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# Have the 3Rs and alternatives been effectively explored?

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【**Abstract**】 *Although the concept of the 3Rs was developed in the 1950s, it took many years before it became a central theme when planning and conducting experiments which might involve animals. There are still many ways in which protocols can be improved to increase both animal welfare and the reliability of the scientific data obtained from the studies.*
*This paper gives some practical advice on how the 3Rs can be implemented more thoroughly in animal research.*
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## Introduction

The idea of alternatives to animal experiments is not new. Already in the 19th century, the English physiologist Marshall Hall (1790 – 1857), working at a time when methods of pain relief and anaesthesia were in their infancy, proposed five principles for refining animal research:

1. Experiments should never be performed if the necessary information could be obtained by observation
2. No experiment should be performed without a clearly defined and obtainable objective
3. Unwarranted repetition of experiments should be avoided
4. Any justifiable experiment should be carried out with the least possible infliction of suffering
5. To avoid needless repetition, every experiment should be witnessed.

The first systematic exploration of the possibility of using alternatives to animal experiments is probably the work of William Russell and Rex Burch. Commissioned by the Universities Federation of Animal Welfare (UFAW), they began by interviewing scientists to collect information which might lead to improvements in research. Their work culminated in the publication of a book, *The Principles of Humane Experimental Technique* in 1959, which summarised their findings in the concept of The Three Rs:

1. Replacement
2. Reduction
3. Refinement

Initially, relatively little attention was paid to this concept. However, by the late 1980s, technological developments and increased focus on the ethics of animal experimentation, both within the scientific community and from outside, brought the 3Rs into the limelight again. The first of a series of World Congresses on Animal Use and Alternatives in the Life Sciences was arranged in Baltimore in 1993. These congresses continue to this day: the 9th congress was held in Prague in 2014 and the next one will be in Seattle in 2017. They now attract around 1,000 participants and ensure that 3R-alternatives are discussed regularly at an international level. The burden is therefore firmly placed on each research institution to ensure that this concept is adequately explored locally, when experi-

ments which might involve animals are planned. It is now normal for legislation on animal experimentation worldwide to incorporate the 3Rs as a requirement.

## What is an alternative?

An 'alternative' to an animal experiment is by definition a method without the use of animals which gives the same answers as the animal would. This means that many "alternatives" are not really true replacements: they do not use animals but they do not give the amount of information which an animal model would do. For example, a cell culture will not reveal how a compound would react if it was given to an intact organism which consists not only of cells but of a large number of organs and systems which may react with the compound. Also, many cell cultures are grown from cells harvested from animals, so they are not actually 100% replacements of animal use, since some animals have been killed to provide the cells.

Furthermore, there are two important facts that have to be remembered when discussing alternatives:

1. Animal experiments are usually needed to develop and validate alternative methods. This is one reason why it can take time to introduce an alternative.

2. It is impossible to discuss whether or not there are alternatives to an animal experiment until the objectives of the experiment have been clarified. This applies to all use of animals, whether it be in research, testing, education or training.

The importance of defining the objectives of an experiment is well illustrated by the debate concerning the use of live animals in education and training. Here is a list of possible objectives for the use of animals in this area:

- To teaching and practise:
- laboratory skills
- general animal handling skills
- preparation-specific animal skills
- To impart good ethical thinking
- To teach new knowledge and reinforce existing knowledge
- To teach data handling skills
- To teach experimental design
- To teach communication skills (oral or written)
- To encourage groupwork
- To improve staff-student interactions

Clearly, very few of these areas actually require the use of animals or animal material. Indeed, the inappropriate use of animals in a class exercise where 'imparting good ethical thinking' is a major element, may well result in a strong negative reaction from the students, who may conclude that the animal use was unnecessary and therefore highly unethical.

