The Three Rs

MARK J. PRESCOTT
National Centre for the Replacement, Refinement and Reduction of Animals in Research (NC3Rs), United Kingdom

Definitions, History, and Examples of the Three Rs

The term "the three Rs" in science refers to replacement, reduction, and refinement, a set of guiding principles for more humane use of animals in research and testing: replacement of animals with nonsentient alternatives; reduction of the number of animals used to the minimum consistent with achieving the scientific objectives; and refinement of scientific procedures and husbandry to minimize pain and distress and improve animal welfare.

The three Rs were conceived in the 1950s by polymath William Russell and microbiologist Rex Burch and elaborated in the book *The Principles of Humane Experimental Technique*, which was sponsored and published by the Universities Federation for Animal Welfare (UFAW) (Russell and Burch 1959). The concepts that underpin the three Rs, though, can be traced back to medical publications in the 1800s. Despite the elegance of the three Rs concept as described by Russell and Burch, the original book had little immediate impact on scientific practice. Gradually over the intervening years, however, the three Rs principles have become accepted as the fundamental ethical framework within which animal research should be conducted. They receive widespread support because they directly address the serious moral dilemma of causing animals harm for scientific purposes, but without impeding scientific or medical progress. Accordingly, the three Rs are now incorporated into national and international legislation and guidelines on the protection of animals used in research and testing. There is broad public support for the three Rs too, with opinion polls consistently showing that acceptance of animal research is conditional on their implementation.

Russell and Bruch recognized that humane research usually results in better science: "the humanest possible treatment of experimental animals, far from being an obstacle, is actually a prerequisite for successful animal experiments." Whilst some procedures will inevitably result in harm to animals, this should be minimized because poor welfare is at odds with good science; it alters behavioral and physiological parameters, introducing unwanted variation into experimental outcomes and potentially confounding the ability to detect changes in the biological parameters under investigation. Experiments using animals whose welfare is optimized, on the other hand, are likely to be more valid, sensitive, and reliable, and often require fewer animals.

The scientific and business benefits of the three Rs are becoming more widely recognized and driving changes in practice outside of the demands of the regulatory framework. There is growing emphasis in the academic and industrial sectors on developing alternative approaches which avoid the use of animals. There are a number of reasons for this, including the need for better research models and tools that more closely reflect human biology and predict the efficacy and safety of new medicines. Some human cell-based approaches enable more precise, detailed, and direct insight into the mechanisms underlying human disease; generate data more quickly; and are cheaper than animal studies when the costs of housing and care and other related factors are taken into account.

In their book, Russell and Burch used the term "*replacement technique*" for any scientific method using nonsentient material to replace methods that use conscious, living vertebrates. They made a distinction between absolute replacements, which do not involve use of animals at any point, and relative replacements, which may involve the use of animals in experiments where they are exposed, probably or certainly, to no distress at all. Examples of absolute (or complete) replacement methods include mathematical and computer (*in silico*) models, which utilize existing biological

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data and information in "virtual" models to predict effects in humans and other animals, use of primitive organisms such as bacteria and amoebae, and *in vitro* models using human cells and tissues. *In vitro* models are rapidly becoming more sophisticated, combining different cells types in complex, three-dimensional (3D) environments (tissue engineering). Some utilize technologies such as microfluidics to innervate the tissue and produce dynamic organ-on-a-chip systems which simulate human organs in miniature. Stem cell technologies (especially induced pluripotent stem cells) also offer huge potential for replacement of animals. A further type of absolute replacement, albeit with a sentient alternative, is the use of human volunteers; for example, for brain imaging studies.

Examples of relative (or incomplete) replacement methods include *in vitro* and *ex vivo* approaches using cells and tissues obtained from vertebrate animals killed by a recognized humane method (sometimes for another research project). Some cell lines can be "immortalized" so that they can grow indefinitely, and hence are considered by some to be an absolute replacement (provided there is no use of animal-derived products such as fetal calf serum to maintain the cultures). Relative replacement also encompasses use of animals not protected under legislation because, based on the available neurophysiological and behavioral evidence, they are considered not to be sentient; this includes use of immature forms of vertebrates and most invertebrates (e.g., *Drosophila* and nematode worms, used extensively in areas such as genetic studies and research on the nervous system). In Europe, under Directive 2010/63/EU (European Union 2010), immature forms are mammal, bird, and reptile embryos up to the last third of their gestation or incubation period, larval forms of amphibians and fish until they can feed independently, and cephalopods (e.g., octopus, squid, cuttlefish) until the point at which they hatch.

