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# Alternatives to use of animal experiments in teaching and research- A review

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## Abstract

The "Alternatives" or "Substitutes" is defined as anything from absolute to partial replacement of live animals in biomedical research and testing. Russell and Burch have given their definition of "alternatives" as "the three Rs-Replacement, Reduction and Refinement". The 4th R i.e. Responsibility was added in 1995. Major areas of medicine and biology where experimental animals are involved include-Developing new treatments for diseases (like multiple sclerosis, cancers, AIDS, Alzheimer disease etc.), preparations of natural products used in medical research, safety testing of chemicals and drugs, study of genetic disorders (like cystic fibrosis, sickle cell anaemia etc.), development of new diagnostic tests for diseases, laboratory mice and stem cells etc. There are some demerits of animal experimental studies like inhumane treatment of laboratory animals and an animals' response to a drug can be different from that of human beings etc. The common alternatives to experimental animals can be physico-chemical techniques, microbiological systems, tissue/organ culture preparation, computer models, epidemiological surveys and plant analysis (e.g. toxicity assays in plants). Research methods superior to using animals to learn about human disease or predict the safety of new drugs are stem cells, microdosing, DNA chips, microfluidics chips, human tissue, new imaging technologies, and post-marketing drug surveillance and alternative organisms [like lower vertebrates (e.g. Zebra fish), invertebrates (e.g. *Drosophila melanogaster)*]. Animal ethics is an issue as important as the human welfare. More efforts need to be undertaken for effective implementation of 4 Rs during laboratory use of animals. The increase in knowledge, improvement of health care and creation of useful products that is derived from experimentation on animals, carries the cost of pain and distress that the animals experience. There are provisions for ensuring that animal use is performed in a humane manner so as to minimize pain, distress or discomfort. Various alternatives to animal use have been suggested, which need to be implemented in an effective manner. The development in the field of science and technology should be brought to its advantage so that in the coming years novel alternatives can be developed and the issue of 'moral values' in experimentations can be sorted out.

**Key words:** Alternatives, 4 Rs., Laboratory animals, Animal Ethics, DNA chips, microdosing, microfluidics, Alternative organisms**.**

## Definition

"Alternatives" or "Substitutes" is defined as anything from absolute to partial replacement of live animals in biomedical research and testing. The use of animals in biomedical research are adjuncts, aids, shortcuts, or supplements which help an investigator to decide whether an experiment on an animal is likely to produce a useful result. Russell and Burch have given their definition of "alternatives" as "the three Rs-Replacement, Reduction and Refinement". The 4th R was added in 1995.

The Four R's:-

1. **Reduction:** It is implemented by animal sharing, improved statistical design, phylogenetic reduction, and use of better quality animals e.g. animals with implanted catheters and flow probes which are used to study physiological functions in major organ system toxicology (MOST) and telemetry systems.
2. **Refinement:** It is done by decreased invasiveness, improved instrumentation, improved control of pain and improved control of techniques used for animal research.

3. **Replacement:** It is achieved through use of non-animal living systems, use of non-living systems and computer simulation.

4. **Responsibility:** The 4th R implies addition of 'responsibility' **(Substantiate with reference/s)** to the original three R's of Russell and Burch. It has grown into a new era of performance-based outcomes, which reflects integrity, honesty, and scientific correctness in appropriate and reasonable use of laboratory animals. This ensures that animal life is required and necessary for biomedical advancement.

## Experimental animals in biological research

Major areas of medicine and biology where experimental animals are involved include:

i. **Developing new treatments for diseases**:- There are many diseases which are yet to have a proper cure like multiple sclerosis, cancers, AIDS, Alzheimer disease etc. All these need initial input in terms of animal experiments (Substantiate with reference/s).

ii. **Preparations of natural products used in medical research**:- Animals can produce useful substances in their blood or milk, like antibodies and hormones, which are important for diagnostic tests, medical treatments, and basic research (Substantiate with reference/s).

iii. **Safety testing of chemicals and drugs**:- A wide range of chemicals and medicines which are used in day-to-day life, need to be tested in experimental animals for their safe use in humans as well as in animals (Substantiate with reference/s).

