



Research Models in Biomedical Sciences: Advantages and Limitations

Isadora Sousa de Oliveira¹, Gabriel Melo Alexandre Silva², Francielle Almeida Cordeiro¹, Ernesto Lopes Pinheiro Júnior¹, Isabela Gobbo Ferreira¹, Felipe Augusto Cerni¹, Umberto Zottich² and Manuela Berto Pucca^{2*}

¹Department of Biomolecular Sciences, School of Pharmaceutical Sciences of Ribeirão Preto, University of São Paulo, Brazil

²Medical School, Federal University of Roraima, Boa Vista, Brazil

ABSTRACT

Research models are routinely used in the biomedical field when we are talking about assays. All these models have advantages and limitations and should be chosen carefully, to avoid, mainly, mistakes in ethical practices. However, it is a great challenge for researchers picking and classifying their experimental models due to the lack of clear literature definition or guidelines. Here, we pioneer describe the overall definition of the main research models and give a gamut of examples from the biomedical field. This review also provides recommendations for aligning experimental practices to meet the necessity of assessing an international research model classification.

KEYWORDS: *Ex situ*; *Ex vivo*; *In silico*; *In situ*; *In vitro*; *In vivo*

ABBREVIATIONS: AIDS: Acquired Immunodeficiency Disease Syndrome; CAM: Chick Chorioallantoic Membrane; CCLE: Cancer Cell Line Encyclopedia; DNA: Deoxyribonucleic Acid; ELISA: Enzyme-Linked Immunosorbent Assay; FISH: Fluorescent *In Situ* Hybridization; HIV: Human Immunodeficiency Virus; iPCR: Immune Polymerase Chain Reaction; ISCT: *In Silico* Clinical Trial; MCL: Mantle Cell Lymphoma; OECD: Organization for Economic Co-operation and Development; PCOP: Porcine Cornea Opacity Permeability; PCR: Polymerase Chain Reaction; SPR: Surface Plasmon Resonance

INTRODUCTION

Latin remains a mainstay in academic literature, particularly in natural and medical sciences [1,2]. Indeed, a plethora of scientific expressions exist that are solely used in Latin [3], e.g. the experimental models *in vitro*, and *in vivo* [4,5], and *in silico* [6,7]. Whilst it is key to select the appropriate research model for a given hypothesis to avoid waste of time and resources [8], choosing a suitable model that is capable of meeting the study objectives, is often complicated by unclear or entirely lacking literature definition of the various models; often, further confusion is added due to semantic differences between languages [9]. This review provides an outline of the most used research models, i.e. *in silico*, *in vitro*, *ex vivo*, *in situ*, *ex situ*, and *in vivo* (Figure 1) and an

in-depth discussion about the advantages and limitations of each model. Furthermore, examples of different technologies applied in biomedical sciences (e.g. drug discovery) surrounding the research models of sciences will be explored, aiming to adopt clear and unambiguous definitions within the employed models.

MAIN RESEARCH MODELS

In Silico

In silico models (i.e. performed on computer) always require digital technology to simulate a specific process (e.g. pharmacologic, physiologic, etc.). Originally, silico referred to the chemical element silicon (Si) or silicium, the main compound of

Quick Response Code:



Address for correspondence: Manuela Berto Pucca; Medical School of Roraima, Federal University of Roraima (UFRR), Av. Capitão Ene Garcez, Brazil

Received: June 26, 2020 **Published:** July 24, 2020

How to cite this article: Oliveira IS, Alexandre-Silva GM, Cordeiro FA, Pinheiro-Júnior EL, Ferreira IG, Cerni FA, Zottich U, Pucca MB. Research Models in Biomedical Sciences: Advantages and Limitations. 2020 - 2(4) OAJBS.ID.000197. DOI: 10.38125/OAJBS.000197

computer circuits [10]. In fact, the scientific expression “*in silico*” was first described in 1989 for experiments carried out entirely on a computer [11]. Nevertheless, some authors still consider *in silico* studies as a virtual extension of *in vitro* and *in vivo* experiments [12,13]. *In silico* approaches are represented by techniques that use

software to analyze data and often involve computational models or simulations based on existing information of closely related phenomena. The output can then be used to make predictions and suggest hypotheses as a basis for *in vitro*, *ex vivo*, and *in vivo* models [14].

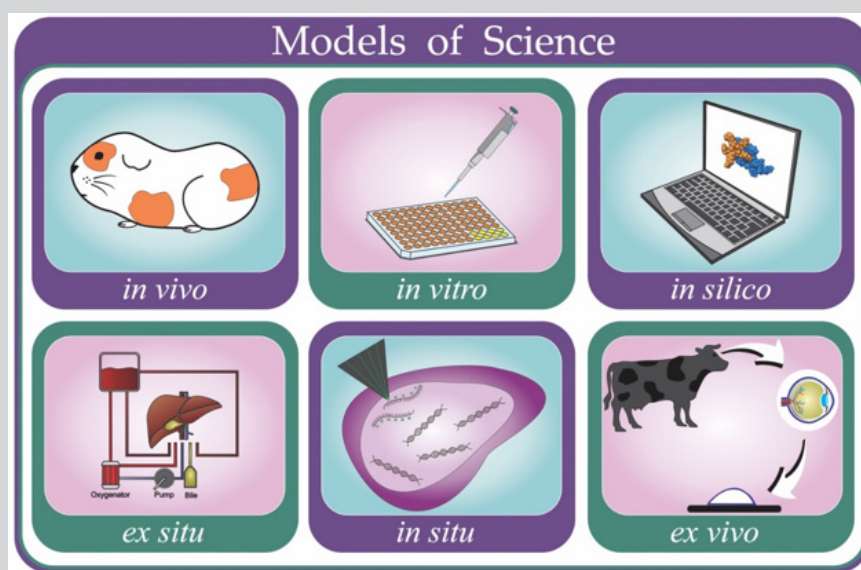


Figure 1: Research models in the biomedical sciences. *In vivo* - represented by a guinea pig; *in vitro*-represented by the 96-well plate; *in silico* - represented by a protein model; *ex situ* - represented by a perfusion system for organ preservation; *in situ* - represented by fluorescent *in situ* hybridization (FISH); and *ex vivo* - represented by ocular tests in bovine corneas.

The greatest advantage of *in silico* methods is that they are usually faster and cheaper than classical tests, whilst also reducing the number of animals to *in vivo* assays [15]. Nevertheless, there are several pitfalls that are commonly encountered when employing *in silico* assays. Mainly, *in silico* studies demand rigorous validation, including experimental verification during model development [16]. Nevertheless, researchers should not be concerned about using *in silico* approaches, which boost medical and pharmaceutical innovation, provide an affordable cost and allow huge business opportunities [17]. Thus, it is not surprising that the usage of *in silico* models has greatly increased in the last years, as demonstrated by pharmacokinetic, oncologic and drug discovery studies [18,19]. Indeed, following the development of ever improving bioinformatic tools, there was a greatly increase in the arsenal of *in silico* methodologies, which have already resulted in significantly improved prediction and elucidation of the dynamics surrounding complex biological phenomena [20,21].

Molecular docking, which explores protein-protein or protein-small molecule binding, is one of the preferred *in silico* methodologies used, especially in drug discovery [22]. Since its discovery in the early 1980s [23], more than 60 different docking tools and programs have been developed for both academy and companies [24]. Other *in silico* methods that are routinely used in research laboratories include molecular modelling (a technique used to model or mimic the structure of molecules) and protein sequencing and its alignment (methods used to evaluate identities and similarities in the amino acid sequence of proteins) [25-28].

In silico toxicity prediction is also being employed to great success [29]. The computational methods employed to predict the toxicity profile of drugs has stood out to design new medicines once as *in vitro* and *in vivo* methods are often limited by ethics

committees, time-consuming, and require a lot of resources [30]. So far, more than 15 sources of *in silico* toxicity model data are available, including ToxCast, Tox21, and ToxBank [30]. Similarly, *in silico* bioprospecting is being explored for searching new molecules for different targets and applications [31]. Detailed *in silico* methods to design biotherapeutics (e.g. antibodies and vaccines) have been discussed in detail elsewhere [32].

Recently, the *in silico* clinical trial (ISCT) came to the light. This approach refers to the development of patient-specific models to form virtual cohorts for testing the safety and/or efficacy of new drugs and of new medical devices. Clinical trials involving new drugs are frequently classified into four phases (I-IV). Phase I recruits about 20 to 100 volunteers and aims to test safety and dose escalation. The use of ISCT can predict the optimal dosage. In Phase II trials, which recruit about 100 to 300 volunteers, ISCT could be used to find representative virtual patients that show adverse effects [33]. In the most expensive Phase III, conducted with 300 to 3,000 patients, ISCT can assess the efficacy in terms of reduced side effects, predict ineffectiveness, and reduce the number of enrolled patients. Thus, ISCT can meaningfully lower the costs related to Phase III clinical trial [33,34].

In Vitro

In vitro (i.e. within the glass) refers to a model in which the assay occurs outside of a living organism and in a controlled environment. In addition, *in vitro* techniques are very valuable for ethical (does not require animals) and economic reasons [35]. Although the number of *in vitro* assays could be uncountable, few examples frequently used will be explored in this section.

All analytical techniques are classified as *in vitro* techniques such as chromatographic and spectrometric techniques,

although they can be used to evaluate samples from *ex vivo* and *in vivo* studies. Chromatography is a century-old technique [36] used to separate and quantify complex mixtures of molecules according to their properties, which can be classified in different types (e.g. liquid chromatography, gas chromatography, ion-exchange chromatography, and affinity chromatography) [37-39]. Moreover, chromatography can be regularly associated with mass spectrometry (MS) analyzes, which is a powerful analytical technique for identifying, quantifying, and exploring molecular structures [40]. Both techniques can be employed to different compounds, including volatile components, lipids, organic molecules, and proteins [41,42]. Another method used to separate proteins according to their Physico-chemical characteristics (charge and size) is electrophoresis [43,44]. Electrophoresis can be performed in a gel [45] or on a filter paper [46], besides been used combined with mass spectrometry to identify protein sequence and mass (i.e. in-gel digestion) [47].

