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INVITED REVIEW

Strategies to apply 3Rs in preclinical testing

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Abstract

Animal experimentation has been fundamental in biological and biomedical research. To guarantee the maximum quality, efficacy and/or safety of products intended for the use in humans in vivo testing is necessary; however, for over 60 years, alternative methods have been developed in response to the necessity to reduce the number of animals used in experimentation, to guarantee their welfare; resorting to animal models only when strictly necessary. The three Rs (Replacement, Reduction, and Refinement), seek to ensure the rational and respectful use of laboratory animals and maintain an adequate projection in terms of bioethical considerations. This article describes different approaches to apply 3Rs in preclinical experimentation for either research or regulatory purposes.

KEYWORDS
non clinical, reduction, refinement, replacement, testing approaches

1 | INTRODUCTION

The experimentation with laboratory animals has contributed to the development of scientific and regulatory areas with a positive impact on human health.

Although animal experimentation dates back to the 4th century BC, the first ethics formal guidelines for the handling of animals for experimentation were written in 1831 by British psychologist Marshall Hall. This author established two main principles. First, that the experiments should be performed with a clear and definite objective; and secondly that the experiments should be done with the least possible animal suffering.[1] Moreover, a milestone in the history of animal testing was achieved in 1876 when the Cruelty to Animals Act was enacted in the House of Commons of the United Kingdom. This Act became known as the first regulation to be published in relation to the use of animal in scientific research.[2]

Nevertheless, it was not until 1947 that the Universities Federation for Animal Welfare (UFAW) published the first handbook entitled *The Care and Management of Laboratory Animals* to assist institutions in the caring and usage of laboratory animals. After that, in 1954, UFAW designated W. Russel to incorporate human methods in biological research.[1] The first formal presentation of the concept of 3Rs (Replacement, Reduction, and Refinement) was at the UFAW Symposium in 1957.[3,4]

Two years later, W. Russell and R. Burch published *The Principles of Humane Experimental Technique*. This book defined three principles. One of the principles is termed *Replacement*, and is defined as "substitution for conscious living higher animals of insentient material." *Reduction* as "reduction in the numbers of animals used to obtain information of a given amount and precision" and the third R stands for *Refinement* as "any decrease in the incidence or severity of inhumane procedures applied to those animals, which still have to be used."[5]

Abbreviations: ADMET, absorption, distribution, metabolism, excretion, and toxicity; AI, artificial intelligence; AOP, adverse outcome pathway; FDCA, Federal Food, Drug, and Cosmetic Act; INVITTOX, In Vitro techniques in Toxicology; ISO, International Organization for Standardization; MIE, molecular initiating event; OECD, Organisation for Economic Cooperation and Development; QSAR, quantitative structure-activity relationship; STAIR, stroke therapy academic industry roundtable; UFAW, Universities Federation for Animal Welfare.

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In 1978, David Smyth proposed the term "alternative" to term those procedures that can completely replace the animals used to perform the experiments, those that reduce the number of animals required or those that diminish the pain or distress suffered by the animals, prompting researchers to justify the use of animals for experimentation.[6,7]

Although animal testing had initially been developed for biomedical research to study physiological processes and diseases, later these procedures started to be employed in the evaluation of efficacy and safety. In this regard, although Paracelsus was the first alchemist to perform toxicity studies, this type of studies did not become relevant until 1927 when Trevan published the LD_{50} test for the biological standardization of potent and potentially dangerous drugs. Later on, during the'30s the adverse events caused by the presence of diethylene glycol in sulfanilamide elixir to treat streptococcal infections and the presence of paraphenylenediamine in an eyelid and eyebrow dye-induced significant changes of regulations. In this regard, the Federal Food, Drug, and Cosmetic Act (FDCA) introduced a significant change as regards consumer protection in America. According to the FDCA, a safety evaluation for marketing became a requirement not only for pharmaceutical drugs and cosmetics, but also for other kind of products such as food additives, household cleaning products, and industrial chemicals, highlighting that effective control systems are essential to protect the health of consumers.[8,9]

It is important to point out that not only the products must be safe to be used, but also toxicological information must be available in order to act properly in case of accidental exposure.

