Review Article

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Alternatives to Animal Experiments: an Overview

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ABSTRACT:
The use of animals for scientific experimental purposes has contributed a great deal to the advancement of biomedical knowledge and their continued use is essential for the continued understanding and maintaining the scientific advancement in various biomedical spheres. However efforts need to continue to ensure that their use is minimized. In this regard the concept of 3 R's ie Replacement, Reduction & Refinement can help researchers to continue their scientific work while addressing the concerns of the animal rights activists and other agencies involved in regulation of experiments on animals. This article gives an overview of the various alternative approaches that can be employed based on the concept of the 3Rs. The review also touches upon the 4th R- Rehabilitation, the importance of which is now being increasingly recognized. Though the ultimate aim is to completely replace the animals, where that is not possible reduction should be aimed for and refinement techniques should be developed to ensure that the distress to the animals is minimal. In this context the principles of the 3R's is important as today's refinement may be tomorrow's reduction which may ultimately lead to complete replacement of animals in experiments.

Keywords: Animal alternatives, Concept of 3 R's

INTRODUCTION:

The use of animals for experimental purpose has its origin in ancient Greece and its development mirrored the development of medicine [1, 2]. Initial works documented are of Aristotle and Hippocrates who published Historia Animalium and Corpus Hippocraticum respectively which described the structure and function of human body based on the dissection performed on animals (2). Since then, there has been a continuous rise in the use of animals for experimentation with various triggers like discovery of anesthetic agents in 1850's, Koch's postulates, and in 20th century advances in the field of pharmacology, toxicology and immunology which further spurted the growth [2]. Currently around 75-100 million vertebrate animals per year are utilized worldwide for experimentation; while mice and rats make up the vast majority (85-90%) , other animals like rabbits, guinea pig, hamsters, dogs, cats, non-human primates and assorted other animals are also frequently used [3].

The laboratory animals are primarily (around 70%) used for research (drug, cancer), testing various biological agents (vaccines) or for diagnostic and teaching purposes (around 30%) [3,4].

Contribution of animal experiments in research & education:

Animals make good research subjects for many purposes in many aspects of research. First and foremost, they are biologically similar to humans in many ways and are susceptible to many of the same health problems. Hence some species may serve as particularly good models for many aspects of human health and physiology. For instance much of our existing knowledge of immune system has been derived from studies in mice while dogs have contributed to a great extent to our knowledge on functioning of the CVS. Many of the surgical techniques such as coronary bypass, artificial heart valve insertion and pace maker implants were first perfected on dogs before being used in humans. Moreover many of the path breaking discoveries in

medicine ranging from development of vaccines and anti-sera, development of insulin and penicillin, discovery of streptomycin etc were possible due to experiments on animals [5,6].In fact it can be argued that in certain aspects animals make better research subjects than even humans. For example, many species have relatively short life cycles, so they can be studied throughout their entire life span or even across generations. Also, scientists can control certain aspects of an animal's environment like diet, temperature, lighting and so on, more easily than that would be possible with human subjects. The last decade has witnessed the introduction of transgenic animals and their potential advantage in developing many novel animal models of diseases [5]. As is evident from above, It will not be wrong to state that experiments in animals have contributed immensely to the development of medicine and biology and continue to do so.

The concept of alternatives: "the three R's":

The rampant use of animals has been questioned from time to time with Jeremy Bentham, an English Barrister who questioned the lack of moral regard to animals as early as 1780. Since then there has been a growing public sentiment against the in-human use of animals for experimental purposes [7]. In this backdrop the concept of alternatives came into foray initially in a symposium by Charles Hume and William Russell in 1957 and later in a book 'The principles of Humane Experimental Technique' by Russell and Burch in 1959[8].This concept of three Rs (replacement, reduction and refinement) successfully provided an acceptable ethical framework to conduct experimental techniques on animals.

The first R: Replacement

Replacements strategies can be as follows:

- Computer simulations of physiological phenomena and audiovisual materials
- Use of Lower organisms & Embryo stages
- Cell, Tissue and organ culture (In-vitro studies)
- Human and animal cadavers
- Computer simulations: Recent advances in computer technology hold a great potential for replacing the use of animals in research, testing, and education.

