retinal layers^ [14] Gliosis^ [14] Capillary dilatation^ [14] Microaneurysms (OPL, INL)^ [14] Increased number of endothelial cells^ [14] **50% galactose diet; ^30% galactose diet*		
BB rat	1	60–120 days (90% hyperglycemic by 90–120 days) [15]	• *6 mo*: increased retinal capillary BM thickness [15, 18] • *7.7 ± 1.1 mo*: reduced and deranged basal infoldings of the RPE basal plasmalemma [16] • *8 mo*⁺: decreased pericyte/endothelial cell ratio [17] Decreased number of pericytes [17] Microinfarctions with nonperfused areas [19]		

Rat model	Type of diabetes	Hyperglycemia onset	Cellular, morphological, and vascular features of human DR displayed in rat models (Age at which correlates are first reported unless otherwise specified) (*Time post treatment: diabetes, galactosemia, or induction of VEGF overexpression)		
			NPDR features	**PDR features**	**Functional changes (ERG)**
WBN/ Kob rat	2	9–12 months of age [20]	• *5 mo*: decreased outer segment thickness [21] • *10 mo*: clustering of capillaries into tortuous knots [22] • *11 mo*: decreased OPL and ONL thickness [21] • *14 mo*: capillary BM thickening [21] Complete photoreceptor cell nuclei and ONL loss ([21, 22], 169) • *15 mo*: decreased number of capillaries [22] Capillary loop formation [22] • *19 mo*: acellular capillaries [24]	*24 mo*: • Intravitreal and choroidal neovascularization [23, 24] • Increased hyalinization of intraretinal vessels [23] • Increased intravitreal proliferation of fibrovascular elements [23]	
ZDF rat	2	6–7 wks of age [25]	• *14 wks*: increased VEGF-beta mRNA levels in retina [26] • *27 wks*: capillary BM thickening [28] (6–7 mo) [27] Increased capillary hypercellularity [28] (6–7 mo) [27] • *33–34 wks*: increased pericyte apoptosis and presence of pericyte ghosts [29] Increased endothelial cell apopotosis [29] Increased number of acellular capillaries [29]		

Rat model	Type of diabetes	Hyperglycemia onset	Cellular, morphological, and vascular features of human DR displayed in rat models (Age at which correlates are first reported unless otherwise specified) (*Time post treatment: diabetes, galactosemia, or induction of VEGF overexpression)		
			NPDR features	**PDR features**	**Functional changes (ERG)**
OLEFT	2	18 wks of age [30]	• *24 wks*: increased leukocyte entrapment [38] • *28 wks*: reduced total retinal and RNFL thickness [32] • *36 wks*: reduced number of RGCs [32] Increased apoptosis in RNFL [32] • *40 wks*: increased GFAP immunoreactivity in Müller cells [33] • *14 mo*: capillary BM thickening [31, 34] Endothelial cell degeneration [31, 34] Reduced pericyte area to total capillary cross-sectional area ratio [34] Caliber irregularity [34] (*64 wks*) [35] Capillary tortuosity [34] (*64 wks*) [35] (*17 mo*) [31] Capillary loop formation [34] (*64 wks*) [35] (*17 mo*) [31] • *64 wks*: capillary narrowing [35] Microaneurysms [35] (*17 mo*) [31] • *19 mo*: decreased INL and photoreceptor thickness [31] Decreased RPE height [31] Poorly developed basal infoldings [31]		*60 wks*: • Prolonged peak latencies of OPs [36]

Rat model	Type of diabetes	Hyperglycemia onset	Cellular, morphological, and vascular features of human DR displayed in rat models (Age at which correlates are first reported unless otherwise specified) (*Time post treatment: diabetes, galactosemia, or induction of VEGF overexpression)		
			NPDR features	**PDR features**	**Functional changes (ERG)**
GK rat	2 (non-obese)	4–6 wks of age [37] (glucose in-tolerance as early as at 2 wks of age)	• *1 mo**: increased retinal mean circulation time [38] • *3 mo**: increased BRB permeability [39] • *28 wks*: increased ocular VEGF concentrations [41] • *8 mo*: Increased endothelial/pericyte ratio [40] • *12 mo**: Activated microglia [42]	*1 mo* (*post hyperglycemia*) • *1 mo**: reduced retinal segmental blood flow [38] • *12 mo*: migration and accumulation of microglia/ macrophages in the subretinal space [42]	
SDT	2 (non-obese)	25 wks of age (male rats) (100% diabetic by 40 wks of age) [44]	• *40 wks*: increased cellular apoptosis in GCL and INL [45] Increased GFAP immunoreactivity expression across the whole retina [45] • *44 wks*: increased retinal leukostasis [46] • *52–60 wks*: increased INL, ONL, PL, choroidal and total retinal thickness [47] • *68 wks*: fluorescein leakage around the optic disc [48] Venous dilation [48] Distorted retina [48] (*70 wks*) [44] Protrusion of optic disc into the vitreous • *70 wks*: retinal thickening [44] • *77 wks*: anterior chamber hemorrhage with fibrovascular changes around the iris [44, 49] • *49–82 wks*: acellular capillaries [49] Pericyte loss [49] Tortuous vessels [49] Capillary nonperfusion [49] • *20 mo*: increased VEGF immunoreactivity in vascular endothelial cells, GCL, INL, border between ONL and OS [50]	• *70 wks*: tractional retinal detachment with fibrous proliferation [44, 49] • *20 mo*: neovascularization (53%) without ischemia [50] • Tractional changes without retinal detachment [50]	*32 weeks* • Prolonged OP peak latencies [48] *44 weeks* • Reduced a-, b-wave and OP1, OP2, OP3 amplitudes [51] • Increased OP2, OP3, OP4 implicit time [51]

Rat model	Type of diabetes	Hyperglycemia onset	Cellular, morphological, and vascular features of human DR displayed in rat models (Age at which correlates are first reported unless otherwise specified) (*Time post treatment: diabetes, galactosemia, or induction of VEGF overexpression)		
			NPDR features	**PDR features**	**Functional changes (ERG)**
TetO rat [52]	2	7–8 days after incorporation of doxy-cycline in drinking water	*4–5 weeks** [52] • RGC loss • Reduced number of pericytes • Increased number of stenotic vessels • Increased number of veins with abnormal vessel caliber (deep microvascular plexus) • BRB breakdown • Increased GFAP expression • Reactive gliosis • Increased inflammatory markers	*P18* • Intravitreal neovascularization in peripheral retina [54, 58]	*ERG Changes 4–5 weeks after hyperglycemia onset* • Reduced a-waves, b-waves, and c-wave amplitude • Elevated b/a-wave ratio
OIR	/	/	• *P13*: perivascular astrocyte loss [54] • *P14–16*: increased vascular permeability (ceased at p18) [55] • *P18*: reduced peripheral INL(and central), IPL and total retinal thickness [56] Increased Müller cell gliosis in peripheral retina [54, 56] Increased tortuosity of arterioles [57] Decreased vessel profiles in outer and inner plexuses in peripheral retina [54, 58] Altered pericyte-endothelial interactions [54] Shortened retinal outer segment length [97]		*ERG changes at p18* • Reduced a-wave and b-wave amplitude [57]

This table has been modified from a review by Lai and Lo [19].

Table 1. Summary of the cellular, molecular, and morphological features displayed in rat models of DR.

Model		Type of diabetes	Hyperglycemia onset	Cellular, morphological, and vascular features of human DR displayed in non-rodent models (Age at which correlates are first reported unless otherwise specified) (+Time posttreatment: diabetes, galactosemia, or induction of VEGF overexpression)		
				NPDR features	**PDR features**	**Functional changes (ERG)**
Dog	Galactose-fed	/	/	• *19 mo*+: pericyte loss [62] (*36 mo*+: increased endothelial/pericyte ratio [63]) • *24 mo*+: uneven distribution of endothelial cells [62] Acellular capillaries [98] (*36 mo*+ [63]) Endothelial proliferation (in areas with pericyte loss) [62] (*36 mo*+ [63]) • *27 mo*+: microaneurysm [62] (*32–36 mo*+ [64]) (*36 mo*+ [63]) • *33 mo*+: dot and blot hemorrhages [62, 64] Capillary varicose enlargement [62] Areas of nonperfusion [99] (*36 mo*+ [65] (*56 mo*+ [62]) • *36–48 mo*+: hemorrhage [62] • *56 mo*+: preretinal hemorrhage [62] IRMAs [62] • *60 mo*+: AV shunts [62] Arterial node formation [62] Gliosis [62]	• *60 mo*+: neovascularization [65] • *84 mo*+: intravitreal retinal vascular growth [62] Partial detachment of posterior vitreous [62]	
	STZ injection [60]			• *1–3 yrs.*+: capillary BM thickening • *4 yrs.*+: pericyte loss Smooth muscle cell loss • *7 yrs.*+: microaneurysms Acellular capillaries IRMAs		
	Alloxan injection			• *5 yrs.*+: microaneurysm [61] Pericyte ghosts [61] Increased endothelial/pericyte ratio [61] Increased acellular capillaries [61] BM thickening [61] Vascular leakage, increased permeability of retinal blood vessels [100]		

Model		Type of diabetes	Hyperglycemia onset	Cellular, morphological, and vascular features of human DR displayed in non-rodent models (Age at which correlates are first reported unless otherwise specified) (⁺Time posttreatment: diabetes, galactosemia, or induction of VEGF overexpression)		
				NPDR features	**PDR features**	**Functional changes (ERG)**
Cat	Pancreatotomy	1	1–2 weeks post surgery	• *3–10 mo*⁺: capillary BM thickening [70] • *5 yrs.*⁺: microaneurysm [69] (*7.7–9.4 yrs.*⁺ [71]) • *6.5 yrs.*⁺: small intraretinal hemorrhages [69] (*7.7–9.4 yrs.*⁺ [71]) • *7.5 yrs.*⁺: IRMAs [69] Capillary non-perfusion areas [69] (*9.4 yrs.*⁺ [71]) • *8.5 yrs.*⁺: microvessel dilation and tortuosity	• *8.5 yrs.*⁺: small foci of neovascularization	*9.4 yrs.*⁺: decreased b-wave (trend) [71]
Rabbit	STZ injection	1		*135 days*⁺: • 10% eyes: "Moderate vasculopathy with hard or soft exudates, widespread hemorrhages" [75] • 40% eyes: "Serious vasculopathy with serious retinal and preretinal hemorrhages, vascular lesions, hemovitreous, venous thrombosis" [75]	• *135 days*⁺: 50% of eyes: proliferative retinopathy [75]	
	Diet-induced [72]	2	12–24 weeks	• *12 wks*⁺: increased number of microaneurysms		
	VEGF implant	/	/	• *7 days*⁺: increased dilation and tortuosity of vessels [73] • *14 days*⁺: vascular leakage [73]	• *14 days*⁺: neovascularization (decreased after 21 days with almost complete regression by 35 days) [73] *VEGF + bFGF* • *48 hrs*⁺: grade + 1 neovascularization [74] • *4 days*⁺: grade 4 neovascularization [74] • *2 wks*⁺: total traction retinal detachment with significant hemorrhage [74]	

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Model		Type of diabetes	Hyperglycemia onset	Cellular, morphological, and vascular features of human DR displayed in non-rodent models (Age at which correlates are first reported unless otherwise specified) (⁺Time posttreatment: diabetes, galactosemia, or induction of VEGF overexpression)		
				NPDR features	**PDR features**	**Functional changes (ERG)**
Swine	STZ injection	1	1 wk [79]	• *18 wks*⁺: capillary BM thickening with rarefaction [79]		
	Alloxan injection	1	15–30 days [76]	• *90 days*⁺: increased GFAP in Müller cells [76] • *20 wks*⁺: capillary BM thickening [77] • *20 wks* (*age*): pericyte loss [78] Reduced total number of BRB capillaries [78] Capillary collapse [78]		
Zebra fish	Glucose-induced	1	1–2 days [90]	• *Day 28*: decreased IPL and INL [90]		
	Glucose-induced (*flk*:EGFP transgenic zebrafish)			• *6 dpf* (*3 days of glucose tx*): increased diameter of hyaloid-retinal vessels [89]		
	Hypoxia-induced	/	/	• *3 days*⁺: increased capillary sprouts in arterioles and veins [91] Increased number of branch points [91] Reduced intercapillary distance [91]	*3 days*⁺: neovascularization (increased vascular area) (plateau at day 12) [91]	
	vhl mutants	/	/	• *5.75 dpf*: increased hyaloid and choroidal vascular networks [101] • *6 dpf*: vascular leakage *dpf = days post fertilization*	*7.5 dpf*: • Severe macular edema [101] • Retinal detachment [101] • Intraretinal neovascularization (predominantly in IPL) [101]	

This table has been modified from a review by Lai and Lo [19].

Table 2. Summary of the cellular, molecular, and morphological features displayed in nonrodent models of DR.

Model		Type of diabetes	Hyperglycemia onset	Cellular, morphological, and vascular features of human DR displayed in non-human primate models (Age at which correlates are first reported unless otherwise specified) (⁺Time post treatment: diabetes, galactosemia, or VEGF overexpression–induction)		
				NPDR features	**PDR features**	**Functional changes (ERG)**
Monkey	STZ injection, spontaneous or pancreatectomy [81]	1	/	*Changes observed in diabetic monkeys with spontaneous or pharmacologically-induced hypertension* • *6–15 yrs.*⁺: focal intraretinal capillary leakage spots Capillary dilatation Capillary dropout Cotton wool spots Microaneurysms Arteriolar dilatation and occlusion Atrophic retinopathy		
	Spontaneous, obese	2	/	• *3–8 yrs.*⁺: cotton wool spots [82] Capillary dropout [82] Microaneurysms Kim, #413} IRMAs [82] Nonperfused areas [82] • *8 yrs.*⁺: RGC loss [83] Decreased ONL, IS, OS thickness [83]	• *3–8 yrs.*⁺: cystoid macular edema [82]	*8 yrs.*⁺: • Reduced OPs [83] • Reduced amplitudes in mfERG • Delayed a-wave [83]
	Intra-vitreal VEGF injection	/	/	• *2 days*⁺: tortuous vessels [85] Vascular leakage [85] • *3 days*⁺: vessel dilatation [84] • *18 days*⁺: microaneurysms [84] Intraretinal hemorrhage [84] Venous beading [84] • *30 days*⁺: intraretinal hemorrhage and edema [84]	• *2 days*⁺: grade 2 iris neovascularization [85] • *8 days*⁺: grade 3 iris neovascularization [85] • *19 days*⁺: neovascular glaucoma [85]	
	VEGF implant	/	/	• *3 wks*⁺: vascular leakage (BRB breakdown) [73] • *2–3 wks*⁺: dilation of retinal vessels [73] *Note*: *vascular changes peaked at 3 wks*	• *1 wk.*⁺: iris neovascularization (regressed between wks 2–3)	

Model		Type of diabetes	Hyperglycemia onset	Cellular, morphological, and vascular features of human DR displayed in non-human primate models (Age at which correlates are first reported unless otherwise specified) (*Time post treatment: diabetes, galactosemia, or VEGF overexpression–induction)		
				NPDR features	**PDR features**	**Functional changes (ERG)**
Marmoset	Galactose-fed (30%) [86]	/	/	• *2.5 yrs.*[+]: microaneurysm Increased acellular capillaries Pericyte loss BM thickening BRB breakdown	• Macular edema and thickening	

This table has been modified from a review by Lai and Lo [19].

Table 3. Summary of the cellular, molecular, and morphological features displayed in nonhuman primate models of DR.

1.1. Rat models

1.1.1. Pharmacological

1.1.1.1. Streptozotocin (STZ) injection

Streptozotocin (STZ) injection diabetic rats reproduce early symptoms of DR, though prominent differences have been identified between rat strains possibly as a result of differing genetic susceptibility to DR development. A report comparing differences in the development of DR lesions in Sprague Dawley, Lewis, and Wistar rats revealed that neuronal loss in the ganglion cell layer (GCL) occurred only in Lewis rats, while both Lewis and Wistar rats showed degeneration of capillaries with pericyte ghosts after 8 months of hyperglycemia [1]. Sprague Dawley rats did not develop any lesions.

In general, neuronal and glial changes appear to precede vascular changes in this model. Retinal apoptosis and retinal thinning developed within 1–2 weeks of hyperglycemia. Müller cell gliosis, as evidenced by increased glial fibrillary acidic protein (GFAP) expression, was prominent beginning at 4 weeks of hyperglycemia [2–5]. Studies have also documented a reduction in astrocyte number and processes [2, 5], decrease in the number of cells in the GCL [1, 3], and an increase in number of microglia [1, 3] after 4–6 weeks of hyperglycemia. Blood-retina barrier (BRB) breakdown was present after 2 weeks of hyperglycemia, while other vascular changes, including acellular capillaries [1, 6], leukostasis [7], and arterial and venous capillary basement membrane (BM) thickening [8], were only present after a longer period of hyperglycemia. Pericyte loss became evident after 4 [6] or 8 months [1] of hyperglycemia. Retinal dysfunction was evidenced by decreased b-wave [4, 6, 9] and OP amplitude [4, 6] and decreased a-wave amplitude [4, 9] with delayed oscillatory potentials (OP) [9] as early as at 2 weeks and 10 weeks, respectively.

1.1.1.2. Alloxan injection

Studies using alloxan injections for diabetes-induction are limited, and none have assessed for neurodegenerative lesions of DR. Alloxan-induced diabetic rats were found to have BRB breakdown within 6 weeks of hyperglycemia. With a longer duration of diabetes, there was retinal microvascular cell death [10], intraretinal microvascular abnormalities (IRMA) [11], pericyte ghosts [12], acellular capillaries [10, 12], and capillary BM thickening [12]. Neovascularization began in the mid-peripheral retina after 2 months of hyperglycemia and extended to all regions of the retina by 9 months [11].

1.1.2. Diet induced

Galactose-fed rats display many retinal microangiopathy features that resemble non-proliferative diabetic retinopathy (NPDR). Four months of 30% galactose-fed diet resulted in increased retinal microvascular cell apoptosis [10]. With a longer duration of galactosemia, pericyte ghosts, acellular capillaries, vessel dilation, capillary BM thickening, and IRMAs became evident in both 30 and 50% galactose-fed rats [12–14]. Long-term galactosemia of 28 months further resulted in gliosis, displacement or disruption of retinal layers, an increase in the number of endothelial cells, and microaneurysm formation in the outer plexiform layer (OPL) and inner nuclear layer (INL) [14].

1.1.3. Transgenic

1.1.3.1. Biobreeding (BB) rats

The diabetes-prone biobreeding (BB) rat is a T1D model derived from outbred Wistar rats. BB rats develop insulitis with selective β-cell destruction, hyperglycemia, hyperketonemia, ketonuria, and hypoinsulinemia. Rapid progression to fatal diabetic ketoacidosis results if exogenous insulin is not administered. The majority of rats develop hyperglycemia by the age of 90–120 days [15]. Existing reports have demonstrated reduced and deranged basal infoldings of the retinal pigment epithelium (RPE) basal plasmalemma [16], pericyte loss, reduced pericyte to endothelial cell ratios [17], capillary BM thickening [15, 18], and microinfarctions [19]. Microaneurysm formation and neovascularization were absent even after 11 months of diabetes [19]. Characterization studies of DR using this model remain limited. Subsequent inbreeding and outbreeding have produced various genetically distinct substrains (named after the origin of the colony), including the BBDP/Wor, BB/OK, and BB/Pfd rat. Care should be taken when comparing results of studies utilizing rats of different substrains due to potential genetic and phenotypic differences.

1.1.3.2. Wistar Bonn/Kobori (WBN/Kob) rats

The Wistar Bonn/Kobori (WBN/Kob) rat is a nonobese T2D strain of Wistar rats that develop exocrine pancreatic insufficiency characterized by α- and β-cell destruction. Male rats develop hyperglycemia at around 9–12 months of age, while females remain unaffected [20].

The retinal changes in this rat model have long been argued to be more degenerative than diabetic. At the age of 5 months, prior to the development of hyperglycemia, the rats showed signs of retinal degeneration with the loss of rods and cones and thinning of the photoreceptor layers [21]. Although retinal capillary changes, such as capillary BM thickening [21], reduced capillary number, formation of capillary loops, presence of acellular capillaries, and clustering of capillaries into tortuous knots [22], were observed after hyperglycemic development, it is uncertain whether these changes arose from the development of diabetes or resulted from further progression of retinal degeneration. WBN/Kob rats survive for a longer duration under diabetic conditions compared to other models. Perhaps, this prolonged survival and hyperglycemia duration had led to the development of intravitreal and choroidal neovascularization, which was accompanied by the hyalinization of intraretinal vessels and increased proliferation of fibrovascular elements in the vitreous at 24 months of age [23, 24]. Such features had not been previously reported in other rats. The proliferative changes were mostly absent in non-diabetic age-matched female counterparts [23], suggesting that the WBN/Kob rat may serve as a diabetic angiopathy model. Nevertheless, the retinal degenerative changes in the WBN/Kob rats are arguably different from that of human DR, making it a less suitable model for DR.

1.1.3.3. Zucker diabetic fatty (ZDF) rats

The Zucker diabetic fatty rat (ZDF/Gmi, fa/fa) is a T2D model derived from the partial inbreeding of the fa/fa line. The rats carry an inherited mutation in the leptin receptor and need to be maintained on a specialized diet to ensure consistent diabetes development. Male rats display marked

hyperglycemia, hyperinsulinemia, insulin resistance, and obesity at 6–7 weeks of age [25]. Studies have reported increased vascular endothelial growth factor (VEGF) expression in the retina that correlated with retinal levels of inflammatory markers at 14 weeks of age [26], capillary BM thickening with increased retinal capillary hypercellularity at 27 weeks [27, 28], and increased pericyte apoptosis, endothelial apoptosis, presence of pericyte ghosts and number of acellular capillaries as compared to the lean controls by 33–34 weeks [29]. The rats have a relatively short lifespan of 1 year but do not require insulin treatment while exhibiting consistently marked hyperglycemia with glucose levels averaging at 500 mg/dL when fed with the appropriate diet. To date, few retinal morphological and therapeutic drug studies have been performed on this rat model.

1.1.3.4. Otsuka Long-Evans Tokushima fatty (OLETF) rats

The Otsuka Long-Evans Tokushima fatty (OLETF) rat is a T2D model originally generated from selective breeding of the Long-Evans rats that spontaneously exhibited polyuria, polydipsia, and obesity. The OLETF rat is characterized by mild obesity, a late onset of chronic hyperglycemia after the age of 18 weeks, male inheritance, pancreatic islet hyperplasia, and the development of T2D as a result of metabolic syndrome [30]. Significant weight gain occurs between the age of 1 and 6 months followed by significant weight loss after the age of 9 months [31].

The rats display an array of retinal cellular morphological changes corresponding to the early stage of DR. Increased leukocyte entrapment was first reported in 24-week-old rats. Spectral domain optical coherence tomography (SD-OCT) revealed significant thinning of the total retinal and retinal nerve fiber layer (RNFL) at the age of 28 weeks [32]. There was retinal ganglion cells (RGC) loss with nonuniform distribution of remaining RGCs and increased apoptosis in the RNFL at 36 weeks [32]. GFAP and VEGF immunoreactivity were upregulated, with VEGF expression extending across all layers of the retina into the OPL [33]. By 14 months of age, there were endothelial cell degeneration, capillary BM thickening, and pericyte loss [31, 34]. A longer duration of hyperglycemia resulted in the reduction in INL thickness, photoreceptor thickness, and RPE height with poorly developed basal infoldings, suggestive of neurodegeneration. Rats aged 14–17 months have consistently exhibited marked microvascular changes, including vessel tortuosity, caliber irregularity, capillary loop formation, capillary narrowing, and microaneurysms [31, 34, 35]. One study further revealed prolonged peak latencies of OPs on ERG, particularly OP1 and OP3, [36], although experimental conditions may have accounted for such abnormalities.

Existing characterization studies have only been performed by a selective number of research groups. Despite the similarities in the pathophysiology of T2D in OLETF rats and humans, the use of OLETF rats in DR studies is limited by the model's late onset of hyperglycemia and absence of late-stage DR features. The presence of hemorrhages, exudates, and acellular capillaries has not been reported in rats up to 19 months of age [31], signifying its unsuitability as an angiopathic DR model.

1.1.3.5. Nonobese Goto-Kakizaki (GK) rats

The GK rat is a nonobese T2D model that develops glucose intolerance at 2 weeks of age and significant hyperglycemia by the age of 4–6 weeks [37]. The rats were generated from repeated

selective inbreeding of normal Wistar rats based on their glucose intolerance indices [37]. After 1 month of hyperglycemia, the rats displayed significantly increased retinal mean circulatory time and reduced retinal segmental blood flow [38]. BRB permeability was increased after 3 months of hyperglycemia [39]. Eight-month-old rats presented with an increased endothelial/pericyte ratio that increased with the duration of hyperglycemia [40]. At 28 weeks, there were increased ocular VEGF concentrations, with significant immunoreactivity in the choroid [41]. This corresponds to the VEGF localization in the eyes of diabetic patients [41]. With 12 months of chronic hyperglycemia, microglia were activated and migrated into the subretinal space between the outer segment and RPE [42], as seen in human proliferative diabetic retinopathy (PDR) with diabetic maculopathy [43]. These retinal microcirculatory changes, however, were not accompanied with retinal vessel diameter or morphological changes. The stable diabetic state of this model allows for the investigation of retinal changes over an extended duration of diabetes.

1.1.3.6. Spontaneously diabetic Torii (SDT) rats

The spontaneously diabetic Torii (SDT) rat is a type 2 diabetic inbred strain of the Sprague-Dawley rat generated by selective breeding of SD rats with polyuria and glucosuria. The rats display glucosuria, hypoinsulinemia, and hyperglycemia without signs of ketonuria. There are prominent sex differences in diabetes development in these rats. Female and male rats develop hyperglycemia by 10 and 20 weeks, respectively, but 100% cumulative incidence of diabetes was only observed in male 40-week-old rats [44]. Merely, 33.3% of female rats developed diabetes by 65 weeks of age [44].

The SDT rat is one of the few rodent models that display advanced lesions and features resembling PDR with prolonged hyperglycemia. Increased cellular apoptosis in the GCL and INL and increased GFAP expression spanning the entire retina [45] were evident at the age of 40 weeks. There was increased retinal leukostasis [46] and retinal thickening [47] by the age of 44 and 52–60 weeks, respectively. Long-standing hyperglycemia in rats older than 60 weeks of age has been shown to result in the distortion of the retina, fluorescein leakage around the optic disc, vessel tortuosity, capillary nonperfusion, neovascularization, and tractional retinal detachment accompanied by proliferative fibrous formations [48] (70 weeks) [44, 49]. Anterior chamber hemorrhage with fibrous proliferation around the iris and posterior synechia formation were also noted in a few cases [44, 49]. A major discrepancy between the rat model and human DR is the development of retinal neovascularization and proliferative DR features in the SDT rat in the absence of retinal ischemia [50]. The presence of microaneurysms and areas of vascular nonperfusion were also rarely reported in this model. Retinal dysfunction was evidenced by delayed OP peak latencies at 32 weeks of age [48] and reduced a-wave, b-wave, and OP amplitudes with increased OP implicit times at the age of 44 weeks [51].

The SDT rat has a relatively long lifespan without requiring exogenous insulin administration. Hence, it may serve as a unique rodent model for PDR, considering that the SDT rate retinae can be exposed to high concentrations of VEGF for a longer period of time. Some researchers have further introduced the *fa* mutation of the leptin receptor gene found in ZF rats into the SDT rat to create a SDT fatty rat that develops diabetes and diabetes-associated complications at a younger age.

1.1.3.7. TetO rat

The NTac:SD-Tg(H1/tetO-RNAi:Insr) (TetO) transgenic rat is a newly reported rat model of T2D exhibiting both hyperglycemia and hyperinsulinemia [52]. The insulin receptor (INSR) gene is reversibly knocked down through the use of tetracycline-inducible small hairpin RNA (shRNA) expression systems [53]. INSR expression is inhibited upon doxycycline administration in drinking water, resulting in insulin resistance. After 4–5 weeks of hyperglycemia, RGC and pericyte loss were observed. The rats displayed an increase in the number of stenotic vessels and veins with abnormal caliber. Blood-retina barrier (BRB) disruption was evidenced by albumin extravasation and reduced zonula occludens-1 expression in the RPE. Increased expression of GFAP and inflammatory markers was also observed in the rat retinae. Functionally, ERG studies revealed significantly decreased scotopic a-waves, b-waves, and c-waves, and an elevated b/a-wave ratio.

The reversible nature of the tetracycline-inducible shRNA system allows for fine modulation of the degree of hyperglycemia to be induced in the rats. This may be useful for the reproduction of clinical situations in an animal model. The rats also project an array of early and late features of human DR within a shorter time span as compared with other rodent models of DR. Given that only one study to date has documented retinopathy changes in the TetO rat, no detailed reports of the timeframe of pathophysiological changes in the retinae are currently available.

1.1.4. Angiogenesis

1.1.4.1. Oxygen-induced Retinopathy (OIR)

In Sprague-Dawley rats, the OIR model predominantly induced vascular changes in the peripheral retina. Two days after pups were transferred to normoxia, there was evident perivascular astrocyte loss [54]. This was followed by significantly increased vascular permeability until P18 [55]. At P18, there was significant reduction in INL, inner plexiform layer (IPL), and total retinal thickness [56] and increased arteriole tortuosity [57]. Increased Müller cell gliosis, as demonstrated by increased GFAP immunoreactivity, was particularly prominent in avascular areas of the retina [56]. Although pericyte apoptosis, a prominent feature of DR, was not observed, pericyte-endothelial interactions were abnormal [54]. This may likewise compromise vascular wall stability. Intravitreal neovascularization was accompanied with decreased blood vessel profiles in the outer and inner plexuses and abnormal vessel tufts extending from the superficial plexus in an extraretinal direction [54, 58]. Functionally, a- and b-wave amplitudes on ERG were reduced in OIR rats [55]. It has been suggested that the degree of vascular hyperpermeability, development of vascular pathologies, and area of avascularity differs, according to the strain of mice used and the degree of VEGF overexpression [55, 59]. Researchers should be meticulous in their selection of rat strains to ensure that desired phenotypes are displayed.

1.2. Nonrodent models

Nonrodent models, including dogs, cats, rabbits, pigs, and zebrafish, have been used for DR studies. Larger animals have eyes that are more similar to human eyes in terms of size and

structure. Larger eyes also allow for the extraction of retinal cells and components of the vitreous for further *in vitro* studies. Nonhuman primates are ultimately most similar to humans anatomically, particularly in regard to the presence of the macula. However, significant costs, ethical challenges, genetic intractability, and feasibility issues regarding the housing and handling of such large animals greatly limit their use. Further studies are required for the establishment of reported features in the following nonrodent models due to the small sample sizes and large intra-species variations in existing reports.

1.2.1. Dog models

Diabetes has been induced in dogs via STZ injections or galactose feeding. Both diabetic and galactose-fed galactosemic dogs display retinal lesions similar to those of human DR, though a long duration of galactose feeding is required for the development of vessel changes associated with PDR. Diabetic dogs displayed capillary BM thickening within 1 year of hyperglycemia, pericyte and smooth muscle cell loss at 4 years, and microaneurysms, acellular capillaries, and IRMAs after 7 years of diabetes [60]. Similar lesions were found in alloxan-induced diabetic dogs after 5 years of diabetes, though such changes were significantly more evident in galactosemic dogs [61]. Galactosemic dogs have been reported to develop features of both NPDR and PDR [62–65], including intravitreal retinal vascular growth and partial detachment of the posterior vitreous after 84 months of 30% galactose feeding [62]. Some have suggested that pericyte destruction and microaneurysm formation are age dependent, with younger animals developing such symptoms at a faster rate than older animals [63].

1.2.2. Cat models

The cat eye has a retinal and choroidal circulation comparable to that of the human eye [66]. The feline lens advantageously does not develop diabetic cataracts, thus allowing for clear visualization of the fundus [67]. The majority of studies of diabetic cats involve induction of diabetes via pancreatectomy. Among the few studies published, there are discrepancies regarding the presence of reported lesions. Within the small sample size, some observed microaneurysms, capillary BM thickening, increased vessel tortuosity, decreased b-waves, capillary nonperfusion, and small intraretinal hemorrhages [68–71], while other cats did not develop microaneurysms or hemorrhage [68]. It is worth noting that the inner half of the diabetic retinae becomes hypoxic prior to the development of apparent capillary nonperfusion. This may be comparable with hypoxia-stimulated VEGF expression in humans prior to the development of DR [67]. The use of cats for DR studies is less feasible due to the slow development of lesions and high costs.

1.2.3. Rabbit models

Although the rabbit globe is significantly larger than that of rodents, rabbits possess a merangiotic retinal circulation, where the inner retina is supplied by choriocapillaris [66]. Humans, rodents, dogs, cats, pigs, and primates have a dual holangiotic circulation, where the inner retina is supplied by central and cilioretinal arteries, and the outer retina is supplied by the

choriocapillaris. Pharmacological, diet-induced hyperglycemia and hypercholesterolemia (lard, sucrose, and cholesterol-added chow [72]), and VEGF injection models (implantation of a human recombinant VEGF pellet into the vitreous [73, 74]) have been used in rabbits for DR studies. However, the presence of DR lesions is highly variable between and within models. Rabbits with STZ-induced diabetes demonstrated varying degrees of retinopathy after 135 days of hyperglycemia. Some showed proliferative retinopathy, while others only demonstrated moderate vasculopathy with hemorrhages and hard or soft exudates [75]. Diet-induced hyperglycemic and hypercholesterolemic rabbits only exhibited an increased number of microaneurysms after 12–24 weeks of special feeding. The development of vascular pathologies and neovascularization in the VEGF implant model was limited by the gradual depletion of VEGF [73]. Neovascularization developed 14 days after the implant was inserted, but there was almost complete regression by 35 days. The addition of basic fibroblast growth factor (bFGF) to the pellet in another study demonstrated a faster induction of retinopathologies [74], but angiogenic responses may vary depending on the strain of rabbits used.

1.2.4. Porcine models

The retinal structure of the pig eye, with its holangiotic retinal circulation and area centralis comparable to the human macula, closely resembles that of the human eye [66]. Few studies have examined morphological changes in the pig retinae. Alloxan-induced pigs developed Müller cell gliosis at 90 days of hyperglycemia [76]. By 20 weeks, pigs developed capillary BM thickening [77], pericyte loss, and capillary collapse with a reduction in the total number of BRB capillaries [78]. STZ-induced pigs showed similar results, with capillary BM thickening and lamellation and rarefaction on electron microscopy at 20-week post-STZ treatment [79]. A proliferative vitreoretinopathy swine model has also been developed, whereby vitreal and retinal detachments were induced surgically prior to the intravitreal injection of RPE cells [80]. However, it is not a suitable angiogenesis model for DR.

1.2.5. Nonhuman primates

1.2.5.1. Monkey models

Monkeys are the most common primate models used for DR studies. Investigations in 39 STZ-induced, pancreatectomy-induced, or spontaneously hyperglycemic T1D monkeys revealed only mild ocular changes after 6–15 years of diabetes [81]. There were no signs of PDR. Only diabetic monkeys that spontaneously developed hypertension or those that were pharmacologically induced to develop hypertension displayed ischemic retinopathy changes, including microaneurysms, capillary dilatation and dropout, focal intraretinal capillary leakage spots, cotton wool spots, and arteriolar lesions. Spontaneously obese type 2 diabetic monkeys developed various retinal lesions and displayed signs of retinal functional loss on ERG, but lesion development was highly variable among individual monkeys [82, 83]. Occurrence of retinopathy was significantly correlated with the presence of hypertension, as seen in the type 1 diabetic monkey models. Angiogenesis monkey models have also been reported, where repeated intravitreal VEGF injections resulted in vessel tortuosity, dilation, leakage, microaneurysm

formation, venous beading, capillary nonperfusion, and preretinal neovascularization [84]. Both VEGF injections and implants resulted in iris neovascularization [73, 85]. Despite their anatomical similarities to human eyes, DR development is highly variable and exceedingly slow, making it an impractical model for DR studies.

1.2.5.2. Marmoset models

An alternate primate model is the marmoset. The marmoset has large eyeballs in respect to its body size with anatomical features closely resembling those of the human eye. The only study to date characterizing the development of retinal DR lesions in these animals reported the development of microaneurysms with increased acellular capillaries, pericyte loss, BM thickening, BRB breakdown, and macular edema in marmosets that were put on a 30% galactose-rich diet for two and a half years [86].

1.2.6. Zebrafish models

Zebrafish (*Danio rerio*) uniquely offers the tractable genetics of rodent models and a retinal structure resembling that of the human eye. They have the distinctive five-layered human retinal structure, though they lack the subretinal plexi found in the inner and outer retina of human retinae [87, 88]. Despite lacking a macula, the density and number of cones in the zebrafish are comparable to that of humans.

Researchers have developed high-glucose diabetic and angiogenic models to mimic DR. Hyperglycemia was induced by immersing zebrafish into glucose-added water [89, 90]. This resulted in reduced IPL and INL thickness [90] and increased diameter of hyaloid retinal vessels [33]. Zebrafish subjected to a hypoxia-induced retinopathy model developed neovascular features after 3 days [91]. One group developed an angiogenesis model using *vhl* (von Hippel-Lindau tumor suppressor gene) mutant fish. By inactivating the *vhl* tumor suppressor gene, hypoxia-inducible factor was upregulated, resulting in overproduction of VEGF. Extensive neovascularization and PDR features were observed in these *vhl* mutant zebrafish, but such a model is not commercially available.

The short lifespan, rapid development, and capability of zebrafish to breed in exceedingly large numbers allow for high-throughput screening [88], genetic screening, and significantly shorter experimental turnover times as compared with that of rodents. Techniques for developing transgenic lines and gene-targeting mutations have also been established. Although the presence of DR-like lesions and exact models ideal for the simulation of DR have not yet been well established, the zebrafish represents a promising short-term model for future DR drug screening.

2. Next-generation DR models and conclusion

The ideal animal model for DR would mimic the complete pathophysiological process of DR in humans, with initial development of NPDR features and gradual progression to PDR with

or without macular edema in the presence of prolonged hyperglycemia. However, the majority of existing animal models only display early or advanced lesions of DR. DR has a complex etiology, with various genetic and environmental factors implicated in its disease susceptibility and progression. Animal models are particularly important for the screening of novel therapeutic interventions. The clinical application of gene therapy for DR has been garnering increasing interest, and this may call for animal models that better reflect the intricacies of DR pathogenesis. Mice and rats are highly genetically tractable, but current transgenic models are based on isolated crosses [92]. Increasing development and use of resources such as The Collaborative Cross [92] may be required to enhance future identification and development of mouse strains with complex traits and epigenetics that are more reflective of the clinical scenario. Only by identifying and manipulating genes that mediate clinically relevant phenotypes can we realize and exploit the full genetic tractability of the mouse to better model DR.

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Chapter 4

Animal Models of Central Diabetes Insipidus: Oxytocin and Low-Sodium Diets as Complementary Treatments

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Abstract

Human central diabetes insipidus (CDI) is a neurobiological syndrome characterized by the presence of hypotonic polyuria, hypernatremia, and polydipsia. CDI can be acquired (aCDI) as the result of brain damage to magnocellular neurosecretory cells or fibers that constitute the hypothalamic-neurohypophyseal system or can be caused by genetic disorders (hereditary CDI). aCDI can be experimentally induced by various surgical interventions, including neurohypophysectomy, pituitary stalk compression (PSC), hypophysectomy, and hypothalamic mediobasal lesions. CDI has been associated with a deficient production of arginine vasopressin (AVP) (the antidiuretic hormone secreted by magnocellular system), while more recently, aCDI animal studies also suggest the possible involvement of oxytocin (OT) (a natriuretic-promoting hormone secreted by neurosecretory systems) and other factors related to serum fluid concentration. Both humans and animals with aCDI may benefit from the combined administration of AVP and OT and, importantly, from a low-sodium diet. Moreover, increased OT levels are observed in Brattleboro rats (with mutated AVP gene), which may explain the regulatory hydromineral capacity shown by these animals after hydromineral challenges. In short, the symptoms shown by the different CDI animal models suggest the involvement of additional factors besides the absence of AVP, which appear to depend on the particular neurobiological systems affected in each case.

Keywords: central diabetes insipidus, hypophysectomy, neurohypophysectomy, pituitary stalk compression, mediobasal hypothalamic lesion, arginine vasopressin, oxytocin, hypernatremia, low-sodium diet

1. Introduction

The term diabetes refers to a wide variety of syndromes, including diabetes insipidus (DI), which share in common the copious production of urine (polyuria). DI is characterized by the

excretion of abundant diluted, "tasteless" urine and was first described by Willis in the seventeenth century. The incidence of this disorder is around 1:25,000 cases, with no statistically significant differences between males and females.

In general, two main DI types can be distinguished: one that is related to deficient secretion of the antidiuretic hormone arginine vasopressin (AVP), which is better documented and referred to as central, neurogenic, neurohypophyseal, or hypothalamic DI, and another characterized by renal insensitivity to the antidiuretic effect of AVP, designated nephrogenic DI [1–5].

This chapter starts with a description of the general characteristics of central diabetes insipidus (CDI), including its symptoms, the brain systems involved, and the etiologies of acquired and hereditary CDI. We then review the main animal models of acquired (neurohypophysectomy, pituitary stalk compression (PSC), hypophysectomy, and hypothalamic mediobasal lesions) and hereditary (Brattleboro rats) CDI. Finally, data from animal and human studies are discussed in relation to the therapeutic usefulness of oxytocin and a low-sodium diet.

2. Central diabetes insipidus: an overview

2.1. General characteristics of human central diabetes insipidus

CDI is a neurobiological syndrome characterized by the excretion of copious amounts of diluted urine, i.e., containing a reduced concentration of electrolytes (mainly Na^+). The excretory response in CDI is associated with a rise in serum osmolality (hyperosmolality) and sodium concentration (hypernatremia) and with the intake of large amounts of water (polydipsia) (see **Table 1**) [2, 5, 6].

2.2. Neuroendocrine control of antidiuresis and electrolyte excretion

Urine and electrolyte excretions by the kidneys are related to the hormones AVP and oxytocin (OT), among other endocrine mechanisms [7, 8]. AVP and OT have similar chemical structures and are both nonapeptides, differing in amino acids at positions 3 and 8 (**Figure 1**). They are synthesized with their corresponding carriers, neurophysins I and II, respectively,

	Diabetes insipidus	**Normal**
Urine volume (liters/day)	Up to 20	1–1.5
Urine osmolality (mOsm/L)	<300	300–1400
Serum osmolality (mOsm/kg)	>300	280–300
Serum sodium (mEq/L)	>145	135–145

Table 1. General characteristics of patients with CDI in comparison to a healthy population.

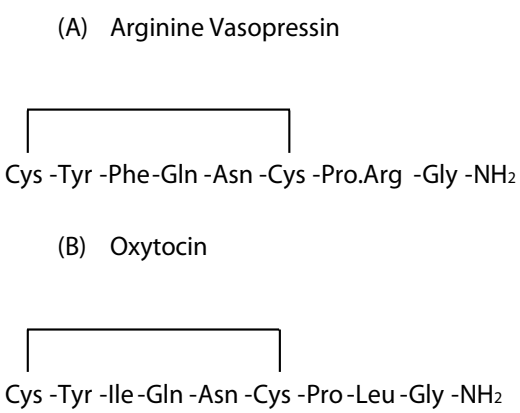

Figure 1. Chemical structure of (A) AVP and (B) OT. AVP is formed by amino acids cysteine-tyrosine-phenylalanine-glutamine-asparagine-cysteine-proline-arginine-glycine-NH_2 and OT by cysteine-tyrosine-isoleucine-glutamine-asparagine-cysteine-proline-leucine-glycine-NH_2. In both cases, a ring of amino acids 1–6 is formed by a disulfide bond.

and human genes for OT-neurophysin I and AVP-neurophysin II are both on chromosome 20, separated by only 12 kb of intergenic sequences [9].

Both hormones are synthesized in the perikarya of the magnocellular neurons of hypothalamic paraventricular (PVN) and supraoptic nuclei (SON). There is a predominance of AVP-producing cells in the SON, while oxytocinergic cells are confined to the rostral and dorsal nucleus region. For its part, the magnocellular PVN possesses a similar number of oxytocinergic and vasopressinergic neurons; its anteromedial portion contains mostly OT neurons, whereas its anterolateral portion contains an inner part with AVP neurons, surrounded by a ring of OT neurons. Less densely packed groups of AVP and OT neurons are found in posterior PVN. The axons of this complex (magnocellular PVN and SON) pass through the inner part of the median eminence, forming the neurohypophyseal stalk, and terminate in the neurohypophysis. Axonal swellings have been identified near fenestrated capillaries in both the median eminence and neurohypophysis, permitting access of these neurohormones to the bloodstream [10].

Hyperosmolality, hypernatremia, and hypovolemia (isotonic loss of fluid and electrolytes) are the main triggers of AVP and OT secretion [7, 11, 12]. After AVP is released into the blood, it acts by binding to AVP receptor 2 (AVPR2) on the basal surface of renal collecting tubule cells, triggering an intracellular signaling cascade. This concludes with activation of a cyclic adenosine monophosphate kinase pathway, increasing the production and insertion of aquaporin-II (AQP2) channels into the cell membrane. This in turn leads to passive water resorption from the lumen of the nephron into the cells of the collecting duct along an osmotic gradient [12–15]. The consequent excretion of concentrated urine is a survival mechanism for prolonged starvation periods. Conversely, diluted urine is excreted in the absence of AVP [8]. The neurohormone OT is especially involved in the excretion of body sodium or natriuresis [11, 16–20]. This secretion appears to be stimulated by increases in the glomerular filtration rate [21] and reductions in tubular sodium reabsorption [22]. These effects are mediated by actions on OT receptors present in the kidney and also, indirectly, by cardiac secretion of

atrial natriuretic peptide (ANP) [11]. OT may also act on AVPR2 to exert antidiuretic effects (**Figure 2**) [23, 24].

Both AVP and OT have been proposed to have synergic effects, with the natriuretic effect of their combined administration having a greater intensity and longer duration than the sum of the effects of each neurohormone [25, 26].

It has been reported that other hormones are involved in hydromineral regulatory processes, such as ANP co-localized with OT in hypothalamic magnocellular neurons (see Bundzikova et al. [27] for a review).

2.3. Etiological bases of human CDI

2.3.1. Acquired CDI

The most common CDI follows brain injury or surgery in the region of the pituitary and hypothalamus with damage to mediobasal hypothalamus (MBH), hypophyseal stalk, infundibulum, or the pituitary gland itself [28–32].

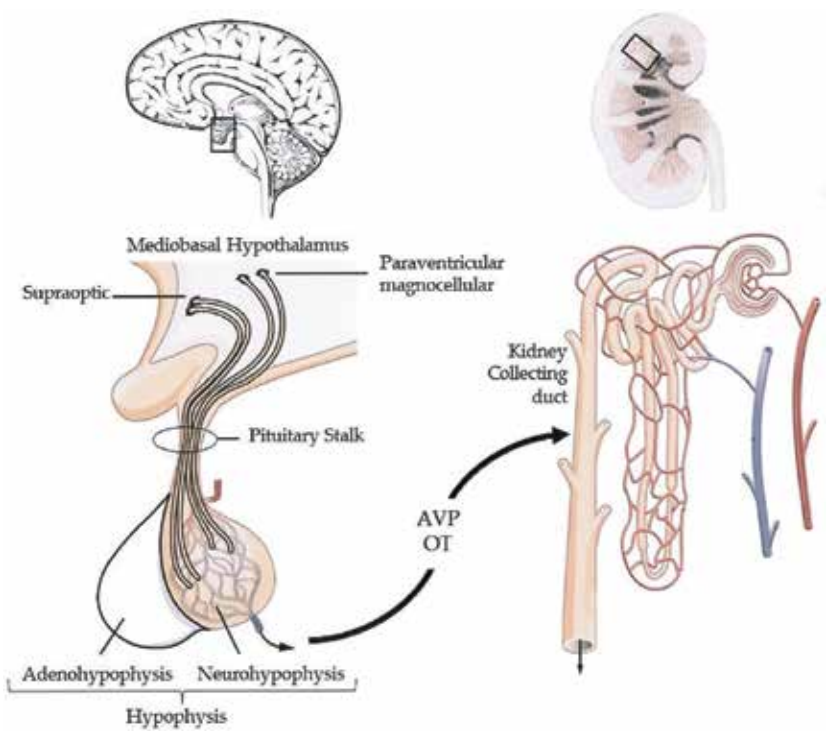

Figure 2. *Upper*: Sagittal section of the brain (*left*) and kidney (*right*). *Lower* (*magnified insets*): AVP and OT neurosecretory system (*left*) and simplified structure of a nephron (*right*).

CDI manifestations can be transient, permanent, or triphasic. Transient CDI starts with an abrupt onset of polyuria and polydipsia within 24–48 h of surgery/trauma and gradually resolves over a 3- to 5-day period [13, 30]. In permanent CDI, polyuric and polydipsic symptoms arise immediately and remain chronic in the absence of treatment [2, 33]. Triphasic CDI (**Figure 3**) was first described by Fisher and Ingram [35]. The first phase, clinically identical to transient CDI, starts with polyuria and polydipsia within 24 h of surgery, followed by an interphase (oliguric phase), with a reduction in urine excretion volume and water intake, and a final persistent phase of polyuria and polydipsia [13, 28, 36].

The regulatory and behavioral symptoms of CDI may be explained by the arrangement of hypothalamic-neurohypophyseal fibers. In this way, CDI with only transient polydipsia may be produced by lesions located ventrally to the median eminence, possibly attributable to secretion into the median eminence area of the hormonal contents of magnocellular PVN and SON neurons. Distinct effects are observed after damage to the whole hypothalamic-neurohypophyseal tract, which results in a consistently increased water intake from the onset (permanent CDI). Finally, the presence of intact/preserved axons or the release of AVP by degenerating terminals is considered responsible for the oliguric interphase in triphasic CDI [28, 37, 38].

2.3.2. Hereditary CDI

Hereditary CDI (hCDI) accounts for only 1–2% of all cases [5, 39]. It is frequently related to mutations in the AVP-neurophysin II gene, located distally at the short arm of chromosome 20 (20p13) and containing three exons. The signal peptide AVP and the NH_2-terminal region of

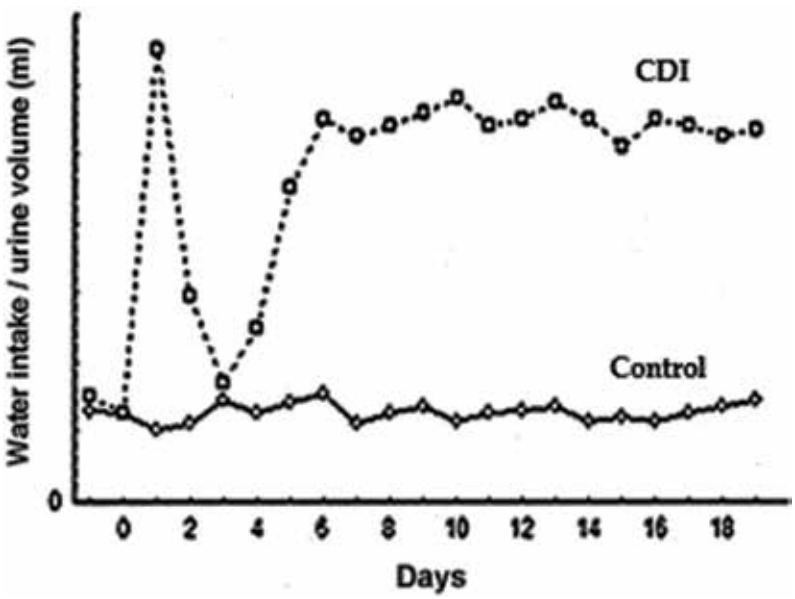

Figure 3. Water intake and urine volume in triphasic CDI (adapted from Ref. [34] with permission from Elsevier).

neurophysin II are encoded by exon A of the AVP-neurophysin II gene, and the central region of neurophysin II is encoded by exon B, while the COOH-terminal region of neurophysin II and glycopeptide are encoded by exon C.

More than 50 AVP mutations segregating with autosomal dominant or autosomal recessive CDI have been described (see http://omim.org/entry/125700). Limited capacity to secrete AVP during severe dehydration is maintained by patients with autosomal dominant CDI, whose polyuric-polydipsic symptoms generally appear after the age of 1 year, when adults are more likely to comprehend the child's requests for water. There have also been reports of three families with autosomal recessive neurohypophyseal DI in which the patients were homozygous or compound heterozygotes for AVP mutations. Two of these families were phenotypically characterized by severe polyuria, polydipsia, dehydration, and early onset in the first 3 months of life.

3. Animal models of CDI

3.1. Animal models of acquired CDI

Acquired CDI (aCDI) can be experimentally induced in animals by surgical interventions that produce a deficit in the secretion of both AVP and OT, including mediobasal hypothalamic (MBH) lesions, sectioning of hypothalamic-neurohypophyseal tract, pituitary stalk compression, hypophysectomy, and neurohypophysectomy (see Bernal et al. [34] for a review).

3.1.1. Neurohypophysectomy and pituitary stalk compression

After exposure of the pituitary gland through a parapharyngeal approach, a small incision is made in the caudal tip of the anterior lobe. This reveals the underlying posterior pituitary lobe, which is then removed by gentle suction. Given the technical difficulty of a specific neurohypophysectomy (neurohypox), selective deafferentation of the neurohypophysis has been induced by some authors (pituitary stalk compression). For this purpose, anesthetized rats are mounted on a stereotaxic frame with nose down 3.5 mm, the skull is opened by removing a small square of the bone, and a triangle-shaped wire is then lowered in coronal plane 4.0 mm caudal to bregma in the midline until it reached the skull floor. The wire is held against the skull for 30 s and then removed, evaluating the absence of tissue damage to the anterior pituitary lobe by microscopic examination and measurements of plasma levels of adenohypophyseal hormones in terminal blood samples.

Neurohypox and pituitary stalk compression (PSC) have been found to interrupt the secretion of AVP and OT, increase urine excretion and water intake, and reduce the excretion of sodium [40, 41]. In addition, salt loading (administration of hypertonic NaCl) increased serum osmolality to a greater degree in PSC animals than in sham-operated animals [42].

In earlier studies in neurohypox dogs, OT administration raised renal clearances [43] and AVP plus OT administration increased sodium excretion [44]. In a study of neurohypox rats, Balment and associates [40] found that AVP administration reduced urine flow and produced a small increase in sodium excretion. Importantly, however, OT administration within the physiological

range enhanced the natriuretic response to AVP, reversing the renal sodium excretion deficit in neurohypox rats. Moreover, the combined use of OT and AVP at low doses that do not separately promote significant body sodium excretion revealed a marked synergic natriuretic effect superior to the sum of their individual effects. Consequently, the reduced natriuretic capacity of neurohypox animals appears to be attributable to a lack of both AVP and OT.

3.1.2. Hypophysectomy

In anesthetized rats, the pituitary gland is exposed by parapharyngeal approach, and a dental drill is used to penetrate the base of the skull. The whole pituitary (anterior and posterior lobes) is then removed by suction.

Hypophysectomy (hypox) interrupts the hormonal secretion of both AVP and OT, depresses sodium excretion, and increases serum sodium concentration and water intake [45]. However, the peak sodium excretion of hypox animals treated with both neurohormones (AVP and OT) remains below that of intact rats, despite the synergic natriuretic effects of the combined treatment. Consequently, it has been proposed that the deficit produced in some adenohypophyseal hormones, such as prolactin (with antidiuretic effects in animals lacking AVP [46], may be relevant in this type of CDI.

Besides their hormone deficit, hypox animals often show marked disorders in their body sodium regulation. Thus, hypox rats consumed larger amounts of NaCl solutions than intact rats [47], an unexpected behavior considering that these animals are hypernatremic. However, hypox animals maintain an adequate hydromineral regulation capacity under homeostatic challenges. Thus, hypox animals significantly reduced their urinary sodium outputs on low-sodium diets [47], while hypertonic NaCl injection into the third ventricle stimulated their sodium excretion and antidiuresis [48, 49]. Given that these capacities could not have been activated by the secretion of AVP or OT, these and other data have been considered to demonstrate the functional integrity of the renin-angiotensin-aldosterone system in hypox rats [47].

3.1.3. Mediobasal hypothalamic (MBH) lesion

Anesthetized rats are mounted on a stereotaxic frame to bilaterally apply an anodic current (1.5 mA) for 15 s through a stainless steel electrode insulated except at the tip. In Wistar rats, stereotaxic coordinates are 6.44 mm anterior to interaural line, 0.4 mm lateral to midline, and 0.2 mm dorsal to interaural line; the extent of lesions is usually estimated by using the rat brain atlas of Paxinos and Watson [50]. Generally, all lesioned animals show extensive MBH lesions in the rostrocaudal dimension with complete damage of the median eminence region. However, partial injury of the arcuate (Arc), ventromedial (VM), and dorsomedial hypothalamic nuclei has also been observed in most rats (**Figure 4**) [51–53].

MBH lesions in animals generate hypernatremia with triphasic polyuric and polydipsic CDI. A distinctive characteristic of MBH-lesioned animals is hyperphagia [51–53], which is not observed in hypox rats [54]. This greater food intake is likely attributable to damage to the Arc and/or VM hypothalamic nuclei [55]. From a neuroendocrine perspective, MBH lesions interrupt AVP, OT, and ANP secretions [49, 56, 57].

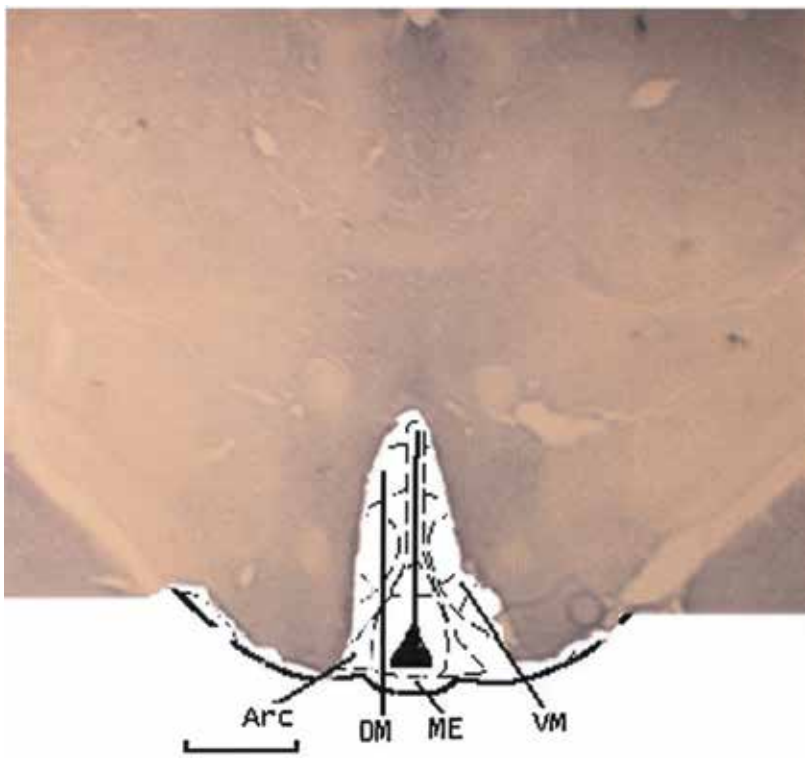

Figure 4. Photomicrograph of coronal brain section of an MBH-lesioned animal stained with cresyl violet at approximately −2.56 mm caudal to bregma. Scale bar, 1 mm (Arc, arcuate nucleus; DM, dorsomedial nucleus; ME, median eminence; VM, ventromedial nucleus) (from Ref. [51] with permission from Elsevier).

Systemic OT administration was found to enhance sodium excretion and reduce urine excretion and the standard polydipsic response of MBH-lesioned animals. However, concomitant food deprivation is necessary to show this effect, and OT-treated MBH animals with food available ad libitum showed no reduction in water intake or urine excretion. Likewise, the effects of OT were only observed when it was administered during the initial diabetic phase, not during the stable phase [51].

Hence, the regulatory capacity of MBH-lesioned animals is closely related to food availability. Specifically, it has been observed that food deprivation generates a transient reduction in water intake of MBH-lesioned animals during the initial DI phase but completely annuls their polyuria and polydipsia during the stable DI phase [51, 52]. According to these results, MBH-lesioned animals appear to retain some antidiuretic capacity, at least under food deprivation conditions. Furthermore, food deprivation on days 20–22 postsurgery was found to produce a lasting reduction in the hyperphagia (besides polydipsia) of MBH animals during the days that followed, when food was available ad libitum. This suggests that the magnitude of the polydipsic response observed after the food deprivation period may be related to the amount of food consumed. This dependence on food intake is in agreement with earlier studies suggesting that the severity of the polydipsia and polyuria of DI is approximately proportional to the amount of sodium ingested in food [58–60].

Given that nephrectomy, which prevents water loss, does not eliminate the polydipsic response of MBH-lesioned animals [61], it seems likely that this response is not exclusively a secondary effect of renal fluid loss. Thus, studies of hydromineral regulation in our laboratory have confirmed that OT administration and food deprivation can reduce water intake without a significant decrease in urine volume [62]. The possible importance of body sodium regulation disorders in the polydipsic behavior of these diabetic animals is supported by findings that low-sodium diets reduce the serum sodium concentration and water intake of MBH-lesioned animals [53] and that OT administration reduces the serum sodium concentration and water intake of food-deprived MBH-lesioned animals [51].

Unlike in hypox animals [48, 49], salt loading did not increase the natriuretic and antidiuretic responses of MBH-lesioned animals [49], suggesting an interruption of neuroendocrine pathways and of autonomous nervous system activity [63–65]. Nevertheless, when MBH-lesioned animals could choose between a hypertonic NaCl solution and water, they preferentially selected the saline solution [52]. These results are consistent with suggestions by other authors that lesions in the median eminence region might affect brain circuits that control body sodium levels [66].

In summary, it appears to be well established that food deprivation reduces body sodium accumulation and consequently the diabetic polydipsia of this animal model. Thus, the combination of the natriuretic effect of OT and the food deprivation may have a normalizing effect on the hypernatremia of MBH animals.

3.2. Animal model of hereditary CDI: the Brattleboro rat

The standard model of hereditary CDI is the homozygous Brattleboro rat, first reported by the Valtin group in the 1960s [67]. This strain was developed from Long-Evans hooded rats, which are unable to synthesize antidiuretic hormone as an autosomal recessive trait of the AVP-neurophysin II gene. The mutated allele encodes a normal AVP but an abnormal neurophysin II, which interferes with the regular transport and processing of the AVP-neurophysin II precursor molecule [39]. Homozygous Brattleboro rats, as would be expected, exhibit polyuric and polydipsic responses as well as chronic hypernatremia and hyperosmolality. However, AVPR2 is preserved in Brattleboro rats, which are therefore reactive to AVPR2 agonists [68].

Nephrectomy, which prevents renal water loss, was found to block the polydipsic response of Brattleboro rats after hypertonic NaCl administration, evidencing the primary character of polyuria in these animals [69], in contrast to the aforementioned observations in nephrectomized MBH-lesioned animals [61]. Thus, it has been observed that Brattleboro rats compensate for the excretion of excessive diluted urine by consuming large amounts of water [69] and reducing their consumption of salty solutions [70], in contrast to hypox and MBH-lesioned animals [47, 52].

However, various hydromineral challenges have been found to modify the renal regulatory capacity and hydromineral behavior of Brattleboro rats. Thus, Wilke and associates [71] reported reductions in urine volume and increases in urine osmolality and AQP2 channels after food deprivation or restriction, whereas Wideman and Murphy [72] observed a drastic fall in the water intake of Brattleboro animals. Conversely, hypertonic NaCl administration

was found to markedly increase their urinary osmolality [73–75], natriuresis [57, 73, 74], and water intake [69]. These preserved regulatory capacities of Brattleboro rats have been related to the presence of increased OT plasma levels [27, 40, 45, 73, 74, 76, 77], in contrast to the AVP and OT deficit of neurohypox, hypox, and MBH-lesioned animals [40, 41, 45, 49, 57].

The OT-induced antidiuretic effect in Brattleboro rats was blocked by treatment with AVPR2 antagonist [23, 78–81] but not by OT receptor antagonist [80], suggesting that OT may act on AVPR2 of Brattleboro rats to activate the antidiuretic response [23, 24, 82]. However, OT does not appear to be the only hormone involved in the antidiuretic effects observed in Brattleboro rats. Morrissey et al. [46] reported that prolactin, an adenohypophyseal hormone that contributes to neurohormone secretion regulation [83], reduced urinary excretion volume in Brattleboro but not control rats.

OT also appears to be critical for the natriuretic capacity of Brattleboro rats. Thus, the suppression of OT secretion by neurohypox greatly diminished sodium excretion rates in Brattleboro rats; moreover, OT administration produced a substantial and sustained natriuresis in the neurohypophysectomized animals [74].

Besides increasing the antidiuretic and natriuretic capacity of Brattleboro rats, OT increases their urine osmolality, AQP2, glomerular filtration rate, and effective filtration fraction [21, 23, 74, 78–81, 84].

Other animal models have been developed in later studies. Thus, it has been found that mutations in the AQP2 gene that interfere with its cellular processing can produce autosomal recessive nephrogenic diabetes insipidus [85]. In addition, the transgenic rat TGR(ASrAOGEN)680, characterized by a transgene-producing antisense RNA against angiotensinogen in the brain and a reduced blood pressure [86], exhibits mild CDI due to a 35% reduction in plasma AVP and, unlike observations in Brattleboro rats, normal plasma sodium and osmolality [87]. With regard to the renin-angiotensin system, low-renin hypertensive animal models (e.g., transgenic TGR(mREN2)27 rat carrying the murine Ren-2 gene) have also proven useful [88].

4. OT and low-sodium diets as complementary treatments of CDI

At the beginning of the twentieth century, patients and animals with CDI were successfully treated with pituitary extracts (which supposedly contained both AVP and OT) (see Qureshi et al. [89] for a review). However, the observation of another oxytocic effect associated with those treatments (stimulation of uterine muscle contraction) reduced the use of pituitary extracts and of OT itself. These data, alongside the discovery of Brattleboro rats, which apparently developed all DI symptoms, including blockage by nephrectomy, focused interest on a vasopressinergic approach to CDI [90]. Thus, it was considered that AVP deficit would be solely responsible for the excretion of large volumes of diluted urine (hypotonic polyuria) and would secondarily increase serum osmolality (hyperosmolality), serum sodium concentration (hypernatremia), and water intake (polydipsia). For this reason, the first-choice treatment for CDI patients is desmopressin (1-deamino-8-d-AVP), a synthetic analog of AVP that is selective for AVPR2 and exerts an even more potent regulatory effect than that of the hormone itself.

However, many studies reviewed in this chapter appear to suggest that other hormonal components besides AVP secretion are also interrupted, including OT in acquired CDI (the most frequent form). In this line, more recent studies [91] compared the effects of OT and desmopressin administration in CDI patients, finding that both treatments had positive effects on urine flow reduction, serum sodium concentration, and osmolality, and increased urine osmolality and urinary AQP2 excretion. Other authors also reported that the administration of minute amounts of pituitrin (containing AVP and OT) appears to control and improve fluid status with minimal adverse reactions [31]. This possible therapeutic option is consistent with the synergic natriuretic effect of low doses of OT and AVP observed in CDI animal models [40, 45]. It therefore seems plausible that the combined administration in humans of low-dose AVP and OT would enhance their effects while minimizing oxytocic side effects. Hence, besides its natriuretic and antidiuretic effects [24], OT administration in patients with CDI may exert a valuable hyponatremic effect [82].

In cases in which relatively large brain lesions are responsible for CDI (MBH model), the polydipsic response cannot be understood exclusively as a secondary effect of fluid excretion, because it continues to be observed in animals whose polyuria is interrupted by nephrectomy [61]. One of the factors that may explain this hyperdipsic response is diabetic hypernatremia, which would also not be solely a consequence of hypotonic excretion, in agreement with reports that lesions in the median eminence region may affect brain circuits that control body sodium levels [66].

Some more recent animal studies have suggested that food deprivation or a low-sodium diet may be potentially useful in CDI patients, always in combination with their habitual pharmacological treatment. In fact, low-sodium diets are frequently prescribed for patients with AVP-resistant or nephrogenic DI [5, 92]. These data also agree with classic studies that have indicated a relationship between diabetic polydipsia and dietary sodium. Thus, substances that increase body sodium levels were reported to exacerbate polydipsia [35, 59, 93], and, conversely, water intake was reduced in animals that were food deprived [59, 89] or on a low-sodium diet [59].

5. Concluding remarks

In summary, although Brattleboro rats are considered as the prototype model of CDI, the hydromineral characteristics of these animals do not seem to be fully comparable with an acquired CDI animal model. Thus, there is an AVP secretion deficit in Brattleboro rats, but OT remains available, whereas the CDI resulting from brain damage blocks the secretion of both hormones. Moreover, the symptoms and characteristics of humans and animals with aCDI indicate the involvement of other factors besides the habitual lack of AVP, which appear to be related to the neurobiological systems affected in each animal model. For instance, natriuresis and antidiuresis are increased after salt loading in hypox animals [48, 49] but not in MBH-lesioned animals [49] or humans with CDI [2].

Individualized therapies that take into account of the specific neurobiological system involved in each type of CDI are evidently desirable to improve the quality of life of these patients.

However, according to the research data reviewed in this chapter, all CDI patients might benefit from a low-sodium diet and from OT administration or, possibly, the combination of low doses of AVP and OT.

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Chapter 5

Animal Models of Obesity Characterized by Non-alcoholic Fatty Liver Disease (NAFLD)

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Abstract

Obesity is one of the major risk factors for the Nonalcoholic Fatty Liver Disease (NAFLD) development, as the leading cause of chronic liver disease. NAFLD is intrinsically related to obesity disorders, especially insulin resistance and dyslipidemia. Interaction between NAFLD and obesity still needs further clarification, and it is necessary to dertemine the mechanisms of these disorders in animal models of disease. Such models are usually the result of genetic and/or nutritional modifications, considering metabolic and histological changes commonly seen in humans. Obesity induced in rodents occur mainly through HFD, HCD, FFD or genetic alterations like in Lep, Acox, KKy models. These models are analogous to NAFLD development, since the increasing visceral fat is highly associated with the accumulation of fat in the form of triglycerides in the liver. Inflammatory markers such as TNF-alpha and IR are active in the predisposition of lipolysis. Hepatic inflammation during NAFLD can also be unleashed by oxidative stress. However, the mechanisms involved in the progression from NAFLD to NASH are not yet elucidated, as some models have shown unexpected outcomes such as severe malnutrition or obesity markers absence and IR after the use of Minimal-change disease (MCD) therapies and drugs, respectively. Thus, it is important to evaluate different animal models of obesity able to induce the profile of NAFLD and NASH disease in humans, assessing their mechanisms of action. The aim of this chapter is to have a comparative analysis of animal models commongly used in the pathophysiology of obesity that present NAFLD/NASH.

Keywords: animal models, obesity, NAFLD, NASH, fatty liver

1. Introduction

The rapid increase in the rate of obesity is a health problem critical in developed countries. Obesity is associated with a number of health problems that are often summarized together as metabolic syndrome and involve the development of insulin resistance, type 2 diabetes, cardiovascular disease and fatty liver disease [1].

Nonalcoholic Fatty Liver Disease (NAFLD) is the leading cause of chronic liver disease affecting 20–30% of the world's adult population, and is characterized by a buildup of fat, mainly in the form of triglycerides, in the hepatocyte cytoplasm, exceeding 5–10% of the cell weight, verified histologically or by imaging techniques. It requires exclusion of other causes of steatosis, such as excessive alcohol consumption, drugs or genetic diseases [2, 3]. About 20–30% of individuals with NAFLD can develop Non-alcoholic steatohepatitis (NASH) [4], a more severe disease condition related to metabolic abnormalities associated with obesity, namely hyperinsulinemia, dyslipidemia, and ectopic lipid accumulation [5]. More specifically, NASH is associated with lobular inflammation, hepatocellular damage and/or hepatic fibrosis [4].

NAFLD is common in Western countries, usually associated with the main characteristics of the metabolic syndrome, such as obesity, insulin resistance and hyperlipidemia [6]. Data from the U.S. National Health and Nutrition Examination Surveys, collected from 1988 to 2008, show a 2-fold higher prevalence of NAFLD, concomitant with the increased or increasing prevalence of metabolic conditions such as obesity and insulin resistance [7].

In this context, obesity induction with the development of hepatic steatosis in animal models is discussed. Animal models of obesity focused on NAFLD and NASH, as well as pathophysiological aspects related to obesity and liver diseases were systematically addressed in this chapter.

2. NAFLD pathophysiology aspects

The main mechanism related to the development of hepatic steatosis are: increased supply of free fatty acids due to increased lipolysis of visceral/subcutaneous adipose tissue and/or increased lipid intake; reduction of free fatty acid oxidation; increased hepatic de novo lipogenesis; and decreased hepatic secretion of very low density lipoprotein (VLDL). In addition, during NAFLD there is an imbalance between the intrahepatic production of triglycerides (TG) (derived mainly from plasma fatty acids delivered to the liver and not oxidized as a fuel) and the removal of intrahepatic TG (mainly exported from the liver to very low density lipoproteins, VLDL-TG) [8, 9], as shown in **Figure 1**.

