Review Article

ALTERNATIVES TO LABORATORY ANIMALS IN EXPERIMENTAL METHODS EMPLOYED IN BIOLOGICAL RESEARCH - A REVIEW

Biswadeep Behera[1], S. Vairamuthu[2] and N. Pazhanivel*[3]

Department of Veterinary Pathology
Madras Veterinary College
Tamil Nadu Veterinary and Animal Sciences University
Chennai – 600 007, Tamil Nadu

ABSTRACT

With the advancement of research and development of medical technology, there is an increase in the number of animals used in research. As millions of experimental animals are being used in different experiments worldwide, there is a lot of debate about the pain, distress, and death experienced by animals during scientific experiments. Experimental animals also require skilled manpower, time-consuming protocols, and are costly. There are various alternatives to animal testing to overcome the drawbacks of animal experiments and avoid unethical procedures. Russell and Burch published principles of the Humane Experimental Technique which includes a strategy of 3 Rs (i.e. reduction, refinement, and replacement) in 1959. They introduced and defined the terms replacement, reduction, and refinement, which subsequently have become known as 'alternatives' or 'alternative methods' for minimizing the potential for animal pain and distress in biomedical research. Therefore, different methods and different alternative organisms are being used to implement the 3 Rs strategy. So, a brief account of these alternatives and the advantages associated is discussed in this review with examples. An integrated application of these approaches would give insight into the minimum use of animals in scientific experiments.

Keywords: Alternative, Biological, Experimental, Laboratory animal, Research

Received : 19.10.2023 Revised : 18.12.2023 Accepted : 18.12.2023

INTRODUCTION

Animals have been used for different purposes like transportation, sports, food, pets, companionship, and recreation for a long time. Animals are also being used for research in an extended way. Different species of animals like fishes (examples – zebrafish, trout), guinea pigs, hamsters, mice, rabbits, rats, birds (mainly chicken), amphibians (Xenopus frogs), primates, dogs, cats, etc. are regularly used in research field since a long time (CULABBR, 1988). However, their numbers being used have gone up with the advancement in medical

[1,3]Professor, Department of Veterinary Pathology, corresponding author Email id: drnpvel@gmail.com
[2]Professor and Head, Centralised Clinical Laboratory

technology. The large numbers of animals used for different experimental purposes are being supplied by different national breeding centers and the centers present in different universities. The term "Alternatives" mainly refers to replacing, reducing, and refining existing techniques with the help of different new methods or techniques.

The main reason for the use of different alternatives instead of laboratory animal use is because

- Laboratory animals are costly.
- Laboratory animal research is more time-consuming.
- Highly skilled/trained manpower is required for handling lab animals.
- Requirement of ethical committee permission for the use of laboratory animals in different experimental trials.

Different organizations such as the Committee for Purpose of Control and Supervision on Experiments on Animals (CPCSEA), the International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH), National Institute of Health (NIH), and Organization for Economic Cooperation and Development (OECD) provide uidelines for animal housekeeping, breeding of animals, their feeding as well as transportation, and more importantly for their scientific use in various experiments (Rollin, 2003). Not only the major ethical concerns but also there are a requirement for highly skilled manpower and the protocols are also time-consuming. Housing, breeding, and the protocols of animal experimentation are not very cost-effective and lengthy procedures (Balls, 1994).

Three Rs: reduction, refinement, and replacement

The use of various alternative methods instead of animal testing was mainly proposed due to the different disadvantages of animal experiments as well as to avoid unethical procedures. A 3 Rs strategy is reduction, refinement and replacement of laboratory animals (Ranganatha and Kuppast, 2012). Different types of methods and various alternative organisms are being used for the implementation of the 3Rs strategy. The main idea of replacement of laboratory animals was given by Hume and Russell in 1957 at the Universities Federation for Animal Welfares (UFAW) (Balls, 1994). For making animal experimentations humane, Russell and Burch, 1959 had given some suggestions in which were later called 3 Rs. Based on this approach, a minimum number of animals are used in various experimental trials which means "Reduction". The animal experimental design should be planned in such a manner that they will feel less pain and distress during that period. Different methodologies and lower organisms may be used in place of higher animals i.e. Replacement (Ranganatha and Kuppast, 2012; Zurlo *et al.,* 1996). Replacement is of two types i.e. 'relative' and 'absolute' replacement. In case of relative replacement, the animals are being used but there is no exposure to any kind of distress

during the experiment. However, in the absolute replacement strategy, no animals are used at any single stage of the experiment (Balls, 1994).

