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Review

A Brief Review of the European Directive on 3Rs and Facilitating Animal Experimentation

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ABSTRACT – The value of animals to humankind extends beyond their provision of food and physical resources; they also serve a wide range of purposes, including their role in experimentation. Given that animals play a substantial part in experimentation, it becomes imperative to improve their living conditions, at the very least as a form of compensation. As a result, the driving force behind the enactment of various legislations, including the European Union Directive (2010/63/EU), is human morality. This directive primarily pertains to specific European member states and is fundamentally aimed at safeguarding the welfare of animals used in experimentation by advocating for the implementation of the 3Rs: replacement, reduction, and refinement. Nevertheless, concerns have arisen, particularly regarding how the provisions of the directive might constrain or influence the advancement of experimentation, notably within the biomedical field. This review explores the nexus between Directive 2010/63/EU and the facilitation of animal experimentation, with a specific focus on the 3Rs. Findings reveal that the judicious application of the 3Rs markedly enhances the progress of experimentation without compromising the quality of outcomes. Furthermore, recent studies propose that the 3Rs alone may not suffice, suggesting the need to introduce additional relevant Rs. Hence, the current decrees are deemed adequate for a certain period, with potential modifications in the future. In order to encourage the advancement of experimentation without jeopardizing animal welfare, it is imperative to conduct periodic, routine reviews.

Keywords: animal welfare, reduction, refinement, replacement, research.

HISTORICAL BACKGROUND ON THE DIRECTIVE

The history of legalization and legislation for animal protection dates back over a century. For instance, in 1876, the United Kingdom introduced an Act to amend laws related to Animal Cruelty (*Wells*, 2011). Since then, animal welfare has become the focus of scientific experimentation worldwide, spanning various domains, including farm animals, animals used for scientific purposes, and captive wild animals. The European Union (EU) has played a crucial role in establishing comprehensive animal protection laws. On September 22, 2010, the EU issued Directive 2010/63/EU, which, following its revision in 2009, out-

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lines the legal framework governing animal protection in scientific experimentation (*EU*, 2009 and 2010). This directive represents the culmination of efforts that began with Directive 86/609/EEC. Its primary objective is to advance animal welfare by promoting the application of the three principles known as the 3Rs: replacement, reduction, and refinement (*Olsson* et al. 2016; *EU*, 1986). *Hartung* (2010) provides a comprehensive discussion of the distinctions between the previous and current directives.

Directive 2010/63/EU set an implementation deadline of January 1, 2013, for all EU member states. In April 2013, Hungary, as a member state, incorporated the directive through Ordinance 40/2013 (II. 14.) and Ordinance 98/2014 (III. 25) of the Hungarian Government (*Hungary*, 2013; *Hungary*, 2014), which replaced the 1998 Hungarian Animal Welfare Act (XXVIII. Act) pertaining to animal experiments (*Hungary*, 1998). The current Directive 2010/63/EU consists of two sections and eight annexes. The first section outlines the intentions (56 points), whereas the second section covers general provisions, the use of particular animals in procedures, authorization procedures, measures to prevent duplication, alternative approaches, and final provisions (a total of 66 articles). The directive chiefly pertains to animals, encompassing whole animals, organs, and tissues intended for use in scientific experiments, regardless of whether the usage involves the entire animal or only a part. Its central aim is to replace animal use with alternative approaches. Thus, EU guidelines mandate that every project, including breeders, suppliers, and users, establish an animal welfare body, be it at the local, regional, or national level, with a consultant and regular supervision and monitoring activities, as specified in Article 27. Within the directive, these animal welfare bodies bear a resemblance to the Institutional Animal Care and Use Committee (IACUC) in the United States (*Van der Meulen-Frank* et al., 2017). Generally, the directive excludes sectors unrelated to scientific experimentation, such as cruel chemical and industrial testing, cosmetic tests, educational demonstrations, and veterinary clinic experiments.

The provisions of Directive 2010/63/EU are fundamentally anchored in the implementation of the 3Rs, which aim to protect animals used in scientific experimentation without compromising the quality of the results. However, the adoption of the 3Rs can pose numerous challenges related to the quality of inputs and outputs in scientific experiments. Restricting methodologies and establishing criteria for animal protection may significantly impact study design, budget, and timelines compared to previous management systems. To address these aspects, this review attempts to assess the directive by investigating the effectiveness of the 3Rs in animal experimentation.