## What types of alternative are available?

All those planning experiments where animals may have to be used, should be aware of the large range of alternatives which are available today. These may roughly be grouped as follows:

- Audiovisual aids (e.g. slides, video films)
- 3D models, mannequins and simulators
- Computer simulations of experiments, including virtual reality
- Construction of new chemical compounds on a computer: QSAR (Quantitative Analysis of Structure/Activity Relationships)

- Cell and tissue cultures, perfused organs
- Biochemical & immunological methods (e.g. radioimmunoassays, ELISA)
- Hybrid DNA technique and genetically modified microorganisms
- Trials on "lower" organisms not covered by animal welfare legislation (e.g. fruit flies, nematode worms and plants)
- Acute experiments (studies performed on animals under terminal anaesthesia)
- Trials on animals needing veterinary treatment
- Experiments on material from ethically sourced cadavers
- Observational studies on animals
- Studies on humans (e.g. microdosing and medical imaging)
- Systematic reviews of the published literature producing new conclusions from animal experiments which have already been performed ("Synthesis of Evidence")

The potential for using alternatives will now be discussed within three major areas of animal use:

1. Education and training
2. Science
3. Breeding of laboratory animals

### *1. Alternatives in Education and Training*

Advances in material technology and multimedia systems have led to the production of a large range of commercial alternatives or supplements to animal use in education and training. Information on these products has been collected in several databases, of which the largest are NORINA and that produced by InterNICHE. Some products can also be loaned, for those who wish to try them before purchase.

In addition, many institutions replace some animal use with simple but effective homemade alternatives, for example to give students and researchers elementary training in handling and injection techniques. There are also films and slide series on the internet showing basic handling, injection and blood sampling techniques for the common laboratory animal species. Some examples of products which are in use in veterinary training can be seen in Figure 1.

A common objection heard about alternatives, particularly models and simulators, is that they are not realistic enough. Dissimilarity is not, however, necessarily a disadvantage. It is important to differentiate between fidelity and discrimination. Fidelity is a description of the overall proportionate difference between the model and the original. A "high-fidelity" music system reproduces the entire range of musical tones equally well. A model showing high discrimination, on the other hand, contains parts that have been deliberately accentuated (and other parts toned down) for educational purposes, so that the student can focus on the parts of the model which the training session uses. This can be an advantage when learning a new technique. Two training models illustrating this difference are shown in Figure 2.

Many studies have been performed to evaluate the use of alternatives, particularly in undergraduate training. Guidance on the use of animals and alternatives in education and training, which summarises the results of these studies, is available.

### *2. Alternatives in Science*

a) Replacement alternatives:

Advances in culturing techniques have led to many scientists no longer beginning with animal models at all, but in-

stead working on cells, tissue or even whole organs *in vitro*. Much of this tissue is human material, avoiding the need for animals altogether.

Among scientists using animal models as well as those opposed to animal research, there is increasing focus on the fact that laboratory animals are not necessarily the best choice for research: the goal is often to learn more about the human body and its reactions to drugs. Animal experiments are therefore at best a step on the path to the ultimate goal, and not always a useful one.

Advances in the combination of cell culture techniques and microchip technology are now making it possible to build functional mini-organs using human tissue. This is particularly relevant for those organs which consist of multiple subunits, such as the lung, liver and kidney. This development, known as organs-on-a-chip, is likely to revolutionise the way in which toxicological studies are performed in the next few years. These artificial organs may well replace mandatory tests on animals, particularly when more organs have been modelled in this way.

The lung-on-a-chip developed at the Wyss Institute at Harvard University, is an example of these developments.

Advances in robotic technology are also benefiting the efforts to replace animal research. Automation can be used to test the effects of large numbers of chemicals on cell cultures, in a fraction of the time it would take to do this manually. High-Throughput Screening using these techniques is becoming an important part of drug discovery and toxicological studies.

Work using robots in other fields can also have interesting side-effects which can benefit animal welfare. Scientists developing new underwater robots for use in marine archeology have in the process of this work studied the way in which fish relate to water currents and perceive pressure changes. This gives valuable data which could be used to predict how fish should be housed in tanks to reduce unnecessary stress and fatigue caused by currents in the tank.

b) Reduction alternatives

Although not a total alternative (since it initially involves the sacrifice of donor animals), it is now possible to grow mini-organs from adult stem cells, opening up the possibility of reducing animal use considerably. For example, functional mini-livers have been produced from adult mouse stem cells, potentially enabling 1000 compounds to be tested on tissue from one mouse.

