IJBI 1 (2), (DECEMBER 2019) 66-72

International Journal of Biological Innovations

Available online: http://ijbi.org.in | http://www.gesa.org.in/journals.php

DOI: https://doi.org/10.46505/IJBI.2019.1206

Review Article

E-ISSN: 2582-1032

Alternative Methods Replacing Animal Testing in Research: An Overview

Abhishek Singh[1*] and Preeti Singh[2]

[1]Department of Zoology, M.D.P.G. College, Pratapgarh (U.P.), India.
[2]Department of Pharmacology and Toxicology
College of Veterinary Science and A.H., DUVASU, Mathura (U.P.). India.
**Corresponding author:* abhizoology20@gmail.com

Received: 17.11.2019 Reviewed: 22.11.2019 Accepted: 05.12.2019

Abstract: The discovery of new drugs involves their testing on animals for efficacy as well as safety before the approval. Millions of animals are being sacrificed to fulfill this need. But experimentation on animals during the past years has created a matter of attention to the Institutional Animal Care and Use Committee (IACUC) for better care and handling of animals. The main aim of these committees is to reduce and finally exclude the animal use from the area of research with appropriate alternatives. Various alternative methods and organisms have been implemented and used respectively in this aspect in the last few years. Alternatives of animals serve the same purpose as that of utilizing whole animal for testing. The techniques such as in vitro method, computer model, stem cell, alternative organisms, use of biotechnology etc. might eventually replace the use of animals for testing and these approaches may provide an insight to minimum utilization of animals in s cientific research. Some alternatives of animal testing have been discussed in this article with some examples.

Keywords: Biological assays, Epidemiological survey, Ethical, *In silico*, Toxicity.

INTRODUCTION

Animals are used for various purposes including research purposes and medical technology development. Mice, rats, guinea pigs, hamsters, rabbits, dogs etc. are commonly used since long time for research purposes. Monkeys and birds are also used in some research purposes (Baumans, 2005). These animals are used in various drug screening and toxicological studies to develop new methods of treatment for infectious and non-infectious diseases and to understand the effect of several procedures of medical strategies and surgical experiments. Additionally, animals are also used in production of vaccines and antibiotics for diagnosis and treatment purposes of different diseases, predicting toxicity and other safety variables. Sometimes animals are euthanized to avoid later pain and distress after experimentation (Rusche, 2003). Several observational and experimental research provide evidence that animal suffer from physical and psychological pain, which is possibly neglected during animal research. The pain, distress and discomfort experienced by animals are critical debating is sue for a long time.

The ethical and scientific issues covering animal research rarely taken into underhand in organized and balanced forum despite of the vast debate of this forum. In addition to the concern of ethics, skilled man power requirement, time consuming protocols and high cost are also the disadvantages of using animals (Balls, 1994). Millions of experimental animals are used all over the world in every year. About 3.71 million animals were used for research in UK in the year 2011. The total number of animals used in Germany was about 2.13 million in 2001, while it was estimated about 1.13 million in USA in the year 2009 (Rusche, 2003). This huge population of experimental animals usually comes from the

breeding centers located in various Universities and National breeding centers. Considering the cruelty and discomfort during experimentation on animals, scientist moved on to think about different methods and alternatives of animals for disease study and testing of different drugs or products (Hendriksen, 2007, 2009; Giacomotto and Segalat, 2010).

Benefits of using animal alternatives

The alternatives used for animal testing are cost effective, easier, effective and reliable. Animal testing replacement does not put the patient at risks or hindering the medical progress. In spite of that, animal testing replacement will bring improved quality and humaneness in our science. Moreover, result of toxicity testing in human tissues is more accurate than of animal models. These are more practical, expedient and last not the least to believe that cruelty free products are more environmental friendly.

Various alternatives of animals testing

1. Computer (*in silico*) models

Computer model helps to design new medicine, studying of human and animal body structure and functions, cardiovascular risk, toxicological studies, body metabolism etc. This technique has value only when the representation of biological effect is done by known equation. These computer models do not form new information but they only simplify the ample amount of data and hypothesis to be tested. The findings obtained from in silico technique require their confirmation in whole animals (Roncaglioni and Benfenati, 2008). Computer Aided Drug Design (CADD) software is used to detect the binding site of a drug molecule. So, this is having advantages of avoiding the test of unwanted molecules possessing no efficacy and helps in reducing the use of number of animals. In CADD, target is first identified by different methods of genetics, molecular biology or bioinformatics. Thereafter, structure determination is done by X-ray crystallography and Nuclear Magnetic Resonance (NMR) spectroscopy. Biological assays are done by Molecular Modeling and Computer graphics. Synthetic chemistry of target is determined by Peptidomimetics and Combinatorial chemistry and clinical trials are finally made in the last (Kore *et al*., 2012).

