THREE RS OF HUMANE ANIMAL EXPERIMENTATION

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Objectives

The objectives of this module are:

- to discuss the Three Rs as they were defined by Russell and Burch in 1959;
- to introduce the concept of alternatives in research, teaching, and testing;
- to discuss the potential and limitations of alternatives; and
- to consider examples of alternatives and how they may be used.

Contents

Introduction

The question of pain and distress in animals used for research, teaching, and testing has concerned both scientists and the public for a long time. It was this concern, together with the increasing use of animals in fundamental and applied research, that motivated the Universities Federation of Animal Welfare to hire W.M.S. Russell and R.L. Burch to examine how research could be carried out more humanely.

The Three Rs stand for reduction, replacement, and refinement. In their book *The Principles of Humane Experimental Technique*, published in 1959, authors Russell and Burch proposed that all research using animals should be evaluated to see whether the Three Rs could be applied. They recognized that while the replacement of animals as research subjects was a desirable goal, considerable gains could be made in humane science through reducing the numbers of animals used and refining the techniques that were applied to animals. Over the past 40 years, the Three Rs have become widely accepted ethical principles and are now embedded in the conduct of animal-based science in Canada and in many countries worldwide.

Many international agencies responsible for setting standards for the care and use of experimental animals, including the Canadian Council on Animal Care (CCAC), require investigators to consider the implementation of the Three Rs during the design of experiments that will use animals. The principal investigator must consider whether or not animals are needed. If an animal must be used, the investigator is required to consider the Three Rs in detail. The protocol submitted to the animal care committee should outline the rationale for using animals and list the databases that were searched to confirm that there are no alternatives to animals. The protocol should also give details of experimental design to ensure that the correct number of animals will be used; note any potential refinements, both in the manner that the animals will be housed and cared for, as well as to the experimental procedures themselves, to minimize adverse impacts on the animals.

The term "alternatives" came into use after 1978, following the publication of the book *Alternatives to Animal Experiments* by David Smyth, a physiologist and President of the UK Research Defence Society. Smyth defined alternatives in terms of the Three Rs: "All procedures which can completely replace the need for animal experiments, reduce the numbers of animals required, or diminish the amount of pain or distress suffered by animals in meeting the essential needs of man and other animals." Although there have been repeated attempts to limit the term "alternatives" to replacement, it is in the broader, original context that alternatives will be discussed in this module.

What is Meant by the Terms Replacement, Reduction, and Refinement?

As previously noted, "alternatives" is used to describe any change from present procedures that will result in the replacement of animals, a reduction in the number of animals used, or refinement of husbandry or techniques that may minimize their pain, stress, or distress.

Replacement refers to methods which avoid or replace the use of animals in an area where they would otherwise have been used. This includes both absolute replacements (e.g., replacing animals with inanimate systems, such as computer programs) and relative replacements (e.g., replacing animals with greater cognitive awareness, such as vertebrates, with animals that current expert peer advice and interpretation of scientific evidence indicate have a significantly lower potential for pain perception, such as some invertebrates).

Reduction refers to any strategy that will result in fewer animals being used to obtain sufficient data to answer the research question, or in maximizing the information obtained per animal, thus potentially limiting or avoiding the subsequent use of additional animals, without compromising animal welfare.

Refinement refers to the modification of husbandry or experimental procedures to minimize the pain and distress experienced by an animal, and to enhance the welfare of an animal used in science from the time of birth until their death.

Satisfying the Replacement Principle

This section describes replacement as it pertains to research, testing, and teaching (training and education).

General Principles Concerning Replacement in Research, Testing, and Teaching

In studies where specific processes, either cellular or molecular, are to be observed or used in isolation, replacement alternatives such as cell, tissue, organ, or bacterial cultures can be excellent options. Some of the variability factors that complicate intact animal research are reduced when cell cultures, bacteria, etc., are used. These include factors such as light, sound, or latent infections. Of course, if inanimate alternatives are used, variability of this type should not be a factor at all.