Computers in research:

Computer based quantitative structure-activity relationships (QSAR) software for pre-clinical testing allow researchers to develop mathematical models and algorithms that can predict the biological effects of new substances based on their chemical structure. For instance, the protease inhibitors were developed very quickly through the use of powerful computers which analysed the viral enzyme and predicted the kinds of chemicals that would block its action. [9, 10]

Expert computer systems [10,11] like 'DEREK' have been developed for the prediction of toxicological hazard. If a new chemical has a structure similar to a known poison in certain key aspects, then the new substance also may be a poison. Such screening can thus replace animal use. Further, the 'METEOR' project has been set up to develop a knowledge-based expert system for metabolism prediction without the use of animals. Furthermore, scientists are developing computer simulations of cells, tissues, fluids, organs, and organ system which allow replacement of some animals [12].

Computers in teaching:

In the field of teaching, alternatives are being developed and implemented [13, 14,15] because they frequently offer learning advantages, are cheaper than animal methods, and satisfy animal welfare concerns. In India software like Ex-Pharm [16] and X-cology [17] have been developed and are being used in undergraduate teaching. As a student advances, animal use at the postsecondary level becomes increasingly tied to research and skill acquisition

Limitations of use of computers:

Limitations on the utility of computer simulations are due to a lack of knowledge of all the parameters involved in the feedback mechanisms that constitute a living system, which means the information on which the computer must depend is incomplete. Moreover, in most cases, research with animals will still be needed to provide basic data for writing computer software, as well as to prove the validity and reliability of computer alternatives. [11,12]

Use of Lower organisms & Embryo stages:

The uses of lower animals with limited sentience/ are not protected by legislation controlling animal experiments and include invertebrates, plants, fungus, bacteria and viruses [18]. Some of them are useful as pre-screen system, especially for agrochemicals and environmental pollutants, and also for genotoxic, studies and endotoxin detection. Though these organisms can respond to certain noxious stimuli but it can be argued that they do not experience pain or suffering in the same way that animals do, particularly in those cases where there is no brain or neural tissue. This makes them a reasonable replacement of animals in experiments [18,19].

Moreover, a major advantage of these organisms is that they can be cultivated much more easily and quickly than most animal or human cells. Their genetic makeup is simple compared with that of animals and humans and the fact that a great deal is known about it also facilitates their use, particularly in toxicological research [18, 19]. A change in genetic material in these organisms is relatively easy to detect and characterize.

There are a variety of non animal organisms ranging from plants to single-celled organisms to invertebrates to embryos that can replace animals in testing. [Table-1].

Alternative Model	Experimental use
An insect test (African locust, Locusta migratoria migratorioides)	To investigate LD50 African locust is used as a test animal instead of mouse. The locust test has proved to be more sensitive than the mouse test.
Salmonella bacteria & *Drosophilia melanogaster (fruit fly)*	Detects mutagenic/genotoxic activity and the test has been validated and accepted for screening purposes in regulatory toxicology.
LAL test for endotoxins	detects the presence of fever-inducing endotoxins, in products for intravenous use, by the ability of the endotoxins to react with amoebocyte lysate derived from blood samples gathered from the horseshoe crab.
Fertilised chicken eggs (HET-CAM test)	Predicts eye irritancy from the effects of a chemical on the chorioallantoic membrane of the egg.
46-day-old chick embryos	Used for studies for estimating venom lethality . Venom is applied to yolk sac membrane on a filter paper disc and its effects on vascular and cardiac function are easily observed throughout the 6 h experiment.
Frog embryo teratogenesis assay xenopus (FETAX) system	used to assess the teratogenic potential of chemicals. Using a dissecting microscope, one can observe any perturbation in the development of the embryo as it is exposed to known teratogens in a petri dish.
Giant squid axon	Useful for the study of the concept of the ionic nature of the electrical action potential in nerve transmission.
Sea urchin & Hydra	can be used to screen for teratogenicity.can be used for basic reproductive research and is favoured for use in screening for reproductive toxicity, teratogenicity, and mutagenicity.

Table 1: Lower organisms & embryo stages as alternatives to animal experiments [19,20,21]

Advances in genetic engineering are opening up further possibilities to replace the use of higher animals, for example by the addition of human drug-metabolising capacity to bacterial test systems. Genetically engineered nematodes (roundworms) which carry human disease genes may prove to be useful in identifying new drugs [20].