iv. **Study of genetic disorders**:- There are many diseases which are inherited fully and partially. Scientists have now made such progress in molecular biology that they can now alter genes and breed strains of mice and other animals for their studies. This may ultimately lead to treatments in genetic disorders like cystic fibrosis, sickle cell anaemia and other diseases (Substantiate with reference/s).

v. **Development of new diagnostic tests for diseases**:- Animal tests have paved the way for many blood tests for the diagnosis of infectious diseases (Substantiate with reference/s).

vi. **Laboratory mice and stem cells**:- Research has been carried out in the creation of mouse embryonic stem cell lines and mice with specific genetic changes. Gene-targeted mice created makes both the mice, and the stem cells used that can act as a model for a similar repository for human embryonic stem cells (Substantiate with reference/s).

## Demerits of animal experimental studies

- Inhumane treatment of laboratory animals.
- The stress meted out to the animals in labs can adversely affect experiments.
- An animals' response to a drug can be different from that of human beings.
- Unpredictable responses of the Guinea pigs are noted to narcotic and anaesthetic agents.
- Rabbits as research animals often encounter problems with anaesthesia.
- Rabbits for antiserum production may show anaphylaxis to foreign antigens.

## Points to be remembered while choosing alternatives

(Substantiate with reference/s)

Certain points that are to be kept in mind while choosing alternatives to experimental animals in biological research are:

- Ideally, the development of replacement alternative methods should be based on a sufficient understanding of the molecular and cellular basis of the phenomenon being measured or studied.
- In many circumstances, an animal test could not be replaced by a single replacement alternative method, the development, evaluation and optimization of stepwise testing strategies and integrated testing schemes should be practiced.
- The acceptance and use of satisfactorily validated replacement alternative methods, and the cessation of use of the equivalent animal procedure, should not be seen as options, but as requirements.

## Common alternatives to experimental animals

(Substantiate with reference/s)

These alternatives can be physico-chemical techniques, microbiological systems, cells/tissue/organ culture preparation, computer models, epidemiological surveys, and plant analysis (e.g. toxicity assays in plants). Research methods superior to using animals to learn about human disease or predict the safety of new drugs are stem cells, microdosing, DNA chips, microfluidics chips, human tissue, new imaging technologies, and post-marketing drug surveillance.

### *(1) Physico-chemical techniques:*

(Substantiate with reference/s)

These help to identify human responses to chemicals and biological substances e.g. Gas chromatography which separates complex substances and solutions into their basic elements which are further identified and measured through the use of mass spectrometry. This is frequently done in vitamin and drug research.

Another example is the use of Chitosan films as a substitute for animal and human epidermal sheets used for *in vitro* permeation of polar and non polar drugs.

Recently, chitosan films have been used as local delivery systems of various plant extracts (*Thymus vulgaris, Matricaria chamomilla, Croton lechleri* and *Calendula officinalis*) to test their antimicrobial activity against the periodontal pathogens, *Porphyromonas gingivalis* and *Aggregatibacter actinomycetemcomitans*, due to their favorable properties such as biocompatibility, biodegradability, and adhesion ability. Together these results indicate that optimized chitosan films have the potential to be developed as a substitute for animal and human cadaver epidermal sheets for preliminary *in vitro* permeation studies.

### (*2) Microbiological systems:*

These are commonly used in toxicology and carcinogenesis (cancer producing) studies (Substantiate with reference/s). These are based on the capability of chemicals to induce mutating changes in a cell's DNA, which is the genetic information center of the cell e.g. Ames Test which can detect 80-90% of all carcinogenic chemicals that have been studied. It is used primarily as a screening system and must be validated with animal studies.