There are also other important *in vitro* methods broadly used for studying structure and biochemical characteristics of molecules, such as circular dichroism [48], protein quantification (e.g. Bradford and Lowry methods) [49,50], crystallography [51], as well as enzymatic assays, both colorimetric and turbidimetric, such as assays employed for phosphodiesterase [52,53] and hyaluronidase enzymes [54,55], respectively.

Other groundbreaking *in vitro* analytical test exhaustively used for research purposes, diagnostics, pharmacokinetic studies (i.e. drug monitoring) and for evaluating quality control of products is the enzyme-linked immunosorbent assay (ELISA), which employs enzyme-mediated color change and respective absorbance to quantify the presence of antigen-antibody mechanism [56]. ELISA method is globally employed because it is cheap, simple, specific, and quick to execute [56]. In connection with antigen-antibody binding, surface plasmon resonance (SPR) is also used to evaluate affinity of immunocomplexes [57].

Most of molecular biology assays are based on *in vitro* experiments, including the prominent polymerase chain reaction (PCR), which allows amplification of specific DNA segments through repetitive cycles of denaturation, hybridization, and polymerase extension [58,59]. In the recent years, the immune-PCR (iPCR) has frequently been employed. The iPCR method provides classical PCR sensitivity to objects traditionally detected by ELISA [60]. Phage display, an elegant *in vitro* technology in which a bacteriophage (i.e. a bacteria and archaea infecting virus) is used to evolve peptides and antibodies has been considered a robust technology for the discovery of therapeutic human antibodies against several diseases (e.g. autoimmune diseases and cancer) [59,61-63]. Still in molecular biology subject, microarray is an *in vitro* renowned technique applied for characterizing protein-protein, protein-nucleic acid, protein-small molecule, and antibody-antigen interactions. The technique has been used in both basic research (e.g. omics analysis) [64] and clinical applications (e.g. tumor markers) [65,66].

Although experiments conducted with living cells removed from humans or experimental animals should be considered an *ex vivo* method, cultures of lineage cells are better defined as *in vitro* approaches. Since 1912, when Carrel and his coworkers developed the first cell line from chicken embryo heart, cell lines have been extensively explored. However, their popularity increased in 1951, after the discovery of the immortalized HeLa cells, obtained from an adenocarcinoma of a patient named Henrietta Lacks [67]. Recently, cell lines, in special human cell lines, are generally used as a source of cells for high-throughput *in vitro* screening assays [68].

For instance, 1457 cancer human cell lines have been explored to predict drug sensitivity, which was defined as a Cancer Cell Line Encyclopedia (CCLE) [69,70]. Nevertheless, the actual number of generated cell lines is not reported and hard to estimate [71].

Similarly, there are other not obvious assays employing cells and organs described as *in vitro* approaches. The *in vitro* chicken enucleated eye test can detect the degree of eye irritation of certain compounds within six hours [72], which is considered an alternative to the *in vivo* Draize eye test method performed in rabbits [73]. Recently, an interesting new concept of *in vitro* assay emerged, named organs-in-a-chip, which describes a cell culture-based model system in which cells of different kinds are placed on small structures (chips). These chip-cells combinations can mimic the role of an organ or tissue, creating the organ-like structures. The organ-like system can be used for different applications such as drug toxicity screening [74], and it is expected that the technique could revolutionize early clinical trials in human patients by clinical-trials-on-chips [75].

Nevertheless, there is a continuous conflict between *in vitro* and *ex vivo* models [76]. Indeed, some authors use *in vitro* to refer to experiments performed within live cells (not cell lines) outside the organism, which should be best classified as an *ex vivo* approach. In any event, *in vitro* models undoubtedly provided an inexpensive and high-throughput alternative to *in vivo* research strategies. However, although *in vitro* models are fruitfully used in biological fields, extrapolation of the observed effects to animal models and humans can be considered weak and may lead to a misinterpretation of the data [77,78]. Indeed, researches that intend to use *in vitro* tests as an alternative to *in vivo* tests should have their approaches validated through scientific studies to assess the reliability and relevance of the method for the given purpose [79].

Ex Vivo

An *ex vivo* model designates a methodology defined by experiments with living cells or tissues taken from an organism and placed in an external environment under controlled conditions [80-82]. The advantage of *ex vivo* experiments is that they are certainly useful to substitute the usage of whole animals as subjects, creating a high output research environment [80,81,83,84]. There are a gamut of studies in the literature employing *ex vivo* assays such as: *ex vivo* human gene therapy [85]; *ex vivo* cell cultivation such as within natural killers [86] and hematopoietic stem cells [87]; and analysis of organs *ex vivo* such as kidneys [88], spleen [89], heart, and lung [90].

Controversially, some authors still recognize an *ex vivo* method as a combination of the *in vivo* and *in vitro* models [78]. Indeed, due to the ambiguity with the terminology, researchers can easily be confused when classifying an *ex vivo* model. To clarify this issue, the well-known *ex vivo* gene therapy is a prime example. The *ex vivo* gene therapy is performed with cells isolated from a living organism, which are expanded/differentiated out of the living (with or without genetic modification) and further administered them back to the same or other living organism [91]. Although this technique clearly involves both *in vivo* and *in vitro* concepts, the best definition for this model should be *ex vivo*.

Other eminent *ex vivo* method is testing ocular corrosives on bovine corneas from slaughtered animals, a test recommended by the Organization for Economic Co-operation and Development (OECD) guideline [92]. An advantage of this assay is its speed, with results usually obtained within 24 hours [93]. The porcine cornea opacity permeability (PCOP) assay is other widely used approach

in the ophthalmic research field, which can be considered better in comparison to bovine corneas, since the porcine cornea better resembles the human cornea [94]. The mouse retina is also an excellent *ex vivo* model for determining autophagic cell function [95]. Also, the use of the same material can provide oxygen consumption information, showing the mitochondrial reserve capacity of photoreceptors [96].

Although *in vitro* and *ex vivo* methods can be considered in many cases a potential scientific tool to study different biological effects, sometimes the obtained results can indicate inconsistencies with *in vivo* models, indicating a potential misleading nature of *in vitro* and *ex vivo* procedures [97]. One example of *in vitro* and *ex vivo* experiment could be seen in Figure 2.

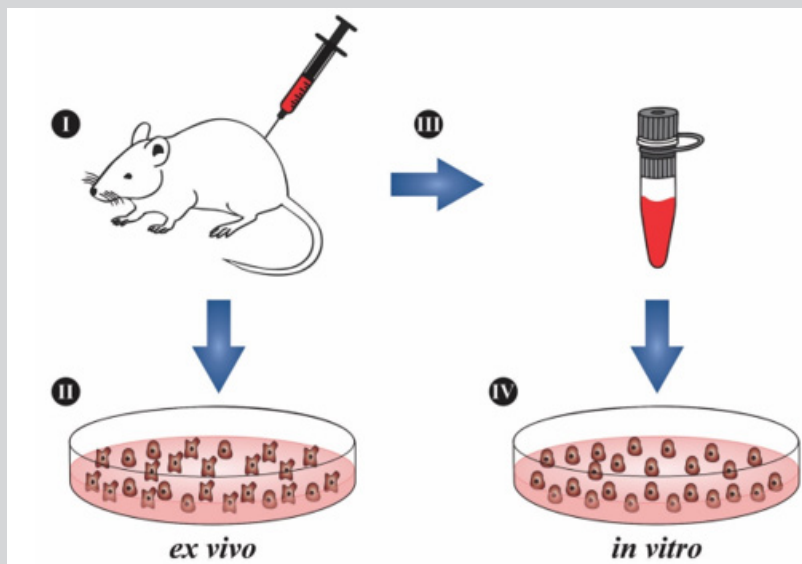


Figure 2: Difference between *in vitro* and *ex vivo* models. (I) Cells (e.g. leukocytes) are collected from an animal such as mouse; (II) differentiated cells are used right after animal collection, characterizing *ex vivo* assays; (III) cells taken from the animal undergo an immortalization process and are commercialized; (IV) immortalized commercial cells (cell lines) are used in assays characterizing *in vitro* models.

In Situ* and *Ex Situ

In situ models (i.e. on site or in position) can also be stated by researchers as *in loco* (i.e. in place), with both referring to studies performed inside its original place or “on site”. *In situ* models are relevant in studying or identifying the active components and mechanisms in their place of origin, such as organs or tissues [98,99]. In medical research, this model is often used to evaluate genes and their respective translated proteins, such as a cytogenetic technique named fluorescent *in situ* hybridization (FISH), in which the genetic material can be assessed without being removed from the cell [100]. Basically, the FISH method uses fluorescently labelled probes that can bind to only those parts of a nucleic acid sequence with a high degree of sequence complementarity [101]. Therefore, FISH is considered a gold standard method for detection of gene alterations, a very useful tool for cancer diagnosis [102-104]. *In situ* neoplasia or carcinoma *in situ* is, by definition, the primal form of cancer and is characterized by being limited to the compartment corresponding to the cell of origin [105]. Notably, studies within *in situ* neoplasia have allowed the elucidation of tumor formation mechanisms [98,106,107]. In fact, in lymphomas, premalignant stages have been identified by evaluating *in situ* follicular lymphoma (FL) and mantle cell lymphoma (MCL) [107]. Several other studies regarding *in situ* carcinoma can be found elsewhere [106,108,109].

