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Bioethics in the Use of Laboratory Animals, Strategies for Reduction, Replacement, and Refinement in rodents

Bioética en el Uso de Animales de Laboratorio, Estrategias para la Reducción, Reemplazo y Refinamiento en roedores en la investigación Científica.

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Abstract:

This literature review addresses the use of animals in scientific research from a bioethical perspective, focusing on the principle of the "3Rs" (Replacement, Reduction, and Refinement). Through a literature search, the strategies needed to reduce the number of animals used, replace them with alternative models, in a search for experimental techniques that minimize their suffering were analyzed. The goal is to promote more ethical and sustainable research, balancing scientific benefits with animal welfare. This review emphasizes the need to adopt innovative and collaborative practices, contributing to science that prioritizes both progress and respect for animal life.

Keywords:

Bioethics, Laboratory Animals, Alternative Models, Animal Welfare

Resumen:

Esta revisión de la literatura aborda el uso de animales en la investigación científica desde una perspectiva bioética, centrándose en el principio de las "3R" (Reemplazo, Reducción y Refinamiento). A través de una búsqueda de la literatura, se analizaron las estrategias necesarias para reducir el número de animales utilizados, reemplazarlos con modelos alternativos, en una búsqueda de técnicas experimentales que minimicen su sufrimiento. El objetivo es promover una investigación más ética y sostenible, equilibrando los beneficios científicos con el bienestar animal. Esta revisión subraya la necesidad de adoptar prácticas innovadoras y colaborativas, contribuyendo a una ciencia que priorice tanto el progreso como el respeto hacia la vida animal.

***Palabras Clave*:**

Bioética, Animales de Laboratorio, Modelos Alternativos, Bienestar Animal

INTRODUCTION

Animal model-based research has been conducted for a long time. Since the 5th century BC, experiments with animals have been documented, but their use increased significantly in the 19th century.[1] In most medical research centers around the world, non-human animals are used as part of scientific studies. These animals help deepen the understanding of diseases that affect humans and explore possible therapeutic solutions.[2] Although some species, such as the fruit fly (*Drosophila melanogaster*), the zebrafish (*Danio rerio*), and the worm *Caenorhabditis elegans*, are evolutionarily distant from humans, they share physiological and genetic characteristics that make them valuable tools for medical progress. Thanks to these similarities, animal research has been fundamental in advancing medical science.[2,3]

The use of animals in biomedical research remains a topic of public and scientific debate. While some people may oppose animal research, social acceptance continues. However, it has also been observed that public support is conditional, varying depending on the availability of alternatives, minimizing harm to animals, and the benefits for human and/or animal health.[4] This variability highlights the importance of guarantees, whether assumed or demanded by different public groups, ensuring that research governance and scientific practices meet expected standards. The relationships between the State, science, and social trust are therefore crucial for the social acceptance of laboratory animal research; however, they are also controversial and ever-changing.[5,6] Ideas about socially acceptable experimental practices involving laboratory animals have evolved over time in response to changes within science and society.[7-10] As laboratory animal research expands

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internationally, with a focus on obtaining medically applicable results and increasing transparency demands, it is essential not to take social support for these practices for granted. Instead, it is crucial to consider these social relationships when designing and planning future research projects, as they will also vary depending on geographical location.[4] The first formal presentation of the 3Rs concept (Replacement, Reduction, and Refinement, Fig. 1) took place at the UFAW Symposium in 1957.[11,12] Two years later, W. Russell and R. Burch published The Principles of Humane Experimental Technique. In this book, three fundamental principles were defined. The first, Replacement, was defined as "the substitution of conscious higher animals with insentient material." The second, Reduction, was understood as "the decrease in the number of animals used to obtain a specific quantity and precision of information." Finally, the third principle, Refinement, was described as "any decrease in the incidence or severity of inhumane procedures applied to those animals that must still be used".[11] The use of animals in research is regulated by ethical committees that evaluate research protocols.[13] National and international laws are based on the 3Rs principle (Reduction, Refinement, and Replacement), proposed by Russell and Burch in 1959. Some researchers have added a fourth "R": Responsibility: promoting animal welfare and ethical discussion about their use.[14,15] To promote and protect animal welfare, Latin American and other countries worldwide have incorporated animal welfare provisions into their laws. Ideally, animal welfare legislation should reflect both science and ethical perspectives, addressing welfare issues in a multidisciplinary manner.[16]