Another example is the use of fungi for studies of the metabolism of drugs. Use of fungi could reduce the over-all need for laboratory animals. It has been seen that selected group of fungi have the ability to metabolize a wide variety of drugs. Most promising is *Cunninghamella elegans*. Variety of drugs including anti-coagulants, diuretics, anticonvulsants, and hemorheologic agents (which make red blood cell membranes more elastic for delivering oxygen to tissues) have been tested using these fungi. This method is being developed by researchers and is not intended entirely to replace the use of mammals such as rats and guinea pigs for the testing of drugs. A recent study has utilized the bacteria *Vibrio vulnificus* to study the modulation of the toxic RtxA1 which induces acute cytotoxicity. This has emerged as a potential for the treatment of infectious diseases. (Substantiate with reference/s)

Ultimate benefit would be the availability of an easily handled, non-mammalian, very predictable system for facilitating the development of drugs. This should result in a reduction in animal demand but will certainly not substitute for doing all animal testing. Researchers have been working with a number of pharmaceutical companies to ascertain the best means of applying the new procedure to drug development. (Substantiate with reference/s)

### *(3) Cells/Tissue/organ culture preparation:*

These tests include experiment with tissues and body fluids of normal animals and humans which can be performed *in vitro* and *in vivo* for measuring absorption, distribution and biotransformation of various drugs e.g. human dopaminergic neurons as substitute for animal models of Parkinson's disease and for transgenic models with modified expression of PARK genes. (Substantiate with reference/s)

Use of *in vitro* cells and tissue cultures which involves growth of cells outside the body in laboratory environment can be an important alternative for animal experiments. The cells and tissues from the liver, kidney, brain, skin etc. are removed from an animal and can be kept outside the body, in suitable growth medium, for few days to several months or even for few years. *In vitro* culture of animal/ human cells includes their isolation from each other and growing as a monolayer over the surface of culture plates/ flasks. Cellular components like membrane fragments, cellular enzymes can also be used. Various types of cultures like cell culture, callus culture, tissue culture and organ culture are used for various purposes. Benefits associated with techniques are, easy to follow, less time consuming and are less expensive. These methodologies are routinely used for preliminary screening of potential drug molecules/chemicals to check their toxicity and efficacy. Almost all cosmetics, drugs and chemicals are tested for their toxicity and efficacy, using these tests. For example, eye irritancy test. To check the irritancy of chemicals previously Draize test was used, which requires animals (mainly rabbit). It is very painful and every time a new animal is used. Scientists suggested an alternative which uses bovine corneal organ culture. The bovine cornea is cultured up to three weeks in laboratory and various analytical methods are used to evaluate the toxicological effect of test-chemical irritancy *in vitro*.

### *(4) Human dopaminergic neurons:*

These can be used as a substitute for animal models of Parkinson's disease and for transgenic models with modified expression of PARK genes. Normally the drug testing is performed exclusively *in vivo* in the so-called MPP, methamphetamine and 6-hydroxydopamine models requiring tens of thousands of animals (ranging from mice to rats and primates) which impose medium to very severe stress on animals. Primary neuronal cultures of rats are used for mechanistic studies, but these are very difficult to handle and human neurons, which would be most relevant, are not usually available.

Researchers have developed a human neuronal model cell line (LUHMES) in which expression of tyrosine hydroxylase will be augmented to make these cells as similar as possible to human brain cells *in vivo*. These can be utilized for study of mechanism of degeneration and mechanism and efficacy of drugs. Calculations show that approximately one lakh animals (used otherwise in severely stressful experiments) could be saved and research data will be immediately usable by others and create an immediate impact if we use this method. (Substantiate with reference/s)

### *(5) Computer models:*

Computers can help to understand the various basic principles of biology. Specialized computer models and software programs help to design new medicines. Computer generated simulations are used to predict the various possible biological and toxic effects of a chemical

or potential drug candidate without animal dissection. Only the most promising molecules obtained from primary screening are used for *in vivo* experimentation. For example, to know the receptor binding site of a drug, *in vivo* experimentation is necessary. Software known as Computer Aided Drug Design (CADD) is used to predict the receptor binding site for a potential drug molecule. CADD works to identify probable binding site and hence avoids testing of unwanted chemicals having no biological activity. Also, with the help of such software programs, we can tailor make a new drug for the specific binding site and then in final stage animal testing is done to obtain confirmatory results. Hence, the total number of experimental animals is lowered and the objectives of Russel and Burch's 3 Rs are achieved.