THE THREE PRINCIPLES (3RS)

When animals are extensively utilized in experiments, and given their status as sentient beings capable of experiencing pain, it becomes our ethical obligation to respect and acknowledge their suffering. This principle underlines the implementation of the 3Rs, a framework governing the use of animals in experimentation. The foundational work in this area is the book titled "The Principles of Humane Experimental Technique," authored by *Russell and Burch* in 1959. This seminal work categorizes animal techniques into what are known as the 3Rs: replacement, reduction, and refinement. The principles laid out in this book have been incorporated into numerous directives worldwide, including Directive 2010/63/EU, the Australian Code for the Care and Use of Animals for Scientific Purposes (*NHMRC*, 2013), and the Canadian Council on Animal Care Policy Statement on Ethics of Animal Investigation (*CCAC*, 1989). With regard to Directive 2010/63/EU, the 3Rs form the core of the directive's framework, with the primary objective of protecting the welfare of animals and eliminating or minimizing their suffering. The directive introduces a classification system for the severity of procedures, categorizing them into four levels: mild, moderate, severe, and non-recovery. This categorization is designed to be flexible, acknowledging the evolving body of knowledge regarding specific animal species. It recognizes that animal species are undergoing genetic modifications due to both environmental (epigenetic) factors and artificial engineering aimed at enhancing productivity.

In contrast to Russell and Burch's book, which primarily focuses on non-human vertebrate animals, both terrestrial and aquatic, Directive 2010/63/EU extends its scope to independently feeding larval and fetal forms of mammals in the last third of their normal development, as well as cyclostomes and cephalopods. Moreover, it permits the use of genetically modified animals, subject to certain constraints, including consideration related to laboratory construction, infection prevention, housing design, management procedures, and animal handling at the endpoint of a project (*Van der Meulen-Frank* et al., 2017).

The 3Rs and animal experimentation progress

The utilization of animals in scientific experimentation confers significant benefits to humanity. Animals serve as vital components in the testing of vaccines, medications, therapeutic approaches, and various other products and protocols (*Barbee & Turner*, 2019; *Gruen*, 2011). The generation of more data from animal experiments can have both positive and negative implications for the 3Rs, although this aspect remains to be thoroughly evaluated. The accrual of

additional data has the potential to diminish the future necessity for animals in experimentation procedures while concurrently augmenting the reliability of these procedures and their associated findings. However, the accumulation of data from animal experiments also underscores the extensive scale of animals involved in scientific research. In 2015, approximately 79.9 million animals were engaged in scientific experiments (*Taylor & Alvarez*, 2019), with nearly 25% of these being used in regulatory studies. After 2019, there has been a remarkable decline in the number of animals involved in experimentation within the EU (*EARA*, 2023), primarily attributed to reduced research activity during the COVID-19 pandemic, which prompted the imposition of restrictive measures. Interestingly, in 2020, the EU used fewer animals in research compared to the United States, China, and Japan (*Statista*, 2020), reflecting the strict theme of the directive. Nevertheless, the total number of animals used in scientific studies is influenced by numerous events. These include the extent of research activities, financial investment in the field, advancements in technology, and the implementation of systematic data gathering methods, among others.

From a political and public standpoint, there are substantial expectations regarding the measurable impacts of the 3Rs principle. Animals participating in scientific experimentation, particularly those involved in biomedical research, frequently undergo invasive procedures that may, in some cases, result in fatality. In light of this, *Louhimies* (2012) raises a pivotal question concerning the assessment of legislative effectiveness: "*Where are we today, and what are the next steps? We have real opportunities ahead of us—are we grabbing them?*". From a logical perspective, it is acknowledged that no single piece of legislation can be considered 100% perfect. This realization has led numerous researchers to propose that the 3Rs may not be entirely sufficient or conceptually suitable for certain animal experimentation groups. In addition, some debate elevates from social and ethical perspectives. Hence, *DeGrazia & Beauchamp* (2019) advocate for the incorporation of social benefit Rs, and *Curzer* et al. (2013) propose the implementation of ecosystem Rs. Moreover, *Slokenberga* (2017) emphasizes the urgent need for comprehensive administrative and criminal liabilities within directives to enhance the protection of animals used in experimentation. Lately, extreme arguments are taking place, merely focusing on the elimination of animal use in research. These arguments have escalated to level of political arguments, such as in the EU, USA and Switzerland (*Han*, 2023; *Bundesrat,* 2022; *European Commission*, 2015). While these proposals and demands aim at improving directives and protecting animals

from experimentation, they may also present potential obstacles to the advancement of scientific experimentation. Given that the directive is founded upon the 3Rs, an examination of the efficacy of the 3Rs in facilitating experiments can offer insight into the directive's adaptability to address emerging challenges in the field. In this review, this approach is employed to highlight contemporary issues within the realm of animal experimentation and assess the directive's capacity to accommodate these concerns.