1. Reduction

Usage of animals as minimum as possible in number i.e. reduction in number of total animals in the experimental trial will be possible through the use of proper statistical support and the design of the experiment. The researcher should ensure the problem is being investigated and will not be repeated. The researcher should also ensure that the model used is the most appropriate one in availability, level of sentience, and potential relevance e.g.- *In vitro* cell culture (Kimber *et al.,* 2001).

2. Refinement

The word "Refinement" refers to alleviating or minimizing discomfort, potential pain, and distress. Some of the alternatives to refinement are putting the earliest possible end point, using appropriate analgesics, anesthetics, tranquilizers, sedatives, etc., proper handling technique, adequate training before experimenting, ensuring drug doses are correct and drugs are not expired, performing various surgeries and procedure aseptically to prevent infection, improve housing system and use of less sensitive species (De Silva *et al.,* 1996).

3. Replacement

Replacement refers to any kind of scientific method that employs non-sentient material that may replace use of the conscious living vertebrates in animal experimentation. It is also classified as 1) Relative Replacement: Animals should not be exposed to any kind of distress during the experiment, 2) Absolute Replacement: Animals should not be used even in any single stage of the experiment (Hendriksen, 2007).

- Various alternative methods to laboratory animals are

  (A) Computer models

  (B) *In-vitro* method

  (C) Microbiological tests

  (D) Alternative organisms

  (E) In-chemico testing

  (F) Epidemiological, clinical, and genetic studies

  (G) Micro dosing and

  (H) Microfluidic chips.

(A) Computer models

Nowadays, a lot of highly specific and sensitive computer models as well as different software programs are being used without animal dissection to identify potential drug candidates and also to predict the toxicological and biological effects of a chemical. After that primary screening, the only most promising molecules are being used for in vivo experimentation. These software programs provide facilities like more speed and cost-effectiveness to preclinical studies.

Computer Aided Drug Design (CADD)

Different computational approaches are gaining rapid exploration, admiration, and implementation mainly in drug design, discovery, and development. The introduction of a new drug into the market is a very complex, risky, and costly process in terms of money, time, and manpower. Discovery of a new drug and its development usually needs a minimum of around 10-14 years and the capital needed for this includes more than 1 billion dollars (Daina *et al.,* 2017). So, to reduce time, cost, and risk-borne factors. CADD method is widely used for the development of a new drug-designing approach. By using CADD approaches, we can also reduce the drug discovery and development cost by up to 50%. CADD mainly consists of the use of different software program-based processes for a standard establishment that relates to structure-activity (Xiang *et al.,* 2012).

Different approaches in CADD

Two different types of approaches are used for new drug design with the help of CADD which are as follows:

1. Direct approach/Structure-based drug design (SBDD)
2. Indirect approach/Ligand-based drug design (LBDD)

Structure-based drug design

In the case of SBDD, the target protein structure is known. After the method of docking, the interaction and bio-affinity of a test compound can be calculated, and based on the result of interactions, a new drug molecule can be designed (Imam and Gilani, 2017).

Overview of the process involved in SBDD

Usually, multiple cycles are to be run in SBDD before the optimized lead reaches the clinical trials. In the first cycle, there is isolation, purification, and target protein structure determination with the help of one of the three key methods i.e. X-ray crystallography, homology modeling, or NMR expand. At first, the virtually screened compounds from different databases were placed in the selected region i.e. active site of protein. Then the score and rank of the compounds are calculated based on the steric, hydrophobic, and electrostatic interaction of these molecules with the target protein active site. After that, the top-ranked compounds are tested with different biochemical assays. In the second cycle, the structure of protein which is complex with the most optimistic leader of the first cycle can be determined. Then the sites of the compound can be identified and optimized towards further increment in potency. Improved lead compounds can be developed by employing multiple cycles of synthesis and crafting their intricate structure with the target protein. This approach can enhance both target specificity and binding affinity (Anderson, 2003).

