Small

Requirement for Animal Experiments: Problems and Challenges

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Please submit a plain text version of your cover letter here.	Dr. Ulf Thomas Scheffler Editor, Small Dear Dr. Scheffler, Thank you very much for the invitation to contribute to the Special Issue entitled "Advanced In Vitro Models for Replacement of Animal Experiments" in Small. I would like to submit our revised version of previous rejected manuscript (smll.202003064) entitled "Requirement for Animal Experiments: Problems and Challenges" to be considered as an Essay Article in Small for the mentioned Special Issue.

First, I would like to thank You and the Reviewers for the time spent in evaluating our paper and also for the very constructive comments, which undoubtedly will improve the overall quality of our work. We also thank You for the possibility given to reply to those comments with a new submission.

However, it was with a bit surprise I saw your rejection letter based on the Rev. #2 comments, taking into account the suggestion to write an Essay in this particular topic was given by You. Nevertheless, we have revised the paper according to the reviewers' suggestions. Please, find enclosed the replies to the reviewers' reports, which also summarize the changes made in the manuscript highlighted in yellow in the main.

The main issues are coming from Rev. #2, mainly stating the paper is not comprehensive and detailed enough. Well, we fully agree with the Rev. #2, because this is not a Review paper, it is an Essay!! However, we can make it a full review, if that's the wish, please let us know. We wrote this work according to the guidelines You sent me in the invitation letter (please see your original invitation text after my signature stating "submission of an Essay on 'Requirement for Animal Experiments / Problems and Challenges'"). Rev. #2 would like to see more details, but this is not what was asked or then cannot be an Essay article, so we would really appreciate if your kind explanation to Rev.#2 what Essay means, since we have followed the Wiley guidelines to draft this particular Essay, or then please do inform if you want us to write not an Essay, but instead a comprehensive review paper. I am sorry for all the confusion, but we hope Rev.#2 could kind understand the difference here.

We truly believe that the changes introduced to the revised manuscript have further improved it and we hope that this Essay can now be consider for publication in your valuable journal.

Thank you very much in advance for your kind consideration!

Yours,
Hélder Santos

_____
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Sent: Friday, November 15, 2019 2:12 PM
To: Almeida Santos, Helder <helder.santos@helsinki.fi>
Subject: Invitation for Small Special Issue on "Advanced In Vitro Models for Replacement of Animal Experiments" (smll.201906638, Santos)

Dear Prof. Santos,

On behalf of Prof. Shareen Doak and Dr. Martin Clift, it is my great pleasure to invite you to contribute an article to the special issue entitled "Advanced In Vitro Models for Replacement of Animal Experiments" in Small.

This special issue aims to highlight the current state of the art in alternative and advanced in vitro models, and test systems, to demonstrate the wide range of

	situations in which they may be applied to replace or reduce the necessity for in vivo testing. Given your highly regarded expertise in this area, we would very much appreciate submission of an Essay on 'Requirement for Animal Experiments / Problems and Challenges'. However, if you would prefer to focus on a slightly different area, then please feel free to contact the guest editors to discuss the topic further. The author guidelines for all article types are available here: http://onlinelibrary.wiley.com/journal/10.1002/(ISSN)1613-6829/homepage/2296_authors.html In its second decade as a top-tier materials science journal, Small (www.small-journal.com) continues to be among the top multidisciplinary journals covering a broad spectrum of topics at the nano- and microscale at the interface of materials science, chemistry, physics, engineering, medicine, and biology. The journal is known for its rapid and fair peer review, quality content, and high impact (2018 Journal Citation Reports: 10.856), making it one of the first choices of the international materials science community. The due date for manuscript submission is 30 Jun 2020. Manuscripts considered for this special issue will be judged using the same high standards as for regular submissions to Small. - we aim for this to be a top-quality selection of the very best research in the field. Accepted manuscripts will be published online as soon as possible, and collected into a dedicated issue of the journal, which will be released summer/autumn 2020. The online publication date will be the official publication date of your manuscript. We would be grateful if you have time to confirm your participation before 25 Nov 2019, by following this personalized link: https://www.editorialmanager.com/small-journal/l.asp?i=297532&l=TFVIH1Z1. Also, please inform the editorial office at small@wiley-vch.de about your chosen article type and a proposed topic or working title. If you do not wish to participate, you can decline by following this link: https://www.editorialmanager.com/small-journal/l.asp?i=297533&l=NKM6C0D3. Manuscripts should be submitted directly to the Small editorial office by following this link: https://www.editorialmanager.com/small-journal/l.asp?i=297534&l=ZZ0HFDKS. Alternatively, submission is possible by logging in to https://www.editorialmanager.com/small-journal/ with your username (hsantos) and password. There will be a link for this invitation in your "Invited Submissions" menu. Please select "By Invitation Only: Advanced In Vitro Models" as the section/category during submission. You are encouraged to suggest suitable referees for your work. If you do not know your password, anticipate at any stage not being able to meet the deadline, or have any other questions, feel free to contact the editorial office at small@wiley-vch.de. We very much hope that you would be interested in contributing to this special issue, and are already looking forward to hearing from you soon. With kind regards, On behalf of the guest editors: Prof. Shareen Doak and Prof. Martin Clift, Dr. Ulf Scheffler, Editor
Do you or any of your co-authors have a conflict of interest to declare?	No. The authors declare no conflict of interest.