Replacement

Replacement intends to substitute animal experimentation with alternative approaches, thereby safeguarding animal species and preserving their natural diversity, particularly endangered species. This approach can only be achieved by minimizing mortality, enhancing living conditions (welfare), and rehabilitating animal reproduction and habitats. According to *Van der Meulen-Frank* et al. (2017), replacement can take two forms: absolute and relative, depending on the requirements of the specific experiment. Furthermore, these authors detail two variants of relative replacement: 1) employing the minimal number of animals, and 2) adopting a minimal-pain approach. In this regard, Directive 2010/63/EU highly encourages the implementation of absolute replacement. However, achieving absolute replacement in several scientific domains, such as *in vivo* toxicological experiments, can be particularly challenging. Hence, the directive permits partial replacement, classifying relative replacement into the following categories: 1) non-recovery experimentation, where animals are fully anesthetized without experiencing any discomfort, and 2) experiments that necessitate the use of animals who are painlessly euthanized. The extent of replacement varies based on numerous variables, including the animal species, the maximum permissible level of pain or stress, and the value pursued by the experimentation within its respective field. According to available literature, numerous biomedical experiments conducted on animals may lack strong justification or direct relevance to the human model (*Van Norman*, 2019; *Bracken*, 2009). Moreover, replacement may appear beneficial in the context of non-research observations, such as restricted measurements relative to the development of treatments. Though the directive upholds the principle of scientific freedom, it also mandates adherence to ethical principles and practices. It stipulates that animal involvement must be a last resort, and experiments must aspire to justifiable goals aimed at potentially improving the quality of life. Consequently, the experimental design must aim to closely replicate the actual pathogen dose range to minimize undesirable scenarios, such as severe physiological implications and an unjustifiably high mortality rate.

Over the past decades, funding for 3R research has facilitated advancements in replacement, predominantly through the establishment of alternative approaches. These approaches are not designed as direct replacements; rather, they represent an alternative approach to addressing a specific research query. In other sections, this term can include animal methods. However, within the scope of this section, these alternative approaches are fundamentally non-animal methods, which is the sector that receives significant funding under the 3Rs development project. Advocacy for these methods plays a crucial role in their implementation, especially since many of these methods are not widely recognized. For instance, *in vivo* experiments reveal the state of actual complex organism reactions to substance(s), albeit their reliability may be hampered by inherent individual genetic variations. To mitigate variability factors and minimize harm in experiments, numerous recognized alternative approaches are available. However, *Genzel* et al. (2020) note that currently, there is no alternative method sophisticated enough to predict organ and body complexity. Therefore, the directive does not mandate the exclusive use of alternative methods but encourages the application of computer models (predictive mathematical methods), *in vitro* studies, *in silico* approaches (such as computational methods: QSARs, systems biology, pathway modeling, PBPK and PKPD modeling), and cell and tissue culture and engineering as alternatives to whole-animal experiments. Though a single alternative approach may not entirely replace *in vivo* experiments for complex endpoints, a combination of alternative methods can provide prediction tools with a certain level of accuracy *(Laroche* et al., 2019). It is important to note that the development and validation of alternative methods are ongoing processes. The European Partnership for Alternative Approaches to Animal Testing (EPAA) promotes the use of read-across tools (predicting a substance's toxicological endpoint based on available data from one or more related substances) and *ab initio* methods (safety assessment based on *in vitro* tests combined with *in vitro* to *in vivo* extrapolation through computational approaches) to enhance the decision-making framework (*Mahony* et al., 2020). Fortunately, substantial data on the risk assessment of thousands of substances in Europe are publicly available online. Much credit goes to the activities of the European Commission, the European Food Safety Authority, the European Medicines Agency, the project EuroMix—European Test and Risk Assessment Strategies for Mixtures, and the project EU-ToxRisk. These risk assessment summaries contribute tremendously to the advancement of 3Rs implementation and the development of alternative approaches.

According to *Goh* et al. (2015), *in vitro* tests exhibited consistent growth in the pharmaceutical industry from 1980 to 2013, significantly contributing to the replacement principle. However, under some circumstances they may not seem sufficient (*Krebs* et al., 2022). Therefore, it's imperative to emphasize that *in vitro* tests should not be viewed as mere supplements to *in vivo* tests; rather, they present valid substitutes. In the field of biomedical research, enormous efforts spanning the past two decades have been dedicated to implementing these alternative approaches. One such approach is tissue-engineered skin, which has been validated and recognized as a viable method for achieving replacement (*Basketter and Gerberick, 2022*; *ESAC*, 2008). Furthermore, omics' technologies, encompassing genomics, proteomics, metabolomics, metagenomics, phenomics, and transcriptomics, have played a remarkable role in promoting the 3Rs in the context of animal experimentation, with a particularly notable impact on replacement. Methods using "omics" technologies involve the analysis of gene expression of metabolites related to treatment (*Kroeger*, 2006), potentially substituting studies that require more time and involve the use of animals. In addition, computational toxicological modeling has emerged as a valuable tool in toxicological investigations, and its adoption in the pharmaceutical field has progressively increased over the last three decades (*Ford*, 2016). Recent technological advancements have been significantly contributing to the revolutionary development of computational models, leading to a paradigm shift from 2D to 3D models. Notably, advancements in machine learning, particularly artificial intelligence, have enabled the development of models capable of predicting hormone receptor binding affinity (*Wong* et al., 2017). It is worth highlighting the substantial contribution of *in silico* simulation in predicting the human response to COVID vaccines. A further marked tool among alternative methods is the meta-analysis approach, a systematic review method involving the statistical treatment of accumulated data. This method serves as a valuable tool in minimizing unnecessary duplication of animal experiments (*Hooijmans* et al., 2014), contributing remarkably to replacement as well as, to a lesser extent, reduction. In contemporary scientific investigations, meta-analysis plays a critical role in preventing similar data redundancy in genomic, clinical, pharmacodynamic, and toxicological investigations. Notably, researchers like *Harding* (2017) have noted the availability of genomic data for non-human primate laboratory animals, representing a promising development in this domain.