Ligand-Based drug design (LBDD)

In LBDD, the target protein 3D structure is not known but the knowledge of ligands which is going to bind to the desired

target site is known. These ligands may be used in the development of a pharmacophore model or molecule that possesses all the necessary structural features for binding to a target active site. Generally, pharmacophore-based approaches and quantitative-structure activity relationships (QSARs) are two important pillars of ligand-based techniques. In LBDD, there is the assumption that the compounds with similar structures also have similar kinds of interaction and biological action and interaction with the target protein (Macalino *et al.,* 2015).

Virtual screening (VS)

Virtual screening is working as a convenient tool now a days to find out the most favorable bioactive compounds with the help of information about protein targets or known active ligands. As an alternative for high-throughput screening, VS can be used as an alternative because of its cost-effectiveness and probability of finding the most appropriate novel hit by filtering the large libraries of compounds. Two different types of virtual screening approaches include structure based virtual screening (SBVS) and ligand-based virtual screening (LBVS). SBVS method mainly depends on the structure of the active site of the target protein whereas the LBVS method is mainly based on the estimation of similarity between the database compounds and known active compounds (Lill, 2013).

Molecular docking

This is an *in-silico* method that predicts the interaction between the ligand and the target protein active site. It is mostly used to accurately estimate the most favorable binding energy and the bio-affinities of ligands with their receptor. Presently, it is applied to virtual screening for the optimization of lead compounds. Three interconnected goals are there in molecular docking methodology i.e. the prediction of the binding pose, bioaffinity, and virtual screening. In the molecular docking method, the basic tools are a search algorithm along with scoring functions for creating and analyzing conformations of the ligand (Guedes *et al.,* 2014).

Computer-Assisted Learning (CAL)

CAL is an interactive computer-assisted learning program without the use of real experimental tools. Different software is Expharm- Developed by JIPMER, India, and X-ecology. In CAL, different procedures such as isolation and mounting animal tissues are demonstrated with the help of videos. We can see the various effects of different drugs on isolated tissue through the screen interface. Content is mainly classified into experimental animals, experimental techniques, and equipment. The procedure involves conducting bioassay experiments on entire organisms (Dewhurst *et al.,* 1994).

(B) *In-vitro* Method

In-vitro culture of cells and tissue mainly involves the isolation of animal/human cells and then growing as a monolayer over the surface of culture flasks/ plates. Various types of cultures like callus culture, cell culture, tissue culture, and organ culture are being

used for various purposes (Shay and Wright, 2000; Steinhoff *et al.,* 2000). Examples of various *in-vitro* models include *the In-vitro* Pyrogen test, embryonic stem cell test, local lymph node assay for skin sensitization, neutral red uptake assay, carcinogenicity test, repeated dose toxicity test, and developmental neurotoxicity test.

- ***In-vitro* Pyrogen Test**

1. ***Limulus* Amoebocyte Lysate (LAL) test**

The fever-inducing substances generally obtained from microorganisms are known as pyrogens and the presence of sufficient amounts of these substances systematically in the body may lead to severe signs of inflammation, multiorgan failure, shock, and sometimes even lead to death in humans. Therefore, it is much needed to test pyrogen presence in all parenteral products including injectable vaccines. Two traditional tests for pyrogenicity i.e. the rabbit pyrogen test (RPT) and the *Limulus* Amoebocyte lysate (LAL) test are being used. The contaminating endotoxin from gram-negative bacteria can be measured quantitatively through LAL which is based on the clotting reaction of the haemolymph of the horseshoe crab. The other product contaminants also cause pyrogenicity and immune activation, but the LAL test is unable to detect them due to high specificity bacterial lipopolysaccharides (Rudbach *et al.,* 1976). Pyrogens present in gram-positive bacteria including lipoprotein, peptidoglycan as well and lipoteichoic acid cannot be detected by LAL test (Raetz and Whitfield, 2002). This is the reason for a complete dismissal of the RPT test in many cases.

