Substitute of Animals in Drug Research: An Approach Towards Fulfillment of 4R's

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The preclinical studies for drug screening involve the use of animals which is very time consuming and expensive and at times leads to suffering of the used organism. Animal right activists around the world are increasingly opposing the use of animals. This has forced the researchers to find ways to not only decrease the time involved in drug screening procedures but also decrease the number of animals used and also increase the humane care of animals. To fulfill this goal a number of new *in vitro* techniques have been devised which are called 'Alternatives' or 'Substitutes' for use of animals in research involving drugs. These 'Alternatives' are defined as the adjuncts which help to decrease the use as well as the number of animals in biomedical research. Russell and Burch have defined these alternatives by three R's - Reduction, Refinement and Replacement. These alternative strategies include physico-chemical methods and techniques utilizing tissue culture, microbiological system, stem cells, DNA chips, micro fluidics, computer analysis models, epidemiological surveys and plant-tissue based materials. The advantages of these alternatives include the decrease in the number of animals used, ability to obtain the results quickly, reduction in the costs and flexibility to control the variables of the experiment. However these techniques are not glittering gold and have their own shortcomings. The disadvantages include the lack of an appropriate alternative to study the whole animal's metabolic response, inability to study transplant models and idiosyncratic responses and inability to study the body's handling of drugs and its subsequent metabolites. None-the-less these aalternative methods to certain extent help to reduce the number of animals required for research. But such alternatives cannot eliminate the need for animals in research completely. Even though no animal model is a complete set of replica for a process within a human body, the intact animal does provide a better model of the complex interaction of the physiological processes.

Key words: Alternatives, DNA chips, *in silico* analysis, micro dosing, micro fluidics

Use of animals in experiments involving scientific research and biological testing has raised concerns in the mind of environmentalists and animal lovers for a long time. In this regard, a number of legislative initiatives have been proposed so as to limit animal research and ensure proper treatment of animals. In the eighteenth century, animal protection movement was started by a group of people known as abolitionists in England who opposed experiments on animals. Another worldwide initiative was started in 1975 by Societies for Protection and Care of Animals (SPCA) who opposed all forms of animal research[1].

In recent times, animal welfare groups or reformers have been opposing the animal research so as to ensure proper treatment of animals. The Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) under the Government of India has been formed to monitor animal experiments through ethics committees set up in respective institutions. Besides the rules and procedures laid down by the CPCSEA, the Indian National Science Academy (INSA), New Delhi has brought out 'guidelines for care and use of animals in scientific research'. These guidelines are widely read and followed by Indian researchers who use animals for experiments. In view of this, non–animal alternatives are being developed wherever necessary[1]. Economic considerations related to the costs of animals and their care, maintenance of animal space and equipment, and appropriate staff also appear to be powerful and effective incentives for the conservative use of laboratory animals and the substitution of less costly approaches when scientifically valid. An apparent 40% decrease in animal use, and the concurrent increase in the use of tissue culture and biotechnology seems to indicate

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that, where scientifically valid, non-animal techniques are being used[2]. For many years now, there has been a carefully planned, well-financed animal rights movement whose goal is the reduction and ultimately the elimination of the use of animals in research. In this regard, groups like scientists centre for animal welfare (SCAW) have been formed so as to ensure proper treatment and human care of animals. Institutional animal care and use committees (IACUC) have been formed in all institutions performing experiments on animals to discuss the ethicality of issues related to the care and proper handling of animals. Two major amendments have been made to the animal welfare act namely, environmental regulations related to the care of animals which help in proper handling of animals, and elimination of calculated LD_{50}. It has been seen that there is less than 50% chance of success of experiments performed on animals which can accurately predict the results in humans. Moreover, researching the human disease using animals is often misleading, thus evolving the need for developing non-animal substitutes in drug research[3].

Definition:
"Alternatives" or "Substitutes" is defined as anything from absolute to partial replacement of live animals in biomedical research and testing[4]. 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[5]. Russell and Burch have given their definition of "alternatives" as "the three Rs - Replacement, Reduction, and Refinement"[6]. The 4th R was added in 1995[7].

THE FOUR R'S

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[8,9].

Refinement:
It is done by decreased invasiveness, improved instrumentation, improved control of pain and improved control of techniques used for animal research[8-10].

Replacement:
It is achieved through use of non-animal living systems, use of non-living systems and computer simulation[8,9].

Responsibility:
The 4th R of Research implies addition of 'responsibility' 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[7].

WHAT ARE THOSE 'ALTERNATIVES OR ANIMAL SUBSTITUTES'?

These alternatives can be physico-chemical techniques, microbiological systems, tissue/organ culture preparation, computer or mathematical analysis (*in silico* testing), 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.

Physico-chemical techniques:
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[11].