TGs are the lipids that accumulate most in hepatocytes in NAFLD. TGs are synthesized through various enzymatic steps of glycerol and fatty acids condensation after activation thereof to their acyl-CoA esters. The liver obtains fatty acids from the circulation from the hydrolysis (lipolysis) of triglycerides in adipocytes in the post-absorptive state and, to a lesser degree, the postprandial lipolysis of triglyceride-rich particles (chylomicrons and VLDL). Fatty acids are released from triglyceride stores in adipose tissue through the action of the sensitive hormone lipase (located in adipocytes). These fatty acids upon their release into circulation are bound to

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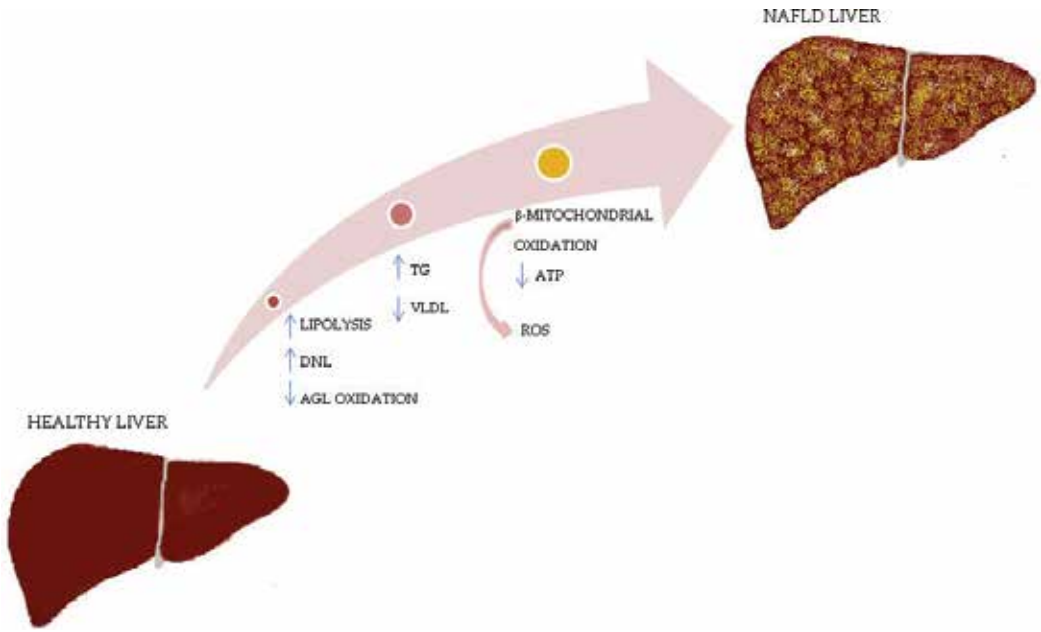

Figure 1. NAFLD mechanisms. Associated factors such as increased lipolysis and de novo hepatic lipogenesis (DNL), decreased free fatty acid oxidation and hepatic secretion of low density lipoprotein (VLDL) aggravate the onset of the disease. The action of Mitochondrial β-oxidation contributes to the synthesis of adenosine triphosphate (ATP), however results in the increase of reactive oxygen species.

albumin. The peripheral tissues, in turn, receive fatty acids as substrates for oxidation (mainly muscle) and storage (adipose tissue), through the action of endothelial lipoprotein lipase on particles rich in circulating triglycerides, either VLDL secreted by the liver, or chylomicrons delivered to lymphatic circulation after intestinal absorption of lipids [10].

During fat digestion, medium and short chain fatty acids are absorbed directly into the portal circulation. Whereas long chain fatty acids (C > 14) are mainly reesterified in chylomicrons, but a proportion of long chain unsaturated free fatty acids (FFA) enters the portal circulation [10].

According to the hypothesis on hepatic lipotoxic lesions, certain FFAs and their metabolites flow through the liver and cause NAFLD/NASH. Dietary intake plays an important role in the generation of FFA. Excess carbohydrate consumption, particularly fructose, leads to *de novo* lipogenesis. Excess calories and fats can result in accumulation of lipids in adipose tissue, and finally, the stored fatty acids are released through lipolysis. Free fatty acids generated by lipogenesis, lipolysis or other mechanisms have three potential destinations: triglycerides formation, oxidation and elimination, or intermediate lipid formation [11].

Hepatic mitochondria play an important role in the oxidation of fatty acids and in the synthesis of ATP. Mitochondrial β-oxidation is a pathway for the elimination of fatty acids, but results in the generation of reactive oxygen species (ROS). In most circumstances, the endogenous antioxidant mechanisms are able to protect against cellular damage caused by ROS. However, in the configuration of impaired mitochondrial function related to obesity

and chronic lipid overload, ROS lead to peroxidation of fatty acids, further interfering in mitochondrial function through oxidative damage to mitochondrial DNA and proteins [12].

The reason some obese individuals are able to regulate mitochondrial function and compensate lipid overload, while others are not, are still unclear. Multiple pathways involving a complex interaction between excess lipids, systemic inflammation and cellular stress probably contribute to the development and progression of NAFLD [13].

2.1. Crosstalk between obesity and NAFLD

Obesity is an important risk factor for non-alcoholic fatty liver disease (NAFLD). Although it is not a risk factor present in all obese individuals, a minority of patients with NAFLD are lean. In the survey conducted by the National Health and Nutrition Examination Survey III, 7.4% of lean adults and 27.8% of overweight/obese adults had hepatic steatosis that could be detected by ultrasound, which highlights the higher prevalence in overweight or obessed individuals. [14].

The pathophysiology of NAFLD is complex, since it is a multifactorial disease, whose disorders contribute to the metabolic syndrome, involving obesity, diabetes mellitus, hypertension and dyslipidemia [15]. Patients with NAFLD and metabolic syndrome (MS) have a higher prevalence and severity of fibrosis and necroinflammatory activity, compared to individuals of NAFLD without MS. In addition, the presence of MS is associated with a high risk of NASH among NAFLD individuals, after correction for gender, age and body mass [15].

Insulin resistance, an important feature of MS and type 2 diabetes mellitus (DM 2), is classified as peripheral insulin resistance and/or hepatic insulin resistance [16, 17]. Peripheral insulin resistance refers primarily to decreased insulin-mediated glucose uptake in skeletal muscle and adipocytes, whereas hepatic insulin resistance relates to the inability of insulin to decrease hepatic glucose production. The accumulation of fat in skeletal muscle has been considered the main pathogenic event leading to resistance to peripheral insulin. Briefly, the accumulation of arachidonic acid fatty acid metabolites in the muscle initiates a series of signaling reactions that increase the phosphorylation of specific serine residues (e.g, S307) on the insulin-1 receptor substrate (IRS-1) in the muscle. However, several researchers have begun to focus on the mechanism of hepatic insulin resistance [16, 17].

The IRS-1 is critical in its signaling, since it is an important target in the inflammatory process and, following serine phosphorylation of IRS-1, there is a reduction in the activation of phosphotidylinositol-3-kinase (PI3k), as well as other proteins involved in the normal insulin signaling process, such as protein kinase B (Akt). These deleterious events are shown to be mediated by proteins that activate inflammatory pathways, such as the c-Jun NH2-terminal kinase (JNK), the kinase IκB (Iκκβ) and PKCθ [18–21]. NFκB is a gene transcription factor that alters insulin signaling and after stimulation, Iκκβ is phosphorylated, leading to the translocation of NFκB to the cell nucleus and subsequent activation of proinflammatory cytokine genes, such as TNF-α, IL- 6 and IL-1β [22–24].

The mechanism of resistance to peripheral insulin point to the fat-induced induction of several inflammatory signaling kinases (PKCθ, IKK-β and JNK1), which may in turn increase serine phosphorylation in IRS-1, thus preventing their participation in the insulin signaling cascade. As a result, the insulin capacity to increase GLUT4 translocation is impaired. The mechanisms of fat-induced hepatic insulin resistance are similar to peripheral resistance.

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Thus, accumulation of fat within the liver results in a blockage in insulin signaling in IRS-2. This proximal blockade in the insulin signaling cascade may limit the ability of insulin to activate hepatic glycogen synthesis and suppress hepatic glucose production, involving the role of PKC, IKK-β and/or JNK1 in the pathogenesis of hepatic insulin resistance induced by fat [17]. Based on a study of patients with diabetes, accumulation of fat in the liver may also be associated with increased hepatic gluconeogenesis [25].

In addition, the hormonal and biological activity of adipose tissue through the secretion of adipokines by white adipose tissue (WAT) contributes to the insulin and metabolic resistance of the disease, including NASH. This is due to the release of inflammatory cytokines by macrophages in the adipose tissue and the increased release of free fatty acids from adipocytes [12].

In the context of chronic overfeeding, white adipose tissue becomes expanded with lipids, leading to adipocyte hyperplasia or hypertrophy. The remodeling of white adipose tissue helps to accommodate adipocyte hypertrophy, but, eventually, impaired innervation and vascularization result in hypoxia and adipocyte dysfunction [26]. This dysfunction would increase adipocyte secretion mediated by JNK1 - inflammatory cytokines [27], resulting in increases and changes of immune cells in adipose tissue and eventual systemic metabolic stress with mitochondrial dysfunction, lipolysis, decreased lipid storage capacity and signaling rupture of insulin [28].

In addition, the Western dietary pattern with excessive intake of high-calorie, rich in fat, sugar and cholesterol and sedentary lifestyle are risk factors for hepatic steatosis. Such factors have an impact on lipid metabolism related to liver diseases, which include decreased conversion of cholesterol to bile acids; increased hydrolysis of cholesterol esters to free cholesterol [29]; increased endogenous cholesterol synthesis [30]; increased absorption of cholesterol-rich lipoproteins [29]; and decreased cholesterol excretion [31]. Excess cholesterol influences the fluidity of the membrane, affecting the function of its proteins [32]. It is also worth mentioning that this excessive consumption activates Kupffer cells and hepatic stellate cells, exacerbating liver inflammation, increasing extracellular matrix synthesis and eventually accelerating progress for NASH [33, 34].

3. Developing animal models of non-alcoholic hepatic steatosis

The progress of obesity with the presence of the NAFLD disorder is characterized as a very complex inflammatory process. Despite the search for new studies in the area, there are a number of challenges in conducting research with humans, especially to investigate genetic and dietary aspects. Controlling the diet and environment of humans becomes difficult for long periods of time, and ethical restrictions limit access to biological samples. Such problems can be circumvented with the use of animal models of disease [35].

Animal models are extremely important for elucidating the etiology of diseases in humans, besides having an integrated view of the pathogenic mechanisms, as well as monitoring the natural evolution of the disease under controlled genetic and environmental conditions. These models constitute important resources in the identification of targets and therapeutic agents that can prevent or revert diseases [36].

The choice of the animal model for studies of obesity is comprised mostly of small animals such as mice, rats, guinea pigs, hamsters, with the genetic, neuroendocrine and dietary alterations.

In these models, the disease can be induced through specific diets or with the use of chemical substances and may result in non-alcoholic hepatic steatosis [37–39].

Despite the diversity of existing models, it is of utmost importance that they can replicate the histological patterns and pathophysiological mechanisms characteristic of each stage of NAFLD in humans, develop comorbidities associated with this disease such as increase of body weight, peripheral resistance to insulin, dyslipidemia, release of adipokines by adipose tissue, among others [40].

Mice and rats are mammals belonging to the Muridae family and the Murinae subfamily, order Rodentia and genus of the mouse *Mus*, whose scientific name *Mus musculus/domesticus* and the rat of the genus *Rattus* and the species *Rattus Norvegicus/rattus*. Both are heavily used in research due to their practical handling and playback performance in short period of time. Its lineage classification may be as genetically variable (heterozygous) termed outbreed or genetically defined inbreed. Outbreds are non-consanguineous and heterogeneous, which brings them closer to representing natural populations, larger litters and lower mortality rate, for example the Swiss colony (NIH, Webster, ICR and CD-1) [41]. Conversely, inbreds are consanguineous and isogenic, from crossbreeding between siblings which determine them to be identical, such as C57BL/ J6, BALB/ C, C3H, FVB, 129, DBA and CBA [42, 43].

Controlled expression of some genes results in the animal model called transgenic, they are susceptible to genome changes with specific DNA fragments, may have a mutated or increased gene, and result in a genetic modification that is transmissible to their offspring. "Knockout" animals, i.e. animals having a knockout gene, have a total or partial sequence of a withdrawn gene, contributing to determine a pathology. And "knock-in" animals acquire a total or partial sequence of a gene introduced in their genome predisposing it to a certain pathology by excess of the gene product [44, 45].

Animals with genetic obesity or induced obesity (**Table 1**) present non-alcoholic hepatic steatosis, either by genetic alteration (**Table 2**) and increased lipid synthesis as in *ob*/*ob* mice, the *db*/*db* rat, agouti (obese yellow); and environmental factors such as diets deficient in methionine or choline, rich in carbohydrates or lipids and can also be combined with genetic alterations [46].

3.1. Genetic models

3.1.1. ob/ob mice

The *ob*/*ob* mice are spontaneous, obese mutants that do not have the Leptin gene (ob) which is autosomal recessive located on chromosome 6. From 4 weeks of age they are able to triple the normal weight when they are exposed to open offer of food due to lack of satiety caused by the absence of the hormone leptin. They are hypothermic and unable to stay warm. Its characteristics go beyond obesity, including hyperphagia, diabetes and non-alcoholic hepatic steatosis that presents with 10–12 weeks of life. The *ob*/*ob* is considered a good model of obesity linked hepatic steatosis, since metabolism of lipids and carbohydrates is related to the amount of white adipose tissue (WAT), which is increased in obesity. WAT in abundance reflects on increased expression of TNF-alpha and lipolysis, whose fatty acids are released to the liver for deposition [47, 48]. In this model, for hepatic steatoectomy, regarded as a second stimulus or agent ("second hit" such as ischemia induction) specific drugs or methionine and choline-deficient diets, is needed [46, 49, 50].

Diet	Concentration/nutrient	Animal model	Follow-up	Obesity	NAFLD	IR	NASH
HFD SFAs +Cholesterol	15% +1%	C57BL/J6	30 weeks	Yes	Yes	No	Yes
	30% +2%	SD	48 weeks	Yes	Yes	Yes	Yes
	49%	C57BL/J6	3 months	Yes	Yes	No	No
HCD	30% fructose	C57BL/J6	4 months	Yes	Yes	Yes	No
	70% fructose	Wistar	5 weeks	Yes	Yes	No	Yes
	65% sacarose	C57BL/J6	8 weeks	Yes	Yes	Yes	No
	70% sacarose	Wistar	4 weeks	Yes	Yes	No	No
	30% fructose/glicose/sacarose	Sprague Dawley	4 months	Yes	Yes	Yes	No
FF	40% HCD + 2% cholesterol e fructose drink	C57BL/J6	25 weeks	Yes	Yes	Yes	Yes

Table 1. Characteristics of obesity/NAFLD diet composition in various animal models.

The ob/ob model behaves similarly to the methionine and choline deficiency models, since they affect oxidative stress, lipid peroxidation, and cell death. They are characterized by a chronic subclinical inflammatory condition with constant release of proinflammatory cytokines, monocyte infiltration of reactive oxygen species (ROS), oxidation of lipid molecules, cholesterol and proteins present in low density lipoprotein (LDL). This is in addition to mitochondrial DNA damage (measured by the mitochondrial levels of 8-hydroxy-2′-deoxyguanosine) and reduced expression of the DNA mismatch repair enzyme [51, 52]. Lipid peroxidation of polyunsaturated fatty acids generates by-products of toxic aldehydes, including malondaldehyde and hydroxynonenal, which are more persistent than ROS and damage more distant intracellular organelles which can cause cell death. These products activate fibrous hepatic stellate cells and are chemotactic for neutrophils [53, 54].

Genetic models	Genetic alteration	Characteristics	Considerations	"Second hit": NASH
ob ob	Absence of the leptin gene	Hyperphagia, Obesity, diabetes, IR and NAFLD	10–12 weeks is confirmed NAFLD.	Hepatotoxic stimuli, specifics diets
db db	Deficient in leptin receptor	Obesity, IR, and NAFLD	3 months is confirmed NAFLD. Features similar to Nafld in humans.	Diet HFD or MCD
KK-AY	knockout for melanocortin receptor 4	Obesity, hyperphagia, hyperinsulinemia, hyperglycaemia and NAFLD	Altered gene expression of lipid metabolism.	Diet MCD, a HFD or use of endotoxin
ACOX	knockout mice for the enzyme acyl-coenzyme A oxidase	Accumulation of fat at liver	1 week presented microvesicular steatosis. At 2 months, livers show extensive steatosis.	Not necessary

Table 2. Characteristics of mouse genetic models for the development of NAFLD.

3.1.2. db/db mice and Zucker fa/fa rats and Koletsky f/f

Obese Zucker fa/fa rats and the *db*/*db* mouse are deficient in leptin receptor function, resulting from mutations in the leptin gene which occur on chromosome 4. Quite close phenotypically to these models is the obese Koletsky *f/f* rat which has the leptin deficiency with reduced energy expenditure and neuropeptidergic alterations of the hypothalamus [55].

Obese fa/fa Zucker rats fed with high fat diet (HFD) made with 60% of saturated lipids for 8 weeks, confirmed the occurance of hyperglycemia and hepatic steatosis. NADPH oxidase activity increased 2.5-fold leading to hepatic liver injury of the animals thus contributing to the progression of NASH [56].

3.1.3. KK-ay mice and knockout for melanocortin receptor 4

The KK-Ay genotype results from the crossing of KK diabetic rats with yellow coat and agouti background (Ay) [57], they develop obesity due to the antagonistic action of the agouti protein in the central nervous system capable of promoting alimentary hyperphagia and consequent obesity [58]. Changes in the genes of the agouti rat and in the encoding gene of the melanocortin receptor 4 (MC4R) are related to the involvement of the melanocortins system in the pathogenesis of NAFLD [59]. They present indicators of resistance to insulin and leptin, conditions that favor the appearance of hepatic steatosis. Also the genetic depletion of MC4R is associated with a severe obesity phenotype such as hyperphagia, hyperinsulinemia, hyperglycemia and hyperleptinemia [60]. To progress to NASH, a "second hit" is needed again as an example: diet MCD, a HFD or use of endotoxin [61].

3.1.4. ACOX and knockout mice for the enzyme acyl-coenzyme A oxidase

The enzyme acyl-coenzyme A oxidase-1 (ACOX 1) is the first enzyme of peroxisomal β-oxidation of long chain fatty acids. Peroxisomal and mitochondrial fatty acid beta-oxidation occuring in mice nullizygous for both peroxisome proliferator-activated receptor alpha and peroxisomal fatty acyl-CoA oxidase (ACOX−/−) exhibits a wide range of microvesicular steatohepatitis. These animals develop NAFLD due to the accumulation of fat generated by impaired β-oxidation [62]. At 1 week of age, the liver of the AOX−/− mice already presented microvesicular steatosis, which intensifies at 2 months of age to inflammatory infiltration [63]. However, when they are 6 to 8 months old, the liver of these mice exhibits reversal of steatosis from hepatocyte regeneration and also exhibits growth retardation [64].

3.2. Animal models induced by diets

3.2.1. High fat diet (HFD)

High fat diet (HFD) treated animals acquire obesity and also increased epididymal fat, hyperglycemia and insulin resistance, because these associated comorbidities are also widely used as a method of inducing hepatic steatosis, as they cause liver changes similar to that of human disease. Usually fat sources such as lard and soybean oil are used, different concentrations of lipids are taken into account in the preparation, varying in proportion from 40 to 60%. The most commonly used rodents are C57BL/6, Swiss, Sprague Dawley, Wistar and SHR [62, 65].

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A higher fat supply leads to increased adiposity in adipose tissue, frequent stimulation of pro-inflammatory cytokines secretion, increased free fatty acids, insulin resistance, and lipolysis, with consequent increase in the transport of free fatty acids to the liver via the portal vein and increased intake of fatty acids. In the liver, hyperinsulinemia inhibits beta-oxidation, reducing the output of fatty acids, leading to unanticipated the accumulation of triglycerides in the cytoplasm of hepatocytes which hallmarks NAFLD. Both actions favor the accumulation of fat in the hepatocytes, a condition that promotes hepatic resistance to the action of insulin. Loss of insulin's ability to suppress hepatic glucose production aggravates overall insulin resistance and exacerbates the manifestation of metabolic syndrome components [66–69].

The addition of cholesterol along with saturated fatty acids has shown to have a good disease predisposition, with progression of NAFLD to NASH. Savard et al. [70] tested the effect of this diet on the respective proportions of saturated fatty acids and cholesterol on the C57BL/J6 model for 30 weeks: control (4% fat and 0% cholesterol); high cholesterol[HC] (4% fat and 1% cholesterol); high fat[HF] (15% fat and 0% cholesterol); and high fat, high cholesterol[HFHC] (15% fat and 1% cholesterol). The animals treated with HCHC showed the highest weight gain, hepatic lipid content, inflammation of adipose tissue and reduction in adiponectin plasma levels, leading to NAFLD in a profound way, as they developed macrovesicular steatosis (grade 3) associated with inflammatory spots (grade 2) and peripheral fibrosis, with these effects being twice as large as in the HC and HF groups [70].

Similarly, adult Sprague Dawley rats fed HFD made with 30% lipids (lard and 2% cholesterol) were evaluated at 4, 8, 12, 16, 24, 36, 48 weeks intervals. At week 8 body weight and epididymal fat weight began to increase, which was associated with increased serum levels of free fatty acids, cholesterol and TNF-α, as well as the development of NAFLD fatty liver. Steatohepatitis occurred between weeks 12 and 48. Apparent hepatic fibrosis did not occur until week 24, and went from week 36 to 48 with insulin resistance reproducing the pathological sequence of events typical of human NASH [71].

Obesity models induced by HFD consumption have also been characterized by inflammation in peripheral tissues as well as in hypothalamic areas critical for energy homeostasis, in an attempt to interrupt body weight control and glucose homeostasis [72]. According to Thaler et al. [73] unlike inflammation in peripheral tissues, which develops as a consequence of obesity, inflammatory signaling of the hypothalamus is confirmed in rats and mice within 1 to 3 days after initiation of HFD treatment, ie before substantial weight gain. In addition, both reactive glucose and markers suggestive of neuronal injury were evident in the arched hypothalamic nuclei of rodents in the first week of dietary feeding, leading to the knowledge that obesity is associated with neuronal injury in an area of the brain, suggesting a crucial aspect for the control of body weight [73]. Induction of FHD obesity in AFasKO mice with death receptor Fas deficiency (also known as CD95), specifically in adipocyte cells, were protected from adipose tissue inflammation and also from hepatic steatosis (more sensitive to insulin, both at the level of the body and in the liver) and hepatic insulin resistance [74].

In C57BL/6 mouse fed with HF diet (49% of lipids) during gestation and/or lactation, or both, the presence of non-alcoholic fatty liver steatosis was verified by expression of protein-1c binding to the sterol regulatory element. There was an exacerbation of NAFLD phenotype in utero and during lactation, demonstrating the development of hepatic steatosis already in fetal life [75].

3.2.2. High carbohydrate diet (HCD)

Prolonged consumption of HCD causes obesity and non-alcoholic fatty liver disease (NAFLD), in addition to oxidative stress in the liver and insulin resistance. The most common sources of simple carbohydrates in the diet are fructose, glucose and sucrose, for example the addition of corn syrup (50–90% fructose) or refined sugar (50% fructose). Fructose is highly lipogenic and has been more widely used than sucrose and glucose, however, induction of chronic models in NAFLD requires longer treatment periods [76–78]. Fructose is primarily metabolized in the liver without the need for insulin, its phosphorylation consumes ATP and accumulates ADP thus stimulating the formation of uric acid and reactive oxygen species (ROS) which rapidly increases the synthesis and hepatic deposition of triglycerides leading to a fatty liver in rodents [79–81].

Treatment with a 30% fructose, glucose and sucrose diet for 4 months in Sprague Dawley rats demonstrated that such treatment induced metabolic syndrome, intrahepatic accumulation of uric acid and triglycerides, increased MCP-1 and TNF-alpha, as well as hepatic steatosis [82]. This simple carbohydrate intake is associated with a greater translocation of the endotoxin from the intestine to the portal vein, ROS formation in the liver (due to the greater oxidation of fatty acids in the hepatocytes) and induction of the TNF-alpha factor. TNF-alpha has been associated with the development of NAFLD because it is involved in the dysregulation of hepatic lipid metabolism and in insulin signaling [83, 84].

The C57BL/6 mouse model fed a high calorie and sucrose diet (65%) for 8 weeks showed obesity, insulin resistance and macrovesicular steatosis [85]. In another study with C57BL/J6 mice with TNF-alpha receptor 1/− TNFR1/− (sterol regulatory element-binding protein 1) were protected against the onset of hepatic steatosis and also the insulin resistance induced by HCD with fructose (30%), this result was associated with increased phosphate levels of adenosine monophosphate-activated protein kinase (AMPK) and protein kinase (AKT), decreased expression of SREBP-1, fatty acid synthesis in the liver and decreased levels of retinol binding protein (RBP4) that behaved differently from the control group [86].

As with Wistar rats, the effect of the sucrose diet (70%) when compared to starch (70%) for 28 days resulted in a significant difference in the group that received sucrose, thus the amount of hepatic fat and serum fructosamine concentration was increased in sucrose diet group and in both hepatic steatosis confirmed in the two groups [87]. However, when using HCD with 70% fructose for 5 weeks, obesity, elevated levels of hepatic triglycerides, macrovesicular steatosis, lobular inflammation (as were observed [88]. Another report with the same animal model in (HCD) added 10% of fructose (corn syrup) in drinking water and evaluated at 7, 14 and 21 days. At the start of treatment, an increase in triglycerides,oxidative stress and hepatic sensitivity to hyperinsulinemia (β-cell reaction) were was observed in serum and liver, suggesting that this increase is related to metabolism that occurred in the liver and probably in the adipose tissue as well [89].

Epidemiological studies have shown that increasing the intake of fructose mono- or disaccharides by humans is a considerable risk factor for NALFD [90, 91], and it is estimated that patients with fructosemia present a fructose consumption about 2–3 times higher than healthy individuals or with other liver diseases [92]. These data stimulated the study of experimental models of NAFLD induced by fructose.

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3.2.3. Fast food diet (FF)

Charlton et al. [93] proposed the mouse fast food diet model. C57BL/6 mice were fed for 25 weeks on a diet composed of 40% fat (12% saturated fatty acids, 2% cholesterol) and the water offered contained corn syrup, it was found that the animals developed obesity and resistance to insulin. I Other studies used the same model and also stated that the intake of FF diet in the form of emulsion for 6 weeks led to the onset of hepatic steatosis and inflammation, and elevated levels of endotoxemia and glycemia [86].

This diet model was very similar to the Western diet, since it presents high concentrations of fat, fructose and cholesterol, appearing as a good model related to human diets and with the capacity to induce obesity, NAFLD among other comorbidities in rodents.

3.3. Combined models (genetic and environmental)

The use of the C57BL LDLr male mouse (low density lipoprotein receptor deficient) treated for 21 weeks with HFD, was associated with weight gain, macrovesicular steatosis and lobular inflammation. Inflammation in adipose tissue and liver provides a positive attenuation for studies of obesity and associated cardiometabolic diseases such as NAFLD and atherosclerosis [94].

db/*db* mice subjected to methionine and choline deficient diet (MCD) showed macrovesicular steatosis, and increased hepatic collagen type 1 mRNA levels in comparison to the control group. This outcome suggests an important model for the study of NASH, i.e. establishing obesity, diabetes, insulin resistance and dietary MCD results in steatohepatitis indicating leptin activity in liver fibrosis. In this way, an interesting comparison is made with the *ob*/*ob* model that is deficient in leptin which also develops steatohepatitis but not hepatic fibrosis when fed with the MCD diet [95].

The verification in the obese and diabetic C57BI/6-A and KK-Ay models of diet treatment (MCD) for 8 weeks demonstrated that KK-Ay rats exhibited increased susceptibility to steatohepatitis and inflammatory infiltration as well as increased levels of TNFα mRNA and lipid peroxidation in the liver where hypoadiponectinemia probably played a key role in the exacerbation of inflammatory and profibrogenic responses [96].

The treatment of the KK-Ay (MC4R) model with hyperlipidic diets at concentrations of 35 and 60% of lipids derived from soybean oil and lard, develop severe hepatic steatosis and show liver changes in the lipogenic gene profile [97]. Another study has shown that the exposure of these animals to 60% HFD for 1 year leads to the appearance of more severe forms of NAFLD such as NASH, fibrosis and hepatocellular cancer, as well as leading to the development of systemic metabolic alterations very similar to those observed in humans [98]. This same model was used for a study of hepatic lesions induced by D-galactosamine/lipopolysaccharide LPS (GalN/LPS) endotoxin, there were significant increases in the levels of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) in the blood, apoptotic and necrotic changes in hepatocytes and/or showed a high degree of lethality. GalN/LPS-induced liver injury was more pronounced in KK-Ay obese than in the control group [61]. LPS is a key component of many bacteria present in the microbiota, plays a central role in innate immune responses and is considered the second hit in NASH models [99].

4. Conclusion

It is very clear that mechanisms responsible for the development of NAFLD, which can occur through a diet, typically western diet, rich in saturated fat and fructose, in addition to other simple sugars, promotes metabolic disorders. This initially affects processes such as liver lipogenesis, resistance to insulin, and even as it is now known, negative consequences due to an unbalanced microbiota, with greater release of LPS. Studies on the mechanisms involved in the development of NAFLD and progression to NASH has steadily increased, due to the increasing number of obesity and liver disorders worldwide. The combination of factors that interfere with etiology such as weight gain, dietary ingredients and genetics are factors that further instigate the urgency to elucidate its effects on the liver using animal models of human diseases.

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Chapter 6

Rodent Models of Obesity and Diabetes

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Additional information is available at the end of the chapter

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Abstract

Genetically modified animals are a widely used tool in biomedical research, since it allows modeling diseases, studying their pathological conditions and identifying and validating new drugs. One of the most common diseases studied in recent years is metabolic disorders resulting from abnormalities in enzyme systems involved in the intermediary metabolism of living organisms with a heavy impact on society. To understand the physiological mechanisms underlying these disorders, animal models, currently the transgenic type, have been employed. It is important to emphasize that there are various metabolic disorders that are not only *congenital* but can be *acquired* or a coexistence of both types. The aim of this chapter, therefore, is to describe the most commonly used rodent models focusing mainly on global emerging pathologies, obesity, diabetes and metabolic syndrome.

Keywords: diabetes, obesity, metabolic syndrome, mice model, rat model

1. Introduction

The use of experimental animals involved in metabolic pathologies goes back a few hundred years. Various mammals such as dogs, rabbits, pigs, primates and rats were frequently used by researchers to understand the physiological mechanism of diseases from the pancreotomy to isolation and purification of insulin in the 1920s to the use of toxic compounds to cause disease and the current use of genetically modified animals [1]. The advantages of using rodents for experimentation are several since it can be improved on besides the reproducibility and reliability of the study results. As expected, animal models for metabolic diseases are as complex and heterogeneous as the diseases themselves, since each one of them has specific modifications incomparable with others. Moreover, inbred lines continue, while new endogenous lines are used in many fields of research [2].

METABOLIC DISORDERS	
CONGENITAL	ACQUIRED AND CONGENITAL
Fabry disease	Diabetes
Phenylketonuria	Obesity
Prader-Willi syndrome	Dyslipidemia
Galactosemia	Hipolipidemia
Tay-Sachs's disease	Hyperthyroidism
Porphyria	Hypoparathyroidism
Pompe disease	Hypothyroidism
Niemann-Pick disease	Cushing's syndrome
Morquio's syndrome	Hyperuricemia
Maroteaux-lamy syndrome	Hemochromatosis
Hunter Syndrome	Hyperparathyroidism
Lesh-Nyhan syndrome	
Hurler syndrome	
Homocystinuria	
Hartnup	
Gaucher disease	

Figure 1. Congenital and metabolic disorders.

Most metabolic diseases in humans are a consequence of the rupture of cellular processes. However, the relationships between genetic defects, underlying molecular interaction networks and phenotypic expression, are little known [3]. Congenital metabolic diseases are disorders produced by a variation in the coding sequence of the DNA resulting in deficiencies or absence of a protein, generally an enzyme, producing metabolic blockages, whereas acquired metabolic diseases are due to diseases of endocrine organs or failure of metabolically active organs [4] (**Figure 1**). In this regard, the chapter focused mainly on both genotypic and phenotypic characteristics of animal models employed in the investigation of emerging metabolic diseases.

2. Mouse models

Current research has seen the increased use of animal models in exploring mechanisms and pathophysiological processes involved in metabolic diseases (**Table 1**) both in congenital and in acquired disease conditions, more importantly in the design and development of therapeutics and drugs for treatment.

Disease	Model	Features	References
Congenital			
Fabry disease	GLAko mouse	Mouse with a complete lack of α-galactosidase A activity. Appear clinically normal, with normal blood and urine analyses and a normal adult lifespan	[5]
Phenylketonuria	PKU mouse	A significantly decrease in level of Phe in the plasma	[6]
Prader-Willi syndrome	$Snrpn^{tm2Cbr}$ mouse	The mouse exhibits postnatal lethality	[7]
Galactosemia	GalT KO mouse	Accumulated some galactose and its metabolites upon galactose challenge but was seemingly fertile and symptom free	[8]
Tay-Sachs's disease	Tay-Sachs (Hexa−/−) mouse	In females there are severe progressive hind-limb weaknesses with impaired motor coordination, balance and mild ataxia	[9]
Porphyria	Uros(mut248) mouse	Production of red urine and shows erythrodontia. Bones are abnormally fragile	[10]
Pompe disease	GAA−/− mouse	A progressive accumulation of lysosomal glycogen in heart and skeletal muscle and diaphragm	[11]
Niemann-Pick disease	ASM (−/−) mouse	A severe, neurodegenerative course and death that occurs by 8 months of age	[12]
Maroteaux-lamy syndrome	MPS VI mouse	A skeletal and chondral dysplasia	[13]
Hunter syndrome	IDS-deficient mouse	A progressive accumulation of glycosaminoglycans (GAG) in many organs and excessive excretion of these compounds in their urine.Neuropathological defects	[14]
Lesh-Nyhan syndrome	HPRT-deficient mouse	Changes in brain dopamine function	[15]
Hurler syndrome	MPS I-H KO mouse	Mice showed no detectable α-L-iduronidase activity	[16]
Homocystinuria	"human only" (HO) mouse	Severe elevations in both plasma and tissue levels of Hcy, methionine, S-adenosylmethionine and S-adenosylhomocysteine and a concomitant decrease in plasma and hepatic levels of cysteine	[17]
Hartnup	HPH2 mouse	A deficient amino acid transport	[18]
Acquired			
Dyslipidemia	LDLR−/− mouse	A severe hyperlipidemia and extensive atherosclerosis	[19]
	apoE−/− mouse	A more severe hyperlipidemia characterized by elevations in VLDL and reductions in HDL, which lead to spontaneous atherosclerosis	[20]
Hyperuricemia	Induced by hypoxanthine	An increase in urinary uric acid	[21]

Table 1. Summary of model rodents of some congenital or acquired metabolic diseases.

2.1. Type 2 diabetes models

Type 2 diabetes (T2D) is a metabolic disorder that is caused by insufficient insulin secretion and/or insulin resistance in peripheral tissues and liver [22]. To help develop new diabetic therapies, it is important to highlight the complex mechanisms of diabetes mellitus. Animal models of T2D are as complex and heterogeneous as the syndrome itself as experienced in humans.

2.1.1. db/db mice

These diabetic mutant mice are modifications of the C57BL strain [23, 24]. The mutation in the leptin OB-R receptor was identified in 1996, and this model has been widely used in the evaluation of antiobesity and antidiabetic compounds and therapies due to its manifestation of hyperphagia and morbid obesity, reproductive failure and severe insulin resistance [25].

2.1.2. BKS db mice

The C57BLKS/J (BKS) consanguine mouse strain is a widely used animal model of T2D. A recessive mutation that occurs spontaneously in the BKS strain produces early-onset diabetes and obesity with moderate initial hyperinsulinemia followed by insulinopenia since pancreatic islets undergo atropia due to degeneration of β cells [25]. Moreover, they show polyphagia and obesity and a marked increase in weight and blood glucose after 3–4 weeks of their birth [26, 27].

2.1.3. KKAy mice

The KKAy mouse, due to its genetic mutation, is the spontaneous animal model of hyperglycemia and hyperlipidemia and its symptoms are similar to T2D [28]. The mutation Ay often becomes obese and infertile within months after birth, and the observed increase in the adipose tissue is due to hypertrophy of fat cells; however, obesity results from a reduction in hypothalamic norepinephrine and dopamine [29]. In this strain of mice, diabetic characteristics such as obesity, hyperinsulinemia and hyperglycemia are observed in early ages, between the 6th and 8th week, and then return to normal at approximately 40 weeks of age. Degranulation, glycogen deposition and hypertrophy of β cells are observed in these animals at 5–10 weeks of age, suggesting that insulin synthesis and release are increased with hyperinsulinemia. Renal lesions such as diffuse glomerulosclerosis, nodular changes and thickening of the peripheral glomerular basement membrane are also observed [29].

2.1.4. BTBR obese mice

BTBR obese mice have more fat mass than most strains of inbred mice [30]. The BTBR model naturally suffers from hyperinsulinemia when compared to other insulin-resistant mice, but when the ob/ob mutation is placed on a BTBR background, the mice are initially insulin resistant with elevated insulin levels, hypertrophy of pancreatic islets and marked hyperglycemia at 6 weeks of age [31]. An important feature of this model is the degree to which it reproduces the essential structural and functional characteristics of the human diabetic glomerular lesion with glomerular hypertrophy, marked expansion of the mesangial matrix, mesangioisis and capillary thickening of the basal membrane as well as loss of podocytes [32].

2.1.5. eNOS−/− mice

The gene-encoding eNOS or endothelial nitric oxide synthase has been considered a potential candidate gene for diabetic nephropathy because of nephropathic changes observed in mouse models of T1D and T2D, thus mimicking many aspects of human diabetic disease [33]. The eNOS −/− mice develop a remarkable albuminuria and pathological change characteristics of T2D such as mesangiolysis, microaneurysms and expansion of the increased mesangial matrix. It is useful to study the role of endothelial dysfunction in the development of diabetes and to facilitate the development of new diagnostic and therapeutic interventions [34].

2.1.6. ALR/LtJ mice

Resistant mouse (R) Leiter (LT) resistant to alloxan (AL) is closely related to the nonobese diabetic (NOD) strain. This model presents an unusual high quantitative expression of molecules associated with systemic dissipation of cell stress from free radicals. The islets of ALR are remarkably resistant to two different combinations of cytokines (IL-1β, TNF-α and IFN-γ) that destroy islets of NOD strains susceptible to alloxan [35], and this mechanism induces diabetes mellitus [36]. The strain derived from Swiss mice is widely used to study insulin-dependent diabetes mellitus in mice.

2.1.7. B6.HRS(BKS)-Cpefat/J

They are homozygous mice obtained by spontaneous fat mutation (Cpefat) on a genetic background, that is, C57BL/6J. They become remarkably obese at 14–15 weeks of age; male homozygous mutant mice develop obesity at a later age than females [37]. Cpefat mice weigh less than wild-type controls before the weaning age. Later, homozygous mutant mice develop a diabetic phenotype characterized by hyperglycemia and insulin resistance [38].

2.1.8. LG/J and SM/J

The large-LG/J and small-SM/J strains are inbred mice derived from selection experiments for large and small body size at 60 days [39]; LG/J animals grow faster at 3–10 weeks and have larger tissues at tails, body and liver and higher content of body fat compared with the SM/J strain. SM/J animals grow faster after 10 weeks of age and have higher fasting glucose levels than LG/J animals. SM/J mice are more sensitive to dietary fat gain than LG/J mice for growth after 10 weeks. Moreover, LG/J mice are more susceptible to develop antinuclear antibodies and rheumatoid factor, as well as renal disease characterized by glomerulonephritis, interstitial nephritis and perivasculitis [40].

2.1.9. NOD/ShiLtJ-Leprdb-5J/LtJ

Nonobese diabetic (NOD) mouse is an endocrine strain developed during a breeding program to establish a cataract-prone subline of non-consanguineous mice [41]. They are homozygous co-isogenic NOD mice, which develop juvenile diabetes and T2D along with the suppression of post-adolescent age-dependent spontaneous T1D. At 5 weeks of age, these mice are hyperphagic; they eat twice the amount of food from a lean control mouse and develop hyperglycemia that does not require insulin therapy for long-term survival [42].

2.2. Type 1 diabetes

After the discovery of insulin, the availability of animal models for the study of the pathogenesis of T1D was delayed by approximately 50 years.

2.2.1. Ins2Akita mutants

A point mutation of the insulin gene 2 in Insta mice (Akita) leads to a pancreatic apoptosis of β cells and hyperglycemia. Thus, these mice are commonly used to investigate T1D complications [43]. Symptoms in heterozygous mutant mice include hyperglycemia, hypoinsulinemia, polydipsia and polyuria beginning at about 3–4 weeks of age. Mice have progressive loss of β-cell function and decreased pancreatic cell density and significant hyperglycemia at 4 weeks of age [44]. It is a model used primarily for the study of retinal complications in T1D [45].

2.2.2. Streptozotocin-treated mice

Streptozotocin (STZ)-induced diabetes mellitus (STZ) offers a very cost-effective and expedient technique that can be used in most rodent strains, opening the field of diabetes mellitus research to a range of genotypic and phenotypic options that would otherwise be inaccessible. Since the initial report of its diabetogenic properties in 1963, STZ has been used alone or in combination with other chemicals or with dietary manipulations for the induction of either T1D or T2D through toxicity of β cells. STZ sensitivity is highly variable in rodents like DBA/2, C57BL/6, MRL/MP, 129/SvEv and BALB/c [46].

2.3. Obesity

The search for new alternatives for prevention and/or treatment to combat global chronic diseases such as obesity is based on the development of new animal models that share characteristics of these human diseases [47].

2.3.1. ob/ob

A spontaneous mutation leading to the markedly obese phenotype in the ob/ob mice was recognized since the 1950s. C57BL/6J mice with a mutation in the obese (ob) gene are obese, diabetic and exhibit reduced activity, metabolism and body temperature. The obesity syndrome of ob/ob mice results from lack of leptin characterized by hyperphagia, reduced energy expenditure and hypothermia; further defects are hypercorticosteronemia, insulin resistance associated with hyperglycemia and hyperinsulinemia, hypothyroidism and growth hormone deficiency leading to a decrease in linear growth. Moreover, ob/ob mice are infertile. The administration of exogenous leptin normalizes all known phenotypic defects in ob/ob mice including obesity, symptoms of the metabolic syndrome and reproductive function [47–50].

2.3.2. C57BL/6J DIO

Diet-induced models of obesity (DIO) are often used to study polygenic causes of obesity. DIO animals mimic the state of common obesity in humans than most of the genetically

modified models and may be the best choice for testing prospective therapeutics [47]. Numerous mouse strains are susceptible to DIO, including C57BL/6 [51]. This mouse develops obesity, hyperinsulinemia, hyperglycemia and hypertension [52].

2.3.3. NONcNZO10/LtJ

The NONcNZO10/LtJ mouse is a polygenic model of T2D that shows moderate obesity and diabetes. This model of obesity-induced diabetes was produced by the combination of quantitative trait loci from the New Zealand Obese (NZO/HlLt) and Nonobese Nondiabetic (NON/LtJ) mice. Interestingly, the NONcNZO10/LtJ males do not exhibit hypercorticism, hyperphagic behavior and obvious thermoregulatory defect. However, they develop visceral obesity, maturity-onset hyperglycemia, dyslipidemia, moderate liver steatosis and pancreatic islet atrophy [53, 54].

2.3.4. TALLYHO/JngJ

TALLYHO/JngJ (TH, formerly TallyHo) is a polygenic mouse that shows obesity, hyperinsulinemia, hyperglycemia (males) and hyperlipidemia at 26 weeks of age [55]. TallyHo mice can be resistant to hypothalamic leptin at 4 weeks of age due to the increased expression of orexigenic neuropeptides in the hypothalamus with no alteration of food intake and neuropeptide expression when intravenously treated with leptin [56]. Male TallyHo mice can develop hyperglycemia, hyperinsulinemia, hyperlipidemia, moderate obesity and enlargement of the islets of Langerhans. Female mice display moderate hyperinsulinemia, hyperlipidemia and obesity but do not manifest overt diabetes (hyperglycemia) [57].

2.3.5. B6(cg)-Tubtub/J

Mice homozygous for the tubby spontaneous mutation B6(Cg)-Tubtub/J develop obesity at the onset of maturity. Specifically, these mice show increased body weight at 3–4 months, whereas the females show this increase at 4–6 months. The increased body weight is due to an increased accumulation of adipose tissue. Blood glucose is normal, but plasma insulin is increased prior to obvious signs of obesity and may rise to 20 times normal at 6 months. The levels of total cholesterol, triglycerides and high-density lipoprotein cholesterol are increased in plasma of homozygous mutant mice; despite this, these mice do not exhibit atherosclerotic fatty streak blood vessel lesions. Importantly, both genders are fertile [58, 59].

2.3.6. Obesity induced by diet

Animal models of obesity that are similar to the human are very important to study the pathophysiology of obesity. The obese mouse model induced by high-fat diet (HFD) is one of the most important tools for understanding the relation between high-fat diets and the pathophysiology of development of obesity.

2.3.6.1. High-fat diet-fed mice

The high-fat diet (HFD)-fed mouse is a model of obesity, impaired glucose tolerance and insulin resistance [60]. The development of disease-induced dietary fat can be divided into

three stages: (1) an early stage in response to a high-fat diet in which mice were sensitive to exogenous leptin; (2) a reduced stage of food intake when mice had an increase in milk production and still retained central leptin sensitivity; and (3) a stage of increased food intake and accompanied by reduced sensitivity to the central leptin [61].

2.3.6.2. High carbohydrate-fed mice

The hypercaloric diets (HCDs) induce hyperglycemia by inducing tolerance to glucose and increasing the levels of TAG, TNF-α and MCP-1/JEin plasma. Moreover, the HCD increases the MCP-1/JE levels in target organs such as the adipose tissue and liver. However, the HC diet also can increase TNF-α concentration in the liver [62]. It is important to mention that the HFD is more effective to induce the body weight gain as compared with the HCD because of the large storage capacities of the adipose tissue and the low satiating effects of HFD as compared to the low capacities of the glycogen stores and of the de novo lipogenesis cost [63, 64].

2.4. Other metabolic syndromes

The metabolic syndrome (MetS) models are important to understand the pathophysiological basis of the MetS and how this syndrome increases the risk to the development of severe complications. The MetS animal model most commonly used is the obese mouse strains with several spontaneous mutations, which have been used for decades and are very well characterized. Moreover, inducing MetS with high-fat diet requires only some months, and these models are useful to study the effects of single genes by developing transgenic or gene knockouts to determine the influence of a gene on MetS [19, 65].

2.4.1. B6.129S7-Ldlrtm1Her/J

This mouse homozygous mutation has an elevated serum cholesterol level of 200–400 mg/dl and they attain very high levels (>2000 mg/dl) when fed with a HFD. Normal levels of serum cholesterol in the mouse are 80–100 mg/dl [66].

2.4.2. B6.Cg-Ay/J

The heterozygote mouse has increased the adipose tissue mass due to fat-cell hypertrophy and later develops insulin resistance and hyperglycemia. Heterozygote mice are also more susceptible to develop tumors than the normal mice, and their spleen cells cause a significantly lower graft versus host reaction. The level of malic enzyme in the liver is elevated [67].

2.4.3. NON/ShiLtJ

These mouse models are extremely useful for research works on obesity, diabetes, dyslipidemia and hypertension. NON/ShitLtJ (nondiabetic obese) mice contain an MHC haplotype resistant to diabetes and demonstrate early impaired glucose tolerance in both genders. These mice do not generate obesity when they are fed a diet containing 6% fat [68, 69].

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3. Rat models

3.1. Type 2 diabetes

3.1.1. Goto-Kakizaki rats

This rat strain was developed by the selective selection of Wistar rats for glucose intolerance over multiple generations, resulting in a polygenic strain that spontaneously develops hyperglycemia with problems in β-cell function. The hyperglycemia that these rats present is due to an increase of gluconeogenesis. Goto-Kakizaki (GK) rats have been considered one of the best nonobese T2D animal models. They are thin rats but present hyperglycemia and increased gluconeogenesis. GK rats present valuable characteristic tools that are commonly and functionally present in human diabetic patients [70, 71].

3.1.2. Streptozotocin-treated rat

This experimental model is useful for studying the regeneration of β cells in which damage to cells is caused by the injection of STZ. In this strain, regeneration of the cells is a complication, which is decreased in adult rats and thus presents a chronic pathological pattern like human T2D, glucose intolerance and low insulin in response to glucose [72, 73].

3.1.3. Pancreatomized Sprague-Dawley rats

To create this model of rats, Sprague-Dawley rats underwent simulated pancreatectomy. One week later, animals develop chronic hyperglycemia that is stable for several weeks without significant alterations in fatty acid levels. It is a strain used for the homeostatic control of the mass of the β cells to produce insulin in both the normal pancreatic growth and during the pathogenesis of diabetes. It is a multipurpose albino model, and primarily evidence of obesity is induced by diet, diabetes and oncology [74, 75].

3.2. Diabetic nephropathy

A very common treatment to obtain this model of rat is the application of streptozotocin, creating rats with diabetic conditions that develop kidney injury, similar to human diabetic nephropathy [76]. The mean urinary volume and protein excretion in these rats are greater than healthy rats; also, the kidney weight increases in this strain, as the immunoreactivity of endothelial nitric oxide in the renal cortex of these rats is much higher [77].

3.3. Obesity

3.3.1. Obesity induced by diet

3.3.1.1. High-fat diet-fed rat

The increase in weight induced by a high-energy diet causes certain defects in the neuronal response to negative feedback signals from circulating adiposity, such as insulin. Insulin

resistance of peripheral tissue involves cellular inflammatory responses that are caused by excess lipids. This model consists of rats fed with a HFD, mainly provoking DIO that has become one of the most important tools to understand the interactions of diets high in saturated fat and the development of obesity [78].

3.3.1.2. Cafeteria diet-induced obese rat

In the above model, body weight increases dramatically and remains significantly elevated in CAF-fed rats. Also, hyperinsulinemia, hyperphagia, hyperglycemia and glucose intolerance are exaggeratedly elevated in CAF-fed rats compared with other models with HFD [79–81]. These models present increased adiposity and hepatosteatosis, brown fat and more inflammation in the adipose tissue and liver. A CAF-fed rat model provides a model of human metabolic syndrome with an exaggerated obesity phenotype with glucose intolerance [81]. With this model, it is possible to study the biochemical, genomic and physiological mechanisms of obesity and disease states related to metabolic diseases [79].

4. Conclusions

Animal research has been and continues to be essential for understanding the underlying mechanisms of most human and veterinary diseases. Metabolic diseases are complex and present heterogeneous clinical forms with significant impact in understanding metabolic disorders. The use of animal genetic models, mainly rodents (mouse and rat), has showed several advantages. However, it is necessary to consider the standards of care of laboratory animals, which are consistent and demand the necessary experimental conditions.

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Section 3

Inflammatory and Viral Infections

Chapter 7

Animal Models of Double Incontinence: "Fecal and Urinary"

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Additional information is available at the end of the chapter

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Abstract

Throughout the world, animal models are being used as simulators of human anatomy and pathophysiology with most of the investigations and treatments first tested on them. Double incontinence (DI) includes both urinary and anal incontinence. This chapter is focused on the use of animals as models to understand pathogenesis, diagnosis and management of double incontinence (DI). DI is a complex disease with variant prevalence around the world which has a severe impact on quality of life (Qol). Many studies are designed to employ rodent and rabbit models to understand the pathogenesis of urinary and fecal incontinence. Urodynamic studies including leak point pressure (LPP) and urethral pressure profilometry (UPP) are used in establishing diagnosis of stress urinary incontinence. Rats have also been used to study fecal incontinence using neurophysiological and sacral nerve stimulation tests. The surgical treatment of double incontinence involves use of mesh, which was initially tested on animals. Animal models have also been used to train surgeons for perineal tear repair surgery. We conclude that the use of animal models provides best approach to learn these specialized surgical skills for medical practitioners and researchers.

Keywords: animal model, double incontinence, stress incontinence, fecal incontinence, human

1. Introduction

The prevalence of female urinary incontinence (UI) in Europe ranges from 14.1 to 68.8% and increases with age [1]. Specifically, stress urinary incontinence (SUI) is highest among all types of UI and is estimated at 23.7% [2]. The prevalence of fecal incontinence (FI) ranges from 2.2 to 50% in women with urinary incontinence or pelvic organ prolapse [3]. FI and UI are pelvic floor disorders (PFD) which lead to social embarrassment and have poor impact

on quality of life. Over $12 billion are spent annually for management of SUI in women [4]. The average annual total cost for fecal incontinence is estimated at $4110 per person [5]. Stress urinary incontinence (SUI) may be defined as involuntary loss of urine on effort or physical exertion (e.g., sporting activities), or on sneezing or coughing. Urgency urinary incontinence (UUI) relates to involuntary loss of urine associated with a desire to void, while anorectal incontinence (AI) is a complaint of involuntary loss of feces or flatus.

The ultimate success of long-term management for double incontinence (DI) is based on an understanding of disease pathophysiology. Little is known about the degree to which UI and FI share risk factors. Animal models have been used to understand pathogenesis of these conditions in humans and for developing novel treatment alternatives. Even though many animal models have been developed to understand pathogenesis, yet many of etiological factors are not explained. Many animal models are used as simulators for teaching surgical skills but long-term studies have not shown the desired improvement in surgical outcome [6]. The surgical procedures in humans were developed through the use and application of animal model as slings and trocar-driven implants [7] for anti-incontinence procedures.

Urinary incontinence is relatively easy to understand when compared to fecal incontinence as anal sphincter defects and FI are complicated surgical problems. Research on use of stem cell for treatment of FI was conducted on rabbits by an iatrogenic sphincter defect, created by cutting of anal sphincter. Human umbilical cord matrix (hUCM) and stem cells from rabbit femur and tibia were harvested and transplanted into injured sphincters which later showed an improvement in their function. Bone marrow-derived stem cells and mesenchymal cells of animals have shown to enhance contractile function of anal sphincter without surgical repair [8]. The limitation of using animals is in their difference with anatomy and size of viscera, which affects the functional outcome. Human cadavers have been used for a long time for teaching anatomy, but due to ethical issues animals were introduced in medical teaching. Animal models were found quite effective, but because of major difference in functional anatomy, mannequins were introduced for medical teaching and learning. There are many centers for simulation-based innovation for medical education (SIME), which probably would give similar results [9].

Most of the studies on new medical and surgical treatment involve the use of animal models for preclinical trials. In this chapter, we discussed use of animal models for relevant research, procedures on pathogenesis and surgical training techniques for DI. We have used standardized terminology for definitions as described by the International Continence Society (ICS) and International Urogynecological Association (IUGA) joint report on terminology [10, 11].

2. Methods of determining SUI in animal models

SUI is a clinical diagnosis mainly by history and physical examination. ICS has defined urodynamic stress incontinence as involuntary leakage of urine during filling cystometry, associated with increased intra-abdominal pressure, in the absence of a detrusor contraction

[10]. The role of urodynamic studies (UDS) is important in identifying types of SUI. Types of SUI can be determined with valsalva leak point pressure (VLPP) and urethral pressure profilometry (UPP). According to Blaivis, SUI types 1 and 2 are related to urethral hyper mobility with VLPP > 90 cm of water for type 1 and between 60 and 90 cm of water for type 2, respectively. Blaivis type 3 SUI is with VLPP < 60 cm water, also known as intrinsic sphincter deficiency (ISD). In addition, a urethral pressure profile (UPP which is urethral pressure–detrusor pressure) < 20 cm water is also seen in the cases of ISD [12]. Animal models that simulate SUI provide an assessment of the mechanism of risk factors, including childbirth injuries, preclinical testing of new treatments and therapies for SUI. Since animals cannot express intent, the use of these animal models has been focused on measuring decreased urethral resistance [13].

2.1. Sneeze testing

SUI is clinically assessed on humans as observation of involuntary leakage from the urethra with effort or physical exertion, or on sneezing or coughing [10]. Based on the urinary leak with a rise in abdominal pressure, sneeze test can be performed in female rat under anesthesia. A whisker cut from anesthetized rat was used to tickle its nose. Even under anesthesia the rat responded with a small sneeze, which transiently increased abdominal pressure. Karl et al. performed cystometry with methylene blue dye in bladder to detect urinary leak. The animal was diagnosed as incontinent if they leaked during the sneeze test and continent if no leak on sneezing was observed [13, 14].

2.2. LPP testing

The human bladder functions by storage and voiding of urine. Voiding is accompanied by an increase in detrusor pressure and a decrease in urethral pressure. In leak point pressure (LPP) testing [15], rats were anesthetized and a transperitoneal catheter implanted in the bladder dome was tunneled subcutaneously from the back of the bladder neck to an exit via the skin. The catheter was capped and the skin incision closed in two layers. The bladder catheter was connected to both a syringe pump and a pressure transducer. The bladder when filled with room-temperature saline through the catheter, the bladder pressure was recorded via a microtip transducer urethral catheter. Pressure and force transducer signals were amplified and recorded on a chart recorder. All bladder pressures were referenced to air pressure at the level of the bladder very similar to LPP assessment in humans with use of external transducers. The three commonly used mechanisms are manual pressure/Crede's LPP, electrical stimulation LPP and table tilt LPP [16–18].

2.2.1. Manual LPP testing

To perform manual LPP testing in rats, they put supine on table and a passive/manual abdominal pressure is applied and increased gradually, thus increasing the vesical pressure until leakage is observed at the urethral meatus. The peak bladder pressure was taken as the LPP. After leak, the external pressure is rapidly removed and bladder pressure quickly returns to baseline [17].

2.2.2. Vertical tilt table LPP

Rat is mounted on a vertical tilt table to keep the bladder erect during UDS, similar to human studies. A saline reservoir is connected to a suprapubic catheter to passively increase bladder pressure by elevating it and maintaining it at a range of pressures (20, 40 and 60 cm H_2O) [19]. In this method, the spinal cord is often transected usually at T8–T9. This transaction eliminates the supraspinal reflex voiding but preserves the urethral reflexes induced by bladder distention, which are predominantly organized in the lumbosacral spinal cord [20]. Studies have shown comparable results of LPP with sneeze test, manual pressure test and vertical tilt table test [21].

2.2.3. Electrical stimulation LPP testing

Electrical stimulation of abdominal muscles for 1 s induces sudden increase in both the intra-abdominal and the intravesical pressure. The lowest intravesical pressure that induced fluid leakage from the urethral orifice (leak point pressure) and the maximal intravesical pressure without urine leakage were recorded and were used to evaluate urethral resistance. However, like tilt table testing, electrical stimulation LPP testing also requires spinal cord transection, suppressing supraspinal continence control [22].

2.3. Urethral closure pressure testing

Effects of stem cell transplantation in rats were evaluated through urodynamic testing, and morphologic changes of the urethra and surrounding tissues were studied [23] both before and after transplantation. The bladder catheter was used as an intraurethral pressure measurement catheter, connecting it to a three-limb tube through a conversion joint. One end of that three-limb tube was connected to the intraurethral pressure sensor, and the other end was connected to the micropump, maintaining the original intraurethral pressure measurement catheter. Pressure was set at 0, and infusion by micropump at rate of 0.25 ml/min was started. Urethral pressure profilometry (UPP) rod was used to pull the intraurethral pressure measurement catheter at 0.1 mm/s traction speed. Meanwhile, intraurethral pressure and intrabladder pressure were recorded. Maximum urethral closure pressure (MUCP) was intraurethral pressure minus intrabladder pressure. Functional urethra length (FUL) was also calculated. Transplantation of adipose-derived stem cells significantly strengthened local urethral muscle layers and significantly improved the morphology and function of sphincters.

3. Establishing the causes of incontinence in animals

3.1. Causes of SUI

The childbirth injury leads to SUI due to musculofascial and neurovascular damage causing weakness in pelvic floor support [24]. Rodents are used to establish the vaginal injury as a leading cause of SUI which occur secondary to vaginal dilatation (VD) during childbirth in human.

Several studies [25, 26] have demonstrated vaginal injury by VD which is induced by using a Foley catheter with cut tip and inflated with different fluid volumes from 2 to 4 ml. This creates pressure in vagina and iatrogenic injury to the urethra, bladder, vagina and levator muscles. Functionally, VD results in decreased urethral resistance, as evidenced by lowered leak point pressures on urodynamic testing done in most of the VD studies [27]. In a study by Lin et al., VD was created in mice by 0.1–0.3 ml balloon in comparison with sham distension. LPP was significantly lower in groups after VD with 0.2–0.3 ml as compared to sham [28]. Research has shown that this procedure has helped in understanding molecular factors like chemokines, neuroregenerative agents and pharmacological agents that contribute to functional recovery including stem cell mobilization following injury [27, 29]. It has also helped in evaluation of the impact of contributing/decompensating factors in the pathophysiology and recovery of continence.

3.2. Causes of UUI

Urinary urge incontinence is observed among patient of overactive bladder (OAB) which is called wet OAB. There are many pathophysiological bases for its explanation including neurogenic and myogenic theories. It has been established through animal studies that urge incontinence is predominantly due to a defect in bladder muscle [30]. In a study on pigs, unstable bladder contractions were produced against induced outflow obstruction, bladder distention and bladder transaction. In affected pigs, stimulation of the spinal roots could no longer alter detrusor contraction. Similarly, sectioning of the spinal roots in these animals did not eliminate the unstable pressure rise explaining myogenic basis of OAB [31]. These manipulations do not eliminate the possibility of increased neuronal firing at the ganglionic level. However, recently, it has been shown that both hexamethonium (which blocks ganglionic transmission) and tetrodotoxin (TTX, which abolishes all neuronal activity) inhibit micturition but do not abolish unstable contractions in the pigs or rats [32, 33], hence supporting myogenic theory. The majority of the structural changes seen were obtained with light microscopic techniques, and local detrusor changes were found similar to those among human with OAB.

3.3. Causes of DI

The innervation of the external urethral sphincter (EUS) from the pudendal nerve is similar between rats and humans [34]. In female rats, the motor pudendal nerve bifurcates within Alcock's canal into separate fascicles that innervate the external anal sphincter (EAS) and EUS. The pudendal nerve controls EUS activity, including tonic activity during continence, and activates to strengthen the guarding response to prevent urinary leakage [35]. It can be trapped and injured during vaginal childbirth because it passes through Alcock's canal in the ischiorectal fossa, especially between the sacrospinous and the sacrotuberous ligaments [36]. Pudendal nerve crush (PNC) injury was induced in rats simulating childbirth injury, leading to deficiency of EUS and causing SUI [37]. Another rat study demonstrated the Pudendal nerve injury effects on external anal sphincter similar to injury during child birth in human affecting EAS and causing FI. In Healy et al.'s study [38], one group of rats used for the experiment had induced bilateral inferior rectal nerve crush (Group A) injury which then acted as a positive control and was observed for EAS effects. In another group (Group B), an intrapelvic retro-uterine balloon

inflation was performed, mimicking the pressure effects of child birth on the pelvic side wall and pelvic floor. Both groups of rats showed signs of EAS muscle atrophy and denervation, leading to FI. However, EMG signs of re-innervation were seen in both groups and recovery of muscle mass at 4 weeks, mimicking human pathophysiology of fecal incontinence.

4. Use of animal model in conservative treatment of incontinence

Since conservative management involves the use of medicine with many side effects, laboratory animals are used in preclinical drug trials. Several animal models have been used to evaluate the best possible conservative remedies for treating both urinary and fecal incontinence. Animal models were to test midurethral slings for surgical treatment of SUI which currently provides the best surgical cure. An outline of conservative management of double incontinence is shown in **Figure 1**.

4.1. Conservative management of SUI

Conservative treatment of SUI includes lifestyle interventions, pelvic floor muscle training, electrical stimulation, vaginal cones, urethral plugs and the drug duloxetine. Medical treatment has been tried and tested on animal models to assess their safety and effects on nervous system in improving SUI.

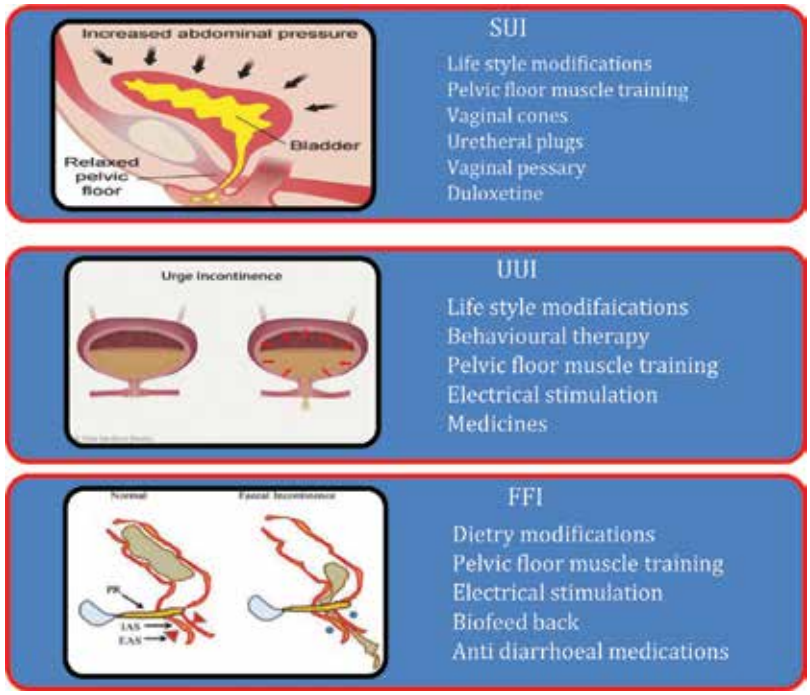

Figure 1. Conservative management of double incontinence.

4.1.1. Selective norepinephrine reuptake inhibitor

Venlafaxine is a selective norepinephrine (NE) reuptake inhibitor, and it significantly decreases the contraction of bladder muscle and increases urethral resistance. This was initially tested on rabbits and rodents. Bladder and proximal urethral muscle strips were electrically stimulated, and their contractile responses were measured both pre- and posttreatment with venlafaxine. It was observed that it significantly increased the contraction of urethral strips ($P = 0.008$) tested by urethral pressure profilometry (UPP) [39].

4.1.2. Norepinephrine (NE) and serotonin (5-HT) reuptake inhibitor

Duloxetine, a norepinephrine (NE) and serotonin (5-HT) reuptake inhibitor, can prevent SUI by facilitating noradrenergic and serotonergic systems in the spinal cord at S3 level (nucleus of Onuf) to enhance the sneeze-induced active urethral closure mechanism. Based on this mechanism, duloxetine is currently being used in humans for conservative management of SUI. Before the human trials, it was tested on cat sphincter [40] and in rat models. Duloxetine caused urethral closing contractions and increased the urethral resistance (leak point pressure) measured using a microtip transducer catheter in the middle urethra of rat models [41].

4.1.3. Stem cell therapy

One of the SUI causes includes urethral sphincter deficiency which is called type III SUI or intrinsic sphincter deficiency (ISD). This occurs usually due to inherent defects in the collagen and elastin of urethral sphincter. Many preclinical trials have investigated whether transplantation of patient's own skeletal muscle-derived cells (SkMDCs) can restore the sphincter musculature. The specific cell type of SkMDCs is myoblasts, satellite cells, muscle progenitor cells, or muscle-derived stem cells. The other stem cell (SC) types used for urethral defects include those from the bone marrow, umbilical cord blood and adipose tissue. These cells are injected as periurethral injections. Herrera-Imbroda et al. used rat models for SC injection, and rats were assessed by LPP testing for therapeutic efficacy of SC treatment [42]. The study also used histological assessment, which revealed the sphincter muscle content, existence of transplanted SCs and possible differentiation of these SCs.

Rodents were also used to explore the feasibility, safety and efficacy of cellular regimen to treat SUI. SUI was induced by vaginal dilatation (VD), and cystoscopic urethral injections of bone marrow or adipose tissue-derived mesenchymal stromal cells (BMSC/ADSC) were given to rats. It was observed that MSCs restored the continence mechanism by improving vascular and connective tissue status of urethral tissues after VD [43]. In another study, human mesenchymal stromal cells were isolated, expanded and characterized. These cells were injected trans-urethrally in immune-suppressed Göttingen Minipigs. The study found this cellular sphincter therapy in Göttingen Minipigs as very safe and effective against SUI [44]. Some animal studies employed dogs with induced SUI and injected SCs therapy to test safety and efficacy for SUI treatment and found similar results [45].

4.2. Conservative treatment of UUI

Clinical observations as well as results from recent studies on murine showed that iatrogenic bladder outlet obstruction leads to a rise in detrusor pressure, mimicking leak in humans secondary to detrusor overactivity (DO) in cases of UUI. Murines were induced DO and then treated by the use of botulinum toxin A (BoNT-A). The therapeutic effects of intramural injections of botulinum toxin A (BoNT-A) into the bladder wall resulted in suppression of detrusor overactivity in murine as seen in human bladder, and the refractory cases of UUI secondary to DO have shown same results with botulinum toxin A (BoNT-A) [46].

4.3. Conservative treatment of FI

Modifying irregular bowel habits is often the first step to manage FI. Pelvic floor exercises with and without biofeedback therapy, reusable bodyworn products and antidiarrheal treatment all play some role in treatment of FI. Sacral nerve stimulation (SNS) and stem cell therapy for improving contractile function of anal sphincter have been studied on animal models.

4.3.1. Sacral nerve stimulation (SNS) therapy

Fecal incontinence is multifactorial in origin. Most of the human studies have focused on anal sphincter functions and its restoration for treatment of FI. There have been numerous animal studies which investigated direct effects of SNS on the muscles of continence. In one study, ten dogs received electrical stimulation of the sacral plexus. Histochemical analysis of the striated external anal sphincter following chronic electrical stimulation demonstrated hypertrophy of stimulated muscle fibers. However, these changes reverted to pre-stimulation level 3 months after the stimulation. Anal tone and reflexes were measured before and during acute stimulation and demonstrated that SNS did not have any significant effect on internal anal sphincter or external anal sphincter force, the recto-anal inhibitory or recto-anal excitatory reflexes, internal anal sphincter slow wave frequency or wave amplitude [47]. The mechanism of action of SNS with the use of surgically implanted interstim is not very clear; however, it was found to be very effective in patients with FI [48].

4.3.2. Stem cell therapy

Stem cell injection at the site of injury can enhance contractile function of the anal sphincter without surgical repair. Human umbilical cord matrix (hUCM) cells have been described as having the characteristics of myofibroblasts, which play a role in healing by producing a wide range of cytokines, growth factors, chemokines and inflammatory mediators. Rabbit bone marrow (rBM) cells are known to secrete many growth factors which contribute to cell propagation and differentiation. Harvested hUCM and rBM stem cells from rabbit femurs and tibias were injected in surgically incised external anal sphincter of the white New Zealand rabbits. Electromyography showed significant improvement in sphincter function 2 weeks after local injection of rBM stem cells, and histopathologic evaluation showed normal

or muscle-dominant sphincter structure in all animals receiving rBM and fibrous-dominant sphincter structure in most animals receiving hUCM cells [8].

5. The role of animal studies in surgical management of DI

5.1. Surgical treatment of SUI

There are a variety of surgical treatment options for SUI. The two most effective procedures are Burch colposuspension and midurethеral slings (MUS), which are available in different synthetic material. Each material has been tested for its efficacy and safety. There are many animal studies regarding the use of mesh before its use in human.

5.1.1. Efficacy of slings tested by LPP

Surgical management including the suburethral sling is one of the most common treatment options for SUI, with an overall objective cure rate of 82% [49]. Suburethral sling therapy provides stability to the supporting layer under the urethra and helps in leak of urine against the rise in abdominal pressure. The urethra remains compressed against the suburethral sling, and continence is maintained. While a sling procedure offers the highest success rate, it also results in the highest morbidity and complication rate among all anti-incontinence procedures. In the last several years, a number of modifications to the sling procedure have been proposed to improve its safety and efficacy while decreasing morbidity. SUI in rats was induced by pudendal nerve transaction (PNT), they were treated by polypropylene suburethral sling and the efficacy of sling was assessed by an increase in LPP [50].

5.1.2. Tissue reaction of different sling materials

Tension-free vaginal tape (TVT) with polypropylene was first introduced by Ulmsten for surgical treatment of SUI and has shown good success rate [51]. The tensile properties of polypropylene used in TVT were studied in rats and found to be significantly greater than cadaveric fascia lata [52]. There have been many other sling procedures using same material with different surgical approaches. Another study on white rabbits has evaluated tissue reactions to five sling materials used in five different procedures like tension-free vaginal tape (TVT), intravaginal slingplasty (IVS) for SUI surgery and polypropylene mesh for hernia repair. The other two procedures to cure SUI included suprapubic approach using suburethral polypropylene tape and cadaveric fascia lata. Rabbit abdominal skin was incised, and a patch of all five sling material was attached with absorbable suture. Study compared the mesh-to-tissue attachment strength of four sling mesh materials on days 2, 7, 15 and 30 after implantation by electron microscopic studies. All five synthetic sling materials produced similar tissue reactions beginning soon after implantation. Cadaveric fascia lata persisted in tissue with remarkable perifascial fibrosis at day 30. When comparing the four polypropylene mesh materials, the attachment capacity of TVT was superior and that of IVS was the least of the four. TVT was statistically better than IVS at all data points. Suprapublic approach with polypropylene and hernia mesh provided results similar to those of TVT [53].