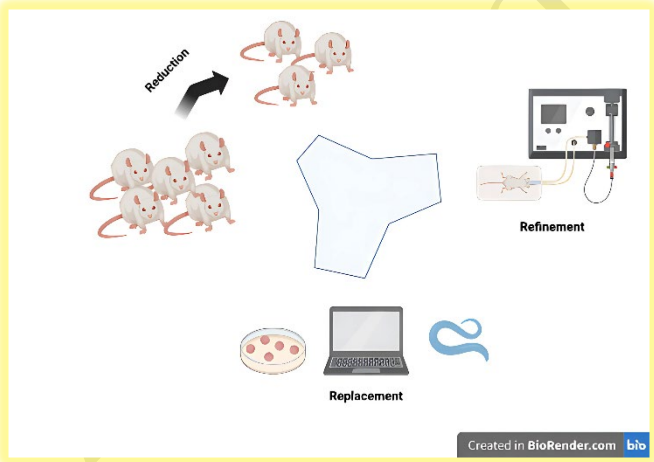

Figure 1. Reduction: Minimizing the number of experimental animals. Refinement: Improving processes and techniques used in laboratory animals. Replacement: Alternatives to the use of laboratory animals.

LAWS, GUIDELINES, AND REGULATIONS ON THE USE OF LABORATORY ANIMALS

Currently, there are guidelines and laws for the management of laboratory animals that guide work with biomodels toward their responsible and ethical use. Some representative examples include: The Guide for the Care and Use of Laboratory Animals, a publication from the National Research Council (USA), Directive 2010/63/EU of the European Union, which protects animals used for scientific purposes, the guidelines from the Canadian Council on Animal Care, and the NOM-062-ZOO-1999 in Mexico. These regulations protect animals used for scientific purposes with the primary objective of achieving high-quality and reproducible results.

REDUCTION ACTIONS

One of the most notable efforts, in addition to being a clear example of reduction in the use of laboratory animals, is the restriction of animal experimentation for the evaluation of cosmetic product safety, a movement that began in Europe during the 1990s. This restriction was fully achieved by 2013. Meanwhile, countries such as Turkey, India, Taiwan, South Korea, New Zealand, and Guatemala have followed the European initiative. Other countries are considering implementing the ban, including Ukraine, Russia, Argentina, Chile, Colombia, Canada, Brazil, Japan, the United States, and Australia.[17] Another example is the case of regulated testing with laboratory animals, such as acute toxicity evaluation to determine the median lethal dose for substances following the guidelines of the Organization for Economic Cooperation and Development (OECD), where the method has been standardized using the lowest possible number of animals in the process.[18]
The use of *in vitro* study methods has significantly contributed to achieving the objective of the reduction principle. The implementation of cell cultures from certain cell lines derived from biomodels such as rodents has led to important advances in both animal and human health.[19] It is important to highlight the use of pilot studies, which help adjust research protocols, as well as recognize their results, allowing for the validation or correction of the experimental approach. Pilot studies with few animals can provide useful information on welfare indicators, especially in unpredictable situations such as new compounds or experimental designs. Their results help define criteria for the main study and potential improvements.[20] In 2022, Schepelmann et al. conducted a pilot study titled: "Colorectal cancer associated with colitis induced by AOM/DSS in 14-month-old female Balb/C and C57BL/6 mice: A pilot study," demonstrating that older animals from both mouse strains can be used for colorectal cancer studies, allowing research into aging in its development and phenotype.[21]

REPLACEMENT ACTIONS

The principle of replacement drove the need to use alternative methodologies that avoid the use of biomodels as experimental subjects. In this context, cell culture models, such as immortal myoblastic cell lines derived from mice (C2C12), rats (L6), and dogs (MyoK9), present an excellent option due to their cost-effectiveness and ease of handling.[22]

The role of laboratory animals is not limited to research; a significant portion of their use occurs in education, particularly at the university level.[23] As a result of this usage, alternatives have been developed, such as InterNICHE, an International Network for Humane Education whose primary objective is to provide high-quality and entirely humane education and training in the fields of human medicine, veterinary medicine, and biological sciences. This organization supports progressive scientific education and the replacement of experimental animals.[24] Another database available online is NORINA (Norwegian Inventory of Alternatives), which contains more than 3,000 audiovisuals that can be used as alternatives or supplements to animal use in education and training, including dissection alternatives, at all levels of education. It also includes resources for laboratory animal care staff and scientists. The database was established in 1991 and is continuously updated.[25] Currently, the use of anatomical models or mannequins is an innovative alternative for learning in the world of laboratory animal use and management. A study conducted in 2021 by Corte et al. titled "Anatomical Evaluation of Rat and Mouse Simulators for Laboratory Animal Science Courses" evaluated different types of rodent simulators, resulting in a lack of realism in the models. The study emphasized that there is little knowledge about their frequency of use, anatomical accuracy, and learning efficiency. This leaves an open opportunity for the development of new and better simulators, where current technological tools can be used to improve them.[26]