2. **Monocyte Activation Test (MAT)**

The main principle of MAT includes that pyrogen will cause *in-vitro* activation of monocytes in whole human blood. The pro-inflammatory cytokines like interleukin (IL)-1β, IL-6, and Tumor Necrosis Factor (TNF)-α are released due to stimulation of pyrogens like bacterial endotoxin. The quantity of cytokines can be measured by ELISA, flow cytometry, or bead-array. In the year 2010, different variants of MAT have been standardized, validated, and adopted by the European Pharmacopoeia. Depending on the diverse immunization strategies and novel vaccines and adjuvants, the use of both the MAT and the LAL test for routine release safety evaluation has been recommended. Though the use of a validated cell culture technique provides a lot of benefits, MAT analysis is highly recommended for monitoring pyrogen contamination (Sandle, 2013). Due to the lack of pyrogenic cytokines and the presence of fever in genetically engineered mice, the MAT assay is unable to provide faithful results. In that case, the RPT may be useful as a complementary tool for monitoring of febrile signals.

- **Local Lymph Node Assay**

This assay is used to test the potential of the test compound for skin sensitization. The main principle of this assay represents that the tested compound is considered a sensitizer when the lymph node (LN) draining the site of the chemical application reveals a primary proliferation of lymphocytes as measured by radioactive labeling in test and vehicle groups (OECD, 1994).

- **Skin Patch Test**

Corrositex is generally used to determine the chemical corrosivity and this test also can be used as an alternative method for the Draize rabbit skin irritation test. The principle of the test revealed that potentially corrosive substances will form colored biomembrane and chemical detection systems. An example of this test is cultured human epidermal keratinocytes which mimic the human epidermis and are mostly used to measure skin irritation and dermal corrosion (Stobbe *et al.,* 2003).

- **Neutral Red Uptake Assay (NRU Assay)**

Neutral assay is also used as an alternative method for the Draize rabbit eye test to screen the chemicals for eye irritation potential. Firstly, neutral red penetrates the cell membrane because of its permeability to the cell membrane and there is intracellular accumulation in lysosomes. If, there is any alteration in the cell surface or lysosomal membrane then the uptake of neutral red will be decreased. NRU assay usually measures the test compound's ability to inhibit the uptake of neutral red dye. NRU 50 / IC 50 serves as the toxicological endpoint (Repetto *et al.,* 2008).

- **Stem cell models**

These models can be used for screening toxicological compounds and also as *in-vitro* models of disease. Genes that are mainly associated with the diseases are inserted into embryonic stem cells and then induced to differentiate into human disease which is used for screening of drugs. E.g. Genes responsible for Parkinson's disease are isolated from the patient and grown into a model of Parkinson's disease and are used for screening potential drugs (Lynch *et al.,* 2019).

(C) Microbiological Tests:

Ames test is one of the most widely used methods to test a chemical's mutation ability in organism's DNA. This is also called the 'Bacterial Reverse Mutation Assay'. Various strains of the bacterium *Salmonella typhimurium* showed different gene mutations involved in histidine synthesis. The strains are mainly auxotrophic mutant's h- (defective organism or deficiency mutant) i.e. there is a requirement of histidine for growth as they cannot produce it. Ames test generally examines the capability of a test compound to create mutation which results in a prototrophic state h+ i.e., the strains can grow on the histidine-free medium (Vijay *et al.,* 2018).

(D) Alternative organisms:

One of the most important and popular model organisms is *Saccharomyces cerevisiae* because of its rapid growth (Generation time is 90 mins, ease of replica plating, mutant isolation, well-defined genetic system, and system of highly versatile DNA transformation. Also, the membrane-bound organelles like mitochondria, nucleus, peroxisome, and organelles of the secretory pathway mimic function of mammalian cells. These organisms are also used as a model to study the mechanism of apoptosis, cell death regulators in humans, and also in cancer studies (Mell and Burgess, 2002).