If it is not possible to use nonsentient alternatives to animals, the next priority is reduction. Reduction refers to methods that minimize the number of animals used per experiment, test, compound, and so on where animal use is necessary. It should not be confused with national targets to drive down the number of animals used in scientific procedures. Reduction can be achieved by obtaining either comparable levels of information (of a given amount and precision) from fewer animals, or greater levels of information from the same number of animals, thereby avoiding the need for further animal experimentation in the future.

Examples of reduction strategies include improved experimental design and statistical analysis (e.g., use of factorial and other efficient designs, sample size calculations), careful selection of animal subjects, and sharing of data and resources (e.g., animals, tissues, equipment) between researchers and institutions. Modern imaging technologies, such as magnetic resonance imaging (MRI) and positron emission tomography (PET), play a major role in reduction. These enable longitudinal studies to be performed in live animals, avoiding the need to sacrifice cohorts of animals at sequential time points to monitor infection or disease progression post-mortem, and allowing individual animals to act as their own controls, thereby reducing variability and the number of animals needed to achieve statistical significance. The ability to study several biomarkers at the same time may also reduce the number of animals or experiments needed. Similarly, use of telemetry devices (implanted or jacketed/external systems) to record physiological measures (e.g., body temperature, heart rate) can lead to reduction through increased data yield per animal and/or experiment. Reduction can also be achieved by careful planning of research strategies and scheduling of animal breeding and experiments. Pilot studies can be useful in the planning stage, for instance by identifying the need to redirect or terminate unprofitable avenues of research. Comprehensive and transparent reporting of animal-based studies in the scientific literature (e.g., following the ARRIVE Guidelines developed by the National Centre for the Replacement, Refinement and Reduction of Animals in Research (NC3Rs)) (Kilkenny et al. 2010) also contributes to reduction by improving reproducibility of experiments and minimizing unnecessary studies.

Re-use of animals in subsequent experiments can decrease the number of animals used overall and may be driven by practical and economic considerations. However, it usually leads to greater harm to the animals that are re-used; hence there are ethical considerations against as well as in favor of re-use. This illustrates that there is interplay between the three Rs and conflicts can arise between reduction and refinement. (Another example would be the serial study of animals using imaging methods that require the animals to undergo repeated anesthetic episodes.) In such cases, the potential harms to the animals concerned (e.g., the effects of continued use in scientific procedures and of long-term housing in the laboratory) must be weighed against the welfare cost of obtaining and housing (and in some cases surgically preparing) naıve animals. Within Europe there are restrictions on the circumstances in which animals can be re-used, in order to limit the harm caused to individual animals (European Union 2010).

The object of refinement to Russell and Burch was to decrease the severity of inhumane procedures applied to those animals that still have to be used. However, refinement encompasses not only the direct harms associated with animal experimentation, such as the scientific procedures (e.g., dosing, sampling, restraint, surgery, anesthesia) and the handling methods applied during the experiment, but also the indirect or contingent harms arising from, for example, breeding, importation and transport, housing, and husbandry. A more contemporary definition of refinement which makes clear the breadth of issues that need to be considered would be "any approach which avoids or minimizes the actual or potential pain, distress and other adverse effects experienced at any time during the life of the animals involved, and which enhances their wellbeing" (Buchanan-Smith et al. 2005). Researchers sometimes misunderstand refinement, thinking it is about improving the quality of a scientific technique or experimental design, or reducing animal use, rather than relating to the animals' experience of the research and minimizing harm/optimizing welfare.

Examples of refinement techniques include use of appropriate anesthetics and analgesics for effective pain relief, comprehensive veterinary care, and accommodation and environmental enrichment which meet the animals' physical and behavioral needs. Welfare assessment scoring systems and implementation of humane endpoints are important concepts in refinement or experiments. A humane endpoint refers to the point at which action is taken (e.g., terminating a procedure, giving treatment, or humanely killing the animal) in order to limit suffering whilst still allowing the experimental objectives to be met.