5.2. Surgical treatment of FI

Obstetric anal sphincter trauma is the most common cause of fecal incontinence with a severe impact on quality of life. Anal sphincter rupture is reported in about 2.5% of vaginal deliveries in centers that practice mediolateral episiotomy and about 11% in centers that practice midline episiotomy [54]. The effect of anal sphincter laceration (with repair) at the time of parturition after term pregnancy on physiologic function of the external anal sphincter was studied on eighty rats [55]. Overall, anal sphincter laceration at time of delivery results in significantly impaired anal function. Rat anal sphincter neurophysiologic functions were assessed. Recovery of sphincter function was evident as early as three months and maintained at six months after injury. The diagnosis and repair of sphincter tear is very important. Unrepaired or badly repaired sphincter can lead to FI. Several simulator models have been developed to provide surgical training to consultants, midwives and trainees. The early diagnosis of anal sphincter injury is very important for effective surgical outcome. The pig model was introduced due to its similarity to both internal and external anal sphincters [56]. The model used cadaveric pig perineum with a clear demarcation between internal and external anal sphincters simulating human sphincters. Another study showed effective teaching of repair of perineal tears using goat perineum model (**Figure 2**), which mimics human female anatomy [57]. Both anal sphincter latex/plastic and cadaveric animal sphincter models have been effectively used for hands on training in different workshops.

Figure 2. Multiperous goat. Cut edge of external anal sphincter (E) held by Allis forceps, (I) internal anal sphincter between anal canal (A) and (E) external anal sphincter.

5.2.1. Artificial anal sphincter

The artificial anal sphincter is used in cases where other treatment modalities fail. It includes an inflatable expander that compresses and flattens the bowel against a pillow. Before its

experiment on humans, it was tried in 16 animals. In experimental animals, anal sphincters were destroyed and artificial sphincter device was implanted. The animals were observed for twenty weeks. The study concluded the safety of implanted sphincter against anal ischemia. Moreover, animals were continent during 85% of activation times [58].

6. Animal genetic models developed for incontinence/SUI studies

During last decade, investigators have developed and tested animal models of SUI in the female rat, seeking to mimic the symptoms of SUI in female patients. Bilateral pudendal nerve crush injury or transection or sciatic nerve transection has been used to cause SUI in rats. The VD model was used by Lin et al. [28] to simulate the damage that occurs in the pelvic floor during vaginal delivery of children. They demonstrated the feasibility of creating a mouse model of acute SUI by VD. Distention volumes of 0.1–0.3 ml in 20 g female mice of strain C57BL/6 resulted in significant reductions of LP, possibly due to partial urethral denervation. This novel model of SUI in mice could be used in future mechanistic studies of female SUI treatment. The childbirth induced vaginal distension, and SUI can be correlated but women recover out of these transient changes with only few remaining symptomatic for SUI. There is a need to develop specific mouse genetic models for incontinence/SUI induced by VD. Further studies can be performed to know the spontaneous cure of incontinence.

7. Conclusion

The use of animal models has helped in understanding the pathogenesis and etiology of both urinary and fecal incontinence. Due to ethical issues related to human cadaveric studies, animal models are good substitute for research related to surgical innovations for treatment of double incontinence. Animal models like sheep, goat and pig have been validated for surgical training for perineal tears. The latest use of animal model is related to studies on mouse for simulated birth trauma-induced SUI and stem cell treatment for double incontinence.

List of abbreviations and acronyms

DI	Double incontinence
FI	Fecal incontinence
UI	Urinary incontinence
SUI	Stress urinary incontinence
UUI	Urgency urinary incontinence
OAB	Overactive bladder

LPP	Leak point pressure
UPP	Urethral pressure profilometry
UDS	Urodynamic studies
VLPP	Valsalva leak point pressure
ISD	Intrinsic sphincter deficiency
PFD	Pelvic floor dysfunction
ICS	International Continence Society
IUGA	International Urogynecological Association
VD	Vaginal dilatation
PNC	Pudendal nerve crush
NE	Norepinephrine
EUS	External urethral sphincter
EAS	External anal sphincter
TTX	Tetrodotoxin
SC	Stem cells
SkMDCs	Skeletal muscle-derived cells
SNS	Sacral nerve stimulation
hUCM	Human umbilical cord matrix
rBM	Rabbit bone marrow
BoNT-A	Botulinum toxin A
PNT	Pudendal nerve transaction
TVT	Tension-free vaginal tape
IVS	Intravaginal slingplasty
BMSC	Bone marrow-derived stem calls
ADSC	Adipose tissue-derived mesenchymal stromal cells

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Chapter 8

Relevance of the CDE and DDC Mouse Models to Study Ductular Reaction in Chronic Human Liver Diseases

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Additional information is available at the end of the chapter

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Abstract

The liver has the remarkable capacity to regenerate through cellular division of hepatocytes. However, following severe injuries that abrogates the replicative capacity of hepatocytes some immature-like cells proliferate around the portal area and invade the parenchyma in a process known as ductular reaction (DR). In humans, DR is observed in virtually all chronic liver disorders although the morphological patterns may vary. DR biology has gained considerable interest because of potential contribution to hepatic cell restoration, fibrosis or carcinogenesis. In humans, observational studies are available but experimental manipulations and lineage tracing are impossible. Animal models represent thus valuable tools to explore such questions. Feeding rodents a choline-deficient, ethionine-supplemented diet (CDE) or a diet enriched in 3,5-diethoxycarboncyl-1,4-dihydrocollidine (DDC) are the most popular models to study DR. They are often used equivalently in the literature although the aspects and outcome of the DR are different and model-specific. Here, we describe experimental procedures and the pathophysiological mechanisms at play; we describe the hepatic lesions and highlight the unique character of DR phenotype, proliferation, lineage commitment and microenvironment in each model. We then compare the models with DR phenotype in human pathologies.

Keywords: liver progenitor cells, ductular reaction, CDE, DDC

1. Introduction

In a healthy liver, hepatocytes are quiescent long-lived cells. Upon mild to moderate hepatocellular injury or depletion, hepatocytes self-duplicate to restore the liver mass. However,

when there is a massive cell loss or a continuous damage to mature hepatic cells, overwhelming the replicative capacity of the remaining hepatocytes, expansion of immature-like cells is observed at the interface between the portal area and the parenchyma in a process called ductular reaction (DR). Expression of biliary markers is a hallmark of DR cells, but nevertheless, DR constitutes a heterogeneous population of proliferating cells ranging from immature stem-like cells to more committed cells with an intermediate hepatobiliary phenotype [1–4]. Cells of the DR are also called liver progenitor cells (LPC) as they have been shown to differentiate into both hepatocytes and cholangiocytes lineages in culture (reviewed in Ref. [5]). In normal livers, no DR are usually observed and LPC are seen, in two-dimensional tissue sections, as single cells located mainly in the canal of Hering, which represents the connection between the smallest ramifications of the biliary tree and the hepatocyte canalicular system [6, 7]. DR/LPC and biliary cells cannot strictly be distinguished at the histological level but based on their location and morphological differences [8]. In a three-dimensional viewpoint, DR and the biliary tree constitute together a contiguous heterogeneous epithelial structure [9]. In humans as in rodents, the histological and morphological patterns of DR vary according to injurious settings and their lineage commitment toward hepatocytes or cholangiocytes has been related to the primary site of cell loss or dysfunction [10].

Over the past decade, there has been a considerable interest in understanding DR/LPC biology. LPC are indeed seen as a potential reservoir for mature hepatocytes. Understanding the nature and differentiation process of LPC may generate cells for liver-cell therapy, which is increasingly under demand due to organ shortage for liver transplantation. Moreover, DR has also been postulated to trigger portal fibrosis [11]. Unraveling the potential mediators of DR could therefore be of great interest to modulate progression of profibrogenic reaction observed in many chronic liver diseases.

Several rodent models of liver injury associate with a DR and are instrumental to study the LPC response and its implication in liver regeneration and wound healing. These models, as in human liver diseases, exhibit a large variety of DR/LPC patterns with different morphological features, kinetics of response, and differentiation potential. The models of liver injury with DR generally combine the damage and loss of epithelial cells (hepatocytes and/or cholangiocytes) with the inhibition of the proliferative capacity (replicative senescence) of mature epithelial cells. Toxins [12, 13], carcinogens [14, 15], or modified diets [16, 17] have been used to induce cell injury, either alone or associated with surgical removal of part of the liver to amplify liver cell depletion. Ethionine, 2-acetylaminofluorene (AAF), and retrorsine are used to block the ability of mature epithelial cells to divide and prevent them from contributing to the liver regeneration process. In mice, dietary manipulations are regarded as convenient, efficient, and reproducible models to induce a robust DR, without need for animal handling, repeated injections, or surgical manipulation. The two most popular dietary DR models are a choline-deficient diet supplemented with ethionine in the drinking water (CDE) or a diet enriched in 3,5-diethoxycarbonyl-1,4-dihydrocollidine (DDC).

In the literature, the DDC and CDE models are often used equivalently to study the LPC response and their role in tissue repair. However, DR in those two models exhibits major

etiological and phenotypical differences. Such differences recapitulate the specificity of the pathophysiological responses to distinct injurious processes. DR activation, expansion, and capacity for differentiation are dictated by the nature of the cellular injury and by the differential microenvironment changes.

In this chapter, we will first describe the pathophysiological mechanisms at play in each model and the experimental procedures to induce DR with the CDE or DDC diet. A description of the hepatic lesions in terms of the cellular compartment injured after CDE and DDC feeding and highlight of the unique character of each model with regard to the DR phenotype, proliferation, lineage commitment, and microenvironment will be explored. Finally, the relevance of these models to study and understand the diversity of DR seen in human chronic liver diseases will be addressed.

2. The CDE model

2.1. The CDE model of hepatocellular injury: pathophysiological mechanisms and DR phenotype

The CDE model consists of *ad libitum* administration of a choline-deficient diet together with procurement of ethionine in the drinking water. Choline is provided by food intake and contributes to the structural integrity and signaling function of cell membranes. A choline withdrawal leads to a decreased synthesis of phosphatidylcholine, a phospholipid crucial for cell membrane and a major building stone of the very low-density lipoprotein particles produced by hepatocytes to export triglycerides. Choline deficiency causes intracytoplasmic fat accumulation, hepatocyte dysfunction, and cell damage [18, 19]. Such (extensive) hepatocellular damage results in high hepatocyte replication ratio, causing their exhaustion and restraining the production of hepatic drug metabolism-related enzymes [20]. Ethionine, a synthetic amino acid, specifically targets the hepatocytes in which, when provided in large excess, it competes with its naturally occurring analog methionine. Competition of ethionine with methionine favors the synthesis of S-adenosyl ethionine (SAE) instead of S-adenosyl methionine (SAM). Consequently, an ethyl group is transferred instead of a methyl group in methylation reactions hereby generating abnormal proteins, lipids, RNA, and DNA molecules, which results in hepatocytic cell damage [21]. Prolonged feeding with ethionine produces liver tumors with extensive LPC proliferation [22]. However, administration of ethionine in supplement to a choline-deficient diet greatly shortens the time required for LPC proliferation [22]. Although, the exact mechanism of action of CDE-induced injury is not well known, it appears that the combined administration of ethionine with choline-deficient chow induces a liver injury in which the hepatocytes are specifically targeted and the replication of the surviving hepatocytes is inhibited [23]. Hepatocyte proliferation to replace damaged liver cells is prevented and activation of the LPC compartment ensues. Several publications characterized the kinetics of the LPC response and liver damage to CDE [24–27]. Briefly, short-term CDE feeding results in steatosis, inflammation, LPC expansion (DR), and fibrosis that progress

in parallel. Cirrhosis and hepatocellular carcinoma may be observed in long-term studies. We intend to describe and analyze in depth the morphology and differentiation capacity of DR after 3 weeks of CDE (except when specified otherwise), at a time when pathological damages are installed and DR robustly established.

After 3 weeks, CDE livers are pale with signs of steatosis throughout the parenchyma. Liver weight is comparable or slightly lower than the deep-red control livers (**Figure 1A** and **B**). Signs of hepatocellular injury are observed with necrotic and apoptotic hepatocytes while bile ducts appear normal within the portal triad (**Figure 1G**) [23, 28]. Also, serum alanine aminotransaminases are increased while bilirubin levels are in the near normal range, indicative of hepatocytic damage (**Figure 1D** and **E**).

In the CDE model, DR expansion, seen on two-dimensional (2D) sections by staining with a biliary marker such as cytokeratin (CK) 19, is observed arising from the portal area and invading progressively the parenchyma (**Figure 1G**). First observable after approximately 1 week of CDE feeding, the DR progressively amplifies to a maximum around 3–4 weeks [23, 24, 29]. DR cells are small cells with a high nuclear-to-cytoplasm ratio, usually uniform in size with a fusiform shape and oval nuclei. On 2D liver sections, they are found as individual cells, grouped in multifocal clusters or organized in a single or double row of cells forming arborizing structures (**Figure 1**) [24]. Architectural three-dimensional (3D) analysis of the biliary tree remodeling in response to CDE reveal that DR are connected to the preexisting bile ducts and that biliary branches intricately split around the portal vein with a random directionality [9]. Moreover, plastination of the bile duct system reveal a denser biliary network after CDE feeding (**Figure 1J**).

Finally, with regard to LPC capacity of differentiation *in vivo*, DR cell-tracking experiments using different transgenic mouse models [23, 28, 30] indicate that, upon CDE diet, a small number of DR cells do differentiate into hepatocytes: in the process LPC lose biliary markers, grow in size, and acquire mature hepatocyte morphological features and functional proteins. Although differentiation is consistently reported, only few DR-derived hepatocytes are reported in this model (<2.5% of hepatocytes).

2.2. The CDE model: practical aspects

Although being widely used, the CDE model is difficult to handle and researchers are confronted with difficulties and ethical issues due to variability in the LPC response, well-being of the animals, morbidity, and mortality. Here, we will review several factors influencing the LPC response to the model. These parameters must be taken into account and controlled to strengthen the model and provide reproducibility.

2.2.1. Dietary variables

Rodent food manufacturers can easily provide food in which the choline content is strictly controlled. Although low choline dietary content could be used [31], we will describe here a model using dietary choline deficiency. The second parameter to adjust for is ethionine supplementation. In the literature, the amount of ethionine in the water varies from 0.05 to 0.165% (wt/vol).

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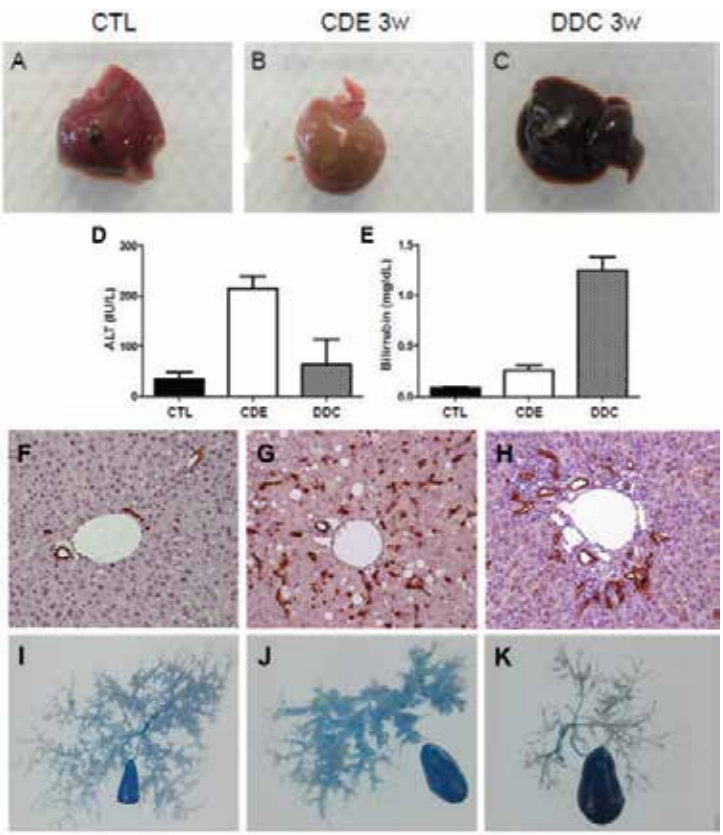

Figure 1. Pathophysiological mechanisms and DR phenotype in the CDE and DDC models. Livers retrieved from control mice (A), mice receiving the CDE (B), or DDC diet (C) for 3 weeks. Serum biochemical measurements for total aminotransferase (ALT) (D) and bilirubin (E) showed increased ALT and near normal bilirubin in the CDE model and increased serum bilirubin with slightly elevated ALT levels in DDC, indicative of hepatocellular damage upon CDE treatment and of primarily biliary injury after DDC diet. Liver sections stained with anticytokeratin 19 (CK19) in control (F), CDE (G), and DDC (H) livers after 3 weeks of diet. In control, CK19 staining reveals bile ducts and LPC as isolated cells close to the periportal tract. In CDE livers, besides bile ducts, DR CK19+ cells are strongly increased in number, forming cells organized in filaments expanding inside the lobule. After DDC feeding, in addition to the larger preexisting bile ducts, CK19+ newly formed DR structures are composed of small cuboidal cells, irregular in size and shape accumulated around the portal area. Plastination of the bile duct system reveals delicately structured biliary tree in control mice (I), a denser biliary network after CDE feeding (J), and dilatation of intrahepatic bile ducts in DDC-fed mice (K).

Mice are not fond of ethionine (due to bad smell), and usually decrease water consumption. This makes it difficult to control effective ethionine intake. Addition of 5% sucrose, choline-free orange juice, or fruit syrup is sometimes used to increase the attractiveness of the drink and this is most of the time not reported in the experimental protocol [9, 23, 28, 29, 32–34]. Ethionine smell increases with exposure to the ambient air and we found that we could maintain stable water intake by replacing ethionine-containing water by a fresh solution every day. Although this sounds trivial, control over ethionine solution consumption is crucial as both variation in ethionine intake and (severe) dehydration may influence LPC response and induce large interindividual variation in the model. To circumvent this, Passman et al. also propose to include ethionine in the chow [29].

2.2.2. Mouse variables

LPC response and morbidity vary according to weight and age of the mice at the time of introduction of the CDE diet. Mice above 25–30 g will be quite resistant to the diet and if they are too old, perhaps because of loss of cell plasticity, LPC response will be discrete. In parallel, if they are too young or too little (<15 g) at the time of dietary exposure, toxicity and ensuing mortality might be excessively high. With the administration of the CDE diet to mice of 6 weeks of age and with a body weight between 18 and 20 g, we and others show a substantial and reasonably reproducible LPC response while maintaining the well-being of the animals [24, 28, 29, 35]. Mice are experiencing the most severe effects of the diet during the first week of administration. Following the first few days, significant weight loss is observed, often associated with mortality [29, 36]. Approximately 1 week after the onset of the CDE treatment, the mice adapt, regain weight, and show (normal) physical activity and behavior. Thus, by respecting the simple rules proposed above, body weight loss may be limited to 10% of the starting body weigh during the first week with weight stabilization thereafter. Importantly, sensitivity to the dietary regimen and magnitude of liver damage and LPC reaction largely vary according to the genetic background of the mice [17]. This imposes the use of an appropriate control group (best being littermates) when comparing the effect of gene deletion or addition in genetically modified animals.

3. The DDC model

3.1. The DDC model of biliary injury: pathophysiological mechanisms and DR phenotype

The DDC model consists of *ad libitum* administration of a diet enriched with the porphyrinogenic agent 3,5-diethoxycarbonyl-1,4-dihydrocollidine (DDC) with normal water. Exposure to a DDC diet provokes the inhibition of the mitochondrial enzyme ferrochelatase, catalyst of the insertion of ferrous iron into protoporphyrin IX to form heme, leading to progressive accumulation of protoporphyrin. This brown pigment first accumulates in the cytoplasm of parenchymal cells and in Kupffer cells. Because of its hydrophobic nature, the excess of protoporphyrin can only exit the liver through biliary excretion, leading to precipitation of this poorly soluble molecule in bile canaliculi and bile ducts, forming crystals increasing in size and number [37]. After 3 weeks of DDC diet, accumulated pigments plug and obstruct the lumen of the smaller branches of the biliary tree and confer a dark coloration to the liver (**Figure 1C**). Bile ducts, usually recognizable as monolayer rings of small cuboidal cholangiocytes delineating a central lumen, show profound morphological alterations while hepatocytes have a normal appearance except for pigment coloration (**Figure 1H**). This indicates that the DDC dietary regimen mostly damage the biliary system, which is additionally supported by increased serum bilirubin (**Figure 1E**). We observed moderately elevated transaminases levels (two to threefold time, **Figure 1D**) although another group reports higher transaminase levels after DDC feeding [16]. In later stages, livers

in DDC-fed mice develop pericholangitis and periductal onion skin-like fibrosis. Our discussion here analyzes DR morphology and microenvironment after 3 weeks of DDC feeding.

In the DDC livers, bile duct damage is associated with a biliary response in which dysmorphic cholangiocytes proliferate in the portal area. In all portal tracts, DR expands as multiple small pseudo-ducts arising next to the larger preexisting bile ducts (**Figure 1H**). These newly formed ductular structures are composed of small cuboidal or more cylindrical cells, irregular in size and shape, assembled in tube-like structures outlining a lumen in most cases, sometime plugged by porphyrin crystals. In contrast to infiltrating DR in CDE livers, DR expansion observed in DDC livers remains enclosed within the portal mesenchyme. No parenchymal invasion crossing the boundaries of portal mesenchyme was observed nor did those reactive cells, always observed as a cluster, adopt a phenotype supporting migration. However, the portal mesenchyme extends and may bridge distant portal spaces (**Figure 1H**). 3D biliary analysis of DDC livers identifies branches randomly directed around the portal vein, connected to the biliary tree but forming apparent distinct structures from the large-diameter bile ducts [9]. Moreover, 3D plastination of the DDC-fed mouse confirms slight focal dilatation of intrahepatic bile ducts and porphyrin plugs while biliary network seems to be less dense [16] (**Figure 1K**).

Finally, concerning LPC capacity of differentiation, upon DDC-induced injury, there is no evidence that cells of the neo ducts undergo hepatocytic cell differentiation [23, 28, 30]. When animals are reversed to a standard chow after DDC diet, the degree of DR expansion decreases, but still with no evidence that DR cells differentiate into hepatocytes. Because a specific LPC marker, that is, exclusively expressed in LPC and not in cholangiocytes, is lacking, we are currently unable to experimentally address the contribution of LPC to biliary regeneration *in vivo* during disease evolution. We can, however, hypothesize that, if not entirely supported by proliferation of mature cholangiocytes, LPC located at the most proximal part of the biliary tree contribute to neo duct formation in the DDC model [9].

3.2. The DDC model: practical aspects

Contrasting with the CDE model, the DDC model is robust and reproducible and has little impact on animal welfare. In all studies, diet (standard rodent chow) is supplemented with 0.1% (wt/wt) of DDC. Similarly, the different mouse strains tested so far develop comparable hepatic phenotype to DDC feeding [16] although differences in susceptibility and kinetics of the response might be expected according to strains. Of note, DDC diet applied to rats does not induce any LPC response [38].

4. Microenvironment-regulating DR expansion and differentiation

The literature brings every day new evidence that the orchestrated interplay between proliferating hepatocytes or cholangiocytes, extracellular matrix-producing myofibroblasts, inflammatory cells (such as macrophages, neutrophils, or lymphocytes), and endothelial cells is pivotal in the regulation of DR expansion and differentiation. We will thus compare the microenvironment accompanying DR in the CDE versus DDC model.

4.1. Extracellular matrix, collagen, and laminin

Extracellular matrix and collagen deposition associates with DR. In the CDE model, a thin and loose web of collagen fibers is associated with invading DR cells, while collagens in DDC livers thicken the portal mesenchyme and abundant extracellular matrix accumulates in clots or thick concentric layers around the neo-formed pseudo-ductular structures (**Figure 2A–D**).

The localization of myofibroblasts, the cells chiefly involved in matrix synthesis and remodeling, adopted a pattern similar to that of the collagen deposition in both models, meaning that DR is at all times associated with myofibroblasts. In CDE livers, myofibroblasts chaperone the DR cells while they penetrate deep into the liver lobule (**Figure 2E**). Conversely, in the DDC model, myofibroblasts densely populate the portal mesenchyme and accumulate rather concentrically around DR (**Figure 2F**).

Laminin is a component of the basal membrane delineating the basal pole of cholangiocytes. Basement membrane is essential to establish the cholangiocytes polarity and to support a tubular structure with a lumen [39]. By contrast, hepatocytes do not lie on a basement membrane. In the CDE model, DR is anchored onto a laminin-rich basal membrane intermingled with collagen. This layer of laminin has been proposed to maintain the immature/biliary phenotype of DR cells and to provide a holding structure facilitating migration of DR into the lobular parenchyma in the CDE model [28, 40]. Moreover, decreased density of laminin and extracellular matrix in CDE livers is associated with enhanced hepatocytic differentiation of DR cells [28]. Indeed, during DR differentiation process, DR cells progressively lose contact with the laminin-rich basement. And when animals are reversed to a standard chow (supply of choline and cessation of ethionine administration) after CDE exposure, the injury reverses, DR, extracellular matrix, and laminin deposition progressively lessen, and concomitantly the number of DR-derived hepatocytes increases.

In DDC livers, laminin deposits as thin basal membrane outlining the DR in a pattern similar to that seen around normal bile ducts, with collagen stacked as separate sheets encircling newly formed DR.

4.2. Inflammatory environment

In response to liver injury, Kupffer cells, the hepatic macrophages, activate and participate to the recruitment of the inflammatory reaction. In CDE livers, enlarged and proliferative Kupffer cells are strongly associated with invading DR within the parenchyma while no portal inflammation is observed [41]. DDC-induced proliferation of the ducts is accompanied by a dense macrophage and neutrophil granulocytic infiltrate around small and larger bile ducts, further supporting that biliary structures are first concerned by the injurious and healing responses in this model [16].

Kupffer cells do not influence DR expansion but modulate its invasive behavior and its specification, through modulation of the density of extracellular matrix as well as via Notch and Wnt signaling pathways [41, 42]. Numb, a direct transcriptional target of Wnt and a negative regulator of Notch, is downregulated in LPC during biliary regeneration, promoting

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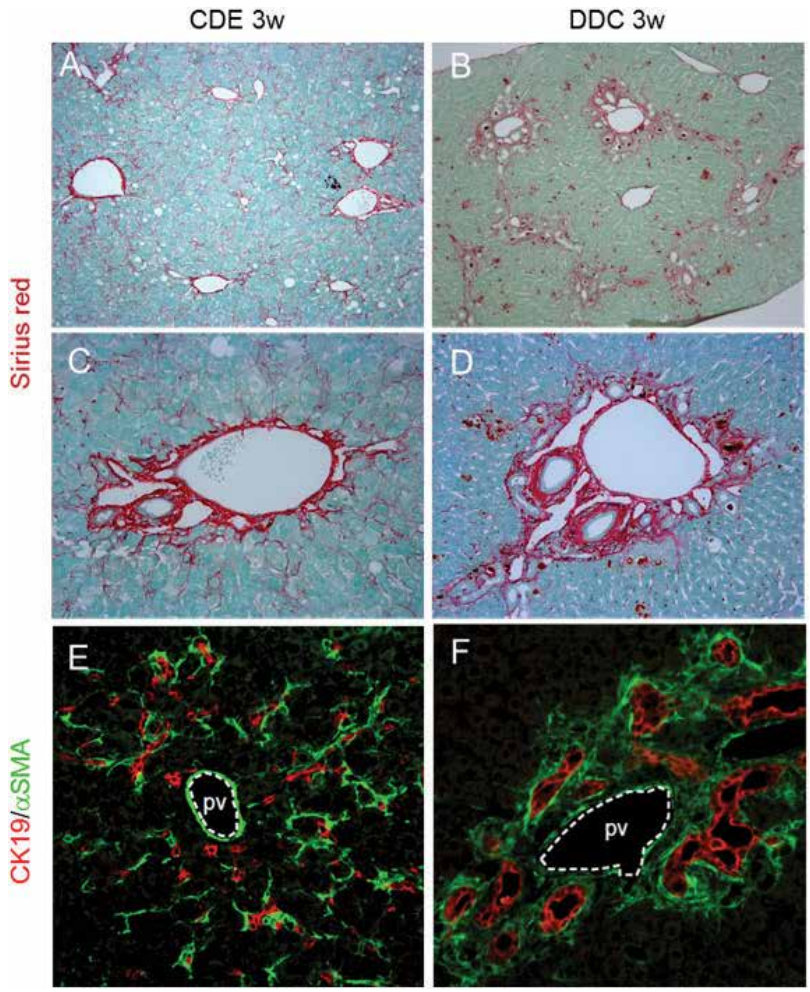

Figure 2. Comparison of the extracellular matrix deposition and the myofibroblast expansion between the CDE and DDC models. Liver sections obtained from mice receiving the CDE (A, C, E) or DDC diet (B, D, F) were stained with Sirius red to highlight fibrillary collagen (A-D), or αSMA and CK19 expression (E and F). In CDE livers, a collagen meshwork covers the whole parenchyma (A) with fibers elongating from the portal area into the lobule (C). In DDC livers, collagen fibers accumulate around the portal area to shape the portal mesenchyma (D), delimiting the boundaries of DR (F). At lower magnification, portal-portal bridging is observed (B). αSMA+ myofibroblasts show a distribution pattern similar as the collagen deposition. αSMA+ myofibroblasts infiltrate the lobule, chaperoning CK19+ DR cells in the CDE model (E), while in the DDC model, αSMA+ myofibroblasts rather accumulate concentrically around the DR structures (F).

biliary specification via the Notch pathway. While during hepatocyte regeneration, macrophage-derived canonical Wnt signaling maintains Numb within LPC and Notch signaling is reduced, promoting hepatocyte specification [42].

Experiments performed in lymphocyte-deficient mice fed on CDE suggest that natural killer cells and T-cells participate also to LPC expansion, presumably through their proinflammatory cytokine production [43]. Moreover, TNF-like weak inducer of apoptosis (TWEAK), produced

by T-cells and activating its receptor fibroblast growth factor-inducible 14 (Fn14), is suggested to be an exclusive LPC mitogen. After both CDE and DDC treatment on Fn14 knockout mice, a significant reduction of the LPC response is observed [31, 44].

4.3. Nearby endothelial cells

As described above, DR requires a typical niche provided by extracellular matrix-producing and inflammatory cells, which are located in the sinusoids closely adjacent to DR. Additionally, sinusoidal endothelial cells themselves could also have an important role in regulating DR. Signaling molecules specifically expressed within the endothelial compartment of the central vein have been shown to have a crucial role in liver zonation [45]. Moreover, in another model of liver injury, hepatocytes divide along the closest microvessel as order principle to restore liver architecture [46]. Either a signaling or a guiding role of endothelial cells on LPC response could be envisaged. However, so far, no experiments have been done to study endothelial regulation of DR in the CDE or DDC model.

5. Comparison of the CDE and DDC models with chronic human liver diseases, HCV and PSC, respectively

In humans, DR is seen in most chronic liver injury, irrespective of the etiology. Historically, DR has been categorized on morphology into "*typical*" and "*atypical*" DR, based on rodent studies [47]. *Typical* DR have a lumen lined by cuboidal cells and are the result of proliferation of preexisting ductules, in analogy with the DR seen after biliary obstruction, while *atypical* describes thin, elongated structures that extend into the lobules and lack discernible lumen as preferentially seen after hepatocytic damage. Therefore, DDC diet best models *typical* DR, while CDE diet replicates pathological pattern of *atypical* DR. However, this dichotomic classification was discouraged some years ago because it could not readily accommodate the range of patterns seen clinically [10]. Another classification schemes attempted to integrate the histologic features, inciting disease and immunophenotyping of DR [48, 49]. However, not all DR fit in this classification, and especially not when biliary obstruction becomes chronic (as in the DDC livers). So far, there is a lack of consensus regarding DR classification in humans, as DRs are diverse, covering a spectrum of features rather than clear subphenotypes [10].

However, based on histological analysis, the DR phenotype in the CDE model resembles the one observed in human chronic Hepatitis C virus (HCV) infection depicting portal fibrosis and in a series of autoimmune hepatitis (AIH) (**Figure 3A–D**). HCV- and AIH-associated DRs have only a vague or no lumen, and comprise small elongated cells with little cytoplasm extending in the periportal parenchyma and associated with dense collagen fibers [10, 11, 50]. In HCV, the extension of DR into the parenchyma and DR severity correlate with the severity of fibrosis and the inflammatory activity, supporting that extracellular matrix and inflammatory signals influence DR [50]. DR in AIH has been proposed to represent a regenerative response as DR persists after the inflammatory activity subsided following immunosuppressive treatment [51]. At the early stage of fibrosing, cholestatic variants of coinfection with hepatitis B and C, expanded DR into the hepatic parenchyma also resemble the DR phenotype seen in CDE livers [10, 52]. With regard

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to LPC fate, the observation of a phenotypic continuum between DR cells and hepatocytes in the livers of patients suffering from HCV supports differentiation of LPC toward hepatocytes [4, 50]. Besides hepatitis, the CDE diet also recapitulates features of the DR associated with lipid accumulation (steatosis) as in chronic alcoholic and nonalcoholic fatty liver diseases [11, 29, 42, 53].

The DR pattern seen in DDC livers is more comparable to that of chronic fibrosing cholangitis such as primary sclerosing cholangitis (PSC) and primary biliary cholangitis (PBC) with DR proliferation restricted within the portal area and accompanied by concentric periportal fibrosis (**Figure 3E** and **F**). In these diseases as in DDC, the primary damage is directed toward cholangiocytes. Intrahepatic bile duct destruction and ductopenia seen in advanced PBC and the fibrous obliterative lesions of PSC do not occur in the DDC model, a phenomenon most likely related to the specific immune component of PBC and PSC which is lacking in the DDC model.

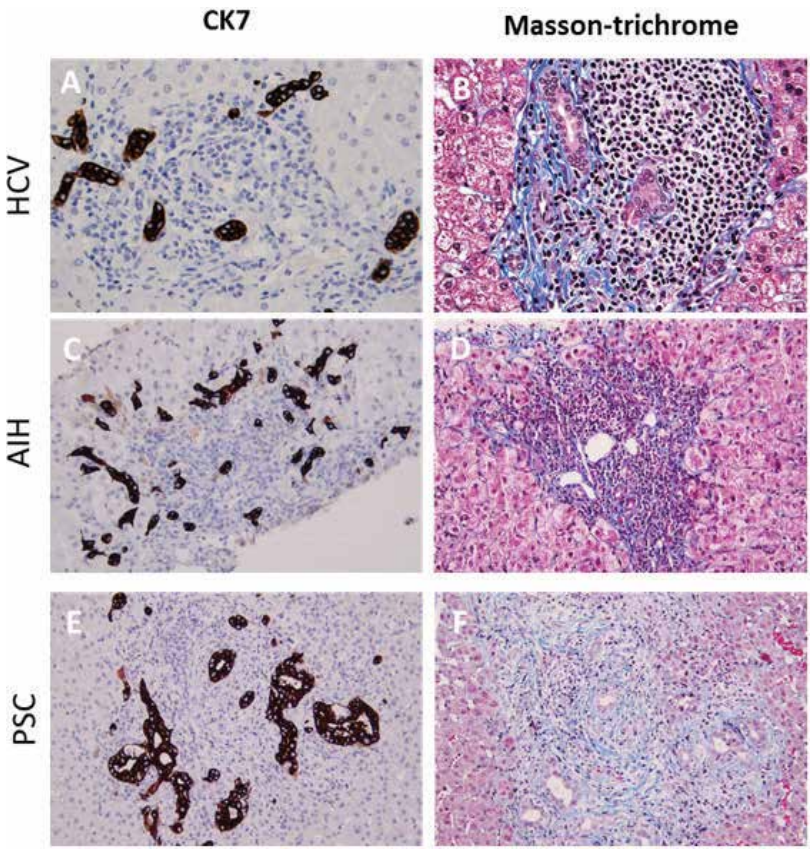

Figure 3. DR observed in human chronic hepatitis C infection and in primary sclerosing cholangitis. Liver stained with anticytokeratin 7 (CK7) and Masson-trichrome of an HCV case with mild inflammation (A and B), AIH with moderate inflammation (C and D), and PSC with cholangitis, edema, and portal fibrosis (E and F). HCV- and AIH-associated CK7+ DRs (A and C) have only a vague or no lumen and comprise and elongated cells with little cytoplasm extending in the periportal parenchyma and associated with dense collagen fibbers (B and D). While the CK7+ ductular proliferation seen in PSC (E) is enclosed in portal mesenchyma and concentric periductular fibrosis occurs (F).

As mentioned above, the Notch and Wnt signaling pathways are involved in the divergence of DR cells fate toward hepatocyte or biliary cells observed in response to CDE versus DDC. Similarly, in human diseases, prevalence of Notch signaling, driving biliary phenotype, is strong in PSC while the expression of Numb, a negative regulator of Notch, is more elevated in HCV samples compared to PSC [54]. Moreover, β-catenin, a component of the Wnt pathway, is found within the cytoplasm and nucleus of human DR cells of HCV-infected livers, signing enhanced Wnt signaling and promoting hepatocyte regeneration, whereas in PSC, β-catenin is predominantly localized to the cell surface, suggesting low activation of the canonical Wnt signaling pathway promoting biliary regeneration [42].

6. Conclusion

In summary, the CDE diet targets specifically hepatocytes and induce DR-containing elongated cells of an undifferentiated and migration-supporting phenotype expanding from portal tracts into the parenchyma. Myofibroblast activation and extracellular matrix deposition precedes this cell expansion, and a laminin-rich sheet sustains those DR while macrophages associate with invading DR. In the DDC model, accumulating protoporphyrin obstructs the hepatobiliary system leading to biliary damage and resulting in highly proliferative cells forming bile duct-like structures remaining restricted to portal mesenchyme, delineated by a thin layer of laminin and accompanied by dense portal inflammation. Moreover, cell-tracking experiments revealed that DR cells are able to generate a small number of functional hepatocytes after CDE but not after DDC exposure. Finally, the DR phenotype and signaling pathways involved in LPC differentiation in the CDE model mirrors the one observed in chronic HCV infection presenting signs of fibrosis and autoimmune hepatitis, while the DDC model could be used to study biliary injury such as PSC or PBC in humans. We believe that characterization of the most widely used dietary DR mouse models will help our understanding of the diversity of DR patterns observed in humans and will help the researchers to select the appropriate model in relation to the specific question addressed.

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Chapter 9

Experimental Animal Models of HIV/AIDS for Vaccine Trials

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Additional information is available at the end of the chapter

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Abstract

AIDS still persists as a relevant disease in public health and scientific research. There have been significant advances in HIV research, notably the development of an effective regimen in antiretroviral therapy. However, the emergence of drug resistance has facilitated continued research in administration of therapy and the development of new antiretroviral drugs. In spite of nearly three (3) decades of intensive research, there still is not an effective vaccine against HIV-1. Animal models have been a crucial tool in drug discovery process for invasive investigation of HIV disease mainly in preclinical evaluation of drugs and vaccines. This undoubtedly is an integral part of successes so far achieved in HIV/AIDS research. Advances in both non-human primate and murine model immunogenetics in response to recombinant viruses have greatly increased the options of animal models available for research. Understanding the pros and cons of these models is imperative for animal study design that could further the development of vaccines and antiretroviral therapies for HIV prevention and treatment of AIDS patients.

Keywords: HIV/AIDS, animal models, clinical trials, humanized mouse model, candidate vaccine testing, primates, HIV reserviour

1. Introduction

To understand the etiology, and mechanistic approach to eliminating disease processes and its attendant challenges, animal models have been indispensable in shortening the length of time, resources and complications inherent in disease prevention and drug discovery. Animal models, however, play a central role in the extensive research of HIV infection since the early 1980 [1].

Numerous animal species could be infected with HIV, but hardly do they develop AIDS-like syndrome that approximates humans. Several reasons have been adduced to this including decreased viral infectivity factor, efficiency of HIV replication in the animal species and host immunologic response proteins thus referred to as host factors [2].

Evolutionarily, the Chimpanzee had been believed to be close to humans thus exploited in AIDS research. Scientists later understood that HIV does not infect the Chimps but SIV besides the high cost attached to its use in HIV diseases research. Maintaining a primate research facility tends to incur huge cost, and most centers are being shut down around the world. These animal models are used to study diseases and infections of lentiviruses specifically Feline Immunodeficiency Virus (FIV) in cats, and Simian Immunodeficiency Virus (SIV) in monkeys. However, these viruses are distinct from HIV and have the problem associated with extrapolating data from experimental studies [2]. Consequently, researchers resorted to intensive search for alternative experimental model for HIV infection. Mouse, being 90% genetically similar to humans poses as a 'go' option hence the intensive and successful development of mouse models for HIV research.

It is worth mentioning though that testing the effectiveness and toxicity of anti-HIV medications such as anti-proteases and HIV-1 Reverse Transcriptase inhibitors (AZT, and 3TC) are experimentally conducted using cell culture techniques derived from human white blood cells [3–5]. No such mechanistic model for HIV pathobiology has been created [6].

Murine experimental HIV models are putatively regarded as the most extensive approach appropriate for evaluating the safety, efficacy and salient aspects of novel drugs or vaccine candidates. These models, to a high degree, have achieved remarkable success thus bridging the gap between preclinical and clinical evaluations on humans. Similarly, they are effectively utilized in toxicological evaluations of drugs, testing of novel anti-HIV small and interfering molecules and pre clinical trials. Bearing that humans cannot be used experimentally, continued development of the animal subjects for use in research has seen tremendous improvement and modifications [2].

2. Initiation and progression of HIV infection

The so-called latent period after the infection by the virus does not mean the virus is inactive [7]. Apart from humans, HIV-1 naturally infects a small number of nonhuman primate species, notably chimpanzees, which have been known to host the virus. Development of AIDS from HIV progression only occurs in humans [8, 9].

To gain insights in the transmission, pathobiology and progression of HIV infection, development of an animal model of HIV-induced immunodeficiency becomes mandatory. Several approaches were adopted to circumvent species tropism, that is, finding a similar lentivirus specific to other species that can cause similar symptoms, that is, immune deficiencies as a results of affinities to CD4+ T-cells and macrophages [9].

2.1. In humans

HIV-1 mainly infects through the genital mucosa with persistence chronic infection even when the virus triggers a notably strong cellular and humoral (both innate and adaptive)

immunity. The reason for this action may stem from the virus genomic integration and subsequent cellular latent activity coupled with its extreme genetic variability, which provides a consistent immune specific escape. It is known that HIV-specific CD8+ lymphocytes are key players involved in initial decrease or suppression of viremia during acute HIV infection conversely this becomes highly dysfunctional and burdened under the strenous condition of chronic viral antigenic persistency [10, 11]. Viral neutralizing antibodies (Abs) are similarly triggered which also accompany immune escape. In some individuals, particularly elite HIV controllers or suppressors, they develop broad neutralizing Abs thereby have an effective control of the virus [12].

HIV infection is hallmarked by massive reduction of CD4+ T cells. During the primary HIV infection, the effector memory CD4+ T cells present in the gut mucosa are consistently and preferentially depleted [13]. The immunopathogenic presentations, in addtion to the systemic and chronic state of immune activation, are believed to contribute directly to HIV disease progression [14]. As a result, persistent antigenic stimulation presents a dysfunctional T-cell population with a loss of functional potential in cytokine production and cytotoxic activity, and the ability to proliferative in response to antigen stimulation. It is believed that this loss of immune balance between Th17 and regulatory T cells (Treg) during HIV disease progression may be the reason for the permeabilization of gut integrity and the pathogenesis of HIV.

Microbial translocation caused by gut permeability is thought to contribute to systemic immune stimulation seen in chronic HIV infection [15]. Additionally, hyper-responsiveness of plasmacytoid DCs during the cause of primary HIV infection, typically results in type-1 IFN excess production which contributes to systemic immune stimulation and HIV-1 disease progression [16].

Previous studies suggest a relationship between CD8+ T cells and the control of chronic HIV replication similar to that of simian immunodeficiency virus (SIV) viremia in non-human primates [17]. There are also rare individuals who control HIV-1 replication to levels which the virus cannot be detected also known as elite controllers with attendant characteristics [18]. This phenotype shows a strong association with certain MHC class I alleles with HIV-specific CD8+ T cells demonstrating superior cytotoxic capacity to kill any HIV-infected target.

Two examples of markers in HIV-1+ patients associated with T-cell exhaustion are Programmed Death-1 (PD-1) and T cell immunoglobulin and mucin domain 3 (Tim-3), which likely are caused by consistent antigenic stimulation [19]. These two molecules have been shown to participate in the downregulation of host immune responses, playing a key role in sustenance of T cell tolerance. It is obvious that Tim-3 is upregulated on virus-specific CD8+ T cells in subjects with chronic progressive HIV infection [19]. Similarly, another report stated the upregulation of Tim-3 on antigen-specific CD8+ T cells in subjects with active TB [20], buttressing similarity in the role played by the inhibitory receptor/ligand interactions with respect to modulation of host immunity to both HIV and M. tuberculosis infections in humans.

2.2. In primates

Several non-human primates are naturally infected with simian T lymphotropic virus (STLV) types I and III. The exogenous type C retrovirus isolated from macaque monkeys in captivity

reported in the USA with an immune deficiency syndrome otherwise known as simian AIDS has been termed STLV-III mac. It is worthy to note that STLV types I and III are similar and/or related to the human T lymphotropic viruses (HTLV-I and LAV/HTLV-III) the causative agent(s) for AIDS. The striking similarities include growth characteristics, similar size of viral structural proteins, morphology, T4 cell tropism and serological cross reactivity of viral proteins [21]. The residing proteins found in the simian virus also have similar molecular weight with respect to the gag and env encoded proteins of LAV/HTLV-III [22]. Both are recognized by reference LAV/HTLV-III human serum and monoclonal antibodies to the core protein, p24, of the human virus. These proteins are basic and have relevant information needed in the development of candidate vaccine, rapid diagnostics and elucidation of HIV virology [21]. Knowledge of the molecular structure and pathobiology of simian viruses yielded a wealth of information and was very useful in the study of the HIV and AIDS in humans [21].

2.3. In rodents

In addition to looking for other lentiviruses, rodents were genetically engineered so that their cells could express both the human version of the CD4 receptor as well as the chemokine co-receptors to which HIV-1 binds, notably the main route of entry to target cells [23]. The envelope glycoprotein 120 (gp120) domiciled on the surface of the HIV-1 virus fuses with the host target cell membrane specifically invoking a cascade of activity involving the CD4+ receptors and chemokines co-receptors thus initiates viral entry. In successfully developed transgenic mice, however, the gp120 will not successfully bind to CD4-expressing T cells thus preventing targeted cell infection. Replacement of the gp120 coding region of the HIV with gp80 region obtained from the murine leukemia virus results in altered virus thus overcoming the problem. This chimeric HIV-1 clone could infect conventional mice cells, but not human cells. Although this has been extensively adopted in research, these models could not produce some disease progression seen in humans, for example, neuro-HIV disease [9].

Humanized mice model also known as humice are mice carrying functioning human genes, cells, tissues, and/or organs engraftment mostly on genetically modified mouse background. They replicate the human HIV immune responses and are currently used to study mechanisms of immune activation, mucosal transmission and prevention, immune pathogenesis and anti-viral drug development.

2.4. Developmental perspectives of HIV animal models

The development of fitting animal models is seen as one of the most important challenges in studies of co-infection, since HIV does not cause disease in rodents and in non-human primates [24].

Lentiviruses specific to other species that also compromise the immune systems in ways similar to HIV-1 have been useful in providing information about the pathogenicity of the virus. In spite of the similarities to HIV, there are species-specific differences in their respective gene products as well as the pathogenesis of the disease fueling the drive for search of better models of HIV disease control.

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3. Defining animal models

3.1. Macaques

SIV in macaques follow a disease course that is similar to HIV in humans. This is useful since it can be exploited for evaluation of drug/vaccine candidates closely related to that being developed for respective human HIV infection. The model thus provides leading and insightful results in and related to drug safety and efficacy of prospective candidates [1]. Certain animal models have been developed over the years through intensive research for insightful and revealing studies on HIV/AIDS and associated cancers. These include both specific rat and mouse models designed for HIV pathogenesis and candidate vaccine development. Scientists have albinitio created the SIV non-human primate(NHP) model (**Figure 1**), for example, the Indian-origin rhesus macaque (*Macaca mulatta*), *Cynomolgous* macaque (*M. fascicularis*), and pigtailed macaque (*M. nemestrina*), for same purposes including development of microbicides. These models are useful in elucidating the mechanism of AIDS pathogenesis as well as in preclinical testing of novel drugs directed at HIV infection and cancer [1, 25, 26].

SIV infection in macaques has been used as a model for AIDS since it was established that non-human primates are resistant to infection by HIV (**Table 1**). Simply put, SIV is a retrovirus causing immunodeficiency similar to AIDS in Asian macaques. More importantly, Macaques also develop TB that is very similar to that of humans, other notable similarities in viral activity and disease manifestation include cavitary lung disease and necrotic lession. The TB latency seems in contrast to humans to have only a small proportion of lately infected Macques develop reactivation [27] though it develops persistent Mycobacterium *bovis bacillus Calmette Guerin* (BCG) [28] and *M. tuberculosis* co-infection [29].

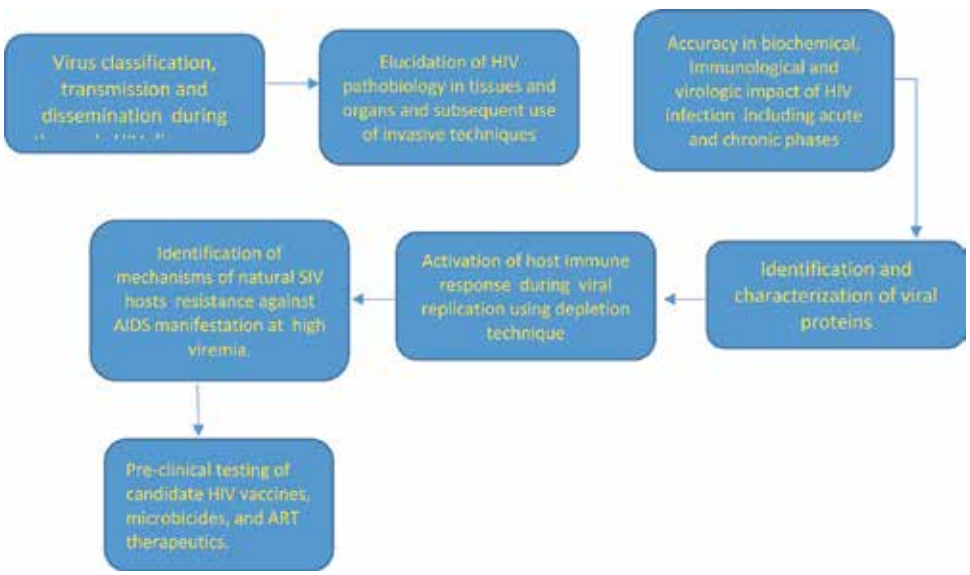

Figure 1. Historical trend and impact of HIV/AIDS research in NHPs.

Animal model	Species	Cons	Pros	Retroviral study sub-types
Macaques	Rhesus macaques (*Macaca mulatta*)	• Resistant to HIV. SIV shows discrepancies to HIV which Macaques are resistance to especially with respect to the association of TB reactivation and viral load. • Low turnaround of the model. • Availability of some rhesus macaques depends on domestic breeding capacity and skill. • Have poorly characterized MHC allelic profiles and may not be suitable for vaccine studies. • May not be appropriate for comparative menstrual cycle-related SIV/SHIV studies.	• Can be infected with SIV which compromises immunity • Ability to causes secondary complications similar to HIV in humans. • Intravenous, intrarectal, intravaginal and penile-exposure models are established • Studies of SIV/TB co-infection models. • Indian macaques have well characterized MHC allelic profiles thus suitable for as a model for vaccine candidates.	SIVmac239 SIVsmPBj6.9 SIVsmPBj6.6 SIVPBj14 SIVmac316 SIVsmE543–3 SIVsmE660 SIVmac251 RT-SHIV SHIV-SF162P3
	Pigtail macaques (*Macaca nemestrina*	• SIVmac infections typically does not refelct HIV-1 infection and is more aggressive. • Usually more expensive to maintain • Not an established model for evaluation of vaccine candidates	• In the female vaginal ecology, physiology and intravaginal virus challenge is similar to that of human. • SIV/SHIV and STI co-infection models can be studied.	SIVmac251 + SHIV/17E-Fr SIVmne SIVmneCl8 SIVmne170 SIVmne027
	Cynomologus macaques (*M. fascicularis*)	• Difficult in sample collection due to smaller size. • Exhibit low viral loads. • Its suitability for vaccine studies is low.	• Has smaller size therefore, easier to handle. • It is more readily available.	RT-SHIV SIVmac251
Baboons		• Limited HIV replication activity in monocytes or macrophages, CSF or brain • Not a good model for heterosexual trans mission as shown in SHIV89.6P chimera model	• Supports HIV-2 replication but not HIV-1 • Baboon microglial cells can be infected by SHIV chimera with strong tropism for baboon PBMC but not for rhesus macaque PBMC.	

Animal model	Species	Cons	Pros	Retroviral study sub-types
Genetic comparison of non human primates in HIV research				
Cons		**Opportunities provided by the NHP models in studies of HIV eradication**	**Pros**	
• High cost, maintenance and availability. • Have limitated suitability for vaccine studies. • SIV differs from HIV in genetic organization especially the Vpx gene of SIV and Vpu of HIV • Simian AIDS generally develops within 6–12 months while the human AIDS develops after several years of infection with HIV		• Identity, dose, and route of virus challenge known. • Control for various clinical parameters that are virtually impossible to control in humans (time of infection, duration of ART etc). • Comprehensive cellular and anatomic characterization of both active and persistent reservoirs (including elective necropsy). • Pilot trials of *in vivo* eradication conducted in a timely and controlled fashion; treatment interruption is possible. • Testing of "risky" interventions (i.e., cell depletion experiments, stem cell-based interventions etc).	• Steady environmental conditions can be applied for long periods therefore enhancing the chances to detect genetic effects • Varying environmental settings can be imposed sequentially on NHP in order to characterize genotype-environment interactions; • Possibility of testing genetic hypotheses in a prospective selective mating • Important and needed invasive and terminal experiments can be conducted and validated	

Table 1. Advantages and disadvantages of non-human primate models used in HIV-1 research.

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In the model, co-infection with BCG and SIV hastened the progression to AIDS [30] and reveals severe diminution of CD4+ T cells, loss of BCG-specific T cell responses, and reactivation of the clinically latent BCG infection into a TB-like disease as reported by Shen et al. [31]. *M. tuberculosis* reactivation in SIV-infected macaques is linked with peripheral T-cell depletion instead of viral load [32].

3.2. Other primates

Chimpanzees support productive infection, but the disease does not occur for at least 10 years. Alter et al.'s [33] investigative study was designed to determine the possibility of using a transmissible agent in humans with capability to induce AIDS in non-humans thus established an animal model in which the pathogenesis, treatment regimen, and prevention of AIDS could be studied (**Table 1** and **Figure 1**). This early attempt pre-dates the virologic investigations that linked human AIDS to a type C retrovirus [33]. The NHP models have recorded tremendous successes, the limitations observed notwithstanding (**Table 2**).

Interestingly, Baboons can support replication of certain strains of HIV-2, but difficult with HIV-1 strains (**Table 1**). It has been shown that HIV infection replicates mainly in the T-cells, with limited or no activity in the monocytes or macropahges, CSF or brain of Baboons and macaque monkeys [34–36].

3.3. Mice

For better assessment of the HIV-linked clinical presentations, murine models have been developed and proved a better tool in elucidating the mechanism of disease progression. Equally giving lead to scientific direction as against non-human primates [37] geared toward the future of HIV drug and vaccine development [38]. Besides that, it is usually costly to work with the non-human primates (large animals), which further underscores the necessity for murine models [9].

Evaluation	Species	Study outcome	Drug
Efficacy and toxicology	Macaques	Long term-highdose HIV treatments had adverse effects not found using short term-high dose treatment	Tenofovir
Prophylactic treatment with anti-virals	Macaques	The effectiveness of prophylaxis in blocking HIV infection as seen in the treatment for occupational exposures.	Various
Mother-to-fetus transmission, and fetal prophylaxis	Macaques	Provided guidelines for antiviral treatment in HIV positive pregnant mothers	Tenofovir and AZT
Vaccine efficacy in SHIV 89.6p, a hybrid SIV, genetically engineered from HIV	Macaques	The Monkeys were not protected against infection with SHIV however, they did have lower viral	MRK-Ad5

Table 2. Successful clinical testing conducted in NHP models.

3.3.1. Murine AIDS

In many ways, murine AIDS (or MAIDS) and human AIDS are similar. Immunological analysis and genetic studies reveal resistant gene(s) in the H-2 complex of mice, an indication that genetic differences in mice could modify features of HIV disease. The defective murine leukemia virus is the major etiologic agent of MAIDS, which seems to be able to induce disease in the absence of virus replication. Target cell proliferation and oligoclonal expansion are induced by the virus, which suggests repressed immunity seen in mice thus referred to as paraneoplastic syndrome. This is further supported by the good response(s) of MAIDS mice to antineoplastic agents. This animal model is useful in demonstrating the emergence of novel hypotheses about AIDS, including the roles of defective HIV and HIV replication in the progression of the disease, and also the importance of identifying the HIV targeted cells *in vivo*.

Although MAIDS and AIDS are triggered by retroviruses of different classes, the availability of a model in small, accessible animal species with elaborated genetics is beneficial in understanding the pathogenesis of AIDS especially in cases where one or more of the affected cellular and molecular pathways are common in both diseases [39].

3.3.2. Genetic modifications/gene manipulations

Potash et al. [40] designed a model of HIV-1 infection of mice for the study of viral replication, its pathogenesis and control. The team substituted the coding region of gp120 in HIV-1/NL4–3 with gp80 from ecotropic murine leukemia virus, which infects only rodents, targeted at infecting rodents with HIV-1 in rodents. The EcoHIV was developed through the chimeric virus construct, which productively infected lymphocytes in mice, but failed to do the same in human lymphocyte culture. It was recorded that immunocompetent adult mice were easily prone to infection by a single dose EcoHIV inoculant as the demonstrated by viral detection in lymphocytes in the spleen, brain cells and peritoneal macrophages. The passage in culture, and induction of antibodies to HIV-1 Gag and Tat showed that the animal produced virus was indeed infectious and immunogenic, respectively.

3.3.3. Transgenic mice

Mice are not susceptible to HIV infection due to the virus specificity for the human cell. These would have otherwise been ideal models, however, owing to the large diverse tools and wide knowledge about the rodent immunity. To circumvent limitation in mice (**Table 3**), complementary mouse models have thus been developed over the years targeting specific genes (**Table 4**). Using these models, the more important features of HIV infections and *M. tuberculosis* can be replicated in mice (e.g. virus replication in splenic lymphocytes, peritoneal macrophages and brain tissue; typical TB granuloma formation; immune repression and/or chronic immune stimulation; and susceptibility to systemic, vaginal, and rectal infection by HIV) [24].

Mice modified genetically are often used for research and/or simply as an animal model of human diseases. The use of genetic engineering tools has greatly improved the ability to develop various mouse models important to preclinical research. With the recent developments in gene editing technologies, it is now possible to generate quickly highly adjustable

Animal Model	Parameters	HIV/SCID-hu	HIV/hu-HSC	HIV/BLT	Reference
Mice	• Sample size	Small	Small	Small	[41]
	• Anatomical comparison with humans	Different same	Different same	Different same	[42–44]
	• Similarity of infective agent to HIV	Similar	Similar	Similar	[45–48]
	• Infection manifestation in comparison with human/HIV	Yes	Yes	Yes	[46, 49]
	• Availability for experimental infection in controlled conditions vis-à-vis route and dose of virus inoculation, drug regimens etc.	Yes	Yes	Yes	[47, 50, 51]
	• Ability to deplete major immune components	Yes	Yes	Yes	
	• Reservoir comparison to human/HIV	Similar	Similar	Similar	
	• Cost of maintenance compared to NHP	Minimal	Minimal	Minimal	
	• Methods mouse model development	SCID mice implanted with fetal human thymus/live.	NOD/ NSG mice irradiated and injected with human HSCs	NOD/SCID mice irradiated & implanted with fetal human thymus/live plus injection with HSCs	
	• Timeframe needed for mouse development	5–7 months from time of birth	2–3 months	5–7 months	
	• Cellular composition during reconstitution	T cells	T & B cells, DCs	T & B cells, monocytes, macrophages, NK cells, DCs	
	• Degree of colonization	Only thymus/live implant	Murine lymph organs and bone marrow	Murine lymph organs, rectum, vagina, gut, bone marrow	
	• Length infection sustained	Grafts last almost 12 months	6–7 months	Over 12 months	

Table 3. Comparison of major mice chimeric models used in HIV-1 research.

mouse models tailored to research needs. Mouse is still putatively the preferred animal model used in drug discovery and therapeutic agent development [52]. Below are some specific examples of genetically modified mouse model backgrounds which resulted from targeted mutations of specific mouse genes as presented in **Table 4**.

3.3.3.1. NOD/SCID mice

Since the early 2000s, a series of immune-deficient mice suitable for developing humice have been successively designed through the introduction of IL-2Rγ^{null} gene (e.g. NOD/SCID/γc^{null} and Rag2$^{null}\gamma c^{null}$ mice) using various genomic approaches. Mouse backgrounds serve as the basic genetic modified rodent from which other disease models are generated mostly by further modification and/or by human tissue engraftment. These mice were generated by genetically introducing human cytokine genes into NOD/SCID/γc^{null} and Rag2$^{null}\gamma c^{nul}$l mouse backgrounds [52]. There are other models that rely on the transplantation of human tissues into the SCID mice, and they are referred to as the SCID-hu mouse model.

Gene name	**Characteristics**
B2m *beta-2 microglobulin*	This is required for normal expression of major histocompatibility class I proteins which displays viral and self-antigens to responsive T cells and secondly for CD8+ T cell maturation and NK cell development.
Foxn1 *forkhead box N1, formerly Hfh11*	*Foxn1*^{nu} mutation is generally known as nude mutation. Homozygote (*nu/nu)* type lack a thymus that is they are 'hypothymic'/'athymic' and thus are T cell deficient. Their responses to thymus-dependent antigens are poor. However, the allogenic and xenogenic grafts though may have NK activity show evidence of leakiness. → Greatly increased susceptibility to infection.
Il2rg *interleukin 2 receptor, gamma chain*	*The Il2rg* is required for IL2, IL4, IL7, IL9, IL15, and IL21 high-affinity binding and signaling. It is required in mediating susceptibility to thymic lymphomas in mice. Mostly observed is the *Il2rg* deficiency that blocks the development of NK cells and the resultant defects in innate immunity.
Myd88 *myeloid differentiation primary response gene 88*	Myd88 is critical adaptor protein utilized by all TLRs (except TLR 3) to activate transcription factor NF-κB in innate immunity signal transduction. Myd88 mutation leads to decreased *innate responsesespecially neutrophils, macrophages,* *hematopoietic, molecular signaling, and apoptotic abnormalities.*
Prf1 perforin 1	*Prf1* is a pore-forming protein that is an important component of the lytic pathway by which NK and CD8+ lymphocytes kill targeted cells.
Prkdc protein kinase, DNA-activated, catalytic polypeptide	The *scid* mutation in the *Prkdc* gene means severe combined immunodeficient. *Prkdc* plays a role in repairing double-stranded DNA breaks and in recombining the variable (V), diversity (D), and joining (J) segments of immunoglobulin and T-cell receptor genes. Homozygous (scid/scid) mutants have no mature T and B cells, cannot mount cell-mediated and humoral adaptive immune responses, do not reject allogeneic and xenogeneic grafts, and are useful cancer research models. The disadvantage is its leakiness as some functional B and T cells as they age, in non-SPF conditions. They cannot be as thoroughly irradiated as other immunodeficient models before being engrafted renders NOD mice diabetes-free.
Rag1 *recombination activating gene 1*	*Rag1* is essential for the V(D)J gene rearrangements that generate functional antigen receptors in T and B cells; homozygous *Rag1*^{tm1Mom} mutants have no mature, functional T and B cells. The *Rag1*^{tm1Mom} mutation on the NOD background renders NOD mice diabetes-free. However, aging NOD.129S7(B6)-*Rag1*^{tm1Mom}/J mice develop B cell lymphomas at a high frequency.
Ticam1 *Trif*	Toll-like receptor adaptor molecule 1. It is an adapter protein used by TLR 3 to activate transcription factor NF-κB in innate i immunity signal transduction. *Its mutation leads to decreased innate responses especially when combined with Myd88 mutation.*

Table 4. Common genetic mutations found in mouse models and their functions (source: Ibeh et al. [52]).

Earlier versions of humanized mice were developed mainly to study HIV-1 infection especially in modeling for immune-pathogenesis [53, 54], although the SCID-hu Thy/Liv model is still used to test for antiviral drugs [55]. In the improved humanized mice strains, several HIV-1 strains have been successfully used for HIV infection in the developed mouse model, and these include CCR5-tropic [56], CXCR4-tropic and dual-tropic (NL4-R3A) viruses [56]. Obviously, HIV-1 infection can be established in immune-deficient mouse models by inoculation through various routes of entry, namely intraperitoneal, intravenous and/or mucosal routes [57, 58]. Various research reports have established sustained viral replication and depletion of CD4+ T-cell using the routes of infection.

Evidently, Nie et al. have shown similar depletion of CD45RAþ naive and CD45RA + effector/ memory CD4þT lymphocytes by CXCR4- tropic HIV-1 in humanized mouse as were observed in HIV-1 patients. Similarly, the preferential depletion of CD45RA + CD4þT lymphocytes by CCR5-tropic HIV-1 was also observed. Further reports on humanized mice have shown its usefulness as a tool for studying various aspects of HIV-1 infection namely the roles of regulatory T cells (Tregs) [57], dendritic cells (pDCs) revealing the pathophysiology of human DC subsets [59] and pDC instigator function during disease initiation [60], HIV-1 immuno-pathogenesis [54, 61, 62], development of new antiviral therapy [6, 63], mucosal transmission, microbicide development [64] and currently in studying latent HIV infections. Latent HIV infections can now be established in a mouse model in the presence of administered ARV [65, 66].

3.3.3.2. BLT mice

Consequently, latency has been successfully generated in humanized BLT mice [67, 68]. Available report has shown that poly lactic-co-glycolic acid (PLGA) nanoparticles with encapsulated rilpivirine (an anti-retoviral drug) coated reproductive tract offered significant protection to BLT humanized mouse model from a vaginal high-dose HIV-1 challenge [68]. Several improvements of humice models with an enhanced human immune cell reconstitution especially the female genital tract tissues create a potential mice, susceptible to intravaginal HIV infection. This type of mouse model will enable studies on mechanisms involved in HIV transmission *in vivo* and represent powerful tool for studying hematopoiesis, inflammatory disease and viral host-pathogen interactions. Several potent HIV vaccines have been put on trial and enjoyed a well-publicized but prematurely terminated results due to high frequency of seroconversions among vaccine recipients [69, 70]. Previously, the only known model for HIV testing is infection of rhesus macaques with simian immunodeficiency virus (SIV) which has provided an excellent non-human primate model for studying HIV pathogenesis [71]. This model, however, has three major disadvantages despite its application in transmitting HIV experimentally to rhesus macaques across the cervicovaginal or rectal mucosa. The established scenario makes it possible to test for microbicides and engages in laboratory study of mucosal HIV transmission. First, they are costly both in procurement and housing (limited number of primate facility globally) and is in high demand; secondly, SIV differs from HIV in genetic organization especially the Vpx gene of SIV and Vpu of HIV and lastly, while simian AIDS generally develops within 6–12 months of infection, the human AIDS develops after several years of infection with HIV (**Table 1**). However, these limitations serve as impediments in the search for an appropriate model of HIV drug testing and disease study, the transgenic mice model has overcome these feared problems associated with the SIV model. Mice have the utmost advantages of being inexpensive, have high reproductive capacity and may be housed in large numbers in a fairly small facility [72]. Furthermore, conduct of experimentation can be done in large numbers and in replicates. The severe combined immune deficiency mouse engrafted with human peripheral blood mononuclear cells (hu-PBL-SCID) could be co-engrafted with xenografts containing the dual of human fetal thymus and liver tissue (SCID-hu thy/liv)/(SCID-hu thy/liv), and this model is widely applied in preclinical evaluation of antiretroviral therapy [55, 58]. In another study, Denton et al. supported these findings and showed that the female reproductive tissues in BLT mice are adequately reconstituted with

HIV-susceptible human CD4þT cells, as well as other relevant populations [67]. Similarly, Dagur et al. in a current study demonstrated dual reconstitution in TK-NOG mouse model as a possible platform to investigate hepatocyte-related HIV-1 immunopathogenesis [73].

3.3.3.3. Other examples

The human hematopoietic progenitor cells (CD34+) from human cord blood are used to reconstitute the immune system of immune-deficient mice also known as humanized mouse [41]. An additional feature or rather advancement incorporates a fragment of the fetal human thymus engraftment, which performs functionally as a human thymus. The significance of this is to allow for a more proper positive/negative T-cell selection previously not obtainable from the original model [74]. Immunologi and virologic parameters such as CD4+ cell depletion, extent of viremia, and co-receptor-mediated tropism were all observed in HIV infection of humanized experimental mice [74, 75]. The humice demonstrated transplanted human cells in mucosal linings therefore, most possibly get infected by intravaginal and/or intrarectal routes [76]. This model is used to evaluate novel approaches in HIV prevention and treatment options including human-neutralizing antibodies, usage of prophylactic anti-retroviral therapies, and T cell-specific siRNA transfer [77]. The effect of M. tuberculosis infection on the induction of HIV gene expression has been studied with HIV transgenic mice integrating the entire viral genome [78]. In this model, viral gene expression was triggered by M. tuberculosis and suppressed after anti-mycobacterial chemotherapy [78].

4. Model suitable for vaccine trials

The question of whether or not there should be a standardized model is the basis on which the current controversies in HIV research rest on [26]. Differences in SIV and SHIV replication in the rhesus macaque, cynomolgous and pigtailed macaques' species have been observed and is favored in the design of experimental models depending on the question raised [40]. For vaccine research, the rhesus does present the ideal for pathogenesis research; however, demanding for its use as a standard does present problems the current wave is to base considerations on the transgenic mice models. Besides, vaccine testing in more than one species of macaques with similar vaccine modalities provides an opportunity to compare outcomes thus increasing confidence of research reproducibility [79].

Regulatory authorities require vaccine candidates to undergo preclinical evaluation in animal models before they enter the clinical trials in humans [80]. The overarching goal of a new vaccine is to stimulate the immune system to elicit an effective immune response against the pathogen it has been designed for, and currently no alternatives to live animal use currently exist for evaluation of this response despite advances in computational sciences for the search of an *in-silico* model [80].

Integral studies such as elucidation of immune protection mechanism, optimizing route and constitutions of vaccines; determining the onset and duration of immunity, as well as satisfying safety and efficacy requirements of the new vaccines, must be done in an integrated

living system [80]. As discussed earlier, a standardized animal model that provides all the information required for advancing a new vaccine through the preclinical stage has still not been met and even if it were, it is still bereft with problems bordering on bioethics. Current trend suggests that humanized mice (**Table 3**) more accurately predict vaccine outcomes that approximate humans.

5. HIV preclinical vaccine trials and predictive biomarker discovery in animal models

To accelerate effort in bridging the translational gap between preclinical evaluation and clinical trials, it is pertinent to make animal model testing more clinical trial like. It is important that clinical endpoints may not be easily established in animal models because of the use of questionnaires to derive the quality of life issues from end users and cannot be replicated in experimental animals; however, there are recent attempts to model pain questionnaires in animals [81]. Obviously, animal models could be designed to use other endpoints that relate or translate into the expected endpoints of clinical trials. The humanized NSG among all other models have been successfully used for multiple *in vivo* preclinical validation studies. Potentially tested areas of validation include: (1) activation of human NK cells with an IL-15 superagonist to inhibit acute HIV infection; (2) delivering anti-CCR5 and antiviral silencing RNAs (siRNA) direct delivery to T cells in order to control viral replication and prevent CD4+ T cell loss; (3) provision of prophylactic protection from HIV infection through induction of neutralizing anti-HIV monoclonal antibodies and (4) suppression of viral replication through introduced engineered HSCs that expresses an HIV-specific T cell receptor (TCR).