However, *in situ* models cannot be applied to all cell types, for example lymphocytes, which exhibit a circulating nature and cannot be studied *in loco* [98]. In the pharmacokinetics field, *in situ* models have also been explored for drug absorption tests [110-112]. In drug absorption evaluation through the intestinal barrier,

the *in situ* intestinal model showed to be even more accurate than the *ex vivo* model [112,113]. This technique consists of a cannula introduction into the small intestine of anesthetized animals for the passage of the drug of interest to be evaluated. By maintaining innervation and blood flow preserved, these absorption models keep a faithful reproduction of what happens *in vivo* [110]. On the other hand, *ex situ* models are those performed outside the original place. For comparative purposes, in a study with bacterial cellulose used for tissue engineering cultures, the bacteria were cultured with *in situ* and *ex situ* modifications. Culture of bacterial cellulose *in situ* were characterized by adding in the medium other material such as an additive, while in the *ex situ* culture modification bacteria just received chemical treatment or absorption of other materials after the bacteria membranes have been formed in culture [114]. However, probably the most relevant *ex situ* method for biomedical sciences is the *ex situ* perfusion for organ preservation, which can restore circulation to regain the organ function (e.g. liver) prior to transplantation [115-117].

Ex situ techniques are also described in quality control of immunobiological products and in biodiversity conservation. In vaccine analysis, we find particle size distribution analysis, zeta potential and settling rate that are performed outside pre-filled syringes [118]. In biodiversity, the *ex situ* is used to describe gene banks, captive breeding, and botanical garden for biological diversity conservation. These methods are an essential part for the reintroduction of endangered species and have some restrictions such as high cost, employee demand and energy resources [119,120]. Examples of *in situ* and *ex situ* models could be seen in Figure 3.

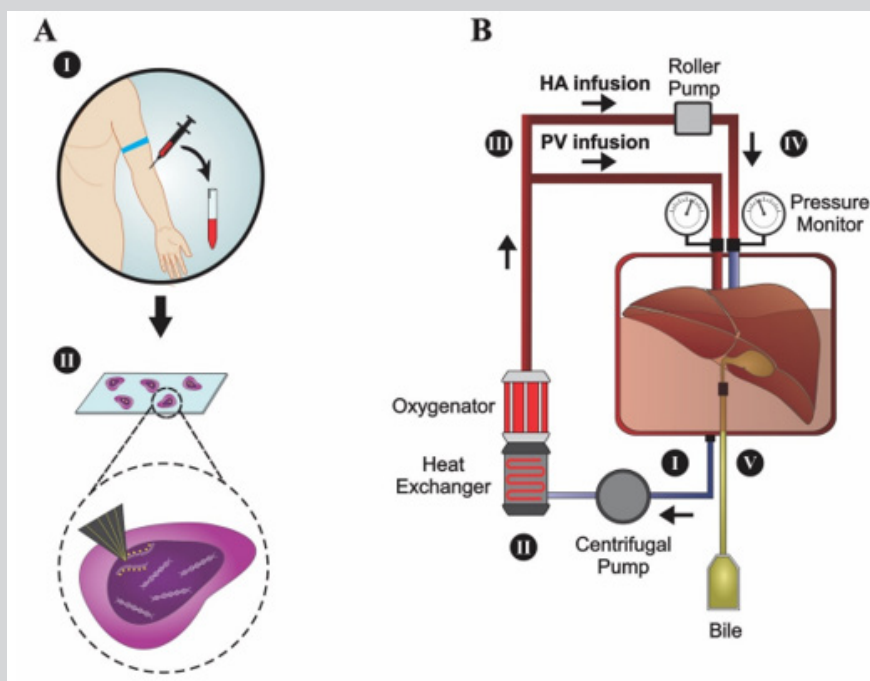


Figure 3: *In situ* and *ex situ* models. (a) Fluorescent *in situ* hybridization (FISH). The biological material is collected (I) and a fluorescent probe (II) is added to label the gene of interest. The material is analyzed using a fluorescence microscopic. (b) *Ex situ* organ perfusion. (I) The organ is placed in a container and submerged in a liquid suitable for infusions. This liquid flows to the centrifugal pump, heat exchanger and oxygenator (II). Through a bifurcation (III), the oxygenated liquid returns to the organ through the portal vein (PV) and part of it (IV) reaches a roller pump, to increase the pressure before returning to the organ through the hepatic artery (HA). (V) Container for the bile storage.

In Vivo

During human history and development, humans always took advantage from numerous scientific researches involving animals. Virtually, from the past century, every drug released on the market was dependent on the use of *in vivo* models in some step of their development [121].

The progress of specific and efficacious treatments of diabetes, several types of cancer, heart surgery, among others, were only possible using animals in scientific research [122]. A recent survey demonstrated that 44% of the population and more than 80% of medical students support the use of animals in research, although they also state that alternative methods should be applied, whenever possible, in order to reduce the number of animals for that purpose [123].

Overall, researchers seek to investigate organisms at multiple levels, starting from their molecules, to cells, tissues, organs, and up to their systems, both in disease and health conditions. For most of them, there is the option to conduct studies using *in silico* or *in vitro* models, such as molecular modeling and cell line culture, respectively. Cell line culture, specifically, has largely evolved in the past 20 years, and became a usual approach to mimic the complex structures of tissues, currently playing key roles in research. In this regard, several animal studies can be replaced due to the advent of such technology [124,125]. Nonetheless, the understanding of physiological processes and systemic interactions still requires *in vivo* experiments.

In vivo model (i.e. within the living) describes a methodology where the whole subject is studied while alive. Although *in vivo* models can be performed with non-animals, such as plants and

seeds [126], *in vivo* experiments using animal models are the most used, reaching more than 100 million animal experiments per year in the world (e.g. Great Britain performed 1,078,738 experimental procedures just in mice in 2018) [127,128]. *In vivo* animal assays are carried out when specific situations are not suitable to be done in humans, due to a possible risk to their physical and/or physiological integrity. The animal use in research is not only due to their similarity with human physiology, but also because human diseases may also affect other animal species [129]. Additionally, it is possible to have *in vivo* models with specific mutations that directly cause or strongly predispose the animal to a desired anomaly or disease, being referred to as "experimental models of a disease" [129-132].

Research with animals have the purpose of providing experimental data in one living organism to study a phenomenon in another species, aiming to obtain preclinical data to determine how a hypothesis would work on humans [133]. On the other hand, the results obtained using animals are not necessarily reproducible in humans. Despite great similarities that a species may have with humans, genetic differences such as gene families, redundancies, and regulation of gene expression patterns may influence the variable responses [122].

Although massive worldwide investments in drug development, the overall success rate of new drugs, especially on clinical trials, remains low. One possible explanation relies on the preclinical research phase, when 'inappropriate animal models' are chosen because of lacking a predictive model, which leads to non-extrapolating conclusions [134]. In this context, it is fundamental to select the correct animal model according to the objectives of the proposed research [134].

Although rodents (rats, mice, and guinea pigs) are mostly used due to their shorter lifespans, which creates the possibility of producing many generations [135], there are many other available animal models, such as insects (*Drosophila* spp.) [136], fish (Danio rerio, or zebrafish) [137], nematodes (*Caenorhabditis elegans*)

[138], frogs (*Xenopus* spp.) [139], rabbits [140], horses [141], dogs [142], cats [143], pigs [144], and even non-human primate models [145], among others (Figure 4). Some examples of preclinical animal models and their use in research are listed in Table 1.

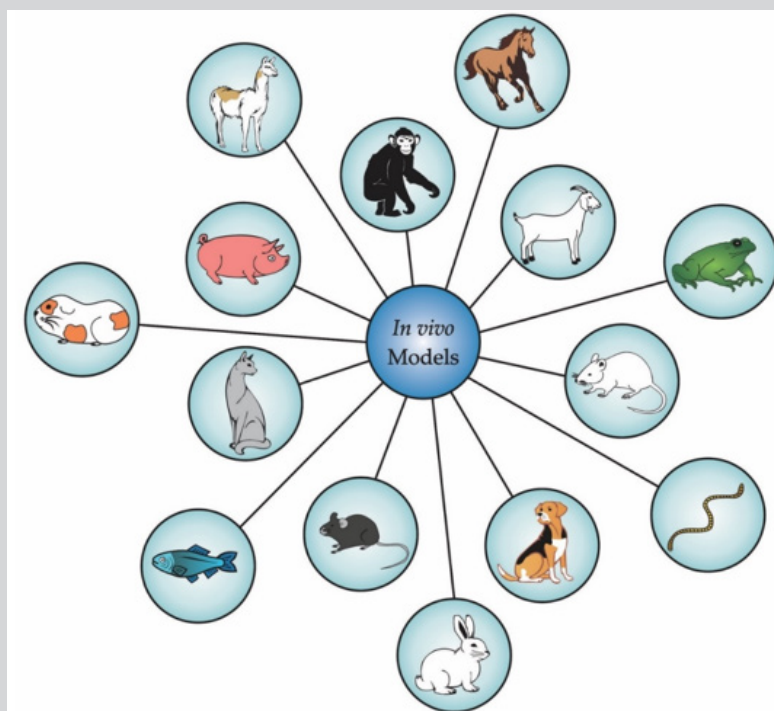


Figure 4: Main *in vivo* models used in preclinical research.

Table 1: *In vivo* models in preclinical research.