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. Chitosan films capable of simulating the flux of model drugs, 5-fluorouracil (5-FU) and indomethacin (INDO) across rat, rabbit and human cadaver epidermal sheets have been developed and statistical design has revealed that concentration of chitosan, crosslinking time and concentration of crosslinking agent, 5% sodium tripolyphosphate (NaTPP) significantly influenced the *in vitro* flux of 5-FU and INDO across chitosan films. *In vitro* permeation of both 5-FU and INDO across optimized film formulations was found to be comparable to that obtained across rat, rabbit and human epidermal sheets.

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[12]. 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[13].

Microbiological systems:
These are commonly used in toxicology and carcinogenesis (cancer producing) studies. 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[14]. A recent study has utilized the bacteria *Vibrio vulnificus* to study the modulation of the toxic RtxA1 which induces acute cytotoxicity[15]. This has emerged as a potential for the treatment of infectious diseases.

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.

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.

Tests for absorption include *in vitro* methods such as percutaneous absorption and everted sac technique. The *in vivo* methods can be isolated rat gut technique and disintegration of solid dosage forms. Tests for distribution include *in vitro* methods like protein binding model and ultrafiltration while the *in vivo* technique is autoradiography. Tests for biotransformation can be *in vitro* like subcellular fraction, isolated hepatocytes and isolated liver perfusion while the *in vivo* methods can be radiolabelled studies and clinical drug metabolism studies[16].

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[17].

Computer or mathematical analysis:
This technique is of value when a biological effect can be represented by a known equation. Virtual

human organs and virtual metabolism programmes can now predict drug effects in humans more accurately than animals can. Computers can be used to design the molecular structure of drugs to target specific receptors. For example, the protease inhibitors for patients with HIV were designed by computer and tested in human tissue cultures and computer models, bypassing animal tests due to the urgent need for a treatment. In 1997, Roche Pharmaceuticals had a new heart drug approved on the strength of data from a virtual heart because the animal data was inconclusive.

Research teams around the world are working on a 'virtual human', which is designed to predict drug metabolism and metabolite interaction with any given organ – information that animal models will never be able to provide. Scientists can simulate experiments *in silico* (on computer) in minutes that could take months or years to do in the lab or clinic. This technique can be applied as a substitute for animals but must be validated with animal studies[18].

Epidemiological surveys:

These use the existing data or previously exposed species data for 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[19]. It has also been reported recently that the consumption of alcohol is associated with the risk of glioblastoma in a dose-response relationship[20].

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[4]. 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[21].

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, liver and cardiac toxicity[22].

Stem cells in toxicological research:

For biotechnology and pharmaceutical firms, using stem cells to test potential drug leads for toxicity could help them avoid wasting time on harmful compounds. Stem cell screens would be part of a prioritization of compounds early in the discovery and development process. Embryonic stem cells with a specific ethnic genome could be used to develop ethnic cell populations that could be screened to check the toxicity of potential drug leads. This could help to develop the drugs to be used in specific races of human populations. Currently, the most successful development of stem cells as *in vitro* model for toxicology testing is in human cardiac tissue. Embryonic stem cells have been differentiated into cardiac tissue that shows potential as a toxicological model of disease (since withdrawal of terfenadine). Developing stem cell models of the liver, the other major organ that animal models fail to emulate, has been going on[22].

Drawbacks of stem cells:

Stem cells fall short in their modeling of systemic toxicity. These cannot predict the effects of a metabolite of drug inside the body since it might

have a different effect on a particular organ than the parent drug. Since stem cells reflect a single organ's response inside a Petri dish, these cannot predict the impact of subsequent metabolites on the whole organism. Stem cells won't replace animals. They will reduce and refine use of them. Besides stem cells themselves can grow into teratomas and grow uncontrolled inside the organism.

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 ultrasensitivity 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[23].

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[24]. 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[25]. 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[25].

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 feed back information for computer analysis. This can mimic what goes on in the body on a micro scale[26].

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[19]. 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[27].

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[11].

Post-marketing drug surveillance:
ADR's 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[11].

Advantages of *in vitro* techniques (animal substitutes)[1]:
The advantages are the reduction in the number of animals used, ability to obtain results more quickly, reduction in the cost of the tests/experiments, and flexibility to change conditions and variables of the experiment.

Disadvantages of *in vitro* techniques (animal substitutes)[1]:
The disadvantages include basic research requires the answers to an animal's metabolic response in order to gain a fuller knowledge of the metabolism of drugs

inside the organism. Non-animal alternatives have been unable to provide such complete information on the fate of metabolite inside the subject, transplant studies involving substitution of an organ, tissue, or device, cannot use alternative techniques, since no alternative has demonstrated the ability to accept or reject an implant, surgical techniques require animal models in which to develop and perfect new techniques before use in humans and non-animal alternatives cannot substitute for these animals, and idiosyncratic responses of a substance which produce an allergic or an unpredicted response cannot be tested in an alternative model. These effects do not fit any pattern or equation, which is the basis for alternative models.

CONCLUSIONS

The closer a proposed therapy is to human use, the more likely it will require animal research. Where feasible, appropriate alternatives are being devised. 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. Even though no animal model is a complete set of models for a process within a human being, the intact animal does provide a better model of the complex interaction of the physiological process than does an alternative technique.

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