It is possible to design an integrated preclinical approach using PDX models organized with systems biology to enable the discovery and development of predictive biomarkers in order to classify clinical tumor responsiveness to a novel agent [82]. Peradventure the classifier achieved a high level of accuracy in the experiment, and biomarker-driven clinical trials could be developed based on the prevalent rate of the identified biomarkers and their link with efficacy.

6. Next generation models

The actual mechanism of sexual transmission is not precisely defined. What is known, however, is that HIV must be transmitted via the mucosal surfaces of the genital tract in both hetero- and homosexual cases [83]. The fact that some individuals remain uninfected despite multiple exposures, whereas others get infected after an exposure to HIV-infected semen further confounds the situation. An animal model adapted to mucosal transmission of the virus would aid elucidation of the mechanisms and dose of virus required for transmission and provide a system for testing pharmacologic and biologic cofactors that may affect HIV transmission.

Although intravenous inoculation of SIV into macaques is a near perfect model for studies of pathogenesis, this route is not appropriate for studying factors involved in the sexual transmission

of HIV. In one of the earliest experiments on the subject(macaques) [73], an animal model for the heterosexual transmission of HIV was developed by applying SIV onto the genital mucosa of both mature and immature male and female rhesus macaques. The study suggests that in the genital tract, the mucous membrane acts as a barricade to SIV infections as well as the non-involvement of spermatozoa and seminal plasma for genital HIV transmission.

Recent animal model research has focused on: (1) refinement of existing models and the development of new ones; (2) development of a model in response to latency especially HIV reservoir and immune perseverance and (3) evaluation of vaccine candidate that would elicit broadly neutralizing antibodies. As discussed in the chapter, a suitable and cost-effective animal model for HIV has been a goal spanning three decades with important milestones accomplished.

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Chapter 10

Animal Inhalation Models to Investigate Modulation of Inflammatory Bowel Diseases

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Additional information is available at the end of the chapter

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Abstract

Inflammatory bowel diseases (IBDs) comprise primarily two disease manifestations, ulcerative colitis (UC) and Crohn's disease (CD), each with distinctive clinical and pathological features. Environmental and clinical factors strongly affect the development and clinical outcomes of IBDs. Among environmental factors, cigarette smoke (CS) is considered the most important risk factor for CD, while it attenuates the disease course of UC. Various animal models have been used to assess the impact of CS on intestinal pathophysiology. This chapter examines the suitability of animal inhalation/smoke exposure models for assessing the contrary effects of CS on UC and CD. It presents an updated literature review of IBD mouse models and a description of possible mechanisms relevant to relationships between IBD and smoking. In addition, it summarises various technical inhalation approaches, in the context of mouse disease models of IBD.

Keywords: inhalation, inflammatory bowel disease, animal models, cigarette smoke, ulcerative colitis, Crohn's disease

1. Introduction

Inflammatory bowel disease (IBD) is a chronic inflammatory condition of the gastrointestinal tract encompassing two main disease manifestations, Crohn's disease (CD) and ulcerative colitis (UC) [1].

CD and UC have many similarities in symptoms and disease phenotypes, making diagnosis challenging [2]. Currently, criteria for distinguishing these two manifestations are based exclusively on histopathological and endoscopic examinations [3]. Thus, UC is defined as a chronic, non-transmural inflammatory disease characterised by diffuse mucosal inflammation

involving only the colon. Its primary clinical symptom is bloody diarrhoea [2, 4–7]. As UC is an inflammatory disease, the state of the immune system is a fundamental aspect of the disorder, with an atypical T helper cell (Th)2 response, mediated by natural killer T cells that secrete interleukin (IL)-13 [1, 8, 9]. CD is a relapsing, transmural inflammatory disease that may affect the entire gastrointestinal tract. Its major clinical symptom is abdominal pain or nonspecific abdominal symptoms and bloody diarrhoea is rare. The T cell profile in CD is different from that of UC and, in fact, a Th1 cytokine profile is dominant in patients with CD [4, 7, 10, 11]. Notably, innate immune responses are similarly activated in both CD and UC [12]. Several studies suggested that IBD pathologies result from an inappropriate inflammatory response to intestinal microbes in a genetically susceptible host, with consequent alteration of the intestinal epithelium.

During IBD development, the paracellular space in the intestinal epithelium becomes more permeable, impacting defensive strategies naturally activated by specialized epithelial cells, including goblet and Paneth cells [13–16]. This process primes a positive feedback loop, with increased exposure to the intestinal microbiota, leading to amplification of the inflammatory response. Observations in patients or animal models show that host-microbiome interactions and microbiome fluctuations play prominent roles in such inflammatory processes [17, 18]. However, whether these alterations contribute to the disease, or simply reflect secondary changes caused by the inflammation, is still under debate.

Indeed, the basic aetiology of IBD is still unclear and the potential factors contributing to the pathogenesis of the disease, such as dysbiosis, epithelial and/or immune system dysfunctions and oxidative stress, represent the major research topics in the IBD field. Moreover, new area of interest arose from the necessity of understanding the potential environmental causes behind the disease onset.

Among the environmental factors associated with IBDs, the most significant causes are cigarette smoke (CS) and nicotine, and these inversely affect the risk and course of UC and CD. The relationship between smoking and IBD has been known for many years, with the first report of a negative correlation between IBD and smoking, in a cohort of UC patients, published 40 years ago [19]. Since then, there have been numerous epidemiological, clinical and pre-clinical studies describing the dual effects of active smoking in the two forms of IBD [20, 21]. CS is associated with a higher risk for developing CD and a worse outcome in CD patients. In contrast, UC is considered a non-smokers' disease, with a significantly lower risk of disease development in current smokers. Despite the considerable research on smoking and IBD, the molecular mechanisms for CS-induced impacts on IBD development, as well as the specific CS components responsible, are not well understood [22].

To better understand the different aetiological factors in the onset of IBD, a variety of disease models were developed. Human and *in vitro* studies have historical limitations because of design complexity, duration and cost or, for *in vitro* studies, the lack of translational applicability. Therefore, animal models are advantageous by allowing *in vivo* experiments to be conducted under more easily controlled conditions than those in human studies, while providing the organism complexity lacking in *in vitro* systems. Increased knowledge of mucosal immunity and host-microbiome interactions and dynamic, as well as the availability of new

genetic engineering technologies, enabled the development of numerous murine models that, in turn, substantially increased the understanding of intestinal inflammatory processes [23, 24]. Arguably, none of these models can completely recapitulate the complexity of human IBD, but they can provide valuable information about major aspects of the disease, thereby enabling a common set of principles of human IBD pathogenesis to be established.

This book chapter reviews key studies conducted in animal inhalation/smoke exposure models aimed at evaluating the different modulation of UC and CD by CS. The application of inhalation technology to rodents, reproducing the clinical effects of smoking on colonic inflammation, will increase the chances of identifying new anti-inflammatory molecular mechanisms and possibly therapeutics, finally increasing the chances of IBDs defeat.

2. Technical aspects of inhalation

2.1. Methods of acute and chronic pulmonary delivery of aerosols to rodents

The technical means for pulmonary delivery of aerosols (either small molecules, proteins or mixtures) may employ either direct intratracheal administration or, alternatively, inhalation exposure, the latter often requiring restraint of animals.

For acute pulmonary delivery of an agent, intratracheal administration may be ideal. Its main advantages are that it requires little infrastructure or equipment and can be performed in a basic *in vivo* lab environment [25]. In addition, dose delivery can be accurately and reproducibly estimated [26]. However, this method also has several shortcomings, such as need for anaesthesia, inability to administer volatile agents or gases and unequal distribution in the lungs, resulting in minimal exposure to the alveoli. Overall, such concerns make intratracheal administration a less suitable method for subchronic or chronic pulmonary delivery.

For subchronic or chronic administration of aerosols to rodents, repeated inhalation exposure systems are preferred. Thus, animals are exposed to aerosols within a confined environment for a fixed daily duration. In the field of toxicology, testing guidelines for repeated dose exposure for toxicological assessments, such as the OECD TG413 guideline, recommend up to 6 h per day exposure for a 90 day exposure period. However, for therapeutic or disease modelling purposes, the exposure period must be determined empirically, based on the effective dose and the time needed for the target biological effect to occur. Importantly, exposure systems must enable consistent delivery of aerosols, at concentrations that are stable during the exposure period, and with appropriate aerosol properties to enable efficient inhalation and uptake [27].

Principally, two types of exposure chambers are routinely used to administer aerosols to rodents, whole body or nose-only exposure chambers, each with its own advantages and disadvantages [27]. Whole body exposure systems are restraint free, as the animals are placed into an exposure chamber, either in a cage or on a mesh or grid surface, depending on the specific system. Both chambers are technically simple, assuming sufficient infrastructure (aerosol

generation and functional chambers). Both also enable exposure of large numbers of animals, for example, chambers of >700 L may each accommodate approximately 200 mice. The freedom of movement of animals during exposure results in minimal stress, although the animals require training to adjust to grid-caging systems and food is typically withdrawn to minimise oral uptake of aerosol constituents. One criticism of whole body exposures is that there is a high potential for compound uptake through non-inhalation routes because animals have surface contact with aerosol deposits on the cage surfaces and on their fur. In historical studies, up to 60% of aerosol constituents on the fur (pelt burden) were ingested following whole body exposures [28] and transdermal uptake may also be significant for some compounds. Because the skin is an effective barrier for drug transport, only potent drugs with appropriate physicochemical properties (low molecular weight and adequate solubility in aqueous and non-aqueous solvents) are suitable candidates for transdermal delivery [29–31]. Such mixed uptake mechanisms potentially occurring in whole body exposure systems complicate both dose estimations and require deconvolution of uptake amounts through oral/transdermal and inhaled routes.

Nose-only exposure chambers require restraint of the animals to permit only the head (nose) to be exposed to the test aerosol. This has the major advantage of decreasing deposition of aerosol constituents on the pelts, resulting in less oral uptake from grooming behaviour [32]. However, there are also disadvantages with this system, including technical asphyxiation (animal movements in the exposure tube may cut off their air supply); therefore, constant monitoring during the exposure period is required. In addition, because of stress associated with restraint in nose-only exposure systems, training is required to adapt animals to the technical procedures. Vehicle or fresh air exposures are also needed to help distinguish such stress-related effects from treatment effects [33]. The daily execution of nose-only exposures requires that animals be individually inserted into the exposure tubes, a technical aspect that may limit the numbers of animals that can be used in the experiments.

2.2. Dose translatability

Measurement of dosages in an *in vivo* inhalation experiment is dependent upon many parameters, including deposition of the agent to the lungs (which itself is dependent upon aerosol droplet size), respiratory minute volume and body weight of the animal. This relationship is generally described by the following formula [34]:

$$DD = \frac{C \times RMV \times D \times IF}{\text{Body weight (kg)}} \quad (1)$$

where DD is the delivered dose (mg/kg); C is the concentration of substance (mg/L); RMV is the respiratory minute volume (L/min) and IF is the inhalable fraction.

Among these parameters, the respiratory minute volume is important to determine the availability of compound for deposition and exchange in the lungs. This parameter may be calculated using allometric formulae relating body weights to minute volumes in laboratory animals [35, 36]. The alternative, direct measurement of the minute volume, as can be

performed when nose-only exposure tubes are used (head-out plethysmography measurements), is preferable as it would enable the researcher to control any effects of test item on the minute volume, when calculating the estimated dosage.

Important for *in vivo* disease modelling is the translation of the animal models to human therapeutics or treatment regimen. This will require an estimation of human equivalent dose (HED), based on the animal data. The most commonly used method to convert to HED is with a body surface area conversion factor [37]. Alternatively, a mg/kg conversion factor may be applied, though this typically will result in a lower safety margin and higher HED values, compared with the body surface area conversion. HED is generally described by the following formula [37]:

$$\text{HED} = \frac{\text{animal dose (mg/kg)} \times \text{animal } K_m}{\text{human } K_m} \quad (2)$$

where K_m is the correction factor reflecting the relationship between body weight and body surface area (e.g. human $K_m = 37$; mouse $K_m = 3$; rat $K_m = 6$ and dog $K_m = 20$).

3. Overview of animal IBD models

The various types of animal models developed to study IBD may be divided into several categories depending on: the method of inducing the pathology (*chemically induced, bacteria-induced or genetically engineered*); the IBD subtype modelled in the animal (*UC or CD*); the site of inflammation (*colon, ileum, both sites or systemic*); and, in genetically engineered models, the gene modification strategy (*conventional transgenic* (*Tg*) *or knockout* (*KO*), *cell-specific conditional Tg or KO*, *inducible KO, knock-in, innate, mutagen-induced or spontaneous models*) [23, 38, 39]. The total number of IBD mouse models is growing, especially because of current genetic engineering approaches that accelerate development of new strains, so far, over 74 genetically engineered mouse models were reported to spontaneously develop intestinal inflammation [38]. The full description of all IBD models is beyond the scope of this chapter. However, **Table 1** summarises the most significant IBD murine models, highlighting their methods of pathology induction, IBD subtypes, sites of inflammation and mechanism of action (**Figure 1**). More detailed reviews of the different mouse models of IBD are available (e.g. see Refs. [23, 40, 41]).

There is a close agreement in many pathological findings among experimental IBD models and human disease. These include the molecular pathways and histological features of tissue injury, dysfunction of the immune system (including impact of the microbiome), genetic heterogeneity and primary defects in mucosal barrier function. All pathologies have been well established in several experimental models of colitis; therefore, these models closely resemble aspects of the human diseases. These common features enable exploration of specific pathological mechanisms, facilitating development of new therapeutic approaches. However, none of these models fully reflects human IBD, with each representing rather a small tile of a mosaic. This hinders a generalised view of the systemic consequences of IBD, often masking possible extra-intestinal implications [42].

IBD model	Model category	IBD subtype	Site of inflammation	Mechanism	References
DSS	Chemically induced	UC	Colon	Epithelial cell damage	[147, 148]
TNBS		**CD**/UC	Colon	Hapten-dependent immunogenic response	[149]
DNBS		CD/UC	Colon	Hapten-dependent immunogenic response	[150]
Oxazolone		UC	Colon	Hapten-dependent immunogenic response	[151]
Acetic acid		UC	Colon	Epithelial cell damage	[152]
Carrageenan		UC	Colon	Epithelial cell damage	[153]
Indomethacin		CD	**Small intestine** Colon	Epithelial cell damage	[154]
Iodoacetamide		UC	Colon	Sulphydryl (SH) compound (e.g. glutathione) blocker	[155]
DNCB		**UC**/CD	Colon	Hapten-dependent immunogenic response	[156]
Salmonella induced	Bacterially induced	UC	Colon	Bacterial colonisation-induced inflammation	[157]
Adherent invasive *E. coli*		UC	**Colon** Small intestine	Bacterial-dependent epithelial cell damage	[158]
C3H/HejBir	Spontaneous	CD	**Small intestine** Colon	Epithelial cell dysfunction	[39]
SAMP1/4it		CD	Small intestine	Epithelial cell dysfunction	[40]
IL-10$^{-/-}$	Genetically engineered/ knockouts (KO)	CD	**Small intestine** Colon	Impaired Treg function	[74]
TGF-β$^{-/-}$		UC/CD	Systemic	Macrophage hyperactivation and impaired Treg function	[159]
IL-2$^{-/-}$		UC	Colon/systemic (no small intestine)	Impaired T cell/Treg function	[160]
NOD2$^{-/-}$		CD	Small intestine Colon	NF-κB and TLR2 signalling dysregulation	[161]
A20$^{-/-}$		UC/CD	Colon Small intestine	TNF-induced NF-κB signalling dysregulation	[162]
MDR1A$^{-/-}$		UC	Colon	Accumulation of bacterial products and increased T cell activation	[163]
Gαi2$^{-/-}$		UC	Colon	Impaired T/B cell function and epithelial cell damage	[164]
TCRα$^{-/-}$		UC	Colon	Th2-type inflammation	[75]
IL-23$^{-/-}$		CD	Small intestine Colon	Impaired Th17 cell function	[165, 166]

IBD model	Model category	IBD subtype	Site of inflammation	Mechanism	References
XBP1$^{-/-}$	Genetically engineered, conditional KO	CD	**Small intestine** Colon	Loss of Paneth and goblet cells with impairment of mucosal defence	[167]
NEMO$^{-/-}$		CD	Small intestine/ colon	NF-κB signalling dysregulation	[168]
IL-7 Tg mice (IL-7 overexpression)	Transgenic mouse	UC	Colon	CD4+ T cell infiltration-dependent inflammation	[169]
STAT4 Tg mice (STAT4 overexpression)		CD	Small intestine Colon	Th1-type inflammation	[170]
HLA-B27 Tg mice		UC/CD	Small intestine Colon	Bacterial sensitisation	[171]
DNN-cadherin/ keratin8$^{-/-}$		CD	Colon	Epithelial cell dysfunction	[172]
TNF$^{\Delta ARE}$	Mutation knock-in	CD	Small intestine	TNF-α overproduction	[64]
CD45RB high-transfer	Adoptive transfer	CD	**Small intestine** Colon	IL-12-driven Th1 hyper-response	[173]

Table 1. Classification of animal models of IBD. IBD subtype and site of inflammation predominantly addressed by the model, where applicable, are shown in bold font. DSS, dextran sulfate sodium; IBD, inflammatory bowel disease; DNBS, 2,4-dinitrobenzene sulfonic acid; TNBS, 2,4,6-trinitrobenzenesulfonic acid; UC, ulcerative colitis; CD, Crohn's disease; DNCB, Dinitrochlorobenzene.

The presence of such a multitude of mouse models indicates that IBD is mediated by complicated, multifactorial mechanisms. As expected, this complexity is greater in human beings, where environmental and clinical factors, such as smoking, diet, drugs, ethnicity, geographical area, social status, gender, stress and appendectomy, further modulate onset of IBD pathologies [43–46].

3.1. Inhalation studies investigating the effect of CS in rodent models of IBD

Clinical and pre-clinical findings suggested divergent effects of smoking or smoke constituents on the pathophysiology of the gut depending mainly on two conditions, the IBD subtype and the route of administration of the active substance (such as nicotine or CS). Active human smoking is difficult to mimic under laboratory conditions, while classical *in vitro* approaches have translational limitations. Thus, several animal models have been used to assess the impact of CS, nicotine or non-nicotine CS constituents on intestinal pathophysiology [47]. Both genetic- and chemically induced IBD models have been used and effects of various treatment regimens on gut inflammation in these systems are summarised in **Table 2**. There is a general consensus that CS and nicotine administration do not cause macroscopic or histological damage or inflammation in the healthy gut. However, differences in immune cell recruitment [48], cytokine secretion [49–51], mucosal barrier [52, 53] and oxidative stress were observed [54, 55], although without evident tissue damage.

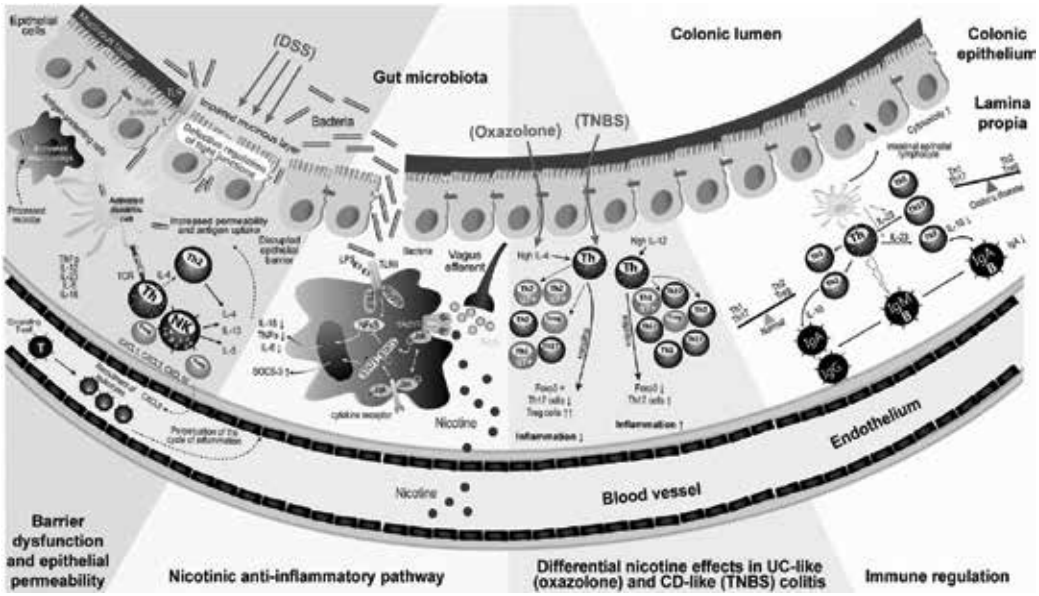

Figure 1. Schematic view of major inflammatory and anti-inflammatory mechanisms implicated in inflammatory bowel diseases and the potential role of a nicotinic anti-inflammatory pathway. Top: altered microbiota in the colonic lumen and/or epithelial-damaging factors (e.g., DSS in experimentally induced colitis) lead to the disruption of the epithelial barrier function and the consequent infiltration of bacteria and other antigens. Middle: various inflammatory processes can be triggered in the lamina propria by the infiltrating bacteria (DSS-induced epithelial barrier; "Barrier dysfunction and epithelial permeability" and "Nicotinic anti-inflammatory pathway" sectors), haptens (oxazolone- and TNBS-induced inflammation, "Differential nicotine effects in UC-like (oxazolone) and CD-like (TNBS) colitis" sector) or by endogenous dysregulation of the balance between Th1/Th17-driven and Th2-driven immune activities, (genetically engineered mouse models; "Immune regulation" section). A hypothetical role of nicotinic receptor-mediated anti-inflammatory response is depicted in the "Nicotinic anti-inflammatory pathway" sector. Bottom: the colonic vasculature is symbolized as a tube running perpendicular to the cross section of the colon. The blood stream delivers leukocytes recruited by cytokine shedding from the local inflammatory sites and enables the perpetuation of the inflammation, e.g., via circulating T-cells. Systemically provided nicotine could increase the anti-inflammatory nicotinic signaling that is naturally transmitted by acetylcholine shed from the efferents of the vagus nerve that innervate the colonic wall. For details of these mechanisms, see Chapter 4.1 to 4.4. Modified from: De Jonge & Ulloah (2007), Ordas et al. (2012), Xu et al. (2014).

Consistent with results of human epidemiological studies, CS had opposing effects on development of CD (negatively) and UC (positively) in several, but not all, of their respective IBD models. Only a few of these studies used inhalation exposure (**Table 2**) and most of their findings mimicked the effects of smoking in humans with IBD.

Thus, the dichotomous effects of CS inhalation, on development of CD versus UC, were perfectly reproduced using two different rat IBD models [54–60]. 2,4,6-trinitrobenzenesulphonic acid (TNBS) and 2,4-dinitrobenzene sulphonic acid (DNBS) were instilled into the rat colon to induce, respectively, CD- and UC-like symptoms. Indeed, pre-exposure of rats to CS increased acute (2–24 h post-induction) intestinal inflammation in the TNBS-induced colitis (CD-like) model [54–57]. The authors used a ventilated smoking chamber filled with a fixed concentration of smoke, delivered by burning commercial cigarettes at a constant rate (2 or 4%, vol/vol, smoke/air) [61]. These results showed that promotion of neutrophil infiltration, as well as free radical production with the accumulation of reactive oxygen metabolites in the intestinal

IBD model	IBD subtype—species	Treatment	Endpoint observed	Effects on intestinal inflammation	References
TNBS colitis	CD—rat	Cigarette smoke (inhalation)	Mucosal damage: ↑ MPO activity: ↑ LTB_4 level: ↑ GSH level: ↓ ROM generation: ↑ TNF-α protein: ↑ SOD activity: ↓ iNOS activity: ↑ COX2 protein: ↑	↑	[54–57]
		Oral nicotine	LTB_4 level: ↓ PGE2 level: = MPO activity: ↓ Histology score: ↓ iNOS protein: ↓ Serum IL-1: =	Low dose: ↓ High dose: ↑ or no effect	[78, 79]
	CD—mouse	Subcutaneous nicotine	Histology score: ↑ DAI scoring: ↑ Treg/Th17 cell ratio: ↓ α7nAChR expression in T cells: no	↑	[77]
		Carbon monoxide (inhalation)	Histology score: ↓ MPO activity: ↓ TNF-α protein and RNA: ↓	↓	[73]
		Oral TCDD	Histology score: ↓ Colon cytokine proteins: ↓ Gene expression Immune cells in MLN and colon	↓	[174]
Iodoacetamide	CD—mouse	Oral nicotine	Mucosal damage: J↑; C↓ iNOS activity: J NA; C= MPO activity: J=; C NA PGE_2 level: J↓; C↓ Histology score: J↑; C↓	Jejunitis: ↑ Colitis: ↓	[175]

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IBD model	IBD subtype—species	Treatment	Endpoint observed	Effects on intestinal inflammation	References
IL-10$^{-/-}$ mice	CD—mouse	Oral nicotine	Mucosal damage: J↑; C↓ Histology score: J↑; C↓ Gene expression	Jejunitis: ↑ Colitis: ↓	[52]
		Carbon monoxide (inhalation)	Histology score: ↓ Colon cytokine proteins: ↓ Gene expression	↓	[71]
DNBS colitis	UC—rat	Cigarette smoke (inhalation)	Histology score: ↑ Mucosal damage: ↑ MPO activity: ↑	↑	[58]
		Subcutaneous nicotine	Mucosal damage: ↓ MPO activity: ↓ LTB_4 level: ↓ ROM generation: ↓ Colon cytokine proteins: ↓	↓	[59]
		Cigarette smoke (inhalation)	Mucosal damage: ↓ MPO activity: ↓ LTB_4 level: ↓ ROM generation: ↓ Colon cytokine proteins: ↓	↓	[59]
		Cigarette smoke (inhalation)	Histology score: ↓ Mucosal damage: ↓ MPO activity: ↓ iNOS activity: ↓ LTB_4 level: ↓ Colon cytokine proteins: ↓	↓	[60]
Oxazolone colitis	UC—mouse	Subcutaneous nicotine	Histology score: ↓ DAI scoring: ↓ Treg/Th17 cell ratio: ↑ α7nAChR expression in T cells	↓	[77]

IBD model	IBD subtype—species	Treatment	Endpoint observed	Effects on intestinal inflammation	References
DSS Colitis	UC—mouse	Oral nicotine	Histology score: ↓ DAI scoring: = MPO activity: = PGE_2 level: ↓	↓	[80]
		Subcutaneous nicotine	DAI scoring: ↓ Histology score: ↓ miRNA expression	↓	[50]
		Cigarette smoke (inhalation)	Colon cytokine RNA: ↓ MPO activity: ↓ Infiltrating immune cells DAI scoring: ↓	↓	[22]
		Cigarette smoke (inhalation)	Mucosal damage: = Colon cell proliferation: = Colon cell apoptosis: = Colon angiogenesis: ↑ Bcl2/VEGF protein: ↑	No effect	[66]
		Subcutaneous nicotine	DAI scoring: ↓ Histology score: ↓ MPO activity: ↓ TNF-α and IL-6 mRNA: ↓	↓	[176]
		Oral nicotine	DAI scoring: ↓ Histology score: ↓ Colon TNF-α protein: ↓ MPO activity: ↓ Colon cytokines mRNA: ↓	↓	[81, 102]
		Oral cotinine	DAI scoring: =	No effect	[81]
		Subcutaneous nicotine	DAI scoring: = Histology score: = Colon TNF-α protein: =	No effect	[81]
		Intraperitoneal nicotine	DAI scoring: = Histology score: ↓ Colon TNF-α protein: ↑	No effect	[81, 82]
		Oral TCDD	Histology score: ↓ Colon TNF-α RNA/protein: ↓ MPO mRNA: ↓	↓	[177]

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IBD model	IBD subtype—species	Treatment	Endpoint observed	Effects on intestinal inflammation	References
TCR$\alpha^{-/-}$ mice	UC—mouse	Carbon monoxide (inhalation)	Histology score: ↓ Colon cytokines RNA/protein: ↓	↓	[72]
Clostridium difficile ToxA	UC—mouse	Intraluminal nicotine	MPO activity: ↓ LTB_4 level: ↓ Luminal fluid: ↓ Substance P release: ↓	↓ Colon; No effect in ileum	[178]

↑, potentiating effect; ↓, attenuating effect; =, no changes; TCDD, 2,3,7,8-tetrachlorodibenzo-p-dioxin; TCR, T cell receptor; NA, not applicable; ROM, reactive oxygen metabolites; DAI, disease activity index (for further details please see the reference), MPO, myeloperoxidase; LTB4, leukotriene B4; PGE2, prostaglandin E2; SOD, superoxide dismutase 2; COX, cyclooxygenase; iNOS, nitric oxide synthase.

Table 2. Effects of cigarette smoke or related compounds in experimental models of IBD.

tissues, contributed significantly to the potentiating effects of CS on intestinal inflammation. In contrast, in DNBS-treated rats (UC-like model), CS inhalation improved macroscopic signs of colitis at the mucosal level and decreased the levels of colonic pro-inflammatory cytokines [59, 60]. In these latter papers, Ko et al. used a similar inhalation method to the aforementioned study [61], but with a different time of exposure and a few "homemade" modifications to the smoking chamber. One study, conducted in DNBS-treated rats exposed to CS for 15 days before and 2 days after DNBS instillation, showed increased macroscopic and histological damage in the CS-exposed rat colon [58]. Noteworthy, this study used a different inhalation method than did the others. Rats were exposed to a rhythmic inhalation of smoke, with only the nose exposed to the specialized chamber [62], and this chamber was filled with mainstream smoke from a high tar, unfiltered cigarette.

Furthermore, the effect of CS on the development of small intestinal inflammation (CD-like pathophysiology) was studied in a $TNF^{\Delta ARE}$ mouse model [63]. In this mouse model, a knock-in mutation determines the deletion of the AU-region of the TNF-α mRNA, resulting in a systemic TNF-α overproduction and the consequent development of chronic Crohn's-like ileitis and inflammatory arthritis [64]. The authors exposed the mice to CS 4 times a day with 30 min smoke-free intervals, 5 days per week for 2 or 4 weeks [65]. Contrarily to what obtain in human and rat CD, in this model CS did not modulate gut inflammation. Both molecular (e.g. inflammatory and autophagy gene expression) and histopathological endpoints were not affected by CS smoke compared to fresh air exposed mice.

In contrast to its effects in CD rodent models, CS exposure for 2 weeks decreased UC-like inflammation in an acute DSS-induced colitis model in mice [22]. Montbarbon et al. showed a significant decrease in macroscopic and histological colon damage, as well as in colonic pro-inflammatory cytokine expression, in DSS-exposed mice after CS inhalation. Interestingly, this study highlighted a pivotal role for a specific intestinal lymphocyte type, iNKT, in the CS-dependent protection of the colon. The authors used a ventilated smoking chamber of the InExpose® System and exposed the mice to the mainstream smoke of research cigarettes 5 days per week (5 cigarettes/day). However, a previous study, in a long-term mouse model of DSS-mediated chronic colitis, showed a CS-dependent increase in inflammation-associated colon adenoma/adenocarcinoma formation. Although specific inflammatory endpoints were not reported, the number of colon adenomas/adenocarcinomas was significantly increased in the CS-exposed mice [66]. This tumour formation was associated with inhibition of cellular apoptosis and supported by increased angiogenesis. As a possible explanation for this discrepancy, this study used Balb/c mice while the protective effects of CS [22] were observed in C57BL/6 mice. Opposite responses in Balb/c mice, compared with C57BL/6 and other mouse strains, were also reported for other chemical inducers of IBD [67]. Moreover, a different inhalation method was applied in the Balb/c mouse study. These mice were exposed to 2 or 4% CS in a ventilated smoking chamber for 1 h per day.

In the context of inhalation studies aimed to understand the major CS component responsible for the observed anti-inflammatory effects in the intestine, three studies on the anti-inflammatory properties of carbon monoxide (CO) in IBD models are notable. Indeed, CO, a prominent component of CS long considered as just being a toxic gas [68], was recently shown to exert

potent cell protective effects because of its anti-inflammatory, anti-apoptotic and anti-oxidant capabilities [69, 70]. In three different studies, inhaled CO consistently decreased inflammation in chemically induced and genetic mouse models of UC and CD, respectively [71–73]. In particular, the same group of researchers [71, 72] exposed two different knockout mouse models, IL-$10^{-/-}$ [74] and TCR$\alpha^{-/-}$ [75, 76], to CO at a concentration of 250 ppm (part per million) or compressed air (control), attempting to recapitulate, at least in part, CS effects on the development of CD and UC, respectively. IL-$10^{-/-}$ mice were generated by gene targeting in 1993 by Kuhn et al. [74], introducing two stop codons in exon 1 and 3 of the IL-10 gene in murine ES cells. These mice are characterised by extensive Th1-mediated enterocolitis originated by an antigen-driven uncontrolled immune response mainly resembling human CD condition. T cell receptor (TCR) α knockout mice were generated with a similar gene targeting approach [76], thus integrating a neomycin cassette in the first exon of the TCRα locus. In these mutant mice, the intestinal mucosal immunoregulatory mechanisms are negatively affected, triggering the development of UC-like symptoms [75]. Surprisingly, CO inhalation suppressed inflammation in both models, regardless of their IBD subtype, through a heme oxygenase (HO)-1 dependent pathway. The anti-inflammatory capabilities of CO were also confirmed in a TNBS-induced mouse model of CD. Mice were exposed to CO at 200 ppm, beginning after TNBS administration and throughout the remaining study period (3 days) [73]. Thus, the increased colonic damage induced by TNBS was significantly inhibited by the CO treatment, with a consistent suppression of inflammatory markers, such as TNF-α levels and myeloperoxidase (MPO) activity.

As highlighted in the aforementioned reports, although CS or CS component inhalation studies in mouse models seem to recapitulate most epidemiological observations in humans, differences in the inhalation methodologies are many and frequent, making impossible a clear and solid comparison between the studies.

The route of administration was relevant on the final effect also when single CS components, such as nicotine, were administered to IBD mouse models or patients [47]. Thus, in a TNBS mouse model of CD, the detrimental effects of subcutaneous nicotine administration [77] contrasted with the dose-dependent bivalent effect of nicotine administered in the drinking water, that is, positive at low and negative at high concentrations [78, 79]. Furthermore, subcutaneous or oral nicotine administration to rats treated with DNBS led to, respectively, decreased or increased colon inflammation [58, 59]. Finally, while oral or subcutaneous nicotine administration attenuated inflammation caused by DSS treatment in mice [50, 80], intraperitoneal nicotine injection had no effects [81, 82]. Inconsistencies related to different routes of administration of CS components were also observed in human studies [83–86]. Overall, these observations suggested that the route of administration of a CS-related compound, such as nicotine, is important to consider in treating colitis. In animal models, it is clear that mimicking the nicotine intake profiles in smokers (inhalation) could result in increased treatment efficacy. This idea was supported in humans by the conflicting results obtained by local nicotine application (enemas) [87]. Therefore, although the colon may be an important site of action for CS components, the responsible molecule for the observed effects might act on many peripheral and central inflammatory pathways, such as vagus-related anti-inflammatory nicotinic signalling, or might require intermediate metabolic transformations.

3.2. Limits and pitfalls of studies using inhalation mouse models

Among the aforementioned studies, only a few used inhalation exposure (**Table 2**) models were observed, although many of the findings mimicked human smoking effects in IBD, the results were still variable. Such heterogeneity in observed CS effects on experimentally induced colitis is not unexpected, given variability in animal species and strains, IBD inducers, CS exposure schedules, endpoints and observation periods.

When comparing such quality-relevant exposure conditions, group sizes were usually sufficient, but most of the studies used only male mice or rats, instead of both genders as recommended by the Organisation for Economic Co-operation and Development (OECD) test guidelines. Only one rat study employed the preferable nose-only inhalation mode [58]. Many of the papers did not describe the exposure chambers sufficiently and explanations of exposure concentration parameters (such as number of puffs, flow rate and chamber volume) often did not enable derivation of the standard Total Particulate Matter (TPM) or smoke constituent concentration values, in a weight per volume unit (e.g. mg/L). The most evident heterogeneity among studies, however, was in exposure schedules and durations. The CS inhalation studies in IBD models typically used daily exposure durations no longer than one hour, with none using the recommended 6 h/day duration. Some studies pre-exposed the animals a few days before IBD induction and discontinued CS exposure after the induction treatment, while others continued exposure until the end of the study or began CS inhalation after IBD induction [59]. To explore more systematically the effects of inhaled CS or CS constituents on IBD in various models, there is a clear need to harmonise exposure conditions to be closer to minimal standards for inhalation toxicity studies. This is particularly true for exposure schedules and durations, as well as for documentation of meaningful concentration measurements in the exposure atmospheres (**Table 3**). Finally, to elucidate the molecular mechanisms of IBD-CS interactions, beyond the current knowledge, it will be necessary to combine robust IBD models (UC and CD), well-controlled, state-of-the-art inhalation exposure design and technology and disease-specific endpoints with systems-wide molecular profiling. We conducted systems toxicology-oriented inhalation studies using mouse models to investigate effects of CS and candidate modified risk tobacco products in chronic obstructive and cardiovascular diseases [33, 88–91]. These studies demonstrated the feasibility and suitability of this approach for identifying the molecular basis of disease mechanisms and the biological impacts of CS. The study design and inhalation exposure technology were based on the OECD guidelines TG412 and TG413 for 28 and 90 days inhalation toxicity studies, respectively [92, 93]. Satellite groups were included to provide material for the additional molecular investigations and a similar study was conducted on rats exposed to nicotine aerosols [33]. A very detailed description of the study design and methodology was provided [94] and this might serve as a template for new IBD inhalation studies. Of course, adaptations will be necessary, based on specifications of the IBD models. For example, most chemically induced IBD models require acute, rather than subchronic or chronic, observation periods, while the genetically engineered IBD models develop the disease in a similar timeframe as the COPD and CVD models.

IBD model, induction	Study design	Exposure duration	Inhalation technology	CS/inhalant characterisation	References
(OECD TG 412 recommendation)	At least 5 males and 5 females per group, 3 dose levels of test article, filtered air and/or vehicle control	6 h/day; 5 (7) days/ week; 28 days	Nose-only preferred, whole body acceptable, detailed description of exposure chamber to be given	Analytical characterisation; respirable particle size (1–3 μm MMAD), nominal and actual test article concentration (mass per volume) to be indicated, constant concentration during exposure period	[92]
Rat (Sprague Dawley), TNBS enema	8–10 rats/group (males only), 1 dose level, fresh air control	1 h/day; 4 days pre-induction	Whole body, ventilated smoking chamber (20 L) with 5–6 rats, smoke generated with peristaltic pump	"Camel" cigarettes, 4% v/v smoke, no characterisation	[56]
Rat (Sprague Dawley), TNBS enema	10–12 rats/group (males only), 2 dose levels, fresh air control	1 h/day; 4 days pre-induction	Whole body, ventilated smoking chamber (20 L) with 5–6 rats, smoke generated with peristaltic pump	"Camel" cigarettes, 2 and 4% v/v smoke, no characterisation	[55]
Rat (Sprague Dawley), TNBS enema	6–8 rats/group (males only), 1 dose level, fresh air control	1 h/day; 4 days pre-induction	Whole body, ventilated smoking chamber (20 L) with 5–6 rats, smoke generated with peristaltic pump	"Camel" cigarettes, 4% v/v smoke, no characterisation	[54]
Rat (Sprague Dawley), TNBS enema	10 rats/group (males only), 2 dose levels, fresh air control	1 h/day; 8 days pre-induction	Whole body, ventilated smoking chamber (20 L) with 5–6 rats, smoke generated with peristaltic pump	"Camel" cigarettes, 2 and 4% v/v smoke no characterisation	[57]
Mouse (C57BL/6), DSS in drinking water	6–10 mice/group (males only), 1 dose level, fresh air control	2 week (5 days/ week) pre-induction and 1 week post-induction	Whole body, InExpose chamber (Scireq) and rotary smoking machine	3R4F reference cigarettes, mainstream smoke from 5 cigarettes (8 puffs per cigarette), no concentration/ characterisation	[22]
Rat (Sprague Dawley), DNBS enema	6–8 rats/group, 3 dose levels, fresh air control (10 rats/group)	5–40 min/day, 15 days pre-induction and 2 day post-induction	Nose-only, puffwise smoke injection into chamber	2R1 reference cigarette, 5, 20 or 40 puffs/day (undiluted), no concentration/ characterisation	[58]

IBD model, induction	Study design	Exposure duration	Inhalation technology	CS/inhalant characterisation	References
Rat (Sprague Dawley), DNBS enema	7 rats/group, 2 dose levels, fresh air control	1 h/day; 3 days post-induction	Whole body, ventilated smoking chamber (20 L) with 5–6 rats, smoke generated with peristaltic pump smoke, no characterisation	"Kings" cigarettes, 4% v/v; no concentration/ characterisation	[59]
Rat (Sprague Dawley), DNBS enema	6–8 rats/group, 1 dose level, fresh air control	1 h/day; 3 days pre-induction, 4 day post-induction	Whole body, ventilated smoking chamber (20 L) with 5–6 rats, smoke generated with peristaltic pump	"Camel" cigarettes, 2 and 4% v/v smoke, no characterisation	[60]
Mouse (Balb/c), DSS in drinking water	5–12 mice/group (males only), 2 dose levels, fresh air control	3 cycles of: 7 days DSS + CS (1 h/day) followed by 14 days recovery	Whole body, ventilated smoking chamber (20 L), smoke generated with peristaltic pump	"Camel" cigarettes, 2 and 4% v/v smoke, no characterisation	[66]
TCR$\alpha^{-/-}$ mouse (C57BL/6)	10 mice/group (5 males and 5 females), 1 dose level, fresh air control	4 week (daily duration not indicated)	Whole body, 3.70 ft^2 plexiglass animal chamber, 12 L/min flow rate	CO gas, 250 ppm in air, continuous measurement	[72]
IL-10$^{-/-}$ mouse (C57BL/6)	12 mice/group (males only), 1 dose level, fresh air control	4 week (daily duration not indicated)	Whole body, 3.70 ft^2 plexiglass animal chamber, 12 L/min flow rate	CO gas, 250 ppm in air, continuous measurement	[71]
Mouse (C57BL/6), TNBS enema	12 mice/group, 1 dose level, fresh air control	3 day (permanent) post-induction	Whole body, acrylic chamber	CO gas, 200 ppm in air, continuous measurement	[73]

Table 3. Comparison of exposure conditions in published inhalation studies using rodent IBD models.

4. Mechanisms of IBD pathogenesis with possible relationship to CS constituents

4.1. Nicotinic anti-inflammatory pathway

The vagus nerve transmits signals by releasing acetylcholine that, in turn, stimulates neuronal and immune cells via their nicotinic acetylcholine receptors (nAChR) [95, 96]. These are ligand-gated ion channels expressed not only in neuronal cells, but also in most mammalian non-neuronal cell types, though different cell type-specific downstream signalling functions [97]. In the nicotinic anti-inflammatory pathway, nAChR activation by acetylcholine or other ligands inhibits the downstream NF-κB pathway, attenuating production of TNF-α and other

cytokines [98, 99]. This pathway was reported to be one of the most likely explanations for CS-associated anti-inflammatory responses in the gut. Mapping the relevant neuronal circuits revealed that efferent vagus nerve fibres innervated the small intestine and proximal colon [100]. Vagotomised mice were more susceptible than normal mice to developing colitis after exposure to DSS and had increased levels of NF-κB and cytokines, such as IL-1β, IL-6 and TNF-α [101–103]. Pretreatment with nicotine reversed these effects through activation of α7nAChR, identified as the major receptor involved in nicotinic anti-inflammatory pathways [99, 104]. Potential therapeutic applications of selective α7nAChR agonists, such as the partial α7 agonists 3-(2,4-dimethoxybenzylidene)-anabaseine (GTS-21) and anatabine citrate, and of α7nAChR-positive allosteric modulators, was explored in pre-clinical and clinical studies [105–109]. Moreover, additional nAChR subtypes, such as α4β2, α3β4, α3β2 and α6, were also proposed as targets for nicotine treatment [110–112], increasing the complexity, but also the therapeutic potential, of this approach. Although research on the mechanisms involved in nicotinic anti-inflammatory pathways has highlighted the pharmacological potential of nAChR agonists, studies showing contradictory results obtained with specific α7nAChR ligands [82] suggested that these compounds should be used with caution in patients with IBD.

4.2. Immune regulation

The immunosuppressive effects of cigarette smoking, on both cellular and humoral immunity, have long been recognised [113–115]. Studies exploring how nicotine or CS can suppress the immune system indicated that, in nicotine-treated animals, T cells did not enter the cell cycle and proliferate as expected. Similar effects were observed in smokers and in animals exposed to CS [116–118]. Several studies described the implications of CS for different immune cell types, as well as the diverse actions of nicotine or CS, depending on the pathological environment, for example, UC or CD, in which the immune cells originated [77, 99, 112, 119–122]. For instance, when stimulated by lipopolysaccharide, peripheral blood mononuclear cells derived from smokers showed decreased IL-8 release only if subjects were also CD patients [122]. Similarly, the same investigators demonstrated that smokers with CD had significantly lower IL-10 (anti-inflammatory)/IL-12 (pro-inflammatory) ratios than non-smokers or smokers with UC. As suggested in some reports, the differential signalling of dendritic cells from CD (Th1-like) and UC patients exposed to cigarette smoke extract (CSE) *in vitro* could play a role in the opposing responses of cigarette smoke exposure, that is, a Th1-like response in CD, with increased Foxp3-positive CD4 T cells [121].

4.3. Barrier dysfunction and intestinal permeability

The intestinal mucosa is one of the most important physical barriers against external threats. Changes in intestinal permeability are crucial for the development of IBD [123] and several studies implicated CS in regulating barrier integrity. However, the effects of smoking on intestinal permeability are controversial. Several *in vitro* and *in vivo* observations, in studies using humans or rodents, suggested that decreased intestinal permeability in smokers might explain the protective effects of smoking in UC [53, 124–127]. In contrast, a recent article reported that mice exposed to CS exhibited increased intestinal permeability and bacterial translocation, intestinal villi atrophy, damaged tight junctions and abnormal tight junction

proteins [128]. However, no intestinal barrier changes were identified in the colons of control or CS-exposed mice, suggesting that there was CS-related organ specificity and, thus, possibly explaining the opposing effects of smoking on CD and UC.

4.4. Gut microbiota

Much evidence supports the strong impact of environmental factors on gut microbiota, and smoking has recently been investigated as a potential factor shaping the microbiota. This potential connection implied new possibilities regarding the role of smoking in IBD development. Thus, studies targeting selected bacterial groups reported that patients with active CD, who also smoked, had microbial profiles different from those of non-smoking patients with CD. Similar results were found in healthy smoking controls, suggesting that the association related not to intestinal inflammation but, instead, to a direct impacts of smoking on the microbiota [129, 130]. Differences between mice and humans at the level of the gut microbiota limit the usefulness of mouse models, relevant to CS, gut microbiota and IBD. However, a few studies using rats and mice were consistent with observations in humans, indicating CS-dependent shifts in gut microbiota compositions [131–133]. These observations supported a possible role for CS in shaping the gut microbiome, with potential, though still unknown, consequences for evolution of inflammation-related disorders, such as IBD.

4.5. Other potential mechanisms

Currently, the processes described in Sections 4.1–4.4 have been those most explored as potential links between CS and IBD development. However, there are several other possible mechanisms, indicative of how environmental factors might exponentially increase complexity of IBD pathology.

4.5.1. Colon motility

In UC, fasting colonic motility increased, whereas motor responses to food significantly decreased [134]. Observations in experimental animals and humans showed that nicotine promoted smooth muscle relaxation, reducing symptoms, such as diarrhoea and urgency without significantly influencing inflammation [135–137].

4.5.2. Eicosanoid-mediated inflammation

Smoking and nicotine may also affect UC by reducing eicosanoid-mediated inflammatory responses. Two studies independently demonstrated this specific effect in humans and rabbits [53, 138].

4.5.3. Rectal blood flow

Patients with UC have significantly higher rectal blood flow than normal controls, but smoking decreased rectal blood flow to within normal ranges [139–141]. However, changes in blood flow can affect intestinal inflammation in opposing ways. Decreasing blood flow can reduce levels of inflammatory mediators that reach the mucosal surface, while long-term impairment

of rectal mucosal microvascular blood flow can result in a higher incidence of anastomotic breakdown in chronic smokers [140].

4.5.4. Non-nicotine-mediated effects

Although nicotine is considered to be the major mediator of CS effects on intestinal inflammation, there is a clear evidence for involvement of other smoke constituents in CS-dependent responses. Both UC and CD mouse models were affected by carbon monoxide (CO) inhalation [71–73, 142]. These studies suggested that the mechanism through which CO protected against intestinal inflammation involved promoting bactericidal activities of macrophages [142]. Nitric oxide (NO) was also suggested as contributing to beneficial CS effects, based on its relaxant effects on colonic smooth muscle from UC patients [143]. Moreover, physiological NO, derived from nicotine-stimulated intestinal neuronal cells, functioned as a mediator in smooth muscle relaxation in the colons of DSS-treated mice [137].

5. Conclusions

Smoking cigarettes is addictive and causes a number of serious diseases, including those of the respiratory and cardiovascular system [144], it also negatively impact on the gastrointestinal tract, such as CD [145]. Many of the adverse health effects of smoking are reversible and important health benefits are associated with smoking cessation [146]. With regard to the other major IBD form, a protective effect of cigarette smoking on the risk of UC development is well documented. However, whether CS constituents have beneficial effects on the course of the disease is less clear and the potential mechanisms are not understood.

CS inhalation studies in IBD mouse models would, ideally, reproduce the clinical effects of CS on colonic inflammation. This would facilitate identification of the mechanisms involved in the effects of CS on colitis and, eventually, lead to the characterisation of new anti-inflammatory processes involved in colon protection [22]. Nonetheless, so far, the results obtained using animal models of IBD following exposure to inhaled CS or to nicotine via non-inhalation routes, reflected the ambiguity of the clinical observations. These inconsistencies often reflect the high variability related to animal models (e.g. strains, IBD inducers, etc.) and inhalation methodologies. A more systematic and standardised approach is required to obtain consistent and reproducible data addressing the mechanisms by which CS interacts with the inflammatory processes in animal models of UC-like and CD-like colitis. Such systematic investigations could provide valuable insights into the possible anti-inflammatory effects of CS constituents in models related to UC. Corresponding studies in CD models would provide more mechanistic detail about how these compounds can enhance inflammation in CD.

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Conflict of interest

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Chapter 11

Use of Animal Models in the Study of Colitis

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Abstract

Inflammatory bowel diseases (IBDs) relate to chronic inflammations in different parts of the gastrointestinal (GI) tract involving both ulcerative colitis (UC) and Crohn's disease (CD). Ulcerative colitis begins in the rectum and extends continuously up the colon. Notably, CD may affect any area of the GIT, from the mouth to the anus. Various conditions may influence the genesis of the disease, such as genetics, environment, intestinal microbiota and the presence of agents of enteric infections. Experimental models are therefore suitably used to investigate the various etiological factors; similarly colitis can be induced by genetic modification, cell transfer, spontaneous inflammation and chemical agents. The objective of this chapter is to present current concept on animal models of inflammatory bowel diseases. These models are crucial for the understanding of inflammatory bowel diseases, development of alternative treatments and more effective therapeutic agents thus contributing to the control of the disease.

Keywords: inflammatory bowel disease, animal models, ulcerative colitis

1. Introduction

The immune system has a mechanism of response to tissue injury and antigenic stimuli, whose main function is to protect the body. This response is called inflammation. It is characterized by the interaction of several types of cells and signaling molecules, to promote the repair of damaged tissue [1, 2]. Despite its protective function, the inflammatory response must not be prolonged, since exacerbated inflammation may cause physical loss of function in the organ [3].

The various manifestations characterized by chronic inflammation include inflammatory bowel diseases (IBDs), such as ulcerative colitis (UC) and Crohn's diseases (CD) [4, 5]. Their clinical presentations can be overlapping as well as distinct [6, 7]. UC is generally associated to superficial inflammation of the colon mucosa and CD comprises transmural damage that may occur in any segment of the gastrointestinal tract (GI) [8].

The IBDs are common among white people between the ages of 20 and 40 years old and the second peak of the disease occurs around 55 years of age. The distribution of this disease in males and females seems similar, though CD is more frequent in women [9].

The genesis of UC and CD involves multiple factors, such as genetic, environmental, intestinal microbiota and the presence of enteric infections. These factors may affect the immune system, by expanding and perpetuating the inflammatory process [10].

The treatment of IBD is considered unsatisfactory, because among other factors, cure may not be possible, only remission of the disease and/or treatment of symptoms. Several classes of drugs are currently used to treat IBD, such as aminosalicylates, corticosteroids and immunosuppressives [11]. The odds remain that these drugs have many side effects, are expensive and lose their efficacy after a certain period of medication [12].

Therefore, concise experimental studies are needed to unravel the etiology and pathophysiological mechanisms of IBD for development of alternative treatments and/or more effective therapeutic agents to treat patients with ulcerative colitis. Thus, the use of experimental animal models becomes essential to elucidate the mechanism(s) involved and accelerate the development of a specific and effective therapy.

2. Animal models for inflammatory bowel disease: colitis

The use of animal models is crucial for the investigation of the processes involved in the genesis of IBD. Although the development of the disease in animals does not present all the mechanisms observed in humans, animal models still are the most effective way to study the factors involved in the pathogenesis of BD. It would be difficult to conduct such a study in humans. Additionally, the animals are very useful for the assessment of new therapeutic approaches [13].

Over time, several experimental models of IBD were developed to conduct experimental studies, particularly mice and rats. The animal models of intestinal inflammation can be divided into four groups according to the type of induction namely: genetic modification, cell transfer, spontaneous inflammation and chemical agents [14–16].

The ideal experimental model is characterized by morphological disorders and intestinal inflammation, in addition to clinical symptoms, pathophysiology and development of the disease similar or equal to human condition IBD [17]. The chapter thus presents animal models developed for the study of and experimentation on colitis.

2.1. Genetic modification

The development and use of genetically modified animals have contributed to the investigation of various issues related to the IBD, and the most frequent are connected to the triggering of inflammation through the impairment of various functions such as epithelial barrier and detection of bacterial components, signaling of the innate immune response, immune regulation and signaling response to stress [18]. Studies involving the human genome have shown the association of more than 160 genes connected to IBD [19] (**Table 1**).

When the intestinal epithelial barrier is impaired, the intestinal mucosa has contact with bacteria from the lumen, which generates the inflammatory responses that will lead to the development of IBD. Studies have demonstrated the relationship between CD and certain genes involved in the intestinal epithelial barrier function. Animal models that express defects in this barrier are discussed and are essential in the investigation of IBD pathogenesis [18].

a. C57BL/6 mouse expressing a dominant negative N-cadherin (NCADΔ)

N-cadherin is a glycoprotein related to epithelial cell adhesion and is essential for normal development. Mice with a dominant-negative mutation of N-cadherin developed porous regions in the intestinal epithelium and intercellular adhesion disorder, developing transmural inflammation of the jejunum similar to CD at 3 months old. The organization of the small intestinal epithelium makes it possible to use chimeric mice, generated from normal blastocyts and genetically manipulated embryonic stem cells, to study cadherin function. Therefore, embryonic stem cells were transfected with a dominant negative N-cadherin mutant (NCAD*Δ*) under the control of promoters active in small intestinal epithelial cells and then introduced into C57BL/6 mouse blastocysts [20].

b. FVB mice

FVB mice originated from Swiss syngeneic strains subjected to processes that triggered intolerance or sensitivity to histamine. FVB mice were derived from the syngeneic Swiss strains, selected from crosses in which progenies received pertussis vaccination and challenged with histamine thus developing resistance or sensitivity to it. From this experimental system, two strains were selected: one resistant to the histamine factor (HSFR/N) and another sensitive (HSFS/N). Within the latter group, in the early 1970s, a group of eight syngeneic generations, HSFS/N, carrying Fv-1b alleles and susceptible to the Friend leukemia virus, lineage B, were selected. They are good breeders with large progenies and the eggs of the FVB/N lineage favor *in vitro* fertilization procedures since it has a huge pro-nucleus that facilitates DNA microinjection and the production of transgenic [21].

c. Muc2 knockout ($Muc2^{-/-}$) and Muc2 heterozygous ($Muc2^{+/-}$) mice

The surface of the intestinal mucosa has many components essential for its protection, such as MUC2 that has a lubricating function and also provides a protective barrier between the contents of the lumen and mucosal layers. Wild-type ($Muc2^{+}/^{+}$), $Muc2^{+}/^{-}$ and $Muc2^{-}/^{-}$ littermates are mostly used and scores recorded weekly until the age of 16 weeks to obtain a disease activity index (DAI). The previously mentioned $Muc2^{-/-}$ mice were backcrossed onto a 129SV

Mutation	Main results	References
Dominant-negative mutant N-cadherin	Transmural inflammation in the jejunum similar to that of patients with CD	[20]
Lack of Muc2	Development of IBD in the distal colon	[22]
Specific deletion of C1galt1 in epithelial cells	Inflammation in the distal colon	[23]
MDR1a gene deleted	Development of colitis	[24]
Absence of IRE1 α	Spontaneous colitis	[26]
Nuclear kappa B (NF-kB) deactivated in intestinal epithelial cells	Development of colitis with apoptosis of epithelial cells	[27, 28]
Specific deletion of type II receptor for TGB-β in CD4+-specific T cells and dendritic cells	Spontaneous inflammation in several organs and colitis	[29, 30]
Absence of SMAD4	Inflammation through the entire gastrointestinal tract	[31]
Absence of IL-2 and deficiency of JAK 3 (component of IL receptor)	Autoimmune disease with colitis and other disorders; spontaneous colitis	[32–34]
Absence of IL-10 and deletion of CRF2-4 (component of IL-10 receptor)	Spontaneous development of colitis; chronic colitis	[38, 39]
Absence of TCR α	Spontaneous triggering of colitis	[40]
Absence of STAT3 in macrophages and neutrophils	Spontaneous onset of colitis	[41]
Super expression of STAT4 and immunization with DNP-KLH/CFA	Development of transmural colitis	[43]
Absence of Gαi2	Triggering of colitis and colorectal cancer	[44–46]
Absence of enzyme A20	Inflammation in the intestine and in other organs	[47]
Absence of T-bet and T and B cells	Onset of colitis similar to UC	[48]
Altered levels of TNF-α	Development of inflammation of the ileum and in the distal colon	[49–51]
Super expression of IL-7	Spontaneous onset of colitis	[53]
Absence of Gpx1 and Gpx2	Triggering of colitis and ileitis	[54]
Specific deletion of XBP1 factor	Development of ileitis	[55]

Table 1. Genetic models of colitis.

(Charles River, Maastricht, The Netherlands) genetic background for nine generations followed by intercrosses to breed animals homozygous for the Muc2 disruption. Mice lacking this component may develop IBD in the distal colon at approximately 5 weeks of age [22].

d. *Intestinal epithelial cell-specific C1galt1$^{-/-}$ (IEC C1galt1$^{-/-}$) mice*

The enzyme β1, 3-galactosyltransferase 1 (C1galt1) is related to the synthesis of O-glycan in mucus-producing epithelial cells. The mucus transports a large amount of O-glycan, since

this substance forms 80% of the mucus molecular weight. Approximately 30% of the individuals with UC have disorder in O-glycan synthesis. To study the role of O-glycans in intestinal tissue, the model was generated by crossing mice with loxP sites flanking C1galt1 (C1galt1$^{f/f}$ mice) with an intestinal epithelium-specific Cre-expressing transgenic line (Villin-Cre mice), giving rise to animals lacking C1galt1 in the intestinal epithelium, referred to as C1galt1$^{-/-}$ (IEC C1galt1$^{-/-}$) mice specific for intestinal epithelial cells. All the animals with deletion of C1galt1 in intestinal epithelial cells developed distal colon inflammation at 12 weeks of age. Deletion of C1galt in epithelial cells was also induced in adult animals and, 10 days later, the development of colitis was also observed [23].

e. *MDR1a$^{-/-}$ mice*

The multiple drug resistance gene (MDR1) codes the multiple drug carrier (P-glycoprotein) in intestinal epithelial cells. Around 25% of FVB mice with the gene MDR1a "deactivated" developed colitis at 1 year of age [24]. In the Japanese population, the MDR1 gene was related to susceptibility to late onset of UC [25]. To obtain FVB.mdr1a−/− mice, FVB control mice and mdr1a$^{-/-}$ mice were bred and maintained in the SPF facility. Mdr 1a$^{-/-}$ mice had been backcrossed onto the FVB strain for at least seven generations for models obtained at Taconic Farms [24].

f. *Ire1 α $^{flox/flox}$-Villin-Cre mice*

Inositol requiring enzyme 1α (IRE1 α) acts as a major stress sensor. The Villin-Cre transgenic mice expressing Cre recombinase specifically in intestinal epithelium is currently available. Floxed mice (Ire1 α $^{flox/flox}$), in which the 121-nucleotide exon 2 of the Ern1 (i.e. Ire1 α) allele was flanked with two loxP recombination sites. Intestinal epithelia-specific Ire1 α knock-out mice (Ire1 α $^{flox/flox}$-Villin-Cre) were produced therefore, by intercrossing the Ire1 α $^{flox/flox}$ mice with Villin-Cre mice. Animals without IRE1 α develop spontaneous colitis and are characterized by the loss of goblet cells, which causes the intestinal epithelial barrier to become nonfunctional [26].

g. *NEMO^{IEC-KO} mice*

Animals with deactivation of nuclear factor-kappa B (NF-κβ) binding in intestinal epithelial cells, through conditional ablation of NEMO or both IKK1 (IKKa) and IKK2 (IKKb)—IKK subunits essential for NF-κβ activation, have an exacerbated response to luminal microbiota, which leads to the development of colitis with apoptosis of epithelial cells at the age of 6 weeks. To investigate the function of NF-κβ signaling in the gut epithelium *in vivo*, this was generated in mice lacking NEMO specifically in intestinal epithelial cells (NEMO^{IEC-KO} mice) by crossing mice carrying loxP-flanked (floxed, FL) NEMO (Ikbkg) alleles with Villin-Cre transgenics. These experiments were performed using mice backcrossed into the C57BL/6 genetic background for at least five generations. They were used as wild-type controls. The mice used were housed in individually ventilated cage systems, in either specific pathogen-free or conventional animal facilities [27, 28]. Therefore, regardless of the immune system, intestinal cells clearly provide protection against bacterial pathogens of the microbiota [18].

2.1.1. Transgenic animal models expressing colitis through modulation of immune regulatory agents

Mice with several genetic disorders associated with immune regulation were examined, revealing the development of colitis that was related to different substances [18]. These models with genetic modifications that affect immune regulation and trigger IBD are discussed below.

a. Type II receptor for TGF-β or SMAD4 absence

The transforming growth factor-beta 1 (TGF-β1) acts both in immune regulation and in the differentiation of regulatory T cells. Transgenic animals with dominant-negative mutant TGF-β type II receptor in specific CD4+ T cells showed spontaneous inflammation at around 3–4 months of age, and there was increased expression of Th1 and Th2 cytokines in several body organs such as colon, liver, stomach, duodenum, pancreas and kidney. These animals were created by using DNA microinjection into (C57BL/6xC3H) F1 fertilized oocytes, which results in six transgene-positive mice. All pups in the experimental cycle appeared normal at 2 weeks of age, but three died between 2 and 4 weeks of age. Three remaining transgene-positive mice appeared healthy and were bred; their progeny was further used to identify transgenic lines expressing a functional dominant-negative TGF-β receptor type II [29].

Animals with specific deletion of type II receptor for TGF-β in typical dendritic cells developed several inflammations, including colitis. Thus, B6.129S6-Tgfbr2^{tm1hlm} mice, carrying homozygous loxP site insertion flanking exon 2 of Tgfbr2 gene is currently available and obtainable from the National Cancer Institute (Frederick, MD) mouse repository (strain 01XN5) and other laboratories. CD11c-Cre transgenic mice (B6.Cg-Tg(Itgax-cre)1-1Reiz/J), OT-II transgenic mice (B6.Cg-Tg(TcraTcrb)425Cbn/J), Rag1$^{-/-}$ (B6.129S7-Rag1^{tm1Mom}/J) and wild-type C57BL/6 J are all obtainable by researchers. The mouse model with CD-specific T*gfbr*2 deletion highlights the critical importance of TGF-β signaling in CDs in the maintenance of immune homeostasis and in the prevention of autoimmunity [30].

SMAD4 acts as a mediator in intracellular signaling of TGF-β. To inactivate specifically Smad4 in T cells, it was necessary to cross mice with a transgene encoding a Cre-recombinase driven by the Lck promoter (Smad4co/co; Lck-Cre) or the CD4 promoter (Smad4co/co; CD4-Cre) with the strain carrying the Smad4 conditional allele (Smad4co/co). Mice without SMAD4 in specific T cells and those without SMAD4 in CD4+ T cells had inflammation through the entire gastrointestinal tract at 3 months of age [31].

b. Mutations affecting the interleukin receptor (IL-2)

Interleukin-2 (IL-2) is related to activated-induced cell death and to the triggering of regulatory T cells function. Deficient IL-2 mice develop spontaneous autoimmune colitis, gastritis, hepatitis, pneumonia, pancreatitis, nephritis and hemolytic anemia [32]. Janus tyrosine kinase 3 (JAK 3) acts as a transducer of interleukin-2 receptor common gamma chain. Deficiency of JAK 3 led to the spontaneous development of colitis in mice [33]. The animal model that uses IL-2 is characterized by association of colitis with the autoimmune mechanism, which was demonstrated in the spontaneous onset of colitis without the presence of microorganisms [34].

Similarly, IL-10 is a regulatory cytokine with IBD susceptibility genes not only in adults but also in children [35–37]. IL-10 deficient mice develop spontaneous colitis [38]. Mice with deleted CRF2-4, a component of the IL-10 receptor, develop splenomegaly and disorders such as chronic colitis [39].

c. Mice with T cell-related mutations

The T cell receptor (TCR) is essential for the recognition of antigens by T cells. Approximately 60% of mice without TCR α, TCR component, developed Th2-mediated spontaneous colitis. The animals used in this study were produced by crossing TCR β × mutant mice with TCR mutant mice, and generated TCR β × gamma double mutant mice, which are deficient both in $\alpha\beta$ and γ cells. By mutating, the recombination-activating gene RAG-1 mice totally deficient in mature T and B lymphocytes were produced [40].

The transcription factor, signal transducer and activator of transcription 3 (STAT3) acts in the control of various innate immune responses such as differentiation of Th17 cells and epithelial renovation. Its gene is susceptible to UC and CD [35, 36]. Animals with intestinal epithelial cells deficient in STAT3 developed spontaneous colitis. In response to inflammatory stimuli, LysMcre/Stat$^{flox/-}$ mice develop enterocolitis even though they produce more IL-10 than normal animals in the same condition. To disrupt the Stat3 gene specifically in macrophages and neutrophils, two types of animals were crossed, mice in which the Stat3 gene is flanked by two loxP sites (Stat3$^{flox/flox}$) and the other mouse in which the Cre cDNA is inserted into the lysozyme M gene by a knock-in approach (LysMcre mice) [41].

STAT4 is a transcription signal transducer and activator factor that acts in the development of Th1 cells. Its gene was considered susceptible to UC in individuals with white skin [42]. Transgenic mice with super expression of STAT4 when immunized with 2,4- dinitrophenol-keyhole limpet hemocyanin/Complete Freund's Adjuvant (DNP-KLH/CFA) to activate the cytomegalovirus (CMV) developed transmural colitis. The murine STAT-4 cDNA was cloned downstream of the CMV promoter into the pcDNA3.1 expression vector (Invitrogen, San Diego, CA) yielding the pcDNA3.1S4 vector. A 3.6-kp *NruI*/*PvuII* fragment of pcDNA3.1S4 containing the STAT-4 expression cassette was microinjected into pronuclei of fertilized eggs of FVB/NHSD mice. Mice were maintained under specific pathogen-free conditions in isolated cages [43].

d. Gαi2$^{-/-}$ mice

Gαi2 signal transduction proteins participate in a wide signaling system. Mice that lack Gαi2 proteins showed dysfunctions related to T cells and developed colitis when they were 3–4 weeks old and colorectal cancer at approximately 3–4 months of age. Gαi2-deficient (Gαi2$^{-/-}$) mice on a 129SvEv X C57BL/6 background (backcrossed four or five generations into 129SvEv and then intercrossed) and on a pure 129SvEv background were used [44–46].

e. A20$^{-/-}$mice

A20 is an enzyme related to ubiquitin, which in turn is associated to NF-κβ1 activation. It acts in the prevention of tissue destruction and inflammation mediated by innate immune cells, in addition to regulating skin differentiation. To study the functions of A20 *in vivo*, A20-deficient

($A20^{-/-}$) was generated by gene targeting. Rodents that lack this enzyme developed inflammation in various organs, including bowel, when they were 3–6 weeks olds [47].

f. T-$bet^{-/-}$ mice

T-bet is a transcription factor that regulates the differentiation of T cells into Th1 cells. To investigate whether the T-bet's protective function is linked to innate immunity, the T-$bet^{-/-}$ mouse developed on the $RAG2^{-/-}$ background was examined. The mice experimental model that lacked the T-bet factor showed no colitis. On the other hand, a new mouse model through which animals without T-bet were crossed with RAG2 mice lacking T and B cells showed the development of colitis similar to what occurs in UC at 4 weeks of age, therefore, T-bet expression protects against colitis and T-$bet^{-/-}$ × $RAG2^{-/-}$ mice develop spontaneous colitis [48].

g. TNF $^{\Delta ARE}$ mice

TNF-α is related to the onset of CD, because of the development of spontaneous inflammation in the ileum of mice with increased TNF-α activity [49]. Animals with intrinsic defect related to post-transcriptional regulation of TNF mRNA (TNF ΔARE rat model) developed inflammation, especially in the terminal ileum and subsequently in the proximal colon [50, 51]. To develop TNFΔARE mice, a 69bp deletion encompassing TNF ARE was conducted in embryonic stem cells in conjunction with the neomycin resistance marker (neo) flanked by loxP sequences. The excision of the neon gene and generation of TNFΔARE rat lines was due to the transient expression of the Cre recombinase in fertilized oocytes from the previously mentioned mice. The TNFΔARE homozygous mice developed early pathologies and also had a short shelf life. To reverse this problem, the effects of the TNFΔARE mutation on TNF biosynthesis were investigated in TNFΔARE/− hemizygous mice generated by the crossing of TNFΔARE/+ to TNFΔARE/− mice. The use of hemizygous TNFΔARE/− mice made it possible to investigate the regulatory role of the ARE element in a monoallelic system without the interference of a wild type TNF allele in the generation of TNF. So, a defective function of ARE may be etiopathogenic for the development of analogous human pathologies, in this case, Crohn's-like inflammatory bowel disease.

h. SRα/IL-7 mice

IL-7 has an important role in the homeostasis of T cells, and the gene linked to the IL-7 receptor was related to UC [35, 36]. To study the IL-7 role, it was critical to develop the model SRα/IL-7 transgenic mice using 2.1 kb SRα/lL-7 DNA fragment that was linearized by AatH and ApaU digestion. Microinjection into the pronuclei of fertilized eggs of C57BL/6 J mice was performed with ~10^3 copies of the SRα/lL-7 DNA fragment according to the standard procedure [52]. Rodents with super expression of IL-7 developed spontaneous colitis at about 4–12 weeks of age [53].

i. Double-KO mice deficient in both Gpx1 and Gpx2 gene expression

Activation of endoplasmic reticulum (ER) stress responses of intestinal epithelial cells to the metabolic by products of bacteria in the intestinal lumen is necessary for intestinal homeostasis and prevention of chronic inflammation [18]. The glutathione peroxidase (GPX) enzyme is

selenium-dependent and can reduce a broad range of hydroperoxides. The inflammatory process is associated with increased lipid peroxidation, and hydroperoxide appears to mediate IBD-related symptoms. To clarify the role of GPX in the intestinal epithelial cells, a homozygous double-KO mice with interruption in the expression of Gpx1 and Gpx2 genes was generated. These animals have signs and symptoms related to inflammatory bowel disease. The Ye-Shih Ho (Wayne State University, Detroit, MI) generated Gpx1-KO mice, as C57BL/6 J and 129Sv/J hybrids. Likewise, the generation of Gpx2-KO mice as B6 and 129S3 hybrids occurred at the City of Hope (COH; Duarte, CA) Transgenic Mouse Core. These mice were housed at the COH Animal Resources Center in ventilated cage racks. So, it was possible to verify that mice deficient in Gpx1 and GPX2 developed spontaneous colitis and ileitis GPX2 at about 3 weeks of age [54].

The transcription factor XBP1 is involved in the response to ER stress. XBP1 was related to CD and UC. To understand the function of this factor, Xbp1 exon 2 was almost completely disrupted in the intestinal tissue (small intestine and colon). Mice with specific deletion of this factor in intestinal epithelial cells developed spontaneous ileitis due to the lack of Paneth cells that secrete protective cells [55].

