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Animal experimentation: Implementation and application of the 3Rs.

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Summary

Despite the development of powerful molecular biological techniques and technologies, studies involving research animals remain a key component of discovery biology, and in the discovery and development of new medicines. In 1959, The Principles of Humane Experimental Technique, the 3Rs (**R**eplacement, **R**eduction and **R**efinement) were developed to provide a framework to ensure animal research was undertaken as humanely as possible. Sixty years since their inception, the extent to which the 3Rs have been adopted and implemented by the global scientific and medical research communities has unfortunately been slow and patchy. However, this situation is changing rapidly as awareness increases, not only of the 3Rs themselves, but of the impact of animal welfare on the reproducibility, reliability and translatability of data from animal studies.

The earliest records of the use of animals for scientific and medical research are from the 4th and 3rd centuries BC. Since then, the number of animals used for research worldwide has progressively increased, particularly in the 18th and 19th centuries, a consequence of the dramatic increase in scientific endeavours during this period. Despite the development of powerful molecular biological techniques and technologies, rightly or wrongly, studies involving research animals, undertaken in parallel with complimentary, non-animal experimental approaches, remain a key component of discovery biology, and in the discovery and development of new medicines.

Animal experiments, by their very nature and purpose, will cause pain, suffering and distress, and in many cases, the death, of the research animal. Morally and ethically, it is critical to minimise these harms, to undertake these studies as humanely as possible, in short, to apply The Principles of Humane Experimental Technique, the 3Rs, first articulated by Russell and Birch in 1959 [1]. Sixty years on, this article does not seek to argue for or against the use of animals for research. Instead, to reflect on the extent to which these Principles have been adopted, applied and developed by the global research community.

What are the Principles of Humane Experimental Technique, the 3Rs? [2]

- **Replacement:** Methods which replace the use of animals in research. These may be absolute i.e. no use of animals or animal tissues at all (e.g. studies involving human participants, computer simulations), or partial, the use of tissues or cells from humanely killed animals. In some jurisdictions, for example the European Union,, replacement could include replacement of protected species with non-protected species (e.g. immature vertebrates or invertebrates).

- **Refinement:** Methods which minimise the pain, suffering or distress of the research animal (e.g. administration of analgesics and anaesthetics, housing animals in social groups with appropriate enrichment of their environments to allow them to express natural behaviours, monitoring of welfare before, during and after experiments).
- **Reduction:** minimising the number of animals required per study (e.g. making multiple measurements, and at multiple time points, from the same animal), whilst maintaining robust experimental design to enable appropriate statistical analysis of the data. Alternatively, power calculations to calculate the optimal number of animals required, although this does require pilot data or access to data from previous studies.

Since their introduction, additional Rs have emerged globally. The "Reuse" of individual animals in further studies where these studies cause no additional suffering for the animal, the "Rehabilitation" or aftercare of the research animal, a legal requirement in India, and in South Africa, the requirement to demonstrate "Respect" for the animal. There is also Relevance, the need to use a specific species if, for example, you wish to explore that particular species' physiology, behaviour or neurobiology, or for the purpose of conservation biology, where replacement with less sentient species, cells or computer modelling approaches would not allow you to address these scientific questions [3].

Which R should take precedence?

Without question, the Replacement of animals with non-animal alternatives has to be the ultimate goal of all those involved in animal experimentation. Any other position is morally indefensible. Whilst considerable resources are being devoted to the development and validation of non-animal alternatives, unfortunately there are still many scientific questions that can only be answered using research animals. If there is no alternative to animal use, opinions on whether Refinement or Reduction takes precedence vary. In some countries, particularly in the Emerging World, Reduction comes before Refinement, a consequence of limited resources and pressure from ill-advised ethical review bodies [4,5], personal communications]. The result: studies that are underpowered, with insufficient numbers of animals used to enable appropriate statistical analysis of the data obtained to be undertaken. The data obtained is meaningless and the animals have suffered and/or died needlessly. We are the voice of the research animal; we should consider it from the perspective of an individual animal, and their individual, specific suffering. For me there is no debate, Refinement has to take precedence over Reduction. If suffering is unavoidable, it is preferable to cause limited suffering to a number of animals rather than a large amount of suffering to an individual animal. Further, multiple small refinements, for example how animals are housed, handled, the presence of stimulatory environments/objects in their enclosure, when provided together, can have a considerable, cumulative impact in reducing animal suffering and distress.

Adoption of the 3Rs globally

Sixty years on since they were first articulated, the extent to which the 3Rs have been adopted and implemented by the global scientific and medical research communities has unfortunately been slow and, at best, patchy or varied, both across continents and between research areas.

Globally, until the publication of the book "Alternatives to Animal Experiments" in 1978 [6], there was very limited awareness and therefore adoption of these Principles. With significant investment in the

development and validation of alternatives, particularly by global Pharma, the creation of national 3Rs Centres, and legislative changes, the situation is changing, particularly in Europe. Elsewhere in the World, progress is less forthcoming. There is still a considerable amount of work to be undertaken, both in raising awareness in those countries where knowledge of the 3Rs is currently limited, and in promoting their adoption and application by the entire global research community.

Critical to this is the enshrinement within robust ethical review processes and in law, and the establishment of national 3Rs initiatives and centres. Many countries do not have research animal welfare legislation, animal ethics committees (Institutional Animal Care and Use Committees) or indeed a legal requirement for the ethical review of animal studies [7]. Research, including animal research, is a global endeavour. There needs to be greater harmonisation, globally, of research animal welfare legislation and ethical review processes similar to that that exists for human clinical trials. There also needs to be a mandatory, legally enforced focus on the application of the 3Rs for all animal studies.