Cell, Tissue and Organ culture (In-vitro studies):

Cells, tissues, and organs can be kept alive outside a living organism and used for testing. This qualifies as a relative replacement [19] as animals are still required as a source for these *in-vitro* systems, however the tests avoid the use of protected animals and also the animal would experience distress for a much shorter time, and perhaps less distress overall, than occurs with whole animal testing because it would be sacrificed before any experimental manipulations were carried out. Some examples of in vitro alternatives are given below: [Table 2]

Alternative Model	Experimental Use
Cultured somatosensory neurons	Are used for testing response to irritants The patch-clamp electrophysiological recording technique is used for cultured adult rabbit trigeminal neurons which respond to known pain-producing irritants (capsaicin and resiniferatoxin).
Cholinergic cell lines	for testing drug efficacy and toxicity in many conditions involving Degeneration and/or malfunction of cholinergic neurons for eg Neuron Disorders, Familial Dysautonomias, Alzheimer's Disease (AD), Tardive Dyskinesia, and Huntington's Chorea
Keratinocytes	Used as model for testing phototoxicity of drugs as Ultraviolet light injury increases phospholipase activity in these cells
Endothelial cells	Used as models for testing anti-coagulants

Table 2: Use of in-vitro models as alternatives to animal experiments [22, 23, 24]

A number of tissue equivalents are also used to replace the use of animals. The models are in widespread use by cosmetic, chemical and pharmaceutical companies worldwide. The old tests called for three animals for each chemical that is evaluated for skin corrosivity and dermal irritation. Since there are more than two thousand chemicals introduced each year, the substitution of animals with these tissue equivalents could save many laboratory animals in a year. Some examples of these alternatives are given as under [Table 3]

Alternative Model	Experimental Use
EpiDerm	consists of normal, human-derived epidermal keratinocytes which have been cultured to form a multilayered, highly differentiated model of the human epidermis
EpiOcular corneal model	consists of normal, human-derived epidermal keratinocytes, which have been cultured to form a stratified, squamous epithelium similar to that found in the cornea.
MelanoDerm	consists of normal, human-derived epidermal keratinocytes and melanocytes which have been cultured to form a multilayered, highly differentiated model of the human epidermis.
EpiAirway	consists of normal, human-derived tracheal/bronchial epithelial cells which have been cultured to form a multilayered, highly differentiated model, which closely resembles the epithelial tissue of the respiratory tract.
Corrositex	Reconstituted skin: This involves the use of a "synthetic skin," called Corrositex, which can be used in place of animals to test chemicals for skin corrosivity--that is, to see whether a substance will corrode or burn the skin

Table 3: Tissue equivalents as alternatives to animal experiments [25, 26, 27, 28]

Use of Human tissues and volunteers:

This is a great alternative and helps to avoid the problem of inter-specific extrapolation from animals to humans and to get more mechanistic information. Some of the examples include use of immortalised human cell lines like keratinocytes for drug studies, and the use of foetal and placental tissues [14,21]. For example, human placentas have proved quite useful in testing the ability of a drug to cross the placenta from mother to foetus [19]. In nutrition studies, and sometimes for dermal toxicology, human volunteers can be utilized.

The second R: "Reduction":

It was initially defined as 'reduction in the numbers of animals used to obtain information of a given amount and precision'. It has been recently modified to define "The use of fewer animals in each experiment without compromising scientific output and the quality of biomedical research and testing and without compromising animal welfare"

Various strategies that can be employed are summarized under the following headings: (29, 30, 31, 32)

- Intra-experimental reduction
- Supra-experimental reduction
- Extra-experimental reduction

Intra-experimental reduction: It is the application of reduction techniques directly at the level of experimentation. It includes improvement in the design of the study and analysis using proper statistical technique.

Statistical analysis:

- Proper training of investigator to apply proper statistical technique to appropriate design (not be underpowered, repeatability)
- Minimizing the number of animals without compromising on the power of the test
- Combining treatment and control groups helps in reducing the number of groups
- Data variability: is the primary reason for increasing the sample size and hence the variability has to be kept minimum which can be done by reducing heterogenecity by the following measures.
  - Using genetically homogenous and pathogen free strains
  - Enrichment of environment which helps to decrease physiological, psychological or behavioural variation due to the stressful conditions they are subjected to in the laboratory.
  - Appropriate standardization of experimental procedure to decrease the sample size
  - Always look into raw data to detect any obvious errors or to exclude the outliers
- Pre-screening of compounds: tested by non-animal methods (in-vitro assays; computerized systems) helps us understand better and focus on intended study and reduce toxicity.

