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REVIEW

Ethical considerations regarding animal experimentation

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Summary

Animal experimentation is widely used around the world for the identification of the root causes of various diseases in humans and animals and for exploring treatment options. Among the several animal species, rats, mice and purpose-bred birds comprise almost 90% of the animals that are used for research purpose. However, growing awareness of the sentience of animals and their experience of pain and suffering has led to strong opposition to animal research among many scientists and the general public. In addition, the usefulness of extrapolating animal data to humans has been questioned. This has led to Ethical Committees' adoption of the 'four Rs' principles (Reduction, Refinement, Replacement and Responsibility) as a guide when making decisions regarding animal experimentation. Some of the essential considerations for humane animal experimentation are presented in this review along with the requirement for investigator training. Due to the ethical issues surrounding the use of animals in experimentation, their use is declining in those research areas where alternative in vitro or in silico methods are available. However, so far it has not been possible to dispense with experimental animals completely and further research is needed to provide a road map to robust alternatives before their use can be fully discontinued.

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Introduction

Animal model-based research has been performed for a very long time. Ever since the 5th century B.C., reports of experiments involving animals have been documented, but an increase in the frequency of their utilization has been observed since the 19th century [1]. Most institutions for medical research around the world use non-human animals as experimental subjects [2]. Such animals might be used for research experimentations to gain a better understanding of human diseases or for exploring potential treatment options [2]. Even those animals that are evolutionarily quite distant from humans, such as *Drosophila melanogaster*, Zebrafish (*Danio rerio*) and *Caenorhabditis elegans*, share physiological and genetic similarities with human beings [2]; therefore animal experimentation can be of great help for the advancement of medical science [2].

For animal experimentation, the major assumption is that the animal research will be of benefit to humans. There are many reasons that highlight the significance of animal use in biomedical research. One of the major reasons is that animals and humans share the same biological processes. In addition, vertebrates have many anatomical similarities (all vertebrates have lungs, a heart, kidneys, liver and other organs) [3]. Therefore, these similarities make certain animals more suitable for experiments and for providing basic training to young researchers and students in different fields of biological and biomedical sciences [3]. Certain animals are susceptible to various health problems that are similar to human diseases such as diabetes, cancer and heart disease [4]. Furthermore, there are genetically modified animals that are used to obtain pathological phenotypes [5]. A significant benefit of animal experimentation is that test species can be chosen that have a much shorter life cycle than humans. Therefore, animal models can be studied throughout their life span and for several successive generations, an essential element for the understanding of disease progression along with its interaction with the whole organism throughout its lifetime [6].

Animal models often play a critical role in helping researchers who are exploring the efficacy and safety of potential medical treatments and drugs. They help to identify any dangerous or undesired side effects, such as birth defects, infertility, toxicity, liver damage or any potential carcinogenic effects [7]. Currently, U.S. Federal law, for example, requires that non-human animal research is used to demonstrate the efficacy and safety of any new treatment options before proceeding to trials on humans [8]. Of course, it is not only humans benefit from this research and testing, since many of the drugs and treatments that are developed for humans are routinely used in veterinary clinics, which help animals live longer and healthier lives [4].

COVID-19 AND THE NEED FOR ANIMAL MODELS

When COVID-19 struck, there was a desperate need for research on the disease, its effects on the brain and body and on the development of new treatments for patients with the disease. Early in the disease it was noticed that those with the disease suffered a loss of smell and taste, as well as neurological and psychiatric symptoms, some of which lasted long after the patients had "survived" the disease [9-15]. As soon as the pandemic started, there was a search for appropriate animal models in which to study this unknown disease [16, 17]. While genetically modified mice and rats are the basic animal models for neurological and immunological research [18, 19] the need to understand COVID-19 led to a range of animal models; from fruit flies [20] and Zebrafish [21] to large mammals [22, 23] and primates [24, 25]. And it was just not one animal model that was needed, but many, because different aspects of the disease are best studied in different animal models [16, 25, 26]. There is also a need to study the transmission pathways of the zoonosis: where does it come from, what are the animal hosts and how is it transferred to humans [27]?

There has been a need for animal models for understanding the pathophysiology of COVID-19 [28], for studying the mechanisms of transmission of the disease [16], for studying its neurobiology [29,30] and for developing new vaccines [31]. The sudden onset of the COVID-19 pandemic has highlighted the fact that animal research is necessary, and that the curtailment of such research has serious consequences for the health of both humans and animals, both wild and domestic [32] As highlighted by Adhikary et al. [22] and Genzel et al. [33] the coronavirus has made clear the necessity for animal research and the danger in surviving future such pandemics if animal research is not fully supported. Genzel et al. [33], in particular, take issue with the proposal for a European ban on animal testing. Finally, there is a danger in bypassing animal research in developing new vaccines for diseases such as COVID-19 [34]. The purpose of this paper is to show that, while animal research is necessary for the health of both humans and animals, there is a need to carry out such experimentation in a controlled and humane manner. The use of alternatives to animal research such as cultured human cells and computer modeling may be a useful adjunct to animal studies but will require that such methods are more readily accessible to researchers and are not a replacement for animal experimentation.

Pros and cons of animal experimentation

ARGUMENTS AGAINST ANIMAL EXPERIMENTATION

A fundamental question surrounding this debate is to ask whether it is appropriate to use animals for medical research. Is our acceptance that animals have a morally lower value or standard of life just a case of speciesism [35]? Nowadays, most people agree that animals have a moral status and that needlessly hurting or abusing pets or other animals is unacceptable. This represents something of a change from the historical point of view where animals did not have any moral status and the treatment of animals was mostly subservient to maintaining the health and dignity of humans [36].

Animal rights advocates strongly argue that the moral status of non-human animals is similar to that of humans, and that animals are entitled to equality of treatment. In this view, animals should be treated with the same level of respect as humans, and no one should have the right to force them into any service or to kill them or use them for their own goals. One aspect of this argument claims that moral status depends upon the capacity to suffer or enjoy life [37].

In terms of suffering and the capacity of enjoying life, many animals are not very different from human beings, as they can feel pain and experience pleasure [38]. Hence, they should be given the same moral status as humans and deserve equivalent treatment. Supporters of this argument point out that according animals a lower moral status than humans is a type of prejudice known as "speciesism" [38]. Among humans, it is widely accepted that being a part of a specific race or of a specific gender does not provide the right to ascribe a lower moral status to the outsiders. Many advocates of animal rights deploy the same argument, that being human does not give us sufficient grounds declare animals as being morally less significant [36].

ARGUMENTS IN FAVOR
OF ANIMAL EXPERIMENTATION

Those who support animal experimentation have frequently made the argument that animals cannot be elevated to be seen as morally equal to humans [39]. Their main argument is that the use of the terms "moral status" or "morality" is debatable. They emphasize that we must not make the error of defining a quality or capacity associated with an animal by using the same adjectives used for humans [39]. Since, for the most part, animals do not possess humans' cognitive capabilities and lack full autonomy (animals do not appear to rationally pursue specific goals in life), it is argued that therefore, they cannot be included in the moral community [39]. It follows from this line of argument that, if animals do not possess the same rights as human beings, their use in research experimentation can be considered appropriate [40]. The European and the American legislation support this kind of approach as much as their welfare is respected.

Another aspect of this argument is that the benefits to human beings of animal experimentation compensate for the harm caused to animals by these experiments.

In other words, animal harm is morally insignificant compared to the potential benefits to humans. Essentially, supporters of animal experimentation claim that human beings have a higher moral status than animals and that animals lack certain fundamental rights accorded to humans. The potential violations of animal rights during animal research are, in this way, justified by the greater benefits to mankind [40, 41]. A way to evaluate when the experiments are morally justified was published in 1986 by Bateson, which developed the Bateson's Cube [42]. The Cube has three axes: suffering, certainty of benefit and quality of research. If the research is high-quality, beneficial, and not inflicting suffering, it will be acceptable.