- **Lower vertebrates**

The use of lower vertebrates as an alternative is the most attractive one due to their genetic relatedness. For example, *Danio rerio* is used as an alternative and it has a transparent body which helps in the direct observation of developmental stages, identification of different phenotypic traits during mutagenesis, assessment of endpoint of toxicity testing, easy screening, and direct observation of gene expression through microscopy. They are having very short life cycles and high fecundity rates. The embryo and larvae of *Danio rerio* can be developed and used for testing in the cell culture plates and also in Petri dishes. The availability of the whole genome sequence of this organism also helps in molecular as well as genetic research (Hill *et al.,* 2005).

- **Invertebrates**

Generally, invertebrates are smaller in size and they have a brief life cycle as well as simple anatomy. Some examples of invertebrates include *Caenorhabditis elegans* and *Drosophila melanogaster.*

1) *Caenorhabditis elegans*: It is a eukaryotic and multicellular nematode. Due to transparent and simple cellular complexity, they are used in the study of various neurological disorders as well as in cancer, and diabetes studies (Wilson-Sanders, 2011).

2) *Drosophila melanogaster*: The complete genome for *Drosophila melanogaster* has been sequenced and annotated. They usually show 75% of generating similarities with human diseases. They can also serve as an alternative model due to their short lifespan and organ functions that are comparable to mammals. The embryo is used mainly in cell fate determination, axon path findings, neuronal development, and organogenesis. Larvae are generally used for studying the physiological and developmental processes as well as the behaviour like forging (Vijay *et al.,* 2018).

(E) *In-chemico* testing

In-chemico testing method can be used as replacements for animal (*in-vivo*) assays". The toxic potential of a compound can be detected by different non-animal test methods by using relatively simple chemistry-based methods (Nepal *et al.,* 2018).

Direct Peptide Reactivity Assay (DPRA)

This assay is mainly used to assess whether a chemical or cosmetic will cause allergy or not. The DPRA is an *in-chemico* method, and can also quantify the remaining concentration of lysine- or cysteine-containing peptide within 24 hrs of incubation with the test chemical compound at 22.5-30°C. The synthetic peptides mainly contain phenylalanine which is important for the detection. By use of high-performance liquid chromatography (HPLC) with gradient elution and UV detection at 220 nm, relative peptide concentration can be measured (Nepal *et al.,* 2018).

(F) Epidemiological, clinical, and genetic studies

Epidemiology: It is a valuable tool in the field of Evidence-based Medicine. The main purpose is to identify the risk factors and determine the treatment. This study reveals an unbiased relationship. However, epidemiological studies cannot replace animal experiments although they can guide the research worker in promising directions and therefore help to reduce the number of animals used in experiments (Hunniford *et al.,* 2021).

Clinical studies: Valuable information regarding disease processes can be obtained from the clinical cases. This will also help to know about the vaccine new therapies or new ways of using known treatments.

Genetic monitoring: This is an important tool that will help minimize the number of animals used. It is also used to detect changes in species abundance or diversity.

(G) Micro dosing

A 'micro-dose' means less than one-hundredth of the pharmacological dose up to a maximum of 100 µg. Micro dosing can be measured in any biological samples including plasma and urine to determine ADME (Absorption, Distribution, Metabolism and Excretion). It can be analyzed by using an accelerator mass spectrometry. Early metabolism data can be obtained before going into human phase-I trials. This tool also allows for testing in relevant species (Combes *et al.,* 2003).

(H) Microfluidic chips

A microfluidic chip is 2 cm wide and contains a series of tiny chambers which contain a sample of tissue from different parts of the body. The chips are made up of inorganic (glass, silicon, ceramic, etc.),polymers[COC(Cyclicolefincopolymer), PMMA (Poly (methyl methacrylate)), PDMS (Polydimethylsiloxane), etc.], or organic materials (paper). The compartments are primarily connected by micro-channels through which the blood substitutes flow. The blood substitute contains the test drugs and is circulated in the device. The sensors present in the chip help in feedback information for computer analysis. This can be used to study the disease process and drug metabolism (Syama and Mohanan, 2021).