Biological systems are known for their complexity and their ability to behave in an unexpected manner with the production of artifacts. A much simpler system such as a cell line is not so likely to produce artifacts, as long as the cells are maintained in the appropriate environment, are correctly defined, and are not contaminated with mycoplasm. A corollary to the artifact problem is the simplicity with which the environment of the cells may be altered and in a manner that could not be repeated in the intact animal. It is easy, for example, to alter the pH, ion content, or oxygen level, etc. of the growth medium to study the effect of these changes. The repeatability of the studies

should be much greater when there is good control of all the potential variables. Where fresh cell lines are required, it should be possible to get many more cultures, and therefore experiments, from each animal than if the whole animal was used for a study.

If the alternative is a video or a computer program, there may still be the need to use a small number of animals to produce the video or develop the program.

Cell cultures, bacteria, and inanimate models cannot be used to study processes exactly as they would occur within the context of a whole, live organism, but recent technological developments have made it possible to generate in vitro organoid models which are being used to study a range of in vivo biological processes including tissue renewal, stem cell or niche functions, and tissue responses to drugs, mutations, or damage. Behavioural responses are more difficult to study in human organoids. The behaviour of simple organisms (e.g., bacteria, nematodes) may provide clues, but it is very difficult to extrapolate the relevance to more complex organisms. Along the same line, it would be impossible to study species-specific and sex-specific phenomena using simple organisms or inanimate models.

Replacement in Research

Basic Research

Animals have been used extensively to study fundamental principles in biology. Often investigators have used species where there was a similarity between the animal's physiology and biochemistry and that of humans. Many fundamental processes are common to a wide range of organisms, including invertebrates.

The Alternatives

The use of animals with lower cognitive awareness, particularly invertebrates, is considered to be an acceptable replacement of higher animals as research subjects, where appropriate to the study. For example, the nematode, *Caenorhabditis elegans*, is widely used to study basic neuronal function as this organism has 302 neurons in its nervous system and the function of each neuron can be studied, as well as its interaction with other neurons. As another example, fruit flies, *Drosophila melanogaster*, which have been used for over a century to study genetics, are also being used to study a wide spectrum of biological processes, including embryonic development, learning, behaviour, and aging. Another important replacement alternative in research is the replacement of rodent-based methods by in vitro methods for monoclonal antibody production.

Replacement in Safety and Efficacy Testing

Regulatory agencies require safety and efficacy testing for medical and veterinary treatments, as well as for common products such as household cleaners and pesticides. Public concern regarding

the use of animals in these tests, as well as concern about the relevance of the animal data, have been driving the need to seek alternatives.

One of the major challenges for proponents of alternative methods for testing new compounds has been to prove that they are as effective as the animal-based tests they are intended to replace. Two organizations created to ensure sound scientific validation and subsequent acceptance by regulatory agencies of proposed alternatives to animals in testing are the European Centre for the Validation of Alternative Methods (ECVAM) in Italy, and the Interagency Coordinating Committee for the Validation of Alternative Methods (ICCVAM) in the United States. Canada is now participating in the validation of alternative methods through the newly formed Canadian Centre for the Validation of Alternative Methods.

Although regulatory agencies throughout the world have been cautious about accepting alternatives, progress continues to be made. The US-based National Toxicology Program Interagency Centre for the Evaluation of Alternative Methods lists alternative methods acceptable to US and other international regulatory agencies.

Replacement in Training and Education

Practical Skills Training

Learning skills, from simple techniques such as blood sampling to complicated surgical procedures such as laparoscopic surgery, is an important part of the training of medical and veterinary personnel. Animals continue to be used for some of this training; however, some skills, such as suturing techniques, may be developed without using animals.

The Alternatives

There are now inanimate models that can be used to practice procedures. The Koken rat, for example, will allow a student to practice tail vein injections many times before it is attempted on a live animal. Mannequins and computer-based technologies are available to allow surgeons to practice laparoscopic surgery. The acceptance of these inanimate objects for training comes when the touch and feel of the training is similar to that experienced when using a living animal.