Structure-based and ligand-based methods are the two general categories of CADD. In structure-based method, calculations of interaction energies of all the tested compounds are done on the basis of information of structure of structural protein. It is mostly preferred for soluble proteins which can be readily crystalized and having availability of high-resolution structural data of target protein. The main goal of structure-based CADD is to design the compounds that tightly bind to the target (Jorgensen, 2010). In ligand-based method, knowledge related to known active and inactive molecules are exploited by chemical similarity searches or by construction of predictive, Quantitative Structure Activity Relationship (QSAR) models (Kalyaanamoorthy and Chen, 2011). This method is generally preferable for membrane protein targets, when negligible or little information about the structural protein is available. QSAR is used to predict the biological activity of a drug molecule (Knight *et al*., 2006). In QSAR-based drug discovery project, different groups of active and inactive ligands are first collected and then set of mathematical descriptors are created which describe the structural and physiochemical properties of the collected compounds. Further, a model is generated to identify the relationship between those descriptors and their experimental activity, which maximizes the predictive value (Zhang *et al*., 2011). These computer models are good in predicting the carcinogenic and mutagenic property of a drug. Molecular structure of drug that is protease inhibitors has been designed by computer data base to perform testing in human tissue cultures for the purpose of development of treatment in HIV patient.

2. *In vitro* testing

In vitro testing can be used as an important alternative of animal testing. In this, cell, tissue and organ of different animals are taken outside and grown in vitro in laboratory environment under suitable growth condition for a period of few years. Although, animals are needed for these in vitro systems, but in this they experience pain, distress and suffering for a shorter period of time in comparison to whole animal testing. This is because animals are sacrificed before experimentation. The benefits associated with in vitro cultures system are that different organs can be collected from a single animal, which makes a perfect use of whole animal, easy to perform, consume less time and less expenditure required. In in vitro culture, animal cells are isolated and grown over the surface of culture plate as a monolayer. Cellular enzymes and membrane component can also be utilized. Although cell or tissue culture method has minimized the use of intact animals for experimentation, but still animal-derived serum are required for the maintenance of these cells in culture. Approximately, every year one million fetal cows are sacrificed to produce the fetal bovine serum to supply all over the world for the growth of cultured cells (Brunner *et al*., 2010). These methodologies are used in drug research, preliminary screening of chemicals, drug molecules, cosmetics etc. to evaluate their efficacy and toxicological effects (Shay and Wright, 2000; Steinhoff *et al*., 2000). Additionally, these techniques are also used in testing of newly produced chemicals and drugs on skin of human, toxicological studies and production of monoclonal antibodies. The isolated components also have disadvantages and the limitations of in vitro testing deals with its inability to generate complete physiological response of whole organism. It is because the components on isolation from the animal become undifferentiated and unable to pursue their special functional capacity. Secondly, it is impossible to determine the variable effects associated with the route of exposure due to which test results may be affected.

Eye irritancy test

Earlier, a test named as Draize test was used to check the irritancy of chemicals in eye. This test requires a new animal (especially rabbit) for each experiment and it is a very painful

process. Considering this pain, an alternative method named as bovine corneal organ culture have been developed for in vitro testing the toxicological effects of chemical's irritancy (Xu *et al*., 2000). Whole eyes of bovine used for in vitro testing are readily obtained from slaughter house (Burton et al., 1981). Additionally, several types of cell cultures such as rabbit and human corneal cells, cells of human hepatoma, hamster fibroblast and mouse macrophages are also used for *in vitro* eye irritation test (Nardone and Bradlaw, 1983; Shopsis et al., 1984).