At the contrary, painful, low-quality research with lower likelihood of success will not be acceptable [42, 43].

Impact of experimentations on animals

ABILITY TO FEEL PAIN AND DISTRESS

Like humans, animal have certain physical as well as psychological characteristics that make their use for experimentation controversial [44].

In the last few decades, many studies have increased knowledge of animal awareness and sentience: they indicate that animals have greater potential to experience damage than previously appreciated and that current rights and protections need to be reconsidered [45]. In recent times, scientists as well as ethicists have broadly acknowledged that animals can also experience distress and pain [46]. Potential sources of such harm arising from their use in research include disease, basic physiological needs deprivation and invasive procedures [46]. Moreover, social deprivation and lack of the ability to carry out their natural behaviors are other causes of animal harm [46]. Several studies have shown that, even in response to very gentle handling and management, animals can show marked alterations in their physiological and hormonal stress markers [47].

In spite of the fact that suffering and pain are personalized experiences, several multi-disciplinary studies have provided clear evidence of animals experiencing pain and distress. In particular, some animal species have the ability to express pain similarly to human due to common psychological, neuroanatomical and genetic characteristics [48]. Similarly, animals share a resemblance to humans in their developmental, genetic and environmental risk factors for psychopathology. For instance, in many species, it has been shown that fear operates within a less organized subcortical neural circuit than pain [49, 50]. Various types of depression and anxiety disorders like posttraumatic stress disorder have also been reported in mammals [51].

PSYCHOLOGICAL CAPABILITIES OF ANIMALS

Some researchers have suggested that besides their ability to experience physical and psychological pain and distress, some animals also exhibit empathy, self-awareness and language-like capabilities. They also demonstrate tools-linked cognizance, pleasure-seeking and advanced problem-solving skills [52]. Moreover, mammals and birds exhibit playful behavior, an indicator of the capacity to experience pleasure. Other taxa such as reptiles, cephalopods and fishes have also been observed to display playful behavior, therefore the current legislation prescribes the use of environmental enrichers [53]. The presence of self-awareness ability, as assessed by mirror self-recognition, has been reported in magpies, chimpanzees and other apes, and certain cetaceans [54]. Recently, another study has revealed that crows have the ability to create and use tools that involve episodic-like memory formation and its retrieval. From these findings, it may be suggested that crows as well as related spe-

cies show evidence of flexible learning strategies, causal reasoning, prospection and imagination that are similar to behavior observed in great apes [55]. In the context of resolving the ethical dilemmas about animal experimentation, these observations serve to highlight the challenges involved [56, 57].

Ethics, principles and legislation in animal experimentation

ETHICS IN ANIMAL EXPERIMENTATION

Legislation around animal research is based on the idea of the moral acceptability of the proposed experiments under specific conditions [58]. The significance of research ethics that ensures proper treatment of experimental animals [58]. To avoid undue suffering of animals, it is important to follow ethical considerations during animal studies [1]. It is important to provide best human care to these animals from the ethical and scientific point of view [1]. Poor animal care can lead to experimental outcomes [1]. Thus, if experimental animals mistreated, the scientific knowledge and conclusions obtained from experiments may be compromised and may be difficult to replicate, a hallmark of scientific research [1]. At present, most ethical guidelines work on the assumption that animal experimentation is justified because of the significant potential benefits to human beings. These guidelines are often permissive of animal experimentation regardless of the damage to the animal as long as human benefits are achieved [59].

PRINCIPLE OF THE 4 RS

Although animal experimentation has resulted in many discoveries and helped in the understanding numerous aspects of biological science, its use in various sectors is strictly controlled. In practice, the proposed set of animal experiments is usually considered by a multidisciplinary Ethics Committee before work can commence [60]. This committee will review the research protocol and make a judgment as to its sustainability. National and international laws govern the utilization of animal experimentation during research and these laws are mostly based on the universal doctrine presented by Russell and Burch (1959) known as principle of the 3 Rs. The 3Rs referred to are Reduction, Refinement and Replacement, and are applied to protocols surrounding the use of animals in research. Some researchers have proposed another "R", of responsibility for the experimental animal as well as for the social and scientific status of the animal experiments [61]. Thus, animal ethics committees commonly review research projects with reference to the 4 Rs principles [62].

The first "R", Reduction means that the experimental design is examined to ensure that researchers have reduced the number of experimental animals in a research project to the minimum required for reliable data [59]. Methods used for this purpose include improved experimental design, extensive literature search to avoid duplication of experiments [35], use of advanced imaging techniques, sharing resources and data, and appropriate statistical data analysis that reduce the number of animals needed for statistically significant results [2, 63].

The second "R", Refinement involves improvements in procedure that minimize the harmful effects of the proposed experiments on the animals involved, such as reducing pain, distress and suffering in a manner that leads to a general improvement in animal welfare. This might include for example improved living conditions for research animals, proper training of people handling animals, application of anesthesia and analgesia when required and the need for euthanasia of the animals at the end of the experiment to curtail their suffering [63].

The third "R", Replacement refers to approaches that replace or avoid the use of experimental animals altogether. These approaches involve use of *in silico* methods/computerized techniques/software and *in vitro* methods like cell and tissue culture testing, as well as relative replacement methods by use of invertebrates like nematode worms, fruit flies and microorganisms in place of vertebrates and higher animals [1]. Examples of proper application of these first "3R2 principles are the use of alternative sources of blood, the exploitation of commercially used animals for scientific research, a proper training without use of animals and the use of specimen from previous experiments for further researches [64-67].

The fourth "R", Responsibility refers to concerns around promoting animal welfare by improvements in experimental animals' social life, development of advanced scientific methods for objectively determining sentience, consciousness, experience of pain and intelligence in the animal kingdom, as well as effective involvement in the professionalization of the public discussion on animal ethics [68].

OTHER ASPECTS OF ANIMAL RESEARCH ETHICS

Other research ethics considerations include having a clear rationale and reasoning for the use of animals in a research project. Researchers must have reasonable expectation of generating useful data from the proposed experiment. Moreover, the research study should be designed in such a way that it should involve the lowest possible sample size of experimental animals while producing statistically significant results [35].

All individual researchers that handle experimental animals should be properly trained for handling the particular species involved in the research study. The animal's pain, suffering and discomfort should be minimized [69]. Animals should be given proper anesthesia when required and surgical procedures should not be repeated on same animal whenever possible [69]. The procedure of humane handling and care of experimental animals should be explicitly detailed in the research study protocol. Moreover, whenever required, aseptic techniques should be properly followed [70]. During the research, anesthetization and surgical procedures on experimental animals should only be performed by professionally skilled individuals [69].

The Animal Research: Reporting of In Vivo Experiments (ARRIVE) guidelines that are issued by the National Center for the Replacement, Refinement, and Reduction of Animals in Research (NC3Rs) are designed to improve the documentation surrounding research involving experimental animals [70]. The checklist provided includes the information required in the various sections of the manuscript i.e. study design, ethical statements, experimental procedures, experimental animals and their housing and husbandry, and more [70].

It is critical to follow the highest ethical standards while performing animal experiments. Indeed, most of the journals refuse to publish any research data that lack proper ethical considerations [35].

INVESTIGATORS' ETHICS

Since animals have sensitivity level similar to the human beings in terms of pain, anguish, survival instinct and memory, it is the responsibility of the investigator to closely monitor the animals that are used and identify any sign of distress [71]. No justification can rationalize the absence of anesthesia or analgesia in animals that undergo invasive surgery during the research [72]. Investigators are also responsible for giving high-quality care to the experimental animals, including the supply of a nutritious diet, easy water access, prevention of and relief from any pain, disease and injury, and appropriate housing facilities for the animal species [73]. A research experiment is not permitted if the damage caused to the animal exceeds the value of knowledge gained by that experiment. No scientific advancement based on the destruction and sufferings of another living being could be justified. Besides ensuring the welfare of animals involved, investigators must also follow the applicable legislation [74, 75].