The Three Rs Applied to Research Using Nonhuman Primates

Because of their close relatedness to humans, nonhuman primates are used in biomedical research to study aspects of human biology and disease, and for the development and safety assessment of new pharmaceuticals and medical devices. Large numbers are also used in cognitive and behavioral research. Opportunities exist to apply the three Rs in all of these research areas, as well as to field studies with natural populations (especially those involving trapping, handling, collaring, and collection of biological samples). This is a legal requirement in most countries for research involving procedures with the potential to cause harm; as is prior evaluation of whether each project is justified by weighing the likely harms to the animals against the potential scientific and/or societal benefits. In addition, some jurisdictions place restrictions on nonhuman primate use (e.g., the European Union has an effective ban on the use of great apes, save exceptional circumstances), and many contain special provisions aimed at improving the health and psychological well-being of these complex animals when housed in the laboratory. This reflects the high level of concern among the general public about the use of nonhuman primates in invasive research.

In silico methods, *in vitro* methods, and human volunteers are used to avoid or replace the use of live nonhuman primates. Nonetheless, finding suitable replacement options for nonhuman primates is difficult in some areas. For example, macaques are the gold standard model in behavioral neuroscience research because their brains are anatomically and functionally akin to those of humans, many of their visual and motor abilities are on a par with humans, and they can be trained to perform a variety of psychological tests similar to those used with people. Many neuroscience experiments aim to explain the performance of a well-defined behavioral task in terms of the activity of the brain's neurons and systems. Typically, such experiments combine behavioral measurements of sensory, cognitive, motor, or other psychological functions with some method of monitoring or manipulating the activity of individual neurons, circuits, or systems to establish a causal relationship. Since many of these procedures used to monitor and manipulate brain elements are invasive, only rarely can they be ethically carried out in humans. Non-invasive imaging technologies that can be used in humans currently lack the spatial resolution to investigate activity at the neuronal level.

In some cases, it is possible to substitute use of nonhuman primates with other vertebrate species, such as mice or rats. Whilst not replacement as defined by Russell and Burch, this approach may be ethically desirable if an assessment of the available evidence suggests that, on balance, use of the non-primate species would result in less harm overall. A successful example of this approach is the use of transgenic mice to replace Old World monkeys for neurovirulence safety testing or live oral polio vaccine (there is now also an *in vitro* assay).

All of the reduction strategies outlined above have been applied to research with nonhuman primates. Sharing of study designs, data, and experience, particularly in industry, has led to significant reduction. For example, a data-sharing collaboration between the NC3Rs and pharmaceutical and biotechnology companies worldwide has identified opportunities to reduce by up to 64 percent the number of macaques used per monoclonal antibody in development as therapy for human disease (Chapman et al. 2015). The reduction is achieved by decreasing the number of dose groups, recovery animals, and chronic studies performed, without affecting the study outcome. This work illustrates the importance of adopting a flexible, case-by-case approach to study design, based on strong scientific rationales.

Many opportunities exist to refine the use and care of nonhuman primates and much guidance is available in the scientific literature (Jennings and Prescott 2009). In addition to the above, key considerations for laboratory-based nonhuman primate research include use of purpose-bred animals, housing in compatible social groups, spacious and complex environments enabling performance of a wide behavioral repertoire, careful study design, and use of non-invasive techniques (e.g., saliva for measures of HPA (hypothalamic–pituitary–adrenocortical) axis function, hair follicles for genotyping).

The high intelligence of nonhuman primates permits behavioral management techniques to be used to elicit their voluntary cooperation with capture, husbandry, and scientific procedures, giving them greater control over their situation and reducing stress (Prescott and Buchanan-Smith 2003). Establishing positive relationships with nonhuman primates is important for animal welfare generally, and is of special relevance to many types of research where the researchers depend on the cooperation of the animal to perform behavioral and cognitive tasks. Guidance on recognizing, interpreting, and responding appropriately to communication signals exists online; see the NC3Rs-funded Macaque Website (www.nc3rs.org.uk/macaques) and Common Marmoset Care (www.marmosetcare.com).

To fully apply the three Rs during the design, conduct, analysis, support, oversight, and review of nonhuman primate research studies, and to maximize the benefits, all staff involved need to receive appropriate training, continuing professional development, and institutional support. As new knowledge and technologies that can advance the three Rs emerge, there should be timely review and evolution of scientific and animal management practices, study designs, and research strategies in order to meet best practice.

SEE ALSO: Animal Welfare Act; Behavioral and Cognitive Research; Captive Care Legislation and Regulations; Captive Management; Environmental Enrichment; Non-Invasive Techniques: Vocalizations; Psychological Well-Being; Telemetry; Veterinary Care

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