Replacement offers the potential to reduce research expenses by minimizing the substantial costs associated with animal experimentation (*Polli*, 2008),

including the costs of animals themselves, housing, transportation, and feeding. However, it is important to note that in certain circumstances, additional costs may be incurred, such as software licenses, equipment (complete or spare), and quality assurance kits. Challenges may also arise due to the accessibility and variability of materials and reagents required for recommended alternative approaches. These challenges may make the implementation of alternative methods and the 3Rs difficult, especially in regions with limited resources, such as low-income and third-world countries. The potential for unaffordable price increases can jeopardize the adoption of replacement methods, leading to limitations in publications and potential compromises in the quality of research outcomes. Reputable journals typically demand high standards as well as article processing charges (APCs). In such cases, the lack of anticipated publications (*DEFRA*, 2010) can be directly attributed to budget constraints and indirectly linked to inadequate animal protection laws. Therefore, effectively implementing the 3Rs framework necessitates international harmonization and collaboration to overcome the challenges faced in advancing experimentation progress. Global adoption of the 3Rs is imperative, thereby ensuring that no scientific organization can outsource unjustified experiments to other nations. Technological advancements have facilitated worldwide collaborations that benefit both humans and animals. Subsequently, the concept of a cooperative framework has gradually gained prominence among scientific institutions. For instance, the "3R Blackboard" is a recognized platform that provides a space for various research groups to distribute excess biological materials obtained from animals (*Czubala* et al., 2022). Notably, the current EU directive, Directive 2010/63/EU, has expanded and continues to broaden the collaboration zone. It genuinely encourages the sharing of technical and non-technical information among scientific communities through open-access database systems, enhancing the prediction and validation of methods, both direct and alternative, as well as promoting more effective directive execution. This strategy is rapidly advancing, with numerous easily accessible databases now available.

One of the most frequently voiced concerns regarding replacement is the reliability of these methods or models. Although alternative approaches offer comprehensive animal protection, not all methods are as reliable or suitable as human models (*Ritskes-Hoitinga*, 2022; *Mikhaylova & Thornton*, 2019; *Kattan & Gerds*, 2018; *Ferdowsian & Beck*, 2011). According to *Hackam & Redelmeier* (2006), only 33% of the most highly cited studies (selected citations) were deemed suitable for human simulation in clinical applications. In numer-

ous instances, the intricate interactions and complexities of organisms and tissues are not readily discernible or fully comprehensible without the inclusion of data obtained from *in vivo* animal studies. This concern is particularly pronounced in certain fields, such as pharmacodynamics, metabolism, epigenetics, and toxicology. For instance, certain medications intended to target specific cell types may elicit unexpected or unconventional responses during experimentation (*Saeidnia* et al., 2015). In this context, the directive does not take an extremist stance. In urgent studies, the replacement principle allows for the utilization of a limited number of animals in experimentation (relative replacement) without compromising the quality of findings. According to *Hopper* (2016), automated microsampling represents one of the approaches in biomedical experimentation to minimize sample sizes without sacrificing the quality of results. The same author asserts that the use of microsampling in animal experimentation aligns with all the criteria of the 3Rs.

Another noteworthy issue is that specific animal data is not always applicable or accurate when extrapolated to other species (*Saeidnia* et al., 2015; *Van der Worp* et al., 2010). This issue is exemplified in cases involving drug withdrawal periods. In this regard, a well-devised plan can ultimately aid in averting crises and mitigating errors. Notably, the directive has established clear obligations for projects to ensure the accuracy of results. These obligations encompass comprehensive feasibility studies, a well-structured work plan that includes a sound understanding of species differences, and the engagement of qualified and competent teams. Moreover, the directive specifies the qualifications required for members at various project phases: project design, procedure design and implementation, animal care, and animal euthanasia. These acquisitions are imperative to verify the correctness of experimental design and the reliability of outcomes, especially regarding their potential applicability to other species.