Another popular tool is the Structure Activity Relationship (SARs) computer programs. It predicts biological activity of a drug candidate based on the presence of chemical moieties attached to the parent compound. Quantitative Structure Activity Relationship (QSAR) is the mathematical description of the relationship between physicochemical properties of a drug molecule and its biological activity. The activities like carcinogenicity and mutagenicity of a potential drug candidate are well predicted by the computer database. The recent QSAR software shows more appropriate results while predicting the carcinogenicity of any molecule. The advantages of computer models over conventional animal models are the speed and relatively inexpensive procedures. A very good example is a study which assessed the effectiveness of computer models versus the traditional laboratory practices. In this comparative study, two groups of undergraduate students performed an experiment with the traditional wet lab approach and computer assisted learning (CAL), respectively. CAL is an interactive computer assisted learning (CAL) program without involvement of real experimental tools. At the end of the study both the groups were assessed for the knowledge gain (through test questionnaires, calculations, and interpretation). It was found that the students performing CAL had a better problem solving attitude. Moreover, the cost of new techniques was much less than the traditional laboratory practices. (Substantiate with reference/s)

### *(6) Epidemiological surveys:*

These survey use the existing data or previously exposed species data for the study of lifestyle factors in populations to find correlations that might be significant. For example, epidemiology has linked smoking to cancer; high cholesterol to heart disease; and folic acid deficiency in pregnancy to spina bifida. These surveys are useful to limit the range of investigations regarding a chemical or other substance. It has also been reported recently that the consumption of alcohol is associated with the risk of glioblastoma in a dose-response relationship. (Substantiate with reference/s)

### *(7) Plant analysis:*

Plant substitution has had limited success in animal research. Some effects of exposure to certain substances have been demonstrated and the effects related to humans. A recent study on the effect of pharmaceuticals and their residues as environmental contaminants was performed on *Brassica juncea*, and demonstrated drug-induced defense responses and activation of detoxification mechanisms as a result of oxidative stress. (Substantiate with reference/s)

### *(8) Stem cell research:*

Stem cells may provide a complementary alternative to animals as *in vitro* models of disease and for toxicological testing. Disease genes are inserted into embryonic stem cells, which are then induced to differentiate into human disease tissues that can be used to screen for drugs. Embryonic stem cells can grow and differentiate in a Petri dish into the variety of cells that build a human organ. These *in vitro* versions of human tissue are superior to dishes of a single cell type to assess the toxicological impact of a drug. They provide a human impact profile, not a mouse's. Researchers have created an embryonic stem cell line using the genes from a Parkinson's patient that shows disease's degenerative symptoms. Diabetes and Alzheimer's disease have been found to be linked with a mix of genetic and environmental roots, and stem cells have been used to screen new drugs for the treatment of these common disorders. Embryonic-stem-cell-derived mouse models of two spinal cord diseases, spinal muscular atrophy and Lou Gehrig's disease have been developed to screen new drugs.

Mammals aren't always a great model especially when it comes to reflecting a drug's potential liver and heart toxicity. Animal models are expensive, and they take time to produce results. Stem cells provide a better substitute to study various cancers as well as liver and cardiac toxicity. (Substantiate with reference/s)

### *(9) Microdosing:*

It is a new method of obtaining human metabolism data which enables potential new drugs to be tested safely in humans at an earlier stage. Microdosing relies on the ultra-sensitivity of accelerator mass spectrometry (AMS) which is a very sensitive device. Currently, 40% of drugs fail in Phase I clinical trials, which take up to 18 months and cost £3-5 million. Microdosing could screen out drugs destined to fail earlier, faster and cheaper. Microdosing takes only 4-6 months and costs £0.25 million per drug. Its accuracy at predicting human metabolism is excellent. (Substantiate with reference/s)

### *(10) DNA chips:*

These enable the study of pharmacogenetics which helps in personalized drug treatment. DNA chips are glass slides studded with an array of genes or fragments of DNA. A sample of DNA tagged with fluorescent dyes is exposed to a new drug, and then washed over the chip. When the

genes on the chip match the DNA in the sample, they stick together and the colours reveal which genes have been activated or suppressed by the experimental drug. This technique helps to design drugs for a particular individual. Recent progress in the advent of microarray whole genome expression profiling have produced prodigious data sets on genetic loci, potential candidate genes, and differential gene expression related to alcoholism and ethanol behaviors. Genetical genomics, which combines genetic analysis of both traditional phenotypes and whole genome expression data, offered a potential methodology for characterizing brain gene networks functioning in alcoholism. (Substantiate with reference/s)