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Small

REPLY TO REVIEWER'S COMMENTS

Reviewer #1:

The manuscript is well written and follow a logic description of the issue. The readers of the journl will appreciate it. The different thematics have been sequentially reported and deal with the possibility to start a deep new discussion to how apporach in vitro and in vivo experiments.

I only suggest to reformulate the abstract. Also cut the number of refs.

R.: We would like to thank the reviewer for the overall appraisal and valuable comments to our essay article. We need to highlight, however, that this is an Essay article, and that the abstract is limited to a short number of words/sentences and, subsequently, to a very brief description of what is covered in the article. We have, however, revised the language of the abstract for clarity purposes.

The references included in the manuscript were carefully selected to support each statement in which they are placed. Cutting on the number of references may lead to a lack of support of the information provided, and may limit the amount of articles suggested for further reading that we see suitable/needed for a deeper understanding of the issues covered herein. We hope the reviewer understand the need of the references for the wide audience of the journal and this multidisciplinary topic.

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REPLY TO REVIEWER'S COMMENTS

Reviewer #2:

Is this article timely? No: There are many articles covering this area that are in the literature. In its present form, the article does not progress the knowledge and understanding of the field.

R.: We would like to thank the Reviewer for the time spent reading and analyzing our article. We would like to clarify that this is an Essay article and not a regular review and, therefore, we are limited to a short amount of information that we can provide. The title and type of article (Essay) were suggested by the editor of this special issue, Dr. Ulf Scheffler, as part of a special issue entitled "*Advanced In Vitro Models for the Replacement of Animal Experiments*", which we believe will further address the issues that are briefly covered in this Essay. Here, we aimed at briefly addressing the current landscape of animal experimentation and shortly review a number of alternative strategies to the *in vivo* studies.

Is the article balanced, fair, and correct? No: There are moments in the article where the language is too casual (e.g. end of abstract 'will be pointed out'; not scientific writing). Further, the authors make inflammatory comments based upon poor understanding (e.g. 'Animals are closest system to the human body'). In fact, they aren't. For example, the reproductive system is not ideal in rodents for humans. Also, pig-skin is the closest thing to human skin, from animals, but the in vitro models of human skin is closer. So such comments in such an article are not helpful to make the conclusion thee authors wish to (which remains unclear).

R.: We have revised the manuscript and addressed these comments by improving the scientific writing and by attenuating the statements that could be perceived as inflammatory and based on poor understanding. Furthermore, we highlighted the differences between human and animals as concerning skin and reproductive system in page 5.

Does the length of the article do justice to the subject matter? No: The different sub-sections are brief and not informative enough. In this context, the article is too short. This is a common theme throughout.

R.: We would like to re-inforce that this is an Essay article with its own guidelines, and that we cannot cover all aspects and perspectives as we would in a review article. The text is continuously supported by a number of references, providing the reader with material for a deeper understanding of the intricacies of the in vivo studies as well as the currently available alternative models.

COMMENTS TO AUTHOR:

This article is interesting, but with the number of 'alternative' focussing articles currently published and being published it is difficult to see where this article progresses the knowledgeable or opinion of this subject matter. The article is too casual in its text, and in places is overly-inflammatory (e.g. Animals are the closest system to humans (stated by the authors)). This is not correct, nor helpful in the discussion overall that the authors try to make. The examples of tables are not thorough enough to be suggested as an overview, nor are the sub-sections detailed enough to give the reader a full understanding of the different topic areas. The article is loose, and does not warrant publication at this time, as it is too premature in its context, detail and provision of information beyond that already accessible within the literature.