<i>In vivo</i> model	Main Species Employed	Models in Research	Reference
Cat	<i>Felis catus</i>	Ophthalmology, Type-2 Diabetes	[170], [171]
Dog	<i>Canis familiaris</i>	Osteoarthritis, Periodontology	[172], [173]
Fish	<i>Danio rerio</i>	Drug development screening, Behavioral studies, Cancer therapy, Autoimmune diseases, Nanomedicine	[174], [175], [176], [177], [178]
Flies	<i>Drosophila melanogaster</i>	Drug development screening, Cancer therapy	[179], [180]
Frog	<i>Rana pipiens</i>	Analgesia and nociception, Electrophysiology, Multiple sclerosis, Teratogenesis	[181], [182]
	<i>Xenopus laevis</i>		[183], [184]
Goat/sheep	<i>Ovis aries</i>	Osteoporosis, Bone tissue engineering	[185], [186]
	<i>Capra aegagrus hircus</i>		
Guinea pig	<i>Cavia porcellus</i>	Cardiovascular disease, Pharmacological characterization	[187], [188]
Horse	<i>Equus ferus caballus</i>	Depression, Heterologous antiserum and antivenom generation	[189], [59,190]

Llama	<i>Lama glama</i>	Nanobody (VHH) generation	[191]
Mice	Wild derived strain:	Cancer therapy, Drug development, Hybridoma technology, Toxicological evaluation, DNA vaccines, Nanotechnology, Inflammation, Chronic stress, Behavioral studies and depression	[192], [193], [194]
	<i>Mus musculus</i>		[62,195], [196]
	Inbred strains:		[197], [198]
	BALB/c, C57BL/6		[199], [200]
Nematode	<i>Caenorhabditis elegans</i>	Drug development screening, Toxicological evaluation	[201], [202]
Non-human primates	<i>Callithrix jacchus</i>	Neuroscience research, HIV, Anxiety, Tuberculosis	[203], [204]
	<i>Macaca mulatta</i>		[205], [206]
	<i>Macaca fascicularis</i>		
Pig	<i>Sus scrofa domesticus</i>	Wound healing, skin graft, Pharmacokinetics	[207], [208]
Rabbit	<i>Oryctolagus cuniculus</i>	Atherosclerosis, Immunogenicity, Bone implants	[140], [209], [210]
Rat	<i>Rattus norvegicus domestica</i>	Anxiety and depression, Toxicological evaluation, Cancer therapy, Hypertension, Neurodegenerative diseases, Drug discovery	[211], [212]
	<i>Wistar</i>		[213], [214]
	<i>Lewis</i>		[215], [216]

Non-human primates have been extensively used as models in animal research, due to their close phylogenetic relationship to humans, with validated similarities in terms of behavioral and biochemical activities, as well as gene expression patterns [146]. Based on that, some non-human primates are considered key *in vivo* models for specific research fields, including studies with the acquired immunodeficiency disease syndrome (AIDS), autoimmune diseases, Parkinson's disease, hepatitis, diabetes, physiological and psychiatric disorders, transplants, toxicological effects, dentistry, drug abuse and vaccine development [133,147].

Nevertheless, there are many regulatory requirements and policies to perform experiments on non-human primates, especially because they are large animals, intelligent, social, long-lived, and non-domesticated animals [148]. Therefore, non-human primates should only be used in specific cases, when the less-sentient species do not meet the requirements of the research.

PICKING THE BEST RESEARCH MODEL

Selecting the best research model can be a real challenge. Based on the efforts to 3Rs principle (Reduce, Refine and Replace the use of animals), many *in vitro* and *in silico* models have been explored as alternatives to *in vivo* methods, such as organs-in-a-chip and toxicity prediction, respectively. Nevertheless, these alternative models should be mechanistically based on the *in vivo* process, scientifically supported, and based on well-known responses described *in vivo*, which means that the model should be validated before being adopted. Fortunately, the availability of *in vivo* alternative tests has increased intensely, and many are very attractive in cost and time [149].

Researchers can obtain information about the alternative validated methods through different regulatory agencies [150-152]. Based on the above, method validation is vital to check the safety and efficacy of the research. In concern to the biopharmaceutical drugs, many regulatory organizations have addressed this issue in the chemical and pharmaceutical industry. For instance, the analytic validation can be assessed at two levels.

1. The pre-study validation that aims to show the method can achieve its objectives; and

2. In-study validation that verifies if the method remains valid over time by including quality control samples in routine runs [153,154].

However, the main challenge in biomedical sciences is when the model cannot be compared to humans. Animal models have supported critical advances in biomedical research, offering deep insights into several diseases. But they have been less successful as a basis for advancing human health as the failure of translation from animal models to human patients has often been disappointing [155].

A common criticism of model-based biomedical research is that while we have gotten very good at curing mouse models, we have made much less progress for human patients and, although the assumption that the animal models are a good proxy for humans is a central tenet of biomedical research, it is not always a reliable one [156-158]. For instance, it is well-known the gap between human and murine inflammation responses. Thus, how can we compare mice models of human immune-related diseases (e.g. diabetes, asthma, and multiple sclerosis)? There are many animal data failing to predict human responses to potential immunological therapies [159].

Choosing of the best experiment to validate a hypothesis is not the only issue for researchers. There is no consensus in the scientific community for the model classification. Misunderstandings with *in vitro*, *ex vivo*, *in situ*, *ex situ*, and *in vivo* models are very frequent, simply because there is no article or review giving satisfactory information about differentiating these models. Although most academy agrees that *in vivo* models are those conducted in animals, the literature presents articles in which assays conducted out of animals (e.g. *ex vivo* and *in vitro*) are referred as *in vivo*. For instance, the chick chorioallantoic membrane (CAM) assay can be defined by researchers either as an *ex vivo* [160] or as an *in vivo* [161] approach.

However, since the assay is developed in the alive chick embryo (which last 21 days to develop), should fit better to classify the assay as an *in vivo* model [162].

Furthermore, it is easy to come across hundreds of research articles, even in highly reputed journals, where cell line culture experiments are referred to as both *in vitro*, and sometimes, *in vivo* [163-166]. Thus, which research model should be the best to classify cultures performed with cell lines? Following the previous definition, we encourage researchers to refer as *in vitro* all experiments developed with cell lines. Histological studies also easily generate confusion among researchers since they can be classified as *in vivo* [167], *ex vivo* [168], as well as *in vitro* [169] models. Basically, the differences between the models are based on the way that the tissue was obtained. *Ex vivo* and *in vitro* histology should follow Sections. On the other hand, *in vivo* histology is a peculiar model, where the tissue is accessed through an intravascular ultrasound radiofrequency technique, named also virtual histology.

CONCLUSION

As can be seen above, researchers can get very confused to pick and classify their research models since there are no guidelines for choosing and classifying them so far. In addition, these models and their classification may vary considerably among the several scientific fields, different research organizations, and individual investigators. This review is the first to clarify the main definitions of the principal research models used in biomedical sciences with marked examples, aiming to guide and align researchers during their experimental practices.

ACKNOWLEDGEMENT

We thank Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP, São Paulo Research Foundation; scholarships to ISO no. 2017/03580-9, ELPJ no. 2016/04761-4, and FAC no. 2017/14035-1), Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq, The National Council for Scientific and Technological Development, scholarships to MBP no. 307155/2017-0 and FAC no. 155276/2018-2), and the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - Brasil (CAPES, Finance Code 001, scholarships to ELPJ no. 88881.186830/2018-01 and IGF).

AUTHORS CONTRIBUTION

ISO, GMAS, FAC, ELPJ, IGF and UZ wrote the review, FAC provided figures and wrote the review and MBP was a major contributor in writing the manuscript. All authors read and approved the final manuscript.

REFERENCES

- Marecková E, Simon F, Cervený L (2002) Latin as the language of medical terminology: some remarks on its role and prospects. *Swiss Med Wkly* 132: 581-587.
- Brunton L (1916) Latin as a universal language. *Nature* 96: 649.
- Beliaieva OM, Lysanets YV, Znamenska IV, Rozhenko IV, Nikolaieva NM (2017) Terminological collocations in medical latin and english: a comparative study. *Wiad Lek* 70: 139-143.
- Soeiro M de NC, de Souza EM, da Silva CF, Batista D da GJ, Batista MM, et al. (2013) *In Vitro* and *In Vivo* studies of the antiparasitic activity of sterol 14 α -demethylase (CYP51) Inhibitor VNI against drug-resistant strains of trypanosoma cruzi. *Antimicrob Agents Chemother* 57: 4151-4163.
- Young VB, Knox KA, Pratt JS, Cortez JS, Mansfield LS, et al. (2004) *In Vitro* and *In Vivo* Characterization of helicobacter hepaticus cytolethal distending toxin mutants. *Infection and Immunity*. 72(5): 2521-2527.
- Becker OM, Marantz Y, Shacham S, Inbal B, Heifetz A, et al. (2004) G protein-coupled receptors: *In silico* drug discovery in 3D. *PNAS* 101(31): 11304-11309.
- Kampmann T, Yennamalli R, Campbell P, Stoermer MJ, Fairlie DP, et al. (2009) *In silico* screening of small molecule libraries using the dengue virus envelope E protein has identified compounds with antiviral activity against multiple flaviviruses. *Antiviral Research* 84(3): 234-241.
- Ahmed V, Opoku A, Aziz Z (2016) *Research methodology in the built environment: A selection of case studies*. Routledge, New York, USA.
- Amano T, González-Varo JP, Sutherland WJ (2016) Languages are still a major barrier to global science. *PLoS biology* 14: e2000933-e2000933.
- Scotti L, Ghasemi J, Scotti MT (2018) Editorial: *In Silico* methodologies applied to drug discovery. *CCHTS* 21(3): 150-151.
- Miramontes Pedro (1989) *DNA and RNA physicochemical constraints, cellular automata and molecular evolution*. Los Alamos, New Mexico, USA.
- Colquitt RB, Colquhoun DA, Thiele RH (2011) *In silico* modelling of physiologic systems. *Best practice & research Clinical anaesthesiology* 25(4): 499-510.
- Amberg A (2013) *In Silico* Methods. In: Vogel HG, et al. (Eds). *Drug discovery and evaluation: safety and pharmacokinetic assays*. Berlin, Heidelberg: Springer Berlin Heidelberg, pp. 1273-1296.
- Ekins S, Mestres J, Testa B (2007) *In silico* pharmacology for drug discovery: methods for virtual ligand screening and profiling. *Br J Pharmacol* 152(1): 9-20.
- Cavaliere F, Cozzini P (2018) *New in Silico trends in food toxicology*. *Chem Res Toxicol* 31(10): 992-993.
- Scior T, Bender A, Tresadern G, Medina-Franco JL, Martínez-Mayorga K, et al. (2012) Recognizing pitfalls in virtual screening: A Critical Review. *J Chem Inf Model* 52(4): 867-881.
- Marchal T (2015) *In Vivo, In Vitro, In Silico!* Best Practices.
- Trisilowati, Mallet DG (2012) *In Silico* experimental modeling of cancer treatment. *ISRN Oncology* 2012: 828701.
- Yan G, Wang X, Chen Z, Wu X, Pan J, et al. (2017) *In-silico* ADME studies for new drug discovery: from chemical compounds to Chinese herbal medicines. *Curr Drug Metab* 18(6): 535-539.
- Sotomayor M, Schulten K (2007) Single-molecule experiments *in Vitro* and *in Silico*. *Science* 316(5828): 1144-1148.
- Fischer HP (2008) Mathematical modeling of complex biological systems. *Alcohol Res Health* 31(1): 49-59.
- Sethi A, Joshi K, Sasikala K, Alvala M (2019) Molecular docking in modern drug discovery: principles and recent applications. *drug discovery and development - New Advances*.
- Kuntz ID, Blaney JM, Oatley SJ, Langridge R, Ferrin TE (1982) A geometric approach to macromolecule-ligand interactions. *J Mol Biol* 161(2): 269-288.
- Pagadala NS, Syed K, Tuszynski J (2017) Software for molecular docking: a review. *Biophys Rev* 9(2): 91-102.
- Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ (1990) Basic local alignment search tool. *J Mol Biol* 215(3): 403-410.
- Marti-Renom MA, Madhusudhan MS, Sali A (2004) Alignment of protein sequences by their profiles. *Protein Sci* 13(4): 1071-1087.
- Pucca MB, Cerni FA, Peigneur S, Bordon KCF, Tytgat J, et al. (2015) Revealing the function and the structural model of Ts4: Insights into the "Non-Toxic" Toxin from Tityus serrulatus Venom. *Toxins (Basel)* 7(7): 2534-2550.
- Bridge LJ, Mead J, Frattini E, Winfield I, Ladds G (2018) Modelling and simulation of biased agonism dynamics at a G protein-coupled receptor. *J Theor Biol* 442: 44-65.
- Myatt GJ, Ahlberg E, Akahori Y, Allen D, Amberg A, et al. (2018) *In silico* toxicology protocols. *Regulatory Toxicology and Pharmacology* 96: 1-17.