2.2. Adoptive transfer model of colitis

The referred methodology induces intestinal inflammation through selective transfer of certain types of immune cells in animals with impaired immune systems (RAG-1 knockout and RAG-2 knockout and SCID pigs). The study based on these models allows the investigation of immune factors, particularly the role of pathogenic T cells and T cell-mediated colitis on mucosal inflammation [18] (**Table 2**).

a. CD4+ CD45RB^{hi} model

The transfer of T CD4+ cells with high levels of protein CD45RB to mice deficient in T cells (RAG$^{-/-}$ or SCID) is the transfer methodology most commonly used to induce colitis, and it increases intestinal inflammation approximately 5–10 weeks after the transfer [56–58]. This method causes an inflammation similar to that of models that use chemical agents and is characterized by hyperplasia and inflammation in the colon, generally causing the death of the animal [59].

Models	Main results	References
CD4 + CD45RB^{hi}	Intestinal inflammation with Th1 responses to antigens of bacteria in the lumen	[56–61]
SheLAg-specific CD4+ T cells	Colitis caused by one single bacterial antigen	[62]
Hsp60-specific CD8+ T cells	Severe inflammation in the mucosa of the small intestine, regardless of the animal's microbiota	[63]

Table 2. Adoptive transfer models of intestinal inflammation.

This model of cell transfer triggers Th1 responses to bacteria in the lumen [60]. After cell transfer, naive T cells react to antigens of the luminal bacteria and expand rapidly in the lymph nodes and colon mucosa, becoming oligoclonal. The presence of pathogenic effector Th1 cells was possible due to the absence of regulatory T cells [18, 61].

b. SheLAg-specific CD4+ T cells

H. hepaticus Ag (SHelAg)-specific CD4+ Th1 clones transfer the disease to RAG1 knockout mice deficient in T cells and infected with a pathogenic bacterium (H. hepaticus) causing colitis between 7 and 8 weeks after the transfer, while the non-infected receptors did not develop colitis. It is inferred, therefore, that pathogenic T cells that respond to a single bacterial antigen may lead to the development of colitis [62].

c. Hsp60-specific CD8+ T cells

In general, heat shock proteins (HSP) are antigens considered potential targets to autoimmune disease. Then, the transfer of CD8+ T cells responsive to HSP60 for immunodeficient animals can trigger severe inflammation in the mucosa of the small intestine regardless of the animal's microbiota [63].

The severity of inflammation by adoptive transfer depends on the donor animal, as well as to the recipient [64, 65]. A study aimed to protocol standardization found that Balb/SCID mice develop a more severe and faster type of colitis C57BL/6 RAG-$1^{-/-}$ mice [66].

2.3. Models of spontaneous colitis

These models concern both the animals that develop spontaneous inflammation in the mucosa and those with inadequate mucosal immune response due to defect or genetic modification [56]. Spontaneous models are of great interest for the study of intestinal inflammation since, as in disease in humans, there is no external manipulation. The murine model C3H/HeJBir, for example, is characterized by chronic inflammation in the colon and in the cecal region [67] (**Table 3**).

a. C3H/HeJBir mouse model

Because of the occurrence of soft, light-colored feces and/or secondary perianal ulceration, and histological evidence of colitis, without pathogens, mice of the C3H/HeJ strain are likely to have a genetic predisposition to develop a form of IBD, with perianal ulceration, it has the pedigree to generate a "high-susceptibility" substrain, C3H/HeJBir, with a high incidence of spontaneous colitis [68].

Models	Main results	References
C3H/HeJBir	Chronic inflammation in the colon and cecal region similar to CD	[67, 69]
SAMP1/YitFc	Severe inflammation in the terminal ileum, similar to CD	[70, 71]

Table 3. Models of spontaneous colitis.

The wounds observed in this mouse model occur in the ileocecal region and the colon. Clinical manifestations begin at 3–4 weeks of age and last up to 10–12 weeks of age, approximately. The characteristics of the colitis developed by this methodology are similar to those of CD [17, 69].

b. SAMP1/YitFc model

Investigation of the mechanisms of CD can be based on the model that uses SAMP1/YitFc mice, where there is spontaneous and/or severe inflammation of the terminal segment of the ileum, which is the primary site of the wounds in CD, this disease model in animals also resembles human CD with regard to histologic features [15, 70, 71]. For the purposes of these experiments, a colony of SAMP1/Yit mice in a barrier facility at the University of Virginia was established from animals provided by Yakult Central Institute for Microbiological Research (Tokyo, Japan). The SAMP1/Yit (H-2^k) mouse strain was originally derived from AKR mice (original parents purchased from The Jackson Laboratories, Bar Harbor, Maine, USA) [70]. The findings from the adoptive transfer research have made it possible to infer that CD4+ T cells that generate a Th1-like cytokine profile, for example, IFN-γ and TNF, act as mediators of intestinal inflammation observed in SAMP1/Yit mice.

2.4. Chemically induced models of intestinal inflammation

Experimental models of colitis induced by chemical agents are used in the analysis of pathological mechanisms of IBD as well as the development of therapeutic agents. The referred method facilitates the induction of colitis. Some chemicals, such as dextran sodium sulfate (DSS) and trinitrobenzene sulfonic acid (TNBS) are the most commonly used substances. Models with such agents have been used for more than 20 years for the investigation and circumstances that affect the development of IBD [16] (**Table 4**).

Induction of inflammation by chemical agents can cause disorders in the barrier of the intestinal mucosa and/or cause hapten-induced hypersensitivity reactions [18]. Several factors are related to the magnitude of the onset of colitis in models induced by chemical substances. Although these models do not have the complexity of human IBD, they can contribute to the study of the processes involved in the control of the disease [72].

Models	Main results	References
DSS	Diarrhea with bleeding, ulcerations and infiltration of granulocytes. Some characteristics are similar to UC in humans	[73, 74]
TBNS	Transmural colitis that resembles CD	[80–82]
DNBS	Extensive tissue damage and acute inflammatory process similar characteristics to CD	[80]
Oxazolone	Inflammation of the distal colon with clinical similarities to UC	[74]

Table 4. Chemically induced models of intestinal inflammation.

a. DSS approach

DSS methodology has been used to induce colitis since 1985 because the disease induced is very similar to the human disease [73]. Its mechanism of action is related to direct epithelial toxicity, which impacts on the epithelial barrier of the intestinal mucosa [18]. In the referred methodology, the substance is diluted in water for animal drinking, for a period ranging from 5 to 7 days. The inflammation induced by DSS has characteristics similar to diarrhea with bleeding, ulcerations and granulocyte infiltration. Dysplasia often occurs during the chronic stage of the inflammation, which is very similar to ulcerative colitis in humans [16, 74].

The inflammatory immune response occurs immediately after disturbance of the intestinal epithelium, due to contact of the antigens in the lumen with the immune cells of the mucosa and submucosa areas [72].

Acute and chronic forms of the disease can be induced, according to the doses and treatment cycles. DSS was also used to induce IBD in transgenic and immunodeficient animals, and most of them are prone to intestinal inflammation. Also, the symptoms and the pathology are more severe with the association of this chemical agent [18]. DSS-induced colitis is frequently used in studies on innate immune processes involved in the onset of IBD and also in restoring the intestinal barrier integrity [72].

b. TBNS

In hapten-induced colitis, the TNBS substances dinitrobenzene sulfonic acid (DNBS) or oxazolone dissolved in ethanol can be administered rectally. The type of response triggered (Th1/Th2) will depend on the hapten selected, as well as on the susceptibility of the animal, and route of administration of the chemical agent. Ethanol is used to disrupt the barrier of the intestinal mucosa, while the agent (TNBS, DNBS or oxazolone) is associated with an autologous protein or microbiota byproducts, becoming immunogenic, and triggering the interaction of specific antigens and cells of the immune system [18, 75, 76]. The IBD models induced by hapten, similar to those that use DSS, can also be applied in transgenic and immunodeficient animals, as well as in those susceptible to immune-mediated colitis [77–80].

In the beginning of the experiment with TNBS-induced model, one single dose can be administered, resulting in acute inflammation, with Th1 response occurring in 2–3 days. Initial sensitization with TNBS can be done by rectal application or via skin, and another administration must be performed 6 days later for the development of delayed hypersensitivity response to TNBS-haptenized colonic proteins [81].

TNBS is administered rectally with ethanol, amidst haptenization of the proteins from the microbiota and the emergence of T CD4+ cells. The severity and the extent of the inflammation depend on genetic factors inherent in the animals and the presence or absence of bacteria that activate T cells [71]. T CD4 + cells are strongly related to Th1-mediated immune responses through IL-12 cytokines, culminating in transmural colitis similar to CD [80, 82].

The IBD model that uses DNBS is similar to the one induced by TNBS, because there is extensive tissue damage and acute inflammatory process. Also, as it occurs in CD, it depends on T CD4+ cells [80].

Oxazolone is generally administered after subcutaneous pre-sensitization (abdomen), which stimulates greater formation of Th2 cytokines and inflammation of the distal colon resembling ulcerative colitis [74].

Like TBNS, oxazolone is also administered rectally. Acute inflammation of the distal mucosa and submucosa generally involves ulcers and infiltrations of neutrophils, macrophages and lymphocytes. The type of response triggered depends on the dose of the substance administered. Overall, this model is useful for the investigation of specific characteristics of the inflammatory process in chronic IBD [72].

Type of induction	Characteristics	Limitations	Advantages	References
Genetic modification	Occurs by gene modification; overexpression or absence of a non-functional gene/protein of interest in all cell types or in a particular cell type, acting on the promotion of inflammation by impairment of epithelial barrier function and bacterial sensing, innate immune signaling, immune regulation, and stress response signaling	It analyzes the pathogenesis of IBD in general It tests the efficacy of new drugs in a lesser than other approached in a preclinical studies	Investigation of certain inflammatory mediators and cellular interactions present in intestinal inflammation Study and better understanding of the complexity of multiple genes and the different types of polymorphisms observed in patients	[14, 18, 19]
Chemical agents	It induces intestinal inflammation by acting on the disturbance of the mucosal barrier, epithelial and/or by triggering reactions of hypersensitivity by hapten	It is not sufficient to understand and characterize the inflammatory and immunological processes involved in intestinal inflammation and the pathogenesis of IBD	It encompasses methodologies that are considered easy to induce, and its simplicity allows it to be used in several experimental protocols	[14, 18]
Cell transfer	It induces intestinal inflammation in an immunodeficient host through the selective transfer of immune cell types	It uses a factor (adaptive immunity) that is not the only necessary requirement for the development of IBD	Investigation of specific route abnormalities and immune gut inflammation To study the role of regulatory T cells and mucosal immune regulation	[14, 18]
Spontaneous inflammation	The crossing of two types of rodents with different genetic contexts provided models that present spontaneous intestinal inflammation, without induced genetic alterations or interventions	Difficulty related to the acquisition of models (availability)	Because there is no intervention, it mimics the complex and multifactorial aspects that encompass the disease in humans	[14, 18, 66]

Table 5. Comparative presentation of the various methods of colitis induction.

Table 5 shows a comparative presentation of the various ways colitis disease is induced as discussed herein. Each of the several models discussed here have similarities with human IBD, and offer advantages for the study of certain aspects involved in the pathogenesis of the disease. Induction by chemical agents is often used widely by researchers mainly because it is easier, although it does not contribute to the elucidation of inflammatory and immunological processes involved in IBD [14, 18]. Genetic modification allows the study of specific aspects present in IBD; however, such form of induction does not allow to analyze the disease in a global way, and contributes less to the test of efficacy of new drugs [14, 18, 19]. Cellular transfer helps to study specific issues, contributing to the investigation of the role of regulatory T cells and mucosal immune regulation, but adaptive immunity alone does not act in the onset of IBD [14, 18]. The spine development of colitis reproduces the disease as in humans (since there is no intervention), allowing the investigation of complex and multifactor situations that are present in the disease, nevertheless, there is a low availability of models reported in the literature [14, 18, 66].

3. Final considerations

The animal models described here can contribute to the understanding of various mechanisms involved in IBD, as well as to the development of new drugs to improve the treatment. It would be extremely difficult to carry out these investigations and experimentation in humans. However, due to a good number of animal models available for this disease, care should be taken in the selection of animal/model choice. Investigators should take into consideration the purpose of their study, for example, the type of compound to be tested and/or pathophysiological mechanisms to be investigated.

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Section 4

Stress, Neurology and Regeneration

Chapter 12

Evaluation of Animal Models Suitable for Hair Research and Regeneration

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Additional information is available at the end of the chapter

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Abstract

Hair loss and regeneration are the subjects of tremendous amount of research for multiple reasons: the well-known importance of hair in individual beauty, the fact that alopecia is a frequent dermatological disease, and that there are limited treatment options. The present work focuses on the evaluation of animal models used for hair research and regeneration. Besides mentioning the option of *in vitro* studies, the chapter analyzes the need of an animal model of alopecia, common used study designs, hair regrowth evaluation methods, and the limitations of the animal models in hair regrowth research. This chapter also discusses the structure of hair, its chemical composition, the properties and functions of hair, consequences of hair loss, the biology of hair loss, and regeneration and existing treatment options for alopecia. By using proper and well thought-out animal models, we aim to refine our knowledge on human hair diseases and hair regrowth. Hair research provides insights into the physiopathological pathways, genetic and cell biochemical mechanisms, and remains a field intensively explored and still inexhaustible.

Keywords: animal models, research *in vivo*, hair regrowth, hair regeneration, alopecia

1. Introduction

Hair loss and regeneration is the subject of tremendous amount of research for multiple reasons. First of all, as hair loss or alopecia is a frequent dermatological disease; second, the treatment options are limited and generate variable rates of success. Last but not least, hair is an important component of human outlook with a strong impact on the overall beauty and attraction of an individual. As several studies have shown, hair plays an interesting part in social and sexual communication.

The chapter addresses several issues: the importance of hair from both personal and social perspective, the structure and chemical composition of hair, hair functions and properties, biology of hair loss and hair regrowth, and consequences of hair loss and treatment options. The authors aim to offer an overview of the hair regrowth *in vivo* and *in vitro* studies, focusing on the animal models, and describing the common study designs and their limitations.

The main reason for hair research on animal models relies on the similarities between human and animal skin biology. New treatments for alopecia with different hair growth-promoting agents and various administration techniques have been tested on animal models to prove efficacy and to minimize possible adverse reactions.

2. Functions of hair

Also known as "fur" in animals, hair is a defining characteristic of mammals. Besides its important thermoregulatory function, it also has a camouflage purpose and offers protection. In animals, hair follicles can modify their type and density during seasonal coat changes [1]. It is noted that in some species, hair provides sensory and defensive functions, while in others it is used for signaling and communication [2, 3].

Although human hair has lost its main thermoregulatory function, on the scalp, it preserves a heat insulation and cooling purpose, by evaporating sweat from soaked hair [4–6]. It also acts as a sunscreen, offering the skin protection against ultra-violet radiation [7, 8].

3. Structure of hair

Hair is defined as an accessory structure of the integument along with the sebaceous glands, sweat glands, and nails [4]. The shaft of the hair (hard filamentous part that extends above the skin surface) consists of three layers, starting from the outside: the cuticle (having several layers of flat, thin cells, overlapping one another), the cortex (containing the keratin bundles in rod-like cell structures), and the medulla (a disorganized and open area at the fiber's center) [9, 10].

In the dermis, we find the bulb of the hair, which contains the dermal papilla. It has an important role in hair formation, growth, and hair cycle [11]. Besides maintaining stem cells that regrow the hair after it falls out, it also nourishes the hair follicle (providing nutrients and oxygen to epidermal cells in the lower layer) due to the blood vessels present at the bottom of the dermal papilla [1].

4. Chemical composition of hair

Hair has a complex chemical structure, containing organic substances (glycogen, acidic polysaccharides, lipids and proteins—amino acids). About 90% of the hair structure consists of proteins, out of which keratin (a combination of 18 amino acids) is the essential component, being produced by the skin keratinocytes. The lipids represent 3% of the hair composition and are supplied by the sebaceous glands or produced in the hair bulb from sterols, fatty acids, and ceramides [12, 13].

Hair also contains inorganic substances (carbon 45.2%, oxygen 28%, hydrogen 6.6%, nitrogen 15%, and sulfur 5.2%) and water. Other mineral components of hair consist of iron, copper, calcium, magnesium, zinc, potassium, and lead, all of them of external sources.

5. Properties of hair

The color of hair depends on the type and quantity of melanin inside the cell. The hair follicle pigmentary unit provides the hair shaft color due to the melanin components (eumelanin and pheomelanin) and the interactions between follicular melanocytes, keratinocytes, and fibroblasts (also involved in wound healing) [11, 14]. In the case of the black hair, the pigment is also found in the extracellular compartment.

Hair is flexible and has elastic properties, being able to get longer by 20–50% under controlled traction. Under heat action, the elasticity decreases and hair can break easily. Hair is also hygroscopic, it can absorb water; a fact which decreases hair elasticity and resistance to a third of its normal value [4, 10].

Hair resistance is mostly due to cysteine amino acid, a substance rich in sulphur, which plays an important role in hair cohesion. Hair resistance seems to be increased to physical and biological agents and decreased to chemicals. Excessive light with UV exposure, repetitive hair-dye, and hair perm generate the alteration of the hair elastic properties by the chemical and photochemical degradation of the amino acids from the keratin structure. Hair resistance equals to a force of 60 kg, but it is decreased in children and elderly people. Hair resistance also depends on the hair diameter [4, 6].

6. Biology of the hair loss and hair regrowth

Human hair is different from hair grown by mammals due to unsynchronized growth cycles and a sensitive response to androgen.

Human hair exhibits a certain seasonal coordination, but the follicles work independently [15–17]. Latest research results sustain the idea that hair follicles act like neurons, being able to interconnect and generate hair loss and hair regrowth in a small region of the scalp. Human hair has a mosaic pattern as it consists of hair in different stages: the majority of the hair follicles (90%) being in growing phase (also known as anagen), 1–2% in regression (catagen phase), and 8–9% of the hair follicles are resting (in telogen phase) [18, 19]. The cyclic changes from anagen to telogen via catagen involve rapid remodeling of both the epithelial and dermal components of hair follicles [20, 21].

In both humans and animals, hair cycle is influenced by stimulatory and inhibitory factors, such as hormones, growth factors, cytokines, neuropeptides, and pharmaceutical products [18, 22, 23]. The dermal papilla supports an increased cell division and growth rate and induces the shift between anagen, catagen, and telogen [18, 19]. In telogen phase, the old hair is lost, but the follicle will be regenerated in early anagen, when new hair grows up [24, 25].

Current concepts of hair loss pathogenesis include genetic, genomic, hormonal, and immune contributions. Furthermore, the patient's behavior influences the hair density and its strength. In recent years, evidence has suggested that hair loss is a multifactorial disease, and the contributing factors include the resistance to insulin, local pathologies (inflammation, hypoxia, and vascular insufficiency), predisposing physiological factors (menopause and aging), association with other diseases (polycystic ovary syndrome, hirsutism, acne, hormonal imbalances, thyroid pathologies, and other autoimmune diseases). Hair loss remains a consequence of the genotype (hereditary information of the organism)-phenotype (morphology, behavior, and development) interaction [25, 26].

The most common form of hair loss is known as "androgenetic alopecia" (AGA), which represents almost 95%. In this case, hair loss is generated by hair cycle abnormalities, such as the shortening of the anagen, within an abnormal hair cycle, and the anagen-telogen rate shifting from 6:1 to 2:1. Also, hair loss can be due to a small-sized dermal papilla. Both situations lead to shortening of hairs, decreasing hair diameter, shaft loss, and an increased number of hairs in telogen phase. In most situations, the changes of hair diameter (hair thinning) are followed by the loss of pigment: final hair (thick and pigmented) can turn back into vellus (thin and white). Studies point out that another cause of hair loss is the fact that the scalp suffers from vasoconstriction and hypoxia [27, 28].

Hair cycle disturbances are mainly caused by an excess of androgens, which alters the production of regulatory factors (soluble paracrine factors and extracellular matrix components) by the dermal papilla cells [13]. Some specific sites of the body (beard, axillary, and pubic hair) react differently than hair from the scalp, as they are androgen-sensitive [4]. Hair miniaturization and thinning, followed by hair fall is most common in the vertex and the crown-frontal area of the scalp [29, 30].

The occipital part of the scalp is an androgen insensitive area that is why in alopecia, hair is still present in this region, and hair follicles are suitable to be used in hair transplants [31, 32]. The androgen effect on hair can be summarized by the metabolization of the testosterone into 5-alpha-dihydrotestosterone by 5-alpha reductase. A good metabolization limits the hair length (in case of the beard, for example) and deficiencies of the 5-alpha reductase generate enlarged hair diameter (thicker hair in the axillary and pubic area) [19, 33–35].

Another form of alopecia is Alopecia areata (AA), a cell-mediated disease directed against active growing hair follicles. It is a nonscarring alopecia, with limited alopecic patches on the scalp or the body, sometimes affecting also the nails. The pathogenesis of AA includes an autoimmune etiology, linked to human leukocyte antigen (HLA) class II alleles and to the T lymphocytic co-stimulatory cascade [30].

7. Genes associated with hair loss

Several studies including recent genome-wide association analyses concluded that a large number of single nucleotide polymorphisms (SNPs) are associated with AGA susceptibility.

So far, only some of the genes involved in hair loss have been discovered: genes AR androgen receptor and EDA2R ectodysplasin A2 receptor from chromosome X, region located at 20p1 on chromosome 20, and additional loci associated with early onset baldness in Europeans, such as *HDAC9* in 7p21.1, *TARDBP* (chr1), *HDAC4* (chr2), *AUTS2* (chr7), *SETBP1* (chr18), q35 (*WNT10A*), chr3q25 (*SUCNR1*), chr5q33.3 (*EBF1*), and chr12p12.1 (*SSPN*) [36].

8. Consequences of hair loss

Alopecia can be a part of the normal aging process. Still, hair loss represents a great concern for patients. Several studies have shown that it generates anxiety and distress especially in females, affecting couple and social relationships [37–39].

Hair loss is defined as a stressful experience for both sexes, patients being unable to cope with the progression of the disease [40, 41]. Stress functions not only as a cause, a risk factor, but also as a consequence of hair loss. Alopecia determines a poor quality of life by the physical and psychological sequelae: low self-esteem, depression, distorted social perception, and psychosocial functioning [42–45].

9. Hair regrowth treatment

Up to the present, although many treatments have been tested, hair loss continues to be a frequent dermatological condition [46].

Two FDA-approved hair loss treatment drugs: Finasteride (acting on the hormonal cause of alopecia—the excess of androgens) and Minoxidil (acting on the physical cause—the hypoxia due to vasoconstriction), are commonly used in clinical practice in order to treat androgenetic alopecia, which represents 95% of all hair loss causes [38, 47–49]. Minoxidil (1 mg per day) is a topical formulation available in 2 and 5% concentration. It stops hair loss and promotes hair growth as it is a vasodilator and potassium channel opener, allowing more oxygen, blood, and nutrients to reach the follicle [50–53]. It has no therapeutic action on the hormonal and genetic causes of hair loss; therefore, it must be used as a continuous support for the hair follicles, otherwise the hair regrowth will cease and hair loss will begin again in 1–2 months [54–57]. Finasteride is a dihydrotestosterone-suppressing 5-alpha-reductase inhibitor, recommended for male use only, decreasing the serum levels of dihydrotestosterone, stopping hair fall (in 48% of the cases), and stimulating hair regrowth (in 51% of the cases). Studies have shown that 1 mg of finasteride oral treatment has an efficacy similar to daily topical application of minoxidil [58–60]. Given the temporary efficacy of finasteride and minoxidil and the limited number of treatments available in alopecia, new therapies are needed to prevent hair loss and enhance hair regrowth [61, 62].

Pharmaceutical hair loss management also includes different substances (arginine, aminexil, caffeine, and taurine), different peptides, B spectrum vitamins, zinc, or different procedures

(application of stem cells or plasma-rich platelets and low-level laser therapy), even if clinical studies in this respect are lacking. A large variety of over-the-counter products claim to treat hair loss pathology: hair tonics, hair balms, hair masks, shampoos, leave in conditioners, topical solutions, or foams function as potential anti-hair loss agents [43, 44, 63–68].

Alternatives to traditional treatment are laser (low-level laser therapy) and platelet-rich plasma (PRP) injections [47, 69].

10. Hair follicle regrowth using gene therapy

Gene therapy aims to deliver genetic material (DNA) into the patients' cells with either a prevention or therapeutic purpose. The therapeutic effect could theoretically be obtained by replacing the mutant gene that causes the disease with a healthy gene, inactivating a mutated gene that causes an imbalance in the organism or introducing a new gene that could fight a particular disease. For the introduction of the gene, a carrier called vector is used, and it usually consists of a modified virus (retrovirus) that will not produce a disease in the organism, but will deliver the gene by integrating the genetic material into the chromosome of a particular cell. The delivery pathway may consist of a direct injection into the tissue or it can be given intravenously, to reach the blood flow [36].

As new evidence shows that 80% of the baldness is genetic, gene therapy could be the solution, although it encounters technical problems that have not been solved up to the present [69]. Most of the hair loss complains in both female and male patients are due to the presence of androgenetic alopecia, caused by hyperandrogenism and sensitivity to dihydrotestosterone (DHT). It has been noticed that people naturally lacking from birth the 5-alpha reductase enzyme (which converts the testosterone to DHT) never develop androgenetic alopecia [50].

Human scalp has DHT-resistant follicles in the occipital area, this location being used to extract the hair follicles for transplant into the vertex or to the fronto-parietal area [53]. Gene therapy may be a solution in this case, if it can trigger the hair follicles with DHT-sensitive cells and change them into DHT-resistant follicles that could regrow hair without being affected by androgen hormones [70]. Another option would consist of the ribonucleic acid (RNA) interference to block the genes responsible for hair loss. Messenger ribonucleic acid (mRNA) represents the carrier of genetic information from the DNA out of the cell nucleus into the cytoplasm, where it is translated into specific proteins, such as receptors, enzymes. Small fragments of nucleic acids, such as small interfering RNAs (siRNAs), can target a specific gene and block the production of any type of protein in a cell. In hair loss, this technology could be used in order to inhibit the androgen receptor (AR) and the 5-alpha reductase enzymes.

Up to the present, an attempt to effectively control delivery of small interfering RNA using biodegradable cationized gelatin microspheres in an animal model of disease was first performed in 2008. Researchers administered local injections of interleukin-4 and neutralizing anti-interferon-γ antibody in C3H/HeJ mice. They concluded that alopecia areata was effectively treated as the treatment suppressed CD8 T cell infiltrates around the hair follicles and

repressed enhanced interferon-γ mRNA expression in alopecic skin. Also, restoration of hair shaft elongation occurred due to Th1 transcription factor T-box 21 small interfering RNAs conjugated to cationized gelatin [71]. Another recent study showed that the sonic hedgehog (*shh*) gene stimulated the hair shaft production and anagen phase in C57BL/6 mice, after being delivered with an adenovirus vector [72].

Gene therapy is currently available only in research settings. It represents a promising therapeutic option for several diseases (especially those with no cure for the moment), but this procedure needs more research and improvement that need to be considered safe and to prove its effectiveness. So far, scientists have encountered difficulties in finding proper delivery pathways of the genes to the body, targeting them to particular cells, controlling the new gene(s) and their effect after they have been inserted into the body [73].

11. Hair regrowth studies *in vitro*

Human hair follicles as research material for hair loss and regeneration involve ethical problems, an invasive collection method and a limited quantity of follicles available for extraction and testing [60, 61].

The first methods of isolation and maintenance of hair follicles in cell cultures go back to 1990, when several researchers used this method in order to study the biology of the hair cycle [74, 75]. Follicles were usually taken during face lifting surgery, but only a third were suitable for the isolation phase of the hair transplant, due to improper collection procedures. The follicles needed to be isolated from human scalp in a few hours, maintained at 2–6°C, in an Earl medium, combined with phosphate buffered saline solution, with calcium and magnesium added. Only the follicles that seemed intact were used.

In vitro hair research was supported by the identification of growth factor function in the process of hair regrowth and differentiation [76–79]. Philpott et al. have reported that in the absence of insulin, follicles prematurely enter the catagen stage [80]. Subsequent *in vitro* and *in vivo* studies, in murine and human models of hair follicles, have demonstrated that IGF-1 level is a regulation factor of hair growth and together with IGF-1 receptor influence hair growth cycle.

Other studies performed in 1990 have shown that transforming growth factor beta 2 (TGF-ß2) promotes anagen to catagen transition. Several inhibitors of hair follicle growth *in vitro* have been identified such as interleukins (IL-1 alpha and beta) and tumor necrosis factor (TNF-alpha). Researchers concluded that these cytokines play a significant part in the pathophysiology of hair inflammatory diseases. Although the factors that perform the transition *in vitro* from anagen to catagen have been discovered, inducing a full hair growth cycle has not been made possible yet. Murine models of hair follicles, isolated at different growth stages *in vitro* seem to maintain their cyclic activity and to illustrate their status *in vivo* [81].

On the other hand, healthy human dermal papilla cells, isolated from hair follicle, lose the ability to produce hair growth when being outside the body. Also, cycling hair follicles cannot be maintained in culture for any length of time [82].

The data that we now possess about the life and function of the hair follicle in health and disease rely on the successful research performed *in vivo* (experiments on natural animals and genetically manipulated models) and *in vitro* (cultures of a cell type—dermal papilla or organ culture of isolated cell follicles). The preference for one of the two experimental alternatives depends on several factors: the purpose of the research and the advantages and disadvantages involved.

12. Hair regrowth studies *in vivo*

12.1. The need of animal models

Animals and humans are remarkably similar at physiological and anatomical levels. Also, genetically speaking, we share 67% of our DNA with earthworms and 99% with mice. Almost 90% of the veterinary medicines used to treat animals are similar to the ones developed for human use. Animal models can mimic human responses, but the differences in species and even in individual animals must be taken into consideration [83]. By recreating human diseases in animal models, we can study and understand the physiopathological processes involved in the disease and maybe find an efficient cure. The first Nobel Prize was awarded in 1901 and other 94 prizes were directly dependent on animal research [84].

Laboratory animals are used when human testing is not available for practical or ethical reasons. Animals represent good research subjects as they have a shorter life cycle that enables scientists to observe the animal throughout the entire life and across several generations. Also, animal models can be easily influenced by the environment, which is controlled by the researcher as far as the diet, temperature, lighting, and other factors are concerned.

Researchers use animal models for short-term objectives (to determine how the animal model responds to a stimuli or a treatment) and long-term purposes (development of a new drug, evaluation of bioavailability or toxicity, genetic study). The animal model should be sensitive, appropriate for the studied condition either by using specific evidence of previous studies or using a new animal model with the risk of generating inaccurate results [69]. Besides the similarity with the human response, other key features of the biomedical research on animal models are specificity to the study purpose, validation of the animal model, and improvement for further research. Animal research has brought many benefits not only to humans but also to animals in disease prevention and treatment [47, 48, 69].

For more than a 100 years, almost all the information obtained in the human and animal health research has been the result of studies performed on animal models. The most common aim of animal models use is the development of new methods for the diagnosis and treatment of diseases, through an understanding of the biology and the physiopathological processes involved [47, 69].

Even though animal models remain a necessity, alternatives consist of computer models, tissue and cell cultures, and other nonanimal-related research methods. In order to minimize the

harmful effect of research performed on animal models, scientists tend to reduce the number of animals used to obtain valid results, to refine the experimental technique, or replace it with nonanimal research methods.

12.2. Animal models used in hair loss and regrowth

A large variety of animals (mice, rats, hamsters, rabbits, sheep, and even stump-tailed macaque) provide useful models for the *in vivo* study of hair loss and regrowth, but 95% of the animals bred for research purposes are rats and mice [85–89].

Mice represent an excellent model to study the hair cycle for several reasons: the first two cycles of the mouse hair follicle are synchronized; the mouse hair cycle is short, lasting for 3 weeks; hair follicles can be easily harvested and examined at specific time points in the cycle. Most importantly, the stages of the hair cycle have been well characterized in the mouse: anagen being morphologically subdivided into six stages and catagen into eight [22]. The periodic intervals of rodent hair cycles (especially the anagen-growing phase) seem to be less susceptible to iatrogenic influences [90]. The mouse hair cycle does not differ structurally from the human hair follicle cycle, except for the fact that during catagen the hair bulb is remodeled, but the vibrissae follicles do not retract. Scientists have recently discovered that a certain progenitor cell population in mice is analogous to the human cells, encouraging research on this particular animal model.

Besides studying the normal hair cycle on mice, scientists also focussed on the growth waves and hormonal control [91]. Significant differences between species regarding the follicular function and limited androgen-sensitive models were noticed [92]. Spontaneous mutations have been discovered and studied on hairless, nude, and tabby mutants, waved and angora animals, leading to the identification of new genes involved in hair loss and opening the path for transgenic technology research [93, 94].

Transgenic mice, also known as "knockout mice," are mice with altered genome through the use of genetic engineering. This gene-targeting technique has revolutionized the biomedical research by offering researchers the ability to create a specific animal model for the most common human diseases. In order to select the most appropriate immunodeficient mouse models for research purpose, scientists also take into consideration: background strain, behavior, husbandry, disease susceptibility, life span, breeding performance, radiosensitivity, functionality of various endogenous immune system components, and leakiness (tendency to produce functional B and T cells as they age).

Up to the present, immunodeficient mice (with T and B cells deficiencies) were used as models for autoimmune disease mechanisms and androgenetic alopecia studies. The androgen action upon the hair follicles has been studied on spontaneous and genetically engineered nude mutant mice [95].

The C57BL/6 mouse is the most popular laboratory rodent, widely used and studied, having its entire genome published. Research applications using this particular type of mice include immunology, cancer, neurodegerative disease, age-related hearing loss, bone density, diabetes,

obesity, and biomarker studies. This black coat mouse has been used for the skin-free pigment and early visible pigmented tips of new anagen regrowth [88]. C57BL/6 represents one of the most well-characterized models available, with a minimum risk of genetic drift. It is also a convenient model for creating transgenic mice, which are recognized by the mixed coat colors.

The C3H/HeJ mouse model was used in a large range of studies: immunology, cancer (especially mammary tumors), inflammation, sensorineural, and cardiovascular disease. This animal model was the most widely reported for hair growth promotion, most possibly due to the fact that C3H/HeJ mice can spontaneously develop alopecia areata (AA) from 6 to 18 months of age. Also, alopecia areata can be surgically induced by skin-grafting from a donor animal with AA onto an isogenic C3H/HeJ recipient (normal haired mice of the same strain) [90, 96].

In 2010, researchers created the first rodent model of AGA, taking into consideration its relationship to androgen metabolism and androgen signaling, mediated by the androgen receptor (AR). They used transgenic mice overexpressing human AR in the skin under control of the keratin 5 promoter and exposed them to high levels of 5-alpha dihydrotestosterone, which led to delayed hair regeneration, mimicking AGA. The scientists concluded that androgen-mediated hair loss is AR-dependent and suggested that AR and beta-catenin mediate this effect [97].

There are many rat strains raised for research purposes, but the albino Wistar Bratislava rat is the most commonly used. Gene knockout techniques are relatively difficult to be applied and successfully achieved in rats. For hair loss and regeneration experiments, the Wistar rats and the Dundee Experimental Bald Rat (DEBR) strain were commonly used. The latter has the ability to spontaneously develop adult onset alopecia areata (AA) at a higher frequency than in the mouse model [98].

In the research field of hair loss and regeneration two major achievements must be mentioned on the rat animal model: coaxing human stem cells to become dermal papilla and producing new hair follicles when transplanted on rat skin [98]. Also, by inhibiting the rejection of foreign skin, human skin grafts were applied and even rat dermal papillae continued to produce hair after reimplantation *in vivo* on a rat model [99, 100].

Research performed on a rabbit animal model, added important data to the field, proving that full thickness transplants, made with full pedicle graft (separated from their original nervous and vascular supply) retain their original *intrinsec* activity and are not modified by the action of the surrounding tissue [101]. Furthermore, the rabbit represents a common animal model used to screen compounds potentially efficient in treating alopecia.

The Golden Syrian hamster (*Mesocricetus auratus*) has been previously used for research purposes, even though it is a very common pet. The hamster flank organ has served as a model to study the effect of testosterone (T) upon the hair follicle, the sebaceous glands, and the dermal pigment. This hamster is known to be useful for the specific and quantitative assessment of different substances on hair growth, being also useful for therapy testing in hirsutism. Macroscopic (hair density evaluation) and microscopic (hair diameter analysis) hair growth assessments have been performed on Golden hamsters [102].

12.3. Common study designs in hair loss and regrowth

12.3.1. Housing conditions

In vivo hair regrowth studies usually use animals of either sex and weight, kept in experimental rooms that are free of pathogens and opportunistic agents. For 7–14 days prior to the experiment, the animals are housed under specific conditions: room temperature of 23°C, controlled humidity, a 12:12 h light, and dark cycle. In order to avoid licking, individual housing is preferred or a maximum of two animals per cage. Standard laboratory diet and water ad libitum are provided. After completing the experiment, animals are euthanized according to the current regulations. For accurate results, most of the studies on animal models are performed in triplicate [47, 69].

12.3.2. Depilation methods

Experimental designs may include one of the depilation methods: shaving, the use of a raisin mixture, or a hair removal cream [91, 103]. The most commonly used is the shaving of a larger skin area (the whole back or body) or of several smaller areas that are denuded for testing. For animal immobilization during procedures, general anesthesia is commonly performed with a combination of ketamine (i.p. 50 mg/kg b.w.) and xylazine (20 mg/kg b.w.) [47, 48, 69].

Some study designs, such as that of Mester et al., required before each successive hair treatment, the shaving of the skin. This procedure can induce mechanic stimulation of hair growth, as previously reported in the literature, and influence the study results. Other experiments done on adult rats point out that after the fur was dyed and shaved, the regrowing hairs formed a system of linear loops that were closely correlated with the shaving process [66, 67].

In order to avoid this effect, it is recommended not to shave the skin of the animal model before each session of therapy. Other factors which influence the hair regrowth are physical factors such as low temperature, which triggers fast regrowth after shaving.

Depilation-induced hair cycle has been studied, and it follows a strict course: nine days after depilation, the hair follicles enter the final stage of the growth cycle (anagen VI). On day 17 after depilation, the follicles enter the regression stage (catagen), while on day 20 follicles get to the resting stage (telogen) [22].

12.3.3. Evaluation of hair loss and hair regrowth

Efficacy of the treatment is screened by observing the presence, rate, and cosmetic acceptability of hair regrowth. More sophisticated assays include determining how the drug induced hair regrowth and exploring the pathogenesis of AA.

Researchers do not possess standardized methods for *in vivo* hair regrowth assessment. New, accurate, and minimally invasive procedures are still needed as the most commonly used tools are qualitative assessments, limited in number. They include macroscopic assessment with the naked eye (visualization and photographs of the area of interest) based on scales that assess the percentage of hair regrowth on the interest area and tricoscopic evaluation (with a

hand-held dermatoscope, with polarized light and magnification abilities) [41]. Trichoscopy allows a correct hair regrowth evaluation, as it can detect decrease of hair diameter up to ten times or diameter variations. Both macroscopic and microscopic methods assess hair regrowth with the help of personalized hair growth scales or standardized, already published scales.

Usually, the dorsal part of the animals is used for the testing. After being depilated and treated, the animal skin is observed and photographed at specific time intervals (day 1, 7, 14, and 21) to record the start of the hair regrowth period and the pattern of hair regrowth, compared to controls. Several hair regrowth potential scores are mentioned by literature. The one described by Matsuda et al., for instance, ranges from 0 to 5: 0 = no hair growth, 1 = less than 20% of hair growth, 2 = 20–39% of hair growth, 3 = 40–59% of hair regrowth, 4 = 60–79% of hair regrowth, 5 = 80–100% of hair regrowth [54]. Researchers also use self-designed scales of hair regrowth that consider: Type IV (high hair density, full, thick fur), Type III (moderate hair density with no visible skin area), Type II (low hair density, with the visualization of the skin), Type I (uneven hair growth on the test area, skin easily seen) [47, 69].

The hair regrowth potential scores can be applied for both macroscopic and microscopic assessments (**Figures 1** and **2**).

On the other hand, quantitative methods, such as hair weight determinations, hair density measurements, or histopathological examination offer more accurate results. For hair weight determination, the regrown hair from an area of 1 cm^2 of skin is cut and weighed with an analytical balance [48].

In order to analyze the histological features at the end of the treatment period, the animals are sacrificed and a skin biopsy is isolated for histopathological examination. The thickness of the skin and the location of hair follicles in the dermis can be assessed by microscopic photography.

Also the hair cycle can be assessed, as the anagen induction can be calculated with the formula: (number of follicles in hypodermis) × 100/(number of follicles in dermis). Literature data showed an association of increasing skin thickness, follicle count, and macroscopic development of skin pigmentation with anagen induction [18, 23]. The study by Liu et al. found that in the anagen phase the bulb of the hair follicles was enlarged and deeply inserted into the dermis. The research also revealed that the hair follicles in the shaved, bare areas were short, small, and in the phases of telogen, anagen, or catagen [69].

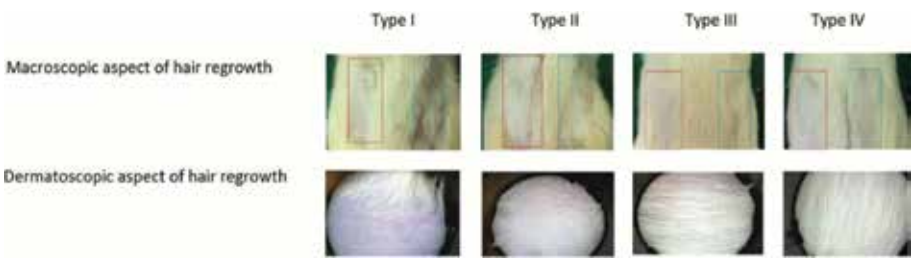

Figure 1. Classification of the hair regrowth effect (type I, type II, type III, type IV) for macroscopic and microscopic assessments—personal study performed on Wistar Bratislava rats. The control area is marked with red (left side of the picture), the test area with blue (right side).

http://dx.doi.org/10.5772/intechopen.69698

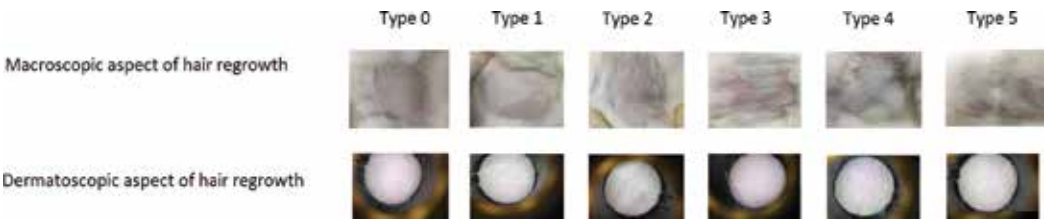

Figure 2. Classification of the hair regrowth effect (type 0, type 1, type 2, type 3, type 4, type 5) for macroscopic and microscopic assessments—personal study performed on New Zealand Rabbits.

The hair growth cycle, consisting of three phases (anagen, catagen, and telogen) is used by both practitioners and researchers to diagnose the hair growth condition and to decide on the hair growth-promoting agent. In human subjects, digital trichoscopy is available, with automatic assessment of the number of follicles in each hair growth phase.

Several studies focused on the validation of Minoxidil 2% treatment on the animal model used, as this topical treatment is thought to be the gold standard treatment for hair loss. This substance affected the normal hair cycle by shortening telogen, causing premature entry of the resting follicles into anagen phase [103, 104].

12.4. Limitations of animal models regarding hair regrowth

The limitation of using an animal model while studying hair regeneration can be briefly summarized as follows. First, synchronized hair cycles generate waves of new hair regrowth, which make the interpretation of result a hard task. Second, the lack of independence of the hair follicles, since they have coordinated regrowth pattern on a precise time scale, as described by Muller-Rover et al. [22]. Third, young mice present the drawback of patchy growth after the second wave of hair growth is completed. Lastly, the increased hair density on an animal model leads to difficulties of assessment by densitometry or cross-section trichometer [69].

The results of our hair growth research performed on Wistar rats showed, besides a normal hair growth in the majority of the animal models, a lack of hair regrowth on the tested area. Other studies performed on black-and-white mice reported that no further hair growth was observed on half of the control animals.

We also noticed a diffuse hair regrowth in some study groups, while in others, the hair appeared to make some specific linear loops that were observed macroscopically. Literature date confirmed our findings. In similar situations, researchers experienced a diffuse hair growth in some animals with an uncharacteristic, diagonal strip [66]. Li-Yaun Liu et al. described four main linear hair regrowth patterns noticed on a rat model: the dorsal loop and the lateral dorsal loop (running along the dorsum and hind limb) and the ventral loop and lateral ventral loop (traveling along the thorax, abdomen, and forelimb). These hair-loop-lines create cranio-caudally-oriented waves of regrowth 2–15 mm wide, symmetrically on both sides of the body, running from the head through the torso to the limbs [105]. Li-Yaun Liu et al. concluded that after shaving the skin, the hair follicles from these new hair lines were always in an anagen phase [106].

Also, the behavior of the animals should be taken into consideration, as it can create issues and interfere with the research results [107]. For example, the C57BL/6 mice show barbering behavior, the dominant mouse in a cage selectively removing hair from its subordinate cage mates. Mice that have been barbered have large bald patches on their bodies, especially around the head, snout, and shoulders [108].

Regardless of the shortcomings of either animal model, most of them validate their usefulness for drug efficacy and safety testing for humans.

13. Conclusion

Although the studies performed on animal models encounter both technical and objective issues, further scientific research is not impeded and continues to remain an intensively explored field. By using proper and well-thought out animal models, we aim to refine our knowledge on human hair diseases and hair regrowth. Hair research provides further insights into the physiopathological pathways and genetic and cell biochemical mechanisms that could promise the cure of hair loss.

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Chapter 13

Animal Models of Rheumatoid Arthritis

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Abstract

Autoimmunity is a condition in which the host organizes an immune response against its own antigens. Rheumatoid arthritis (RA) is an autoimmune disease of unknown etiology, characterized by the presence of chronic inflammatory infiltrates, the development of destructive arthropathy, bone erosion, and degradation of the articular cartilage and subchondral bone. There is currently no treatment that resolves the disease, only the use of palliatives, and not all patients respond to pharmacologic therapy. According to RA multifactorial origin, several in vivo models have been used to evaluate its pathophysiology as well as to identify the usefulness of biomarkers to predict, to diagnose, or to evaluate the prognosis of the disease. This chapter focuses on the most common in vivo models used for the study of RA, including those related with genetic, immunological, hormonal, and environmental interactions. Similarly, the potential of these models to understand RA pathogenesis and to test preventive and therapeutic strategies of autoimmune disorder is also highlighted. In conclusion, of all the animal models discussed, the CIA model could be considered the most successful by generating arthritis using type II collagen and adjuvants and evaluating therapeutic compounds both intra-articularly and systemically.

Keywords: autoimmune disease, rheumatoid arthritis, animal models, biomarkers, therapeutic alternative

1. Introduction

Traditionally the immune system has been considered as a set of structures (molecules, cells, and specialized tissues) and biological processes responsible for the defense against aggression by a variety of infectious agents, chemicals, and tumor cells. One of the fundamental characteristics of the immune system is its ability to discriminate foreign antigens [1]. Autoimmunity is a multifactorial condition in which the host organizes an immune response against its own antigens [2]. Autoimmunity is associated with genetic, immunological, hormonal, and environmental factors, with it being classified as organ-specific and systemic, of which RA is one of the most representative.

RA has an incidence of 5 per 100,000 adults and occurs in 0.5–1% of the population in industrialized countries [3]. The RA can manifest as pain, stiffness, swelling, and loss of mobility. There are different strategies for the study of RA, including experimental animal models that help elucidate different aspects of the disease, as well as evaluate compounds that can reduce the inflammation that triggers the disease. An ideal animal model for RA should reproduce as close as possible the complex pathogenesis and symptoms that underlie the disease, including the presence of chronic inflammatory infiltrates, the development of destructive arthropathy, bone erosion, and degradation of the articular cartilage and subchondral bone [4]. Current RA animal models are highly reproducible and of short duration, having similar patterns to those occurring in human disease although they present some differences, such as (1) faster progression of the disease, characterized by an acute inflammatory response and (2) rodents have a tendency to marked resorption and bone formation (especially of the periosteum/endosteum) in response to joint inflammation. The use of animal models has contributed significantly to the knowledge of processes and mediators that generate inflammation and bone and cartilage damage and, in this sense, can be used as an intermediary to provide knowledge and for the evaluation of therapeutic molecules to correct these disorders [5]. There are numerous therapeutic alternatives for RA; however, the duration of these therapies and the side effects associated with some of these drugs mean that until now, there is no effective therapy for this disease.

2. The human immune system and manifestations of RA

2.1. Primary and secondary lymphoid organs and defense mechanism

The immune system is responsible for protecting the human body from external and potentially pathogenic organisms. It is made up of a series of cells, tissues, and organs distributed widely throughout the body. From the point of view of its structural characteristics, there are organs such as the thymus, spleen, and lymph nodes and tubular structures such as the lymphatic vessels that are intercommunicated. If the functions performed are taken into account, they can be classified into primary and secondary lymphoid organs. The primary lymphoid organs

(thymus and bone marrow) produce T and B lymphocytes, while secondary lymphoid organs include lymph nodes (LNs), spleen, Peyer's patches (PPs), and mucosal tissues, nasal-associated lymphoid tissue (NALT), adenoids, and tonsils, which also harbor perifollicular areas [6].

The immune system has two lines of defense, specific and nonspecific (adaptive and innate), which are responsible for keeping the body free from pathogens or, if present, can eliminate them as well as their residue [7, 8]. The innate, nonspecific antigen response destroys microorganisms and triggers an inflammatory process that blocks the spread of infection. If microorganisms get past this first barrier, antigen-specific adaptive immunity composed of T and B lymphocytes can produce antibodies and killer cells that destroy infected cells [9]. The innate immunity is constituted of external barriers such as mucous and skin; the inflammatory process; cells such as macrophages, natural killer (NK) cells, and phagocytic cells; and chemicals. In relation to adaptive immunity, this is generated only after exposure of inducing agents, and two distinct responses are generated, the cellular response, in which T lymphocytes are responsible for generating this reaction and the humoral response, carried out by B lymphocytes, which in turn are responsible for producing antibodies against the agents that cause damage [10].

2.2. Autoimmune disease as an imbalance in immunoregulation

Autoimmune diseases have been classified according to the organ and tissues affected by the impaired immune response. Nearly 80 autoimmune diseases have been reported to date, and a prevalence of 5–6% worldwide has been stated [11].

An autoimmune manifestation is triggered by antigens that are generated naturally in an individual, and most of the time, these autoimmune events are devoid of pathological character. A large number of people worldwide have autoantibodies to different parts of the body, which are activated when a viral or pathogen infection is present. These diseases are usually the result of an imbalance of immunoregulation [12].

The process by which autoimmune disease is triggered follows different pathways including infection with viruses or bacteria, the use of drugs, the use of irritant chemicals, and environmental factors that damage health. The infection is capable of generating enzymatic changes which in turn alter cell membranes exposed to hidden antigens or may expose new antigenic sites [13]. Also the viral infection can induce new antigens that are released or expressed on its cell surface [14].

Among the first signs of autoimmune disease are pain, swelling, heat, and inflammation [15]. The affection of these diseases will depend on the target organ, since any part of the body, such as the eyes, heart, joints, or brain, can be attacked [16].

Aging and molecular haptens are also associated with the formation of new antigens or the appearance of those that are hidden; in the haptens a response is triggered both against them and the protein to which they bind [17]. Such antigens may be obtained from diet or generated from virus cross reactions with antigens that are present in the individual which force the immune system to react against them.

There are several theories which consider that these types of autoimmunity are due to the loss of tolerance of T lymphocytes or their inability to effectively suppress the reaction that is generated against their own antigens or harmless agents [18].

Autoimmunity is triggered when the response is persistent and leads to the development of uncontrolled cells that react aggressively against any component of the body [19]. These processes require the entry of effector cells into the target organ, whereby there are changes in blood vessels due to the inflammatory substances that are released into the blood [20]. There are different associated factors that trigger an autoimmune disease such as those shown in **Figure 1**.

Recognition of the major histocompatibility complex (MHC) II by the T-lymphocyte receptor (TCR) will produce a clustering with other surface receptors that activate a signaling cascade, which in turn alters the T-lymphocyte transcriptional program. These events produce tissue destruction and loss of function of affected organs during the course of the autoimmune disease. There is evidence that CD4+ T cells are active in local inflammation and cell infiltration that result in inflammation [21].

2.3. Rheumatoid arthritis, its causes and prognosis

RA is a chronic inflammatory disease characterized by synovitis with a symmetrical distribution that causes severe joint destruction [22]. RA is a common autoimmune systemic inflammatory disease that affects approximately 1% of the worldwide population and its incidence is 0.5–1% [3]. Cohort studies have shown that people with arthritis are 54% more likely to die than a healthy person, and there are data that indicate that this frequency is directly associated with the severity of the disease [23]. The process was reported in 1909 by Nichols and Richardson, and among the symptoms of RA are a prodromal period preceded by overt asthenia, general malaise, diffuse myalgia, fever, and anorexia, weight loss, pain, stiffness, and swelling in affected joints [24].

RA initiates as an inflammation of synovial fluid, in which rheumatoid factor (RF), IgM and IgG, and anti-CCP antibodies are present in serum and joints. Complement is activated within the synovial fluid, with C3a and C5a being the most important components found [9]. The disease is perpetuated by the production of cytokines and the action of extracellular matrix metalloproteinases (MMP) [25].

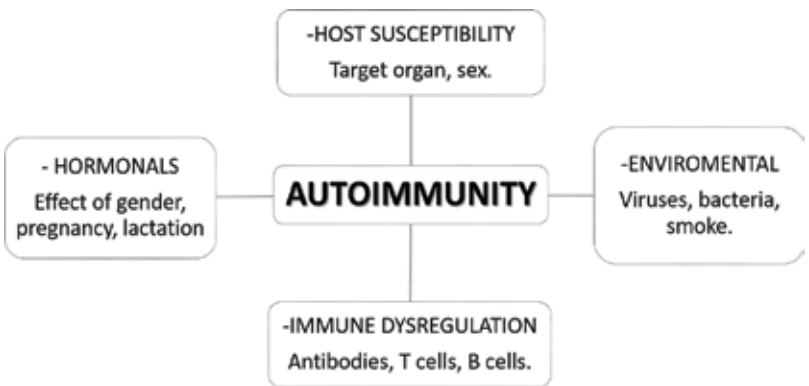

Figure 1. Factors associated with the development of autoimmunity. There are several important factors that are considered the trigger of an autoimmune disease, since the true etiology is not currently known. These include hormonal, immunological, environmental, or genetic factors (susceptibility).

After the onset of the disease, the synovial membrane of patients with RA, which is generally hypocellular, becomes hyperplastic, containing a large number of cells, such as polymorphonuclear leukocytes surrounding immune complexes and complement molecules [26].

Chronic inflammation in hypertrophic synovium is maintained by activated cell groups such as synovial fibroblasts and macrophages, as well as an area containing a clear cell infiltrate: mast cells, CD4+ and CD8+ T lymphocytes, NK cells, B lymphocytes, and plasma cells (the latter produce RF against altered IgGs), along with invasive mesenchymal cells. This accumulation of complexes and cells forms what is called pannus [26].

Once the presence of inflammatory arthritis is recognized, a preliminary diagnosis is made; other diagnoses of arthritis (lupus, psoriatic arthritis, spondyloarthritis, among others) are ruled out. Finally, the risk of developing persistent and/or erosive arthritis is assessed. Patients with early RA develop symmetrical polyarthritis, and its appearance is associated with the presence of positive rheumatoid factor (RF) and/or anti-citrullinated (anti-CCP) antibodies. RF corresponds to antibodies that are directed against immunoglobulins IgG, IgM, and IgA and are usually present in 80% of the patients who suffer this disease [27]. In RA, the inflammatory process is mediated primarily by the action of pro-inflammatory cytokines. In addition to enhancing the activity of IL-1 and TNF-α, IL-17 has a direct effect on the evolution of the disease, since it stimulates osteoclast differentiation and promotes the destruction of the cartilage and bone [28].

Until a few years ago, Th1 cells were considered to be the main cause of tissue damage in autoimmune diseases, but Th17 is currently considered to be the major inducer of autoimmune disease. It migrates more rapidly than Th1 to the areas of lesion. Once there, it stimulates the inflammatory response and is able to recruit other complementary cells, including Th1, which is necessarily associated with Th17 for inflammation and tissue destruction [29].

The onset of RA involves certain components such as T cells (CD4+), monocytes, fibroblasts, B cells, dendritic cells, mast cells, and neutrophils [30]. The synovium of patients with RA usually presents a certain red coloration due to the strong inflammation that is present. Chemokines are usually very important in the pathogenesis of the disease, being the most representative of the CXC family which is a strong promoter of angiogenesis [31].

Cytokines are implicated in the pathogenesis of RA, which triggers and perpetuates autoimmunity, maintaining chronic inflammatory synovitis and directing the destruction of connective tissue. Therefore, they integrate the regulatory immune events and destruction of the tissues that are observed in the clinical progression of RA. Cytokines that play an important pro-inflammatory role in arthritis are TNFα, IL-1β, IL-6, and IL-17 [32].

3. In vivo models for RA

RA is a cosmopolitan disease that affects 60 million people, making it a big problem for the health sector [33]. The etiology and pathophysiology of RA remain poorly understood, but it is generally accepted that genetic, immunological, hormonal, and environmental factors could lead to chronic inflammatory infiltrates, the development of destructive arthropathy, and the manifestation of clinical symptoms [34].

Since 1806, RA has been associated with a certain degree of inheritance; its relationship with genetic and environmental components has not been neglected in greater proportion [35]. There are different experimental models that generate basic knowledge of the pathophysiology of RA for the development of diagnostic kits through the discovery of biological markers. These are grouped in genetic, immunological, hormonal and environmental characteristics; that is, these factors are always associated with most autoimmune diseases.

3.1. Genetic models

The first RA-associated gene appeared in 1978 with the elucidation of HLA association with the disease. Nowadays, it is a clear complex of predisposition to suffer RA [36].

The first set of studies aimed at demonstrating the genetic susceptibility to RA was performed in twins, in which the environment and the genome were similar. This led to the hypothesis that 60% of the changes are attributable to genetic components. One of the best strategies has been to link polymorphisms with RA; despite these efforts, only HLA and PTPN22 factors have been linked with accurate results, hence the importance of genetically modified animal models [3, 37].

The collagen-induced arthritis (CIA) model was developed through the induction of type II collagen although the usefulness of other types of collagen has been demonstrated [38]. This model has become the most effective genetic biomarker. Among the targets that have been evaluated are immunoglobulin-producing B cells which attack type II collagen. Additionally, TCR transgenic mice in which the development of arthritis has been generated by immunoglobulins have also been evaluated [39, 40].

The way in which the transgenic mice model is developed is by injecting a construct that weighs an expression promoter, the gene or molecule of interest, and, commonly, a reporter gene, which will facilitate the exact location of the construction generated by immunofluorescence techniques. Once this construction is obtained, it is introduced into a pronucleus of a fertilized egg. The mouse that will be obtained will be able to express the gene of interest and transmit it to its descendants [41].

The therapeutic targets evaluated in CIA models or transgenic mice with adjuvants (from DBA/1 strain) are genes that code for T lymphocytes, within which the generation of a polymorphism has been associated with the development of RA. Finally, CMH, whose association with RA was reported in 1978, has been assigned to different loci such as HLA-DQ and HLA-DR, as well as to the H-2q region of HCM, demonstrating involvement in animal models with transgenic mice. Despite efforts to detect altered genes in this complex, to date, the exact pathway in which the pathogenesis is produced and the precise effect of this complex on the disease are unknown [42]. **Table 1** summarizes the animal models used for the genetic model of RA.

3.2. Immunological models

Among the immunological factors, commonly considered biomarkers are cytokines, chemokines, immune response cells, and adhesion molecules.

Model	Inbred strain and mechanism	Genotypic characteristics	Limitation	Advantage	Reference
IL-6R	The genetic background is with strain C57BL / 6 and the model is generated due to a mutation generated in the amino acid tyrosine in the IL-6 receptor.	gp130$^{F759/F759}$	A high frequency of B cells is generated, which could result in an increase in the humoral response.	Mutation in GP130 enhances IL-6 expression.	[4, 43, 44]
IL-1Ra	The strain used is C57BL / 6J and said mice are deficient in the gene that enconding for the IL-1Ra receptor antagonist.	B6.129S-*IL1rn*^{tm1Dih}/J	Fail to respond to IL-1 and exhibit an altered immune response to many different target proteins.	Exhibit altered inflammatory responses.	[4, 43, 45]
K/BxN	For this model, the strains of NOD and C57BL/6 are used, in which autoimmunity against the GPI is generated after its crossing.	B6.TCR. C$\alpha^{-/-}$.H-2^{bxg7}	-Number of experimental mice obtained from very small crosses. -Controls may generate arthritis -Additional care due to complexity of the model.	-Obtain control mice, arthritic and experimental at the same time. -Development of early arthritis and RF production.	[4, 43, 46, 47]
SKG	A mutation occurs in the SH2 region of the ZAP 70 protein, which is the result of an error in the selection of T cells, the mouse model is the BALB / c	ZAP-70^{W163C}	-Do not develop lymphadenopathy or lupus-like diseases. -Arthritis is significantly reduced when the mice are rendered TNF-α, IL-1 or IL-6 deficient.	-Develop RF and anti-CCP antibody. -Mutation in the SH2 domain of Zap 70. -Spontaneous arthritis.	[4, 43, 48]
TNF-α-transgenic	The animal strain used is C57BL / 6 in which an overexpression of the human TNF-α gene is generated	Tg197	Have impaired fertility and therefore it is very difficult to obtain offspring from this model.	-Increased TNF-α production. -May lead to the discovery of novel treatment and prevention alternatives. -Develops an erosive polyarthritis.	[4, 43, 49, 50]

CCP, cyclic citrullinated peptide; RF, rheumatoid factor; TNF, tumor necrosis factor; IL, interleukin; α, alpha; SKG, Sakaguchi; BL, black; ZAP 70, zeta-chain associated protein 70; SH_2, Src homology 2; GP130, glycoprotein 130; R, receptor; Ra, receptor antagonist; NOD, nonobese diabetic; TCR, T cell receptor.

Table 1. Genetic models for RA.

Cytokines often provide valuable information within the role of soluble factors that develop in RA. IL-1 β, TNF-α, IL-6, IL-15, and IL-18 are the most documented cytokines that play a regulatory role in RA, depending on the immune response or inflammatory processes.

The mice strains that are used for evaluation of some cytokines or regions of the TCR are knockout mice or transgenic mice, which have a deficiency of the molecule to be studied. Typically, these strains develop the CIA model more easily than other strains such as C57BL/6 [51].

In addition, different therapeutic targets such as chemokines, signaling molecules, and cellular trafficking can be evaluated in arthritis models like collagen-induced arthritis (CIA), streptococcal cell wall (SCW), adjuvant-induced arthritis (AIA), and models in chemokine-deficient knockout mice. On the other hand, immune response cells (NK, monocytes, etc.) are evaluated in K/BxN mice with CIA, which must be pre-stimulated with LPS to inhibit Fc receptors. Adhesion molecules, whose function resides in leukocyte trafficking, have been referred to be evaluated in SCW models with BALB/c mice and among the markers found are P-selectin, ICAM-1, and VCAM-1 [52].

For this model, K/BxN mice, which will jointly express the cell receptor T and the CMH II allele, are usually used. These mice typically develop no problem with a severe form of arthritis, when they are inoculated with serum antibodies due to high levels of GPI. Two mice are required to generate the model: one from the C57BL/6 strain that has the KRN and NOD/Lt transgene carrying the CMH II allele [53].

3.3. Environmental models

One marker that has been associated with RA is a low socioeconomic level, from which unhealthy diets are derived. It has recently been found that the consumption of certain vitamins and minerals from healthy food provides protection and reduces the effects of RA in patients [54, 55].

In animal models, the first link in which the association of the environment in arthritis was observed was when the HLA-B27 complex was evaluated in transgenic rats with spondyloarthritis. The rats did not develop the disease, so it was deduced that normal intestinal flora in B27 plays an important role [56]. Among the main environmental links with RA are smoking, the presence of infections, antibodies to rheumatoid factor, and anti-cyclic citrullinated peptides [57].

Cigarette smoke is strongly associated with RA, and during a study in 2011 in which mice of the DBA/1 J strain were in contact with condensed smoke, an increase in the induction of RA mediated in the CIA model. This strongly suggests that smoking may be an etiologic cause of this pathology [58].

4. Implication of hormones in animal models

Sex hormones are linked to RA because they function normally as inhibitors or suppressors of the immune response. Over the last few years, it has been speculated that the appearance

Animal model	Characteristics	Species	Limitations
Streptococcal cell wall arthritis	Peptidoglycans in the cell walls of bacteria (*Streptococcus* sp., *Lactobacillus* sp.) are responsible for inducing the model. Biphasic arthritis is generated, and it persists for several months. The model is generated in Lewis rats	Rat	The most important limitation is that the arthritis that is generated is monoarticular, and because the inflammation is generated by injecting intra-articularly, the systemic effects cannot be effectively evaluated
Passive transfer of CIA antibodies	The induction is performed by injecting type II collagen. The generation of specific antibodies for type II collagen is well characterized. Mice used are B-cell deficient and resistant to developing CIA	Mouse	It does not generate B- or T-cell responses, which are important factors to evaluate in RA despite having macrophages and polymorphonuclear cell infiltrates
Collagen-induced arthritis	This model is inducible in susceptible mice and rats; it is the gold standard and causes polyarthritis. Animals are inoculated with collagen II and coadjuvant; a re-restriction of MHC II is generated. The most commonly used strain of mice is DBA 1/J	Rat, mouse	Prior experience is required to perform the injection of the agents to evaluate intra-articularly. Arthritis characterized by polyarthritic does not meet all the characteristics, and results generated are variable. The arthritis that is triggered is acute
Immune complex-induced arthritis	Is generated for passive transfer of anti-isozyme antibodies in mice, which are injected into the knee with poly-L-lysine-lysozyme	Rat, mouse	The model does not comply with endogenous factors that are generated, and it is very difficult to resemble certain models such as CIA. Reactivity is generated by the presence of type II collagen and increases the production of factors that trigger inflammation
Spontaneous arthritis in knockout or transgenic mice	Mutations generated in the model are only generated in mice 4–5 weeks of age. Arthritis is mild and can be induced by serum transfer and induces changes in the MHC	Mouse	These models require specific environmental stimulants, which are very sensitive to the changes; therefore they are usually models with considerably high costs. Another limitation is that antibodies to anti-glucose-6-phosphate isomerase are generated that mostly cause pathogenic effects

RA, rheumatoid arthritis; CIA, collagen-induced arthritis; MHC, major histocompatibility complex.

Table 2. In vivo models for the study of RA.

of polymorphisms in genes encoding testosterone, progesterone, and androgens is what trigger an imbalance in the hormone complex with immune system and thus be associated with RA [59].

A higher prevalence of arthritis is present in females, but in murine models, it has been shown that for CIA and antibody-induced arthritis models, male mice and rats (CIA only) exhibit a higher prevalence. Castration of male mice produces a high prevalence of arthritis in the SCW model [60].

Another hormone that can also be a marker is cortisol secreted during periods of stress. In mice it has been shown to reduce the sensitivity to be induced in the CIA model; therefore, it usually confers some protection [61]. **Tables 2** and **3** summarize the in vivo models for the study of RA and the information of the genetic, immunological, and environmental animal models used in RA, respectively.

Characteristic	**Model**		
	Genetic	**Immunological**	**Environmental**
Model genetic background	Transgenic mice deficient in a specific gene to evaluate the effect of this in the murine model.	Arthritis is induced by transfer of antibodies in the serum to susceptible mice, transgenic mice and induced by adjuvants	When in contact with external pollutants, in the animal model, the induction of RA in the CIA model increases.
Advantages	-A specific genetic model allows studying how the disease is developed during prolonged periods and with possibility of repetition. -It allows knowing the functioning of the genes involved in RA.	Generates knowledge about several inflammation-related molecules and their implications in its signaling pathways.	This model gives the facility to know how the environmental influences the intestinal flora and the development of RA.
Year of development	In 1991 the first genetic model was developed, which was of mice that overexpressed the human TNF-α gene	The model of adjuvant arthritis was the first to be described in 1956	The main environmental risk factor for the development of arthritis is smoking and the first murine model of this factor was described in 1992.
Limitation	- Place of indeterminate integration. - Expensive model. -Variable gene expression	- Evaluation of a single gene at a time. - It is generated in inducible models and less in transgenic mice	- There are very few genes or alterations that are evaluated in this model for RA so it is almost obsolete.
Phenotypic expression	Transgenic mice	C57BL/6, K/BxN	DBA 1/J
Reference	[42, 62]	[51, 62]	[58, 63, 64]

RA, rheumatoid arthritis; CIA, collagen induced arthritis.

Table 3. Comparison of animal models for the study of RA.

5. Therapeutic strategies

Over the years, different RA therapies have been developed; one of the most representatives is TNF blockers, which, although effective, generate some notable side effects. The importance of animal models serves to generate the knowledge and evaluation of new therapies [52]. All these models are intended to induce inflammation and subsequent destruction of one or more peripheral joints in different animal species, with rodent species, such as mice and rats, being the most frequently used. The most common administration routes for evaluation of compounds in murine RA models are two, as shown in **Figure 2**. The most common administration route used for molecules including viral vectors such as lentivirus, retrovirus, and adeno-associated virus is intra-articular, which consists of administering a vector that contains a gene with regulatory effects on some mechanism of RA in the joint of the animal model being evaluated. Recently, murine models have been evaluated in which the viral vector is administered systemically (intravenously), and these have been able to reduce the inflammation generated by the disease as well as other clinical symptoms, without decreasing the effectiveness associated with the route of administration [52].

Some of the therapeutical goals of drugs, irrespective of their route of administration, include regularizing cytokine levels, since there is an overproduction of these, adjusting the expression levels of transcriptional products that cause inflammation and bone degradation, and decreasing chemokines and adhesion molecules, all of which are determinants of RA pathogenesis [52].

The models most used for the evaluation of exogenous substances that help reduce the disease are varied [65]; besides they depend on the animal model, as thus illustrated.

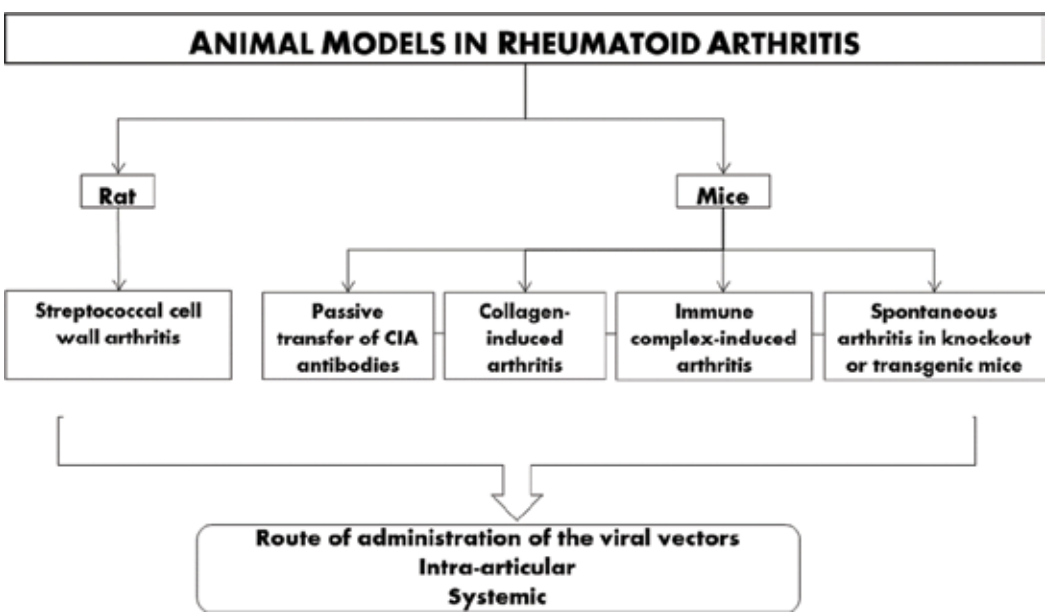

Figure 2. Animal models in RA. The models that are currently used for the generation of RA in mice and rats are varied and depend on the purpose. This can be generated by adjuvants with susceptible animals or spontaneously with genetically modified animals. There are several routes of administration which depend on the vehicle to be administered; for gene therapy the most used are the intra-articular and systemic routes.

5.1. Rat models

5.1.1. Streptococcal cell wall (SCW) arthritis

The SCW arthritis rat model is an experimentally induced inflammatory model with many features which resembles RA in humans. This model is used for studies of pathogenesis, therapy, and obtaining genetic knowledge in the acute and chronic phases of inflammation. Arthritis is induced in strains of rats that are susceptible (Lewis rat strain) and is performed by intraperitoneal injection of peptidoglycan polysaccharide polymers, which are obtained from the cell wall of *Streptococcus pyogenes* group A, D58 strain. This model representatively generates the severity of the arthritis and also generates granulomas in the liver, spleen, and peritoneum, granulomatous enterocolitis, and uveitis [66].

Commonly the streptococcal walls are formed by peptidoglycans (PG) bound to specific polysaccharides. These peptidoglycans have the function of triggering chronic arthritis, followed by a systemic response. Once these PGs are deposited in aqueous solution, three events may occur, which may be deposited in articular tissues. These may last a long time that stimulate the macrophages and pro-inflammatory mechanisms of T-cell activation [67].