To guarantee product safety, some particularities must be borne in mind, such as the product nature, its intended use, and its chemical composition. For example, is it acceptable to apply the same strategy to analyze the safety of pharmaceutical drugs or pesticides? The answer is certainly no, mainly due to ethical concern. For these reasons, over the past years, there has been an increase of new strategies that reduce or do not involve the use of laboratory animals to evaluate the efficacy and/or safety of products intended for human use. Although the scenario is complex, it is necessary to have scientific and regulatory knowledge for the safe use of certain products, thus avoiding serious deleterious effects as was the case of the misuse of humidifier disinfectants in Korea. This disinfectant contained polyhexamethylene guanidine (PHMG), which has been widely used in industrial and consumer products as biocides and its use was extended as humidifier disinfectant in Korea. But, it was not considered that humidifiers could generate not only fog (micrometres in size) but also smaller particles (nanometers in size), becoming inhalation as a relevant route of exposure since it could be possible to penetrate deeply in the respiratory tract. In this sense, inhalation toxicity must be mandatory to analyze, but the product was sold without any data related to this information and induced fatal lung disease, demonstrating that a compound could become toxic when the exposure is changed.[10,11]

Taking into account that the strategies to analyze the efficacy and/or safety of different products such as pharmaceutical drugs, cosmetics, medical devices, and chemicals, among others may vary from product to product, the need for approaches and methods to evaluate such products and improve the predictability has increased. The pressure of the public opinion on governments and companies, and also on the scientific area, to reduce the use of animals is an important driver in the efforts to use alternative methods, although economic factors also play an important role in the area.

Based on the main idea presented in OECD 405,[12] this review discusses the different strategies complying with the 3Rs principles. These strategies can be applied sequentially and adapted to different scenarios in preclinical studies both in basic research and in the regulatory area, reducing animal experimentation to a minimum possible.

2 | BIBLIOGRAPHIC APPROACH

The first approach includes the analysis of current literature to obtain information about the characteristics of a specific product. This is crucial to avoid the performance of unnecessary experiments, which would go against scientific and ethical principles.

In this approach, the first step is to formulate a clear and concise research question, identifying key words and related descriptors. The use of Boolean operators might be useful to refine the search. Web-based search engines or electronic databases are crucial to identify relevant literature. In this sense, and as regards bibliographic resources, not only international archives (https://www.ncbi.nlm.nih.gov/pmc/, http://www.elsevier.com/online-tools/embase/, etc.) must be searched, but also regional ones (https://lilacs.bvsalud.org/es/, http://www.scielo.org.za/) because certain areas of study might only be relevant locally. Librarians play a central role in this phase.[13]

In order to identify the essential attribute of found bibliography, it is necessary to carry out an exhaustive reading, analysis, and sorting of the available literature. In this sense, the CASP (Critical Apraisal Skill Programme) checklist for clinical research and the STAIR (Stroke Therapy Academic Industry Roundtable) recommendations to increase the translational potential of experimental stroke models are useful recommendations. It is advisable to create a suitable list of published reports to perform a critical reading of the selected material. After a first analysis, the original search may be either expanded or reduced, for example, by limiting the search to the last 10 years. This search can be done in other databases and/or refining the selection by choosing the most relevant authors in the field under study.[14]

The information thus obtained provides a secondary qualitative analysis. However, a critical analysis of evidences is difficult to perform when information is obtained by this method, mainly due to the amount of information available. For this reason, certain strategies like the performance of systematic revisions, which establish explicit and reproducible criteria to statistically analyze the bulk of primary sources, allow the weighing of the information and validate the experimental conclusions obtained. Although the bibliographical approach is a useful tool, the lack of standardized methodologies

described in the primary sources, the diversity of experimental designs, the scarcity of reports of negative results, among others, may lead to an overestimation of the conclusions obtained in this type of meta-analysis. If these drawbacks are taken into account, the need for transparency policies for scientific publications becomes obvious.