R.: We thank the Reviewer for the comments and input. We understand the concerns raised by the Reviewer on this subject. However, we would like to highlight that the article should follow the structure of an Essay, and that both length and structure must obey the guidelines of the journal. Further details can be found in the references selected to support the statements. The title/theme of the article has been proposed by the Editor, which we believe was thought to fit the outline of the issue that it is being prepared.

The language of the text was carefully revised, and the scientific writing was improved. The tone set by the arguments utilized was also revised.

The Table was included to summarize some of the most common *in vitro*, *in silico* and *in vivo* models used in preclinical drug development, their advantages and disadvantages, and we have not referred

to it as an overview. The title of the Table and the way it is introduced in the text was changed for clarification purposes.

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Requirement for Animal Experiments: Problems and Challenges

*Flavia Fontana, Patrícia Figueiredo, João P. Martins, and Hélder A. Santos**

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Keywords: in vivo; animal experiments; in vitro; alternative organisms, in silico

Abstract

In vivo models remain a principle screening tool in the drug discovery pipeline. This Essay discusses the challenges associated with the need for animal experiments, as well as their impact on research, individual/societal and economic contexts. A number of alternatives that, with further development, optimization and investment may replace animal experiments are also revised.

1. Introduction

Preclinical evaluation of the efficacy and safety of new drugs and therapeutic strategies is pivotal in the process of drug development. It relies on an extensive series of in vitro, ex vivo, in silico and in vivo tests, which are intended to predict the physiological responses of drug therapies in humans, and therefore, determine the way a therapy is initially implemented.[1] The dynamic and complex microenvironments of living animals have made in vivo tests a regulatory requirement to validate preliminary experimental findings.[2,3] However, only about 10% of the drugs that enter clinical trials end-up being approved by regulatory agencies,[4] putting in evidence that animal models are not close enough to the human organism and often result into low predictability. Therefore, an improved understanding of both the pathological mechanisms and efficacy and safety of the tested drugs are needed to improve the translation of medicines from bench-to-bedside. Yet, the broad use of alternatives to in vivo models, capable of recapitulating the human biology complexities, while giving robust, reproducible, predictive and clinically relevant data, remain to be successfully introduced at larger scale.[5]

While the pharmaceutical research community must strongly commit to the continuing upwards trend in developing and adopting alternatives that can reduce and even replace animal testing, in vivo models remain as a necessary element of preclinical development. However, the hitherto unavoidable need for in vivo studies brings together a number of controversial issues inherent to the use of animals.[6] These include the fact that animals are sentient beings, the elevated costs of the experiments, genetic animal manipulation, the arguable translatability of the obtained data, as well as concerns about the mental wellbeing of the researchers and animal caretakers.[7-12]

In this article, we revise the main problems and challenges associated with the implementation of in vivo tests in the current landscape of drug development in academia, including their impact on research, individual/societal and economical contexts. A number of possible alternatives and solutions to overcome these limitations are also discussed.

2. Problems and challenges

Preclinical testing is still considered as of upmost importance in the drug discovery pipeline, despite the extremely pronounced rate of drug failure in clinical trials.[4,13] This failure rate is even more accentuated for innovative formulations like nanoparticles and drug delivery systems, as shown by some exemplary cases as BIND-014.[14] Innovative in vitro alternatives are being developed and tested, currently also assisted by the use of machine learning.[15,16] Nevertheless, at least for the present and near future, in vivo testing over animal models will still be required and widely used in preclinical experiments. However, several concerns can be raised, including the complexity of performing preclinical studies, the costs, reliability and translatability when using small animals, their impact on the society and one the single individual, the researchers directly working with animals. These concerns grapple with some of the issues related with experiments on living, sentient beings. The issues can be summarized into three broad categories: research, societal and personal, and economic, and are further discussed below.