1) Passive Microfluidics: Passive microfluidics mainly control techniques like capillary forces which play their role in moving, mixing, separating, or otherwise processing.

2) Active Microfluidics: This method refers to the defined manipulation of working fluid with the help of active components such as micro-pumps or micro-valves.

CONCLUSION

Ethics of animals is an important issue like human welfare. Therefore, a lot of efforts have to be undertaken for the effective implementation of the 3 Rs strategy mainly during the experimentation of laboratory animals. Various alternatives to animal use

are there but we have to implement them effectively and scientifically. Therefore, different bioinformatics tools, computer models, *in-vitro* cell cultures, and various alternative model organisms are necessary. To obtain reliable results from various alternative protocols, employ modern techniques, ensure proper data acquisition, and apply accurate statistical procedures. For getting dependable outcomes from different alternative protocols, modern techniques, proper data acquisition, and accurate statistical procedures. With the help of the above different alternatives, there will be minimum involvement of laboratory animals used in various scientific experiments.

REFERENCES

Anderson, A.C. (2003). The process of structure-based drug design. *Chemistry and Biology*, **10**(9): 787 - 797.

Balls, M. (1994). Replacement of animal procedures: alternatives in research, education, and testing. *Laboratory Animals,* **28**: 193 - 211.

Combes, R.D., Berridge, T., Connelly, J., Eve, M.D., Garner, R.C., Toon, S. and Wilcox, P. (2003). Early micro dose studies in human volunteers can minimize animal testing: proceedings of a workshop organized by volunteers in research and testing. *European Journal of Pharmaceutical Science,* **19**: 1 - 11.

CULABBR (Committee on use of laboratory animals in biomedical and behavioural research), national research council and institute of medicine, (1988). Use of laboratory animals in biomedical and behavioral research. National Academy Press, Washington, DC.

Daina, A., Blatter, M.C., Baillie Gerritsen, V., Palagi, P.M., Marek, D., Xenarios, I., Schwede, T., Michielin, O. and Zoete, V. (2017). Drug Design Workshop: A Web-based educational tool to introduce computer-aided drug design to the general public. *Journal of Chemical Education,* **94**(3): 335 - 344.

De Silva, O., Basketter, D.A., Barratt, M.D., Corsini, E., Cronin, M.T., Das, P.K. and Ponec, M. (1996). Alternative methods for skin sensitization testing. *Atla Nottingham,* **24**: 683 - 706.

Dewhurst, D.G., Hardcastle, J., Hardcastle, P.T. and Stuart, E. (1994). Comparison of a computer simulation program and a traditional laboratory practical class for teaching the principles of intestinal absorption. *American Journal of Physiology,* **267**: 95 – 104.

Guedes, I.A., de Magalhães, C.S. and Dardenne, L.E. (2014). Receptor–ligand molecular docking. *Biophysical Reviews*, ***6***: 75 - 87.

Hendriksen, C. (2007). Three Rs achievements in vaccinology. *AATEX Special*, pp. 14.

Hill, A.J., Teraoka, H., Heiderman, W. and Peterson, R.E. (2005). Zebrafish as a model vertebrate for investigating chemical toxicity. *Toxicological Science,* **86**: 6 – 19.

Hume, C. and Russell, W. (1957). The strategy and tactics of experimentation. *Lancet*, **23**: 1049 –1052.

Hunniford, V.T., Montroy, J., Fergusson, D.A., Avey, M.T., Wever, K.E., McCann, S.K., Foster, M., Fox, G., Lafreniere, M., Ghaly, M. and Mannell, S. (2021). Epidemiology and reporting characteristics of preclinical systematic reviews. *PloS Biology*, **19**(5): e3001177.

Imam, S.S. and Gilani, S.J. (2017). Computer aided drug design: A novel loom to drug discovery. *Organic and Medicinal Chemistry International Journal*, **1**(3): 113 - 118.

Kimber, I., Pichowski, J.S., Betts, C.J., Cumberbatch, M., Basketter, D.A. and Dearman, R.J. (2001). Alternative approaches to the identification and characterization of chemical allergens. *Toxicology In-vitro,* **15**: 307 - 312.