Education

In recent years, there has been a significant reduction in the numbers of animals used for teaching and demonstrating biological principles, due to the adoption of alternatives.

The Alternatives

A wide range of materials may be substituted for animals in teaching. Audiovisual aids and computer-based programs allow the student to see the effects of manipulating various organ

systems. Many of the computer programs are interactive, allowing the students to participate in the experiments. For example, an interactive program on anesthesia allows the student to assess the depth of anesthesia, to calculate the dose and route of different anesthetic agents, etc.

Satisfying the Reduction Principle

Literature searches are vital in preventing unnecessary duplication of experiments. Some duplication of studies is required to ensure that the results from one study are reproducible by other investigators in different laboratories. However, it is not necessary to repeat studies over and over again.

There are several ways in which an investigator may be able to reduce the number of animals required in a study: ensuring good experimental design, ensuring personnel are appropriately trained, and taking measures to control variability.

It is important to ensure that appropriate numbers of animals (both experimental animals and the controls) are used. This means that the statistical design of the study should be carefully evaluated before the study starts, possibly through consultation with a statistician. Good experimental design with proper data collection and analysis will minimize the number of animals required. The refinement of statistical analytical techniques has allowed investigators to use fewer animals without losing significant information.

A well-trained research team, extending from the principal investigator to the animal care technicians, will ensure that all procedures related to and peripheral to the study will be standardized. It is important that the team members are trained in their specialty and additional expertise brought on as needed. For example, if the project requires a particular surgical procedure for which no one has been trained, an experienced surgeon should assist. Training in all procedures applied to the animals should be done before the project starts.

For teaching laboratories using animals, the value of the laboratory session is greatly increased if trained instructors, rather than untrained students, set up the animal preparations.

One reason that large group sizes are needed for animal-based studies is to address the variability that can occur when the conditions of the experiments are poorly controlled. Group size may be reduced if, for example, a genetically homogeneous population of animals is used, the animals are protected from diseases, and the husbandry conditions are consistent. This is further explained in the section of the NC3Rs website on experimental design.

Targeted Animal Models

In the past, it was difficult to find animal models that accurately mimicked human conditions, such as many cancers. There were animal models of breast cancer but the cause and the biological

behaviour of the cancer differed from that in humans. Thus, treatments for the animal model were not necessarily applicable to humans.

The Alternatives

The identification of immune-compromised animals meant that cells of human origin could be grown in animals without the need for immune suppression of the host. Now, the behaviour and treatment of the tumour in the animal model could reflect the situation in the human. Such precisely targeted animal models will result in an overall reduction in animal use through a reduction in the variability of the model and the increased usefulness of the results.

Genetically modified animals (transgenic, knockout, and mutant) represent alternatives that promise to provide more relevant results for understanding human disease. Initially, there may be little reduction or replacement because the production of foundation stocks of genetically modified animals requires large numbers of animals. The refinement of methods for the creation of genetically modified animals should lead to a more rapid advance in the understanding and treatment of human diseases with the use of smaller numbers of animals.

Satisfying the Refinement Principle

Refinement may be the most subtle of the Three Rs because it produces the least obvious changes in animal use in terms of numbers. Refinement has its greatest impact in the reduction of pain and distress in animals. Appropriate use of anesthetics, analgesics, and other therapeutic measures are very important refinement measures in invasive studies. The refinement of husbandry, particularly by increasing the complexity of social and physical environments, has improved the well-being of research animals. The establishment of scientific and appropriate endpoints for many studies (e.g., vaccine testing) has meant that animals have had to suffer less without affecting confidence in the results.

The refinement of techniques also plays a significant role in both the reduction and replacement of animals in science, as refined techniques can result in less variability and improve experimental outcomes. For example, the introduction of new and safer anesthetic agents, together with better training of investigators in their use, has reduced the number of anesthetic deaths.