Noteworthy, if the research is conducted to comply with regulations related to experimental procedures, the methods selected must be standardized, validated and, if possible, accepted by either national or international regulatory authorities, such as the OECD Guidelines, INVITTOX, pharmacopoeias, ISO documents.

This bibliographical approach is mandatory for any kind of research, and should be the first step to apply in order to know the context of the research area at that moment. Studies published in Open Access journals for example, promote global knowledge flow and the available budget for the research would not be a limiting factor. However, it is important to note that research groups with fewer financial resources have greater difficulties in publishing the results of their research in open access journals, limiting the exchange of experiences.

Taking into account the bibliographic approach, it is possible to replace animal experimentation, in reports with strong evidence, such as meta-analysis giving adequate information to answer the research question or it is possible to reduce or refine, improving the research design.

3 | VIRTUAL COMPOUND SCREENING APPROACH

In case, the previous approach does not give enough information to solve the research question in order to carry out predictions, the virtual screening method can be used. This approach requires information on the physicochemical characteristics of compounds. Although it is not a common scenario, under certain circumstances, the physicochemical properties alone could be enough to determine the potential environmental and/or health risks, reducing the use of animal experimentation. A good example of this can be found in the OECD 405 guidelines,[12] in which the physicochemical properties and chemical reactivity; for example, the buffer capacity, can predict the potential of a chemical compound to induce acute eye irritation or corrosion, since either low or high pH values (≤2.0 or ≥11.5) of solutions containing these compounds may have severe local effects. However, the acid/alkaline reserve must be analyzed too, since it has shown to have a better correlation than the pH measurement alone. But, if the result is inconsistent, further testing should begin to evaluate it.

Moreover, in the drug discovery process, the attributes of a compound could be useful to determine its potential as a candidate drug. For example, in order to select a lead candidate, the logarithm of the *n*-octanol partition coefficient (log P) is a good predictor of lipophilicity and represents a main physicochemical factor influencing bioavailability, permeability, and frequently the toxicity of a chemical structure. Considering that high lipophilicity values may lead to failure in the drug development process so, the lipophilicity efficiency has been proposed as a measurement of goodness of interaction between a compound and its target protein. This parameter represents the binding energy of a compound normalized by the compound's size and can be taken as the minimum acceptable lipophilicity per unit of in vitro potency. But considering that logP describes the partition coefficient of uncharged molecules, logD was introduced as a better descriptor of lipophilicity of charged molecules at specific pH. It represents the ratio of equilibrium concentration of un-ionized compound in octanol phase to concentration in aqueous phase buffered to different pHs. A particular interest is the $\log D_{7.4}$ that represents the partition coefficient of a compound using aqueous phase buffered to pH 7.4, knowing as physiological pH.[15] Also, it was developed predictor for log D using molecular signature descriptors and a support-vector machine, allowing interactive modification of molecules and also, it is possible to view the prediction structure, highlighting chemical structures that increase or reduced the predicted log D.[16] This information could be required as a first step to perform the virtual screening of compound libraries to select lead candidates with adequate physicochemical properties.[17–19]

Previous information could require bioinformatics methods to gather input from different resources in order trying to predict in vivo effect, therefore the purpose of the virtual screening approach is to use fast and cost-effective in silico methods to predict for example, human toxicity or environmental pollution. In this regard, QSAR (Quantitative Structure-Activity Relationship) studies correlate the chemical structure with the biological activity of a compound, playing a crucial role in the selection of the best compounds for synthesis or biological evaluation. The golden rule of QSAR studies is that similar chemicals have similar physical properties and toxicity, being this premise highly suitable to predict these characteristics. In this regard, the European Parliament and European Union Council have established that for the safety profile registration of chemicals, it is required that registrants demonstrate that the application of alternative methods to test safety have been considered. Thus, QSAR studies represent a good option to replace in vivo testing. In this sense, defined endpoint, unambiguous algorithm, defined domain of applicability, robustness, predictivity, and data curation must be mandatory principles to obtain an adequate and reliable QSAR result to ensure the success of this approach.[20–22]

Although current risk assessment is performed on a single chemical; compound mixtures are often evaluated. Considering the considerable bulk of available information about toxicity of chemicals and that the number of possible compound combinations is huge, the determination of the safety profile of a mixture remains challenging.[23] Although the potential risk of compound mixtures can experimentally be determined, this information can be derived from data obtained from each compound assessed separately and considering their percentage contribution in the mixture. However, this method does not take into account the potential interactions between chemicals, such as synergistic or antagonistic effects.