The SWC model with Lewis rats (endogenous strain that is highly susceptible) is initiated after injection of the PGs in aqueous form. Once the inoculation takes place, it is necessary to observe the animals daily to verify the development of arthritis during the first 6 days, after this they can be checked every 2–3 days for at least 6–8 weeks. The acute response of this model develops from the first 48 hours to the next 10–21 days; finally, the chronic phase is triggered, which persists for several months in the animal model [67].

The conventional way of generating the model is using a systemic approach, which is obtained by intraperitoneal injection. But there are other alternatives that have been used in later years, such as injection of the cell wall intra-articularly. Strategic application will cause local inflammation with edema in 24 hours and after 3 weeks; the inflammation will be very little noticeable but will be accompanied by synovial infiltration of monocytes and remnant lymphocytes. The presence and importance of Th1 cells in the generation of arthritis for this inflammation model have been strongly suggested [68]. A single intraperitoneal administration of the cell wall is sufficient to generate arthritis and cause remission, although early experiments suggest that systemic administration was performed [69]. Among the main therapeutic targets to be evaluated in this model are direct components of the immune system such as cytokines. There are different studies in which inhibiting the IL-37 and P2X7 receptor, regulating the function of IL-10, and decreasing IL-21 production will produce resistance to joint inflammation when presented with this SCW model [70–72].

5.2. Mouse model

5.2.1. Passive transfer of CIA antibodies

The gold standard animal model for the study of RA is arthritis induced by collagen (CIA). The induction is performed by injecting type II collagen, which is the most predominant constituent

protein of the joint cartilage, and its induction with native collagen and adjuvants produces polyarthritis of a cross-immune response to the homologous collagen. The susceptibility of CIA is linked to the expression of certain complexes such as MHC II, which plays a primordial role in T cells [73].

The generation of antibodies specific for type II collagen is highly characterized. Mice used are B-cell deficient and resistant to developing CIA. The antibodies were shown to induce CIA in the DBA1/J mice strain although the arthritis generated is very mild [74].

For the generation of this AR model, it is necessary to obtain anti-collagen type II antibodies, which are obtained from a CIA model. These antibodies can be extracted from different biological matrices such as serum.

These antibodies are important for the generation of CIA pathogenesis. This transfer shows that arthritis is identical in mice with restriction of T cells. Several studies confirm that the development of the model depends on the joint response of B and T cells toward anti-CII antibodies [75].

The importance of knowing RF in RA is not well understood. It is believed to serve in the formation of immune complexes and in the formation of the complement system, which in turn attracts neutrophils to the site of inflammation [76]. The information is unclear to date, and arthritis is also believed to be induced by the binding of citrullinated peptide epitopes or by cross-reactivity [77].

This model is discussed in DBA1/J type mice, and since it is generated from a serum with antibodies in a CIA model, its targets are generally the same as IL-1β and TNF-α, although other therapies have also been discussed and approved, such as the inhibition of CTLA41g-stimulated T cells and the decrease in B cells with anti-CD20 [78].

5.2.2. Collagen-induced arthritis

CIA is an autoimmune disease that is generated in rodents that are usually genetically predisposed to present features very similar to arthritis, with these being immunized with type II collagen emulsified with complete Freund's adjuvant [34]. CIA in mice exhibits a restriction to MHC II in animals expressing H-2q and H-2r molecules [79]. Trentham et al. (1980), described the administration of type II collagen from chicken, emulsified in complete Freund's adjuvant (CFA) is capable of producing rat arthritis [80]. It was later described that this disease can also be induced in mice [81].

This experimental model of arthritis was designed and described primarily in rats, but currently a mouse strain with genetic predisposition to arthritis, strain DBA/1, is used [11]. Due to its restriction to MHC II, it is obvious that T lymphocytes play a prominent role in the autoimmune response, both in the production and regulation of pro-inflammatory cytokines and in the modulation of the B-cell response [79]. Activated T lymphocytes also activate macrophages to produce pro-inflammatory cytokines, TNFα, IL-1, and IL-6, among others, inducing the expression of chemokines and adhesion molecules with the consequent infiltration of polymorphonuclear cells, MN and formation of pannus.

Histologically, the CIA presents intense synovitis that correlates with the development of the disease. About 5 weeks after application of type II collagen, the animals develop a polyarthritis

similar to RA that occurs in humans. In general, there is fibrosis and ankylosis of the involved joints, and among the histological alterations, erosion of the subchondral bone and formation of pannus can be observed, also autoantibody formation.

All these immunopathological processes of CIA involve a response of T and B lymphocytes. The administration of type II collagen induces a strong activation of cooperating T lymphocytes, which stimulate B lymphocytes to produce antibodies against this protein, and later, a series of antibodies that will interact with cartilage constitutive proteins also activate the complement system. In the serum of arthritic animals, antibodies to different proteins have been detected, in addition to type II collagen [82].

The first antigenic determinants of type II collagen, in the case of DBA 1 mice, have been identified with the H-2q haplotype; the antigenic determinant is between amino acids (aa) 257 and 270 of the protein and in mice with the haplotype H-2r; it is between aa 442 and 456 [83].

Pro-inflammatory cytokines induce synovial cells such as chondrocytes, fibroblasts, and osteoclasts to produce MMPs and other effector molecules, all of which are responsible for cartilage degradation, bone erosion, and fibrosis [84].

B cells also play a key role in the development of CIA. It has been shown that the transfer of immune serum from arthritic mice to healthy mice induces severe inflammation, and although the antibody is no longer detected, the inflammatory response persists. This indicates that the humoral response is capable of triggering more factors that have an important role in establishing the autoimmune response in this model [77].

For the CIA model in rats, the therapeutic agents that have been evaluated are methotrexate and corticosteroids, and for biological targets, it is most common to evaluate the soluble receptor of TNF and IL-1, which have had effective results in decreasing the inflammation generated in the model [85].

CIA model but with mice which is currently the most commonly used with the DBA1/J strain because it has been shown that IL-1β boosts inflammation and perpetuates TNF-α, which have become the main therapeutic targets. Analogous collagen system peptides are currently evaluated for good results by reducing the symptoms of the disease in the murine model [86, 87].

The main route of administration for evaluation of therapeutic compounds, used for this model, is intra-articular; despite being the most used, it has caused controversy in its applicability when used in patients. Therefore, in recent years, efforts have been made to replace it by other means such as the system, which, despite being used recently, has shown favorable results.

5.2.3. Immune complex-induced arthritis (IC)

There are certain important events in RA as the formation of immunocomplexes. These immunocomplexes have been found in the synovium and joint cartilage of patients with RA [88]. The appearance of such complexes has been associated with the severity of the disease.

The most commonly used experimental models of RA, the antigen-induced arthritis, and the adjuvant-induced arthritis exhibit close resemblance to the pathophysiology of RA. However, they do not allow a precise investigation of the isolated contribution of IC to the development

of arthritis. In the case of articular lesions, it is important to note that there is no evidence of a significant increase in the prevalence of this type of disease on IC formation although the recruitment and activation of these cells in IC-induced lesions are considered essential to the full-blown development of the reaction [89].

This model has the advantage that its mechanism of action or effector molecules are generated in a simple way and knowledge of the pathogenesis of the disease is obtained quickly. Among the associated molecules are cytokines IL-1 and TNF-α, essential for the development of the disease, as well as macrophages, neutrophils, and mast cells [90, 91].

To generate the induction of RA, FcR, present in mast cells, neutrophils, and macrophages are very important. Activation of the complement pathway, mainly the C5a fragment, which functions as a chemoattractant factor, is also necessary [92, 93]. Also, innate components of the immune system often play an important role in the development of the CIA model. One example is the specificity of the K/BxN model, despite the omnipresence of autoantigen GPI [94].

In this model, the cationic retention principle is used in the passive transfer of anti-lysozyme antibodies in mice, which are injected into the knee with poly-L-lysine-lysozyme. This compound is large enough to be retained in the joints for a prolonged time and contributes to the joint destruction and chronicity of the disease produced by the model. The models with DBA/1 mice show a strong dependence on IL-1, and TNF blockade is usually very ineffective [95].

In this model, immunocomplexes will generate arthritis-like inflammation, which can be triggered in mice and rats (although in a lesser proportion). It is known that complement factors are usually a therapeutic target in this model and have recently added neutrophils, FcRy, and opsonic components which play a key role in RA inflammation [96].

5.2.4. Spontaneous arthritis in knockout or transgenic mice

Mice that are genetically modified will have various uses, such as the removal or introduction of genes for some receptor, cytokines, or other factors that help trigger immune mechanisms in the etiology of RA. Sometimes, spontaneous inflammation occurs, resulting in arthritis or another inflammatory disorder [52].

These models are much discussed at present, due to their ability to generate the disease, without the need to inoculate the animal with adjuvants, antibodies, or some external agent as in the CIA, SWC, and AIA models.

The K/BxN mouse (transgenic KRN T-cell receptor mouse on the background C57BL/6 x NOD) spontaneously develops chronic and progressive inflammation [97]. Clinically visible joint inflammation is observed from the third week of disease onset and thereafter evolves into a chronic and severe type of problem. T cells and B cells generate autoantibodies that promote the perpetuation of disease and joint destruction [98]. In addition to T-cell involvement, B cells secrete autoantibodies that promote joint destruction. Like arthritis with immunocomplexes or passive antibody transfer, and similarly to the CIA model, arthritis can be induced by serum transfer and induces changes in the MHC. Arthritis is mild and nondurable and requires persistence in the injection of serum with anti-CII antibodies. The fragment of Fc immunoglobulins plays an important factor in this model. The K/BxN model is thus an important tool to

study the role of antibodies in the development of RA [66]. This model alone demonstrates that a specific molecule or antibody is not required to generate arthritis. The receptor of the T cells of the KRN strain recognizes the GPI and, on the other hand, the MHC [99]. This immune recognition of G6PI gives rise to autoantibodies to the isomerase which, when purified, can transfer disease. The relevance of this reactivity to the etiology of RA is unclear. Recognition of G6PI is first obtained by antibodies, and subsequently these can be transferred, although the relevancy of this discovery does not really generate knowledge toward RA.

The transgenic T-cell receptor of the krn strain was originally generated to recognize a bovine-type antigen; its discovery was totally serendipity. Different authors have performed different series of studies to define the mechanism of action, the effector phases, as well as the evaluation of cells involved in this animal model of RA [99].

On the other hand, immunization with G6PI induces an inflammatory arthritis that is dependent on T cells in mice that have not been studied, generating clues about the knowledge and use of such a model [100].

In this model, arthritis is produced by transfer of serum antibodies from a previous arthritis model; mice are genetically modified to develop RA when it is induced. The targets in this model are cytokines and chemokines. The absence of these molecules is analyzed to verify if they generate protection or induction of RA and additional information such as the signaling pathway that it alters. The TRANCE/RANKL factor was one of the most used for evaluation of the NF-kB pathway with TNF [101]. The phenotypic characteristics of the major animal models are shown in **Figure 3**.

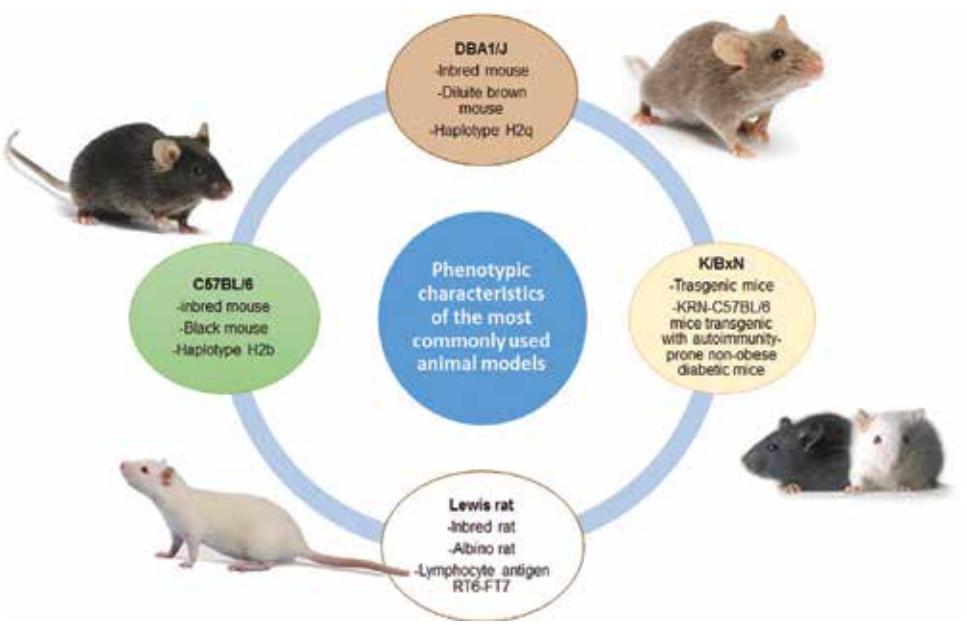

Figure 3. Phenotypic characteristics of animal models. There are different strains of mice and rats used as models for the evaluation of RA; among them the most representative strains are Lewis, DBA 1/J, C57BL/6, and K BxN, which respond to different genotypic and phenotypic characteristics.

6. Future prospect

Animal models, as we have already commented, still are the strongest link to evaluate therapeutic compounds, at least in autoimmune diseases, such as rheumatoid arthritis. In general, each model provides different information about the disease, and it is suggested that for more reliable results, the evaluated compounds have been tested in two different models to have a little more certainty of the effects generated by the molecules that are determined in each experiment.

Efforts are currently directed to find a model that can more accurately reproduce the symptoms and signs of RA taking the security of continuing to obtain reliable results. Another strategy is that the route of administration is as aggressive and invasive as possible but reproduces precise results, so other routes are being evaluated such as systemic to thus discontinue intra-articular injection.

These studies are carried out with the aim of using as few animals as possible and, in turn, with the least suffering, due to the techniques to inoculate the therapeutic agent to be evaluated, in addition to the fact that the population decline does not interfere in the test results.

7. Conclusion

Despite decades of research to eradicate the disease, RA remains one of the most prevalent diseases within its scope and even with a well-defined and unknown etiology. Currently, animal models continue to be an effective and necessary tool for the generation of knowledge on most autoimmune diseases including RA. Currently the animal models are still the main link to understand how the immune system attacks its own components (cells or organs), as well as the evaluation of molecules with therapeutic objectives.

Animal models, inducible (CIA models, passive antibody, or streptococcal wall transfer) or spontaneous (knockout mice), have been shown to be useful in understanding unknown processes. For spontaneous models, it is difficult to perceive the mechanism by which the disease in which the therapeutic compounds are evaluated is triggered. In the same way, no animal model shows all the characteristics of the disease and the route by which the therapeutic agent is introduced can generate variation in the results, so this information should be complementary with the evaluation of more routes of administration. It is envisaged that the generation of new molecular techniques will help to determine the complexity of the functioning of RA as well as to evaluate the different routes of administration of therapeutic compounds pending the attainment of low toxicity and wide benefit therapies that are determined and used for clinical phases.

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Chapter 14

Animal Models for Chronic Stress-Induced Oxidative Stress in the Spleen: The Role of Exercise and Catecholaminergic System

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Abstract

We examined the effects of daily exercise on the gene expression of catecholamine biosynthetic enzymes (tyrosine hydroxylase (TH), dopamine-β-hydroxylase (DBH), and phenyl ethanolamine N-methyltransferase (PNMT)), vesicular monoamine transporter 2 (VMAT 2), antioxidant enzymes (superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx)), concentrations of catecholamines (noradrenaline (NA) and adrenaline (A)) and malondialdehyde (MDA), activities of monoamine oxidase (MAO), and antioxidant enzymes in the spleen of chronically psychosocially stressed rats. Exposure of chronically stressed rats to exercise increased the levels of PNMT protein by 19%, VMAT 2 mRNA by 100%, NA by 160%, and A by 140%; decreased/unchanged MAO enzyme activity; returned concentrations of MDA to control level; and increased CAT and GPx mRNA levels (50% and 150%, respectively). Exercise induced the accumulation of the catecholamines and a decrease of stress-induced oxidative stress in the spleen, which may significantly affect the immune-neuroendocrine interactions in stress conditions. Also, exercise induced the catecholaminergic system and antioxidant defense to become more ready to a novel stressor, which indicates that exercise may induce potentially positive physiological adaptations. Our combined model of chronic social isolation and long-term daily treadmill running in rats may be a good animal model in the research of therapeutic role of exercise in human disease caused by chronic stress.

Keywords: treadmill running, chronic social isolation, catecholamine, antioxidant enzymes, spleen, rats

1. Introduction

Many studies have shown that stress disturbs homeostasis, which induces various disorders. A number of diseases and pathological conditions are related to the long-term adaptive response to stress, particularly under conditions of chronic stress when allostasis can shift from a healthy toward a pathological state [1]. Chronic stress induces behavioral, endocrine, and immune changes in animals [2, 3]. It is known that stress affects a rapid rise of plasma and tissue catecholamines, including the spleen [4]. Data from literature indicate that the sympathetic nervous system (SNS) is one of the major pathways involved in immune-neuroendocrine interactions. The regulation of immunity by sympathetic noradrenergic nerve fibers in the lymphoid organs has been demonstrated by the distribution of tyrosine hydroxylase (TH) nerve fibers, by the presence of adrenoreceptors on the immune system cells, and by immunomodulatory role of noradrenaline (NA) [5]. For example, adrenaline (A) and NA produced by sympathetic nerves may modulate cellular function by acting on β-2 adrenergic receptors of B and Th1 cells [6]. In addition, catecholamine biosynthetic enzymes are expressed in the lymphoid organs [7], as well as in neutrophils and macrophages [8]. It is known that normal catecholaminergic turnover results from balance among synthesis, release, and reuptake of catecholamines. Because of the significant role of catecholamines in neuroendocrine-immune network in stress response, detection of regulatory mechanism for catecholamine synthesis, degradation, release, and reuptake in the spleen in conditions provoked by chronic stress is exceptionally relevant in stress biology, due to its potentially negative impact on immune functions and health. Effective management of stress depends on the ability to identify and quantify the effects of various stressors and determine if individual or combined stressors have distinct biological effects [9]. Animal models have contributed considerably to the current understanding of mechanisms underlying the role of stress in health and disease [10]. It is known that animal model of chronic stress isolation (CSI) produces increased concentrations of catecholamines in the plasma and decreased gene expression of catecholamine biosynthetic enzymes in the spleen, which can modulate the immune function [11]. However, very little is known about the impact of long-term exercise on the catecholaminergic turnover and the antioxidant defense system in the spleen of chronically psychosocially stressed rats. Because of the potential therapeutic role of physical exercise, we investigated whether a combined animal model of chronic isolation and treadmill running in rats (CSITR) may be a good animal model for chronic stress research as well as the benefits of exercise on neuroendocrine and immune functions in stress conditions. Our CSITR animal model was achieved by exposing the individually housed rats to the daily treadmill running for a 12-week period. We opted for long-term daily treadmill running because the short intensive physical activity may induce oxidative stress, while it is not the case with sport-specific activity of longer duration [12]. In addition, we exposed the experimental animals to additional acute immobilization stress, because we wanted to examine whether daily treadmill running induced potentially positive adaptations of the splenic catecholaminergic turnover and antioxidant protection in stress conditions.

We investigated how long-term daily 20 min treadmill running affected the gene expression of key enzymes involved in catecholamine biosynthesis (TH, dopamine-β-hydroxylase (DBH) and phenylethanolamine N-methyltransferase (PNMT)), storage (vesicular monoamine

transporter (VMAT) 2) and degradation (monoamine oxidase (MAO)), as well as the concentrations of catecholamines (NA and A) in the spleen of chronically psychosocially stressed adult rats. Transcription factor cAMP response element-binding protein (CREB) plays a major role in regulation of TH and DBH gene expression during exercise [13]. This chapter discusses the effect of physical exercise on the level of CREB mRNA in the spleen of chronically stressed rats. As the rise in catecholamine catabolism results in increased reactive oxygen species (ROS) production, we measured the concentration of malondialdehyde (MDA), as well as gene expression and activity of the antioxidant enzymes (superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx)). Also, we examined how the additional acute immobilization stress changed the mentioned parameters. In the study, we presumed that physical exercise in chronically stressed rats may induce the potentially positive physiological adaptations of the splenic catecholaminergic turnover, as well as antioxidant protection and oxidative damage repair.

Detecting regulatory physiological mechanism by which physical activity changes catecholaminergic turnover and antioxidant defense system in the spleen in conditions provoked by chronic stress is important in the prevention of immune diseases caused by chronic stress. Also, these results may confirm whether CSITR could be a good animal model in the search for beneficial impact of exercise on neuroendocrine and immune functions in stress conditions.

2. Animal model of chronic social isolation in rats

Many authors have confirmed that animal models are essential to biological research. Chronic individual housing of rats, frequently termed "isolation stress," represents a very strong psychosocial stress [3, 14], which can induce neuroendocrine changes [15] and increased activity of the antioxidant defense system [16, 17] in animals. Also, isolation stress affects different behavioral processes in animals. For example, social isolation led to a reduced duration of grooming and a prolonged latency period to the onset of grooming [18]. In addition, social interactions are an important source of human stress. Social isolation has deleterious effects on health and therefore is regarded as one of the most relevant causes of diseases in mammalian species [14]. For example, it is a risk factor for human depression [19].

Animal models of chronic social isolation (CSI) consisted of 11-week-old Wistar male rats that were subjected to social isolation, with a single animal per cage for 12 or 3 weeks [11, 15]. The visual and olfactory communication among the isolated rats was reduced to the minimal level.

It is known that exposure of an organism to a social isolation leads to the engagement of several hormonal and neurotransmitter systems in the stress response. Chronic social isolation of adult rat males produces a depletion of brain catecholamine stores but no changes in heart auricles and adrenal glands [15]. In addition, CSI of adult rat males decreases the gene expression of catecholamine biosynthetic enzymes in the adrenal medulla [20] and increases concentrations of catecholamines in the plasma [11]. Also, CSI induces an increase in the gene expression of noradrenaline biosynthetic enzymes in stellate ganglia, which may be connected to the increased

risk of cardiovascular diseases [21, 22]. In addition, the gene expression of splenic catecholamine biosynthetic enzymes is decreased after CSI. This might reduce catecholamine synthesis in the spleen and deplete the immunocompetent tissues of catecholamines which cause an impairment of immune response [11]. Key question in adaptive response to stress is how the addition stressor can elicit a variant or altered response depending on prior experience with the current or different stressor. A potentiation of the sympathoadrenal system activity in socially isolated rats upon exposure to novel acute stressors has been reported [23]. The additional acute immobilization does not affect the gene expression of catecholamine biosynthetic enzymes in both auricles of long-term socially isolated rats. This suggests that the response to stress depends on prior experience with stressors [24]. Data from literature indicate a possible adaptation of catecholamine-synthesizing system at the level of gene expression in the heart auricles of chronically socially isolated rats exposed to acute immobilization stress [24]. However, protein levels of catecholamine biosynthetic enzymes in both ventricles of socially isolated rats increased after additional acute stress [25]. With regard to the role of cardiac catecholamines in physiological and pathophysiological processes, it could be hypothesized that increased catecholamine synthesis in the ventricles after acute stress indicates sensitivity of the heart to subsequent stress [25].

It could be concluded that animal model of chronic social isolation (CSI) in rats is a good animal model in the research of neuroendocrine and immune functions in stress conditions. Also, the described results indicate the potential application of CSI animal models in understanding of stress in humans.

3. Animal model of long-term treadmill running in rats

Physical exercise produces modulation of neuroendocrine and immune functions [26] and increases the activity of the antioxidant defense system [27]. Long-term treadmill running in rats is forced exercise which has the propensity to induce both psychological and physical stress [28].

Long-term treadmill running animal model (TR) consists of 11-week-old Wistar male rats that are exposed to long-term treadmill running. Long-term treadmill running is achieved by the rats' daily running on the treadmill for a period of 12 weeks. The treadmill running intensity is gradually increased from week to week and from the initial 10 min—10m/min up to 20 min—20m/min at 0^0 incline [22, 29, 30]. Animals are being exposed to treadmill training 5 days a week for 12 weeks [22].

Treadmill running may induce physiological adaptations, which can be reflected in increased plasma catecholamine concentration, as well as in the change of the synthesis of catecholamine biosynthetic enzymes in rats [31]. It is a very strong stressor, which activates the sympathoadrenomedullary system and increases the synthesis of splenic PNMT protein catalyzing the conversion of NA to A, which both can modulate the immune functions [31]. It is known that cardiovascular diseases, such as hypertension and heart failure, are often associated with sympathetic nervous system overreactivity [32, 33]. The increase of the noradrenaline biosynthetic enzyme expression in stellate ganglia, which causes the increase of plasma NA levels, due to chronic forced running, may play a role in the growing risk for cardiovascular diseases [22, 34].

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It could be concluded that TR shows adaptations that are indicative of chronic stress and that this animal model in rats is good for the study of neuroendocrine and immune functions in stress conditions.

4. Combined animal model of chronic social isolation and long-term daily treadmill running in rats and "cross stressor adaptation hypothesis"

Data from literature confirm that exercise has been widely used in the last years with therapeutic and preventive purposes in a series of pathophysiological conditions. Exercise training reduces the risk of developing diseases related to chronic stress. For example, a physically active lifestyle is associated with decreased risks of coronary heart disease and high blood pressure [35]. In addition, in humans, regular exercise has a beneficial impact on depression [36]. It is known that the theory of "cross stressor adaptation hypothesis" suggests that exercise training, as a stressor on the body, may alter responsiveness to other types of stressors [37]. Mueller [38] suggests that exercise training appears to reduce sympathoexcitation to a variety of centrally mediated sympathoexcitatory stimuli. Reduction in sympathoexcitation may contribute, in part, to the reduced incidence of cardiovascular disease in physically active individuals [38]. In addition, physical activity prevents splenic NA depletion, or spillover, typically observed in sedentary rats following periods of intense sympathetic drive [39]. Also, physical activity may prevent stress-induced suppression of splenic immunity by reducing sympathetic drive to the spleen during stress [40, 41].

Treatment of chronic social isolation and long-term daily treadmill running (CSITR) consists of exposing the individually housed Wistar male rats to the daily treadmill running during 12 weeks [42].

Understanding the mechanisms by which CSITR training alters control of the SNS in health and disease could be important for developing new strategies in the prevention and treatment of cardiovascular diseases. Treadmill exercise leads to a decreased gene transcription of catecholamine biosynthetic enzymes in stellate ganglia in stressful conditions. This may suggest the beneficial effects of treadmill exercise on cardiovascular system in stressed animals [22].

5. Animal model of acute immobilization stress in rats

Immobilization is a standardized procedure frequently used as an additional acute stressor and is considered as one of the most intensive stressors that significantly changes gene expression [43]. It is known that immobilization results in well-characterized catecholamine responses [44]. In this model, animals were restrained in a prone position on a board for periods of 120 min [45]. The head was restricted from movement by a metal loop over the nose, and the feet were taped to raise supports with bandage tape [45].

It is known that the acute immobilization stressor (IMM) triggers an exaggerated elevation of the plasma catecholamines [42]. One of the key questions in adaptive response to stress is how the additional acute stressor can provoke a variant or altered response depending on prior experience with the current or a different stressor [43]. Additional acute immobilization increases plasma catecholamines in animals previously exposed to chronic social isolation (CSI+IMM) [46] and in animals previously exposed to long-term treadmill running (TR+IMM) [34]. This could mean that prior experience may condition physiological systems to "expect" a problem and, therefore, be more ready to respond to a novel additional acute stressor [43]. In addition, immobilization stress more significantly elevates TH and DBH protein levels in the stellate ganglia in rats previously exposed to long-term treadmill running (TR+IMM). Continuous accumulation of their proteins is an adaptation on applied stress regime [34]. Also, chronically stressed rats exposed to novel stressors exhibit exaggerated responses in gene expression of PNMT enzyme catalyzing conversion of NA to A [34]. The increased release of A from stellate ganglia during the additional acute immobilization in rats previously exposed to long-term treadmill running may be caused by the increased synthesis of PNMT. Increased levels of PNMT enzyme in stellate ganglia may have pathophysiological impact, especially on the cardiovascular system, since A is a powerful β2 adrenergic receptor agonist [34]. Also, the heterotypic novel additional acute immobilization stressor elevates the plasma catecholamines but not excessively in the animals previously exposed to CSITR [42]. This finding might be explained by the quality and especially by the intensity of the stressor used. The novel stressors elicit exaggerated responses in prestressed animals, when the novel stressor is of equal or greater intensity or duration and/or it is repeated [43]. Animals exposed to CSITR are already prepared to manage the new situation evoked by a novel stressor, and the exaggerated response is not necessary [42]. Animals exposed to CSITR treatment have statistically more significant expression of TH, DBH, and PNMT genes in the adrenal medulla after additional acute immobilization stress compared with the animals exposed to acute immobilization stress [42]. The increased catecholamine synthesis in the adrenal medulla of chronically stressed animals after additional acute stress is an important adaptive phenomenon of the sympathoadrenomedullary system in rats [43].

6. Other animal models

6.1. Chemical manipulation

Data from literature indicate that chronic stress produces the activation of SNS and hypothalamus-pituitary-adrenal (HPA) axis. In our previous study, we found that exposure of rats to daily treadmill running increased plasma concentrations of NA, A, and ACTH and decreased CORT concentration [34]. It is known that stress hormones via adrenergic and glucocorticoid receptors of immune cells inhibit secretion of the proinflammatory cytokines, such as interleukin 1 beta (IL-1β), interleukin 6 (IL-6), tumor necrosis factor alpha (TNF-α), and interferon-y (INF-γ), while promoting the secretion of the anti-inflammatory cytokines, such as interleukin-4 (IL-4), interleukin-10 (IL-10), and interleukin-13 (IL-13) [47]. Lasting stress exposure induces HPA "fatigue," glucocorticoid resistance, nuclear factor kappa B (NF-Kb)

activation, and negative feedback, which in turn promote the proinflammatory cytokines [47]. The increased proinflammatory cytokines ultimately cause inflammation, which may induce various diseases [47]. For example, the proinflammatory cytokines alter the metabolic processes of neurotransmitters [48], whose secretion suppression and the reuptake block activity play a role in the pathogenesis of depression [47].

6.2. Genetically modified models

During oxidative stress high concentrations of ROS modify nucleoside triphosphates which are incorporated into the DNA during DNA synthesis and may give rise to mutations. Mutations in the genes of regulatory enzymes, transporters, and receptors of the neurotransmitters in the central nervous system (CNS) have been associated with aggression [49].

6.2.1. Target genes

Mutation in human MAO A gene is associated with impulsive aggression in male humans [50], juvenile delinquency [51], impulsivity [52], and female panic and depressive disorders [53, 54]. In addition, mutations in the TH and DA receptor 4 genes influence impulsivity [55–57], and polymorphism in the glutamate transporter (VGLUT) gene is significantly associated with increased "aggression to strangers" [58]. Polymorphism in the tryptophan hydroxylase (Tph2) gene as a causal factor in 5-hydroxytryptamine (5-HT) deficiency is associated with depression [59].

6.2.2. Transgenic mouse/rat models

Genetically modified mouse and rat models are used in the research of human diseases. It is known that the low activity of MAO A enzyme consequently increases catecholamine levels [49]. Reduced levels of the MAO A enzyme, as well as increased NA levels, were observed in aggressive men [49]. MAO A knockout mice showed increased aggression in adulthood [60] and for this reason were used in the research of behavioral disorders.

7. Materials and methods for studying the protective role of exercise against deleterious effects of oxidative stress

7.1. Animal models

Eleven-week-old Wistar male rats were maintained under standard laboratory conditions with water and food ad libitum and kept three to four per cage [25]. The care was taken to minimize the pain and discomfort of the animals according to the recommendations of the Ethical Committee of the Vinča Institute of Nuclear Sciences [25], Belgrade, Serbia, which follows the guidelines of the registered "Serbian Society for the Use of Animals in Research and Education." Animals were divided into four groups in accordance with our previous protocol [42]. The **control group** (n = 10) was not exposed to stress. The animals in **CSITR group** (n = 10)

were exposed to chronic combined social isolation and treadmill running. CSITR was achieved by exposing the individually housed rats to the daily treadmill running during 12 weeks [42]. Chronically stressed animals were exposed to treadmill training ran on the treadmill 5 days a week for 12 weeks [22]. During that period, the exercise time was increased from 10 min to 20 min/day and treadmill speed gradually increased from 10 to 20 m/min by the end of the second week, without incline [22]. The animals ran according to the protocol for 10 additional weeks (20 min/day at a speed of 20 m/min, 5 days a week) [22]. The treadmill training protocol used in our studies involved a gradual increase in running intensity and is commonly used in the similar studies [29, 30]. Animals were exposed to a low-intensity treadmill training [31], which is in accordance with the protocol of Erdem et al. [30] who suggested that low exercise intensity is a key factor in the effect of submaximal endurance training on adrenomedullary catecholamine biosynthesis. During the exercise, we monitored the animals continuously. In **IMM group** (n = 10), the animals were exposed to acute stress immobilization, for a period of 2 hours [42]. Immobilization stress was elicited as described by Kvetnansky and Mikulaj [44]. In **CSITR+IMM group** (n = 10), the animals were exposed to CSITR during 12 weeks, and after CSITR, these animals were exposed to additional acute IMM stress for 2 hours [42]. The animals were sacrificed 3 hours after the acute immobilization. Data from literature show that 3 hours after the acute immobilization, changes in gene expression of catecholamine biosynthetic enzymes in the peripheral tissues are expected [43, 61]. To confirm the presence of oxidative stress in chronically stressed animals, we have introduced a **CSI** group. **CSI group** (n = 10) consisted of animals exposed to treatment of chronic social isolation for a period of 12 weeks. The rats were individually housed. The visual and olfactory communication among the isolated rats was reduced to the minimal level. In this group we measured the concentration malondialdehyde (MDA) in the spleen. Measurement of MDA is widely used as an indicator of lipid peroxidation. Increased levels of lipid peroxidation products have been associated with a variety of chronic diseases. The spleens were rapidly dissected and frozen. To avoid potentially confounding acute effects of exercise, animals were sacrificed 48 hours after the last training session, which is in accordance with protocol of Gavrilović et al. [31].

7.2. Spleen tissue homogenization, RNA isolation, and cDNA synthesis

Total RNAs were isolated from 0.08 g spleen tissues by using TRIZOL reagent (Invitrogen, USA) as described previously by Gavrilović et al. [31]. Reverse transcription was performed using Ready-To-Go You-Prime First-Strand Bead (Amersham Biosciences, UK) and pd $(N)_6$ Random Hexamer (Amersham Biosciences, UK) primer according to the manufacturer's protocol, which is in accordance with protocol of Gavrilović et al. [31].

7.3. Quantitative real-time PCR

TH, DBH, PNMT, CREB, VMAT2, CuZn SOD (SOD1), Mn SOD (SOD2), CAT, and GPx mRNA levels were quantified by quantitative real-time RT-PCR as described previously by Gavrilović et al. [42]. TaqMan PCR assays were carried out using Assay-on-Demand Gene Expression Products (Applied Biosystems, USA) for TH (Rn00562500_m1), DBH (Rn00565819_m1), PNMT (Rn01495589_g1), CREB (Rn01441386_g1), VMAT2 (Rn00564688_m1), SOD1 (Rn00566938_m1),

SOD2 (Rn00690587_g1), CAT (Rn00560930_m1), and GPx (Rn00577994_g1). The reference gene (endogenous control) was included in each analysis to correct the differences in the inter-assay amplification efficiency, and all transcripts were normalized to cyclophilin A (Rn00690933_m1) expression [31]. The results are reported as a fold change relative to the calibrator and normalized to cyclophilin A as previously described [31].

7.4. Spleen tissue homogenization and measurement of protein concentration

The spleens were homogenized in 0.05 M sodium phosphate buffer (pH 6.65). Subsequently, the protein concentration was determined using bicinchoninic acid (BCA) method (Thermo Scientific Pierce, USA), described by Stich [62].

7.5. Western blot analysis

The TH, DBH, and PNMT proteins were assayed by Western blot analysis as described previously by Gavrilović et al. [42]. Antibodies used for quantification of proteins were for TH the monoclonal primary antibody against mouse TH (monoclonal antibody against TH from mouse-mouse hybrid cells, clone 2/40/15, dilution 1:5000, Chemicon International, USA); for DBH the anti-dopamine-ß hydroxylase (N-terminal) antibody, sheep (dilution 1:5000, Sigma, USA); for PNMT the polyclonal ant-PNMT primary antibody, rabbit (dilutation 1:1000, Protos Biotech Corporation, USA); and for β-actin the rabbit polyclonal anti-β-actin (ab8227, dilutation 1:5000, Abcam, USA) [31]. After that, the membranes were incubated in the secondary antimouse, anti-rabbit (dilution 1:5000, Amersham ECL™ Western Blotting Analysis System, UK) and anti-sheep (dilution 1:5000, Calbiochem, Germany) antibodies conjugated to horseradish peroxidase [31]. A secondary antibody was then visualized by the Western blotting enhanced chemiluminescent detection system (ECL, Amersham Biosciences, UK) [31]. The result was expressed in arbitrary units normalized in relation to β actin, which is in accordance with protocol of Gavrilović et al. [31].

7.6. Concentrations of catecholamines

Spleen tissues were homogenized in 0.01 N HCl in the presence of EDTA and sodium metabisulfite. Catecholamine concentration in spleen fractions was determined using 3-CAT Research ELISA kits (Labor Diagnostica Nord, Nordhorn, Germany) according to the manufacturer's protocol. Absorbance was determined at 450 nm using a microplate reader (Stat Fax 2100). Concentrations were normalized to 1 g of tissues in homogenate. Values were expressed as ng of catecholamine per g of tissues.

7.7. Monoamine oxidase enzyme activities

Determination of MAO A and MAO B activity was performed using the Amplex Red Monoamine Oxidase Assay (A12214, Molecular Probes, USA), described by Zhou and Panchuk-Voloshina [63]. This assay is based on the detection of H_2O_2 in a horseradish peroxidase-coupled reaction using N-acetyl-3, 7-dihydroxyphenoxazine (Amplex Red), a highly sensitive and stable probe for H_2O_2. Fluorescence was measured with a fluorometer using

excitation at 560 ± 10 nm and fluorescence detection at 590 ± 10 nm. Monoamine oxidase activity was expressed as U/mg of protein.

7.8. Malondialdehyde measurement

Malondialdehyde concentration in the spleen fractions was determined using Spectrophotometric Assay for Malondialdehyde BIOXYTECH® MDA-586 (OXIS Health Products, Inc., USA) according to the manufacturer's protocol. The MDA-586 method is based on the reaction of a chromogenic reagent, N-methyl-2-phenylindole, with MDA at 45°C. Malondialdehyde concentration was expressed as μM/mg of protein.

7.9. Antioxidant enzyme activities

SOD, CAT, GPx, and GR activities were determined using methods previously described by Stojiljković et al. [64]. Determination of total SOD activity was performed using Oxis Bioxytech SOD-525 Assay (Oxis International, Inc., Portland, OR, USA). CAT activity was determined by the method of Beutler [59], and GPx activity was assessed using the Oxis Bioxytech GPx-340 Assay (Oxis International, Inc., Portland, OR, USA). The final result for enzyme activity was expressed as units per milligram of protein (U/mg).

7.10. Data analysis

The data are presented as means ± S.E.M. Differences of gene expression (mRNA and protein levels) of catecholamine biosynthetic enzymes (TH, DBH, and PNMT); levels of CREB, VMAT 2, SOD 1, SOD 2, CAT, and GPx mRNA; concentration of NA, A, and MDA; as well as enzyme activities (MAO A, MAO B, total SOD, CAT, and GPx) in the spleen were analyzed by one-way ANOVA. The effects of CSITR and IMM compared to control animals, as well as the effects of CSITR+IMM compared to CSITR, were tested by Tukey post-hoc test. Statistical significance was accepted at $p < 0.05$.

Correlations of mRNA levels, protein levels, hormone levels, and enzyme activity were analyzed by the Pearson test, using the Sigma Plot v10.0 (with SigmaStat integration).

8. Results

8.1. Changes of the TH, DBH, PNMT, CREB, and VMAT 2 mRNA levels and TH, DBH, and PNMT protein levels in the spleen

The animals exposed to CSITR showed a decreased level of TH mRNA by 22% ($p < 0.05$, Tukey test, **Figure 1a**), DBH mRNA by 11% ($p < 0.05$, Tukey test, **Figure 1b**), PNMT mRNA by 29% ($p < 0.05$, Tukey test, **Figure 1c**), CREB mRNA by 69% ($p < 0.01$, Tukey test, **Figure 1d**), and increased levels of VMAT 2 mRNA by 100% ($p < 0.01$, Tukey test, **Figure 1e**) and PNMT protein by 19% ($p < 0.05$, Tukey test, **Figure 2c**), whereas levels of TH and DBH protein (**Figure 2a** and **b**) were unchanged compared with the controls.

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IMM stress does not change significantly gene expression of catecholamine biosynthetic enzymes (**Figures 1a–c** and **2a–c**) and levels of VMAT 2 mRNA (**Figure 1e**) 3 hours after immobilization. However, the additional exposure of CSITR animals to acute immobilization stress led to increased levels of PNMT protein by 33% ($p < 0.05$, Tukey test **Figure 2c**) and VMAT 2 mRNA by 100% ($p < 0.01$, Tukey test, **Figure 1e**) 3 hours after immobilization.

8.2. Changes of the NA and A concentrations in the spleen

CSITR significantly increased the spleen concentrations of NA by 160% ($p < 0.01$, Tukey test, **Figure 3a**) and A by 140% ($p < 0.01$, Tukey test, **Figure 3b**), compared with control animals. The significant positive correlation was found between the levels of PNMT protein and A concentration in the spleen of animals exposed to CSITR (Pearson $R = 0.631$, $p < 0.05$, **Figure 4a**).

The exposure of the control animals to acute immobilization stress significantly increased NA concentration by 250% ($p < 0.01$, Tukey test, **Figure 3a**) and A concentration by 240%

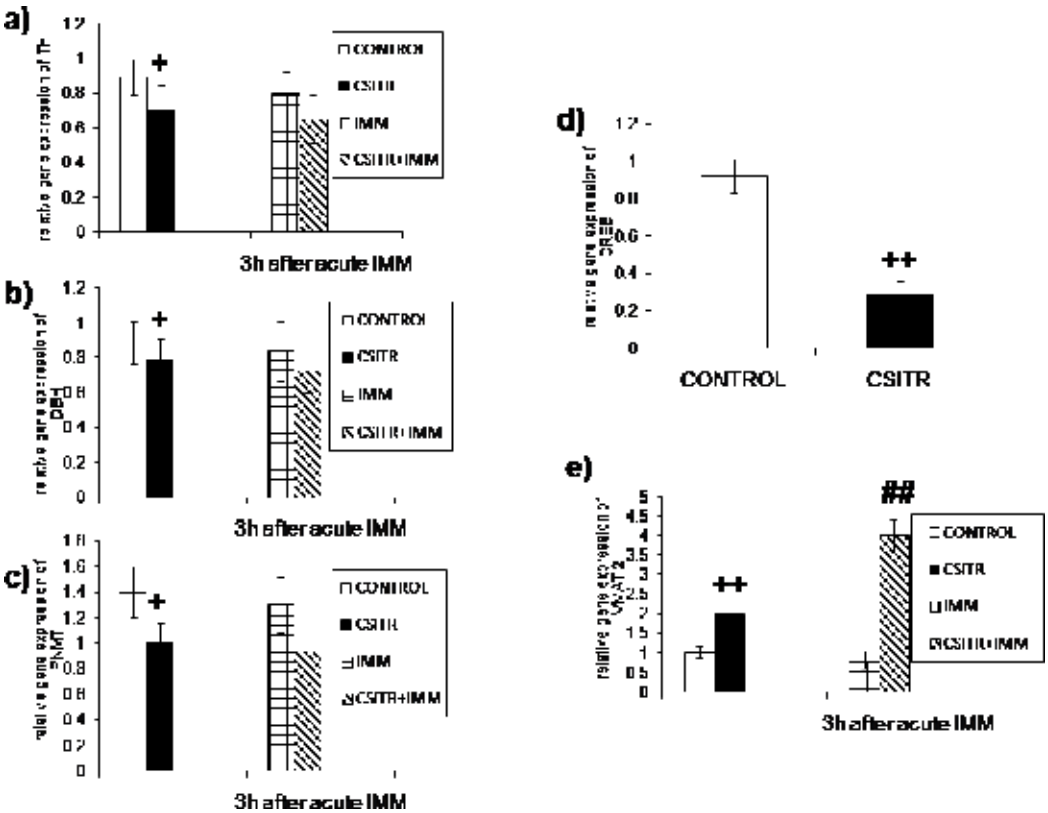

Figure 1. Effects of CSITR and CSITR+IMM models on tyrosine hydroxylase (TH) [a], dopamine-ß-hydroxylase (DBH) [b], phenylethanolamine N-methyltransferase (PNMT) [c], cAMP response element binding (CREB) [d], and vesicular monoamine transporter 2 (VMAT2) [e] mRNA levels in the spleen. Data are shown as mean ± SEM of 10 rats. Symbols: +$p < 0.05$, ++$p < 0.01$ CSITR animals compared to control animals (Tukey test) and ##$p < 0.01$ CSITR+IMM animals compared to CSITR animals (Tukey test).

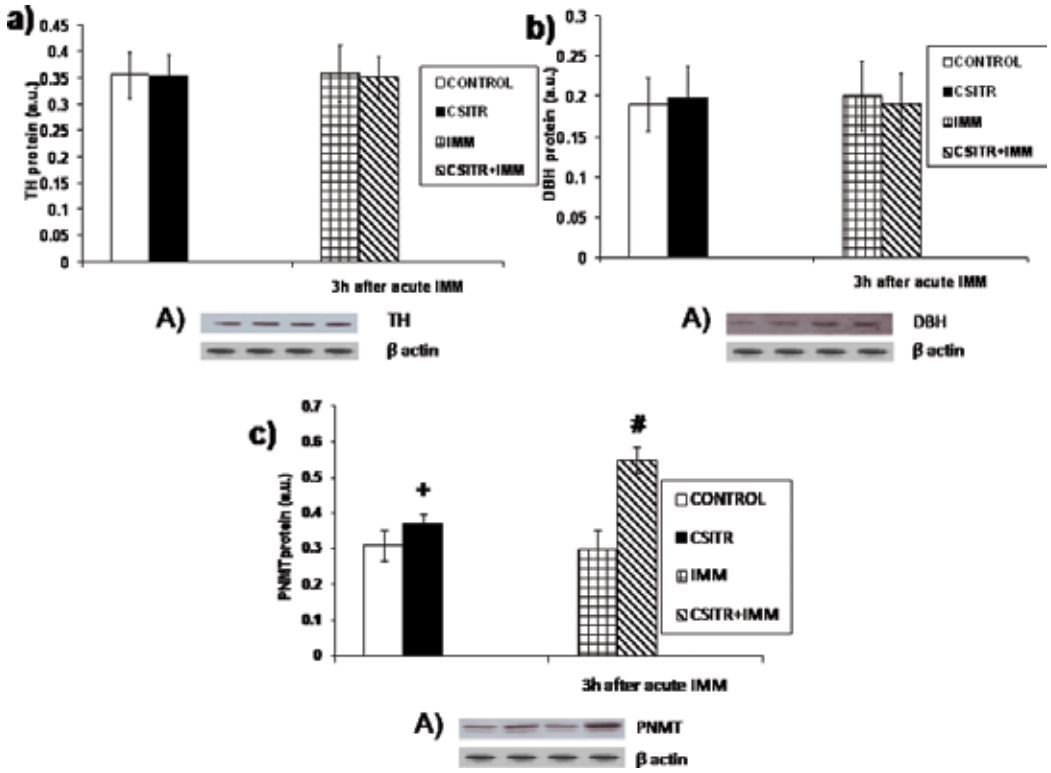

Figure 2. Effects of CSITR and CSITR+IMM models on tyrosine hydroxylase (TH) [a], dopamine-ß-hydroxylase (DBH) [b], and phenylethanolamine N-methyltransferase (PNMT) [c] protein levels in the spleen. Data are shown as mean ± SEM of 10 rats. Symbols: +p < 0.05 CSITR animals compared to control animals (Tukey test) and #p < 0.05 CSITR+IMM animals compared to CSITR animals (Tukey test).

($p < 0.01$, Tukey test, **Figure 3b**), whereas the additional acute immobilization of CSITR animals decreased NA concentration by 17% ($p < 0.05$, Tukey test, **Figure 3a**) and increased A concentration by 15% ($p < 0.05$, Tukey test, **Figure 3b**) 3 hours after immobilization. The significant positive correlation was found between the levels of PNMT protein and A concentration in the spleen of animals exposed to CSITR+IMM (Pearson $R = 0.721$, $p < 0.05$, **Figure 4b**). However, the significant negative correlation was found between the levels of NA concentration and A concentration in the spleen of animals exposed to CSITR+IMM (Pearson $R = -0.661$, $p < 0.05$, **Figure 4c**).

8.3. Changes of the MAO A and MAO B activity in the spleen

The animals exposed to CSITR showed a decreased enzyme activity of MAO B by 34% ($p < 0.05$, Tukey test, **Figure 5b**), whereas enzyme activity of MAO A (**Figure 5a**) was unchanged, compared with control animals.

IMM stress significantly increased the enzyme activities of MAO A by 1000% ($p < 0.001$, Tukey test, **Figure 5a**) and MAO B by 376% ($p < 0.001$, Tukey test, **Figure 5b**) 3 hours after the cessation

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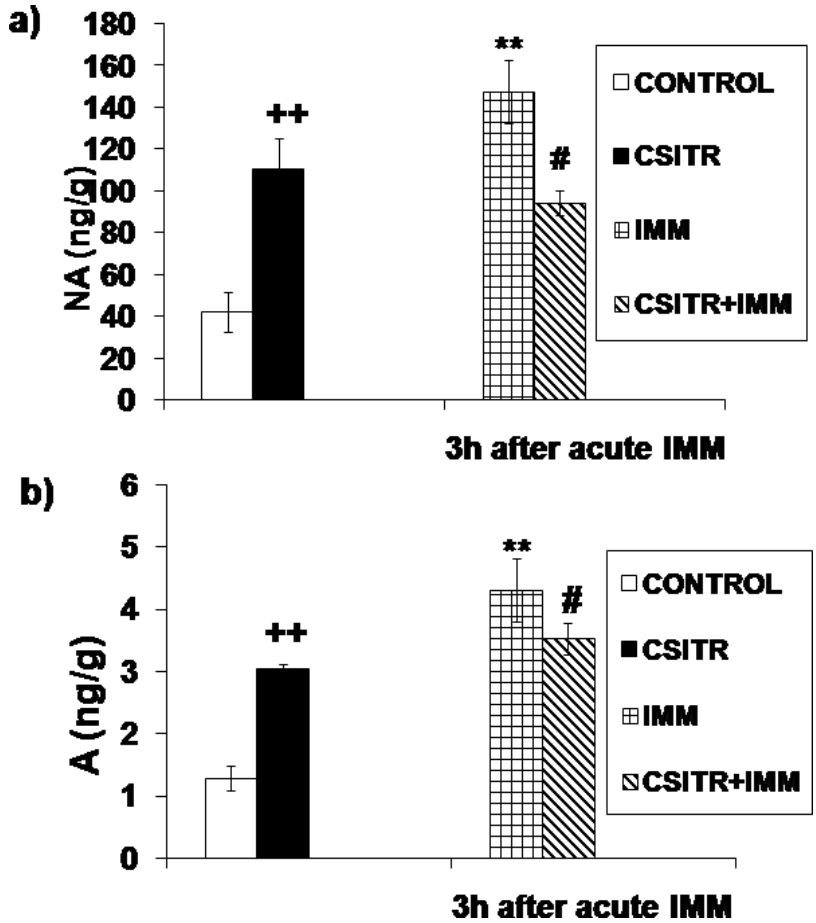

Figure 3. Effects of CSITR and CSITR+IMM models on the concentration of noradrenaline (NA) [a] and adrenaline (A) [b] in the spleen. Data are shown as mean ± SEM of 10 rats. Symbols: ++p < 0.01 CSITR animals compared to control animals (Tukey test); **p < 0.01 IMM animals compared to control animals (Tukey test); and #p < 0.05 CSITR+IMM animals compared to CSITR animals (Tukey test).

of immobilization. The additional acute immobilization of CSITR animals increased enzyme activities of MAO A by 116% ($p < 0.01$, Tukey test, **Figure 5a**) and MAO B by 107% ($p < 0.01$, Tukey test, **Figure 5b**) 3 hours after the cessation of immobilization.

8.4. Changes of the MDA concentrations in the spleen

Chronic social isolation (CSI) significantly increased concentrations of MDA by 21% ($p < 0.05$, Tukey test, **Figure 6**) compared with control animals. The animals exposed to CSITR showed unchanged levels of MDA compared with control animals (**Figure 6**).

The exposure of the control animals to acute immobilization stress significantly increased MDA concentration by 26% ($p < 0.05$, Tukey test, **Figure 6**), whereas the additional acute immobilization of CSI animals increased MDA concentration by 50% ($p < 0.01$, Tukey test,

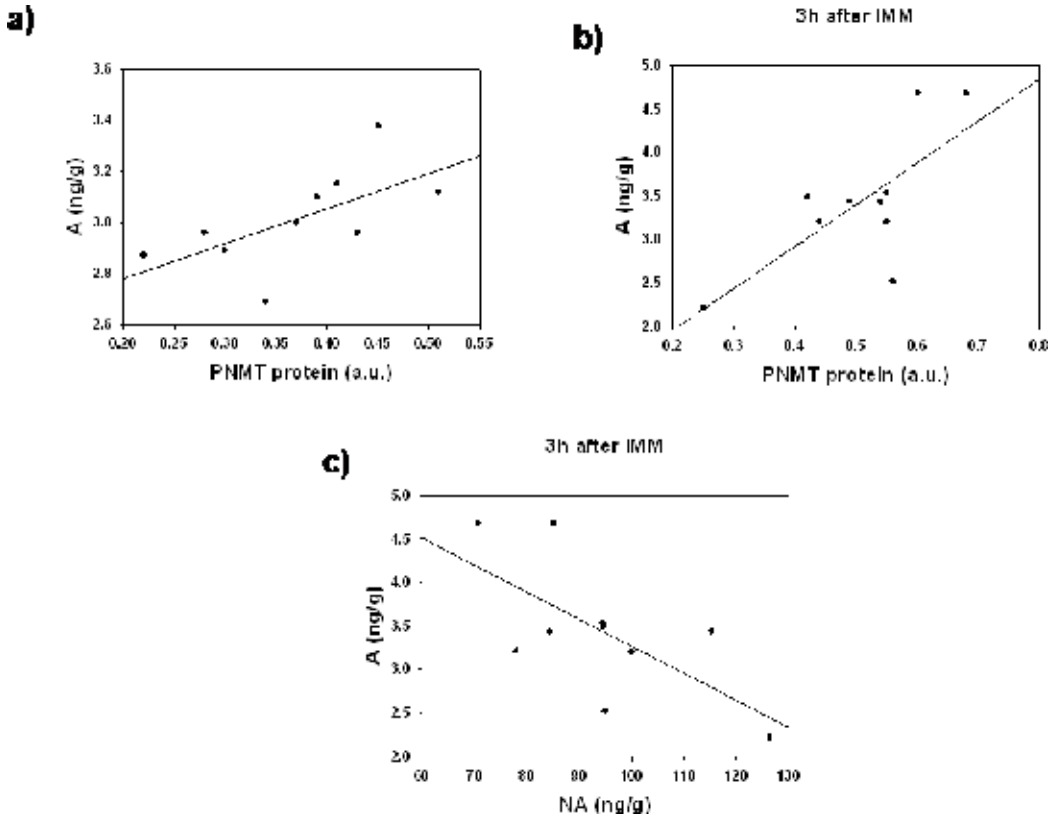

Figure 4. The correlation between PNMT protein level and concentrations of A and NA in the spleen of animals exposed to chronic social isolation and daily treadmill running, as well as of animals exposed to additional acute 2h immobilization stress after chronic social isolation and daily treadmill running (Pearson). **(**a) The correlation in the levels of PNMT protein and A concentrations in the spleen of animals exposed to CSITR (Pearson). (b) The correlation in the levels of PNMT protein and A concentrations in the spleen of animals exposed to CSITR+IMM (Pearson). (c) The correlation between NA and A concentrations in the spleen of animals exposed to CSITR+IMM (Pearson).

Figure 6) 3 hours after the cessation of immobilization. Also, the additional acute immobilization of CSITR animals increased MDA concentration by 16% ($p < 0.05$, Tukey test, **Figure 6**) 3 hours after the cessation of immobilization.

8.5. Changes of the SOD 1, SOD 2, CAT, and GPx mRNA levels as well as total SOD, CAT, and GPx activity in the spleen

The animals exposed to CSITR showed unchanged levels of SOD 1 and SOD 2 mRNA (**Figure 7a** and **b**), as well as significantly increased levels of CAT mRNA by 50% ($p < 0.05$, Tukey test, **Figure 7c**) and GPx mRNA by 150% ($p < 0.01$, Tukey test, **Figure 7d**) compared with control animals. However, CSITR treatment significantly decreased the enzyme activities of total SOD by 36% ($p < 0.05$, Tukey test, **Figure 8a**) and GPx by 30% ($p < 0.05$, Tukey test, **Figure 8c**) compared with control animals, whereas CAT activity remained unchanged (**Figure 8b**).

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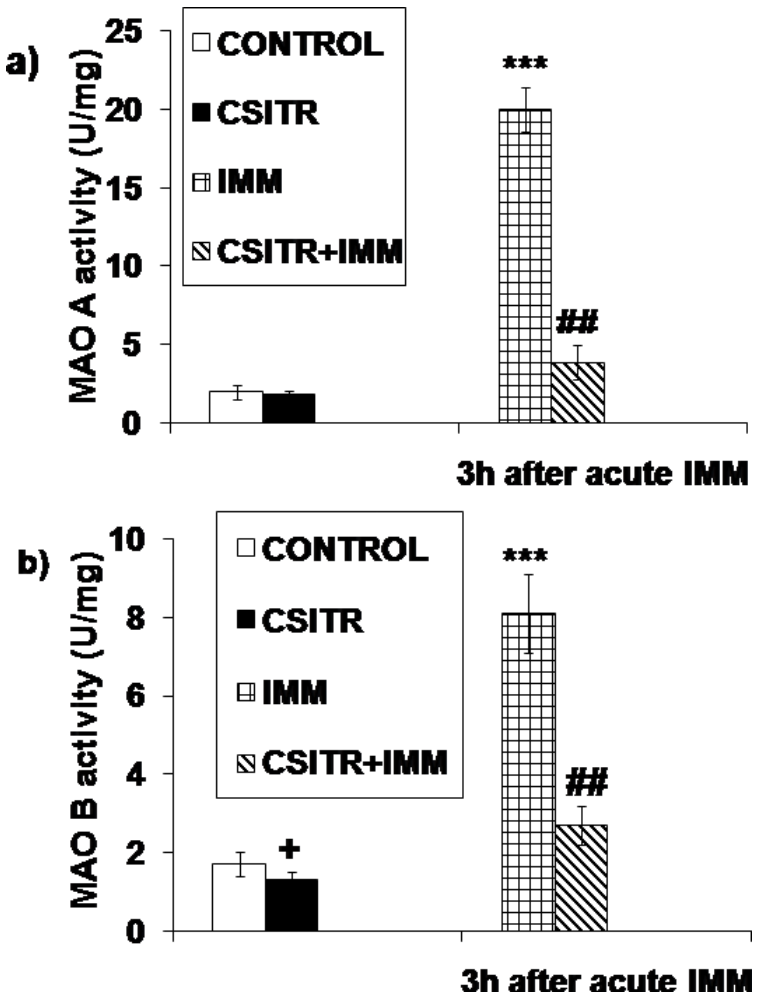

Figure 5. Effects of CSITR and CSITR+IMM models on the enzyme activity of the monoamine oxidase A (MAO A) [a] and monoamine oxidase B (MAO B) [b] in the spleen. Data are shown as mean ± SEM of 10 rats. Symbols: +$p < 0.05$ CSITR animals compared to control animals (Tukey test), ***$p < 0.001$ IMM animals compared to control animals (Tukey test), and ##$p < 0.01$ CSITR+IMM animals compared to CSITR animals (Tukey test).

IMM stress does not change mRNA levels of SOD 1, SOD 2, and CAT (**Figure 7a–c**) as well as enzyme activity of total SOD and CAT (**Figure 8a** and **b**) 3 hours after the cessation of immobilization. However, IMM treatment significantly increased mRNA levels of GPx by 20% ($p < 0.05$, Tukey test, **Figure 7d**) as well as enzyme activity of GPx by 135% ($p < 0.01$, Tukey test, **Figure 8c**) 3 hours after the cessation of immobilization. The additional acute immobilization of CSITR animals increased mRNA levels of SOD 1 by 37% ($p < 0.05$, Tukey test, **Figure 7a**), SOD 2 by 115% ($p < 0.01$, Tukey test, **Figure 7b**), CAT by 57% ($p < 0.05$, Tukey test, **Figure 7c**), and GPx by 18% ($p < 0.05$, Tukey test, **Figure 7d**) as well as enzyme activities of total SOD by 68% ($p < 0.05$, Tukey test, **Figure 8a**), CAT by 13% ($p < 0.05$, Tukey test, **Figure 8b**), and GPx by 576% ($p < 0.01$, Tukey test, **Figure 8c**) 3 hours after the cessation of immobilization.

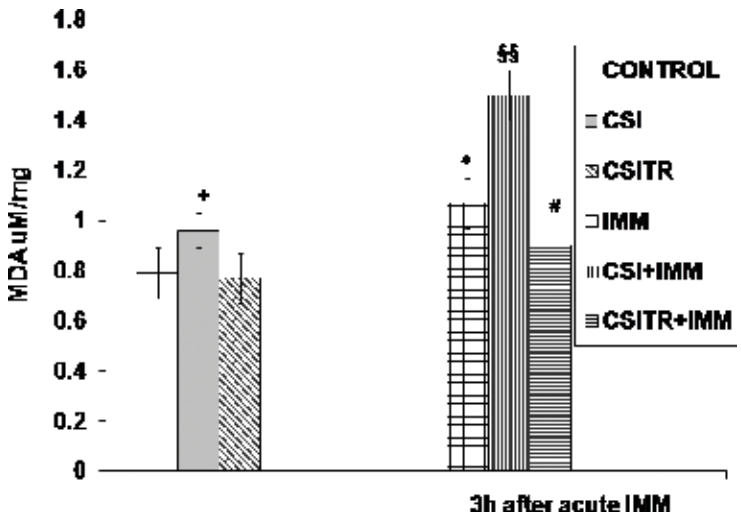

Figure 6. Effects of CSITR and CSITR+IMM models on the concentration of malondialdehyde (MDA) in the spleen. Data are shown as mean ± SEM of 10 rats. Symbols: +p < 0.05 CSI animals compared to control animals (Tukey test), *p < 0.05 IMM animals compared to control animals (Tukey test), §§p < 0.01 CSI+IMM animals compared to CSI animals, and #p < 0.05 CSITR+IMM animals compared to CSITR animals (Tukey test).

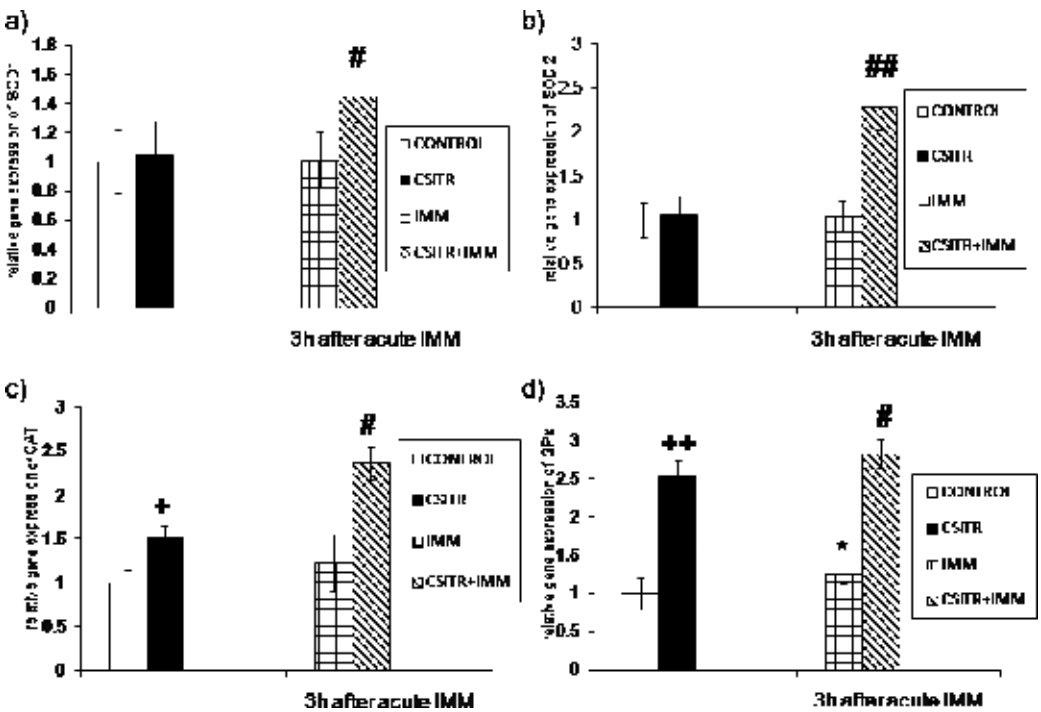

Figure 7. Effects of CSITR and CSITR+IMM models on CuZn superoxide dismutase (SOD1) [a], Mn superoxide dismutase (SOD2) [b], catalase (CAT) [c], and glutathione peroxidase GPx [d] mRNA levels in the spleen. Data are shown as mean ± S.E.M. of 10 rats. Symbols: +p < 0.05, ++p < 0.01 CSITR animals compared to control animals (Tukey test), *p < 0.05 IMM animals compared to control animals (Tukey test), and #p < 0.05, ##p < 0.01 CSITR+IMM animals compared to CSITR animals (Tukey test).

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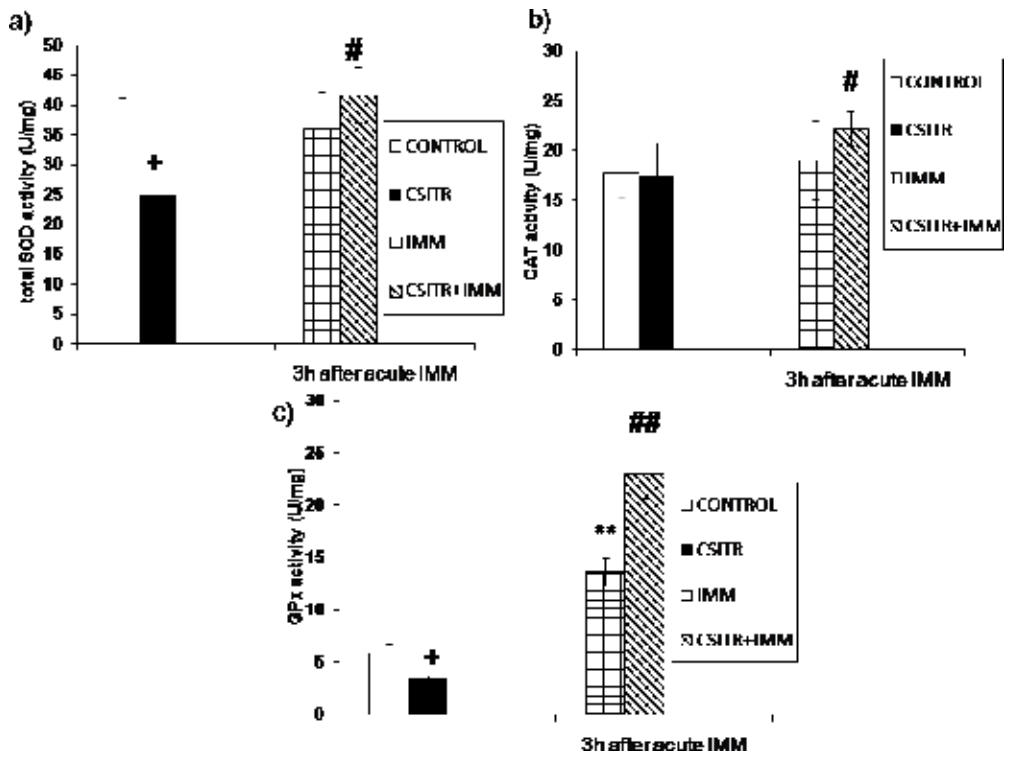

Figure 8. Effects of CSITR and CSITR+IMM models on total superoxide dismutase (SOD) [a], catalase (CAT) [b], and glutathione peroxidase GPx [c] enzyme activity in the spleen. Data are shown as mean ± SEM of 10 rats. Symbols: +$p < 0.05$ CSITR animals compared to control animals (Tukey test), **$p < 0.01$ IMM animals compared to control animals (Tukey test), and #$p < 0.05$, ##$p < 0.01$ CSITR+IMM animals compared to CSITR animals (Tukey test).

9. Discussion

It is known that chronic social isolation induces a reduction of gene expression of noradrenaline biosynthetic enzymes in the spleen [11]. Since the data from literature confirm that the treadmill running stimulates concomitantly peripheral catecholamine secretion and central noradrenergic activity, i.e., NA turnover and release [65], it was tentative to expect that treadmill running would change the splenic catecholamine synthesis of chronically psychosocially stressed rats. However, the results presented in this chapter show that the treadmill running does not lead to further modulation of gene expression of splenic noradrenaline biosynthetic enzymes (TH and DBH) and that reduced level of CREB mRNA coincides with the reduced TH and DBH mRNA levels of chronically psychosocially stressed rats. Also, the treadmill running does not change levels of splenic TH and DBH protein of chronically stressed rats. This finding indicates the decrease of de novo synthesis of NA in the spleen and that the CREB plays a major role in regulating the expression of TH and DBH genes during treadmill running, which is in accordance with the reports of Erdös et al. [13]. Therefore, the treadmill exercise does not affect the synthesis of splenic NA biosynthetic enzymes of chronically stressed rats. Although levels of splenic noradrenaline biosynthetic enzymes are unchanged,

concentration of NA in the spleen of chronically stressed animals exposed to daily exercise is increased. This finding indicates exogenous source of NA in the spleen of chronically stressed rats exposed to daily exercise. These findings strengthen the idea that the sympathetic nervous system (SNS) participates in the NA response to CSITR, which is in accordance with results of Blandino et al. [66], who have confirmed that the noradrenergic system plays an integral role in modulations of splenic IL-1 beta response to stress. In addition, exposure of chronically stressed rats to daily treadmill running reduces PNMT mRNA level. However, CSITR treatment leads to continuous accumulation of PNMT protein catalyzing the conversion of NA to A, suggesting the possibility of the conversion of sympathetic neurotransmitter NA to A in the spleen (**Figure 4a**). This is indicated by significant positive correlation between the levels of PNMT protein and A in the spleen. It is known that catecholamine via adrenergic receptors induces modulation of many immune functions like splenic cytokine production [4]. Moreover, catecholamines might be stored into vesicles by VMAT or degraded by MAO and catechol-O-methyltransferase (COMT) [67]. Expression of VMAT, which plays an important role in the transport of newly synthesized catecholamines into vesicles, positively correlated with norepinephrine levels in both T and B cells which might suggest increased capacity for intracellular catecholamine production [68]. Endogenous catecholamines can modulate function of lymphocytes themselves by a paracrine and autocrine pathway [69]. O'Donnell et al. [70] found that increase of catecholamine levels coincided with reduction of splenic B and NK cells and a concomitant increase in T cells. As reported in this chapter, the treadmill running increases splenic VMAT 2 gene expression, and that increased level of VMAT 2 mRNA coincides with the increased splenic NA and A levels of chronically psychosocially stressed adult rats. A high splenic VMAT 2 transcript level suggests increased capacity of the splenic catecholamines. Therefore, exercise induces accumulation of catecholamines in the spleen of chronically stressed rats, indicating higher readiness of catecholaminergic system to a novel stressor (**Figure 1e**). Brown et al. [71] found that endogenous catecholamines might further initiate intracellular oxidation and apoptosis. However, daily treadmill running does not change enzyme activity of MAO A and decreases enzyme activity of MAO B in the spleen of chronically stressed rats (**Figure 5**). Decreased or unchanged enzyme activities of MAOs indicate that daily treadmill running decreases catecholamine degradation of chronically stressed rats. Therefore, these results indicate that the treadmill running induces accumulation of the splenic catecholamines and that the SNS probably plays a major role in accumulation of the splenic catecholamines in chronically stressed rats.

Chronic social isolation significantly increases concentrations of MDA in the spleen (**Figure 6**). The literature data confirm that exercise training has beneficial effects on oxidative stress and antioxidant defense systems in multiple organs [72]. Meguid et al. [73] showed a significant decrease in serum level of malondialdehyde (MDA) in Down syndrome individuals after treadmill exercise for 3 months. Exposure of chronically stressed rats to daily treadmill running induces return of MDA concentration in the spleen to basal level (**Figure 6**). This confirms that the chronic exercise training induces adaptations that decrease stress-induced oxidative stress. It is in line with the reports of Belviranli et al. [74], who observed that chronic exercise has protective role because the decreased oxidative damage is associated with improved aerobic metabolism induced by physical training.