2.1. Research Issues

Animals are generally considered as the intermediate step between bench and the human body, presenting comparable physiology, genetic similarities, and known behavior. However, why do drugs, materials, nanomedicines, or biomedical materials show promising or even extraordinary efficacy in preclinical models, but then fail in humans? Just taking into consideration cancer research, for example, the murine models widely used derive from cell lines, and are focusing on primary, easily accessible tumors and not on advanced tumor stages with metastases.[7] Moreover, the experimental designs vary widely between different papers, as well as the way how to report the results obtained in each study (e.g., tumor volume, tumor weight, and threshold of controlled tumor growth). Similar issues are reported also in preclinical research for cardiovascular diseases, psychiatric disorders, neurodegenerative diseases and autoimmune diseases.[8-11] Moreover, the physiology of animals is quite different from the human one. Porcine

skin is routinely employed as model of human skin, despite the differences which make alternative models better suited.[17] Moreover, the research into diseases of the reproductive organs as polycystic ovary syndrome or endometriosis is impaired in the selection of suitable animal models; only primates have comparable menstrual cycles, while rodent models are only presenting some of the symptoms and pathological changes.[18,19] Thereby, promising results in a simple murine model can easily then fail in human clinical trials. The failure of drugs in clinical trials creates a strong attrition rate for the pharma companies, promoting a constant research into preclinical models with better predictability or into alternative methods to animal studies.[20,21]

2.2. Societal and Personal Issues

The regulation of animal research has increased over the years with the increase in the societal interest and participation in animal ethics.[22] Multiple laws and guidelines are providing the set rules for institutions and researchers involved in preclinical studies.[23-25] The common silver lining between the different countries is the pledge to apply the 3R approach, where 3R stands for replacement, reduction, and refinement.[26]

Replacement focuses on the possibility to use alternative techniques (e.g., advanced in vitro models) to replace and avoid the use of animal models entirely. However, animal testing is still required for safety and efficacy studies before human clinical trials, and the currently available replacements for animal models are considered not suitable by the regulatory authorities.[27]

Reduction promotes a conscious and rational planning of the minimum number of animals required for gaining significant information. A careful biostatistical analysis is needed to merge the ethical requirements with the experimental demands.[28] However, many researchers do not have a solid background in biostatistics and do not have access to a biostatistician to calculate for them.

Finally, refinement demands a continuous effort to improve the quality of life of the animals, minimizing pain and suffering.[26] In the academic world, this requires a continuous discussion with the veterinarian staff at the animal facility to provide the knowledge needed on painkillers, anesthetics, and enrichments useful to minimize the stress of the animal.

The similarities between the different legislations regard mainly the 3Rs. The application of the rules and the organisms deemed to control the procedures vary amongst countries between "in-house" ethical committees, to external committees, to different levels of committees within the same institution.[24] The differences can hinder the collaboration between scientists where particular requirements from one country can leave visiting researchers puzzled. This is the case of the training requirements for researchers: each different country may have different requirements. The EU requires the researcher to obtain a Federation of European Laboratory Animal Science Association (FELASA) certification with functions A, B and D deemed essential for each researcher.[29] However, a research visit to USA will require the researcher to recertify according to the American standards and so on.

Moreover, the legislation requires knowledge and competences to draft research plans, applications for licenses to work with animals and statistical reports.[29] A further layer of complexity may be the need for some of those documents to be in the language of the country.[30,31] The universal language of Science is English and scientists are nomads, thereby it is surprisingly easy to be working in a foreign country without having a proficient knowledge of the local language.[32] However, these two knowledge barriers may prevent the researcher from applying for an animal license, or may make the application process quite frustrating.

Finally, animals are sentient beings and each researcher or animal caretaker is responsible for their wellbeing and to ensure minimal pain and suffering by euthanizing the animals when needed. While most of the societal ethical concerns focus on the animals, one should consider also the mental wellbeing of the researchers and caretakers. The relationship between humans and animals is complex and social, and the benefits of pet therapy have been demonstrated from

autism to re-educational purposes for convicts.[33-35] Being responsible for animals and for their death can induce depression, difficulty to cope with your work and disenfranchised loss in researchers and animal caretakers.[12] These feelings are related to the ones of people losing their pets, but they are repeated often, due to the nature of the occupation.[12]

2.3. Economical Issues

The use of animals in preclinical studies brings along several costs related both to the animals and their care, and also to the societal requirements. Each license or permission to work with animals is subject to a charge fee upon approval by the authorizing party (committee or agency). The animals are then purchased from authorized breeder with price ranges changing, depending on the type of animal, its age, and the presence of specific modifications in the genetic background.[36,37] The animals, upon arrival, are to be housed in appropriate animal facilities, in enriched holdings respecting the "Refinement" rule.[37] The daily care, assured by animal caretakers, is usually paid by the researchers to the animal facility. The type of experiment to be performed will then dictate the materials and instrumentation needed, as well as painkillers, anesthetics or other drugs to be administered. The materials needed for injections or for surgery need to be sterile in order to minimize any risk for both the animal and the researcher.