### *(11) Microfluidics chips:*

These are just 2 cm wide and contain a series of tiny chambers each containing a sample of tissue from different parts of the body. The compartments are linked by microchannels through which a blood substitute flows. The test drug is added to the blood substitute and circulates around the device. Sensors in the chip feedback information for computer analysis. This can mimic what goes on in the body on a micro scale. (Substantiate with reference/s)

### *(12) Human tissue:*

Alzheimer's and Parkinson's diseases have been studied using the human tissues of patients. HIV/AIDS treatment has come from studying humans and human tissue, particularly blood. New drugs can be tested in human tissues, ethically obtained with fully informed consent. Many researchers work exclusively with human tissue because it is more appropriate than animal tissue and moreover these disorders occur in humans. In a recent study, human cardiac microvascular endothelial cultured cells were used to determine the ability of a comprehensive array of pro-inflammatory stimuli to modulate cell adhesion molecule (CAM) expression, in which different donors showed different CAM expression profiles, confirming genetic variability in the endothelial cells. (Substantiate with reference/s)

### *(13) New imaging technologies:*

Magnetoencephalography (MEG), magnetic resonance imaging (MRI), functional MRI (fMRI), magnetic resonance spectroscopy (MRS), positron emission tomography (PET), single-photon emission computed tomography (SPECT), event-related optical signals (EROS) and transcranial magnetic stimulation (TMS) are the techniques offering a view of the human body –in particular, the brain – that cannot be gained by studying animals. (Substantiate with reference/s)

### *(14) Post-marketing drug surveillance:*

ADR's (expand this term) are currently the fourth leading cause of death in the western world. Post marketing drug surveillance could help to identify unexpected side effects of new drugs much sooner, thereby reducing the burden of adverse drug reactions.

### *(15) Alternative organisms:*

The ethical issues have posed many restrictions over the experimental use of higher model vertebrates like guinea pig, rats, dogs, monkeys etc. Therefore, use of alternative organisms has been proposed. Different model organisms are used to replace experimental animals. (Substantiate with reference/s)

## (A) Lower vertebrates

Lower vertebrates are an attractive option because of the genetic relatedness to the higher vertebrates including mammals. Moreover, there are less ethical problems involved in the experimental use of lower vertebrates.

**Example –** *Danio rerio*

*Danio rerio*, commonly called as zebra fish, is a small freshwater fish with an approximate length of 2–4 cm. It has a nearly transparent body during early development, which helps easy visual access to the internal anatomy. The optical clarity allows direct observation of developmental stages, identification of phenotypic traits during mutagenesis, easy screening, assessment of endpoint of toxicity testing and direct observation of gene expression through light microscopy. Small size, short life cycle and high fecundity favour its laboratory use.

The working space, cost of laboratory solutions, test chemicals and the manpower involved are reduced by opting *D. rerio* as an alternative to animals. Its embryos and larvae can be developed and used for testing in cell culture plates and Petri dishes. Whole genome sequence availability makes Zebra fish an attractive option for molecular and genetic research. From infancy to the adult stage it is used in a variety of applications, mainly for the detection of various toxicological studies of chemicals and pharmaceuticals. It is also having wide applications in the investigation of cancer, heart diseases, neurological malfunctions, behavioural diseases and to observe the mutations and problems in organ development due to exposure to test molecules. Modeling of certain human diseases in zebra fish could be used to ameliorate the disease phenotype and malfunctions in organ development. (Substantiate with reference/s)

## (B) Invertebrates

Invertebrate organisms are widely used as an alternative for laboratory use of animals. They have been used to study various diseases like Parkinson's disease, endocrine and memory dysfunction, muscle dystrophy, wound healing, cell aging, programmed cell death, retrovirus biology, diabetes and toxicological testing. They hold numerous benefits, such as a brief life cycle, small size and simple anatomy, so that a large number of invertebrates can be studied in a single experiment within a short period with less ethical problems. Their cost of housing is less compared to the animals. For example, thousands of flies could be accommodated in a shelter where only few mice can be kept.