Reduction

The primary objective of reduction is to minimize and restrict the number of experimental animals without compromising the project's objectives. Thus, within the confines of the existing legal framework, the principle of the 3Rs is a crucial component of responsible research conduct. However, it does not constitute an attempt to alter the legal foundation itself. It is crucial to note that reduction does not imply the absolute elimination of animals from experiments. Hence, the in situations where alternative approaches are lacking, reduction becomes of fundamental importance, and its value becomes more pronounced when implemented in conjunction with refinement (*Van der Meulen-*

Frank et al., 2017). For instance, during the uncharted COVID-19 pandemic, diverse animal models were indispensable in examining the disease's pathological mechanisms and immune responses (*Kiani* et al., 2022; *Genzel* et al., 2020). To curtail animal exploitation in similar cases, the strategy outlined in Directive 2010/63/EU revolves around eliminating needless duplication and incorporating the findings of preliminary experiments, such as *in vitro* tests. This emphasizes the significance of careful planning and well-defined objectives in the reduction process (*Das* et al., 2009). In this regard, the directive stipulates minimal requirements for application approval, encompassing the proposal, technical and non-technical summaries, and 3Rs-related information. Furthermore, it demands the maintenance of project records, including historical and current information, qualifications of team members, stock replacement approaches, and veterinarian activities to ensure an accurate assessment. According to *Felsmann* et al. (2014), conducting animal experiments in practice may differ from what the protocol outlines. In this case, it is essential to have continuous monitoring and supervision to ensure the fulfillment of all obligations by the project.

The assessment performed before and during the project, as described by *Van der Meulen-Frank* et al. (2017), might appear insufficient, prompting the need for a critical retrospective severity evaluation after the project's conclusion. The primary objective of this retrospective assessment is to provide recommendations for subsequent projects. Fortunately, the current EU directive has adopted the "upper limit to harm" approach, as seen in the first report in the Danish law on animal protection (*Olsson* et al., 2020). In addition, it compels member nations to conduct a retrospective assessment considering objective attainment, harm, and factors relevant to the 3Rs. This indirectly underscores the recognition that the complete elimination of harm is often unattainable. Under such a context, it is desirable to broaden the scope of some models, adopt novel updates, and assure accurate execution. *Grimm* et al. (2017) have noted that existing animal welfare directives and their decrees may negatively impact the credibility of experimentation. Consequently, numerous European and non-European organizations are actively engaged in developing, improving, validating, and implementing models that reduce animal exploitation in experiments. Remarkably, the outputs of these entities have exhibited steady increases and have contributed to the reduction of animal cohorts in experiments, notably in the biomedical sector.

A recent study has proposed an alternate model for evaluation that centers on the potential knowledge gains as an outcome of a project rather than a prospective assessment of potential societal benefits (*Eggel & Grimm*, 2018a). The

harm-benefit analysis report, which plays a crucial role in the appropriate evaluation, is one of the most significant elements requested by Directive 2010/63/EU from scientific projects. In fact, the directive places a high value on harm-benefit analysis, setting it as an obligatory purpose for research. Nevertheless, according to certain reports, the 3Rs might not be sufficient to provide comprehensive insights into the cognitive and emotional capacities of animals, their distinct interests, and the evolving understanding of harm-benefit analysis in animal experimentation (*Eggel & Grimm*, 2018a and b; *Grimm* et al., 2017; *Ferdowsian & Beck*, 2011). Given these considerations, it is advisable that Directive 2010/63/EU undergo periodic revisions. Such reviews should encompass new outcomes, including severity categorization, the reliability of current and newly developed alternative approaches, and harm-benefit analysis. The performance of periodic reviews must be carefully balanced to ensure they do not compromise the quality of experimentation output or animal comfort, which remains the directive's primary objective.

Replacement and reduction in animal experimentation are closely related, with each principle influencing the outcomes of the other. Notably, the expansion of reduction, as well as refinement, seems to be accompanied by the impracticability of eliminating animals from experiments. For instance, the measurement of the median lethal dose (LD_{50}) has alternative methods, such as improved UDP, modeling, and algorithm approaches. However, the reliability of estimated findings can sometimes be compromised when the reduction principle is not appropriately implemented. In such circumstances, validation is often necessary, and this is typically accomplished through animal studies (*Akhila* et al., 2007). The occurrence of this type of validation (re-investigation) is notably attributed to inadequate knowledge in the specific scientific field. This requirement for validation is particularly evident in fields with high reliance on toxicological experiments and genomic investigations (*Spencer* et al., 2019; *Brannen* et al., 2016), notably requiring a large number of animals for validation. In such cases, proper planning and validation can provide a degree of reliability in reduction and/or replacement within the designated method (*Grimm* et al., 2023).