30. Idakwo G, Luttrell J, Chen M, Hong H, Zhou Z, et al. (2018) A review on machine learning methods for *in silico* toxicity prediction. *J Environ Sci Health C Environ Carcinog Ecotoxicol Rev* 36(4): 169-191.
31. Kamble A, Srinivasan S, Singh H (2019) *In-Silico* bioprospecting: Finding better enzymes. *Mol Biotechnol* 61(1): 53-59.
32. Roy A, Nair S, Sen N, Soni N, Madhusudhan MS (2017) *In silico* methods for design of biological therapeutics. *Methods* 131: 33-65.
33. Pappalardo F, Russo G, Tshinanu FM, Viceconti M (2019) *In silico* clinical trials: concepts and early adoptions. *Brief Bioinform* 20(5): 1699-1708.
34. Brown D, Namas RA, Almahmoud K, Zaaqoq A, Sarkar J, et al. (2015) Trauma *in silico*: Individual-specific mathematical models and virtual clinical populations. *Science Translational Medicine* 7: 285ra61-285ra61.
35. Pearson RM (1986) *In-vitro* techniques: can they replace animal testing? *Hum Reprod* 1(8): 559-560.
36. Weil H, Willams TI (1950) History of chromatography. *Nature* 166: 1000.
37. Iler RK (1979) Wiley-interscience publication. The chemistry of silica solubility, polymerisation, colloid and surface properties, and biochemistry. New York, USA.
38. Lehninger AL, Nelson DL, Cox MM (2013) Lehninger principles of biochemistry, (6th edn), WH Freeman, New York, USA.
39. Mesbah M, Premachandran U, Whitman WB (1989) Precise measurement of the G+C Content of deoxyribonucleic acid by high-performance liquid chromatography. *International Journal of Systematic Bacteriology* 39(2): 159-167.
40. Maher S, Jjunju FPM, Taylor S (2015) Colloquium: 100 years of mass spectrometry: Perspectives and future trends. *Reviews of Modern Physics* 87: 113-135.
41. Lazzari E, Arena K, Caramão EB, Herrero M (2019) Quantitative analysis of aqueous phases of bio-oils resulting from pyrolysis of different biomasses by two-dimensional comprehensive liquid chromatography. *Journal of Chromatography A* 1602: 359-367.
42. Morsa D, Baiwir D, La Rocca R, Zimmerman TA, Hanozin E, et al. (2019) Multi-enzymatic limited digestion: The next-generation sequencing for proteomics? *J Proteome Res* 18(6): 2501-2513.
43. O'Connell TX, Horita TJ, Kasravi B (2005) Understanding and interpreting serum protein electrophoresis. *Am Fam Physician* 71: 105-112.
44. Tiselius A (1937) A new apparatus for electrophoretic analysis of colloidal mixtures. *Trans Faraday Soc* 33: 524-531.
45. Hames BD (1998) Gel electrophoresis of proteins: A practical approach. OUP Oxford.
46. Kunkel HG, Tiselius A (1951) Electrophoresis of proteins on filter paper. *J Gen Physiol* 35(1): 89-118.
47. Rosenfeld J, Capdevielle J, Guillemot JC, Ferrara P (1992) In-gel digestion of proteins for internal sequence analysis after one- or two-dimensional gel electrophoresis. *Anal Biochem* 203(1): 173-179.
48. Yao H, Wynendaale E, Xu X, Kosgei A, De Spiegeleer B (2018) Circular dichroism in functional quality evaluation of medicines. *J Pharm Biomed Anal* 147: 50-64.
49. Bonjoch NP, Tamayo PR (2003) Protein content quantification by bradford method. In: Reigosa Roger MJ, (Ed.) *Handbook of Plant Ecophysiology Techniques*. Dordrecht: Kluwer Academic Publishers, pp. 283-295.
50. Peterson GL (1977) A simplification of the protein assay method of Lowry et al. which is more generally applicable. *Analytical Biochemistry* 83(2): 346-356.
51. McREE DE (1999) *Practical protein crystallography*. Elsevier.
52. Valério AA, Corradini AC, Panunto PC, Mello SM, Hyslop S (2002) Purification and characterization of a phosphodiesterase from bothrops alternatus snake venom. *J Protein Chem* 21(8): 495-503.
53. Bjork W (1963) Purification of phosphodiesterase from Bothrops atrox venom, with special consideration of the elimination of monophosphatases. *J Biol Chem* 238: 2487-2490.
54. Di Ferrante N (1956) Turbidimetric measurement of acid mucopolysaccharides and hyaluronidase activity. *J Biol Chem* 220(1): 303-306.
55. Pukrittayakamee S, Warrell DA, Desakorn V, McMichael AJ, White NJ, et al. (1988) The hyaluronidase activities of some Southeast Asian snake venoms. *Toxicon* 26(7): 629-637.
56. Selvanayagam ZE, Gopalakrishnakone P (1999) Tests for detection of snake venoms, toxins and venom antibodies: review on recent trends (1987-1997). *Toxicon* 37(4): 565-586.
57. Piliarik M, Vaisocherová H, Homola J (2009) Surface plasmon resonance biosensing. *Methods Mol Biol* 503: 65-88.
58. Mullis K, Faloona F, Scharf S, Saiki R, Horn G, et al. (1986) Specific enzymatic amplification of DNA *in vitro*: the polymerase chain reaction. *Cold Spring Harb Symp Quant Biol* 51 (Pt 1): 263-273.
59. Pucca MB, Cerni FA, Janke R, Bermúdez ME, Ledsgaard L, et al. (2019) History of envenoming therapy and current perspectives. *Front Immunol*.
60. Ryazantsev DY, Voronina DV, Zavriev SK (2016) Immuno-PCR: achievements and perspectives. *Biochemistry Mosc* 81(13): 1754-1770.
61. Dal Ferro M, Rizzo S, Rizzo E, Marano F, Luisi I, et al. (2019) Phage display technology for human monoclonal antibodies. *Methods Mol Biol* 1904: 319-338.
62. Laustsen AH, Karatt-Vellatt A, Masters EW, Arias AS, Pus U, et al. (2018) *In vivo* neutralization of dendrotoxin-mediated neurotoxicity of black mamba venom by oligoclonal human IgG antibodies. *Nature Communications* 9(1): 3928.
63. Ledsgaard L, Kilstrup M, Karatt-Vellatt A, McCafferty J, Laustsen AH (2018) Basics of antibody phage display technology. *Toxins (Basel)* 10(6): 236.
64. Karahalil B (2016) Overview of systems biology and omics technologies. *Curr Med Chem* 23(37): 4221-4230.
65. Kononen J, Bubendorf L, Kallioniemi A, Bärnlund M, Schraml P, et al. (1998) Tissue microarrays for high-throughput molecular profiling of tumor specimens. *Nat Med* 4(7): 844-847.
66. Zhang D, Salto TM, Putti TC, Do E, Koay ESC (2003) Reliability of tissue microarrays in detecting protein expression and gene amplification in breast cancer. *Mod Pathol* 16: 79-84.
67. Jędrzejczak-Silicka M (2017) History of cell culture. New insights into cell culture technology.
68. Horrocks C, Halse R, Suzuki R, Shepherd PR (2003) Human cell systems for drug discovery. *Curr Opin Drug Discov Devel* 6(4): 570-575.
69. Barretina J, Caponigro G, Stransky N, Venkatesan K, Margolin AA, et al. (2012) The cancer cell line encyclopedia enables predictive modelling of anticancer drug sensitivity. *Nature* 483: 603-607.
70. Ghandi M, Huang FW, Jané-Valbuena J, Kryukov GV, Lo CC, et al. (2019) Next-generation characterization of the cancer cell line encyclopedia. *Nature* 569: 503-568.
71. Risbridger GP (2015) Human cell lines as tools of our trade: "Laying It on the (Cell) Line." *Mol Endocrinol* 29(1): 1-2.
72. Prinsen MK, Koëter HB (1993) Justification of the enucleated eye test with eyes of slaughterhouse animals as an alternative to the draize eye irritation test with rabbits. *Food Chem Toxicol* 31(1): 69-76.
73. Prinsen MK (1996) The chicken enucleated eye test (CEET): a practical (pre)screen for the assessment of eye irritation/corrosion potential of test materials. *Food Chem Toxicol* 34(3): 291-296.
74. Mummery C, van de Stolpe A, Roelen BAJ, Clevers H (2013) Chapter 13 - Human Stem Cells for Organs-on-Chips: Clinical Trials Without

