Module 03 - The Three Rs of Humane Animal Experimentation

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 potentials and limitations of alternatives
- To consider examples of alternatives and how they may be used

Introduction

The question of pain and distress in animals used for research, teaching and testing has concerned the general public and thoughtful researchers for a long time. It was this concern, together with increasing use of animals in fundamental and applied research, that motivated W.M.S. Russell and R.L. Burch to examine how decisions should be made about such use of animals. The Three Rs stand for **R**eduction, **R**eplacement and **R**efinement. In the book *The Principles of Humane Experimental Technique*, published in 1959, the authors Russell and Burch proposed that all research using animals should be evaluated to see if 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 by refining the techniques that were applied to animals. Over the past 40 years the Three Rs have become widely accepted ethical principles to be embedded in the conduct of animal based science. Many agencies responsible for setting standards for the care and use of experimental animals, including the Canadian Council on Animal Care, require investigators to consider the implementation of the Three Rs during the design of experiments that will use animals. The principal investigator must consider the question of whether animals are needed or not and if an animal must be used, then 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 word "alternatives" came into use after 1978 following the publication by David Smyth, a physiologist and President of the UK Research Defence Society,

of *Alternatives to Animal Experiments*. In this book, Smyth provided a Three Rs definition of alternatives: " 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 context that alternatives will be discussed in this module, as originally intended.

What is meant by the Terms "Replacement" "Reduction" and "Refinement"?

As has been noted, the word alternatives is used to describe any change from present procedures that will result in the replacement of animals, a reduction in the numbers used or a refinement of techniques that may reduce or replace animals or reduce the pain, stress or distress of the animals. ***Replacement*** often means the use of an inanimate system as an alternative (e.g., a computer model or program, a mannequin). It can also mean the replacement of sentient animals (usually vertebrates) with less sentient animals (usually invertebrates such as worms, bacteria, etc). It also includes the use of cell and tissue cultures. The cells must come from somewhere and often this means animals. ***Reduction*** means a decrease in the number of animals used previously with no loss of useful information. This may be achieved by reducing the number of variables through good experimental design, by using genetically homogeneous animals or by ensuring that the conditions of the experiment are rigorously controlled. ***Refinement*** means a change in some aspect of the experiment that results in a reduction or replacement of animals or in a reduction of any pain, stress or distress that animals may experience. The establishment of early endpoints for intervention in a study that has the potential to cause pain or distress is an example of refinement. Few of the alternatives completely fulfill the definition of the R category in which they are placed. For example, although the use of tissue cultures will replace many animals, some will be required as a source of cells. In the following sections on Replacement, Reduction and Refinement, examples of each will be given.

Satisfying the Replacement Principle

In this section, we will consider replacement as it pertains to three different areas of research, teaching and testing.

General Principles Concerning Replacement in Research and Testing

Cell cultures, bacteria and inanimate models cannot be used to study processes as they would occur within the context of a whole, live organism. Thus a culture of heart cells is not comparable to heart cells *in situ*, as it cannot reveal the interactions between all the various heart cells as they are normally situated within a whole heart, nor those with the nervous, endocrinologic and immune systems that normally affect them, nor the effects of blood flow and pressure and of the many other factors and signals that exist in a live, whole organism. Behavioural responses cannot be studied in simple cultures of cells. The behaviour of simple organisms (e.g., bacteria, nematodes) could be studied; however it would be 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. In cases where specific processes, either cellular or molecular, need to be looked at or used in isolation, replacement alternatives such as cell/tissue/organ cultures or bacterial cultures become excellent tools. 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, latent infections, etc. Of course, if totally inanimate alternatives are used, variability of this type should not be a factor at all. 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 the study. If the alternative is inanimate (e.g., a computer) there may still be a need to use a small number of animals to get data to feed into the computer. The quality of that data needs to be excellent or it becomes a case of garbage in and garbage out. Biological systems are known for their complexity and their ability to behave in an unexpected manner with the production of artefacts. A much simpler system such as a cell line is not so likely to produce artefacts, as long as the cells are maintained in the appropriate milieu. A corollary to the artefact 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, the ion content, the 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.

The cost of using alternatives is likely to be less than the cost of using intact animals although this may not be inevitable. The costs of computers, software, cell/tissue/organ culture equipment, etc., may exceed the costs of animals.

Replacement in Research

Basic research. Animals have been used extensively to study fundamental principles in biology. Usually investigators tried to use animals where there was a similarity between the animals physiology and biochemistry and the human's. It is recognized that many of the more fundamental processes are common to a wide range of organisms including invertebrates.

The alternatives. The use of lower, less sentient animals, particularly invertebrates is considered to be an acceptable means of replacing higher animals as research subjects. The nematode, *Caenorhabditis elegans*, is widely used to study basic neuronal function. This organism has 302 neurons in its nervous system and so it is reasonable to study the function of each neuron and its interaction with other neurons. In a similar vein, geneticists have used fruit flies for many years. There are other important replacement alternatives in research: one of the most common and useful ones is the replacement of rodent-based methods by *in vitro* methods for monoclonal antibody production.