The variability in reduction across scientific reports is influenced by categorical preferences and the reliability of findings. Therefore, Directive 2010/63/EU acknowledges the need for proper plans and operational goals to enhance validation and minimize re-investigation. Reusing animals has been recognized as an effective strategy for reduction and is commonly employed in pharmacological and toxicological investigations. However, its effectiveness

is more pronounced in the context of reduction than refinement. Properly supervised (under veterinarian) animal reuse, taking into account the animals' lifetime experiences, can significantly contribute to reduction. Screening assays and *in vivo* imaging are also valuable pharmaceutical experimentation approaches that enhance statistical power and reduce the number of animals required for validation (*Brannen* et al., 2016; *Campbell* et al., 2016). The utilization of appropriate statistical evaluation methods with higher powers has led to a reduction in the animal cohort of 20–40% (*Akkermans* et al., 2020) by minimizing the use of invalid methods.

Directive 2010/63/EU encourages researchers to develop, seek, and evaluate similar scientific approaches that reduce error-related noise and enhance the planning process and the quality of outcomes. In order to promote similar approaches, a non-professional summary of the project, including its purposes, techniques, and circumstances, must be available to the public. Furthermore, documentation and method transparency are fundamental practices and essential components of the professional project summary. Transparency is a central objective of Directive 2010/63/EU, aimed at increasing public awareness, especially among advocates against animal exploitation and liberationists. Given that public funding plays a substantial role in the majority of the research sector, transparency is key. While not all animal protection authorities have equal access to research facilities and data (*Clark* et al., 2019), the underlying concept emphasizes the importance of greater transparency. Transparency not only enhances the perception of scientific experimentation but also improves the image of the commercial sector. According to an extensive survey conducted by the European Commission in 2005 across over 25 member states, 74% of the products derived from animals raised in accordance with ethical recommendations were deemed ethically acceptable, while 58% and 57% predicted improved food quality and safety, respectively (*Bonafos* et al., 2010). It's worth noting that with the recent expansion of internet networks and social media (which are rapid, widespread, and user-friendly), as well as the current shortage of animal protein sources, public perceptions may have evolved compared to a few years ago.

Refinement

Refinement has a profoundly positive impact on animal welfare, thereby promoting animal comfort and enhancing the quality of research outcomes. It implies that all compatible methods are suitable for the intended purpose of the procedure, while minimizing and eliminating any discomfort to animals. Ac-

complishing this approach involves measures such as breeding animals, enriching housing systems and facilities, and employing proper animal care methods. In this context, the highest level of refinement achieved correlates with the highest scientific quality. Directive 2010/63/EU strongly emphasizes the need for environmental enrichment, encompassing proper housing conditions, area, facilities, and equipment. This favorably enriches the lives of research animals, broadens their range of activities, and enhances their coping processes. According to *Van der Meulen-Frank* et al. (2017), environmental enrichment contributes more to refinement than to reduction. The implementation of refinement, along with transparency, improves the public perception of animal use in scientific experimentation and industry.

The directive also establishes minimum standards for individuals involved in experiments, especially those responsible for procedure design, method execution, animal care, and euthanasia. Member governments are further obliged to implement training programs for research team members. The establishment of an online educational system in the EU, featuring lectures and webinars, offers an effective means of promoting awareness and understanding of the 3Rs, particularly refinement. Courses and training significantly enhance the expertise of research staff (*Abelson* et al., 2023; *McCormick-Ell & Connell*, 2019). However, as found by *Franco & Olsson* (2013), participation in these courses may not alter participants' perceptions of the current and future requirements for animal use in experiments. This discrepancy in outcomes is largely attributed to improper planning and implementation. Therefore, effective courses or trainings must be constructed to fulfill defined goals and consider the attendee's knowledge level. This approach has the merit of not limiting the utilization of human resources through refinement, enabling young researchers (who may not yet be considered experts) to contribute novel ideas to scientific projects without causing unnecessary discomfort or pain to animals. Accordingly, the directive permits the involvement of young researchers under specific criteria and qualifications, a necessary step toward fulfilling the requirements of refinement. This process plays a crucial role in optimizing the conduction and consistency of scientific investigations (*Kirby*, 2004) and minimizing unjustified discomfort or pain.