To promote the comfort of experimental animals in England, an animal protection society named: 'The Society for the Preservation of Cruelty to Animals' (now the Royal Society for the Prevention of Cruelty to Animals) was established (1824) that aims to prevent cruelty to animal [76].

ANIMAL WELFARE LAWS

Legislation for animal protection during research has long been established. In 1876 the British Parliament sanctioned the 'Cruelty to Animals Act' for animal protection. Russell and Burch (1959) presented the '3 Rs' principles: Replacement, Reduction and Refinement, for use of animals during research [61]. Almost seven years later, the U.S.A also adopted regulations for the protection of experimental animals by enacting the Laboratory Animal Welfare Act of 1966 [60]. In Brazil, the Arouca Law (Law No. 11,794/08) regulates the animal use in scientific research experiments [76].

These laws define the breeding conditions, and regulate the use of animals for scientific research and teaching purposes. Such legal provisions control the use of anesthesia, analgesia or sedation in experiments that could cause distress or pain to experimental animals [59, 76]. These laws also stress the need for euthanasia when an experiment is finished, or even during the experiment if there is any intense suffering for the experimental animal [76].

Several national and international organizations have been established to develop alternative techniques so that animal experimentation can be avoided, such as the UK-based National Centre for the Replacement, Refinement and Reduction of Animals in Research (NC3Rs) (www.caat.jhsph.edu), the European Centre for the Validation of Alternative Methods (ECVAM) [77], the Universities Federation for Animal Welfare (UFAW) (www.ufaw.org.uk), The Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) [78], and The Center for Alternatives to Animal Testing (CAAT) (www.caat.jhsph.edu). The Brazilian 'Arouca Law' also constitutes a milestone, as it has created the 'National Council for the Control of Animal Experimentation' (CONCEA) that deals with the legal and ethical issues related to the use of experimental animals during scientific research [76].

Although national as well as international laws and guidelines have provided basic protections for experimental animals, the current regulations have some significant discrepancies. In the U.S., the Animal Welfare Act excludes rats, mice and purpose-bred birds, even though these species comprise almost 90% of the animals that are used for research purpose [79]. On the other hand, certain cats and dogs are getting special attention along with extra protection. While the U.S. Animal Welfare Act ignores birds, mice and rats, the U.S. guidelines that control research performed using federal funding ensure protections for all vertebrates [79, 80].

Living conditions of animals

CHOICE OF THE ANIMAL MODEL

Based on all the above laws and regulations and in line with the deliberations of ethical committees, every researcher must follow certain rules when dealing with animal models.

Before starting any experimental work, thorough research should be carried out during the study design phase so that the unnecessary use of experimental animals is avoided. Nevertheless, certain research studies may have compelling reasons for the use of animal models, such as the investigation of human diseases and toxicity tests. Moreover, animals are also widely used in the training of health professionals as well as in training doctors in surgical skills [1, 81].

Researcher should be well aware of the specific traits of the animal species they intend to use in the experiment, such as its developmental stages, physiology, nutritional needs, reproductive characteristics and specific behaviors. Animal models should be selected on the basis of the study design and the biological relevance of the animal [1].

Typically, in early research, non-mammalian models are used to get rapid insights into research problems such as the identification of gene function or the recognition

of novel therapeutic options. Thus, in biomedical and biological research, among the most commonly used model organisms are the Zebrafish, the fruit fly *Drosophila melanogaster* and the nematode *Caenorhabditis elegans*. The main advantage of these non-mammalian animal models is their prolific reproducibility along with their much shorter generation time. They can be easily grown in any laboratory setting, are less expensive than the murine animal models and are somewhat more powerful than the tissue and cell culture approaches [82]. *Caenorhabditis elegans* is a small-sized nematode with a short life cycle and that exists in large populations and is relatively inexpensive to cultivate. Scientists have gathered extensive knowledge of the genomics and genetics of *Caenorhabditis elegans*; but *Caenorhabditis elegans* models, while very useful in some respects, are unable to represent all signaling pathways found in humans. Furthermore, due to its short life cycle, scientists are unable to investigate long term effects of test compounds or to analyze primary versus secondary effects [6].

Similarly, the fruit fly *Drosophila melanogaster* has played a key role in numerous biomedical discoveries. It is small in size, has a short life cycle and large population size, is relatively inexpensive to breed, and extensive genomics and genetics information is available [6]. However, its respiratory, cardiovascular and nervous systems differ considerably from human beings. In addition, its immune system is less developed when compared to vertebrates, which is why effectiveness of a drug in *Drosophila melanogaster* may not be easily extrapolated to humans [83].

The Zebrafish (*Danio rerio*) is a small freshwater teleost, with transparent embryos, providing easy access for the observation of organogenesis and its manipulation. Therefore, Zebrafish embryos are considered good animal models for different human diseases like tuberculosis and fetal alcohol syndrome and are useful as neurodevelopmental research models. However, Zebrafish has very few mutant strains available, and its genome has numerous duplicate genes making it impossible to create knockout strains, since disrupting one copy of the gene will not disrupt the second copy of that gene. This feature limits the use of Zebrafish as animal models to study human diseases. Additionally they are rather expensive, have long life cycle, and genomics and genetics studies are still in progress [82, 84].

Thus, experimentation on these three animals might not be equivalent to experimentation on mammals. Mammalian animal model are most similar to human beings, so targeted gene replacement is possible. Traditionally, mammals like monkey and mice have been the preferred animal models for biomedical research because of their evolutionary closeness to humans. Rodents, particularly mice and rats, are the most frequently used animal models for scientific research. Rats are the most suitable animal model for the study of obesity, shock, peritonitis, sepsis, cancer, intestinal operations, spleen, gastric ulcers, mononuclear phagocytic system, organ transplantations and wound healing. Mice are more suitable for studying burns, megacolon, shock, cancer, obesity, and sepsis as mentioned previously [85].

Similarly, pigs are mostly used for stomach, liver and transplantation studies, while rabbits are suitable for the study of immunology, inflammation, vascular biology, shock, colitis and transplantations. Thus, the choice of experimental animal mainly depends upon the field of scientific research under consideration [1].

HOUSING AND ENVIRONMENTAL ENRICHMENT

Researchers should be aware of the environment and conditions in which laboratory animals are kept during research, and they also need to be familiar with the metabolism of the animals kept in vivarium, since their metabolism can easily be altered by different factors such as pain, stress, confinement, lack of sunlight, etc. Housing conditions alter animal behavior, and this can in turn affect experimental results. By contrast, handling procedures that feature environmental enrichment and enhancement help to decrease stress and positively affect the welfare of the animals and the reliability of research data [74, 75].

In animals, distress- and agony-causing factors should be controlled or eliminated to overcome any interference with data collection as well as with interpretation of the results, since impaired animal welfare leads to more animal usage during experiment, decreased reliability and increased discrepancies in results along with the unnecessary consumption of animal lives [86].

To reduce the variation or discrepancies in experimental data caused by various environmental factors, experimental animals must be kept in an appropriate and safe place. In addition, it is necessary to keep all variables like humidity, airflow and temperature at levels suitable for those species, as any abrupt variation in these factors could cause stress, reduced resistance and increased susceptibility to infections [74].

The space allotted to experimental animals should permit them free movement, proper sleep and where feasible allow for interaction with other animals of the same species. Mice and rats are quite sociable animals and must, therefore, be housed in groups for the expression of their normal behavior. Usually, laboratory cages are not appropriate for the behavioral needs of the animals. Therefore, environmental enrichment is an important feature for the expression of their natural behavior that will subsequently affect their defense mechanisms and physiology [87].