- Patients?11Part of this chapter was adapted from an Organs-on-Chips meeting report, Lab on a Chip 2013, DOI: 10.1039/c3lc50248a. Stem Cells (2nd Edn). Boston: Academic Press, p. 343-361.
75. Hodsden S (2015) Human organ-mimicking chip could revolutionize clinical trials. Med Device Online.
 76. Martins M, Schelz Z, Martins A, Molnar J, Hajös G, et al. (2007) *In vitro* and *ex vivo* activity of thioridazine derivatives against Mycobacterium tuberculosis. International Journal of Antimicrobial Agents 29(3): 338-340.
 77. Saeidnia S, Manayi A, Abdollahi M (2015) From *in vitro* experiments to *in vivo* and clinical studies; pros and cons. Curr Drug Discov Technol 12(4): 218-224.
 78. Clift MJD, Gehr P, Rothen-Rutishauser B (2011) Nanotoxicology: a perspective and discussion of whether or not *in vitro* testing is a valid alternative. Arch Toxicol 85(7): 723-731.
 79. Griesinger C, Desprez B, Coecke S, Casey W, Zuang V (2016) Validation of alternative *In Vitro* methods to animal testing: Concepts, challenges, processes and tools. Adv Exp Med Biol 856: 65-132.
 80. Tacke PJ, de Vries IJM, Torensma R, Figdor CG (2007) Dendritic-cell immunotherapy: from *ex vivo* loading to *in vivo* targeting. Nat Rev Immunol 7(10): 790-802.
 81. Dusinska M, Rundén-Pran E, Schnekenburger J, Kanno J (2017) Toxicity tests: *In Vitro* and *In Vivo*. adverse effects of engineered nanomaterials. Elsevier 51-82.
 82. Parente RJA, Tomazett MV, Pigosso LL, Bailão AM, Ferreira de Souza A, et al. (2018) *In vitro*, *ex vivo* and *in vivo* models: A comparative analysis of *Paracoccidioides* spp. proteomic studies. Fungal Biology 122(6): 505-513.
 83. Gregory EK, MA Emran Bashar, Tan M (2012) *Ex Vivo* gene therapy and vision. CGT 12(2): 103-115.
 84. Snijder B, Vladimer GI, Krall N, Miura K, Schmolke AS, et al. (2017) Image-based *ex-vivo* drug screening for patients with aggressive haematological malignancies: Interim results from a single-arm, open-label, pilot study. The Lancet Haematology 4(12): e595-e606.
 85. Gowing G, Svendsen S, Svendsen CN (2017) *Ex vivo* gene therapy for the treatment of neurological disorders. Prog Brain Res 230: 99-132.
 86. Granzin M, Wagner J, Köhl U, Cerwenka A, Huppert V, et al. (2017) Shaping of natural killer cell antitumor activity by *Ex Vivo* cultivation. Front Immunol 8: 458.
 87. Kumar S, Geiger H (2017) HSC niche biology and hsc expansion *Ex Vivo*. Trends Mol Med 23(9): 799-819.
 88. Zotti A, Banzato T, Gelain ME, Centelleghè C, Vaccaro C, et al. (2015) Correlation of renal histopathology with renal echogenicity in dogs and cats: an *ex-vivo* quantitative study. BMC Veterinary Research 11: 99.
 89. Chung WY, Wanford JJ, Kumar R, Isherwood JD, Haigh RD, et al. (2019) An *ex vivo* porcine spleen perfusion as a model of bacterial sepsis. ALTEX 36(1): 29-38.
 90. Schraufnagel DP, Steffen RJ, Vargo PR, Attia T, Elgharably H, et al. (2018) Devices for *ex vivo* heart and lung perfusion. Expert Rev Med Devices 15(3): 183-191.
 91. Naldini L (2011) *Ex vivo* gene transfer and correction for cell-based therapies. Nat Rev Genet 12(5): 301-315.
 92. OECD. Test No. 437: Bovine corneal opacity and permeability test method for identifying i) chemicals inducing serious eye damage and ii) Chemicals not requiring classification for eye irritation or serious eye damage. OECD 2017.
 93. Wilson SL, Ahearne M, Hopkinson A (2015) An overview of current techniques for ocular toxicity testing. Toxicology 327: 32-46.
 94. Van den Berghe C, Guillet MC, Compan D (2005) Performance of porcine corneal opacity and permeability assay to predict eye irritation for water-soluble cosmetic ingredients. Toxicology *in Vitro* 19(6): 823-830.
 95. Gómez-Sintes R, Villarejo-Zori B, Serrano-Puebla A, Esteban-Martínez L, Sierra-Filardi E, et al. (2017) Standard assays for the study of autophagy in the *Ex Vivo* Retina. Cells 6(4): 37.
 96. Kooragayala K, Gotoh N, Cogliati T, Nellissery J, Kaden TR, et al. (2015) Quantification of oxygen consumption in retina *Ex Vivo* demonstrates limited reserve capacity of photoreceptor mitochondria. Invest Ophthalmol Vis Sci 56(13): 8428-8436.
 97. Gotlieb N, Rosenne E, Matzner P, Shaashua L, Sorski L, et al. (2015) The misleading nature of *in vitro* and *ex-vivo* findings in studying the impact of stress hormones on NK cell cytotoxicity. Brain Behav Immun 45: 277-286.
 98. Oishi N, Montes-Moreno S, Feldman AL (2018) *In situ* neoplasia in lymph node pathology. Semin Diagn Pathol 35(1): 76-83.
 99. Edlefsen KL, Greisman HA, Yi HS, Mantei KM, Fromm JR (2011) Early lymph node involvement by mantle cell lymphoma limited to the germinal center: report of a case with a novel "follicular *in situ*" growth pattern. Am J Clin Pathol 136(2): 276-281.
 100. Langer-Safer PR, Levine M, Ward DC (1982) Immunological method for mapping genes on Drosophila polytene chromosomes. Proc Natl Acad Sci U S A 79(14): 4381-4385.
 101. Huber D, Voith von Voithenberg L, Kaigala GV (2018) Fluorescence *in situ* hybridization (FISH): History, limitations and what to expect from micro-scale FISH? Micro and Nano Engineering 1: 15-24.
 102. Onozato ML, Yapp C, Richardson D, Sundaresan T, Chahal V, et al. (2019) Highly Multiplexed Fluorescence *in Situ* Hybridization for *in Situ* Genomics. J Mol Diagn 21(3): 390-407.
 103. Nguyen HT, Dupont LN, Cuttaz EA, Jean AM, Trouillon R, et al. (2018) Breast cancer HER2 analysis by extra-short incubation microfluidics-assisted fluorescence *in situ* hybridization (ESIMA FISH). Microelectronic Engineering 189: 33-38.
 104. El-Menoufy MAM, Mourad ZI, Farahat NM (2018) The prognostic impact of loss of chromosome 7 material detected by fluorescence *in situ* hybridization (FISH) in myeloid malignancies. J Egypt Natl Canc Inst 30(4): 133-138.
 105. Chang AE, Ganz PA, Hayes DF, Kinsella TJ, Pass HI, et al. (2006) Oncology: an evidence-based approach. Springer Science Business Media Inc: Springer e-books, New York, USA.
 106. Parikh U, Chhor CM, Mercado CL (2018) Ductal carcinoma *in situ*: The whole truth. AJR Am J Roentgenol 210(2): 246-255.
 107. Karube K, Scarfò L, Campo E, Ghia P (2014) Monoclonal B cell lymphocytosis and "*in situ*" lymphoma. Semin Cancer Biol 24: 3-14.
 108. Thomas JA, Buchsbaum RN, Zimniak A, Racker E (1979) Intracellular pH measurements in Ehrlich ascites tumor cells utilizing spectroscopic probes generated *in situ*. Biochemistry 18(11): 2210-2218.
 109. Kunishige JH, Doan L, Brodland DG, Zitelli JA (2019) Comparison of surgical margins for lentigo maligna versus melanoma *in situ*. J Am Acad Dermatol 81(1): 204-212.
 110. Stappaerts J, Brouwers J, Annaert P, Augustijns P (2015) *In situ* perfusion in rodents to explore intestinal drug absorption: Challenges and opportunities. Int J Pharm 478(2): 665-681.
 111. Lozoya-Agullo I, Zur M, Fine-Shamir N, Markovic M, Cohen Y, et al. (2017) Investigating drug absorption from the colon: Single-pass vs. Doluisio approaches to *in-situ* rat large-intestinal perfusion. Int J Pharm 527(1-2): 135-141.
 112. Luo Z, Liu Y, Zhao B, Tang M, Dong H, et al. (2013) *Ex vivo* and *in situ* approaches used to study intestinal absorption. J Pharmacol Toxicol Methods 68(2): 208-216.
 113. Yang H, Zhai B, Fan Y, Wang J, Sun J, et al. (2018) Intestinal absorption mechanisms of araloside A *in situ* single-pass intestinal perfusion and *in vitro* Caco-2 cell model. Biomedicine & Pharmacotherapy 106: 1563-1569.