Reproducibility issues in experimentation can undermine the credibility and legitimacy of research in some disciplines (*Macleod & Mohan*, 2019; *Johnson*, 2013; *Richter* et al., 2009). Implementing refinement measures can remarkably enhance experimental accuracy and reproducibility. Directive 2010/63/EU established guiding principles to bolster the credibility and progress of experimentation, including considerations like animal homogeneity,

environmental enrichment (facilities consideration), randomization in experimental design, appropriate statistical methods, qualifications of individuals handling animals, and the potential for animal reuse or rehoming. Notably, these principles are undergoing assessments and recommendations. In a recent assessment, *Ecuer* et al. (2023) suggested a comprehensive framework for the rehoming of laboratory animals, which includes aspects of socialization. They also offered actionable guidance on the necessary procedures in a rehoming program. These guiding principles, referred to as the "hidden Rs" (*Louhimies*, 2012), strongly contribute to high reproducibility. Improving the consistency of animal coherence in scientific experimentations is crucial for achieving precise reproducibility. These hidden Rs not only enhance animal welfare and experimental reproducibility but also reduce the ecological footprint. The directive positively contributes to the advancement and sustainability of a wide range of scientific disciplines, extending beyond animal protection, with climate change being one of the most prominent fields in social media.

The animal endpoint serves as an early indicator (biomarkers) or a potential source of pain and/or distress in an animal experiment. This discomfort can often be minimized or avoided by applying a framework of ethical justification and scientific endpoints. One measure for mitigating pain and discomfort involves the humane euthanasia of the animal. According to *Van der Meulen-Frank* et al. (2017), the endpoint is deliberated as a refinement option with the primary goal of maintaining a balance to align with ethical justifications. Various approaches, such as biomarkers, temperature measurements, biochemical assessment, and clinical indicators, are commonly used to determine endpoints (*Kendall* et al., 2019). In smaller-scale experimental designs, a pilot study is an effective tool for achieving refinement and reduction goals. Its importance is particularly pronounced in certain fields, such as nuclear medicine studies involving PET, SPECT, CT, and MRI scans (*Van der Meulen-Frank* et al., 2017). Moreover, it aids in the development of experimental procedures and the refinement of the number of animals involved, as multiple data points are collected from individual animals (*Hassan* et al., 2006; *Hudson*, 2006). The true value of a pilot study lies in its ability to identify shortcomings in the proposed experimental design or protocol. This knowledge enables researchers to make necessary improvements to environmental conditions and methodologies, thus preventing the unnecessary use of animals in experiments lacking reliable or validated approaches (*Van der Meulen-Frank* et al., 2017). However, during the planning phase, researchers and animal welfare bodies must critically assess whether the small-scale design of the pilot study is suitable for the

large/full-scale study. An additional tool that markedly benefits environmental enrichment is home cage monitoring for mice (*Giles* et al., 2018), which allows for improved assessment of pain, animal welfare, and their impact on experimental outcomes.

OTHER RELEVANT "R" PRINCIPLES

Directive 2010/63/EU has solely adopted the 3Rs of Russell and Burch. This decision has sparked controversial discussions and led to the establishment of other principles, referred to as relevant Rs. It is essential to differentiate between the hidden Rs of refinement and other relevant Rs. While the 3Rs are the core of the directive and the primary focus of experiments, other relevant Rs are additional principles that complement and enhance the credibility of experimentations. The concept of implementing other relevant Rs was observed even before the formulation of Directive 2010/63/EU, with advocates like *Dolan* (1999) proposing their inclusion. However, the 3Rs remain the essential foundation of the directive (mandatory to implement), and strengthening experiments in line with these principles is the current ultimate goal. Expanding the directive by incorporating other relevant Rs can markedly promote a reduction in animal use. Hence, other relevant Rs do not replace the 3Rs stated in the directive; rather, they are considered as aspects that have been identified to be associated with the enhancement of animal experimentation. In contrast, there are initial concerns that the implementation of other relevant Rs might make animal experimentation more complex due to their challenging integration and the need for additional legislative considerations. The adoption of relevant Rs heavily depends on the constraints within various research disciplines, which affect their acceptability levels. Therefore, any future adoption of additional Rs within the European Union should proceed with caution, considering the specific field of experimentation and the diverse national legislations across member nations.

Ongoing investigations on other relevant Rs and hidden Rs have been conducted, with notable efforts to adopt other relevant Rs over the last decade. For instance, in 2013, Mandal and Parija advocated for a 4th R known as "rehabilitation," focusing on the post-experiment rehabilitation of animals (*Mandal & Parija*, 2013). *Curzer* et al. (2013) also urged for a 4th R called "removal" or "refusal," which involves rejecting experimental protocols that could cause unjustified harm. There are ongoing efforts to expand the 3Rs into 6Rs, which include "responsibility" (referring to the management tasks within the experiment), "respect" (designing and performing experiments with the utmost re-