The adverse outcome pathway (AOP) approach might overcome this drawback. The AOP offers a framework to integrate and illustrate hazard and/or risk assessment of chemicals, providing knowledge to establish a relationship via key events (KEs) between a molecular initiating event (MIE) and an adverse outcome (AO) in a biological organization.[24] Although in silico models are a simplistic representation of a system, they can be useful if applied within an AOP construct. The interactions between two compounds can be predicted from the chemical structure and can be used to develop structural alerts, which may represent the probable effect of the chemical inducing MIE. For example, an electrophilic moiety can react with a biological nucleophile forming a covalent bond with either proteins or nucleic acids, and this interaction may lead to downstream adverse effects. Nonetheless, these predicted effects will not necessarily occur, or they may manifest differently depending on the species; for example, a compound mixture may cause respiratory irritation in fish and skin sensitization in humans, thus reflecting that AOPs must be considered as malleable tools that should be constantly refined by introducing new data. Therefore, the capacity of structural alerts associated with molecular initiating event to induce a toxicity in a specific species and also the epigenetic effects to long-term health outcome are matter of continuous study and development with this strategy.[25]

Nevertheless, technological information through high-throughput screening and combinatorial chemistry plays a crucial role not only in risk assessment, but also in efficacy studies assessing the capacity of new structures to produce beneficial effects.

It is known that the drug development process is time consuming and requires the collection of a great amount of information related to the pathophysiology of the disease, the target identification and its validation, the lead discovery and its optimization, preclinical studies, and clinical trials. In this long process, any undesirable effect could justify the rejection of the candidate drug so, computer technologies, such as combinatorial chemistry and high-throughput screening can offer effective strategies to speed up the process.

In the drug development process, parameters such as absorption, distribution, metabolism, excretion, and toxicity (ADMET) must be assessed since they determine the potential effect of the compound under study. In this regard, molecular modeling allows the prediction of ADMET properties through the application of different approaches such as pharmacophore modelling, molecular docking, dynamic simulations, and quantum mechanics calculation.[26–30]

Although this approach can speed up the discovery process by decreasing the number of candidates to be tested experimentally and improving the rationality of each choice, current knowledge indicates that this approach can replace neither in vitro nor in vivo assays. Nonetheless, using artificial intelligence (AI) can be possible design new drugs in a short time, almost 12 months, DSP-1181, a drug to treat obsessive-compulsive disorder, DSP-0038, a dual targeted 5-HT1A agonist and 5-HT2A antagonist to treat Alzheimer's disease psychosis or EXS21546 as adenosine antagonist for anticancer immunotherapy, are examples of that.[31–33] Although artificial intelligence for predicting adverse effects and efficacy have generated optimistic expectations, challenge in developing robust and predictive AI models could be related not only to the quality of the input data, but also to accuracy of the applied preclinical model.[34] In this sense, it was reported a strategy based on Boolean computational model to identify AI-guided disease target, studying rules of gene expression patterns at the outset and throughout a disease´s course. Moreover, it was possible to validate the best human-like animal model, facilitating the design of a patient-derived diseased organoid model,[35] showing the possibility to establish the pharmacological precise target, personalize the choice of the best therapy for a specific patient or even reduce or avoid the use of animal models.

Computer technology allows the generation of a great amount of chemically-related compounds; however, the development, and implementation of new machine learning algorithms and data curation methods capable of handling millions of compounds are urgently needed.