REFINEMENT ACTIONS

The procedures used for substance administration in animals can have a significant impact on their well-being and the scientific value of the results. By refining techniques, opportunities arise to simultaneously improve both animal welfare and scientific outcomes. Important considerations include tissue irritation levels, solubility, biocompatibility, and sterility of substances, as well as the proper selection of needles and injection techniques. It is also essential to ensure accurate handling and precise dosing of administered substances.[27]

TABLE 1 *Techniques for substance administration in animals: procedures, risks, and refinements.*[27]

Technique	Procedure	Risks	Refinement
Topical-Dermal	The dermal route is used to investigate local and systemic effects after absorption and dermal metabolism.	Risk of irritation or sensitization. Possible adverse effects if the dose is increased beyond the adequate level.	Start with a test in a single animal, evaluating the physicochemical properties and avoiding irritating substances. Use controlled volumes and ensure skin cleanliness. Abrasions should only be performed if necessary.
Oral Routes	The oral route is used to expose the animal systemically by including substances in food or water, or by oral administration of capsules/pills.	Palatability problems may arise, leading to insufficient intake. Risk of damage to the gastrointestinal mucosa if the substance is irritating.	Food can be microencapsulated or masked with gelatins to improve palatability. Offer small amounts at regular intervals and use specialized feeders to measure the exact dose. Consider the balance between dosage precision and the social well-being of the animal.
Oral Tube	A feeding tube is passed through the esophagus into the stomach, where the substance to be dosed is expelled at a controlled rate.	Risk of gastric or pulmonary irritation if the contents enter the lungs. Possible damage if the tube is incorrectly positioned, which may perforate the trachea or esophagus.	Use a tube suitable for the species, preferably flexible, and lubricate it for easier passage. Ensure proper positioning to prevent aspiration into the lungs. Monitor the animal for any adverse effects, such as regurgitation. Avoid administering irritating substances.

Intraperitoneal	The intraperitoneal injection is used to administer relatively large volumes of soluble substances, such as anesthetics, when rapid absorption is needed.	Should not be used routinely. May damage internal organs. Irritating substances can cause severe adverse reactions such as pain, fibrosis, and adhesions in the peritoneal cavity.	Use this route only when absolutely necessary. Avoid use in pregnant animals, birds, or for irritating substances. Frequent technique checks should be performed to avoid harm to the animal. Apply controlled volumes and follow precise procedures to avoid needle penetration into vital organs. Ensure correct needle positioning. Not recommended for animals older than rodents.
Subcutaneous	The subcutaneous injection is used to administer many substances, providing slow release and avoiding first-pass metabolism by the liver.	Pain if the pH or osmolarity is not appropriate or if the substance is irritating. Can cause tissue necrosis. Incorrect needle insertion can damage blood vessels.	Perform a preliminary study of the substance to detect possible adverse reactions. Ensure that substances are sterile. Keep the animal still and avoid movements. In repeated dose studies, use small volumes and rotate the injection site to prevent damage to skin or tissues.
Intramuscular	The intramuscular injection is used to administer systemic substances, in slow-release studies, implants, or oily formulations.	Causes more pain than other routes, possibly resulting in temporary lameness. Risk of nerve damage (e.g., sciatic) or muscle damage, causing inflammation. Can cause tissue necrosis if irritating substances are administered. There is a risk of injecting into a blood vessel or fascia instead of the muscle.	Only use if there are no less painful alternatives. Avoid administering irritating substances. Shave the injection area to observe reactions. Avoid sudden movements and ensure the needle is not near nerves or blood vessels. Avoid injecting large volumes in one place and distribute the dose among several areas if necessary. Perform a gentle post-injection massage to disperse the substances. In large animals, proper restraint facilitates the technique.