114. Stumpf TR, Yang X, Zhang J, Cao X (2018) *In situ* and *ex situ* modifications of bacterial cellulose for applications in tissue engineering. *Materials Science and Engineering: C* 82: 372-383.
115. Bral M, Gala-Lopez B, Bigam DL, Freed DH, Shapiro AMJ (2018) *Ex situ* liver perfusion: Organ preservation into the future. *Transplantation Reviews* 32(3): 132-141.
116. Liu Q, Nassar A, Buccini L, Grady P, Soliman B, et al. (2018) *Ex situ* 86-hour liver perfusion: Pushing the boundary of organ preservation. *Liver Transplantation* 24(4): 557-561.
117. Werner NL, Alghanem F, Rakestraw SL, Sarver DC, Nicely B, et al. (2017) *Ex Situ* Perfusion of Human Limb Allografts for 24 Hours. *Transplantation* 101(3): e68-e74.
118. Lewis LM, Guo J, Torres E, Wang J, Billones H, Kolhe P, et al. (2017) *Ex Situ* and *In Situ* Characterization of vaccine suspensions in pre-filled syringes. *Journal of Pharmaceutical Sciences* 106(8): 2163-2167.
119. Mounce R, Smith P, Brockington S (2017) *Ex situ* conservation of plant diversity in the world's botanic gardens. *Nature Plants* 3: 795-802.
120. Kasso M, Balakrishnan M (2013) *Ex Situ* conservation of biodiversity with particular emphasis to Ethiopia. *International Scholarly Research Notices* p.11.
121. Worker T (2004) The use of non-human animals in research: A guide for scientists. *Alternatives to Laboratory Animals* 32: 119-120.
122. Barré SF, Montagutelli X (2015) Animal models are essential to biological research: Issues and perspectives. *Future Sci OA* 1(4): FSO63.
123. Joffe AR, Bara M, Anton N, Nobis N (2016) The ethics of animal research: a survey of the public and scientists in North America. *BMC Medical Ethics* 17: 17.
124. Chen FM, Liu X (2016) Advancing biomaterials of human origin for tissue engineering. *Progress in Polymer Science* 53: 86-168.
125. Zhang YS, Yue K, Aleman J, Moghaddam KM, Bakht SM, Yang J, et al. (2017) 3D Bioprinting for Tissue and Organ Fabrication. *Ann Biomed Eng* 45(1): 148-163.
126. Kumari M, Ernest V, Mukherjee A, Chandrasekaran N (2012) *In vivo* nanotoxicity assays in plant models. *Methods Mol Biol* 926: 399-410.
127. Ebel I (2013) Pesquisa usa 115 milhões de animais por ano no mundo, diz ativista.
128. UAR news team (2019). Animal research numbers in 2018. Understanding animal research.
129. Perlman RL (2016) Mouse models of human disease: An evolutionary perspective. *Evol Med Public Health* 2016(1): 170-176.
130. Council NR (2004) Science, medicine and animals.
131. Guan C, Ye C, Yang X, Gao J (2010) A review of current large-scale mouse knockout efforts. *Genesis* 48(2): 73-85.
132. Kim IY, Shin JH, Seong JK (2010) Mouse phenogenomics, toolbox for functional annotation of human genome. *BMB Rep* 43: 79-90.
133. Andersen ML, Winter LMF (2019) Animal models in biological and biomedical research - experimental and ethical concerns. *An Acad Bras Cienc* 91(suppl 1): e20170238.
134. Denayer T, Stöhr T, Van Roy M (2014) Animal models in translational medicine: Validation and prediction. *New Horizons in Translational Medicine* 2(1): 5-11.
135. Vandamme TF (2015) Rodent models for human diseases. *Eur J Pharmacol* 759: 84-89.
136. Alaraby M, Annangi B, Marcos R, Hernández A (2016) *Drosophila melanogaster* as a suitable *in vivo* model to determine potential side effects of nanomaterials: A review. *J Toxicol Environ Health B Crit Rev* 19(2): 65-104.
137. Dooley K, Zon LI (2000) Zebrafish: a model system for the study of human disease. *Curr Opin Genet Dev* 10(3): 252-256.
138. Kaletta T, Hengartner MO (2006) Finding function in novel targets: *C. elegans* as a model organism. *Nat Rev Drug Discov* 5(5): 387-398.
139. Nutt LK (2012) The *Xenopus* oocyte: a model for studying the metabolic regulation of cancer cell death. *Semin Cell Dev Biol* 23: 412-418.
140. Fan J, Chen Y, Yan H, Niimi M, Wang Y, Liang J (2018) Principles and applications of rabbit models for *atherosclerosis* research. *J Atheroscler Thromb* 25(3): 213-220.
141. Raweerith R, Ratanabanangkoon K (2005) Immunochemical and biochemical comparisons of equine monovalent and polyvalent snake antivenoms. *Toxicon* 45(3): 369-375.
142. Berglundh T, Abrahamsson I, Lang NP, Lindhe J (2003) De novo alveolar bone formation adjacent to endosseous implants. *Clin Oral Implants Res* 14(3): 251-262.
143. Topolnik L, Steriade M, Timofeev I (2003) Hyperexcitability of intact neurons underlies acute development of trauma-related electrographic seizures in cats *in vivo*. *Eur J Neurosci* 18(3): 486-496.
144. Barbero AM, Frasch HF (2009) Pig and guinea pig skin as surrogates for human *in vitro* penetration studies: a quantitative review. *Toxicol In Vitro* 23(1): 1-13.
145. Machado CJ, Bachevalier J (2003) Non-human primate models of childhood psychopathology: the promise and the limitations. *J Child Psychol & Psychiat* 44(1): 64-87.
146. Phillips KA, Bales KL, Capitanio JP, Conley A, Czoty PW, et al. (2014) Why primate models matter. *Am J Primatol* 76(9): 801-827.
147. Bontrop RE (2001) Non-human primates: essential partners in biomedical research. *Immunological Reviews* 183: 5-9.
148. Tardif SD, Coleman K, Hobbs TR, Lutz C (2013) IACUC review of nonhuman primate research. *ILAR J* 54(2): 234-245.
149. (2017) National academies of sciences, Studies D on E and L, Toxicology B on ES and Evaluations C on 21st CS into R-B. Model and assay validation and acceptance. National Academies Press USA.
150. O'Connor L (2017) EU reference laboratory for alternatives to animal testing. EU Science Hub-European Commission, Belgium.
151. (2019) AltTox.org. animal testing alternatives | Non-Animal Methods.
152. NIEHS (2019) Interagency coordinating committee on the validation of alternative methods.
153. Stöckl D, D'Hondt H, Thienpont LM (2009) Method validation across the disciplines--critical investigation of major validation criteria and associated experimental protocols. *J Chromatogr B Analyt Technol Biomed Life Sci* 877(23): 2180-2190.
154. Oliva A, Fariña JB, Llabrés M (2016) Pre-study and in-study validation of a size-exclusion chromatography method with different detection modes for the analysis of monoclonal antibody aggregates. *J Chromatogr B Analyt Technol Biomed Life Sci* 1022: 206-212.
155. van der Staay FJ, Arndt SS, Nordquist RE (2009) Evaluation of animal models of neurobehavioral disorders. *Behav Brain Funct* 5: 11.
156. Pound P, Ebrahim S, Sandercock P, Bracken MB, Roberts I (2004) Where is the evidence that animal research benefits humans? *BMJ* 328(7438): 514-517.
157. Seok J, Warren HS, Cuenca AG, Mindrinos MN, Baker HV, et al. (2013) Genomic responses in mouse models poorly mimic human inflammatory diseases. *Proc Natl Acad Sci USA* 110(9): 3507-3512.
158. Ioannidis JPA (2012) Extrapolating from animals to humans. *Sci Transl Med* 4(151): 151ps15.
159. Mestas J, Hughes CCW (2004) Of mice and not men: differences between mouse and human immunology. *J Immunol* 172(5): 2731-2738.
160. Jedelská J, Strehlow B, Bakowsky U, Aigner A, Höbel S, et al. (2013) The chorioallantoic membrane assay is a promising *ex vivo* model system for the study of vascular anomalies. *In Vivo* 27(6): 701-705.