To apply virtual screening, a high-performance computing infrastructure is needed to test a high number of druggable targets and chemical compounds, calling for cooperative work between researchers from both the academia and the industry. In this sense, interdisciplinary development employing shared computer facilities and expertise through cloud-based computational platforms has proved to be more cost-effective.[36]

Such platforms are being developed to help researchers with various types of applications to prepare and guide them in the drug discovery and development pipeline, providing remote services that may be used in the cloud or offering integration with a self-implemented workflow. Some examples of this are 3decision.discngine.com, a collaboration forum for researchers to exchange data on ligand-receptor interactions, 3d-qsar.com, a web-based platform that allows the generation and validation of 3D-QSAR models, and www.playmolecule.com /BindScope, a structure-based binding prediction tool, among others.[36]

4 | IN VITRO APPROACH

If the previous strategies are not enough to give adequate information to answer the research question, in vitro approach could be used to study efficacy and safety of compounds. These methods employ immortalized cell lines, which have been induced to proliferate for long periods of time. These cell populations are genetically identical and fairly well characterized, thus assuring reproducibility of results. Yet, they might express unique gene patterns that are not present in primary cells. Therefore, to compensate for these drawbacks, the characteristics of cultured cells must be assessed periodically to assure the reliability of results.[37]

In this approach, two- or three-dimensional (2D or 3D) cell cultures can be used. In 2D cultures cells are grown as monolayers, while in 3D cultures cell–cell and cell–extracellular matrix interactions are included in the final response, thus better mimicking the complexity of the in vivo microenvironment.[38] In this sense, liver tumor cells growing in 3D cultures have shown a similar gene expression profile and treatment response, as compared to in vivo models. Model spatial organization, adding different types of cells

and facilitating the interaction between cell and its microenvironment, can be obtained using different strategies such as, 3D co-cultures embedded in a Matrigel, spheroid methods, microfluidic systems, or 3D bioprinting. Nevertheless, 3D cultures have some disadvantages, 3D spheroids are difficult to standardize and 3D homogeneous organoids are difficult to obtain in large numbers and also 3D bioprinting requires expensive consumables and equipment.[39] Although 3D cultures represent a significant advance in comparison with 2D cultures, they do not fully behave as higher-level target systems, which have a more complex anatomical and physiological organization. Validation of these methods represents a huge challenge since it must demonstrate its reliability and relevance for the given purpose, identifying mechanisms that are causative for downstream and finding appropriate reference data for the test under study.[40] In spite of that, this strategy might be useful to test the potential toxicity of xenobiotics in humans and other species, as shown by the use of 3D HepaRG spheroids to predict genotoxicity in vivo.[41]

5 | EX VIVO APPROACH

Ex vivo and in vitro techniques are similar, since both types of experiments are carried out outside the animal, but the former uses cells obtained directly from the living organism.

In ex vivo experiments, either isolated cells, whole organs, or fractions of them can be used as long as viability is maintained. In ex vivo models, tissues maintain their architecture, thus replicating the in vivo conditions. Ex vivo models can be used to analyze the potential pharmacological effect of new compounds, for example, those tested in isolated human umbilical vessel or animal aortic rings on which the effect of drugs on the smooth muscle contractile activity, angiogenesis or extracellular matrix signaling can be tested. Human skin explants obtained at surgery can also be used to study the effect of compounds on wound healing, chronic inflammatory disease, or on viral, fungal or bacterial infections.[42–45]

Ex vivo experiments are also suitable to assess the potential adverse effects and compound toxicity under normal or pathological conditions. It should be kept in mind that protein expression patterns as those of the enzymes of the metabolism can vary from species to species; in this regard, the results obtained ex vivo could be more representative of in vivo conditions, such as ex vivo skin explants from elective human plastic surgery as strategy to understand the mechanism involved in several human cutaneous disorders,[46] or for testing safety and efficacy of cosmetic products, instead of using rabbits or genetically engineered mouse models.[47,48]