Adoption and application of each R

Replacement: There has been some notable recent successes in the development of non-animal alternatives, for example the development of "Organ on a Chip" technologies and the widespread use of databases for the toxicological evaluation of potential new medicines. Indeed, the UK Governments strategy is to become the world leader in the development of alternatives over the next 25 years [8]. However, despite the considerable effort and resource being devoted globally to the development of alternatives, there is considerable reluctance amongst the research community to adopt them, the so called "3Rs valley of Death"- development but limited uptake or use. The reasons for this lack of uptake could be many: a lack of awareness amongst the research community; an unwillingness to let go of, or acknowledge, the limitations of animals preparations/techniques on which they have published extensively; limited published historical or background data from these alternatives; minimal external pressure to change. Driving forward replacement is going to take a concerted and more forceful effort from all involved; Ethical review bodies, funders, regulatory authorities, journals, raising awareness but also refusing to fund, approve or publish studies involving animals unless the researcher fully justifies why animals have to be used.

Our goal is to replace animals but with what? Replacement of vertebrates with non-animal alternatives, for example, computer simulations and ethically sourced human cell cultures, does not raise additional ethical issues, but what about with animals of lower sentience, those thought to be less able to perceive pain e.g. flies, insects, worms? Most jurisdictions that place restrictions on species require the use of the species of lowest sentience provided it is capable of addressing the scientific question under investigation [7]. However, it is becoming increasing apparent that invertebrates, particularly insects, exhibit aversive responses that do not meet our current "criteria" for a response to painful stimuli [9,10]. Is it morally justifiable to provide protection for one species but not another? Should we treat all living creatures equally? In India, animal welfare legislation provides protection for all living beings, including insects, except man.

Refinement: There is an increasing realisation that animal welfare, the minimising of pain suffering and distress, can have a substantial impact on the reproducibility and reliability of the data obtained [11]. The data from the majority of pre-clinical animal studies is not reproducible in humans [12]. Morally, should we continue with such studies if the data obtained cannot be trusted?

Until recently, it was believed that standardisation of everything was the answer. However, it is becoming increasing apparent that this is not the case; housing, environment, handling, diet, gut microbiome and many other factors can have a substantial impact on the data obtained, and its reproducibility [13,14]. Animals, like humans, are a heterogeneous population, living beings rather than research tools; we should view them as such if we want to increase the validity of animal studies. We also need to adopt many of the practices involved in clinical research: randomisation of studies, double blinding, multicentre studies, minimisation of bias and robust experimental design [15]. Without these interventions, data is only reproducible in the laboratory in which it was gathered at that particular moment in time.

There is a need to raise awareness in the research community of all the known factors that could influence the reproducibility and reliability of their data, for research into the impact of each intervention on experimental outcomes, and greater communication and collaboration between researchers and animal welfare experts, and the sharing and promotion of each other's 3Rs resources. Increased use of PREPARE guidelines [16] in designing studies, and a requirement to fully detail, as per the ARRIVE [17] or similar guidelines, all welfare interventions in publications. Researchers should treat with caution and question the validity of any data where the 3Rs have not been applied or fully described.

Current initiatives, led by animal welfare organisations, to abolish studies that cause severe suffering to animals [18] or the use of procedures, such as the forced swim test [19,20], which have questionable validity or translatability to human disorders should be encouraged and implemented. Similarly, there should be more non-regulatory expert working groups working collaboratively to develop recommendations to refine existing animal models or procedures in specific areas of research, for example animal models of sepsis and septic shock [21], in order to reduce animal use and suffering,

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Reduction: The number of animals used for scientific and medical research worldwide is unknown; most countries do not keep records. Animal activist organisations estimate that over 100 million animals may be used worldwide every year. Because of the lack of records, nobody knows whether this figure is increasing or decreasing, whether researchers are implementing principle of Reduction.

In the UK, one of the few countries to collate and publish the numbers of research animals used annually within its jurisdiction, from the late 1930s to the present day, the numbers have cycled between 1 million and 5.5 million [22]. Whilst the number of wild-type or non-genetically altered animals is falling, which is to be encouraged, the total number of procedures on animals in the UK has increased over the last 15 years, plateauing at the current level of approximately 4 million per year [22]. This increase in numbers is due to the creation, breeding but not necessarily use of genetically altered animals or animals with a harmful spontaneous genetic mutation, the number of wild-type animals used is falling [22]. Genetically altered animals, which are bred but not used, are included as procedures in both UK and EU statistical reports. In addition to running counter to the principal of reduction, this increased creation, breeding and use of genetically altered animals raises other significant ethical issues - the excessive wastage of animals, the increased likelihood of them experiencing suffering including severe suffering, whether they are accurate models of human disease to name but a few.

The way forward

Whilst the 3Rs are increasingly being implemented by the global research community, for the sake of the research animals and the validity of the ensuing data, there needs to be far greater momentum and impetus.

There has to be far greater raising of awareness, globally, of the 3Rs and their impact, mandatory education, training and continuing professional development, and increased collaboration between all involved- students, early career and established researchers, vets, animal caretakers and technologists, animal welfare experts and animal ethics committee members. There needs to be more funding for 3Rs interventions, greater sharing and promotion of 3Rs resources including those developed by other agencies or bodies, the global harmonisation of animal welfare legislation and the strengthening of ethical review processes. Collectively, we should all be striving to go beyond what is legally required, developing and fostering a "Culture of Care" [23,24], a commitment to improving animal welfare, scientific quality, care of staff and transparency for stakeholders, within our organisations and practices when animals have to be used.

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