The features of environmental enrichment must satisfy the animals' sense of curiosity, offer them fun activities, and also permit them to fulfill their behavioral and physiological needs. These needs include exploring, hiding, building nests and gnawing. For this purpose, different things can be used in their environment, such as PVC tubes, cardboard, igloos, paper towel, cotton, disposable masks and paper strips [87].

The environment used for housing of animals must be continuously controlled by appropriate disinfection, hygiene protocols, sterilization and sanitation processes. These steps lead to a reduction in the occurrence of

various infectious agents that often found in vivarium, such as Sendai virus, cestoda and Mycoplasma pulmonis [88].

EUTHANASIA

Euthanasia is a term derived from Greek, and it means a death without any suffering. According to the Brazilian Arouca Law (Article 14, Chapter IV, Paragraphs 1 and 2), an animal should undergo euthanasia, in strict compliance with the requirements of each species, when the experiment ends or during any phase of the experiment, wherever this procedure is recommended and/or whenever serious suffering occurs. If the animal does not undergo euthanasia after the intervention it may leave the vivarium and be assigned to suitable people or to the animal protection bodies, duly legalized [1].

Euthanasia procedures must result in instant loss of consciousness which leads to respiratory or cardiac arrest as well as to complete brain function impairment. Another important aspect of this procedure is calm handling of the animal while taking it out of its enclosure, to reduce its distress, suffering, anxiety and fear. In every research project, the study design should include the details of the appropriate endpoints of these experimental animals, and also the methods that will be adopted. It is important to determine the appropriate method of euthanasia for the animal being used. Another important point is that, after completing the euthanasia procedure, the animal's death should be absolutely confirmed before discarding their bodies [87, 89].

Relevance of animal experimentations and possible alternatives

RELEVANCE OF ANIMAL EXPERIMENTS
AND THEIR ADVERSE EFFECTS ON HUMAN HEALTH

One important concern is whether human diseases, when inflicted on experimental animals, adequately mimic the progressions of the disease and the treatment responses observed in humans. Several research articles have made comparisons between human and animal data, and indicated that the results of animals' research could not always be reliably replicated in clinical research among humans. The latest systematic reviews about the treatment of different clinical conditions including neurology, vascular diseases and others, have established that the results of animal studies cannot properly predict human outcomes [59, 90].

At present, the reliability of animal experiments for extrapolation to human health is questionable. Harmful effects may occur in humans because of misleading results from research conducted on animals. For instance, during the late fifties, a sedative drug, thalidomide, was prescribed for pregnant women, but some of the women using that drug gave birth to babies lacking limbs or with foreshortened limbs, a condition called phocomelia. When thalidomide had been tested on almost all animal models such as rats, mice, rabbits, dogs, cats, hamsters, armadillos, ferrets, swine, guinea pig, etc., this terato-

genic effect was observed only occasionally [91]. Similarly, in 2006, the compound TGN 1412 was designed as an immunomodulatory drug, but when it was injected into six human volunteer, serious adverse reactions were observed resulting from a deadly cytokine storm that in turn led to disastrous systemic organ failure. TGN 1412 had been tested successfully in rats, mice, rabbits, and non-human primates [92]. Moreover, Bailey (2008) reported 90 HIV vaccines that had successful trial results in animals but which failed in human beings [93]. Moreover, in Parkinson disease, many therapeutic options that have shown promising results in rats and non-human primate models have proved harmful in humans. Hence, to analyze the relevance of animal research to human health, the efficacy of animal experimentation should be examined systematically [94, 95]. At the same time, the development of hyperoxaluria and renal failure (up to dialysis) after ileal-jejunal bypass was unexpected because this procedure was not preliminarily evaluated on an animal model [96].

Several factors play a role in the extrapolation of animal-derived data to humans, such as environmental conditions and physiological parameters related to stress, age of the experimental animals, etc. These factors could switch on or off genes in the animal models that are specific to species and/or strains. All these observations challenge the reliability and suitability of animal experimentation as well as its objectives with respect to human health [76, 92].

ALTERNATIVE TO ANIMAL EXPERIMENTATION/
DEVELOPMENT OF NEW PRODUCTS
AND TECHNIQUES TO AVOID ANIMAL SACRIFICE
IN RESEARCH

Certainly, *in vivo* animal experimentation has significantly contributed to the development of biological and biomedical research. However it has the limitations of strict ethical issues and high production cost. Some scientists consider animal testing an ineffective and immoral practice and therefore prefer alternative techniques to be used instead of animal experimentation. These alternative methods involve *in vitro* experiments and ex vivo models like cell and tissue cultures, use of plants and vegetables, non-invasive human clinical studies, use of corpses for studies, use of microorganisms or other simpler organism like shrimps and water flea larvae, physicochemical techniques, educational software, computer simulations, mathematical models and nanotechnology [97]. These methods and techniques are cost-effective and could efficiently replace animal models. They could therefore, contribute to animal welfare and to the development of new therapies that can identify the therapeutics and related complications at an early stage [1]. The National Research Council (UK) suggested a shift from the animal models toward computational models, as well as high-content and high-throughput *in vitro* methods. Their reports highlighted that these alternative methods could produce predictive data more affordably, accurately and quickly than the traditional *in vivo* or experimental animal methods [98].

Increasingly, scientists and the review boards have to assess whether addressing a research question using the applied techniques of advanced genetics, molecular, computational and cell biology, and biochemistry could be used to replace animal experiments [59]. It must be remembered that each alternative method must be first validated and then registered in dedicated databases.

An additional relevant concern is how precisely animal data can mirror relevant epigenetic changes and human genetic variability. Langley and his colleagues have highlighted some of the examples of existing and some emerging non-animal based research methods in the advanced fields of neurology, orthodontics, infectious diseases, immunology, endocrine, pulmonology, obstetrics, metabolism and cardiology [99].

IN SILICO SIMULATIONS AND INFORMATICS

Several computer models have been built to study cardiovascular risk and atherosclerotic plaque build-up, to model human metabolism, to evaluate drug toxicity and to address other questions that were previously approached by testing in animals [100].

Computer simulations can potentially decrease the number of experiments required for a research project, however simulations cannot completely replace laboratory experiments. Unfortunately, not all the principles regulating biological systems are known, and computer simulation provide only an estimation of possible effects due to the limitations of computer models in comparison with complex human tissues. However, simulation and bio-informatics are now considered essential in all fields of science for their efficiency in using the existing knowledge for further experimental designs [76].

At present, biological macromolecules are regularly simulated at various levels of detail, to predict their response and behavior under certain physical conditions, chemical exposures and stimulations. Computational and bioinformatic simulations have significantly reduced the number of animals sacrificed during drug discovery by short listing potential candidate molecules for a drug. Likewise, computer simulations have decreased the number of animal experiments required in other areas of biological science by efficiently using the existing knowledge. Moreover, the development of high definition 3D computer models for anatomy with enhanced level of detail, it may make it possible to reduce or eliminate the need for animal dissection during teaching [101, 102].