In this sense, electrophysiology studies can be performed using single cells, such as disaggregated cardiomyocytes, or multicellular preparations, as those obtained from papillary muscle or intact heart isolated from laboratory animals, and occasionally from humans. In this regard, the QT interval (time from the beginning of the QRS complex to the end of the T wave) on the electrocardiogram is a measure of the duration of the ventricular depolarization and repolarization events. The interaction of a compound under study with a specific potassium voltage-gated channel related to subfamily H member 2 of the human ether-a-go-go-related gene (hERG, KCNH2) can induce an abnormal rapid cardiac delayed-rectifier potassium current leading to a fatal cardiac failure. In the early stages of investigation of a new compound, the proarrhythmic effect can be determined through a non-clinical evaluation of the capacity of the drug to produce a delayed ventricular repolarization. The latter strategy allows researchers to either dismiss the compound for further studies or to set up a strict pharmacovigilance schedule to prevent adverse effects after the drug is approved for clinical use.[49]

Both in vitro and ex vivo strategies can collaborate to reduce animal experimentation. In this sense, different international societies try to influence the public opinion, taking legislative action, and seeking governmental collaboration to diminish in vivo experimentation. It was described that animal experimentation can produce misleading information, is time consuming, and also ethically controversial, also, some institutions claim that in vitro and ex vivo methods can be performed at a much lower cost than in vivo ones (https://www.hsi.org/news-media/time_and_cost/). These changes have prompted a great number of companies to offer alternative methods in the portfolio of contract research organizations (CROs), thus representing a sizable industry that takes part in national and international validation processes. Market research companies monitor relevant technologies to analyze safety profile of a compound to comply with national or international regulations. In this sense, the estimated growth by MarketsandMarkets™ showed the major compound annual growth rates (CAGR) belong to organ on chip (36.6%) and 3D cell culture (23.6%) and the minor CAGR belonging to in vitro toxicology (6.6%) and human liver model (3.6%). Regretfully, accessing this information is costly, and sometimes the critical and impartial analysis of the market carried out by the academia is not taken into account in such decision-making process.[50]

6 | IN VIVO APPROACH

Finally, after having gone through the previous steps, but the information obtained does not have adequate validity to be considered relevant, it may be necessary to carry out an in vivo test. Considering the overwhelming necessity to assess the efficacy and safety of new products, one could assume that, over the recent years, a great number of animals have been used to conduct the experiments; however, the number of laboratory animals employed in the whole world has remained undetermined. Although institutions from some countries such as Canada or countries belonging to European Union, must report metrics related to animal research, teaching, and testing conducted, in other countries this information is only partially reported to government agencies such as United States of America, meanwhile in various Latin American countries neither reports nor local regulation related to animal experimentation are available.[51,52] But, based on the statistical analysis of data from 37 countries belonging to the European Union and

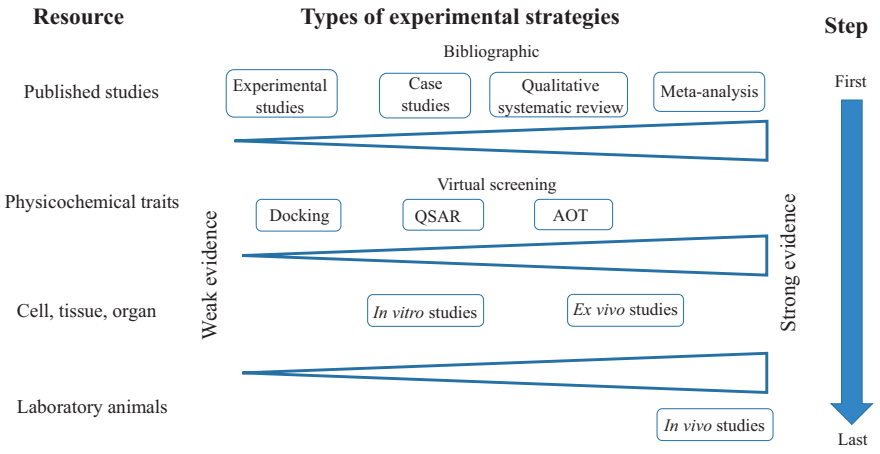

FIGURE 1 Schematic guide based on the 3Rs for investigation designing

using a prediction model based on the publication rates from 142 countries, Taylor and Alvarez (2015)[53] estimated that 192.1 million of animals worldwide were used in 2015. Taking into account these figures, it would be advisable to apply an in vivo approach only in cases in which previous approaches have failed to address the topic under study or when the experiments performed in animals prove to produce a significant knowledge based on harm-benefit analysis. Local and/or international regulations might also call for the performance of in vivo experiments. Proper planning, development, and reporting are the key points to accomplish the principle of the 3Rs when in vivo strategies are used.[54,55]