There are many examples of refinements that have made a difference both to the animals (in terms of minimizing pain and distress) and to the results of scientific investigations.

Husbandry

In the past, research animals were often singly housed in cages or pens that provided very little substrate or space for normal behavioural activities. Most research animals are social and isolation is stressful for them.

The Alternatives

Most animals may be kept in social groups in complex environments that allow them to behave in a normal manner. There are many reports documenting the beneficial effects of this type of housing. For example, rats living in a socially and physically complex environment develop a thicker cerebral cortex with more dendritic connections compared to rats that are kept in isolation. Young rabbits that were kept in small cages developed skeletal abnormalities because they were unable to hop and run during the time when their muscles and bones were maturing.

Alternatives to Previously Used Blood Sampling Techniques

The retro-orbital sinus of some small species (particularly rodents) was a convenient site from which to collect fairly large samples of blood. The procedure had risks (e.g., the eye could be damaged, especially if samples were taken repeatedly) and was painful. Several alternatives have been developed, including blood sampling from the tail vein, the saphenous vein, and the jugular vein. Although some skill is required to perform these efficiently, the risk of causing severe damage to the animal is greatly reduced.

Experiments That Cause Severe Suffering or Death

For studies involving vaccine testing, infectious diseases, tumours, organ rejection, etc., the endpoint for the animal may in the past have been death from the disease. As an animal approaches death, it stops eating and drinking, rapidly becomes dehydrated, and, except in a small number of instances, death can be predicted to occur within a short period of time from the point at which the animal stops eating and drinking.

The Alternatives

When an experiment is expected to cause severe suffering or the death of an animal, endpoints should be established to limit the extent of the suffering and to anticipate death. If possible, pilot studies should be used to demonstrate the earliest point at which the scientific goals are reached, and pain and distress are minimized. At a minimum, the pilot studies should be used to determine which clinical signs are most appropriate to indicate that the endpoint has been reached or when the death of the animal becomes inevitable.

Toxicity Testing

The LD_{50} test was required by regulatory agencies as an assessment of toxicity of new compounds. The LD_{50} is the dose that will kill 50% of the animals. Many animals were used to accurately find this dose, although its relevance to human toxicity has not been established.

The Alternatives

A number of refinements to toxicity testing have been developed and have become accepted as Organisation for Economic Co-operation and Development (OECD) guidelines. For acute toxicity testing, the fixed dose procedure (TG 420), the acute toxic class method (TG 423), and the up-and-down procedure (TG 425) have now been accepted by OECD-member countries. Fewer animals and earlier endpoints are part of the refinements. The LD_{50} test (TG 401) has now been withdrawn and regulatory agencies from the OECD-member countries are required to accept data generated using one of the three alternative guidelines. In addition, recommendations from the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) have been published, describing how in vitro data may be used to select the starting dose for the test, further limiting the numbers of animals needed and increasing the predictivity of the data.

Summary

The Three Rs tenet guides scientists on the ethical use of animals in science. The authors of the book *Principles of Humane Experimental Technique*, Russell and Burch, intended that each of the principles should be applied to any program of work, in the order: replacement, reduction, and refinement. Like other national organizations responsible for the care and ethics of animal-based science, the CCAC requires adherence to the Three Rs, as stated in the *CCAC policy statement on: ethics of animal investigation*: "Animals should be used only if the researcher's best efforts to find an alternative have failed. A continuing sharing of knowledge, review of the literature, and adherence to the Russell-Burch) '3R' tenet of 'Replacement, Reduction and Refinement' are also requisites. Those using animals should employ the most humane methods on the smallest number of appropriate animals required to obtain valid information".

Implementation of the Three Rs has been seen as occurring through the use of "alternative methods." Considerable progress has been made in the development and validation of non-animal methods, in the implementation of good experimental design techniques and statistical analysis, and in improving housing conditions and procedures in animal-based studies. Adherence to the principles is now considered to be sound science, reaping benefits for human, animal, and environmental health, as well as improving the quality of life for the animals used in the studies.

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