The greatest single potential for reduction, however, which is applicable to almost all animal studies, is the use of statistical methods to limit animal numbers to those which are absolutely necessary to achieve significant results at a given power level.

To further this aim, it is vital that the laboratory animal facility does everything possible to reduce unwanted variations in factors which can create experimental "noise", since this will otherwise increase the number of animals needed to produce a significant result. Some of the most important sources of experimental noise are:

- Variations in age, sex and weight of the animals
- Stress
- Subclinical disease
- Variations in room temperature
- Differences between animal cages (contents and position in the room)
- Temporal differences between treatments
- Changes in environmental factors
- Exposure of the animals to different researchers
- Exposure of the animals to different technicians (for example at weekends)

There are a number of good guidelines for the design and analysis of animal studies:

- Guidelines for the Design and Statistical Analysis of Experiments Using Laboratory Animals
- NC3Rs Design Assistant

- FRAME Training Schools
- NC3Rs websiteon experimental design

Another potential for reducing animal numbers is to share material with other researchers. The EU Directive 2010/63 (Article 18) requires Member States to facilitate, where appropriate, the establishment of programmes for the sharing of organs and tissues of animals that are killed.

c) Refinement alternatives

Even after application of the principles of Replacement and Reduction, there is an enormous potential to improve the welfare of animals in research using methods of Refinement. These are often paid too little attention and left by researchers to the discretion of the laboratory animal facility. Many of the practical techniques in a study which can be refined (e. g. blood sampling techniques) will, however, have enormous influence on the quality and validity of the scientific results.

For example, if injections are painful or if they consist of excessive amounts of fluid, they will cause stress to the animals which in turn will then respond differently to the treatment. Some techniques, for example intramuscular injection, which are standard practice in humans and larger animals, may be impossible to perform in rodents because of the sheer limitations of size. Likewise, common injection techniques such as intraperitoneal injection can easily be performed differently from time to time, if the operator is not skilled. This can result in a drug being deposited in very different places (e. g. the intestine, the liver or the peritonal cavity) which in turn will markedly affect absorption time and the clinical effect of a drug.

The same applies to methods of identification. The first question should always be whether it is necessary to mark the animal at all, or whether visual clues can be used instead.

If the animal has to be physically marked, much thought should be given to the effect of the identification method. A ring, eartag, flipper band or collar must be of a weight and size which do not affect the animal's normal behaviour. Even apparently harmless details such as the colour of a tag should be considered, in case this triggers attacks (mobbing) from other members of the species.

There are indeed many parameters which can cause so-called contingent suffering, i. e. pain, distress and lasting harm caused not by the experimental procedure itself but by the fact that the animal is being housed outside its preferred environment. Contingent suffering may be generated by factors such as transport, housing, husbandry and the formation of social hierarchies which the animal does not really want to take part in. Whether an animal is housed singly or in groups should also be based upon knowledge of what they prefer to do in the wild. Individual housing of social animals, even though it solves problems of aggression, may be extremely stressful. There is evidence from studies on singly-housed male mice that they can develop symptoms which in humans would be characterised as depression.

There are many other areas with the potential for improved animal welfare. One obvious example is in the design of behavioural experiments, where it is clearly better for the animal to be given a reward for performing as the researcher wants, rather than being given punishment for making a "mistake".

Animals that are free from stress and at harmony with both their environment, fellow animals and human contacts, will automatically deliver research results which are more valid and easier to detect, since the effect of a treatment will be more easily visible on top of a background of normal, non-stressed behaviour. Conversely, it may be difficult or impossible to register any effect of a treatment if the animal is already highly stressed before treatment starts.

Increased awareness of this has led to great interest in the detection of an animal's state of mind using facial expressions. So-called Grimace Scales have now been established for a range of species. These can be used in connection with treatments likely to cause pain, such as ear tattooing in rabbits, to see if, for example, application of a local anaesthetic cream prevents pain during the procedure.

## Guidelines for Replacement, Reduction and Refinement

Many institutions have developed standard operating procedures, especially for more complex techniques. These may be valuable, but in themselves they are no guarantee of good animal welfare. There are, however, now available a large number of guidelines and position statements written by expert groups and published in journals with a system for peer review. A worldwide collection of such guidelines is available.