3D CELL-CULTURE MODELS AND ORGANS-ON-CHIPS

In the current scenario of rapid advancement in the life sciences, certain tissue models can be built using 3D cell culture technology. Indeed, there are some organs on micro-scale chip models used for mimicking the human body environment. 3D models of multiple organ systems such as heart, liver, skin, muscle, testis, brain, gut, bone marrow, lungs and kidney, in addition to individual organs, have been created in microfluidic channels, re-creating the physiological chemical and physical microenvironments of the body [103]. These

emerging techniques, such as the biomedical/biological microelectromechanical system (Bio-MEMS) or lab-on-a-chip (LOC) and micro total analysis systems (lTAS) will, in the future, be a useful substitute for animal experimentation in commercial laboratories in the biotechnology, environmental safety, chemistry and pharmaceutical industries. For 3D cell culture modeling, cells are grown in 3D spheroids or aggregates with the help of a scaffold or matrix, or sometimes using a scaffold-free method. The 3D cell culture modeling conditions can be altered to add proteins and other factors that are found in a tumor microenvironment, for example, or in particular tissues. These matrices contain extracellular matrix components such as proteins, glycoconjugates and glycosaminoglycans that allow for cell communication, cell to cell contact and the activation of signaling pathways in such a way that the morphological and functional differentiation of these cells can accurately mimic their environment *in vivo*. This methodology, in time, will bridge the gap between *in vivo* and *in vitro* drug screening, decreasing the utilization of animal models during research [104].

ALTERNATIVES TO MICROBIAL CULTURE MEDIA AND SERUM-FREE ANIMAL CELL CULTURES

There are moves to reduce the use of animal derived products in many areas of biotechnology. Microbial culture media peptones are mostly made by the proteolysis of farmed animal meat. However, nowadays, various suppliers provide peptones extracted from yeast and plants. Although the costs of these plant-extracted peptones are the same as those of animal peptones, plant peptones are more environmentally favorable since less plant material and water are required for them to grow, compared with the food grain and fodder needed for cattle that are slaughtered for animal peptone production [105].

Human cell culture is often carried out in a medium that contains fetal calf serum, the production of which involves animal (cow) sacrifice or suffering. In fact, living pregnant cows are used and their fetuses removed to harvest the serum from the fetal blood. Fetal calf serum is used because it is a natural medium rich in all the required nutrients and significantly increases the chances of successful cell growth in culture. Scientists are striving to identify the factors and nutrients required for the growth of various types of cells, with a view to eliminating the use of calf serum. At present, most cell lines could be cultured in a chemically-synthesized medium without using animal products. Furthermore, data from chemically-synthesized media experiments may have better reproducibility than those using animal serum media, since the composition of animal serum does change from batch to batch on the basis of animals' gender, age, health and genetic background [76].

ALTERNATIVES TO ANIMAL-DERIVED ANTIBODIES

Animal friendly affinity reagents may act as an alternative to antibodies produced, thereby removing the need for animal immunization. Typically, these antibodies are obtained *in vitro* by yeast, phage or ribosome display.

In a recent review, a comparative analysis between animal friendly affinity reagents and animal derived-antibodies showed that the affinity reagents have superior quality, are relatively less time consuming, have more reproducibility and are more reliable and are cost-effective [106, 107].

Conclusions

Animal experimentation led to great advancement in biological and biomedical sciences and contributed to the discovery of many drugs and treatment options. However, such experimentation may cause harm, pain and distress to the animals involved. Therefore, to perform animal experimentations, certain ethical rules and laws must be strictly followed and there should be proper justification for using animals in research projects. Furthermore, during animal experimentation the 4 Rs principles of reduction, refinement, replacement and responsibility must be followed by the researchers. Moreover, before beginning a research project, experiments should be thoroughly planned and well-designed, and should avoid unnecessary use of animals. The reliability and reproducibility of animal experiments should also be considered. Whenever possible, alternative methods to animal experimentation should be adopted, such as *in vitro* experimentation, cadaveric studies, and computer simulations.

While much progress has been made on reducing animal experimentation there is a need for greater awareness of alternatives to animal experiments among scientists and easier access to advanced modeling technologies. Greater research is needed to define a roadmap that will lead to the elimination of all unnecessary animal experimentation and provide a framework for adoption of reliable alternative methodologies in biomedical research.

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Conflicts of interest statement

Authors declare no conflict of interest.

Author's contributions

MB: study conception, editing and critical revision of the manuscript; AKK, DP, GH, RB, Paul S, Peter S, RM, BF, NC, SM, LL, DD, GMT, MCE, MD, SM, Daniele M, GB, AD, KD, MCM, TB, MS, STC, Donald M, AM, AB, KLH, MK, LS, LL, GF: literature search, editing and critical revision of the manuscript. All authors have read and approved the final manuscript.

References

[1] Fernandes MR, Pedroso AR. Animal experimentation: a look into ethics, welfare and alternative methods. Rev Assoc Med Bras 2017;63:923-8.

[2] LaFollette H. Ethics in practice: an anthology, 4th ed. New York: Wiley & Sons 2020.

[3] Franco NH. Animal experiments in biomedical research: a historical perspective. Animals 2013;3:238-73. https://doi.org/10.3390/ani3010238

[4] Animal Research at Stanford.. Avalable at: https://med.stanford.edu/animalresearch/why-animal-research.html. Accessed on: 30/05/2021.

[5] Simmons D. The use of animal models in studying genetic disease: transgenesis and induced mutation. Available at: https://www.nature.com/scitable/topicpage/the-use-of-animal-models-in-studying-855/. Accessed on: 30/05/2021.

[6] National Research Council (US) Committee on Developmental Toxicology. Using Model Animals to Assess and Understand Developmental Toxicity In: scientific frontiers in developmental toxicology and risk assessment. Washington (DC): National Academies Press (US) 2000. https://doi.org/10.17226/9871

[7] Regenberg A, Mathews DJ, Blass DM, Bok H, Coyle JT, Duggan P, Faden R, Finkel J, Gearhart JD, Hillis A, Hoke A, Johnson R, Johnston M, Kahn J, Kerr D, King P, Kurtzberg J, Liao SM, McDonald JW, McKhann G, Nelson KB, Rao M, Siegel AW, Smith K, Solter D, Song H, Sugarman J, Vescovi A, Young W, Greely HT, Traystman RJ. The role of animal models in evaluating reasonable safety and efficacy for human trials of cell-based interventions for neurologic conditions. J Cereb Blood Flow Metab 2009;29:1-9. https://doi.org/10.1038/jcbfm.2008.98

[8] Williams ED. Federal protection for human research subjects: an analysis of the common rule and its interactions with FDA regulations and the HIPAA Privacy Rule. Congressional Research Service 2005. Available at: https://fas.org/sgp/crs/misc/RL32909.pdf. Accessed on: 30/05/2021.

[9] Fotuhi M, Mian A, Meysami S, Raji CA. Neurobiology of COVID-19. J Alzheimers Dis 2020;76:3-19. https://doi.org/10.3233/JAD-200581

[10] Iadecola C, Anrather J, Kamel H. Effects of COVID-19 on the nervous system. Cell 2020;183:16-27. https://doi.org/10.1016/j.cell.2020.08.028

[11] Marshall M. How COVID-19 can damage the brain. Nature 2020;585:342-3. https://doi.org/10.1038/d41586-020-02599-5

[12] Marshall M. COVID's toll on smell and taste: what scientists do and don't know. Nature 2021;589:342-3. https://doi.org/10.1038/d41586-021-00055-6

[13] Paolo G. Does COVID-19 cause permanent damage to olfactory and gustatory function? Med Hypotheses 2020;143:110086. https://doi.org/10.1016/j.mehy.2020.110086

[14] Reichard RR, Kashani KB, Boire NA, Constantopoulos E, Guo Y, Lucchinetti CF. Neuropathology of COVID-19: a spectrum of vascular and acute disseminated encephalomyelitis (ADEM)-like pathology. Acta Neuropathol 2020;140:1-6. https://doi.org/10.1007/s00401-020-02166-2

[15] Taquet M, Geddes JR, Husain M, Luciano S, Harrison PJ. 6-month neurological and psychiatric outcomes in 236379 survivors of COVID-19: a retrospective cohort study using electronic health records. Lancet Psychiatry 2021;8:416-27. https://doi.org/10.1016/S2215-0366(21)00084-5

[16] Lakdawala SS, Menachery VD. The search for a COVID-19 animal model. Science 2020;368:942-3. https://doi.org/10.1126/science.abc6141