161. Lokman NA, Elder ASF, Ricciardelli C, Oehler MK (2012) Chick Chorioallantoic Membrane (CAM) assay as an *In Vivo* model to study the effect of newly identified molecules on ovarian cancer invasion and metastasis. *Int J Mol Sci* 13: 9959-9970.
162. Ribatti D (2010) The chick embryo chorioallantoic membrane in the study of angiogenesis and metastasis: The CAM assay in the study of angiogenesis and metastasis. Springer Science & Business Media, Germany.
163. Antoni D, Burckel H, Josset E, Noel G (2015) Three-dimensional cell culture: A breakthrough *in Vivo*. *Int J Mol Sci* 16(3): 5517-5527.
164. Lelièvre SA, Kwok T, Chittiboyina S (2017) Architecture in 3D cell culture: An essential feature for *in vitro* toxicology. *Toxicol In Vitro* 45(3): 287-295.
165. Kutscher HL, Morse GD, Prasad PN, Reynolds JL (2019) *In vitro* pharmacokinetic cell culture system that simulates physiologic nanoparticle exposure to macrophages. *Pharm Res* 36(3): 44.
166. Allen DD, Caviedes R, Cárdenas AM, Shimahara T, Segura AJ, et al. (2005) Cell lines as *in vitro* models for drug screening and toxicity studies. *Drug Dev Ind Pharm* 31(8): 757-768.
167. Nasu K, Tsuchikane E, Katoh O, Vince DG, Virmani R, et al. (2006) Accuracy of *in vivo* coronary plaque morphology assessment. *Journal of the American College of Cardiology* 47(12): 2405-2412.
168. Li L, Pahwa S, Penzias G, Rusu M, Gollamudi J, et al. (2017) Co-Registration of *ex vivo* Surgical Histopathology and *in vivo* T2 weighted MRI of the Prostate *via* multi-scale spectral embedding representation. *Sci Rep* 7(1): 8717.
169. Alam K, Al Ghaithi A, Piya S, Saleem A (2019) *In-vitro* experimental study of histopathology of bone in vibrational drilling. *Med Eng Phys* 67: 78-87.
170. Narfström K, Holland DK, Menotti RM (2011) The domestic cat as a large animal model for characterization of disease and therapeutic intervention in hereditary retinal blindness. *Journal of Ophthalmology*.
171. Samaha G, Beatty J, Wade CM, Haase B (2019) The burmese cat as a genetic model of type 2 diabetes in humans. *Anim Genet* 50(4): 319-325.
172. Bergknot N, Rutges JPHJ, Kranenburg HJC, Smolders LA, Hagman R, et al. (2012) The dog as an animal model for intervertebral disc degeneration? *Spine* 37: 351-358.
173. Struillou X, Boutigny H, Soueidan A, Layrolle P (2010) Experimental animal models in periodontology: A Review. *Open Dent J* 4: 37-47.
174. Parnig C, Seng WL, Semino C, McGrath P (2002) Zebrafish: A preclinical model for drug screening. *ASSAY and drug development technologies* 1(1): 41-48.
175. Piatto ÂL, Capiotti KM, Tamborski AR, Oses JP, Barcellos LJG, et al. (2011) Unpredictable chronic stress model in zebrafish (*Danio rerio*): Behavioral and physiological responses. *Prog Neuropsychopharmacol Biol Psychiatry* 35(2): 561-567.
176. Manni I, de Latouliere L, Gurtner A, Piaggio G (2019) Transgenic animal models to visualize cancer-related cellular processes by bioluminescence imaging. *Front Pharmacol*.
177. Martínez NFJ, Martínez MT, Mulero V, Galindo VJ (2019) Models of human psoriasis: Zebrafish the newly appointed player. *Dev Comp Immunol* 97: 76-87.
178. Sieber S, Grossen P, Bussmann J, Campbell F, Kros A, et al. (2019) Zebrafish as a preclinical *in vivo* screening model for nanomedicines. *Adv Drug Deliv Rev* 151-152: 152-168.
179. Pandey UB, Nichols CD (2011) Human disease models in *Drosophila melanogaster* and the Role of the fly in therapeutic drug discovery. *Pharmacol Rev* 63(2): 411-436.
180. Gonzalez C (2013) *Drosophila melanogaster*: a model and a tool to investigate malignancy and identify new therapeutics. *Nat Rev Cancer* 13(3): 172-183.
181. Stevens CW (2011) Analgesia in *amphibians*: Preclinical studies and clinical applications. *Vet Clin North Am Exot Anim Pract* 14(1): 33-44.
182. Wagner CA, Friedrich B, Setiawan I, Lang F, Bröer S (2000) The use of xenopus laevis oocytes for the functional characterization of heterologously expressed membrane proteins. *Cell Physiol Biochem* 10(1-2): 1-12.
183. Mannioui A, Zalc B (2019) Conditional demyelination and remyelination in a transgenic *Xenopus laevis*. *Methods Mol Biol* 1936: 239-248.
184. Fort DJ, Mathis M (2018) Frog embryo teratogenesis assay-xenopus (FETAX): Use in alternative preclinical safety assessment. *Cold Spring Harb Protoc* 2018: pdb.prot098319 .
185. Dias IR, Camassa JA, Bordelo JA, Babo PS, Viegas CA, et al. (2018) Preclinical and translational studies in small ruminants (Sheep and Goat) as models for osteoporosis research. *Curr Osteoporos Rep* 16(2): 182-197.
186. McGovern JA, Griffin M, Huttmacher DW (2018) Animal models for bone tissue engineering and modelling disease. *Dis Model Mech* 11(4): dmm033084.
187. Madsen CS, Janovitz E, Zhang R, Nguyen TV, Ryan CS, et al. (2008) The guinea pig as a preclinical model for demonstrating the efficacy and safety of statins. *J Pharmacol Exp Ther* 324(2): 576-586.
188. Grigoleit HG, Grigoleit P (2005) Pharmacology and preclinical pharmacokinetics of peppermint oil. *Phytomedicine* 12(8): 612-616.
189. Fureix C, Jegou P, Henry S, Lansade L, Hausberger M (2012) Towards an ethological animal model of depression? A Study on Horses. *PLOS ONE* 7(6): e39280.
190. Sapsutthipas S, Leong PK, Akesowan S, Pratanaphon R, Tan NH, et al. (2015) Effective equine immunization protocol for production of potent poly-specific antisera against *calloselasma rhodostoma*, *Cryptelytrops albolabris* and *Daboia siamensis*. *PLoS Negl Trop Dis* 9(3): e0003609.
191. Vercruyssen T, Pardon E, Vanstreels E, Steyaert J, Daelemans D (2010) An intrabody based on a llama single-domain antibody targeting the N-terminal α -helical multimerization domain of HIV-1 Rev prevents viral production. *J Biol Chem* 285(28):21768-21780.
192. Pritchard JB, French JE, Davis BJ, Haseman JK (2003) The role of transgenic mouse models in carcinogen identification. *Environ Health Perspect* 111(4): 444-454.
193. Sharpless NE, DePinho RA (2006) The mighty mouse: genetically engineered mouse models in cancer drug development. *Nat Rev Drug Discov* 5(9):741-754.
194. Glukhova XA, Prusakova OV, Trizna JA, Zaripov MM, Afanaseva GV, et al. (2016) Updates on the production of therapeutic antibodies using human hybridoma technique. *Curr Pharm Des* 22(7): 870-878.
195. Gad SC (2016) Animal models in toxicology. 3rd edn, Boca Raton: CRC Press, USA, pp. 1152.
196. Pinto PBA, Assis ML, Vallochi AL, Pacheco AR, Lima LM, et al. (2019) T cell responses induced by DNA vaccines based on the DENV2 E and NS1 Proteins in Mice: Importance in protection and immunodominant epitope identification. *Front Immunol* 10: 1522.
197. Greish K, Alqahtani AA, Alotaibi AF, Abdulla AM, Bukelly AT, et al. (2019) The effect of silver nanoparticles on learning, memory and social interaction in BALB/C Mice. *Int J Environ Res Public Health* 16(1): 148.
198. da Silva BAF, da Costa RHS, Fernandes CN, Leite LHI, Ribeiro FJ, et al. (2018) HPLC profile and antiedematogenic activity of *Ximenea americana* L. (Olacaceae) in mice models of skin inflammation. *Food Chem Toxicol* 119: 199-205.
199. Borrow AP, Heck AL, Miller AM, Sheng JA, Stover SA, et al. (2019) Chronic variable stress alters hypothalamic-pituitary-adrenal axis function in the female mouse. *Physiology & Behavior* 209: 112613.

200. Nisar S, Farooq RK, Nazir S, Alamoudi W, Alhibshi A (2019) Exposure to early life adversity alters the future behavioral response to a stressful challenge in BALB/C mice. *Physiol Behav* 210: 112622.
201. Kim W, Hendricks GL, Lee K, Mylonakis E (2017) An update on the use of *C. elegans* for preclinical drug discovery: screening and identifying anti-infective drugs. *Expert Opin on Drug Discovery* 12: 625-633.
202. Dengg M, van Meel JCA (2004) *Caenorhabditis elegans* as model system for rapid toxicity assessment of pharmaceutical compounds. *J Pharmacol Toxicol Methods* 50(3): 209-214.
203. Kishi N, Okano H (2017) Neuroscience research using non-human primate models and genome editing. In: Jaenisch R, Zhang F, Gage F, (Eds.) *genome editing in neurosciences* (CH): Springer, New York.
204. Manickam C, Shah SV, Nohara J, Ferrari G, Reeves RK (2019) Monkeying around: Using non-human primate models to study nk cell biology in HIV Infections. *Front Immunol* 10: 1124.
205. Pagliaccio D, Pine DS, Leibenluft E, Monte OD, Averbek BB, et al. (2019) Cross-species convergence in pupillary response: Understanding human anxiety *via* non-human primate amygdala lesion. *Soc Cogn Affect Neurosci* 14(6): 591-599.
206. Peña JC, Ho WZ (2016) Non-human primate models of tuberculosis. *Microbiol Spectr* 4(4).
207. Tapking C, Popp D, Branski LK (2019) Pig model to test tissue-engineered skin. In: Böttcher HS, Biedermann T (Eds.) *skin tissue engineering: methods and protocols*, Springer New York, p. 239-49.
208. Dhondt L, Croubels S, Paepe PD, Cock PD, Devreese M (2019) P27 the juvenile pig as animal model for unraveling renal drug elimination processes in children. *Archives of Disease in Childhood* 104(6): e28-e28.
209. Ferreira RN, Machado de ARA, Sanchez EF, Maria WS, Molina F, et al. (2006) Antibodies against synthetic epitopes inhibit the enzymatic activity of mutalysin II, a metalloproteinase from bushmaster snake venom. *Toxicol* 48(8): 1098-1103.
210. Mapara M, Thomas BS, Bhat KM (2012) Rabbit as an animal model for experimental research. *Dent Res J (Isfahan)* 9(1): 111-118.
211. Souza D, Sadananda M (2017) Anxiety and depressive-like profiles during early- and mid-adolescence in the female Wistar Kyoto rat. *Int J Dev Neurosci* 56: 18-26.
212. Merlo E, Schreider IRG, Simões MR, Vassallo DV, Graceli JB (2019) Mercury leads to features of polycystic ovary syndrome in rats. *Toxicol Lett* 312: 45-54.
213. Zhang RK, Wang C (2018) Effect of matrine on tumor growth and inflammatory factors and immune function in Wistar rat with breast cancer. *Zhongguo Ying Yong Sheng Li Xue Za Zhi* 34(4): 375-378.
214. Pinheiro JEL, Boldrini FJ, de Campos ALMP, Santos NA, Bendhack LM, et al. (2018) LmrBPP9: A synthetic bradykinin-potentiating peptide from *Lachesis muta rhombeata* venom that inhibits the angiotensin-converting enzyme activity *in vitro* and reduces the blood pressure of hypertensive rats. *Peptides* 102: 1-7.
215. Turek A, Olakowska E, Borecka A, Janeczek H, Sobota M, et al. (2016) Shape-memory terpolymer rods with 17- β -estradiol for the treatment of neurodegenerative diseases: An *In Vitro* and *In Vivo* Study. *Pharm Res* 33(12): 2967-2978.
216. Kadhum WR, Hada T, Hijikuro I, Todo H, Sugibayashi K (2017) Development and optimization of orally and topically applied liquid crystal drug formulations. *J Oleo Sci* 66(9): 939-003950.