Experienced research groups should be encouraged to write and publish their own position statements and guidelines if there is little available in the literature. Norecopa has, for example, published position statements on food deprivation, toe-clipping and pain relief.

The introduction of EU Directive 2010/63 has led to the construction of a website with a lot of relevant information and guidelines for the refinement of animal experiments. These include advice and examples of how to assess the severity of an experiment. All the same, such guidance may not always be relevant to all types of animal research. The EU advice is less applicable for experiments being performed on fish, since in those cases other techniques are often used, and research on animals in an aquatic environment poses potential threats to them which are not experienced by terrestrial animals. Norecopa, therefore, commissioned an expert working group which published guidance on the severity classification of procedures involving fish to supplement this.

### *3. Alternatives in the breeding of laboratory animals*

Animal research produces inadvertently a great many animals at breeders that are surplus to requirements, for a variety of reasons which include the wrong gender, age, strain or weight. A study in the Netherlands showed that roughly the same number of animals were killed as surplus as were actually used for research. Strong efforts should be made to reduce this surplus. This can be achieved in part by researchers being less specific, if possible, when they order animals. Again, it is important to rememberthe EU Directive's Article 18: *Member States shall facilitate, where appropriate, the establishment of programmes for the sharing of organs and tissues of animals killed.*

### Incentives to introduce 3R-alternatives

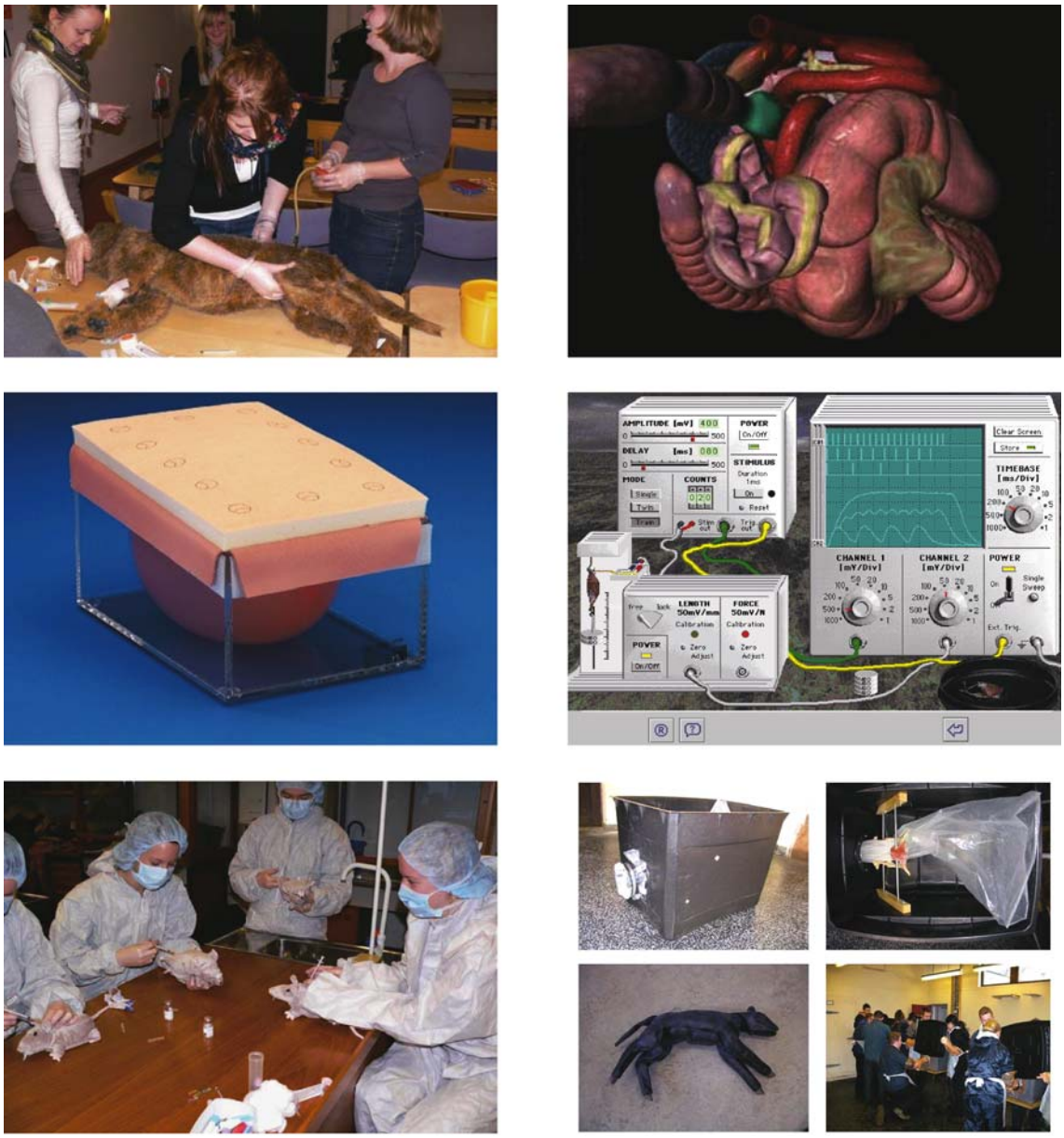
Technicians and researchers who advance the implementation of the 3Rs can be rewarded and further motivated by the award of a 3R Prize. Many organisations, such as Norecopa, now award such prizes annually.