The decreased oxidative stress resulting from chronic training may originate from the elevated antioxidant system [75]. Powers et al. [76] observed that different combinations of intensity (low, moderate, and high) and duration (30, 60, and 90 min/day) produced different effects on the regulation of the antioxidant enzymes SOD, CAT, and GPx in the left ventricle. Exposure of chronically stressed rats to daily treadmill running induces an increase in CAT and GPx mRNA levels, while SOD1 and SOD2 mRNA levels remain unchanged (**Figure 7**). It is known that the adaptive response of the antioxidant system is specific to either the type of tissue or the different antioxidant systems involved [77, 78]. Ordonez et al. [79] found that a 12-week exercise significantly increased erythrocyte glutathione peroxidase activity which resulted in reduced oxidative damage. Sprint training caused an increase in the cardiac activity of glutathione redox cycle-related enzymes (GPx and GR) without inducing any changes in glutathione S-transferases (GST) and SOD activities or glutathione (GSH) levels in the myocardium [80]. It is important to notice that in CSITR the level of CAT activity remains unchanged, whereas total SOD and GPx activities are decreased (**Figure 8**). After 12 weeks of training process, changes in mRNA levels of antioxidant enzymes are not consistent with the changes in enzyme activities in the spleen of chronically stressed rats. Discrepancies between mRNA levels and activities may be related to differences in mRNA stability or translational efficiency [81]. García-López et al. [82] suspect that it is possible that the expressions of antioxidant enzymes mRNA were initially upregulated and then downregulated. In addition, regulation of expression might act on individual mRNAs to block their translation and thereby lead to their degradation [82]. Therefore, message degradation may be the primary target of regulation of expression [82]. Discrepancies between mRNA levels and activities of MnSOD may be in a kinase/phosphatase signal transduction pathway that may exert a fine control over posttranscriptional regulation of MnSOD expression [83]. In addition, CAT may be inactivated by its substrate, hydrogen peroxide, due to formation of complex II or complex III of CAT at high peroxide concentrations [84]. Nilakantan et al. [85] found that NO or NO-derived products inhibit both CAT and GPx enzyme activities. The results presented in this chapter confirm that daily treadmill running induces high splenic antioxidant enzyme transcript levels probably for immediate translation whenever necessary in chronically stressed rats, which is in accordance with the results of García-López et al. [82]. A high splenic CAT and GPx transcript levels suggest that exercise could induce the antioxidant defense system to become more ready to a novel stressor.

To confirm whether exercise is optimal stimulus to regulate expression levels of splenic catecholamines and antioxidant enzymes and whether the exposure of chronically stressed rats to daily treadmill running induces potentially positive adaptations of the splenic catecholamines and antioxidant protection, this chapter discusses the effects of additional acute immobilization stress. Detection of regulatory mechanism for catecholamine metabolism and antioxidant protection in the spleen in conditions provoked by the additional acute immobilization of chronically stressed animals exposed to daily exercise is exceptionally relevant in stress biology, because of the significant role of catecholamines and oxidative stress in modulation of immune function. The acute immobilization (IMM) and additional acute immobilization (CSITR+IMM) do not affect the synthesis of splenic noradrenaline biosynthetic enzymes (TH and DBH) 3 hours after a termination of immobilization stimulus (**Figure 2**). Also, acute

immobilization (IMM) does not change the level of PNMT mRNA and PNMT protein 3 hours after the termination of immobilization stimulus (**Figure 2**). Wong et al. [86] reported that PNMT protein and enzyme activity changes require additional time of approximately 18–20 hours to reach maximum stimulated levels. Three hours after the additional acute immobilization (CSITR+IMM), the increased synthesis of splenic PNMT protein (**Figure 2**) affects the increase of A (**Figure 3**) in the spleen of chronically stressed animals exposed to daily exercise. These data raise the possibility that 3 hours after additional acute stress, the spleen only converts sympathetic neurotransmitter NA to A of chronically stressed animals exposed to daily exercise. This is confirmed by the significant positive correlation between the levels of PNMT protein and A (**Figure 4b**), as well as negative correlation between the levels of NA and A in the spleen (**Figure 4c**). The acute immobilization (IMM) triggers an exaggerated elevation of splenic catecholamines, while the additional acute immobilization (CSITR+IMM) elevates only splenic A in chronically stressed animals exposed to daily exercise. This data confirm that the chronically stressed animals exposed to daily exercise show high readiness to convert sympathetic neurotransmitter NA to A. In addition, significantly elevated levels of VMAT 2 mRNA 3 hours after additional acute immobilization in chronically stressed animals exposed to daily exercise were found (**Figure 1**). Chronically stressed animals exposed to daily exercises have statistically less significant activation of MAO enzymes after additional acute immobilization compared with the animals exposed only to acute immobilization stress (**Figure 5**). These results confirm that the additional acute immobilization (CSITR+IMM) reveals high readiness of chronically stressed animals exposed to daily exercise for the accumulation of splenic A.

Additionally, it was proven that 3 hours after the acute immobilization, concentration of splenic MDA increased, which is in accordance with the reports of Belviranli et al. [74], who showed that the acute stress triggers oxidative stress. The acute immobilization (IMM) does not change either the levels of SOD 1, SOD 2, and CAT mRNA (**Figure 7**) or the total SOD and CAT enzyme activity (**Figure 8**) in the spleen 3 hours after a termination of immobilization stimulus. This finding is in line with the reports of Pajović et al. [16], who confirm that the acute immobilization does not change the levels of SOD enzyme activity. The increased oxidative stress produces inhibitory effects on CAT and SOD activity, which is evident from decreased enzyme activity of CAT and SOD (**Figure 8**) and increased concentration of MDA (**Figure 6**). These results are in accordance with the reports of Haider et al. [87] who showed that increased oxidative stress produced inhibitory effects on CAT activity. However, the acute immobilization (IMM) increases only the levels of mRNA and enzyme activity of GPx (**Figures 7** and **8**), but that increase was not sufficient to reduce oxidative stress. These results, together with the above mentioned data, confirm that acute immobilization induces oxidative stress. In addition, elevated levels of MDA 3 hours after the cessation of immobilization in chronically stressed animals exposed to daily exercise are observed (**Figure 6**). However, additional acute immobilization (CSITR+IMM) induces an increase of SOD 1, SOD 2, CAT, and GPx mRNA (**Figure 7**), as well as total SOD, CAT, and GPx enzyme activity (**Figure 8**) 3 hours after the cessation of immobilization in chronically stressed animals exposed to daily exercise. These data suggest high readiness of splenic antioxidant enzymes to repair or prevent damage by reactive oxygen species in chronically stressed animals exposed to daily exercise after additional acute immobilization stress. This could mean that exercise may condition physiological systems to “expect” a problem and, therefore, be more ready to respond

to a novel additional acute stressor by increased antioxidant protection. The readiness of the chronically stressed organism exposed to exercise to respond to a heterotypic stressor by an exaggerated expression of splenic antioxidant enzymes is an important adaptive phenomenon of the antioxidant defense system. Therefore, these results confirm that exercise have an important protective role in the splenic antioxidant defense system.

10. Conclusions

The exposure of chronically stressed rats to daily exercise induces the increase in the synthesis of splenic PNMT protein catalyzing the conversion of sympathetic neurotransmitter NA to A. In addition, the increased levels of splenic VMAT 2 mRNA and decreased/unchanged MAO enzyme activity suggest that daily exercise leads to accumulation of splenic catecholamines in chronically stressed rats. The accumulation of the splenic catecholamines provoked by exercises may have an important impact on the immune-neuroendocrine interactions in stress conditions. The return of the splenic MDA concentrations to basal levels confirms that exercise may decrease stress-induced oxidative stress, while the increased splenic antioxidant enzyme (CAT and GPx) transcript levels suggest that exercise could induce the antioxidant defense system to become more ready to a novel stressor, which indicates that exercises may repair oxidative damage in chronically stressed rats. Moreover, it can be concluded that exposure of chronically stressed rats to daily exercise causes high splenic antioxidant enzyme transcript levels and catecholamine levels and that the exercise can be beneficial, inducing an adaptive response to possibly other stressors that may be encountered later.

The remarkable anatomical and physiological similarities between humans and animals, particularly mammals, have prompted researchers to investigate a large range of mechanisms and assess novel therapies in animal models before applying their discoveries to humans [88]. Our combined model of chronic social isolation and long-term daily treadmill running may be a good animal model in the research of the preventive role of exercise on neuroendocrine and immune functions in stress conditions, suggesting the potential application of CSITR animal model in understanding of human stress, as well as the potential therapeutic role of exercise in human diseases.

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Conflict of interest

The authors report no conflict of interest. The authors alone are responsible for the content and writing of the paper.

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Chapter 15

Studying Side Effects of Tyrosine Kinase Inhibitors in a Juvenile Rat Model with Focus on Skeletal Remodeling

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Additional information is available at the end of the chapter

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Abstract

The tyrosine kinase (TK) inhibitor (TKI) imatinib provides a highly effective treatment for chronic myeloid leukemia (CML) targeting at the causative oncogenic TK BCR-ABL1. However, imatinib exerts off-target effects by inhibiting other TKs that are involved, e.g., in bone metabolism. Clinically, CML patients on imatinib exhibit altered bone metabolism as a side effect, which translates into linear growth failure in pediatric patients. As TKI treatment might be necessary for the whole life, long-term side effects exerted on bone and other developing organs in children are of major concern and not yet studied systematically. Here, we describe a new juvenile rat model to face this challenge. The established model mimics perfectly long-term side effects of TKI exposure on the growing bone in a developmental stage-dependent fashion. Thus, longitudinal growth impairment observed clinically in children could be unequivocally modeled and confirmed. In a "bench-to-bedside" manner, we also demonstrate that this juvenile animal model predicts side effects of newer treatment strategies by second generation TKIs or modified treatment schedules (continuous vs. intermittent treatment) to minimize side effects. We conclude that the results generated by this juvenile animal model can be directly used in the clinic to optimize treatment algorithms in pediatric patients.

Keywords: juvenile, growth, bone, tyrosine kinase inhibitor, side effects, CML

1. Introduction

The introduction of tyrosine kinase inhibitors (TKIs) for targeted treatment of chronic myeloid leukemia (CML) marked a paradigm shift in the field of hemato-oncology [1, 2]. However, soon after CML became most successfully treated cancer—first in adults and thereafter in

children—it was learned that chronic exposure to TKIs impaired modeling of the osseous skeleton as an off-target effect [3]. This skeletal side effect resulted in impaired longitudinal growth in not outgrown minors [4, 5]. With regard to a potential lifelong necessity of TKI intake, children with CML differ from a typical patient with CML who is about 60 years old [6]. Thus, the rational of the research of TKIs' off-target effects is to generate a clear picture of early and late sequelae of long-term drug intake.

On this background, the essential objective of this chapter is the description of a juvenile (still growing) rat model that allows a chronic administration of TKIs via the drinking water in order (i) to mimic osseous changes observed in humans, (ii) to further characterize and investigate the causative pathophysiologically mechanisms resulting in impaired bone growth, (iii) to test approaches in growing animals for ameliorating the off-target effect resulting in growth impairment, and (iv) to check further organs beside bone for long-term TKI toxicity.

In this chapter, sections describe i) the highly effective role that TKIs play in standardized attempts to operationally cure CML in adults as well as in children, ii) elucidate the role of the established juvenile male Wistar rat model to investigate with ease the skeletal changes at all developmental stages, and iii) focus on the administration of TKI via the drinking water over many weeks as an adequate and convenient way resulting in the achievement of therapeutic drug blood levels. TKI-induced changes in long bones, as well as vertebrae, can be investigated with dedicated small imaging devices while blood levels of bone turnover markers, growth hormone, and vitamin D metabolites can be followed at different stages of development. The results of these investigations as well as the derived hypothesis on the pathophysiological cascade, specifically how TKIs impair longitudinal bone growth, are in excellent agreement with clinical observations. In addition, the juvenile animal model is of value to monitor other long-term TKI side effects on the heart and fertility to generate an overall picture on all possible side effects.

2. Role of tyrosine kinase inhibitors in chronic myeloid leukemia treatment

The principal function of tyrosine kinases (TKs) involves the regulation of multicellular aspects of the organism. By transferring a γ-phosphate group from adenosine triphosphate (ATP) to the hydroxyl group of tyrosine residues on signal transduction molecules, cell-to-cell signals, including growth, differentiation, adhesion, motility, and death, are transmitted [7]. Around 90 TK genes have been identified in the human genome [8]. Based on kinase domain structure, 58 are of transmembrane receptor type and can be grouped into 20 subfamilies and 32 are of cytoplasmic non-receptor type which falls into 10 subfamilies [8, 9]. TK receptors play a role in either transmembranous or intracellular signal transduction as they act as relay points controlling intracellular signaling pathways. Non-receptor TKs exhibit no transmembrane protein domain and are located in the cytoplasm. Generally, they are involved in signaling downstream of the receptor TKs.

In humans, TKs have been demonstrated to play significant roles in the development of many malignant diseases like chronic myeloid leukemia (CML) [8]. CML results from a reciprocal chromosomal translocation involving the c-abl proto-oncogene 1 (*ABL1*) on chromosome 9 and the breakpoint cluster region (BCR) on chromosome 22, thus forming the *BCR-ABL1*

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oncogene [10, 11]. This t(9; 22) translocation or Philadelphia chromosome (Ph+) is a characteristic cytogenetic abnormality seen in 95% of patients with CML and in 15–30% of adult patients with acute lymphoblastic leukemia (ALL) [12, 13]. The *BCR-ABL1* oncogene codes for two forms of fusion transcripts: $p190^{BCR\text{-}ABL1}$ and $p210^{BCR\text{-}ABL1}$, which are constitutively highly activated and subsequently dysregulate intracellular signaling by enhancing proliferative capability and resistance to apoptosis of hematopoietic stem or progenitor cells, leading to a massive increase in myeloid cell numbers.

About 1–1.5/100,000 residents are diagnosed with CML every year with an age peak between 50 and 60 years [14], representing around 20% of all cases of leukemia in adulthood [15]. Concerning pediatric patients, the frequency of diagnosis is about 0.05–0.40/100,000 residents per year within the age of 0–18 years [16]. Thus, CML represents one of the rarest leukemic disorders in childhood and adolescent age, accounting for only 2–3% of all children suffering from leukemia [16]. In terms of morphological characteristics, childhood CML is not different from adult CML. However, it is a matter of an ongoing debate whether and to what extend molecular differences exist between CML diagnosed at childhood or older age [6]. For example, pediatric CML shows a breakpoint distribution in the *BCR* gene more similar to adult Ph+ ALL [17].

Still, as the *BCR-ABL1* oncogene is the single molecular aberration causing the development of CML, specific TKIs like imatinib (Gleevec®, Novartis) have been developed to inhibit the BCR-ABL1 TK [19]. By achieving hematological and cytogenetic response in over 90% of the patients after a few months of imatinib treatment, imatinib has been very effective in inhibiting progression of CML (**Figure 1**) [1, 20–23].

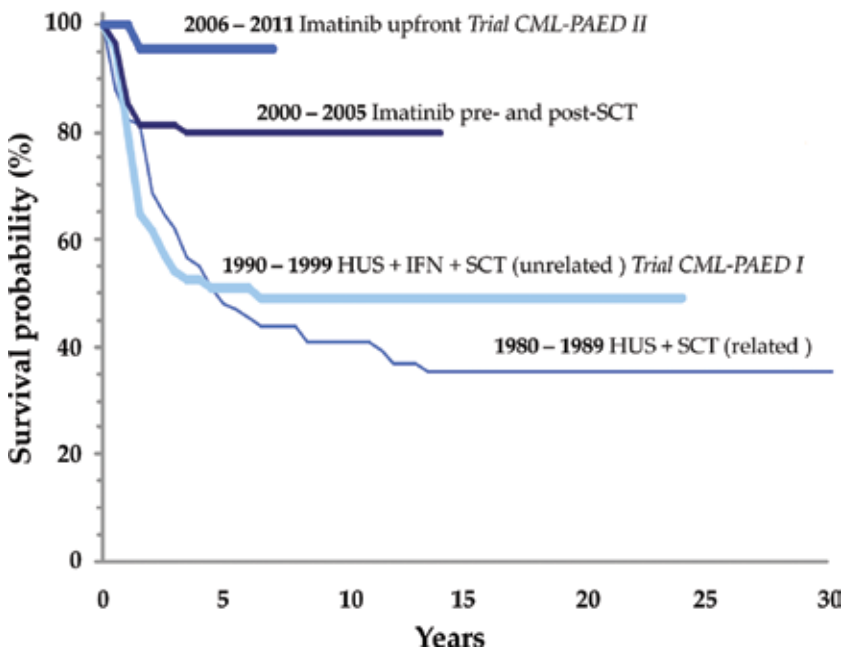

Figure 1. Survival probabilities by year of diagnosis (1980–2013) of pediatric patients with CML in Germany [18].

However, some patients develop resistance to imatinib resulting in loss of treatment response or even leukemic relapse. Among other underlying mechanisms, BCR-ABL1 kinase domain mutations can cause varying degrees of drug insensitivity [24]. In order to counter these mechanisms, next generation TKIs have been developed like dasatinib (SPRYCEL®, Bristol-Myers-Squibb), nilotinib (Tasigna®, Novartis), bosutinib (BOSULIF®, Pfizer), and ponatinib (ICLUSIG®, Ariad Pharmaceuticals) with different affinities to the ATP-binding pocket of the BCR-ABL1 TK [25].

3. Imatinib as front-line treatment for pediatric CML

As imatinib has proven very effective in adult CML, its accelerated clinical approval was given in the year 2001 for adults with CML and without age restriction in 2003. In several studies, imatinib showed similar antileukemic efficacy in children compared to adults (**Figure 1**) [26, 27]. Typically, standard dose of imatinib (300 mg/m^2) achieved a complete hematologic remission in 95% of the pediatric patients after 3 months, a complete cytogenetic remission in 80% after 12 months, and a major molecular remission (MR3.0 = 0.1 % ratio copy number of gene transcripts BCR-ABL1/control gene) in 60% after 18 months of treatment [16, 28].

Although imatinib acts relatively specifically against the dysregulated BCR-ABL1 TK, it is known that imatinib exerts off-target effects at therapeutic blood levels on other TKs (**Table 1**). The reason for this is the affinity of imatinib to the ATP-binding pocket of the kinase domain. This domain is a characteristic feature of many members of the kinome, including TKs (review of the human kinome: [29, 30], review structure of the TK: [8, 9]).

Nevertheless, imatinib treatment is generally well tolerated, showing mostly mild side effects. Neutropenia, thrombocytopenia, and anemia occur in up to 45, 20, and 10% of patients, respectively, who are in the chronic phase of CML and receive standard dose imatinib [2]. Nonhematologic adverse effects include nausea, skin rashes, peripheral edemas, muscle cramps, and elevated liver transaminase levels [2].

Studies with imatinib in adult patients also showed disturbed bone metabolism as a specific side effect [3] comprising altered calcium metabolism as well as increased trabecular mineralization and increased bone density in stamp biopsies [32]. In children, imatinib therapy has been associated with severe longitudinal growth retardation [4, 5, 33–41] (**Figure 2**), but the detailed mechanism how imatinib interferes with bone metabolism and the final consequences are not fully understood.

Regardless the type of kinase, imatinib binds to all structurally accessible ATP-binding pockets. Accordingly, other membrane-bound and cytosolic TKs, e.g., c-abl, PDGF-R α/β, c-KIT, and c-FMS [31, 42–44], are inhibited which play a major role in bone remodeling.

Tyrosine kinase	BCR-ABL1	c-abl	c-Kit	PDGF-Rα	PDGF-Rβ	c-FMS
IC_{50}(μM)	0.25	0.19	0.15	0.10	0.39	1.42

IC, inhibitory concentration.

Table 1. Inhibitory effect of imatinib on selected TKs [31].

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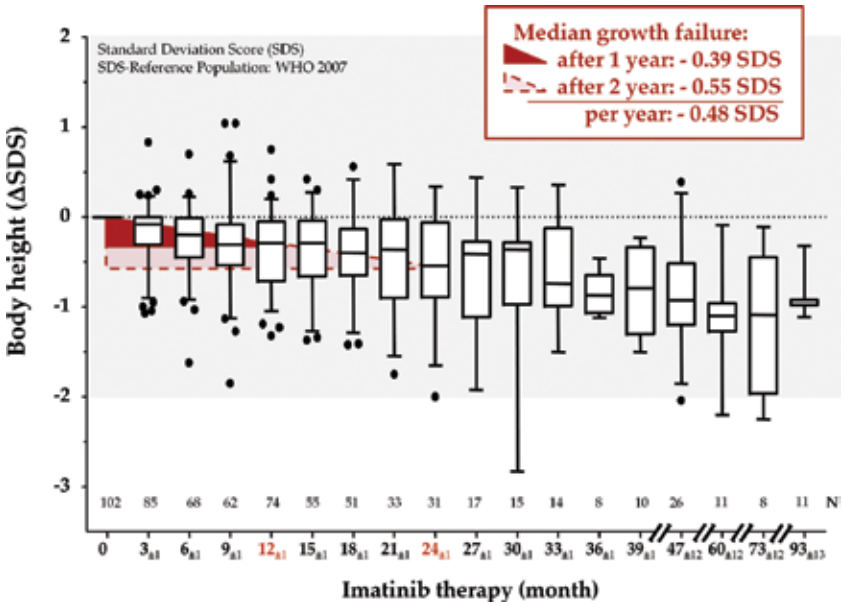

Figure 2. Growth failure in pediatric CML patients during imatinib treatment [4]. SDS: Standard deviation score. One hundred and two patients (54 male/48 female; median age 12 years, range: 1–18 years) at diagnosis of CML receiving imatinib as upfront treatment were enrolled retrospectively in the trial CML-PAED II during the period 02/2006 to 06/2014. Height standard deviation scores (SDS) were derived from WHO-AnthroPlus, version 1.04 software, a global growth-monitoring tool providing normal range values for the age cohorts from birth till 19 years. Eighty-one out of 102 patients fulfilled the criteria for continuous assessment of growth scheduled at 3- months intervals during imatinib exposure. Twenty-one patients were analyzed at intervals ≠ 3 months. Calculation: Δ SDS = $SDS_{TKI\ therapy}$–$SDS_{Diagnosis}$; data are shown as Whiskers box plot (median ± 5^{th}/95^{th} percentile).

Underlying dynamic processes of the growing skeleton are subject to strict regulation/communication of bone formation and resorption and can be easily influenced by interfering factors. At present, it is not possible to study simultaneously these complex bone remodeling processes such as the interaction of bone forming osteoblasts and bone resorbing osteoclasts by culturing systems *in vitro*. Therefore, it is only possible to study bone breakdown, bone structure, changes in the mineral content, and the overall structure of the bone *in vivo* in appropriate juvenile animal models.

In addition, TKI treatment for CML is not curative in most patients. Although first results from stopping TKI trials in adult patients after achieving sustainable deep molecular remission look promising most patients probably require a lifelong TKI treatment. This poses an increased risk to pediatric CML patients exposed to TKI treatment for decades as the long-term side effects on bone or other organs in a still growing organism presently are totally unknown.

4. Juvenile animal model for chronic TKI exposure

All regulatory authorities (Food and Drug Administration (FDA), Health Canada, European Medical Agency (EMA)) require animal tests to be conducted before humans are exposed to a new molecular entity. In drug developmental process, every potential new therapeutic agent

has to pass clinical phase I-III studies in humans to verify safety, dosage, efficacy, side effects, and monitoring adverse reactions. All these studies are done in adult volunteers or adult patients if the disease under study occurs not exclusively at pediatric age [45]. Thus, in order to gain insight into side effects occurring specifically in the still growing organism during the preclinical research phase, *in vivo* studies in young growing animals are of main importance.

However, almost in all instances, primarily adult animal models are used in preclinical research (for reviews about the ongoing debate about animal models in clinical research see Refs. [46–48]). Adult animals were also used to study the influence of imatinib on the skeletal system [49, 50]. But as the growth process of the juvenile bone differs significantly from a mature bone, results described so far in adult patients/animals cannot readily be transferred to pediatric cohorts. Furthermore, Juvenile animal models cannot easily be selected as they are not established to match every single "research question" or disease on a routine basis.

Therefore, we describe here our established juvenile animal model to study side effects of a **chronic exposure** of imatinib primarily on the **growing bone** and to a lesser extent on other organs.

When establishing a juvenile animal model, several issues should be considered like the rodent species itself (mouse vs. rat), the strain (inbred vs. outbred), the overall speed of development (age when puberty starts), and convenient ways of drug administration in the situation of long-term exposure (intraperitoneal vs. subcutaneous vs. oral gavages vs. micro-osmotic pumps). Overall, the developmental stages must be comparable to human life.

4.1. Mice versus rat

Most of the animals used in biomedical research are mice and rats because of their availability, ease of handling, and fast reproduction rate. Mice are an excellent model for human diseases because genetically they share 98% homology with human genome as well as a similar organization of their DNA and gene expression. However, the genome of a rat is smaller than its human equivalent but larger than that of a mouse.

Compared to mice, rats offer many advantages as, for example, their physiology is easier to monitor and is more like the corresponding human condition. But the most important advantage of the rat is its bigger size, not just because of the added ease to perform surgical procedures, but because of larger substructures (e.g., bone growth line, metaphysis) in organs thus influencing (i) which ratio of the organ is prone to an experimental lesion and (ii) the distance effects drugs exert to a specific anatomical area [51].

As we questioned about side effects of a chronic imatinib exposure on the growing organism, we were interested in the side effects on the long bones, which are much bigger in rats as compared to mice. As an additional benefit, we could also monitor side effects on other growing and developing organs like heart and testis as rats are a preferred model in cardiac and reproduction questions [52].

4.2. Inbred versus outbred strain

In general, the difference between outbred and inbred strains lies in their genetic background. Inbred strains are characterized by almost 99% homogeneity of the genome resulting from a

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long inbreeding of this strain, whereas outbred strains have a diverse genetic background. Due to this genetic characteristic, animals of inbred strains react nearly identical to a specific intervention, like medical treatment, surgery, etc., wherefore the influence of this intervention on a particular parameter can be identified more precisely. However, outbred strains reflect the natural situation more accurately as every individual is genetically different from the other. Every animal of an outbred strain will react slightly different to a specific intervention, which discloses all possible effects of this intervention on the metabolism and mimics more the situation in the clinic. Therefore, depending on the experimental question and if you need a genetic diversity in your test population, inbred or outbred strains are used. Our study focused on side effects of long-term TKI treatment on bone remodeling and to mimic the human situation, we choose juvenile rats of the outbred strain "Wistar."

4.3. Male versus female

We exclusively studied Wistar rats of male gender, as males tend to be more sensitive to bone influencing agents than female animals due to more rapid weight development and gender-specific hormones.

Prepubertal young Wistar rats triple their body weight, regardless of sex, from about 60 to 180 g in 14 days from the 3rd to 5th week of life due to the increasing growth hormone (GH) pulse amplitudes. The duration of GH pulses is significantly longer in males versus females, a pattern that continues throughout adulthood. Between 5th and 7th week of life, GH pulse amplitudes are similarly increased in both sexes [53, 54]. The rapid skeletal growth associated with this is particularly strongly influenced by interfering factors. In postpuberty, the growth slows down, especially in female rats, who weigh 200 g in the 8th week of life and 220 g in the 10th week of life. Contrary, male animals reach a body weight of 300 g postpubertally in the 8th week of life and 390 g in the 10th week of life. These differences in growth dynamics should also make postpubertal bone alterations due to TKI exposure more prominent in male animals.

Nevertheless, additional factors especially endocrine changes in hormones, such as testosterone, 17ß-estradiol, and corticosterone, inducing and associated with the onset of puberty and puberty itself may be more important than GH to decide about the sex when setting up an animal model. It is commonly considered that puberty lasts until the 8th week of age [54]. However, onset of puberty in the rat (as measured by the age at vaginal opening and the onset of estrous cyclicity) occurs between 4th and 5th week in females, whereas in males (as measured by preputial separation which is an androgen-dependent event) occurs around 7th week of life depending on the strain used [55]. The onset of puberty in male Wistar rats based on the increase in plasma testosterone levels starts at 46–50 days of age and progressively increases until 76 days of age [56–58]. However, related to the increased production of estrogen and its positive influence on bone formation, trabecular bone density increases significantly both in women and in female rats with the onset of puberty [59, 60]. Because of this hormonal influence, effects on the bones, which are only mild, would be more difficult to detect in the female organism.

4.4. Drug administration

For chronic drug exposure, we choose administration via the drinking water. Drug application via subcutaneous (s.c.) or intraperitoneal injection (i.p.) or oral gavage is the most

accurate type of body weight-related exposure. However, young animals are prone to risks of injury and subsequent infection in the pharynx and/or esophagus [61, 62]. Micro-osmotic pumps could also be considered for s.c. administration but repeated implantation and removal of the pumps combined with the increased risk of infection should be taken into account [63]. For a detailed review of routes for chronic drug administration, see ref. [64]. However, due to the pharmacodynamics of the TKI, single shot by s.c. or i.p. administration would need at least two TKI applications daily over 10 weeks. Considering animal ethics as well as personal resources over several weeks including shifts on weekends, these numerous manipulations are hardly tolerable and affordable. For these reasons, the chosen intake of the drug via the drinking water was the most adequate and convenient form of chronic TKI exposure. Also, the stability of the TKIs in aqueous solution at room temperature facilitated this approach. Hence, the drug intake is dependent on the daily drinking volume considering age and associated body weight. Other possible interfering factors are loss of liquid when changing the water bottles or leaking water bottles and changes in the drinking behavior due to changes in the environment like fluctuation in the room temperature or humidity, or social conflicts between the animals. To counteract this, the care of the experimental animals, the measuring of the drinking volume, and the determination of weight gain were always carried out on a fixed schedule and by identical staff members including weekends.

Age-dependent drinking behavior of mammals varies. According to body weight, higher volumes are ingested by younger animals [65, 66]. Furthermore, rodents show a circadian rhythm of their food and drinking water intake. About 80% of the maximum daily intake of liquids occur at night [64]. This allows the conclusion that by administration via the drinking water, a peak level was achieved during the night, comparable to the single administration in human patients during the daytime.

4.5. Developmental stages

Due to the well-documented developmental stages of the rat, it is possible to carry out a comparison with human developmental stages in order to interpret the generated data in an orientated manner (**Table 2**).

Developmental stages	Rat	Human
Weaning	3 weeks	6 months
Puberty	7 weeks	12–14 years
Adolescent	8–11 weeks	15–20 years
Adult	>12 weeks	>20 years
Death	2–3 years	70–80 years

Table 2. Developmental stages of rat and human [67].

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Considering the rapid maturation of the rats and the objective of examining the development (infancy, puberty, and young adulthood), we selected an exposure period of 10 weeks starting at 4 weeks of age.

Summing key issues in the juvenile animal model described, we chronically exposed healthy 4-week-old male Wistar rats to varying concentrations (low dose vs. high dose) of imatinib via drinking water over a period of 10 weeks while growing. We applied different treatment schedules to mimic possible new treatment strategies (continuous vs. intermittent). During the entire exposure time, the developmental stages from the end of weaning until young adolescence were covered (**Figure 3**). During ongoing imatinib exposure, a defined number of animals from each cohort were humanely sacrificed at prepubertal stage (age 6 weeks; after 2 weeks of exposure), at pubertal stage (age 8 weeks; after 4 weeks of exposure), and at postpubertal stage (age 14 weeks; after 10 weeks of exposure) [68].

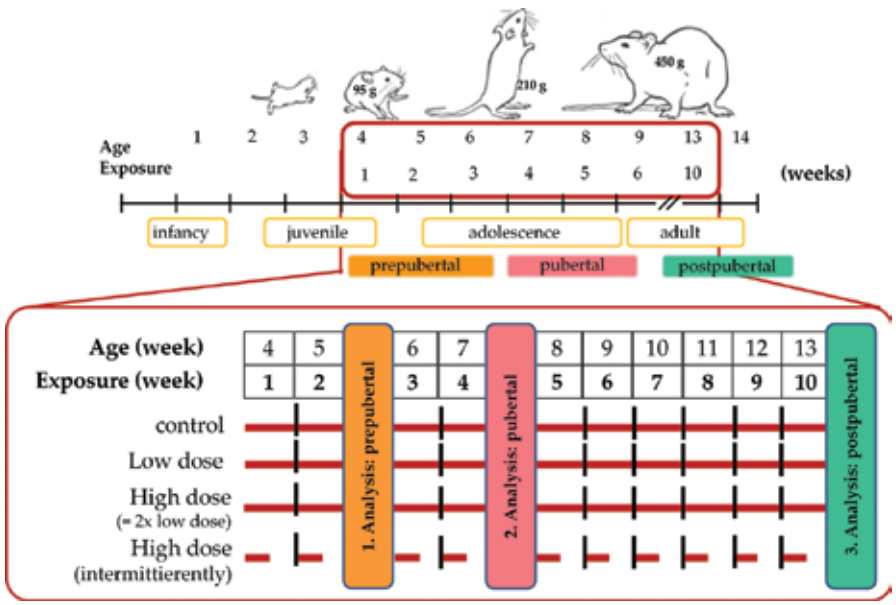

Figure 3. Experimental design of the juvenile animal model.

5. Side effects of chronic imatinib treatment on growing bone

At defined time points of analysis (**Figure 3**), blood serum was collected to measure TKI concentration by high-performance liquid chromatography (HPLC), biochemical markers of bone turnover, and hormone levels by ELISA technique. Long bones (tibia and femur) and lumbar vertebrae L1–L4 were isolated to determine bone length, vertebral height, bone mass,

and strength by using quantitative computed tomography (pQCT), micro-computed tomography (μCT), and biomechanical testing [68].

5.1. TKI serum concentration

Imatinib mean serum levels of 1600 and 5600 ng/mL were achieved by continuous drug exposure via the drinking water to either low or high dose, respectively [68]. These serum concentrations match well with therapeutic imatinib levels of pediatric patients ranging from 2000 to 8000 ng/mL on imatinib administered at doses of 260–570 mg/m^2 daily [26], whereas in adult patients, serum levels in the range of ~1000–3400 ng/mL on imatinib doses of 400–600 mg daily were measured [69, 70]. Reflecting the half-life of imatinib in rats reported to be 12.3 h [71], serum levels of animals receiving high dose imatinib intermittently were below the detection limit of the assay (10.0 ng/mL) when serum was collected at the end of a 4-day period without drug exposure.

5.2. Long bone length and bone quality

During growth, a 10-week exposure to imatinib caused a significant reduction of the long bone length dose-dependently (**Figure 4**) [68]. These findings match with clinical data in children indicating that continuous administration of imatinib—even in high doses—does not result in a complete stop of growth, rather in a decelerated growth rate of the long bones [5, 36, 38–41, 72]. During growth, pQCT analysis of the bones revealed significantly reduced

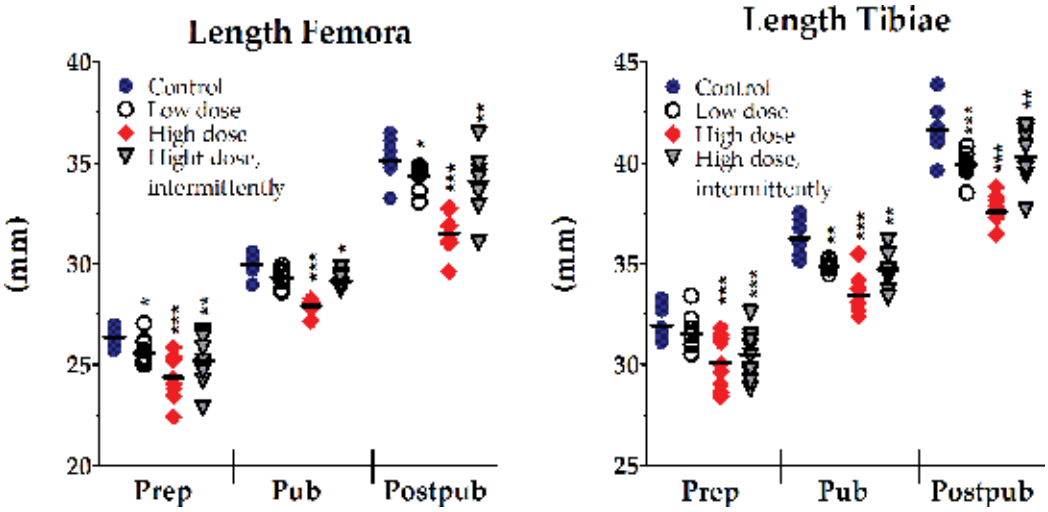

Figure 4. Growth impairment of long bones by imatinib is dependent on the cumulative dose [68]. Prep: Prepubertal; Pub: Pubertal; Postpub: Postpubertal. Compared to controls, high dose imatinib (1000 mg/L daily) causes stronger longitudinal growth impairment than low dose exposure (500 mg/L daily). "On/off" exposure (3 days "on", 4 days "off") to high dose imatinib mitigates this effect. Of note, the cumulative dose resulting from 1000 mg/L administered "on/off" is approximately identical to 500 mg/L daily administered continuously. The resulting reduction in length reflects the cumulative dose administered.

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trabecular bone mineral density (BMD) by imatinib exposure. Analysis of the 3D trabecular structure by μCT emphasizes these findings by demonstration of reduced bone volume density in combination with reduced trabecular number and connectivity [68]. Furthermore, our findings also indicated unchanged cortical BMD and cortical thickness during growth dose- and time-independently, whereas the bone strength of the femora was decreased after long-term exposure to high dose imatinib. This could be explained by decreased cross-sectional area, periosteal, and endosteal circumference of the femora, suggesting a blunted radial appositional bone growth [68]. With regard to pediatric patients, BMD measurements or increased fracture rates under long-term imatinib treatment are not published yet. However, intermittent treatment of the high dose mitigated all bony side effects of the long bones, which might offer a new perspective for pediatric patients.

5.3. Vertebrae height and quality

Concerning lumbar vertebra, 10-week imatinib exposure significantly reduced vertebral height combined with reduced trabecular BMD dose-dependently, whereas total BMD, cortical BMD, cross-sectional area, and cortical thickness were not affected [68, 73]. At the moment, only limited data are available on the effect of imatinib on vertebrae. In adult patients with CML, O´Sullivan et al. observed significantly increased lumbar spine BMD after 24 months of imatinib treatment as assessed by dual energy x-ray absorptiometry (DXA) [74], whereas Vandyke et al. observed unchanged BMD [75]. We predict from our animal model that imatinib also alters vertebral properties, but not to the same extent as in long bones [68].

5.4. Bone turnover markers

The bone resorption marker tartrate-resistant acidic phosphatase (TRAP) revealed significantly decreased serum levels under continuous imatinib exposure indicating reduced osteoclast activity at all developmental stages [68]. This is confirmed by *in vitro* studies showing that imatinib impairs osteoclastogenesis leading to diminished numbers of TRAP-positive osteoclasts [49, 76]. However, bone resorption marker C-terminal collagen cross-links (CTX-I) revealed by trend elevated serum levels prepubertally, but normal levels during the ongoing exposure time, indicating nearly unchanged osteoclast activity during growth [68]. This is consistent with data from pediatric patients with CML describing by trend elevated CTX-I levels prepubertally while on imatinib [77].

Under imatinib exposure, bone formation marker osteocalcin was decreased but procollagen type I (PINP) levels were by trend elevated, pointing to improved bone formation and mineralization [68]. *In vitro* assays using human isolated mesenchymal stem cells, primary rat osteoblasts, and mouse osteoblast-like cell line MC3T3-E1 revealed all increased mineralization combined with reduced proliferation under therapeutic imatinib concentration [50].

However, bone turnover markers of pediatric patients with CML exhibited a biphasic response during imatinib therapy with increasing levels within the first 3 months of treatment and a significant decline during long-term treatment (**Figure 5**) [77, 78].

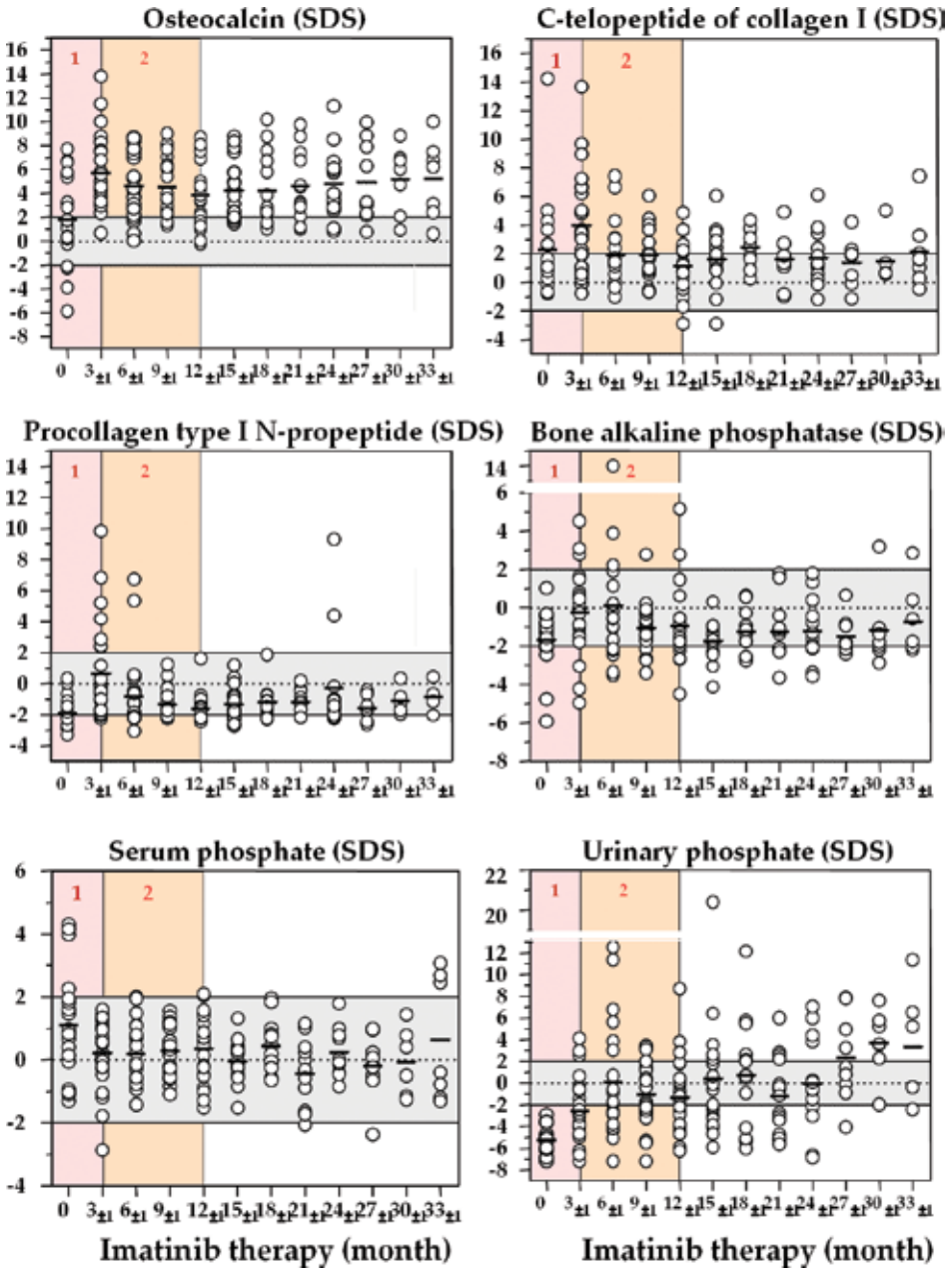

Figure 5. Biphasic response (time period 1 [pink background], period 2 [brown background]) of bone remodeling to imatinib treatment in pediatric patients with CML. Data depicted from CML-PAED II study [78]. One hundred and nineteen patients (70 male/49 female, median age 12 years, range 1–18 years) received 260–340 mg imatinib/m² daily within 1 week after diagnosis of CML (0). Up to 30 patients (range 20–30) out of this cohort could successfully be monitored repeatedly over a median period of 3 years for all parameters planned to be analyzed by collecting blood and urine for 3- months under appropriate circumstances. Assays were performed in a central laboratory as described previously [77]. Age normalized reference values were used as standard deviation scores (SDS).

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6. Non bone-related side effects of imatinib treatment

6.1. Growth hormone

Main length growth regulating factors at childhood and adolescence are GH and "insulin-like growth factor 1" (IGF-1), thyroid hormone (T3, T4), glucocorticoids, and sex hormones during puberty [79].

GH is secreted by pituitary somatotrophins in a pulsatile manner and acts on peripheral tissues, either directly or indirectly, through the stimulation of IGF-1 synthesis and secretion [80–82]. As reported, the increase in body height during childhood is initiated by promoting chondrocyte proliferation and endochondral ossification in the growth plate or induction of osteoblastogenesis, leading to linear bone growth [80].

Owing to growth, children and/or adolescents going through puberty are particularly vulnerable to a possible GH deficiency (GHD) under long-term imatinib treatment [5, 41]. Mimicking those findings in children on imatinib treatment, the juvenile animal model disclosed significantly lowered serum levels of IGF-1 binding protein 3 (IGF-BP3)—a stable and more accurately measurable degradation product of IGF-1—at all concentrations applied and at all ages investigated [37, 83, 84]. Data of clinical studies in pediatric CML patients under TKI therapy revealed IGF-1 and IGFBP-3 levels almost exclusively in the very low or deep pathological range when compared to age-matched controls, independent of treatment duration [83, 84].

6.2. Vitamin D and bone

Within the bone remodeling cycle, vitamin D plays a crucial role by influencing the overall mineralization and bone turnover of the skeleton. The main effects of the active vitamin D metabolite $1.25(OH)_2D_3$ comprises of stimulating the absorption of calcium/phosphorus from the gut to create optimal circumstances for bone mineralization, as well as stimulation of the osteoblast-mediated mineralization and osteoclast differentiation [85]. The consequences of vitamin D deficiency are secondary hyperparathyroidism and bone loss, leading to osteoporosis and fractures, mineralization defects, which may lead to osteomalacia in the long-term, and muscle weakness, causing falls and fractures [86].

Hypophosphatemia, associated with low serum levels of $25\text{-}(OH)D_3$, $1.25(OH)_2D_3$, calcium, and secondary hyperparathyroidism are known side effects in adult patients with CML under imatinib treatment [3]. An explanation for these findings is that imatinib directly stimulates bone formation while restraining resorption, resulting in a net flux of calcium from extracellular fluid into bone, a decreased serum calcium level, and a compensatory rise in the level of parathyroid hormone, which causes phosphaturia and modest hypophosphatemia [50]. Pediatric patients with CML also exhibit moderate secondary hyperparathyroidism in conjunction with pathologically low $25\text{-}(OH)D_3$ and $1.25(OH)_2D_3$ levels but normal serum calcium and phosphate levels under imatinib therapy [77, 78]. Thereby these effects were independent of the duration of imatinib therapy, which underlined once again that

regulation and compensatory mechanisms on the growing skeleton are different from those in the adult skeleton.

How imatinib interferes with vitamin D synthesis and metabolism is poorly understood yet. So far only one study investigated *in vitro* the effect of imatinib on keratinocytes yet and revealed a competitive inhibition of CYP27B1, a vitamin D hydroxylating enzyme, by imatinib [87].

6.3. Fertility

TKs like c-kit and PDGF-R, which are inhibited "off-target" by imatinib, are involved not only in the bone remodeling process but also in the regulation of spermatogenesis [88], raising the question of testicular toxicities by imatinib treatment. Up to now, the influence of TKIs on the male reproductive endocrine system in pediatric patients with CML is still controversially discussed [89].

The first study in neonatal rats revealed that imatinib interferes with postnatal testicular development [90]. Investigations in the juvenile animal model starting at an older age (4 weeks) depicted unchanged testis weight but reduced testosterone levels under long-term imatinib exposure until young adulthood. Inhibin B, a protein that is predominantly produced in the testis controlling follicle stimulating hormone (FSH) [91], did not significantly differ from controls, at all doses, and by all application schemes tested [92]. A clinical study conducted in a small cohort of boys (age: 7.8–18.9 years) with CML receiving TKI treatment revealed testosterone and inhibin B levels within normal age-related reference ranges [83, 84, 92]. Therefore, severe testicular toxicity by imatinib seems to be unlikely.

However, a closer look on spermatogenesis in the juvenile animal model revealed that the spermatogenic cell counts were significantly decreased by high dose imatinib exposure (**Figure 6**). Additionally, during spermatogenesis cell cycle, the stage of the dominant cell proportion was shifted to more immature stages. Low dose and intermittent imatinib exposure attenuated these findings. Interestingly, spermatogenic cell proliferation was significantly lowered at all imatinib doses applied [93]. Thus, a delayed negative effect of long-term imatinib exposure on spermatogenesis cannot be excluded.

6.4. Cardiac side effects of TKI treatment

In the literature as well as indicated by the manufacturers in the specialist information, cardiotoxic and vascular side effects of imatinib and the next-generation TKIs are of special concern [94–99]. However, this primarily may play a role in older adult patients with CML (age > 65 years) under TKI treatment.

The juvenile animal model under discussion disclosed an increase in the relative heart weight ratio (= ratio of the heart weight to total body weight at sacrifice) under imatinib exposure. Another study found that imatinib treatment led to mitochondrial-dependent myocyte loss and cardiac dysfunction, occurring more severely in older mice, in part due to

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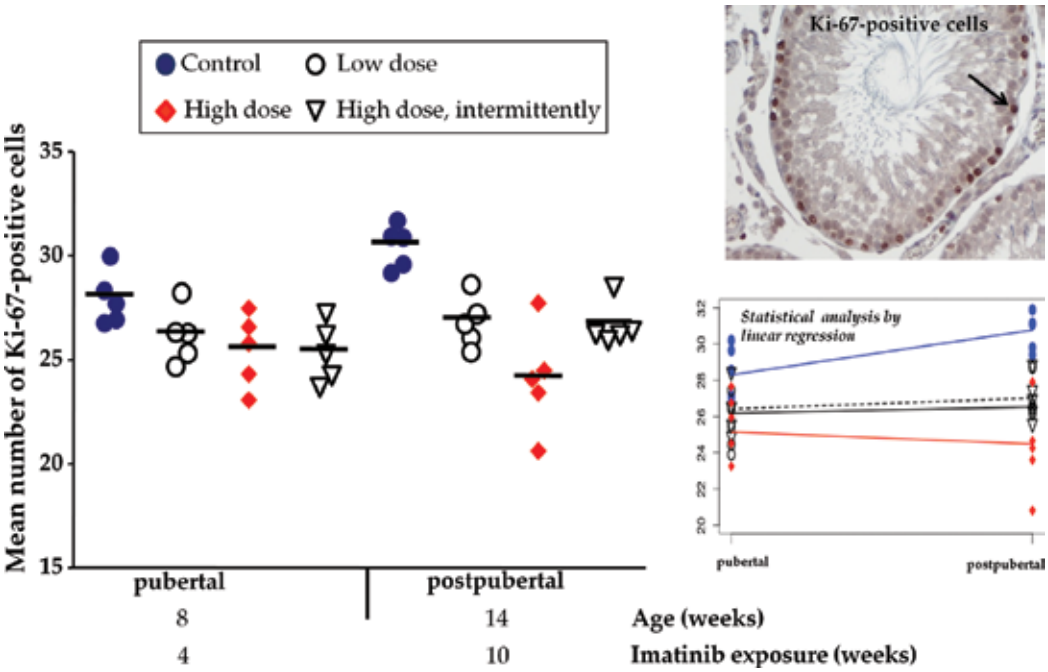

Figure 6. Number of proliferating testicular epithelium cells (Marker Ki67) in Wistar rats under imatinib exposure [93].

an age-dependent increase in oxidative stress [100]. This suggests that cardiac monitoring of older patients receiving imatinib therapy may be especially warranted.

As cardiac side effects were also observed with the use of dasatinib, in the experiment conducted with the juvenile animal model, this 2nd-generation TKI was tested for safety, efficacy, and dose response. Surprisingly, animals died spontaneously in a dose- and exposure time-dependent manner (**Figure 7**). Data of the surviving animals that were sacrificed according to the experimental set-up schedule (**Figure 3**) disclosed—dependent on the cumulative dose administered—increased relative heart weights, impaired heart ejection fraction as assessed by echocardiography, and elevated brain natriuretic peptide (BNP) serum levels, an indicator of cardiac dysfunction [101]. Data of this unexpected high toxicity can be explained by the serum elimination half-life time of dasatinib which is rather short and in the range of 2–3 h in rodents [102]. As known from clinical data on treatment of CML by dasatinib, it is not mandatory to achieve steady state drug blood levels as the intracellular concentration of dasatinib is responsible for efficacy, which is sufficiently achieved by once daily drug administration. Initial trials in humans based on drug administration twice daily were characterized by high toxicity requiring treatment interruption or reduction to once daily dosing [103]. Thus, the juvenile rat model also mimics this situation as a continuous intake of small doses of dasatinib via the drinking water evidently is associated with higher toxicity.

Initially, inhibition of the c-abl kinase was assumed to be the reason for cardiac toxicity by TKI [94]. But an extensive *in vitro* study of 18 TKIs on myocytes showed that their relative ability to inhibit ABL1 or ABL2 did not correlate with myocyte damage, revealing that inhibition

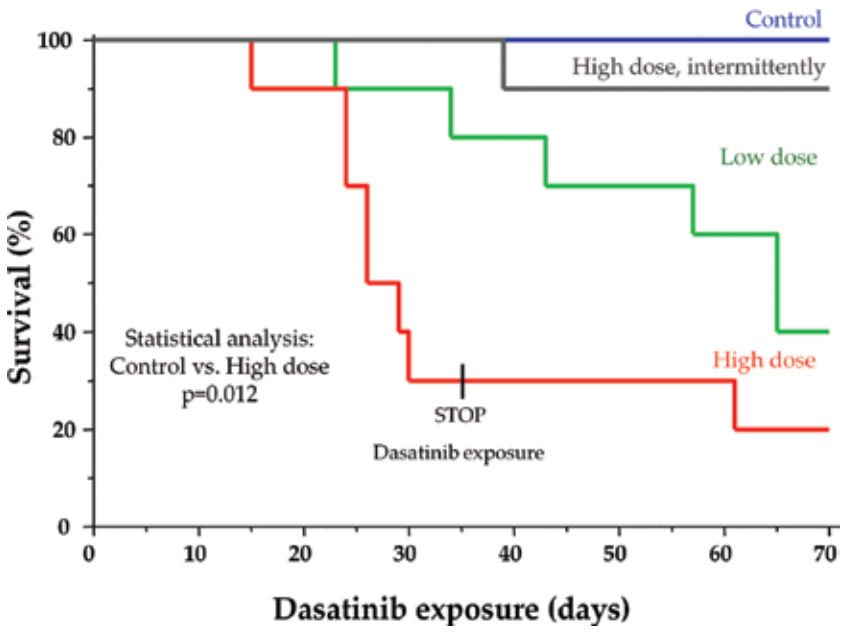

Figure 7. Survival rate of juvenile Wistar rats under chronic dasatinib exposure [101].

of other kinases like MEK1 and MEK2 could be responsible for the cardiotoxicity. However, it was reported that all TKIs induce myocyte damage correlating with their kinase inhibitor selectivity [97]. So, we conclude that it might be prudent to carefully monitor cardiac function in still growing individuals with CML if treated with TKI continuously over long periods.

7. Hypothesized model of osseous damage and clinical relevance

7.1. Model of action of imatinib on bone remodeling

Despite the knowledge accumulated so far, the detailed mechanism how imatinib impairs bone remodeling and growth remains yet speculative. In *in vitro* studies, it was shown that imatinib impairs osteoblastogenesis as well as osteoclastogenesis revealing its effect on bone remodeling [49, 50, 104, 105]. However, long bone growth is not only based on the balanced action of bone formation and bone resorption but also depends on the endochondral bone formation at the epiphyseal line of the long bones. Here, the column structure of the epiphyseal line, achieved and maintained by chondrocytes, is of main importance. In general, the epiphyseal line or growth plate is divided into different zones: reserve zone (RZ), proliferative zone (PZ), and the hypertrophic zone (HZ) followed by the primary spongiosa (PS)—the initial trabecular bone. The transition zone between HZ and PS is the osteochondral junction (OJ) (**Figure 8A**).

During growth, new cartilage is formed at one side of the epiphyseal growth plate and is gradually replaced by bone. The work by Nurmio et al. disclosed a disorganization of the epiphyseal line by imatinib treatment of neonatal rats (1–15 days old) (**Figure 9**) [106].

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Instead of the typical long, smooth proliferating chondrocyte columns at the epiphyseal line, a thin, disorganized layer of proliferative cells was detected after imatinib treatment resulting in a decreased thickness of PZ and increased the thickness of the HZ. This is in line with an *in vitro* study revealing an inhibitory effect of imatinib on chondrocyte proliferation [107]. Nurmio et al. also observed that imatinib treatment led to a bone resorption arrest and increased bone formation at the OJ [106].

However, combining our data [68] with data from Nurmio et al. [106] and Vandyke et al. [107], it can be hypothesized that imatinib exposure alters metabolism and remodeling of the growing bone in a temporal-spatial stepwise fashion (**Figure 8B**). In the first instance, migration, proliferation, and activity of chondrocytes will be impaired by imatinib leading to a disturbed organization of the growth plate impairing longitudinal bone growth [106]. Altered growth hormone secretion under imatinib treatment as shown before in the growing organism [39, 41, 83] may aggravate this growth impairment. Thereafter, ongoing drug exposure causes a spatial activity shifting of bone remodeling: initially, the formation will be elevated and shifted to the area of the osteochondral junction, whereas the activity of bone resorption remains unchanged but will be spatially shifted to the distal area of the trabecular bone [106]. Finally, under long-term imatinib treatment, osteoblastogenesis and osteoclastogenesis will be impaired [104, 108], hampering bone remodeling during growth.

As an interesting approach, our juvenile animal model demonstrated that intermittent imatinib exposure will ameliorate growth impairment in rats. The inhibitory effect is not irreversible and we assume that during the days "OFF" imatinib exposure catch-up growth occurred. Therefore, drug administration following a schedule with "days on drug" and "days off drug" might reduce some skeletal side effects in pediatric patients. A single trial in older adults has already proven that intermittent TKI treatment is sufficient to control CML once remission

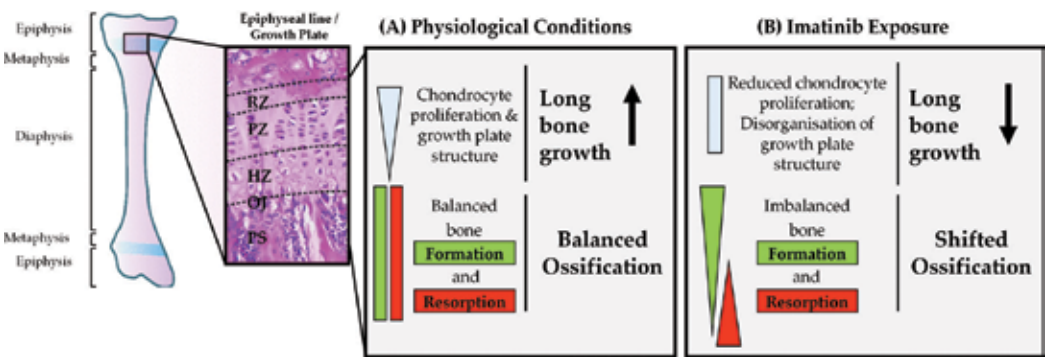

Figure 8. Schematic overview of physiologic bone growth (A) and under imatinib exposure (B). On the left side, longitudinal section of the epiphyseal line of a rodent proximal tibial metaphysis is depicted [109]. The epiphyseal plate separates the epiphysis from the metaphysis and is important for endochondral bone formation. The growth plate is divided into reserve zone (RZ), proliferative zone (PZ), and the hypertrophic zone (HZ). The transition of HZ to the primary spongiosa (PS)—the initial trabecular network formed after the vascular invasion and matrix calcification—is the osteochondral junction (OJ). Under physiological conditions, longitudinal growth occurs by endochondral ossification. In this process, new cartilage is formed at one side of the epiphyseal growth plate and is gradually replaced by bone. Chondrocytes of the growth plate are initially in a resting state in the RZ. They differentiate through proliferative and hypertrophic stages (PZ, HZ) as the growth plate moves past. This programmed differentiation pathway ends in cell death in the HZ and the replacement of cartilage by bone by osteoblasts in the OJ resulting in the PS. (For detailed review, see Ref. [110]).

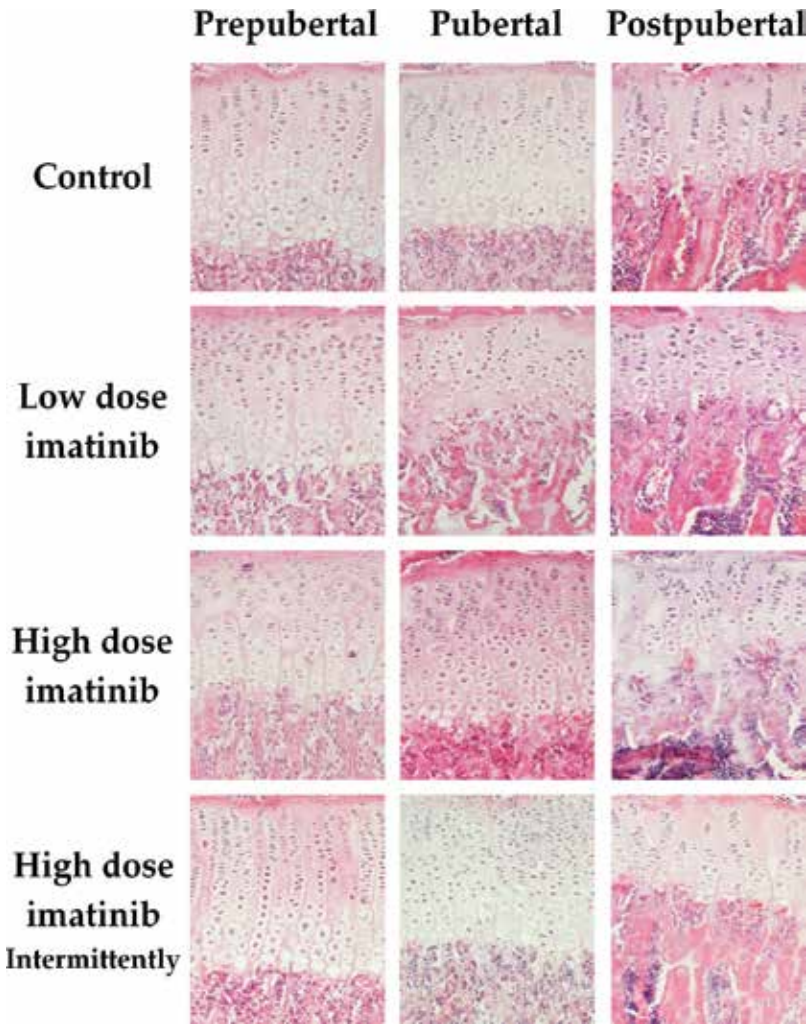

Figure 9. Disorganization of the femoral epiphyseal line by long-term imatinib exposure. 2 μm sections of decalcified femora were stained with hematoxylin-eosin (magnification 100 ×). Controls show the typical "column" structure of the epiphyseal line and its physiological narrowing with increasing age. However, under imatinib exposure, the cellular architecture is more disorganized in a dose- and time-dependent manner.

has been achieved [111, 112]. However, the length and frequency of intervals to allow catch-up growth in children on TKI treatment still have to be defined and at least in our rat model, this approach did not recover the biomechanical strength of the long bones.

7.2. Clinical relevance

The established juvenile rat model mimics to a gross extent side effects of long-term TKI exposure on the growing bone in a developmental stage-dependent fashion. Impairment of longitudinal growth, as observed in children under imatinib treatment, could be unequivocally modeled and confirmed.

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Our hypothesis of spatiotemporal shifting of skeletal formation and resorption under imatinib is supported by clinical observations of a biphasic reaction of corresponding osseous metabolism serum markers in adult and pediatric patients with CML. In adult patients, an increase in bone formation occurred accompanied by elevated bone formation markers in the serum within the first months of therapy [74]. Pediatric CML patients display a biphasic response of bone formation and bone resorption by increasing levels within the first 3 months of imatinib treatment followed by a significant decline until 12 months of treatment (**Figure 5**) [77, 78].

Furthermore, we could show that long-term imatinib exposure may result in reduced bone strength possibly posing an elevated fracture risk in pediatric patients. Since 2001, adult CML patients are treated with imatinib but until now, no elevated fracture rates have been described in these patients [113]. As pediatric CML patients are treated with imatinib only since the beginning of this millennium, there is still no long-term experience. Our animal model also revealed that intermittent imatinib treatment mitigated skeletal effects on the growing bone, thus pointing toward a possibility to improve the risk-benefit ratio of long-term TKI exposure in pediatric patients. First clinical data in adults look promising but further studies must be carried out to determine whether the intermittent exposure is also sufficiently effective for the control of CML [111, 112]. Regarding pediatric patients, the results from the juvenile animal model and the clinical experience from adult patients with CML should be combined. This approach can be expected to harbor great potential in translational research.

8. Other animal models

The aim of the animal model described in this chapter was to evaluate side effects on bone remodeling rather than gaining further insight into the biology of CML (e.g., to study elementary mechanisms of CML disease progression) or on a more efficient antileukemic treatment exerted by new drugs (e.g., exploring why resistance develops under TKI therapy) [114, 115]. For these essential questions, the reader is kindly referred to the detailed body of literature on establishing and maintaining acute lymphatic or myeloid leukemic cells in xenograft models, transgenic models, and syngeneic models using a broad range of species [116–119], whereas mice are used mostly in orthotopic animal models [120–123].

Our research described, focused on the question how bone metabolism is affected by TKI treatment as an off-targeted side effect and therewith induced structural and mechanical osseous changes in healthy not-outgrown animals [124]. Bone remodeling has been studied in many species and resulted in the current available knowledge [125–131]. Evidently, the financial burden of animal maintenance and drug doses to be administered when sequelae of chronic exposure are investigated are much lower using small animals like mice and rats. Especially in these species, the time periods concerning defined stages of development are shorter, thus requiring drug exposure only for 2–3 months in order to mimic one to two decades in humans [132].

Most importantly, any intervention on the bone during chronic TKI exposure of the animals was minimized. Bone growth and repair is governed by regulatory mechanisms other than that of the outgrown organism. Therefore, the model described here differs principally from

experiments investigating bone healing and growth after surgical procedures performed on the skeleton (for a comprehensive review see Refs. [131, 133, 134]).

Ethical concerns in the last decades resulted in the establishment of studying bone growth and development preclinically in *ex vivo* cultures mostly making use of embryonic bone of mouse or rat strains [135, 136]. For an overview on conventional versus static versus 3D dynamic bioreactor models as well as a chorioallantoic membrane (CAM)-culture systems, the reader is kindly referred to a comprehensive review by Abubakar et al [137]. The composition of the nursing cell culture medium in these models is a crucial step. However, concerning TKIs whose metabolism in juvenile rodents is still poorly characterized and pleiotropically influences bone remodeling (e.g., impact on synthesis of growth hormone and insulin-like growth factor, liver metabolism, vitamin D metabolism, renal function, etc.) evidently not all components can be added to a cell culture medium mimicking correctly the *in vivo* situation. Therefore, our investigations had to be restricted to a genetically unchanged—"healthy"—animal model to study the side effects of long-term TKI exposure on bone remodeling during growth and in addition on other developing organs.

9. Conclusion

Long-term toxicity resulting from off-target effects of TKIs can be assessed conveniently by administering TKIs via the drinking water to juvenile male Wistar rats over a prolonged period. During all developmental phases (prepubertal, puberty, postpubertal, and adult), drug blood levels are obtained corresponding to data in humans. The juvenile animal model disclosed reduced long bone length and diminished vertebral height combined with reduced bone mass density and reduced breaking strength dose-dependently after chronic exposure to imatinib. Thus, the juvenile animal model depicted here mimics perfectly clinical observations on osseous changes observed in pediatric patients with CML. Furthermore, intermittent exposure of the high TKI dose mitigated the skeletal side effect and therefore represented a possible treatment option for pediatric patients suffering from longitudinal growth retardation under imatinib therapy. The juvenile animal model might also be of value to predict sequelae of TKI treatment in other human organs following exposure over decades.

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Chapter 16

Animal Models of Fetal Medicine and Obstetrics

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Abstract

Animal models remain essential to understand the fundamental mechanisms occurring in fetal medicine and obstetric diseases, such as intrauterine growth restriction, pre-eclampsia and gestational diabetes. These vary regarding the employed method used for induction of the disease, and differ in relation to the animal characteristics (size, number of fetuses, placenta barrier type, etc.). While none of these exactly mirrors the human condition, different pregnant animal models (mice, rats, guinea pigs, chinchillas, rabbits, sheep and pigs) are here described with respect to advantages and limitations. The ability to employ noninvasively diagnostics varies among species, specifically for ultrasound and clinical magnetic resonance imaging procedures. Management of feeding, handling, care and anesthesia are particularly important factors in the pregnant animal.

Keywords: animal models, fetal medicine, diagnostics and imaging, handling of pregnant animals

1. Introduction

Obstetrics deals with pregnancy, childbirth and the post-natal period, whereas gestation (from Latin: "to carry") is the time between conception and birth. Gestation is typical for mammals, where an embryo/fetus develops in the uterus.

Research in the pregnant human is problematic and may pose significant ethical restrictions as the well-being of the mother and her unborn baby is critically important. Thus, the use of animal models provides a way to gain insight into the improved understanding of the human pregnancy. Animal models remain essential to understand the fundamental mechanisms underlying the onset of obstetric diseases, and to discover improved methods for prevention, diagnosis and treatment. However, the translatability between animals and humans should be carefully considered. In obstetric research, several factors may contribute in the selection of the most appropriate animal model, concerning the mother, fetus and placenta. In addition, the timing of the study during the gestation needs to be considered, as pregnancy is a dynamic process.

The aim of this chapter is to review the advantages and limitations of relevant animals, including mouse, rat, chinchilla, guinea pig, sheep and pig models, and their use in studying fetal growth disorders (intrauterine growth restriction, IUGR), preeclampsia and diabetes in pregnancy. Furthermore, available imaging modalities for studying pregnant animals including fetal and placental characteristics are presented. Finally, ethical and welfare considerations are described, as well as how physiological effects of pregnancy pose special requirements to the management of feeding, handling, care and anesthesia.

2. Considerations

Knowledge of the different characteristics of the animal and its gestation is a prerequisite in order to select the most suitable animal model, interpret experimental findings and reach appropriate translational conclusions. In obstetric research, in particular, it is necessary to consider fetal/neonatal characteristics and the physiological changes during the gestation period. Several species have been used to study the normal pregnancy and related pathological conditions [1]. First, the human gestation is described for comparison.

3. Human pregnancy and fetal development

The human gestation is about 280 days and is divided into three trimesters, each of which is marked by specific fetal developments and embryonic changes. The first trimester is from gestation week (GW) 1–12, including the conception, second trimester is GW 13–28 and third trimester is GW 29–40. In other mammals, the gestation is defined as the time between conception/fertilization and birth, which for comparison is 266 days in humans. A single fetus is carried in 97–98% of all human pregnancies. The human newborn is extremely dependent on the mother and has immature motoric skills which traditionally placed the neurodevelopment of the human newborn as *altrical* (from Latin: "to nurse"), referring to the undeveloped motoric system. However, the advanced development of the human brain at birth rather places the human newborn as *precocial*, meaning well-developed at birth [2]. The human brain at birth is more advanced than all other animal models used in research [3].

The placenta is the interface between the maternal and fetal circulation, facilitating an exchange of oxygen, nutrients, waste products and other molecules, for example, certain drugs. The fetal trophoblast cells form the external component of the placenta, the *chorionic plate*. The nomenclature of the placenta barrier refers to the degree of erosion of the maternal tissue in the uterine cavity and the interface between the maternal and fetal circulation. The placental interface differs greatly between species (**Figure 1**). Humans have *hemomonochorial* placenta barrier because the maternal blood (*hemo-*) is in direct contact with only one layer of trophoblasts (*−mono*) in the chorion plate (*−chorial*). Thus, the human placenta is implanted completely within the uterus with a deep invasion of the trophoblasts and erosion of the uterine epithelium [4]. In rodents (mice and rats), the placenta barrier is *hemotrichorail* with three layers of trophoblasts dividing the maternal blood from the fetal capillaries in the chorionic plate [5]. Another nomenclature used for the microscopic structure of the placenta exchange area refers to the villous or labyrinth type. The human placenta is of the villous type where chorionic vessels branch out with few interconnections. In a placenta of the labyrinth type, the fetal vessels, the trophoblasts and the maternal blood space branch out and are interconnected in a complex labyrinthine pattern [5].