The list of chargeable items increases with the complexity of the experiment, and it represents a hefty investment of resources from the research group. At industrial level, the investments into such experiments are in a completely different scale compared to academia.[38,39] Nevertheless, given the high attrition rate experienced by pharma industries, preclinical testing in animals is expected to provide the maximum amount of information with the minimum associated costs.[20]

All the issues presented in this section are currently tackled both at research laboratory and industrial levels to minimize the need for animals, providing accurate data, avoiding waste of money, and responding to societal demands.

3. Possible Alternatives/Solutions

Over the years, different approaches for testing efficacy and toxicity of drugs and drug delivery systems as alternatives to animal testing have been investigated and adopted. In this regard, the European Union Reference Laboratory for alternatives to animal testing (EURL ECVAM) has been coordinating and conducting validation studies of alternative methods for the safety assessment of different chemicals, and promoting their use in an international context.[40] The integration of bioinformatics tools and various computer models, along with in vitro cell cultures and model tissues/organisms, can provide alternative protocols that result in the minimization of the number of animals used for scientific procedures (**Figure 1**).[41] **Table 1** summarizes the advantages and disadvantages of the most common in vitro, in silico and in vivo models currently in use.

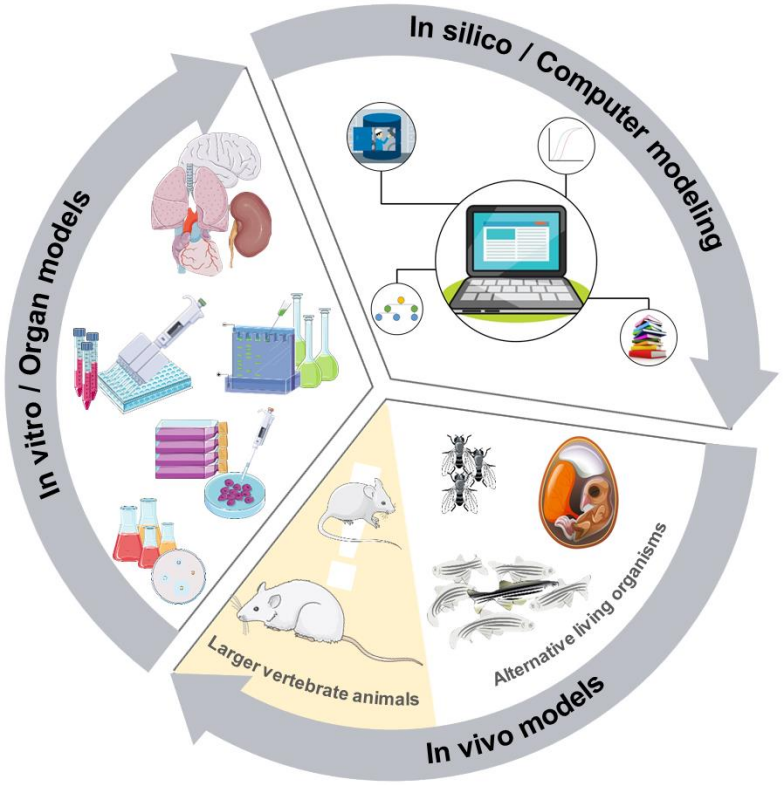

Figure 1. Schematic illustration of the different models and techniques available in drug development. A higher importance and relevance should be placed into cells, organ models and alternative living organisms as alternative to the use of larger vertebrate animals, along with in

silico and other computer models, reducing the need for extensive screening experiments. In vivo studies are currently still needed and demanded for from the regulatory authorities, but researchers in academia and industry should aim to reduce their use by improving the alternative methods currently available. Figure prepared with elements from Servier Medical Art, Servier, licensed under a Creative Commons Attribution 3.0 Unported License.