### Conclusions

3R-Alternatives should become a theme for discussion at all stages of a research programme, including the phase before the animals actually arrive at the laboratory. Critical points along this path are, among others:

- Breeding
- Transport
- Acclimation to the new environment
- Procedures, e. g. choice of
  - dose
  - method of administration
  - blood sampling
  - methods of data collection (e. g. measurements of body temperature, heart rate, blood pressure)

- Pilot studies




Note:

a. A mannequin which can be used to train auscultation of heart and lung sounds, blood sampling and fracture management (http://www.rescuecritters.com)

b. A three-dimensional model of the digestive system of the horse (http://www.3dglasshorse.com)

c. Surgery training pad (http://www.limbsandthings.com)

d. A computer simulation of the classic frog nerve-muscle preparation (http://www.virtualphysiology.com)

e. Rats from IKEA which can be used to train basic handling and injection techniques (http://www.ikea.com/cn/en/catalog/products/60169350)

f. A home-made simulator of the birth canal of the ewe, together with an artificial lamb, which can be used to give veterinary students training in obstetrics (http://oslovet.norecopa.no/produkt.aspx? produkt=9266)

**Fig. 1** Examples of products for use in veterinary training.

Research technicians should be involved already at the first planning meeting. They know the potential and limitations of the animal facility better than others, they possess a large range of practical skills and are good at thinking

Note. a. shows a model of a human heart with high fidelity (https://www.wardsci.com/store/catalog/product.jsp? catalog_number=813015).

b. shows a model of a rat designed to teach microsurgery, where the organs to be operated upon are discriminated positively in relation to the rest of the rat (http://www.microdev.nl/index.php/products/md-pvc-rat).

**Fig. 2** Examples of the difference between fidelity and discrimination.

laterally when novel experiments are to be performed, and they have the most contact with the animals. Early involvement will also prevent rumours circulating and will ensure that 3R improvements are implemented as early as possible.

Scientists do not necessarily have sufficient insight into the daily challenges of running an animal facility and the indirect costs of their research. Time must be set aside to inform them of this and to ensure that there are sufficient material and human resources to conduct the study and provide adequate monitoring 7 days a week.

Statisticians should also be involved in the planning process from the earliest stages.

The European Commission has built a comprehensive website with information on the 3Rs and alternatives. The EU Directive's articles 4, 13, 16 and 47 provide valuable guidance on how to implement the 3Rs, which is highly relevant even outside the EU

Finally, *The three S's of* Carol M. Newton (1925 – 2014) provide a very useful supplement to the 3Rs.

- *Good Science*
- *Good Sense*
- *Good Sensibilities*

Where it is not possible to find evidence in the scientific literature when discussing a procedure, it is legitimate to ask whether it makes good sense, and also to apply "critical anthropomorphism": would the procedure be stressful if it was applied to a human being? By doing this, we will indeed follow "Principles of Humane Technique" which was the title of Russell and Burch's book where the 3Rs were first described in detail.

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