**Example – (i)** *Drosophila melanogaster*

*Drosophila melanogaster*, also known as fruit fly is one of the most widely studied invertebrates in research. It has a well studied genome which enables study of molecular mechanisms underlying the human diseases. Its complete genome has been sequenced and annotated, which encodes more than 14,000 genes on four chromosomes. Only three genes carry the bulk of genome of *D. melanogaster*. Nearly 75% of the genes involved in human diseases are believed to have a functional homolog in the fly. *D. melanogaster* requires extremely low cost of maintenance, propagation and screening as compared to the other mammal based models. It also produces the results very rapidly due to a short life cycle. Fruit fly possesses four stages in life cycle – the embryo, the larva, the pupa and the adult. Each stage of fly has its own advantage, hence considered as a multiple model organism to study the various concepts.

The response of flies to many drugs which are acting on CNS is similar to that observed in mammals. The brain of the adult fly is quite extraordinary because more than 100,000 neurons form the discreet circuits, which mediate various complex behaviors like circadian rhythms, learning and memory, feeding, sleep, courtship, aggression, grooming and flight navigation.

Fruit fly serves as an important tool to investigate neurodegenerative diseases like Alzheimers, Parkinson's, disease and Huntington's disease. (Substantiate with reference/s)

**(ii) *Caenorhabditis elegans***

*Caenorhabditis elegans* is a eukaryotic nematode. This multi cellular organism is approximately 1 mm in length and has a very short generation time. As a model, *C. elegans* have been used to study various neurological disorders like Huntington's disease, Parkinson's disease, Alzheimer's disease; various immune disorders as well as cancer and diabetes. It has served in developing and testing of the therapeutic agents for treatment of these diseases. (Substantiate with reference/s)

### (C) Microorganisms

**Example – *Saccharomyces cerevisiae***

Whole genome of this unicellular fungus has been sequenced in 1996. The nuclear genome contains about 16 chromosomes with more than 13 million base pairs. It also contains an extra nuclear genome in the mitochondria. The budding yeast carries its genetic information in the form of 6000 genes. The number and size of genes are relatively small and the density of genes is very high. Best characterized and studied genome makes *S. cerveisiae* one of the most ideal eukaryotic microorganisms for the biological studies. Presence of similar cellular architecture and rudimentary life cycle like multi cellular eukaryotes is another advantage. The numerous membrane-bound organelles like nucleus, peroxisome, mitochondria and the organelles of secretary pathway also mimic the functions of mammalian cells.

This brewing yeast is used to understand programmed cell death, cell death regulators in humans and is very useful in cancer research. *S. cerevisiae* helps to understand the fundamental aspects of cellular biology in neurodegenerative diseases like Alzheimer's, Parkinson's and Huntington's diseases by studying the endogenous or heterologous proteins that lay at the root of these diseases. (Substantiate with reference/s)

## Conclusion

Animal ethics is an issue as important as the human welfare. More efforts need to be undertaken for effective implementation of 4 Rs during laboratory use of animals. The increase in knowledge, improvement of health care and creation of useful products that is derived from experimentation on animals, carries the cost of pain and distress that the animals experience. There are provisions for ensuring that animal use is performed in a humane manner so as to minimize pain, distress or discomfort. Various alternatives to animal use have been suggested, which need to be implemented in an effective manner. For this integration of various computer models, bioinformatics tools, in vitro cell cultures, enzymatic screens and model organisms are necessary. These integrated approaches can result in minimum involvement of animals in scientific procedures. No new drug can be used in patients until it has been extensively tested in animals. Alternative methods do help to reduce the number of animals required for drug research, but there is no way they can completely eliminate the need for animals in preclinical studies. There should be an emphasis on reducing the overall use of experimental animals and also finding newer alternatives that can suffice the research needs. The development in the field of science and technology should be brought to its advantage so that in the coming years novel alternatives can be developed and the issue of 'moral values' in experimentations can be sorted out.

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