Table 1. Common in vitro, in silico and in vivo models used in preclinical drug development.[a]

	Model	Advantages	Disadvantages	Refs.
In vitro	**2D cell cultures** Cell monolayers, sandwich cultures, micro-patterning, transwell culture systems	Fast, simple and cheap set up; Reproducibility; Output data can be easily analyzed (e.g. imaging, FACS);	Changes in physiology lead to altered phenotypes not representing clinics; Lack of interaction with ECM; Unnatural cell growth kinetics and attachment; Data collected often does not correlate to what occurs in vivo;	42-46
	3D cell cultures Organoids, spheroids, organs-on-chips, hydrogels, bioreactors, cell sheets	Cell growth in 3D physical shape; Physiologically more relevant than 2D cultures; Closer to living tissues; Reproducibility;	Lack of standardized approaches; Higher costs compared to 2D;	43,47,48
In silico	**IATA** QSAR models, PBK models, in vitro tests, chemical analogues (read-across)	High throughput screening of compounds at lower cost than in vitro and in vivo; Predictions based on human or animal data, so the results are directly translatable; Useful in polypharmacology, multitarget drug discovery and repurposing of older drug; Availability of large datasets; More cost-effective.	Lack of precise information to build the models; Drawbacks or lack of clarity in the algorithms used	49-51
In vivo	**Large vertebrates** Mice, rats, rabbits, dogs, pigs, non-human primates	Relatively short gestation period; Shorter life cycles; Genetic similarity to humans; Lessen the risk for unforeseen complications in humans; Animals can be genetically modified to study specific physiology and pathophysiology;	Ethical issues; Animals are purposely bred for experiments; Impact on researchers and animal caretakers; High costs; 90% of drugs fail in clinical trials.	12,52
	Alternative living organisms Microorganisms, lower vertebrates, invertebrates, CAM	No ethical issues for embryos or invertebrates; cheaper models; good for study of mechanisms.	Animals are purposely bred for experiments; Lack translatability to higher organisms and humans; Lack of mammalian physiology;	52,53

[a] ADME: Adsorption, Distribution, Metabolism, Excretion; CAM: chick embryo chorioallantoic membrane; ECM: extracellular matrix; FACS: fluorescence-activated cell sorting; IATA: Integrated Approaches to Testing and Assessment; PKB: physiologically based kinetic; QSAR: Quantitative structure-activity relationship;

3.1. In Vitro Cells/Tissue/Organ Models

In vitro models involve the use of different types of culture (e.g., conventional 2D cell cultures, tissues or organ cultures) for preliminary screening of potential chemicals/materials, allowing the evaluation of their efficacy and toxicity, along with various other endpoints.[41,54] As these techniques are easy to perform and less expensive and time consuming, they represent an important alternative for animal experiments.[41,54] Furthermore, in some cases, the in vitro models are closer to human than the animals. Porcine skin is routinely employed as model of human skin to assess drugs or medical devices.[17] However, porcine skin presents anatomical differences, which can reduce the predictivity and translatability of the model.[55]

Comparing to the well-established 2D cell cultures, the 3D cell culture models resemble better the complex organization of living tissues and physiological functions at the organ level, as they enable the crosstalk between different cell types and extracellular matrices, as well as other organs. Therefore, they allow the evaluation of more complex biological responses upon contact with the tested compounds.[56] Moreover, different microfluidics devices have been developed as an in vitro diagnostic tool for culturing adherent and non-adherent cell lines, since such devices provide accurate and controllable biochemical/biophysical environments, along with high resolution spectroscopies and real-time imaging techniques.[57,58] Microfluidic cell cultures have been used to investigate a variety of biological processes, including cell signaling and dynamic cell-to-cell interactions, as well as drug screening and optimization, and toxicological testing.[57,58]

Among others, 3D organ models of almost all organs of the human body have been reconstructed, and are typically produced by sequential cell seeding into cell culture inserts or porous 3D scaffolds, and can differ in their complexity and, consequently, predictivity.[59] The microfabrication of more sophisticated systems, such as organs-on-chips, allowed to produce biomimetic systems comprising microfluidic channels lined by living human cells, replicating key functional units of living organs that integrated human organ- level pathophysiology in

vitro.[60,61] With this approach, it was possible to fabricate models, for example, of blood vessels, bones, skin, cartilage, muscles, kidney, liver and brain.[60,61] Therefore, these mechanically active microdevices offer a low-cost alternative to the animal studies for screening and toxicological evaluation of new compounds.[61] A tumor-on-a-chip model was developed by Carvalho et al., which allowed to simultaneously perform viability studies to evaluate efficacy and dose-response effect of the cells exposed to a drug-loaded nanoparticles gradient, and also evaluate expression levels of certain genes (e.g., MMP-1, Caspase-3, and Ki-67).[62]

Nevertheless, there are still quite numerous complications with in vitro models. Thereby, worldwide continuous efforts should focus on the optimization of these models and on the promotion of their widespread implementation both in academia and industry.