3.1. Gestation length

A short gestation time (or rapid reproduction) is sometimes considered an advantage to obtain a high experimental productivity or for economic reasons. However, if repeated procedures are required during the gestation time, a longer gestation period is usually preferred. A longer interval between the experiments allows for longer restitution and thereby, reduces the induced stress response in the animals. In addition, surgical manipulation might be difficult to employ in animals with a short gestation. During a long gestation time, the response of environmental or physiological influences on the fetal development could also become more pronounced.

3.2. Number and size of fetuses

Occurrence of a single fetus in uterus is obviously preferred for individual fetal monitoring. A small number of fetuses will often correlate with bigger fetal size [6]. A bigger fetus makes it possible to receive a higher spatial resolution and sensitivity using non-invasive diagnostic tools, for example, clinical magnetic resonance imaging (clinical MRI) and computerized axial tomography (CT or CAT). Furthermore, surgical procedures are easier to perform. However, larger litter sizes provide a higher sampling size per gestation, and thus, the number of animals used can be reduced in accordance with the "3 R's" (see Section 9).

3.3. Placentation

Many differences exist in relation to placentation in the different animal models, such as the development and changes of the placenta during the time of gestation, blood flow, transfer of oxygen, nutrients and waste products, metabolic, endocrine and immunologic function [4].

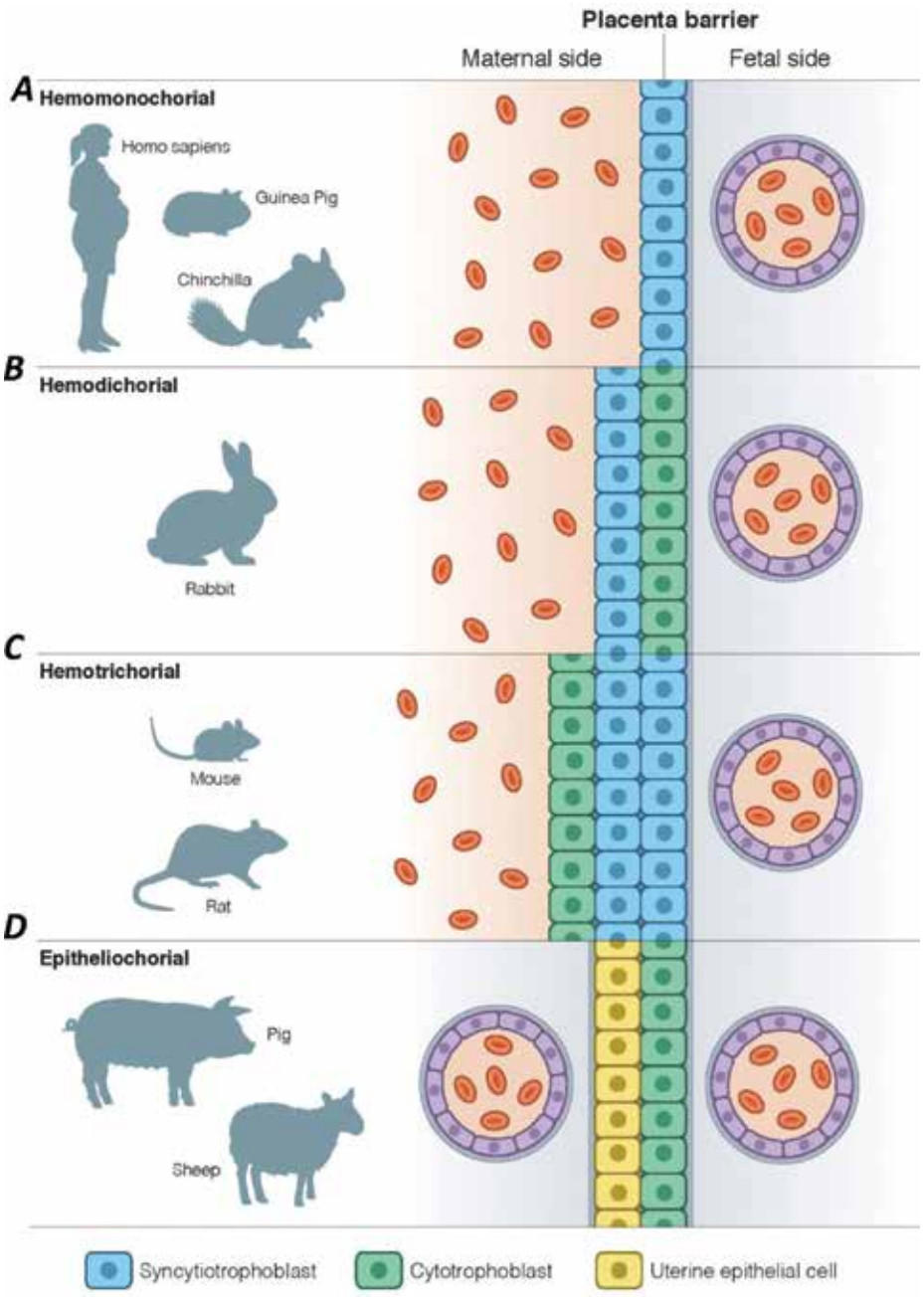

Figure 1. Schematic presentation of different placenta barriers as seen in a microscope. (A) Hemomonochorial placenta barrier as seen in, for example, human, guinea pigs and chinchillas. Only one layer of syncytiotrophoblasts separates the maternal blood space from the fetal capillaries. (B) Hemodichorial placentabarrier as seen in the rabbit. One layer of syncytiotrophoblasts and one layer of cytotrophoblasts separate the maternal blood space from the fetal capillaries. (C) Hemotrichorial placenta barrier as seen in, for example, mice and rats. Three layers of trophoblast cells separate the maternal blood space from the fetal capillaries. (D) Epitheliochorial placenta barrier as seen in, for example, sheep. One layer of uterine epithelium cells and one layer of trophoblast cells separate maternal and fetal capillaries. Furthermore, in all three cases, maternal and fetal blood is separated by connective tissue and basal laminae.

In relation to drug transfer, it seems obvious to use animal models to study the passage across placenta and potential teratogenic or toxic effects, but unfortunately the transplacental transfer and the placental metabolic demand vary greatly among species [7]. Drugs may transfer across placenta by passive transport, active transport or facilitated transport, whereas lipid-soluble molecules with a small molecular size can cross the placenta by passive diffusion. In that regards, the guinea pig seems as a more human translatable model compared to, for example, the sheep, because the guinea pig has a thin *hemomonochorial* placenta barrier compared to the thicker *epitheliochorial* placenta barrier in the sheep [7]. For hydrophilic molecules, the passive diffusion is negligible, and the transport capacity varies widely between species depending on the transport proteins located in the trophoblast cells. For example, the antidiabetic drug metformin, a hydrophilic molecule, is in humans transported across the placenta by organic cation transporters (OCTs). When studied in an animal model, it is highly relevant to identify the specific OCT transporters in the pregnant animal to verify the expression of the transport proteins [8]; otherwise the translatability has little value.

4. Animal models

Table 1 shows the average gestation length, number of fetuses, maternal weight, neonate weight and the placental barrier type in human and relevant species.

Basal gestation parameters of the laboratory animals

Animal species *Latin*	Gestation length (days)	Number of fetuses	Maternal pre-pregnancy weight (g)	Neonate weight (g)	Placenta barrier type
Human *Homo sapiens*	266	1	5900	3183	Hemomonochorial villous
Mouse *Mus musculus*	20	5–6	19	1	Hemotrichorial labyrinth
Rat *Rattus norvegicus*	22	9	283	6	Hemotrichorial labyrinth
Guinea pig *Cavia porcellus*	67	3–4	728	80	Hemomonochorial labyrinth
Chinchilla *Chinchilla lanigera*	113	1–2	480	40	Hemomonochorial labyrinth
Rabbit *Oryctolagus cuniculus*	30	5	1591	39	Hemodichorial labyrinth
Sheep *Ovis aries*	153	1–2	39.100	2376	Epitheliochorial
Pig *Sus scrofa*	115	5–14*	84.000*	400–1900[a]	Epitheliochorial

Data are acquired from the PanTheria database [82].*

Dependent of the breed of pig (domestic pig or mini-pig) [20].

Table 1. Basal gestation parameters in pregnant animal models.

4.1. Mice

Mice and rats are the most used species in research. Practical advantages include relatively low costs, an easy maintenance and a long tradition in scientific research. Mice have an important advantage in many genetic manipulated models along with inbreed strains. The mouse has a short gestation period of around 20 days, and it carries a litter size of 5–6, which allows for quick data collection. The placenta is of the *hemotrichorial* labyrinth type [5]. The newborn mice are neurodevelopmental immature with closed eyes. Because of the large litter size, it is difficult to measure and follow individual fetal and placental progress. In addition, the small size makes surgical procedures difficult.

4.2. Rats

Rats have a long tradition as research models because the intrinsic properties, like the physiology and macro- and microanatomy, are well-known [9]. Rats pose some of the same advantages and disadvantages as mice; a short gestation period (around 22 days), large litter size (around 9 fetuses) and placental structure of a *hemotrichorial* labyrinth type (**Figure 1**). The considerable larger size of rats compared to mice makes them more suitable for surgical procedures and diagnostic imaging. Unfortunately, the genetic manipulation is much less developed in rats than in mice, but this may become more pronounced in the future [10].

4.3. Guinea pigs

The guinea pig has a gestation length of around 67 days and gives birth to 3–4 precocious offspring with a well-developed nervous system at birth [2]. These characteristics make newborn guinea pigs suitable for research in fetal development. The placenta barrier is *hemomonochorial* (**Figure 1**), and it is histologically comparable with the human placenta barrier. In fact, the guinea pig is a well-established model to study placentation, and suggested to become one of the most important animal model for new placental studies in obstetric research [4]. They are affordable and easy to maintain in research environments. Intravenous approaches can be more complicated than for mice and rats due to the lack of a long tail.

4.4. Chinchillas

The chinchilla is not a traditional animal model in obstetric and fetal medicine. The chinchilla has mainly been used to study diseases of the ear due to similarities with human anatomy and function [11]. However, several characteristics of the gestation make the chinchilla a suitable model to imitate human pregnancy. Like the guinea pig, the chinchilla gives birth to 1–2 precocious offspring and has a *hemomonochorial* placenta barrier. Chinchillas have the longest gestational period (around 113 days) of any rodent, which is advantageous in longitudinal studies. The chinchilla has recently been used to study the placenta metabolism using hyperpolarized magnetic resonance imaging (MRI) [12]. Genomic and RNA sequencing information are available in this species [13]. Chinchillas are relatively cheap and easy to maintain in a research environment. However, the chinchilla has so far not been used to investigate intrauterine growth restriction (IUGR), preeclampsia or diabetic pregnancy.

4.5. Rabbits

The rabbit is known for its rapid reproduction with a short gestation of only 30 days and a litter size of around five cubs. Because coitus induces the ovulation, it is possible to time the gestation and obtain a precise age of the fetuses, which is of practical experimental advantage. In particular, the rabbit has been used to study reproduction and early embryogenesis [14]. The larger size of rabbits compared to rodents facilitates various diagnostic techniques, such as ultrasound imaging, allowing structural information about the fetal size and hemodynamic characteristics [15], and even fetal and placental vasculature and hemodynamics can be studied by Doppler ultrasonography [16]. The rabbit has a *hemodichorial* placenta barrier of the labyrinth type (**Figure 1**). The thickness of the trophoblast cells alternates, resulting in thick and thin areas of the barrier [5].

4.6. Sheep

The sheep has a gestation length of 153 days and gives birth to 1–2 neurodevelopmentally matured lambs with about the same weight as a human newborn [17]. Therefore, the sheep is a translatable model for investigating fetal physiology. However, the placenta structure is very distinct from the human placenta. The placenta barrier is of the *epitheliochorial* type where the uterus remains intact without invasion of the trophoblast cell. Thus, the fetal and maternal blood are divided by an intact uterine epithelium (**Figure 1**). The missing trophoblast invasion and no erosion of the uterine epithelium lead to a description of the placenta as "superficial" [5]. Sheep are easy to handle, and pregnant sheep tolerate invasive procedures [17].

4.7. Pigs

The anatomical and physiological similarities to humans make the pig an excellent animal model in, for example, research of metabolic, cardiovascular, infectious diseases, xenotransplantation and neurological disorders. Surgical and anesthetic procedures are well established in the pig [18], and the genome is today fully sequenced in parallel with the existence of an important homology between the human and pig genome [19]. However, regarding the gestation, the pig has some important differences from the human pregnancy. The pig, like sheep, has an *epitheliochorial* placenta barrier (**Figure 1**), where the uterine epithelium remains intact during the entire gestation period [5]. Depending of the type of pig, it gives birth to 5–14 piglets. The domestic pig has a litter size of 10–14 and a birthweight of 1.3–1.9 kg, whereas breeds of minipigs, like the Yacatan and Göttingen, has a litter size of 5–8 with a birthweight of 0.4–1.0 kg [20]. They have the same gestation length of around 115 days. Piglets are well-established models in fetal and neonatal research [21] and have been used, in particular, to study neonatal physiology in response to physical activity and nutrition [22].

5. IUGR models

Intrauterine growth restriction or retardation (IUGR) occurs when a fetus does not reach its genetic growth potential, mostly due to placental insufficiency with limited offer of oxygen

and energy; caused by multiple factors, that is, smoking, preeclampsia or multiple pregnancy. IUGR affects up to 8% of all human pregnancies and may lead to serious complications in the newborn. Similarly, IUGR also initiates late-onset diseases, such as diabetes and cardiovascular diseases. The most commonly used IUGR animal model is the rat, but pigs, guinea pigs, mice, rabbits and sheep have also been studied for this purpose (**Table 2**). Six different methods have been reported to obtain an IUGR animal model: (1) diet-induced IUGR, (2) heat-induced IUGR, (3) IUGR induced by artery ligation, (4) hypoxia-induced IUGR, (5) embolization-induced IUGR and (6) glucocorticoid-induced IUGR. The most frequently used methods are the diet and ligation approach.

5.1. Diet-restriction IUGR

Diet-induced IUGR has mainly been performed using either calorie restriction or low-protein diet. Calorie restriction is often provided via a 50% restriction diet as notably programs insulin resistance and hypertension [23]. This approach has been adopted by López-Tello, demonstrating a diet-induced IUGR rabbit model, where animals were offered 50% of daily global nutrition, allowing investigations of the early changes in fetoplacental hemodynamics [24]. Interestingly, they found that neonates from this group were significantly smaller than those in the control group, which were offered food *ad libitum* throughout the pregnancy, and that the IUGR-induced animals showed asymmetrical growth and brain sparing. Furthermore, the restriction diet provided a significant altered blood flow perfusion. Hawkins et al. investigated the impact of maternal malnutrition in early gestation on the ovine blood pressure and cardiovascular reflexes, also by reducing maternal global nutrition, but in this study only by 15% in the first 70 days of gestation [25]. This study showed that even mild maternal undernutrition altered fetal cardiovascular development and produced a low blood pressure. However this redcution was not sufficient to induce IUGR.

Low-protein diet in fetal programming features different compositions of macronutrients. The Southampton diet (SH) and the Hope farm diet (HF) is often used in fetal programming (**Table 3**). The main difference between these two diets is the amount of starch, simple sugars (sucrose and glucose) and lipids (corn oil and soy oil) vary, whereas SH has high starch

Animals	**Methods**					
	Diet induced	**Artery ligation**	**Heat induced**	**Embolization**	**Hypoxia**	**Glucocorticoid**
Rats	[83]	[84]			[31]	[85]
Mice	[86]	[27]			[30]	
Guinea pigs	[87]	[28]				
Rabbits	[24]	[88]				
Sheep	[25]		[32]	[33]	[89]	[90]
Pigs	[91]					

Table 2. IUGR models (number refers to reference list).

Diet	Protein	Fibre	Starch	Simple sugars	Lipid	Methionine
Southampton diet 9%	Casein 9%	5%	48.5%	Sucrose 23.4%	Corn oil 10%	0.5%
Southampton diet 12%	Casein 12%	5%	46.5%	Sucrose 24.3%	Corn oil 10%	0.5%
Hope Farm diet	Casein 9%	5%	8%	Glucose 66,7%	Soy oil 4,3%	0.2%

Table 3. Different low-protein diets used for fetal programming.

content (42–51%), corn oil (10%) and sucrose (21–24%) and HF has low starch (8%), soy oil (4.3%) and glucose (53–67%). They also differ in their impact on the offspring; the SH has shown to program hypertension whereas HF programs insulin resistance [26].

5.2. Ligation-induced IUGR

IUGR induced by artery ligation is frequently used in animal research. Ligation intends to reduce blood flow and thereby oxygen and nutrition to the fetus. This approach has been introduced in relevant animals (**Table 4**). Notice that all the listed animals have bicornated uteruses while humans have a simple pyramid-shaped uterus [1]. These animals have two large horns and each have their own blood supply, allowing animal to act as both control (one horn) and case (another horn). Ligation is performed on the uterine vessel and can be performed either unilaterally (on only one of the horns) or bilaterally.

The timing and the site of ligation is of important matter in the ligation-induced IUGR model. Ligating at the distal portion of the uterine vessel implies a complete blockage of the iliac artery and the uterine blood supply is then solely dependent on the ovarian artery. Conversely, when ligating at the central portion of the uterine vessel, the blood supply comes from both the ovarian and iliac artery, resulting in a less affected uterine blood delivery.

Animals	Methods				
	Artery ligation	NO reduction	RAS-related models	Immunological	Transgenic models
Rats	[37]	[40]	[92]	[47]	
Mice		[42]	[43]	[48]	[50]
Guinea pigs	[93]		[94]		
Rabbits	[95]				
Sheep	[96]				
Pigs				[97]	

Table 4. Preeclampsia models (number refers to reference list).

Janot et al. demonstrated that ligating on the central portion of the uterine vessel was necessary to maintain a viable pregnancy, by establishing IUGR models in mice with ligation at either positions [27]. Mice ligated at the distal portion had a 100% abortion rate and a 50% mortality rate. In contrast, mice ligated at the central portion had an abortion rate of 75% (but still inducing a characteristic IUGR profile) and no maternal mortality. Herrera et al. used an ameroid occlusion to ligate the uterine artery bilateral in guinea pigs at day 35 of gestation [28] (**Figure 2A**). The occlusion led to an increased placental vascular resistance associated with a decreased fetal and placental weight, and the study also showed asymmetrical growth of the fetal organs.

5.3. Hypoxia-induced IUGR

Hypoxia has been shown to affect the size of the offspring pathologically and functionally [29]. Hypoperfusion of placenta increases the amount of reactive oxygen species, causing oxidative stress and a reduced vasodilation. Ligation, as described earlier, also causes hypoperfusion of placenta creating hypoxia, but in this section hypoxia will be refered to as reduced environmental oxygen saturation. Rueda-Clausen et al. studied the impact of hypoxia on IUGR and preeclampsia in mice [30]. Mice were mated and randomly assigned to either cases or controls. Cases were placed in a sealed chamber for 3 days with an oxygen concentration of 10.5% ± 0.3% (normal oxygen content is 20% in atmospheric air) and then placed in clean cages. This prolonged lack of oxygen significantly induced IUGR, but the pub survival was down to approximatly 10%. Tapanaien et al. found that rat dams having an oxygen concentration of 13–14% induced IUGR with a birthweight of 24% lower than controls (20% oxygen), but without significant fetal death, suggesting that an oxygen concentrations of 13–14% may become beneficial for inducing of IUGR [31].

5.4. Additional methods for IUGR

5.4.1. Hyperthermia

Galan et al. initiated a study by exposing five pregnant ewes to hyperthermic conditions for 80 days, initiated from the 40th gestation day [32]. The ewes were exposed to 40°C during the day and 35°C during the night. The study established an interesting IUGR model with some similarities with the human IUGR (asymmetrical growth, hypoxia and hypoglycaemia). Even though this method successfully induced IUGR, a more widespread use of this hyperthermic-based IUGR model could become difficult due to animal ethical restrictions, and this method has only been reported in sheep.

5.4.2. Embolization

Duncan et al. induced IUGR by injecting microspheres of 15–30 μm into the umbilical-placental vascular bed from day 120 of gestation in a sheep model [33]. This procedure reduced the fetal oxygen saturation to 50%, resulting in significantly reduced growth and significant altered pH, SaO_2 and pO_2.

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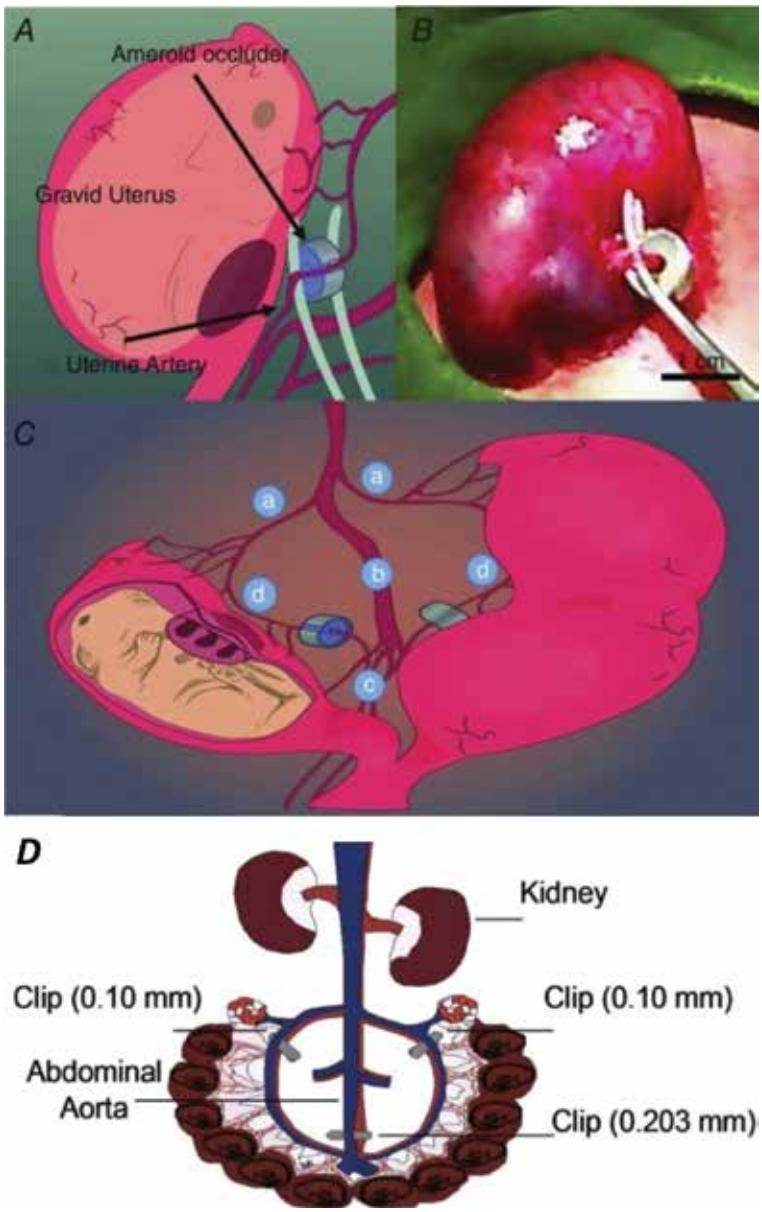

Figure 2. Ameroid occludder placement (reproduced from ref. [28]). Schematic representation (A and C) and photograph (B) of the placement site of the ameroid constrictors in the uterine artery of a pregnant guinea pig at 35 days of gestation. C shows the maternal artery supply to the uterus in gunea pigs; a, ovarian arteries; b, aorta; c, uterine arteries; d arcade arteries. D shows induction of reduced uterine perfusion pressure (RUPP) model in pregnant rats (reproduced from ref. [37]). In the rat RUPP model, laparotomy is performed through an abdominal incision on day 14 of gestation. A silver clip with a 0.203-mm internal diameter is placed around the aorta right above the iliac bifurcation, and silver clips with 0.1 mm internal diameter were placed around the left and right uterine arcade at the ovarian artery before the first segmental artery. Uterine perfusion pressure in the gravid rat is reduced by ~40%. Blood pressure is measured via a carotid arterial catheter.

5.4.3. Glucocorticoid

Exposure to glucocorticoid during pregnancy has long been known to be associated with a low birthweight and concomitant adult diseases. When investigating the effect of glucocorticoid, it is necessary to distinguish between natural cortisol and synthetic glucocorticoid. Previous studies have shown that dexamethasone induces hypertension in rodents whereas cortisone acetate and betamethasone do not [23]. Additionally, different species and sex may react differently to glucocorticoid exposure. When looking at long-gestation mammals, the timing of glucocorticoid exposure is essential. Exposure to glucocorticoid in the pregnant ewe in the early gestation has shown to induce hypertension in adulthood of the offspring, whereas exposure late in gestation promoted insulin resistance rather than hypertension in the offspring. Glucocorticoid exposure can be administered subcutaneously, through maternal drinking water or intraperitoneal injections [23].

6. Preeclampsia models

Human preeclampsia is a multiorgan disorder with onset after the 20th week of gestation. The dignostic criteria includes a blood pressure that exceeds 140 mmHg (systolic) and 90 mmHg (diastolic) and simultaneous dection of proteinuria. The condition can lead to kidney failure, liver rupture, stroke, eclampsia with seizures and HELLP syndrome [34]. The definitive pathogenesis of preeclampsia is yet to be found but may be associated with oxidative stress, angiogenic factors, an immunological response between mother and placenta or superficial placentation [35]. Appropriate animal models of preeclampsia must meet the following criteria (**Table 4**): they should initiate hypertension, proteinuria and endothelial dysfunction, and furthermore, resolve after delivery of the placenta [34]. Preeclampsia is presumably caused by reduced uterine blood flow due to abnormal trophoblastic invasion in spiral arteries. This has implicated the need of an animal model of reduced uterine perfusion pressure to study the mechanisms within preeclampsia. In 1940, one of the first studies describing this correlation was performed [36], demonstrating pregnancy-mediated hypertension in dogs following partial ligation of the infrarenal abdominal aorta. This ligation procedure has subsequently been performed in rabbits, monkeys, sheep, primates, guinea pigs and rabbits [37]. One of the best-described studies was performed in baboons [38], showing that hypertension occurred in parallel with renal changes due to uteroplacental ligation, supporting the view that hypoxia/ischemia participates in the potential mechanisms underlying the pathogenesis of preeclampsia. Rodents are also reported as important ligation-induced models of preeclampsia. Preeclampsia in rats has been established by clipping around aorta, above the iliac arteries, and at both uterine arteries, at day 14 of gestation (**Figure 2D**), providing characteristic pathological conditions, including hypertension, proteinuria and renal impairment [37].

6.1. Additional methods

6.1.1. Nitrogen oxide reduction

Another approach to stimulate the conditions of preeclampsia is to manipulate genes thought to influence the pathogenesis. NO production is reduced in preeclampsia [39], and several

studies have been performed to mimic this pathogenesis [40],[41]. A study by Molnár et al. inhibited the NO synthase in pregnant rats [40], resulting in hypertension, proteinuria, thrombocytopenia and IUGR; all characteristic findings that were considered consistent with preeclampsia. However, one study in eNOS knockout mice showed, controversially, a decreased blood pressure [42].

6.1.2. RAS-related models

Women with preeclampsia have general elevated levels of autoantibodies (AT_1-AA) that bind and activate the angiotensin II type 1a receptor, mediating augmented blood pressure. To imitate this pathogenesis, Zhou et al. successfully injected purified AA_1-AA into pregnant mice, resulting in hypertension, proteinuria, placental abnormalities, glomerular endotheliosis and small fetus size [43]. This study also showed that co-injection of losartan (AT_1-antagonist) prevented these conditions. However, losartan in human pregnancies is contraindicated due to teratogenicity.

6.1.3. Anti-angiogenic factors

In pregnancy, VEGF plays an important role in angiogenesis, while placental growth factor (PIGF) plays an important role in placentation. Preeclampsia in women, however, shows elevated levels of sFlt-1, a VEGF receptor binding and inactivating both VEGF and PIGF. sFlt-1 has been introduced to both mice and rats by a adenoviral vector, demonstrating preeclampsia characteristics (increased BP, proteinuria and glomerular endotheliosis) [44-46] . A limitation of this method is that the reported results were not specific to pregnancy and were dose dependent [44].

6.1.4. Immunological methods

A host of immunological mediators, thought to be a part of the pathogenesis in preeclampsia, have been studied in animal models, including TNF-α (tumor necrosis factor), IL-6 and anti-IL-10, and all mediators provoked elevated blood pressures [47] [45] [46]. A study by Zenclussen et al. injected T-helper-1-like-cells into mice, causing increased blood pressure, proteinuria and glomerular fibrosis [48]. This method is interesting as it exhibits the inflammatory pathway, but they are considered unlikely to participate in the primary events of preeclampsia.

6.1.5. Transgenic models

It is well-known that a genetic predisposition exists in relation to preeclampsia [34]. Transgenic mice models can be generated to study the influence of relevant genes. The APOL1 gene encodes apolipoprotein L1, and variants of the gene (APOL1-G1 and -G2) are associated with kidney disease [49]. As the gene is only found in humans and some primates, transgenic mice models were developed to study the gene variants *in vivo*. Beckerman et al. found an association between the APOL1 gene and a preeclampsia phenotype that occurred during the second half of pregnancy with significant blood pressure elevation, loss of litters and maternal death from eclampsia [50]. Mice with the G2 gene variant were affected more severely. Importantly, also wild type mice carrying transgenic litters developed eclampsia, which is consistent with the known influence from the fetal genotype and the placenta.

There are several other ways to induce preeclampsia, including adriamycin-induced, chatechol-O-methyltransferase-deficient and BPH/5 mice strain. However, these methods have only been used in mice and will not be discussed further [51].

7. Diabetic pregnancy

Diabetes in pregnancy is divided in two groups, pre-gestational diabetes mellitus (PGDM) and gestational diabetes mellitus (GDM). In PGDM, the pregnant woman suffers from diabetes acquired prior to onset of pregnancy. PGDM is subdivided in type 1 (insulin deficiency) or type 2 (insulin resistance). Type 1 diabetes (DM1) is caused by an autoimmune reaction against the insulin producing pancreatic β cells [52]. DM1 is often diagnosed in early childhood, and DM1 patients will require exogenous insulin. Type 2 diabetes (DM2) is the most common, less severe type of diabetes. In DM2, the skeletal muscle and adipose tissue are insensitive to insulin, and the β cells fail to compensate.

In normal pregnancy, maternal tissues become progressively insensitive to insulin. This effect is likely caused by hormones from the placenta. In order to maintain a euglycemic state, the woman must increase her insulin secretion by 200–250%. About 3–10% of the pregnant population is unable to produce an adequate insulin response to compensate this insulin resistance and they develop GDM. The choice of animal model should depend on the type of diabetic pregnancy that the research aim to study. Diabetes can be induced pre-gestationally or gestationally as either an insulin-resistant or insulin-deficient model and the following methods can be used to induce PGDM or GDM in animals: (1) surgical induced (partial pancreatectomy), (2) chemical induced (streptozotocin or alloxan), (3) diet induced and (4) genetic models.

7.1. Surgical-induced diabetic pregnancy by partial pancreatectomy

Partial pancreatectomy is provided by removal of up to 95% of pancreas prior to mating, leading to onset of PGDM with concomitant insulin deficiency [53]. This model was introduced in female rats in 1970 [54], but the model is hampered by several factors: surgical complexity, high post-surgical mortality, a long time between surgery and development of diabetes (2–3 months) and sequelae like digestive problems from the missing exocrine pancreas. However, partial pancreatectomy in sheep fetuses in late gestation has been used to study fetal insulin and glucose metabolism *in utero* [55].

7.2. Chemical-induced diabetic pregnancy

A widely used method for induction of experimental diabetes is chemical destruction of pancreatic β cells, resulting in insulin deficiency. This approach resembles a DM1 model, but it has been used to mimic GDM. Streptozotocin and alloxan are the most used drugs, especially in rats and mice. The amount of time required to induce diabetes and the phenotype (mild to server diabetes) depend on factors such as animal species, strain, dose and mode of administration (sc, iv, ip or im) [56]. In rats, streptozotocin has shown to cause ovarian dysfunction

[57], and untreated diabetes generally results in subfertility. For these reasons, streptozotocin is often administered on the day of mating in order not to interfere with a successful mating and where the risks of direct toxic effects on the embryo are little [56].

7.3. Diet-induced diabetic pregnancy

Obesity is a well-known risk factor for DM2 and GDM [58]. Feeding with high-fat diets and/or high concentrations of sucrose and fructose induces insulin resistance, and this approach is used to create animal models of DM2 and GDM in rats, mice and sheep (**Table 5**) [56] [59]. This method is cheap and accessible, but relatively more time-consuming than chemical induction. Holemans et al. fed female rats with a diabetogenic diet 4 weeks prior to mating and during gestation [59]. They found that diabetes was not present prior to mating, but was confirmed at gestation day 20, resembling a GDM model. Liang et al. used a similar protocol in mice, but diabetes was developed pre-gestational in this study [60]. In sheep, a 60 days of diabetogenic diet before mating resulted in insulin resistance and increased fetal adipose tissue and β cell mass in mid-gestation (gestation day 75) [61]. Another way to study hyperglycemia and hyperinsulinemia and the impact on the fetus is by continuous iv glucose infusion during gestation [62]. However, this method is considered too simple and lacks the complexity of a diabetic pregnancy.

7.4. Genetic models of diabetic pregnancy

Several genetic mice models of diabetic pregnancy exist. Genetic engineering and inbreeding are unfortunately impossible in several species [56]. The "non-obese diabetic" mice and "bio breeding" rats are inbreed strains spontaneously developing DM1. They are used to study fertility and fetal complications in DM1 diabetic pregnancy [53]. The "db/db" mouse is a classic DM2 model with a mutation in the leptin receptor gene (ObR) resulting in excessive appetite and hence obesity [63] [56]. These mice are infertile, but the heterozygote "db/+" mouse are fertile and develops insulin resistance during gestation, and they are therefore providing a model of GDM [64]. Newborns of "db/+" mice show complications related to GDM like

	Methods				
	Partial pancreatectomy	**Chemical**		**Diet**	**Genetic**
		STZ	**Alloxan**		
Rats	[54]	[98]	[99]	[59]	[100]
Mice		[101]	[102]	[60]	[64]
Guinea pigs		[103]	[104]		
Rabbits		[105]	[106]		
Sheep	[107] fetal surgery *in utero*	[108]	[109]	[61]	
Pigs		[110]	[111]		

Table 5. Diabetic pregnancy models (number refers to reference list).

macrosomia regardless of fetal genotype. An important factor is that the diabetic phenotype of the "db/+" mouse is not present prior to gestation, making this model more transferable to GDM than many other models [56].

With genetic models of diabetic pregnancy, it is important to remember the genetic predisposition to diabetes in the fetus. Embryo transfer can be used to study the influence of maternal diabetes separately from the fetal genotype [65]. Many genes affecting β cell function in pregnancy can be mutated in mice to induce a diabetic phenotype [56].

8. Imaging diagnostics of the pregnant animal

Ultrasound imaging is widely used in small animal practice for the evaluation of the pregnancy and determination of number of fetuses, and it is also used to monitor abnormal pregnancies, such as poorly fetal development for gestational age and to identify pregnancies in which there is embryonic resorption or fetal abortion. In the placenta, ultrasound-based Doppler is the first-line technique for the evaluation of uteroplacental blood flow. The Doppler technology is based on analysis of the change in frequency or intensity of ultrasound waves when they are reflected by a moving target such as erythrocytes. Ultrasound exposure is considered harmless; and in fact, animal experiments subjected to fetal ultrasound imaging in various mammalian species showed no pathological effects for the embryo, no congenital malformations or adverse neurobehavioral effects [66]. The technique is often combined with simultaneous administration of a sonographic contrast agent, resulting in an enhanced gray scale or color Doppler signal, facilitating visualization of microvascular structures down to the microvascular perfusion. The mean diameter of micro-bubbles ranges from 2 to 10 μm, less than that of a red blood cell but sufficiently large to be trapped within the vascular space [67]. Thus, ultrasound imaging allows discrimination between fetal and maternal circulatory systems by imaging the intervillous space alone, and it could be used to diagnose the abnormalities of placental blood flow [68] (**Figure 3A**).

MRI is another non-invasive method for diagnostic information. MRI uses the body's natural magnetic properties to produce detailed images from any part of the body. For imaging purposes, the hydrogen nucleus is used because of its abundance in water and fat. What makes MRI so powerful is the exquisite soft tissue and anatomic details. MRI has been increasingly used for detailed visualization of the fetus *in utero* as well as placental structures. While small rodents have fetal sizes that are difficult to investigate with most MRI systems, recent development in very high-field MRI systems now allows visualization of fetal anatomical structures down to a mouse fetus. Wu et al. demonstrated how embryonic mice brain structures could be delineated *in vivo* at embryonic day 17 using an 11.7 T MRI system. *In utero*, 3D MRI has been extensively used in larger animals in clinically available MRI systems. As an example, high-resolution MRI of the inner ear structures of fetal sheep *in vivo* has been demonstrated [69]. Pregnant domestic pigs, on the other hand, are too large to fit in a standard MRI machine bore, precluding MRI as a diagnostic tool in this animal model. Non-brain investigations of the fetus have been increasingly performed using MRI.

Similar to the ultrasound-based contrast-enhanced method, an excellent soft tissue image contrast can be obtained by MRI contrast agents; usually a paramagnetic (gadolinium) molecule that alters

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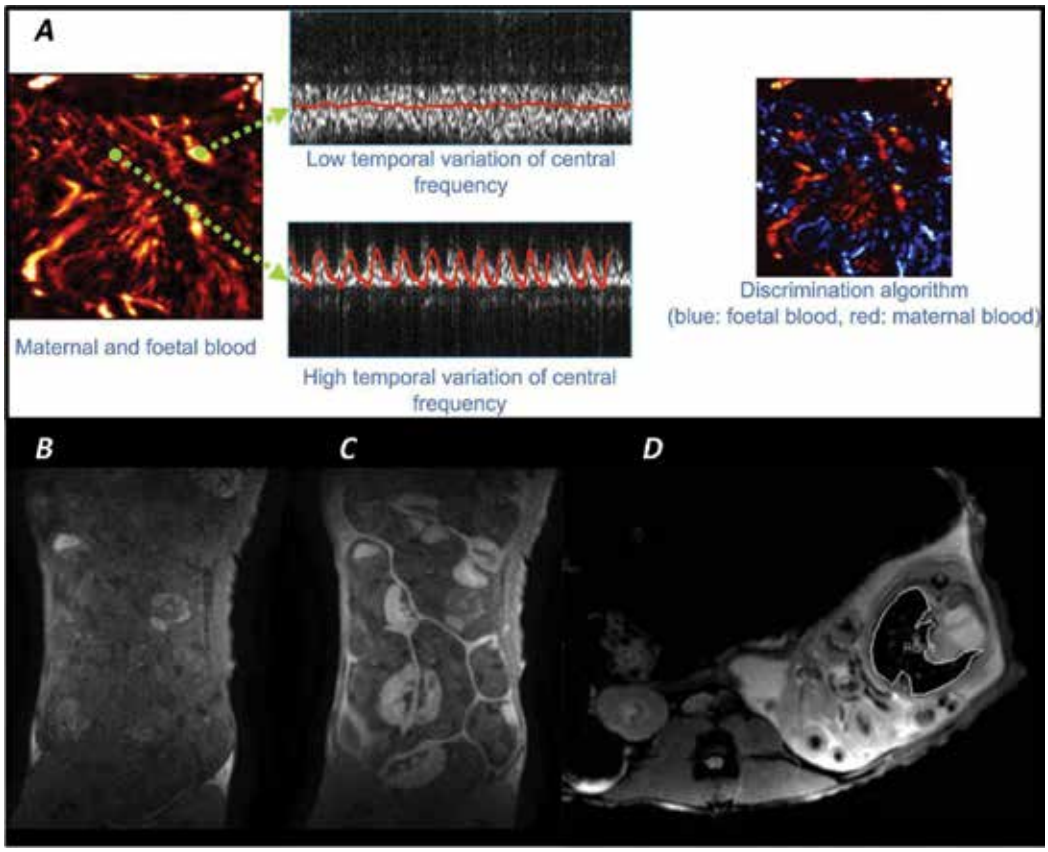

Figure 3. Placental blood flow mapping with discrimination of fetal and maternal circulation using ultrafast ultrasound Doppler in the rabbit model. With this technique, the pulsatility of each placental vessel is analyzed. Discrimination between maternal and fetal blood flows is performed using advanced analysis (A). Evaluation of placental blood flow with magnetic resonance imaging (MRI) in the rabbit model before (B) and after (C) injection of contrast product. Blood oxygen level-dependent MRI showing an axial view of the fetus with the fetal liver selected as the region of interest (D). (A–C are reprinted from ref. [66]; D is reprinted from ref. [71]).

the intrinsic T1-relaxation times of various soft tissues where the contrast agent accumulates. For example, Mourier et al. have evaluated placental blood flow with MRI in a rabbit model before and after injection of a paramagnetic contrast agent [68] (**Figure 3B–C**). Animal studies have shown that small-size gadolinium agents cross the placenta and are extracted by the fetal kidneys into the amniotic fluid [70] [71]. Mikkelsen et al [12] revealed a high signal of [1-13C]-pyruvate and its derivative [1-13C]-lactate in the chinchilla placenta using hyperpolarized MRI; a non-harmful imaging modality using non-ionizing endogenous substrates for interrogating accumulation and metabolic pathways. In parallel, Friesen-Waldner et al. examined noninvasively the fetoplacental metabolism and transport of pyruvate in guinea pigs using the same technique [72]. The relation between maternal oxygen challenge and fetal oxygenation has recently become possible to study using the blood oxygen-level dependent (BOLD) MRI sequence; a non-invasive technique for evaluating organ tissue oxygenation that requires no contrast exposure (**Figure 3D**). Studies in sheep fetuses have shown that changes in cotyledon and fetal BOLD MRI signals are closely related to changes in fetal oxygenation estimated by fetal arterial hemoglobin saturation [73]. MRI

has also shown promises in the fetal heart. As example, Yamamura et al. have demonstrated the applicability of MRI for future evaluation of fetuses with complex congenital heart defects [74].

Moreover, more sophisticated MRI sequences, such as diffusion-weighted imaging (DWI), MR spectroscopy and diffusion tensor imaging allow for visualization of inherent structural, metabolic, cellular and microvascular characteristics. While these techniques have potential applications in fetal imaging, the familiarity with fetal MRI is still limited within researchers working with animal pregnancies.

9. Animal ethics and handling

From an ethical viewpoint, animal experiments involving pregnant animals do not differ from other types of animal experimentation, but the ethical aspects of using fetuses for experiments should be considered. The 3Rs should be considered in all other types of animal experimentation [75], including the options for replacement, which is the first R. One obvious alternative option is to use the placenta from women who have just given birth [76]. The maternal and the fetal circulations are re-established with a pump system, and this option has been used to study the passage of chemical substances from mother to fetus [77]. In addition to the fact that this alternative can replace the use of live animals, it also has the advantage of avoiding problems related to species differences in the maternal-fetal barrier. However, it can only represent transport in the last part of the third trimester. The second R, reduction, should not only include the number of mothers, but also the number of fetuses. The studies should be conducted so that no more experimental animals are used than is necessary to obtain statistically safe results [75]. The third R, refinement, should importantly include the accommodation, feeding and care of the experimental animals based on the specific requirements of the pregnant animals.

9.1. Physiological effects of gestation

The physiology of pregnancy can create stress problems in animals which therefore pose special requirements for their handling, care and anesthesia. Most studies on this topic have been performed in ewes, but the same changes are expected in other species. When the size of the fetus increases, more energy and blood must flow to the uterus, and therefore maternal blood volume, cardiac output and contractility approach their maximum [78]. Additionally, the lungs should be able to deliver an increased amount of oxygen while the uterus presses on the diaphragm so that the thoracic cavity volume is decreased. As a result, respiratory rate increases, but at the same time the risk of hypoxia increases even during short-term apnea. The expanding uterus further delays gastric emptying and decreases the esophageal sphincter tone, which increases the risk of regurgitation and aspiration pneumonia during anesthesia. Special conditions apply to sheep and other ruminants, as the last part of the gestation period is characterized by very little space in the abdominal cavity, which may limit the volume of the fermenting compartment so that feed intake is limited. Furthermore, pregnancy may also prolong plasma half-lives of anesthetics and other drugs, and the high level of progesterone will have a sedative effect, and therefore anesthesia doses must be reduced to prevent overdosing [79]. The fasting period prior to anesthesia should be minimized to prevent metabolic disturbances.

9.2. Feeding pregnant animals

Many females will have a temporary loss of appetite in the first part of the pregnancy, and an increased appetite later. In general, the nutrition requirements are the same as for non-pregnant females. However, in the last part of the gestation period, the fetus growth will increase dramatically, and so will the nutrition requirements for the mother [80]. For the species with largest litters and heaviest fetuses, the need for energy will increase most. Pregnant rodents should typically be fed with a special breeding mix, which has a slightly higher content of proteins, vitamins and minerals. They are typical feed *ad libitum*, so the increased amount of feed is not observed, but for *restricted* fed animals, like pigs, the amount should be adjusted. Pregnant sows should be fed individually so that they maintain a normal weight and body mass, and their energy needs will specially increase during the last 4 weeks [81]. The composition of the sow feed does not need to be changed once it has been ensured that it contains sufficient amino acids; this is particularly important in young sows. Sheep are fed normal maintenance diet during the first 2/3 of the gestation period, and it should be ensured that they maintain a normal weight and body mass. In the last 1/3 of the pregnancy, the feed requirement increases, and as the space in the abdominal cavity is limited, it is important to feed them with a high quality feed that does not overload the rumen.

9.3. Handling and care of pregnant animals

Pregnancy poses special requirements for the handling of experimental animals. Generally, pregnant animals tolerate less stress than non-pregnant animals, and should be transported as little as possible during the first and last part of the pregnancy. At the beginning of pregnancy, the implantation process of ovarian eggs is sensitive to stress, and ultimately in pregnancy, the mothers are physiologically stressed and therefore have a low threshold of stress tolerance. Mice and rats are bred in monogamous (one male and one female) or polygamous mating systems (one male and two to six females). In guinea pigs, the polygamous mating system can be practiced with 1 male to 10 females. In polygamous systems, the females are removed from the male before they give birth. For pigs, the gilts will go into estrus after contact with a boar, and after mating, the pregnant sows are group housed. The sheep differs by being seasonally polyestrous. Ewes are typically paired in autumn so that they lamb in the spring. If the animals should give birth, they must have access to pre-birth material during the last days of gestation.

9.4. Anesthesia of pregnant animals

The anesthesia risk is higher in the pregnant than in non-pregnant animals due to physiological alterations described above [79]. In general, anesthetics can cross the blood-brain barrier and will usually cross placenta. Therefore, in some species, local anesthesia, such as epidural anesthesia, is preferred due to their minimal systemic effects; this applies especially to cows, sheep and other ruminants where general anesthesia furthermore can lead to tympanitis. In most other species, it is necessary to use general anesthesia. The choice of anesthetics depends on the animal species, but drugs generally have to be selected for their minimum effects on cardiac output, renal blood flow and fetus physiology [79]. Drugs with major depression effects on the fetus should be avoided. Inhalation drugs can be used, but as the degree of neonatal depression depends on the maternal anesthesia depth, higher doses should be avoided. Furthermore, they

can induce decreased uterine blood flow and fetal acidosis. Both sevoflurane, isoflurane and nitrous oxide are safe to use. Caution should be taken when using opioids as these are only slowly eliminated in the fetus. Xylazine and other alpha-2-agonists are also problematic as they have major depression effects on the fetus. During cesarean section, anticholinergic drugs should be given for inhibition of vagal tone during uterus traction, and ketamine can be used in combination with other drugs, such as thiopental, as long as ketamine is administrated in low doses. Propofol induces a rapid anesthetic phase and is rapidly cleared from the neonates blood circulation [79].

10. Conclusion

Because there is no animal model equal to the human situation, caution should be taken to extrapolate the results to human diseases. As one animal model may have advantages in one study, it may have disadvantages in others. The careful choice of model is therefore crucial. Mice and rats are currently the most used animal models to study pregnancy, including pregnancy-related diseases like IUGR, preeclampsia and diabetic pregnancy. However, larger animal models, like the guinea pig or sheep, have advantages making them more translational to human pregnancy. Introduction of new diagnostic techniques has facilitated (non-invasive) imaging of physiological, hemodynamic and metabolic measures, even in the smallest animal models. In parallel, the increasingly better management of feeding, handling, care and anesthesia of the pregnant animals reduce physiologically stress. These factors contribute to an increasingly translatability to the human pregnancy. The use of animal models provides a way to gain insight into the improved understanding of the human pregnancy, and there are today available pregnancy-related animal models that facilitate experimental studies that cannot be made in humans.

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Chapter 17

Animal Model of Parkinson Disease: Neuroinflammation and Apoptosis in the 6-Hydroxydopamine-Induced Model

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Additional information is available at the end of the chapter

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Abstract

6-Hydroxydopamine (6-OHDA), a synthetic neurotoxin, has been used to generate animal models of Parkinson's disease (PD). Even though 6-OHDA induced neurodegenerative model in rat, it does not reproduce all the symptoms of the disease, but it does replicate most of the cellular processes such as oxidative stress, neurodegeneration, neuroinflammation and apoptotic neuronal death. The knowledge of the mechanisms involved in neurodegeneration is relevant to define possible therapeutic targets for PD.

Keywords: neurodegeneration, *substantia nigra pars compacta*, cellular stress, Parkinson's disease, therapy

1. Introduction

Parkinson's disease (PD) is a chronic-neurodegenerative disorder that presents motor and non-motor symptoms. The bradykinesia, resting tremor, rigidity and postural instability are caused by neurobiological defects [1]. PD affects a wide variety of nuclei in the central nervous system (CNS), including the dorsal motor nucleus of the vagus, nuclei of the Rafe, locus coeruleus, pontine peduncle nucleus, retrorubral nucleus, parabrachial nucleus, ventral tegmental area (VTA) and the *substantia nigra pars compacta* (SNpc) [2]. PD could be sporadic or due to genetic alterations (alpha-synuclein, parkin, PINK1, dardarin, and oxDJ-1). Despite the fact that PD is multifactorial; an indisputable sign of the disease is the

progressive degeneration of the dopaminergic neurons of the nigrostriatal pathway, neuroinflammation, the presence of Lewy bodies and generalized damage of the neuronal circuits that control the movement [3].

2. Animal models for PD

Cellular processes associated with PD such as oxidative stress, neurodegeneration, neuroinflammation and cell death, has been successfully evaluated in rat and mice. Till date, there exist two general types of experimental murine models: genetically manipulated and chemically induced.

2.1. Genetically manipulated

The induction of gene mutations, alterations in protein functionality and sub- or overexpression of proteins have generated models for PD. These innovative genetic engineering strategies have been developing for PARK2, alpha-synuclein, PINK1, and oxDJ-1. The results are diverse. For example, the genetic deletion of exon 3 of PARK2 in mice increases extracellular striatal dopamine contents but the DAT levels are decreased [4, 5]. These facts do not alter the nigrostriatal pathway because the number of dopaminergic neurons remains normal. A key factor for Parkinson's disease progression is the formation of Lewy bodies [6], due to which, α-synuclein has been incorporated as a gene or peptide to produce amyloid-like composed fibrils. Other strategy involves the incorporation of drugs to modify alpha synuclein aggregation in mice and in *in vitro* models [7, 8]. In mice, it causes dopaminergic neuronal death [2]. But the deleterious effect is dependent on the site of administration, type of particle (gene, peptides, and oligomers), dose, and molecular vector used.

2.2. Chemically induced

The most commonly used neurotoxins are: (a) 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) [9], which is converted to 1-methyl-4-phenylpyridinium (MPP+) by monoamine oxidase (MAO-B), (b) 6-hydroxydopamine (6-OHDA) [6, 10], (c) herbicides such as paraquat or rotenone [11] and (d) metals (manganese, iron) [12]. MPTP crosses the blood-brain barrier (BBB) [13], which in addition to cause damage to the nigrostriatal pathway, causes neuronal loss of the GABAergic neurons [14], catecholaminergic neurons (VTA, *locus coeruleus*, retrorubral nuclei) [15], reduction of serotonine receptor in the cortical and subcortical regions and reactive gliosis [16]. The toxicity of herbicides and metals is characterized by mitochondrial dysfunction due to peripheral and brain cellular stress [6, 17]. The neurotoxin 6-hydroxydopamine is more selective for the dopaminergic neurons of the SNpc [18, 19] because it causes specific degeneration of dopaminergic neurons in the SNpc [19–21] and does not cross the BBB. The advantages and limitations of 6-hydroxydopamine model are showed in **Table 1**.

Feature	Advantages	Limitations
Animal(s) used [6, 22]	The injection of 6-OHDA can be performed in rats (most common), mice, cats, guinea pigs, dogs and monkeys (uncommon)	None
Usage of the model [1, 20, 23, 24]	Unilateral (standardized and most common) or bilateral (uncommon) injection into the nigrostriatal pathway	None
Mode of administration [20, 25]	As the 6-OHDA does not cross BBB, intracranial injection by stereotaxis needs precise administrations on nigrostriatal pathway	Stereotaxis procedure needs special equipment
Type of lesion [20, 26]	Reproducible; retrograde; relatively progressive. Dose and site dependent	Cannot reproduce complete pathophysiology
Transporter mediated entry [13, 27]	Selective entry into the target using Dopamine transporter (DAT), can cause selective destruction of brain dopaminergic neurons	Noradrenaline transporter (NAT) mediated entry causes damage and destruction of brain noradrenergic neurons
Dopaminergic neuronal loss [6, 28]	More in SNpc, nucleus specific to dopaminergic neuronal population, than in VTA, nucleus containing glutamatergic neuronal populations, representing a good model for PD	Toxic for other catecholaminergic neurons
Progressive and age-dependent effects of PD [6, 22]	None	Absent due to acute neurodegenerative property of 6-OHDA injection
Circling motor behavior [12, 20]	Quantifiable depending on the dosage of methamphetamine or apomorphine injected and severity of the lesion; correlates with the magnitude of nigrostriatal lesions	None
Non-motor behavioral phenotypes [3, 6]	None	Causes cognitive, psychiatric and gastrointestinal disorders
Survival rate [27]	High survival	5 in 100 die due to lack of proper post-surgery recovery
Cellular process associated to the cytotoxicity [3, 13, 29–31]	Oxidative/nitrosative stress, apoptosis, autophagy, necrosis, neuroinflammation	No Lewy body formation

Table 1. Characteristics of 6-OHDA model.

3. Vulnerability of dopaminergic neurons to 6-OHDA

6-Hydroxydopamine (6-OHDA) is a highly oxidizable dopamine analog, which can be captured through the dopamine transporter (DAT) [25]. Till date, three mechanisms have been proposed to explain the cytotoxic effect of 6-OHDA: (1) intra- or extracellular auto-oxidation,

which favors the production of hydrogen peroxide, superoxide and hydroxyl radicals [13]; (2) formation of hydrogen peroxide by the effect of monoamine oxidase [32]; and (3) direct inhibition of the mitochondrial respiratory chain I complex [33].

These mechanisms can act independently or in combination to generate reactive oxygen species (ROS) [30]. Injection of 6-OHDA increases iron levels in the SNpc, which further induces the generation of ROS and cytochrome c release [13]. ROS and quinones derived from 6-OHDA diminishes the antioxidant capacity of the cell, resulting in oxidative damage to proteins, lipids and DNA [34]. Miyama and colleagues observed that 6-OHDA treatment decreased cellular glutathione content in a time-dependent manner before the oxidation of DJ-1 (oxDJ-1), a PD-related endogenous protein [35]. The oxidative stress generated can be amplified by the increase of free calcium in the cytoplasm, which is the product of glutamate excitotoxicity or by the loss of mitochondrial membrane permeability [36].

The dopaminergic neurons of the SNpc are vulnerable to oxidative stress induced by 6-OHDA, because they have increased basal levels of ROS, as well as low levels of glutathione peroxidase, an enzyme that reduces hydrogen peroxide to water [37]. The dopamine neurotransmitter has a high susceptibility to auto-oxidize and to become neuromelanin, which promotes the formation of hydroxyl radicals. This when combined with iron accumulated normally at high concentrations in dopaminergic neurons [3, 38], affects its elimination capacity. Also, during the oxidation of dopamine, several transient metabolites are formed such as dopamine o-quinone, aminochrome and 5,6-indolequinone [39]. These metabolites induce the formation of superoxide and adducts with several proteins like parkin [40, 41], tyrosine hydroxylase (TH) [42], glutathione peroxidase 4 [43] and several others. Indeed, it has been proposed that 5,6-indolequinone is the most reactive species that could form adducts with alpha-synuclein generating neurotoxic oligomers [7].

However, not all dopaminergic neurons of SNpc are vulnerable to 6-OHDA toxicity because there are subpopulations of dopaminergic neurons in SNpc expressing calcium-binding proteins such as calretinin and calbindin-D28k, which prevent the accumulation of intracellular calcium, avoiding the consequent excitotoxicity due to glutamate, and the cytotoxic action of 6-OHDA [44, 45]. The redox system plays an important role in protecting the dopaminergic neurons against oxidative stress. The thioredoxin and glutaredoxin systems directly mediate reduction of the 6-OHDA-quinone *in vitro* and protect neurons against dopamine-induced cell death [46].

4. 6-OHDA model

Ungerstedt and colleagues demonstrated that intracerebral stereotaxic injection of 6-OHDA causes degeneration of the nigrostriatal pathway [10]. To evaluate the 6-OHDA toxicity *in vivo*, three models of injury have been developed: (1) the medial forebrain bundle injection [47, 48], (2) the intranigral lesion [21, 49] and (3) the intra-striatal injury [20, 50–52]. Although injury to the medial forebrain bundle and the intranigral lesion is useful to demonstrate the immediate neurotoxic effects, it has the disadvantage of causing rapid and generalized degeneration of the injured nucleus [53], being unfavorable models to study the cell death type generated by long-term oxidative stress. However, the unilateral or bilateral intra-striatal model does cause

the progressive loss of dopaminergic neurons of the SNpc, emulating the nigrostriatal damage observed in PD (**Figure 1**) [23, 24, 54–56].

4.1. Intra-striatal model

Kirik and colleagues [20] described that the ventrolateral region of striatum in the rat that receives afferents from the motor and the sensorimotor areas of the cortex and exclusive innervations of the SNpc. The dorsomedial region of the striatum has a mixture of innervations of the SNpc, the VTA, the frontal cortical area and the limbic system. Therefore, 6-OHDA lesions involving the dorsomedial region have general effects on locomotion and drug-induced (such as amphetamine and apomorphine) rotational behavior, while lesions affecting the ventrolateral region show effects pronounced at the beginning of the movement, sensorimotor orientation and fine motor behavior [20]. In addition, they observed that a single dose given at one striatal site causes 80% reduction in striatal innervation, and a loss of about 90% of the nigral dopaminergic population; while the dose administered at several sites of the striatum generates damage in extra-striatal innervation [20]. The effect of intrastriatal injection depends on the site of injury and dose.

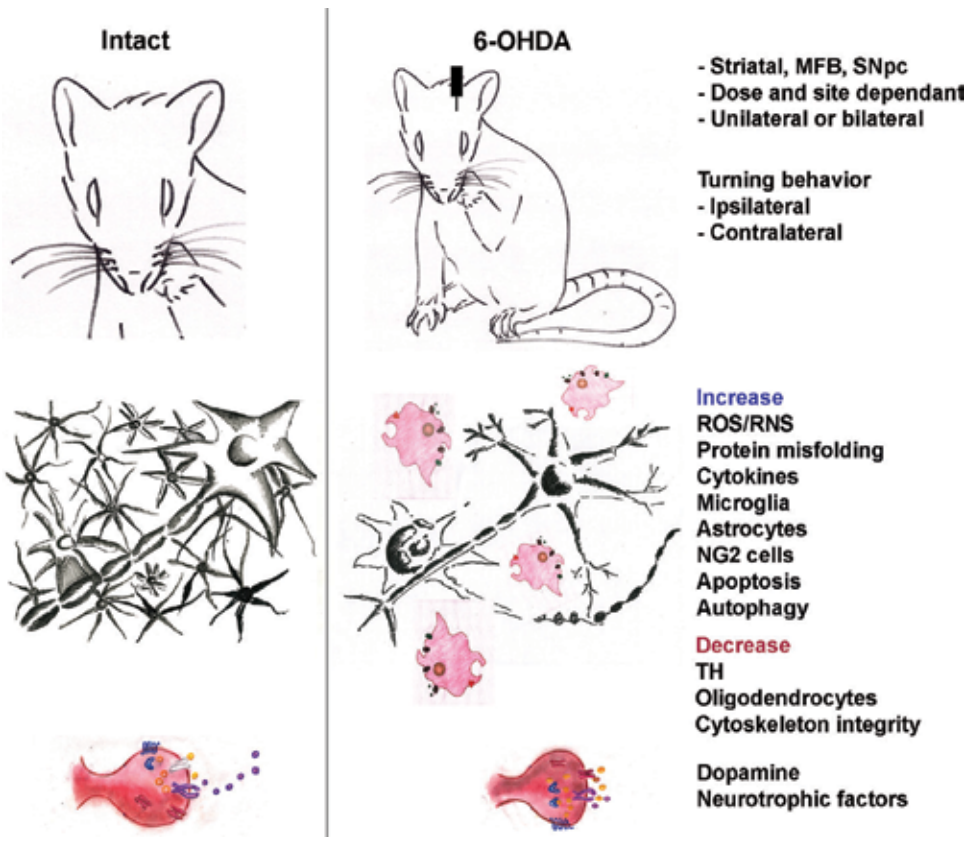

Figure 1. Overview of cellular processes promoted by 6-OHDA in rat.

The intra-striatal injection of 6-OHDA mainly affects dopaminergic neurons of the SNpc, and it also generates a reduction of dopaminergic neurons in the VTA, which form the mesolimbic pathway and innervate to the nucleus accumbens [28, 57]. The loss of dopaminergic neurons in the VTA does not exceed 20% of the population, and the damage does not progress over time, as observed in the SNpc. The 6-OHDA model does not replicate the presence of Lewy bodies [8], and for this reason, murine models with alpha-synuclein have been established. These approaches are based on gene knockout models [58], or gene overexpression [59] and intracerebral injection of alpha-synuclein [60]. These approaches might be the relevant in understanding the degeneration of the nigrostriatal pathway and its impact on other brain nuclei, but further research is still needed.

5. Neuroinflammation

Neuroinflammation in PD is characterized by microgliosis and astrogliosis increased around the dopaminergic neurons in SNpc [61]. These cellular process promotes high levels of expression of major histocompatibility complex type II (MHC-II) [62], chemokine receptors, integrins, neurotrophins and several other markers [63]. Elevated levels of pro-inflammatory cytokines, inducible nitric oxide synthase (iNOS), cyclooxygenase 2 (COX2), nitric oxide and reactive oxygen or nitrogen species (ROS/RNS) by NADPH oxidase system or by mitochondria are also observed in PD patients [31, 64]. Recently it has been demonstrated that copper-zinc superoxide dismutase (SOD1) released by microglial cells, or a TNF receptor 2 selective agonist, could confer neuroprotection against 6-OHDA toxicity *in vivo* [65, 66].

Injury of CNS leads to cell death, cellular swelling, excitotoxicity and the release of free radicals and nitric oxide, which triggers a strong glial response [67, 68] referred as reactive gliosis, involving the activation of microglia, astrocytes, oligodendrocytes and Neuron/glial 2 (NG2) cells [69, 70]. After injury, mature astrocytes proliferate and acquire stem cell properties suggesting their capacity to promote regeneration [71]. Depending on the stimulus and intensity of the lesion, all the three types of glia directs the cell either toward the neuroprotection by producing neurotrophic factors or toward the neurodegeneration by producing apoptotic mediators and ROS/RNS. However, NG2 cells, with their neurogenic [72], oligodendrogenic [73], astrogenic [74] and microgliogenic properties play indirect role in directing the cell toward apoptosis or protection. The presence of NG2-positive cells has been identified in SNpc but not in the striatum of the rat [75]. A recent study in a murine paradigm showed that conversion of NG2 cells to astrocytes to produce cerebral dopamine neurotrophic factor (CDNF) is anti-inflammatory in 6-OHDA-induced rat PD model [76]. However, studying the role, mode of activation and conversion of NG2 cells could give further clues to the field of neuroinflammation.

The neuroinflammatory process has been evaluated through glial cell markers such as glial fibrillary acidic protein (GFAP) for astrocytes [77, 78] and OX-42 or Iba-1 antibodies to microglia [79, 80]. The temporal course of activation of these glial populations has been determined by the neurotoxic effect, from day 3 post-injury [51], and even its activation was observed up to 3 weeks after injury with 6-OHDA [78]. The neuroinflammatory process to that precedes the death of nigral dopaminergic neurons (2 weeks post-injury) is probably a mechanism indicating

cell damage. Another body of evidence suggests that the increase in the activation of glial cells, and the consequent release of pro- and anti-inflammatory cytokines at the site of damage, could increase the cytotoxicity of 6-OHDA [26]. Overexpression of human alpha-synuclein in a mouse model of PD showed enhanced expression of proinflammatory cytokines and microglial activation [81]. Recently, the studies focused on NG2 cells, mitochondrial dysfunction or Lewy body accumulation (trend topic based in alpha-synuclein model) has been relevant to understand neuroinflammatory process and define alternative therapeutic targets for PD.

6. Apoptosis

The majority of studies indicated that apoptosis is the main type of cell death produced by 6-OHDA, but necrosis and autophagy contribute on neurodegenerative process also [29, 82, 83]. Given the variety of experimental models, it is not still possible to determine the proportion of dopaminergic neurons of the SNpc affected by one or other types of cell death. However, the convergence of several types of cell death could explain the time course of degeneration and the activation of the neuroinflammatory process [84].

Cell death has been highlighted as the final effect of 6-OHDA cytotoxicity. Several techniques are used to determine cell death type in dopaminergic neurons in rats (TUNEL, silver staining, and immunostaining to caspase-3, GSK-3β, Bax, Bad) [85–87]. Interestingly TUNEL technique is unspecific to identify apoptosis because on *in vitro* studies the 6-OHDA induces necrosis at same dose used *in vivo* [88, 89]. So the use of other apoptotic markers is recommended to show the loss of cellular integrity or specific chromatin condensation on the dopaminergic neurons of the SNpc [51].

Caspase-3 is the major effector caspase in neurons and its activation has been demonstrated by applying neurotoxins *in vitro* and *in vivo.* This cysteine protease is enrolled both in intrinsic and in extrinsic apoptotic pathway [90–92]. In *in vivo* studies, its presence has been evidenced 1 week after intra-striatal injection of 6-OHDA in rats [78, 93]. Most *in vivo* studies have demonstrated the expression of caspase-3 in different cell death models, suggesting that caspase-3 activation is involved in programmed cell death of the SNpc [92, 94, 95]. However, some recent studies are unable to confirm the presence of active caspase-3 or caspase-9 and, based on this, state that these caspases are not involved in the apoptosis of dopaminergic neurons of the SNpC [96, 97]. This controversy is further exacerbated by recent findings demonstrating the involvement of caspase-3 in non-apoptotic functions, such as the activation of microglia [98, 99]. Although most authors agree with the involvement of caspase-3 in the 6-OHDA-induced neurodegeneration, the doubt still remains if caspase-3 expression only leads to neuronal death. It has therefore been necessary to explore other markers of the apoptotic process and in this regard, scientists have highlighted the study and role of glycogen synthase kinase 3β (GSK-3β).

GSK-3β is involved in the signaling pathway of neuronal apoptosis activated by oxidative stress [100], a central factor in the neuropathological process of PD [101]. GSK-3β is activated by phosphorylation of the tyrosine residue 216 (Y216), located in the kinase domain and inactivated by the phosphorylation of serine 9 (S9) [100]. It was observed that a single dose of 6-OHDA administered in the neostriatum of the rat causes caspase-3 and GSK-3β expression,

loss of cytoskeletal integrity, TH levels decreased and activation of apoptotic process in dopaminergic neurons of SNpc [51, 85, 92].

Other authors demonstrated atrophy and progressive death of dopaminergic neurons dependent on translocation to the nucleus of the inducing factor of Apoptosis-inducing factor (AIF), in which there was no activation of caspase-3 or release of cytochrome C or signs of apoptosis. These researchers further demonstrate that death induced by 6-OHDA in dopaminergic neurons is mediated by activation of AIF-dependent Bax [97]. In this work, AIF activation suggests the involvement of regulated necrosis. The controversy between dependent or independent death of caspase-3 could be explained by the dose, study model and site of injury employed. However, since most evidence includes the involvement of caspase-3 in the 6-OHDA-induced apoptotic process, studies that contradict this fact suggest that 6-OHDA could also lead to neuronal death by apoptosis (independent of caspase-3) or other cell death processes (necrosis and autophagy) *in vivo*.

All the toxin-induced PD models had scant attention when it comes to the neuroprotective or regenerative strategies. Neuropathology and studies related to the correlation between inflammation and immune cells need to pay much more attention. It is of great interest to know the stimulus by which glial cells respond to the microenvironment and how do they decide whether to release neuroprotective or apoptotic mediators. It would be of interest to know if all the activated glial cells arise from a limited number of precursor cells or if all glia have equal potential to proliferate. It is also most important to study in detail about the types of receptors which are present on glial cells that play a major role in the field of neuroinflammation.

7. Relevance of 6-OHDA model in gene therapy

The 6-OHDA injury model has been used to demonstrate the benefits of neurotrophic therapy (NT) [102]. NT consists of directed delivery of genes encoding neurotrophic factors such as brain derived neurotrophic factor (BDNF) [103], glial cell line-derived neurotrophic factor (GDNF) [104–109], cerebral dopamine neurotrophic factor (CDNF) [76, 110], mesencephalic astrocyte-derived neurotrophic factor (MANF) [111], vascular endothelial growth factor (VEGF) [112] through nanoparticles [113, 114], or through viral or non-viral gene vectors [76, 104–107, 115]. The purpose of NT assessed in the 6-OHDA model is to prevent the progression of neurodegeneration and to stimulate the functional regeneration of the nigrostriatal system [116, 117]. The recovery of dopaminergic populations could improve motor function. It is therefore important to identify further underlying mechanisms of oxidative stress, neuroinflammation, neurodegeneration and neuronal death caused by 6-OHDA. This knowledge is the key to discovery novel therapies to treat PD.

8. Conclusion

The 6-OHDA model reproduces several cellular processes identified in the PD, therefore it is a key model to explore the molecular bases of cytotoxicity, as well as to study the cellular

processes activated by oxidative stress (neuroinflammation and neuronal death), and consequently a useful model to understand the mechanisms of novel therapies for PD.

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Abbreviations

6-OHDA	6-hydroxydopamine
AIF	apoptosis-inducing factor
BBB	blood–brain barrier
BDNF	brain derived neurotrophic factor
CDNF	cerebral dopamine neurotrophic factor
CNS	central nervous system
COX2	cyclooxygenase 2
DAT	dopamine transporter
GDNF	glial cell line-derived neurotrophic factor
GFAP	glial fibrillary acidic protein
GSK-3	glycogen synthase kinase-3
Iba-1	ionized calcium binding adaptor molecule 1
iNOS	inducible nitric oxide synthase
MANF	mesencephalic astrocyte-derived neurotrophic factor
MHC-II	major histocompatibility complex type II
MPP+	1-methyl-4-phenylpyridinium
MPTP	1-methyl-4-phenyl-1, 2, 3, 6-tetrahydropyridine
NADPH	nicotinamide adenine dinucleotide phosphate
NAT	noradrenaline transporter
NG2	neuron/glial 2

NT	neurotrophic therapy
OX-42	CD11b antibody (integrin, alpha M)
oxDJ-1	oxidized DJ-1 protein
PD	Parkinson's disease
PINK1	PTEN-induced putative kinase 1
ROS	reactive oxygen species
ROS/RNS	reactive oxygen or nitrogen species
S9	serine 9
SN	*substantia nigra*
SNpc	*substantia nigra pars compacta*
SOD1	superoxide dismutase 1
TNF	tumor necrosis factor
TUNEL	terminal deoxynucleotidyl transferase mediated X-dUTP nick end labeling
VEGF	vascular endothelial growth factor
VTA	ventral tegmental area
Y216	tyrosine residue 216

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The world has recorded losses in terms of human life as well as extensive time spent in experimentation with development of new drugs, elucidation of disease mechanism(s), and therapeutic agent discovery. Ethical and legal issues cojoin in slowing down scientific discoveries in medicine and biology. The past two (2) decades, therefore, have seen tremendous attempts that largely are successful in developing animal models with the characteristics of mimicking, approximating, or expressing transplanted human organs/tissues. These models or rather approaches seem to be fast, cost-effective, and easy to maintain compared to primates. This book is a collection of expert essays on animal models of human diseases of global interest. A visible objective of the book is to provide real-time experimental approach to scientists, clinicians, ethicists, medicolegal/medical jurisprudence workers, immunologists, postgraduate students, and vaccinologists and informative and multidisciplinary approach for the identification of new therapeutic targets and biomarkers using animal models as well as investigating the pathogenesis and therapeutic strategies of human diseases. An increased understanding of the genetic, molecular, and cellular mechanisms responsible for the development of human diseases has laid out the foundation for the development of rational therapies mainly with animal models.

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