Replacement in Safety and Efficacy Testing

The use of animals for safety and efficacy testing new products has increased greatly over the past forty years or so. Companies producing the products, regulatory agencies and consumers want to be sure that the products are safe to use. While medical treatments make up the greatest bulk of these products, just about anything we use must be proven to be safe, for example, the cars we drive and the products we use, including household cleaners, pesticides, cosmetic products, etc. Once upon a time baboons were used in crash tests. The alternatives, instrumented mannequins (crash test dummies), provide much more precise information than did the animal model. Public concerns for safety of products drove the need for increased testing, and public concerns about how animals are used in safety testing are now driving the need to seek alternatives. One of the major challenges for the proponents of alternative methodologies 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 USA. Although regulatory agencies throughout the world have been cautious about accepting these alternatives, progress continues to be made. As of 2002, there are three in vitro tests accepted by the European regulatory agencies and three by the USA regulatory agencies and there are several more being evaluated.

Replacement in Education and Training

Practical Skills Training

Learning skills, from simple techniques such as blood sampling to complicated surgical procedures such as laparoscopic surgery, are an important part of the training of medical and veterinary personnel. Animals continue to be used in this training. However, some skills such as suturing techniques may be developed without using animals. Discarded placentas may be used to practice microsurgery techniques.

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. 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 anaesthesia allows the student to assess the depth of anaesthesia, to calculate the dose and route of different anaesthetic agents, etc. Mannequins and computer-based technologies are available to allow surgeons to practice laparoscopic surgeries. 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 organism.

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

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 attempt to reduce the number of animals required in a study. It is important to ensure that appropriate numbers of animals are used, both the experimental animals and the controls. This means that the statistical design of the study should be carefully evaluated before the study starts. Perhaps a statistician should be consulted. Good experimental design with proper data collection and analysis will minimize the number of animals required. 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 success of the laboratory session is greatly increased if trained instructors rather than untrained students set up the animal preparations. One cause of large group sizes comes from the variability that can occur when the conditions of the experiments are poorly controlled. Large group sizes may be reduced if, for example, a genetically homogeneous population of animals is used, or the animals are not subject to intercurrent diseases, or the husbandry conditions are stable. The issue of variability is considered in more detail in Module 04. Control animals may represent up to 50% of the animals in a study. The investigator should try to minimize the number of control animals. Using one control group with several test groups rather than one control group for each test group may do this. If a particular procedure is used repeatedly in a laboratory, there will be a historical record of controls for that procedure. For a study using the procedure, it may be possible to use a

very small number of controls and show that they fall within the historical limits of the controls, rather than use a full complement of controls.

Targeted animal models. In the past, it was difficult to find animal models that accurately mimicked human conditions like many cancers. There were animal models of breast cancer but the cause and the biological behaviour of the cancer differed from that in the human. Thus treatments for the animal model were not necessarily applicable to humans.

The alternative. The development 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 (GM) animals (transgenic, knockout and mutant) represent alternatives that promise to provide more relevant results for human disease understanding. Initially there may be little reduction or replacement because the production of foundation stocks of GM animals still requires large numbers for breeding. The refinement of results from the GM 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 has been the least glamorous of the Three Rs because it produces the least obvious changes in animal use if numbers are the most important statistic. The refinement of techniques has a significant role to play in both the reduction and replacement of animals in research, teaching and testing. Refined techniques will result in less variability and improve the outcome in terms of results obtained. For example, the introduction of new and safer anaesthetic agents together with better training of investigators in their use has reduced the number of anaesthetic deaths. Refinement has its greatest impact in the reduction of pain and distress in animals. Appropriate use of anaesthetics, 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. There are many examples of refinements that have made a difference both to the animals (in terms of minimizing pain and distress), and 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 in behaviour 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 husbandry. For example, rats living in a socially and physically complex environment develop a thicker cerebral cortex, with more dendritic connections compared with those 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 and 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 so that the experiment can be terminated before the animals suffer. 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 LD50 test was required by regulatory agencies as an assessment of toxicity of new compounds. The LD50 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 OECD guidelines. For acute toxicity testing the fixed dose procedure (Tg420); the acute toxic class method (Tg423) and the up and down procedure (Tg425) have now been accepted by OECD member countries. Fewer animals and earlier endpoints are part of the refinements. The LD50 test (Tg401) 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 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 use of animals in research, teaching and testing is not a right but a privilege. It is incumbent upon every researcher to ensure that privilege is not abused. Even though animals are, in most cases, bred for research, that does not mean that we may use as many as we like in whatever way we like. Each animal is an individual and should be treated as such. We must be careful that they are not subjected to needless pain or suffering. Excessive numbers should not be used just because they are there. They should not be used at all if an equally suitable model

system could be used to obtain the same results. Every possible step must be taken to reduce or prevent pain and suffering.