spect for animals), and "reproducibility" (ensuring that test outcomes are reproducible) as complementary Rs for fostering a culture of care (*6Roundtable*, 2022; *Akkermans* et al., 2020). Though the concept of a culture of care has been gaining global attention, the term is most commonly recorded in the EU guidelines and published workshops (*Hawkins & Bertelsen*, 2019). Other common relevant Rs, such as "reason" (justifications for conducting animal experimentation), "recognition" or "relevance" (considering the possible implementation of appropriate alternative approaches), "reflection" (sincerely searching for alternative approaches that align with 3Rs), "reconsideration" (evaluating the feasibility of applicable alternative approaches), and "relief" (minimizing animal harm as much as possible) are also being advocated. Intriguingly, recent conferences and workshops have not merely focused on Rs but have also considered elements that influence the quality of research. For instance, in a recent conference, the so-called "mental health and work wellbeing" were proposed for consideration (*Ferrara* et al., 2022), as a strong connection has been identified between a culture of caring in animal experimentation and individual performance.

CALL FOR GLOBAL 3RS IMPLEMENTATION

Scientific, legal, political, economic, and cultural barriers all contribute to the implementation challenges of the 3Rs. Most of these obstacles have been addressed in the aforementioned sections and can be overcome by advocating for the benefits derived from the implementation of the 3Rs. The advocacy for global adoption of the 3Rs is not a recent development; it predates the establishment of Directive 2010/63/EU. Nevertheless, it is essential to emphasize this historical perspective to expedite the worldwide implementation and harmonization of the 3Rs. One of the primary challenges in achieving global 3Rs implementation is the divergence in guiding provisions across nations, which is primarily founded on national-level social, cultural, and ethical grounds. *Grimm* et al. (2023) identified four major challenges related to these premises, including moral foundations point of view, highlighting practical recommendations, establishment of national legislation, and actual implementation. Hence, some opinions assert that 3Rs directive decrees may not be suitable or feasible in certain countries where cultures rely more on guidance within a limited legal framework established by lawmakers (*Ormandy & Schuppli*, 2014). However, this notion is not entirely accurate since the 3Rs can be readily adopted across continents. For instance, Directive 2010/63/EU allows for modifications to national and secondary legislation (which leads to minimal disparities in laws among member states). Hence, it respects stringent

measures and cultural values when there's no viable alternative and no undue harm incurred. It is noteworthy that legislation comparable to the Directive can also be observed in non-member states, such as Switzerland (*Hehemann*, 2019). This similarity is evident both in terms of major provisions and specific details.

Though recent organized initiatives strongly promote the adoption of relevant Rs, the primary emphasis should be on achieving global 3Rs harmonization. A fundamental understanding of the 3Rs, as defined by *Bayne* et al. (2013), makes their incorporation feasible in nations lacking a well-developed directive. The global adoption of the 3Rs provides a legal framework that nurtures scientific advancements and ethical evolution, considering cultural diversity (*Louhimies*, 2012). Furthermore, the global adoption of the 3Rs can lead to harmonization, minimizing errors (reducing discrepancies and eliminating duplication), and thereby improving reliability and reproducibility.

Despite the absence of complete global 3Rs harmonization, several initiatives aimed at international harmonization have emerged. Across Europe, the European Partnership for Alternative Approaches to Animal Testing plays a substantial role in fostering international collaborations. Additional joint efforts involving universities and small or medium-sized enterprises are also contributing to the global development, validation, dissemination, and implementation of the 3Rs. However, further endeavors, including international meetings and discussions, collaborations, and sponsorships, are imperative to achieving global 3Rs adoption. These efforts should be established within a defined framework with adaptable specifics, adhering to a structured timeline.

CONCLUSION

To date, the EU's legislative framework has mandated the incorporation of the 3Rs within its member states. This mandate has remarkably enhanced the conditions and protection of animals involved in scientific investigations. Directive 2010/63/EU has, therefore, elevated the credibility and quality of experimentation through the promotion of best practices, streamlined workflows, data dissemination, public health safeguarding, and the development of alternative approaches. The directive's inherent flexibility safeguards our ethical justifications at present; however, it is critical to evaluate the extent to which the 3Rs principle fulfils its designated ethical objective. This is extreme necessary for altering the viewpoints on societal norms and research methodologies, which can enhance the comprehensiveness of policies that regulate animal research.

By embracing the 3Rs, Directive 2010/63/EU aspires to promote the value and quality of scientific experiments, which are the cornerstones of the project evaluation process. Although the 3Rs have made immense contributions to the scientific community and the public at large, the expansion of these principles to include other relevant Rs can potentially enhance animal protection practices. However, it is crucial to acknowledge that such an expansion might also impede experimental progress, especially from a quantitative perspective. Therefore, while the current provisions of Directive 2010/63/EU based on the 3Rs should be rigorously upheld, any future adjustments should be made without undermining the directive's foundational principles. Nevertheless, such adjustments should only occur following a comprehensive assessment of the EU directive's efficacy and the attainment of its objectives, which depends on future reports.

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