Lill, M. (2013). Virtual screening in drug design. In silico models for drug discovery, humana Press, Totowa, NJ, pp.1 - 12.

Lynch, S., Pridgeon, C.S., Duckworth, C.A., Sharma, P., Park, B.K. and Goldring, C.E. (2019). Stem cell models as an *in vitro* model for predictive toxicology. *Biochemical Journal*, **476**(7): 1149 - 1158.

Macalino, S.J., Gosu, V., Hong, S. and Choi, S. (2015). Role of computer-aided drug design in modern drug discovery. *Archives of Pharmacal Research*, **38**(9): 1686 - 1701.

Mell, J.C. and Burgess, S.M. (2002). Yeast is a model genetic organism. In: Encyclopedia of Life Sciences. Mcmillan Publishers Ltd.

Nepal, M.R., Shakya, R., Kang, M.J. and Jeong, T.C. (2018). Example in the chemical method for testing skin sensitizing potential of chemicals using endogenous molecules, *Toxicological Letter,* **289**: 75 - 85.

OECD. (1994). *OECD Guidelines for the Testing of Chemicals*. OECD.

Raetz, C.R.H. and Whitfield, C. (2002). Lipopolysaccharide endotoxins. *Annual Review of Biochemistry*, **71**: 635 – 700.

Ranganatha, N. and Kuppast, I.J. (2012). A review of alternatives to animal testing methods in drug development. *International Journal of Pharmacy and Pharmaceutical Science*, **4**: 28 – 32.

Repetto, G., Del Peso, A. and Zurita, J.L. (2008). Neutral red uptake assay for the estimation of cell viability/

cytotoxicity. *Nature Protocols*, **3**(7): 1125 - 1131.

Rollin, B.E. (2003). Toxicology and new social ethics for animals.

Rudbach, J.A., Akiya, F.I., Elin, R.J., Hochstein, H.D., Luoma, M.K., Milner, C.B., Milner, K.C. and Thomas, K.R. (1976). Preparation and properties of a national reference endotoxin. *Journal of Clinical Microbiol*ogy, **3**(10): 21 – 25.

Russell, W.M.S. and Burch, R.L. (1959). The principles of humane experimental technique, London, UK.

Sandle, T. (2013). Sterility, sterilization and sterility assurance for pharmaceuticals: technology, validation, and current regulations. Cambridge: Wood head Publishing, pp. 171 – 188.

Shay, J.W. and Wright, W.E. (2000). The use of telomerase cells for tissue engineering. *Nature Biotechnology,* **18**: 22 - 23.

Steinhoff, G., Stock, U., Karim, N., Mertschin, H., Timke, A., Meliss, R.R. and Bader, A. (2000). Tissue engineering of pulmonary heart valves on allogenica cellular matrix conduits *in vivo* restoration of valve tissue. *Circulation,* **102**: 50 - 55.

Stobbe, J.L., Drake, K.D. and Maier, K.J. (2003). Comparison of *in vivo* (Draize method) and *in vitro* (Corrositex assay) dermal corrosion values for selected industrial chemicals. *International Journal of Toxicology*, **22**(2): 99 - 107.

Syama, S. and Mohanan, P.V. (2021). Microfluidic based human-on-a-chip: A scientific research. *Trends in Food Science and Technology,* **110**: 711 - 728.

Vijay, U., Gupta, S., Mathur, P., Suravajhala, P. and Bhatnagar, P. (2018). Microbial mutagenicity assay: Ames test. *Bio-protocol*, **8**(6): e2763 - e2763.

Wilson-Sanders, S.E. (2011). Invertebrate models for biomedical research, testing, and education. *ILAR Journal,* **52**: 126 - 152.

Xiang, M., Cao, Y., Fan, W., Chen, L. and Mo, Y. (2012). Computer-aided drug design: lead discovery and optimization. *Combinatorial chemistry and high throughput screening*, **15**(4): 328 - 337.

Zurlo, J., Rudacille, D. and Goldberg, A.M. (1996). The three Rs: the way forward. *Environmental Health Perspectives,* **104**: 878.