In blood sampling techniques, welfare parameters and blood volumes associated with different bleeding sites in mice (*Mus musculus*) have been compared, including methods such as submandibular (facial), retro-orbital, saphenous, sublingual, and tail sampling. However, there is not enough high-quality evidence available to determine the optimal blood sampling route. Among the newer techniques is the use of the submental site, known as "chin bleeding." Meanwhile, the use of the retro-orbital sinus, despite being supported by previous studies, is no longer recommended by some institutions, and it is suggested that this procedure be performed only under terminal or general anesthesia, according to NC3Rs and NIH guidelines. On the other hand, sublingual blood collection and tail tip amputation also require anesthesia. Therefore, options for collecting large blood volumes without anesthesia are limited, with saphenous, facial, and chin bleeding considered viable, although the welfare impact of chin bleeding has not yet been thoroughly compared with other sampling sites.[28]

One of the most consistent alternatives in refinement for blood sampling is the so-called "microsampling," which represents a significant advancement, as it allows for the collection of biological samples with considerably smaller blood volumes compared to traditional methods. This not only reduces the physiological impact on animals, minimizing stress and potential alterations in results, but also optimizes experimental design by decreasing the need for satellite groups. By requiring less blood per sample, it facilitates the integration of additional evaluations within the same experiment, such as biomarkers or metabolic analyses, maximizing the information obtained from each animal and promoting a more ethical and efficient use of animals in safety studies.[29] Another measure used for refinement is the use of environmental enrichment. Environmental enrichment is a type of modified environment that has been used in studies on animal welfare and diseases for over seventy years. It has been shown that modifications in the environment, such as enrichment, lead to lasting changes in the behavior of rodents. Environmental enrichment includes mechanical modifications in the housing space or additional objects that provide cognitive and physical stimulation to the animal. This can be achieved by incorporating elements that encourage activities such as play, nesting, or foraging, as well as creating larger and more complex cages.[30] An enriched cage, which includes both social and physical elements, is designed by researchers to be safe, attractive, and varied. This cage can offer opportunities for movement and other forms of physical activity. It can also allow animals to interact with each other, either directly or indirectly, with ecologically relevant or even novel inanimate objects. This type of enrichment, when applied during the early stages of development, can influence the development of rodents in various ways. When applied in the early stages of life, it is known as developmental environmental enrichment.[30]

FOURTH R

In order to protect the welfare of laboratory animals, Russell and Burch published The Principles of Humane Experimental Technique in 1959, where they introduced the "3R" principle: Reduction, Replacement, and Refinement. However, years later, the International Foundation for Ethical Research in the United States added a fourth R: Responsibility, an ethical principle that ensures animals are treated with the utmost respect and care during their use in scientific research.[31]

Another concept proposed by Indian legislation regarding animal experimentation is Rehabilitation as a fourth R, driven by the need to provide relief and well-being to animals subjected to experimentation. Rehabilitation is carried out with the primary objective of mitigating any type of suffering or pain in animals and, in some cases, preserving their lives. It refers to the post-experimentation care given to animals that have been: 1) bred for experimentation, 2) exposed to experimentation, and 3) housed in animal facilities for research and educational purposes. Its purpose is to reduce the impact of physical, physiological, or psychological trauma they may have experienced and to ensure they can live under the best possible conditions until their natural death.[32]

Meanwhile, Reproducibility has been considered as a possible "fourth R" in animal research, complementing the classical principles of Replacement, Reduction, and Refinement. This concept not only refers to the inability to replicate results but also to the validity of methods and conclusions, including their generalization and robustness. To improve reproducibility, detailed planning and the use of efficient research designs, such as the split-plot design, are necessary. This approach reduces the number of animals required while enhancing the study's capability without compromising data quality. Cooperation among researchers, funding bodies, and regulatory agencies is essential to ensure that studies are reproducible, ethical, and responsible, with the goal of optimizing the data obtained and minimizing unnecessary animal use in research.[33]

CONCLUSION

The use of animals in scientific research has been fundamental for advances in medicine and biological sciences, but it has also sparked debates regarding animal welfare and the ethics involved. The 3Rs principle (Replacement, Reduction, and Refinement) has guided experimental practices toward greater responsibility and humanization, promoting alternatives that minimize suffering and the number of animals used. The inclusion of a fourth "R," such as Responsibility, underscores the importance of treating animals with the respect they deserve, ensuring their well-being throughout the research process. Furthermore, rehabilitation and reproducibility emerge as key concepts in the current context, aiming to mitigate post-experimentation suffering and ensure the validity and ethics of studies. As research progresses, it is essential to continue integrating innovative and ethical alternatives that reduce animal use without compromising scientific quality, fostering a balance between scientific advancement and respect for animal life.

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