[17] Neff EP. Meeting the need for COVID-19 models. Lab Anim 2021;50:111-2. https://doi.org/10.1038/s41684-021-00759-2

[18] Dinnon KH 3rd, Leist SR, Schäfer A, Edwards CE, Martinez DR, Montgomery SA, West A, Yount BL Jr, Hou YJ, Adams LE,

Gully KL, Brown AJ, Huang E, Bryant MD, Choong IC, Glenn JS, Gralinski LE, Sheahan TP, Baric RS. A mouse-adapted model of SARS-CoV-2 to test COVID-19 countermeasures. Nature 2020;586 560-6. https://doi.org/10.1038/s41586-020-2708-8

[19] Gurumurthy CB, Quadros RM, Richardson GP, Poluektova LY, Mansour SL, Ohtsuka M. Genetically modified mouse models to help fight COVID-19. Nat Protoc 2020;15:3777-3787. https://doi.org/10.1038/s41596-020-00403-2

[20] Nainu F, Rahmatika D, Emran TB, Harapan H. Potential application of Drosophila melanogaster as a model organism in COVID-19-related research. Front Pharmacol 2020;11:588561. https://doi.org/10.3389/fphar.2020.588561

[21] Galindo-Villegas J. The zebrafish disease and drug screening model: a strong ally against Covid-19. Front Pharmacol 2020;11:680. https://doi.org/10.3389/fphar.2020.00680

[22] Adhikary PP, Ul Ain Q, Hocke AC, Hedtrich S. COVID-19 highlights the model dilemma in biomedical research. Nat Rev Mater 2021;17:1-3. https://doi.org/10.1038/s41578-021-00305-z

[23] Hein WR, Griebel PJ. Road less travelled: large animal models in immunological research. Nat Rev Immunol 2003;3:79-84. https://doi.org/10.1038/nri977

[24] Hild SA, Chang MC, Murphy SJ, Grieder FB. Nonhuman primate models for SARS-CoV-2 research: infrastructure needs for pandemic preparedness. Lab Anim 2021;50:140-1. https://doi.org/10.1038/s41684-021-00760-9

[25] Kumar S, Yadav PK, Srinivasan R, Perumal N. Selection of animal models for COVID-19 research. Virus disease 2020;31:1-6. https://doi.org/10.1007/s13337-020-00637-4

[26] Muñoz-Fontela C, Dowling WE, Funnell SGP, Gsell PS, Riveros-Balta AX, Albrecht RA, Andersen H, Baric RS, Carroll MW, Cavaleri M, Qin C, Crozier I, Dallmeier K, de Waal L, de Wit E, Delang L, Dohm E, Duprex WP, Falzarano D, Finch CL, Frieman MB, Graham BS, Gralinski LE, Guilfoyle K, Haagmans BL, Hamilton GA, Hartman AL, Herfst S, Kaptein SJF, Klimstra WB, Knezevic I, Krause PR, Kuhn JH, Le Grand R, Lewis MG, Liu WC, Maisonnasse P, McElroy AK, Munster V, Oreshkova N, Rasmussen AL, Rocha-Pereira J, Rockx B, Rodríguez E, Rogers TF, Salguero FJ, Schotsaert M, Stittelaar KJ, Thibaut HJ, Tseng CT, Vergara-Alert J, Beer M, Brasel T, Chan JFW, García-Sastre A, Neyts J, Perlman S, Reed DS, Richt JA, Roy CJ, Segalés J, Vasan SS, Henao-Restrepo AM, Barouch DH. Animal models for COVID-19. Nature 2020;586:509-15. https://doi.org/10.1038/s41586-020-2787-6

[27] Pandey K, Acharya A, Mohan M, Ng CL, Reid SP, Byrareddy SN. Animal models for SARS-CoV-2 research: a comprehensive literature review. Transbound Emerg Dis 2020;68:1868-85. https://doi.org/10.1111/tbed.13907

[28] Pechanova O. Why we still need animal models. Pathophysiology 2020;27:44-5. https://doi.org/10.3390/pathophysiology27010006

[29] Natoli S, Oliveira V, Calabresi P, Maia LF, Pisani A. Does SARS-Cov-2 invade the brain? Translational lessons from animal models. Eur J Neurol 2020;27:1764-73. https://doi.org/10.1111/ene.14277

[30] Mahajan A, Mason GF. A sobering addition to the literature on COVID-19 and the brain. J Clin Invest 2021;131:e148376. https://doi.org/10.1172/JCI148376

[31] Renn M, Bartok E, Zillinger T, Hartmann G, Behrendt R. Animal models of SARS-CoV-2 and COVID-19 for the development of prophylactic and therapeutic interventions. Pharmacol Ther 2021;228:107931. https://doi.org/10.1016/j.pharmthera.2021.107931

[32] Shi J, Wen Z, Zhong G, Yang H, Wang C, Huang B, Liu R, He X, Shuai L, Sun Z, Zhao Y, Liu P, Liang L, Cui P, Wang J, Zhang X, Guan Y, Tan W, Wu G, Chen H, Bu Z. Susceptibility of ferrets, cats, dogs, and other domesticated animals to SARS-coronavirus 2. Science 2020;368:1016-20. https://doi.org/10.1126/science.abb7015

[33] Genzel L, Adan R, Berns A, van den Beucken JJJP, Blokland A, Boddeke EHWGM, Bogers WM, Bontrop R, Bulthuis R, Bousema T, et al. How the COVID-19 pandemic highlights the necessity of animal research. Curr Biol 2020;30:R1014-R1018. https://doi.org/10.1016/j.cub.2020.08.030

[34] Deb B, Shah H, Goel S. Current global vaccine and drug efforts against COVID-19: Pros and cons of bypassing animal trials. J Biosci 2020;45:82. https://doi.org/10.1007/s12038-020-00053-2

[35] Delahaye P. Animal studies, animal ethics. In: a semiotic methodology for animal studies. 1st ed. Cham: Springer 2019.

[36] Singer P. All Animals are Equal. Available at: https://spot.colorado.edu/~heathwoo/phil1200,Spr07/singer.pdf. Accessed on: 30/05/2021.

[37] Machan TR. Why human beings may use animals. J Value Inq 2002;36:9-14. https://doi.org/10.1023/a:1014993828953

[38] Gruen L. The Moral Status of Animals In: Zalta EN, ed. Stanford Encyclopedia of Philosophy. Metaphysics Research Lab, Stanford University 2021. Available at: https://plato.stanford.edu/entries/moral-animal/. Accessed on: 30/05/2021.

[39] Cohen C. The case for the use of animals in biomedical research. N Engl J Med 1986;315:865-70. https://doi.org/10.1056/NEJM198610023151405

[40] Cohen AI, Wellman CH. Contemporary debates in applied ethics. Malden, 1st ed. MA: Blackwell Pub 2005.

[41] Liou S. The Ethics of Animal Experimentation. 2010. Available at: https://hopes.stanford.edu/animal-research/. Accessed on: 30/05/2021.

[42] Bateson P. When to experiment on animals. New Sci 1986;109:30-2.

[43] Bateson's cube. Available at: https://en.wikipedia.org/wiki/Bateson%27s_cube. Accessed on: 30/05/2021.

[44] Monsó S, Benz-Schwarzburg J, Bremhorst A. Animal morality: what it means and why it matters. J Ethics 2018;22:283-310. https://doi.org/10.1007/s10892-018-9275-3

[45] Proctor H. Animal sentience: where are we and where are we heading? Animals 2012;2:628-39. https://doi.org/10.3390/ani2040628

[46] Langford DJ, Bailey AL, Chanda ML, Clarke SE, Drummond TE, Echols S, Glick S, Ingrao J, Klassen-Ross T, Lacroix-Fralish ML, Matsumiya L, Sorge RE, Sotocinal SG, Tabaka JM, Wong D, van den Maagdenberg AM, Ferrari MD, Craig KD, Mogil JS. Coding of facial expressions of pain in the laboratory mouse. Nat Methods 2010;7:447-9. https://doi.org/10.1038/nmeth.1455

[47] National Research Council (US) Committee on Recognition and Alleviation of Pain in Laboratory Animals. Recognition and alleviation of pain in laboratory animals. Washington (DC): National Academies Press (US) 2009.