As for planning and development, different tools to help scientists are available. The Experimental Design Assistant (EDA) offers a stepwise visual representation of the experiment to be performed including a specific feedback and advice in each step is relation with the project objectives (https://eda.nc3rs.org.uk/). PREPARE (Planning Research and Experimental Procedures on Animals: Recommendations for Excellence) is a guide for good practices in animal experimentation[56,57] and ARRIVE (Animal Research: Reporting of In Vivo Experiments) is a guideline to improve the rigor and transparency of the scientific report.[58,59]

These recommendations help designing in vivo experiments with adequate internal and external validation procedures performing a correct study design and animal model, which will ultimately assure a translational value of the animal experiments to humans.

Useful knowledges, strategies and tools are offered by different websites, such as https://norecopa.no/more-resources/culture-of-care, in order to improve animal welfare and scientific quality, but also in relation to alternative methods https://norecopa.no/alternatives.

Finally, there is a great interest to evaluate risk and hazard of different products for human health, so it could be useful to develop test systems that allow to predict, in short or medium term, the long-term toxic or carcinogenic potential of a compound. Omics technologies provide scientists with the ability to probe biologic variance with high sensitivity in simple systems (cell culture, isolated organ, etc.) but also in more complex ones (in vivo models). In this sense, this strategy, can detect tissue-specific changes with increasing sensitivity, analyzing thousands of genes, proteins, or metabolites in order to detect changes induced by a treatment on transcriptional or translational expression levels in laboratory animals. Therefore, omics technologies such as toxicogenomics, toxicoproteomics, and toximetabolomics could predict specific endpoints of toxicity after short-term in vivo exposure due to the fact that these methods can detect even the smallest changes at the molecular level that still precede morphological and clinical endpoints.[49,60] This strategy can reduce the chronic safety studies in animals, such as chronic toxicity tests or carcinogenicity studies, with short-term animal experiments evaluated by omics technologies. Therefore, it is possible to improve chemical safety estimation, replacing, reducing, or at least refining animal experimentation.[61] Although this strategy was described as a superior scientific understanding with increased efficiency, the insufficient validation, the complexity of its interpretation and lack of standardization has been described as the main weakness.[62,63]

7 | CONCLUSION

The global spending on research and development (R&D) has reached a record of almost US$ 1.7 trillion, but only 10 countries account for 80% of the total cost. EEUU, Japan, Germany, Korea, between others are spending relatively more in terms of their GDP. As part of the Sustainable Development Goals of the UNESCO, countries have pledged to substantially increase public and private R&D spending as well as the number of researchers by 2030 (http://uis.unesco.org/apps/visualisations/research-and-development-spending/).

Improvements in biomedical research related to 3Rs application can differ between countries and different areas of research, but there is need for a more structural and effective exchange of method developments as well as more investment, especially in some countries and areas, in order to promote the real access to strategies that adhere to ethical principles of the 3Rs.[64] Moreover since virtual screening and in vitro methods as part of new approach

methodologies (NAM) are promoted by numerous independent organizations in order to replace animal testing, especially in the context of chemical hazard and risk assessment, more efforts must be done globally in order to determine their suitability for potential regulatory application.[65]

In this context, this manuscript tries to highlight different strategies to allow researchers find the best way to answer their research inquiry, fulfilling the ethical requirements for a responsible experimentation, even when their budget is not the highest (Figure 1).

DISCLOSURE

The authors declare that they have no conflict of interest. No datasets were generated or analyzed during the current study.

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ETHICS STATEMENT

Not applicable.

AUTHOR CONTRIBUTION

SG conceptualized the idea, reviewed literature and wrote the final version. ARB critically reviewed the initial draft and contributed to revising the manuscript, All authors have read and approved the final version for publication.

DATA AVAILABILITY STATEMENT

Data sharing is not applicable to this article as no new data were created or analyzed in this study.

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