3.2. Alternative Living Organisms

The use of alternative organisms has also been suggested to overcome the ethical issues and restrictions of using higher model vertebrates in animal experiments.[41] In this category, several model organisms have been proposed, including: (1) microorganisms, such as Saccharomyces cerevisiae, as rapid growth and versatile model for molecular and genetic studies for several diseases;[63] (2) lower vertebrates like Danio rerio and chick embryo; and (3) invertebrates organisms, including Drosophila melanogaster and Caenorhabditis elegans.[41,52]

Danio rerio, also called zebrafish, has become increasingly popular as a replacement method for larger vertebrate animal experiments. This model can be easily handled in the laboratory, and presents a nearly transparent body during early development, offering a low-cost option for direct observation of developmental stages (embryos and larvae), direct observation of gene expression using light microscopy, and an easy screening of new molecules and assessment of endpoint of toxicity testing.[64,65] Additionally, the whole genome sequence is already available, making zebrafish an appealing alternative for molecular and genetic studies in cancer research, heart diseases, neurological malfunctions, and to observe the mutations and other problems

during organ development upon exposure to the testing compounds.[41] Moreover, zebrafish have emerged as a promising preclinical in vivo model for screening nanomedicines, as reviewed by Sieber et al..[66] They highlighted the benefits of this model in the nanomedicine development pipelines, including biological conservation, availability of genetic tools, imaging modalities, and disease models, which allow the quick screening of nanomedicines as a bridge between in vitro and rodent studies.[66] Chick embryo is another cost-effective and less sentient in vivo model that is gaining popularity for screening of chemicals and biomaterials in a short time. The chorioallantoic membrane (CAM) assays of the chick embryo is highly vascularized, containing both mature vessels and capillaries, and is easily accessible for orthotopic implantation of different biomaterials.[67,68] For example, Moreno-Jiménez et al. used the chorioallantoic membrane (CAM) model as a bioreactor to study the regeneration of a human living bone for tissue engineering applications.[69] Furthermore, CAM assay allows the study of antitumor therapies on the tumor growth, along with the molecular pathways associated.[70]

Invertebrate organisms can also be used as an alternative for animal experiments, although their use might be limited for certain diseases, because they do not have adaptive immune system. The most commonly used models are the fruit fly Drosophila melanogaster and the nematode Caenorhabditis elegans, and they present less ethical problems and cost of housing compared to larger animals.[41,71] The fruit fly Drosophila melanogaster represents a unique and sensitive model to study of human genetics, as nearly 75% of the genes involved in human diseases present a functional homolog in the fruit fly.[41,71] Additionally, the fruit fly has four stages in its short life cycle, i.e. embryo, the larva, the pupa and the adult, which offer different benefits to study various processes: (1) the embryo can be used to study the cell fate determination, organogenesis, and neuronal development; (2) the larva can be useful to assess physiological and developmental processes; and (3) the adult fly is more complex, allowing to study the functions of various organs (e.g., heart, gut, lungs, and kidney).[41,72] Caenorhabditis elegans (C. elegans) is a small nematode with metabolically active and intact digestive, endocrine,

reproductive, sensory and neuromuscular systems.[73] As an intermediate between in vitro testing and larger animal experiments, C. elegans assays have been used to predict toxicological data of testing compounds or nanomaterials obtained in mammals.[73,74] Due to the various complex developmental stages (i.e., embryogenesis, morphogenesis and growth to an adult), C. elegans represents a good model to study numerous various neurological disorders, such as Parkinson's, Alzheimer's and Huntington's diseases, as well as cancer and diabetes.[41] Moreover, Rive et al. used C. elegans to evaluate the adverse effects of acute and chronic exposure to small size graphene oxide and the amino-functionalized counterpart, which was assessed by characterizing fecundity, physiology, lifespan and developmental timing after treatment.[75]