[48] Carbone L. Pain in laboratory animals: the ethical and regulatory imperatives. PLoS One 2011;6:e21578. https://doi.org/10.1371/journal.pone.0021578

[49] Gregory G. Physiology and behavior of animal suffering. United Kingdom: Blackwell Publishing Company 2004, pp. 25-50.

[50] Panksepp J. Affective neuroscience: the foundations of human and animal emotions. USA: University Press 2004.

[51] Bradshaw GA, Capaldo T, Lindner L, Grow G. Building an inner sanctuary: complex PTSD in chimpanzees. J Trauma Dissociation 2008;9:9-34. https://doi.org/10.1080/15299730802073619

[52] Balcombe J. Animal pleasure and its moral significance. Appl Anim Behav Sci 2009;118:208-16. https://doi.org/10.1016/j.applanim.2009.02.012

[53] Graham KL, Burghardt GM. Current perspectives on the biological study of play: signs of progress. Q Rev Biol 2010;85:393-418. https://doi.org/10.1086/656903

[54] Hecht EE, Mahovetz LM, Preuss TM, Hopkins WD. A neuroanatomical predictor of mirror self-recognition in

chimpanzees. Soc Cogn Affect Neurosci 2017;12:37-48. https://doi.org/10.1093/scan/nsw159

[55] Veit L, Pidpruzhnykova G, Nieder A. Associative learning rapidly establishes neuronal representations of upcoming behavioral choices in crows. Proc Natl Acad Sci USA 2015;112:15208-13. https://doi.org/10.1073/pnas.1509760112

[56] Emery NJ, Clayton NS. The mentality of crows: convergent evolution of intelligence in corvids and apes. Science 2004;306:1903-7. https://doi.org/10.1126/science.1098410

[57] Gamble JR, Cristol DA. Drop-catch behaviour is play in herring gulls, Larus argentatus. Anim Behav 2002;63:339-45. https://doi.org/10.1006/anbe.2001.1903

[58] McCance D. Critical Animal Studies: An Introduction. 1st ed. USA: SUNY Press 2012.

[59] Ferdowsian HR, Beck N. Ethical and scientific considerations regarding animal testing and research. PLoS One 2011;6:e24059. https://doi.org/10.1371/journal.pone.0024059

[60] Hansen LA. Institution animal care and use committees need greater ethical diversity. J Med Ethics 2013;39:188-90. https://doi.org/10.1136/medethics-2012-100982

[61] Tannenbaum J, Bennett BT. Russell and Burch's 3Rs then and now: the need for clarity in definition and purpose. J Am Assoc Lab Anim Sci 2015;54:120-32.

[62] Lee KH, Lee DW, Kang BC. The 'R' principles in laboratory animal experiments. Lab Anim Res 2020;36:1-3. https://doi.org/10.1186/s42826-020-00078-6

[63] Di Salvo L. The Ethical and Moral Status of Invasive Animal Research: The Dilemma and Alternative Approaches. Rome: LUISS Thesis Guido Carli 2017. Available at: https://tesi.luiss.it/19067/. Accessed on: 30/05/2021.

[64] Dondossola D, Santini A, Lonati C, Zanella A, Merighi R, Vivona L, Battistin M, Galli A, Biancolilli O, Maggioni M, Villa S, Gatti S. Human red blood cells as oxygen carriers to improve ex-situ liver perfusion in a rat model. J Clin Med 2019;8:1918. https://doi.org/10.3390/jcm8111918

[65] Dondossola D, De Falco S, Kersik A, Maggioni M, Di Girolamo L, Biancolilli O, Busana M, Lonati C, Carù F, Zanella A, Gatti S. Procurement and ex-situ perfusion of isolated slaughterhouse-derived livers as a model of donors after circulatory death. ALTEX 2019. https://doi.org/10.14573/altex.1909131

[66] Furka I, Brath E, Nemeth N, Miko I. Learning microsurgical suturing and knotting techniques: comparative data. Microsurgery 2006;26:4-7. https://doi.org/10.1002/micr.20201

[67] Tolba RH, Czigány Z, Osorio Lujan S, Oltean M, Axelsson M, Akelina Y, Di Cataldo A, Miko I, Furka I, Dahmen U, Kobayashi E, Ionac M, Nemeth N. Defining standards in experimental microsurgical training: recommendations of the European Society for Surgical Research (ESSR) and the International Society for Experimental Microsurgery (ISEM). Eur Surg Res 2017;58:246-62. https://doi.org/10.1159/000479005

[68] Banks RE. The 4th R of research. Contemp Top Lab Anim Sci 1995;34:50-1.

[69] Neal JM, Bernards CM, Butterworth JF 4th, Di Gregorio G, Drasner K, Hejtmanek MR, Mulroy MF, Rosenquist RW, Weinberg GL. ASRA practice advisory on local anesthetic systemic toxicity. Reg Anesth Pain Med 2010;35:152-61. https://doi.org/10.1097/AAP.0b013e3181d22fcd

[70] Kilkenny C, Browne W, Cuthill IC, Emerson M, Altman DG. NC3Rs Reporting Guidelines Working Group. Animal research: reporting in vivo experiments: the ARRIVE guidelines. Br J Pharmacol 2010;160:1577-9. https://doi.org/10.1111/j.1476-5381.2010.00872.x

[71] The Capacity of Animals to Experience Pain, Distress and Suffering In: The Ethics of Research Involving Animals. Available at: https://www.nuffieldbioethics.org/wp-content/uploads/Animals-Chapter-4-The-Capacity-of-Animals-to-Experience-Pain-Distress-and-Suffering.pdf. Accessed on: 30/05/2021.

[72] Peterson NC, Nunamaker EA, Turner PV. To treat or not to treat: the effects of pain on experimental parameters. Comp Med 2017;67:469-82.

[73] Canadian Council on Animal Care. Guide to the Care and Use of Experimental Animals. Available at: https://www.ccac.ca/Documents/Standards/Guidelines/Experimental_Animals_Vol1.pdf. Accessed on: 30/05/2021.

[74] Singh VP, Pratap K, Sinha J, Desiraju K, Bahal D, Kukreti R. Critical evaluation of challenges and future use of animals in experimentation for biomedical research. Int J Immunopathol Pharmacol 2016;29:551-61. https://doi.org/10.1177/0394632016671728

[75] Buchanan K, Burt de Perera T, Carere C, Carter T, Hailey A, Hubrecht R, Jennings D, Metcalfe N, Pitcher T, Péron F, Sneddon L, Sherwin C, Talling J, Thomas R, Thompson M. Guidelines for the use of animals: guidelines for the treatment of animals in behavioural research and teaching. Anim Behav 2013;85:285-97. https://doi.org/10.1016/j.anbehav.2011.10.031

[76] Rai J, Kaushik K. Reduction of animal sacrifice in biomedical science & research through alternative design of animal experiments. Saudi Pharm J 2018;26:896-902. https://doi.org/10.1016/j.jsps.2018.03.006

[77] Marafante E, Smyrniotis T, Balls M. ECVAM: the European centre for the validation of alternative methods. Toxicol in Vitro 1994;8:803-5. https://doi.org/10.1016/0887-2333(94)90072-8

[78] Stokes WS, Schechtman LM, Hill RN. The Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM): a review of the ICCVAM test method evaluation process and current international collaborations with the European Centre for the Validation of Alternative Methods (ECVAM). Altern Lab Anim 2002;30:23-32. https://doi.org/10.1177/026119290203002S04

[79] Animal and plant health inspection service. Available at: https://www.aphis.usda.gov/aphis/ourfocus/animalwelfare/ct_awa_enforcements. Accessed on: 30/05/2021.