Supra-experimental reduction: it involves various measures taken to change the setting in which experiments take place (i.e. changes at institutional levels)

- Training researchers in experimental design and statistics
- Including a statistician in the institutional ethics committee(IEC)
- Reducing surplus breeding
- Framing policies regarding re-use or recycling of animals.
- Inter-departmental co-operation/ co-ordination & sharing research animals: can be very effective in reducing the use of animals within the institution. For example if one researcher is carrying out a study on one organ (viz liver), other researchers can make use of other organs of the same animal e.g. kidneys, brain tissue etc. Some labs alert all their researchers when any animal is going to be killed to facilitate sharing of various tissues/organs among its investigators.

Extra-experimental reduction: it includes the reduction which can be achieved through more-distantly related developments. Includes improvement at research levels to ensure superior quality, consistent and safe pharmaceuticals, so as to reduce the animal use for safety and efficacy testing e.g.: by following Good manufacturing practices (GMP) during production of various vaccines.

The third R: "Refinement":

It is defined as "Any decrease in incidence and severity of inhumane procedures applied to those animals which still have to be used in experiments". These are alternatives which minimize pain, potential suffering or distress and which enhance animal well being. It can be applied to any stage in the life cycle of a laboratory animal (from its birth to death). For implementing reforms it is very crucial to understand the kinds of sufferings which an animal can encounter as it forms the basis of applying the refinement techniques. It is although a wide area but areas of primary interest are housing, husbandry and care, experimental procedure, pain management and humane endpoints. Various recommended steps to refine animal use in experiments have been recommended [33, 34].

- Animals should be handled only by properly trained staff and adequate training should be compulsory to users before they handle lab animals
- It is the responsibility of ethics committee to look into research protocol and bring in refinement at their level
- Refinement should not be a single event but a continuous process with adequate checks at regular intervals
- Refinement must be considered by editors when any research is being considered for

publications to ensure priority to this important aspect.

- Lack of awareness among researchers is a potential problem which can be solved by including ethical aspects in care and use of laboratory animals in curriculum of undergraduate and postgraduate students
- Finally a refinement database should be established at various levels
- Specific examples: [35,36,37]
- Refining experimental procedures:
  - Use of smallest possible size needles for injection
  - Volume and frequency of dose to be kept minimal
  - Avoid repetition of procedure
  - While performing abdominal surgeries in rats instead of waiting for audible vocalisation by rats, subtle signs like flank twitching can be picked to provide additional pain relief
- Refining endpoints:
- various clinical signs can be picked up to define humane endpoint:
  - Decrease in body temperature
  - Flank twitchings
  - Haematological signs
- If it can be predicted that suffering will be severe from an experiment it is best to sacrifice the animal at the end of experiment (humane endpoint);
- Other possible refining procedures:
  - Use of faecal analysis in place of blood
  - Positive reinforcement for eg. motivating animals to participate rather than by using force
  - Using non-invasive imaging modalities
  - Enriching the environment in which they live
  - Socialization of species whenever possible

The Fourth R: "Rehabilitation"

The concept of the fourth R ie "Rehabilitation" has also been gaining recognition. This is based on the realization that the personnel who perform the experiments on animals have a moral obligation for the animals even after their use (38). Investigators are responsible for the after-care and/or rehabilitation of animals, post-experimentation. The animal shelters / refuges can be established to take care of these animals and in case of dogs/cats; suitable homes can be explored where these animals can spend their post experimentation period [39]. The costs involved in the after-care and/or rehabilitation of animals have to be factored in as part of research costs and should be appropriately increased commensurate with the level of sentience of the animals [39, 40]. In some situations it may be permitted to euthanize the animals when rehabilitation of the experimental animals is not possible for instance; when the animal is paralysed and is not able to perform its natural functions or the animal is left permanently in a state of extreme distress /pain or it is threatening to other animals or human beings.

Conclusion:

The use of animals for scientific experimental purposes has contributed a great deal to the advancement of biomedical knowledge and their continued use is essential for the continued understanding and maintaining the scientific advancement in various biomedical spheres. However efforts need to continue to ensure that their use is minimized. Modern techniques have helped us a great deal in cutting down the extent of their use as for instance now it is possible to use organ slices in basic studies where once the only option was to use the whole animal. Moreover, newer non-invasive techniques make it possible to study blood flow or nerve activity in the living human brain instead of animals.. Though the ultimate aim is to completely replace the animals, where that is not possible reduction should be aimed for and refinement techniques should be developed to ensure that the distress to the animals is minimal. In this context the principles of the 3R's is important as today's refinement may be tomorrow's reduction which may ultimately lead to complete replacement of animals in experiments [41, 42].

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