3.3. Computer Models

In addition to the traditional in vitro and in vivo testing approaches, Integrated Approaches to Testing and Assessment (IATA) offer a means of integrating and translating the data obtained using toxicity testing methods, being a flexible and suitable tools for toxicological decision making. IATA concept has been proposed by the Organization for Economic Cooperation and Development (OECD) member countries, in order to progressively shift from traditional drug assessment using animal models.[76] IATA uses new approaches like high content screening and high-throughput screening methods, together with different computational methods used for data generation, interpretation and integration.[77,78] The use of adverse outcome pathways (AOP) in developing IATA can be useful to organize and understand the key events within biological pathways that lead to adverse outcomes induced by chemicals. The general framework on how AOP can be used for this purpose is the following: (1) formulation and identification of the problem (e.g., regulatory question, end- point of interest, and decision context); (2) gathering and evaluating the existing information available for the chemical of interest; (3) assessing the weight of evidence of the gathered information and evaluate whether this information is adequate for decision- making regarding a potential risk; (4) if additional information is needed,

the AOP can help to develop a testing strategy to generate supplementary information; and (5) when the information is adequate, make a regulatory decision or final conclusion.[76,79] Several computation methodologies have been used in IATA, including Quantitative structure-activity relationship (QSAR) models, physiologically based kinetic (PBK) models, in vitro tests, and integrating existing sources of data on chemical analogues (read-across).[78,80] QSAR modeling represents one of the most popular computer-aided tools utilized for drug discovery and lead optimization, being particularly powerful when no 3D structures of specific drug targets are available.[81,82] The QSAR-based drug design project commonly involves the selection of suitable molecules, from which a set of chemical descriptors is created. Then, a model is constructed to establish the association between the descriptors and the bioactivity of interest, followed by the model validation to select those that present greatest performance, and finally, the model application to predict the activity of the tested molecules.[81,83] However, an incorrect data selection, inappropriate molecular descriptors and unsuitable model validation, can lead the constructed model to fail.[81]

PKB models are used to assess the chemical's pharmacokinetic properties, such as absorption, distribution, metabolism and elimination, since it presents compartments that represent plasma and various organs in the body. Therefore, these models can help to evaluate the dosimetry related to observed toxic effects in humans or other species.[80,84] Furthermore, PBK models offer a scientific basis to extrapolate across population or species, as well as routes of exposure based on physiology.[80,85] Additionally, physiologically based kinetic and dynamic (PBK/D) modelling combines kinetic and dynamic interactions of a specific chemical upon exposure by applying differential equations, providing an additional description of the interaction of the compound or its reactive metabolite with the receptor mediating the adverse effect.[86]

Grouping/category approaches for read-across have emerged as alternative tools for hazard assessment of an untested chemical, using the available experimental data for structural and physicochemically similar compounds.[87,88] These tools are particularly useful for complex

endpoints (e.g., repeated dose or developmental and reproductive toxicity). Different tools can complement each other, and when appropriately applied, read-across approaches can replace de novo animal testing.[87,89]

4. Conclusions

While animal testing and experimentation are a regulatory requirement for validating preliminary data and animals will remain indispensable in research for some time, the development, optimization and investment in alternative solutions merits further commitment. As the legitimate interests of animal welfare, elevated experimental costs, and individual and societal consequences raise, animal experimentation must be conducted under stricter policies of reduction and refinement. Simultaneously, researchers, industry partners and regulatory agencies should commit towards the development, implementation and wide use of alternatives to animal testing, until advanced in vitro experiments, computer-modeling based approaches or any other serendipitous options can replace animal studies.

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Although animal experiments are still required to validate preliminary data on safety and efficacy of new compounds, high costs and ethical issues related with animal welfare are usually associated. Here, the main problems and challenges related to the use of animals in research are addressed, along with a variety of possible alternatives to animal testing.

Keyword: in vivo; animal experiments; in vitro; alternative organisms, in silico

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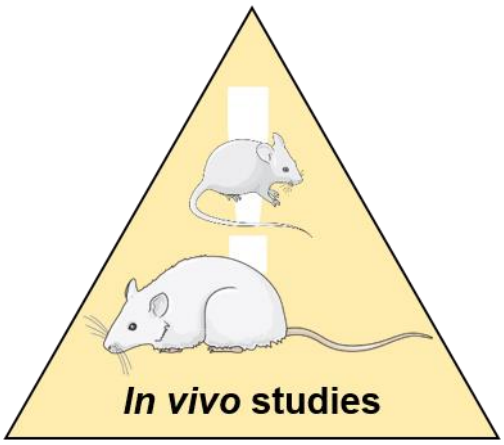
Requirement for Animal Experiments: Problems and Challenges



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