[80] Rollin BE. The regulation of animal research and the emergence of animal ethics: a conceptual history. Theor Med Bioeth 2006;27:285-304. https://doi.org/10.1007/s11017-006-9007-8

[81] Kehinde EO. They see a rat, we seek a cure for diseases: the current status of animal experimentation in medical practice. Med Princ Pract 2013;22:52-61. https://doi.org/10.1159/000355504

[82] Clovis Y. Worms. Flies or fish? A Comparison of common model organisms-part 1: models for biomedical research. Available at: https://invivobiosystems.com/disease-modeling/worms-flies-fish-comparison-common-model-organisms/. Accessed on: 30/05/2021.

[83] Prüßing K, Voigt A, Schulz JB. Drosophila melanogaster as a model organism for Alzheimer's disease. Mol Neurodegener 2013;8:1-12. https://doi.org/10.1186/1750-1326-8-35

[84] Milan DJ, Peterson TA; Ruskin JN, Peterson RT, MacRae CA. Drugs that induce repolarization abnormalities cause bradycardia in zebrafish. Circulation 2003;107:1355-8. https://doi.org/10.1161/01.cir.0000061912.88753.87

[85] Petroianu A. Aspectos éticos na pesquisa em animais. Acta Cir Bras 1996;11:157-64.

[86] Iki Y, Ito T, Kudo K, Noda M, Kanehira M, Sueta T, Miyoshi I, Kagaya Y, Okada Y, Unno M. Animal ethics and welfare education in wet-lab training can foster residents' ethical values toward life. Exp Anim 2017;66:313-20. https://doi.org/10.1538/expanim.17-0026

[87] Neves SM. Manual de Cuidados e Procedimentos com Animais de Laboratório do Biotério de Produção e Experimentação. São Paulo: Universidade de São Paulo 2013. Available at: http://www.fo.usp.br/wp-content/uploads/Manual-Cuidados-com-Animais.pdf. Accessed on: 30/05/2021.

[88] Muller CA, Ramos S, Saisse AO, Almosny NRP. Videocâmeras em biotérios de experimentação: importante ferramenta no controle da contaminação ambiental na microbiota de

camundongos. Arq Bras Med Vet Zootec 2015;67:689-97. https://doi.org/10.1590/1678-4162-7334

[89] Guia brasileiro de boas práticas para eutanásia de animais. Conselho Federal de Medicina Veterinária do Brasil Brasilia 2013. Available at: https://www.cfmv.gov.br/guia-brasileiro-de-boas-praticas-para-a-eutanasia-em-animais/comunicacao/publicacoes/2020/08/03/#1. Accessed on: 30/05/2021.

[90] O'Collins VE, Macleod MR, Donnan GA, Horky LL, van der Worp BH, Howells DW. 1,026 experimental treatments in acute stroke. Ann Neurol 2006;59:467-77. https://doi.org/10.1002/ana.20741

[91] Schardein J. Drugs as Teratogens. 1st ed. Cleveland, Ohio: CRC Press. Inc. 1976.

[92] Akhtar A. The flaws and human harms of animal experimentation. Camb Q Healthc Ethics 2015;24:407-19. https://doi.org/10.1017/S0963180115000079

[93] Bailey J. An assessment of the role of chimpanzees in AIDS vaccine research. Altern Lab Anim 2008;36:381-428. https://doi.org/10.1177/026119290803600403

[94] Akhtar AZ, Pippin JJ, Sandusky CB. Animal studies in spinal cord injury: a systematic review of methylprednisolone. Altern Lab Anim 2009;37:43-62. https://doi.org/10.1177/026119290903700108

[95] Lane E, Dunnett S. Animal models of Parkinson's disease and L-dopa induced dyskinesia: how close are we to the clinic? Psychopharmacology 2008;199:303-12. https://doi.org/10.1007/s00213-007-0931-8

[96] Mole DR, Tomson CR, Mortensen N, Winearls CG. Renal complications of jejuno-ileal bypass for obesity. QJM 2001;94:69-77. https://doi.org/10.1093/qjmed/94.2.69

[97] Balls M, Combes R. Animal experimentation and alternatives: revealed preferences. London: SAGE Publications Sage 2017.

[98] Hartung T. Toxicology for the twenty-first century. Nature 2009;460:208-12. https://doi.org/10.1038/460208a

[99] Langley G, Evans T, Holgate ST, Jones A. Replacing animal experiments: choices, chances and challenges. Bioessays 2007;29:918-26. https://doi.org/10.1002/bies.20628

[100] Washio T, Okada J, Takahashi A, Yoneda K, Kadooka Y, Sugiura S, Hisada T. Multiscale heart simulation with cooperative stochastic cross-bridge dynamics and cellular structures. Multiscale Model Simul 2013;11:965-99. https://doi.org/10.1137/120892866

[101] Azer SA, Azer S. 3D anatomy models and impact on learning: a review of the quality of the literature. Health Prof Educ 2016;2:80-98. https://doi.org/10.1016/j.hpe.2016.05.002

[102] Adler S, Basketter D, Creton S, Pelkonen O, van Benthem J, Zuang V, Andersen KE, Angers-Loustau A, Aptula A, Bal-Price A, Benfenati E, Bernauer U, Bessems J, Bois FY, Boobis A, Brandon E, Bremer S, Broschard T, Casati S, Coecke S, Corvi R, Cronin M, Daston G, Dekant W, Felter S, Grignard E, Gundert-Remy U, Heinonen T, Kimber I, Kleinjans J, Komulainen H, Kreiling R, Kreysa J, Leite SB, Loizou G, Maxwell G, Mazzatorta P, Munn S, Pfuhler S, Phrakonkham P, Piersma A, Poth A, Prieto P, Repetto G, Rogiers V, Schoeters G, Schwarz M, Serafimova R, Tähti H, Testai E, van Delft J, van Loveren H, Vinken M, Worth A, Zaldivar JM. Alternative (non-animal) methods for cosmetics testing: current status and future prospects-2010. Arch Toxicol 2011;85:367-485. https://doi.org/10.1007/s00204-011-0693-2

[103] Huh D, Torisawa YS, Hamilton GA, Kim HJ, Ingber DE. Microengineered physiological biomimicry: organs-on-chips. Lab Chip 2012;12:2156-64. https://doi.org/10.1039/c2lc40089h

[104] Edmondson R, Broglie JJ, Adcock AF, Yang L. Three-dimensional cell culture systems and their applications in drug discovery and cell-based biosensors. Assay Drug Dev Technol 2014;12:207-18. https://doi.org/10.1089/adt.2014.573

[105] Wright AK, Ferreira DM, Gritzfeld JF, Wright AD, Armitage K, Jambo KC, Bate E, El Batrawy S, Collins A, Gordon SB. Human nasal challenge with Streptococcus pneumoniae is immunising in the absence of carriage. PLoS Pathog 2012;8:e1002622. https://doi.org/10.1371/journal.ppat.1002622

[106] Gray AC, Sidhu SS, Chandrasekera PC, Hendriksen CFM, Borrebaeck CAK. Animal-friendly affinity reagents: replacing the needless in the haystack. Trends Biotechnol 2016;34:960-9. https://doi.org/10.1016/j.tibtech.2016.05.017

[107] Dübel S, Stoevesandt O, Taussig MJ, Hust M. Generating recombinant antibodies to the complete human proteome. Trends Biotechnol 2010;28:333-9. https://doi.org/10.1016/j.tibtech.2010.05.001

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