Skin corrosion and irritation

In vitro techniques are also used to find the toxic effects of a substance to skin on topical exposure. Uses of human skin equivalent tests are being adopted in place of corrosive and irritating studies on skin of animals. Cells of human skin have been cultured to produce human skin models such as Epiderm, Episkin and Skin Ethic RHE. These replacements have been accepted worldwide including Canada and European Union. European Union has accepted another method to measure irritation of skin and dermal corrosion to replace Draize rabbit skin test in which Human epidermal keratocytes is cultured to mimic the human epidermis (Schäfer-Korting *et al*., 2008).

Skin absorption

Percutaneous absorption and everted sac methods are some in vitro techniques of absorption testing (Arora *et al*., 2011). Several tissue culture methods which measure the rate of chemical absorption by the skin have been approved by Organization for Economic Cooperation and Development (OECD). Excised skin from different sources including human is collected and proper care is taken, avoiding damage to the stratum corneum, so that permeability of skin is maintained through diffusion barrier after excision from the body. It has been proved that skin has the capability to metabolize some of chemicals during percutaneous absorption (Bronaugh and Maibach, 1991). Test substance, including radio-labeled substance, is applied on skin surface sample which separate the two different chambers of diffusion cells. The test substance remains on the skin for a definite time under a specific condition until removed by a suitable chemical. The fluid of receptor is collected at different point of experiment throughout the experiment and test chemical is analyzed. Finally distribution of test chemicals and its metabolites are quantified using a ppropriate methods. Analysis of receptor fluid and treated skin is done to determine the absorption of the test substance.

Phototoxicity

Phototoxicity is a condition in which toxicity of drugs and chemical occurs following exposure to sunlight. OECD has approved 3T3 neutral red phototoxicity test in which mouse-embryo derived cell lines are used to measure the degree of cell toxicity of the cultures or to compare the cytotoxicity of a chemical in the culture after exposure, in the presence and absence of ultraviolet-A light (Ranganatha and Ku ppast, 2012). There is concentration-dependent decrease of uptake of 3T3 in the test animal after the treatment with the test substances and UV-A light and degree of cytotoxicity is measured in this test (Borenfreund and Puerner, 1985). Neutral red is a weak cationic dye that readily accumulates in lysosomes by penetrating cell membrane in normal condition but in xenobiotic toxicity, alteration of cell surface receptors occurs causing lysosomal frangibility and other changes.

As a result of such changes of xenobiotic, there is decrease uptake and binding of neutral red. 3T3 cells are maintained in culture in monolayer formation to a period of 24 h and pre-incubation of 96 well plates per test chemicals with eight different concentrations of test chemicals is done for 1 h. Further one plate is exposed to non-cytotoxic irradiation dose and the other one is placed in dark. Then, treatment medium of both the plates is replaced by culture medium and cell viability is determined by uptake of neutral red after an incubation period of 24 h.

Pyrogenicity

Sacrificing of crabs and rabbits to study the fever producing products or pyrogens is avoided now days. Presently, use of human isolated cells, cell lines and incubation of donated whole blood are in demand (Ranganatha and Ku ppast, 2012). These can be also used to study immuno-stimulant and immuno-suppressant drugs.

Mutagenicity test

The *in vitro* mutagenicity test is done by using culturing mammalian cells which are exposed to toxigenic substances. Mouse lymphoma cell line or hamster ovary cell line are also generally used (Słoczyńska et al., 2014). These cell cultures are exposed to a test substance and surviving ability of cell is determined by metabolizing 8-azaguanine or 6-thioguanine, which indicates capability of test substance in causing mutation.

Hepatotoxicity

In vitro use of perfused liver, liver cell suspension and liver cell cultures have been developed as an alternative, but limited focus has been given in this aspect. In this, viability period for cell is limited and it is not a reproducible phenomenon (Rowan and Goldberg, 1985).

Stem cells

Stem cells can be used as an alternative of animal testing in disease and toxicological study. Petri dish is used for the growth and differentiation of embryonic stem cells into different type of cells that leads to the generation of a human organ. Genes which causes disease are inserted into embryonic stem cells, further induced to human disease tissue for differentiation that can be used for drugs screening. These have values in assessing the toxicological effect of a drug. Many scientists have developed different embryonic stem cell line genes using the genes from Parkinson's disease, Alzheimer's disease and diabetes for the screening of different drugs so that treatment can be done. Stem cell models give a better alternative to study the various types of cancers, liver and cardiac toxicity (Bremer and Hartung,

2004). Stem cells provide a potential for testing the drug toxicity in biotechnological companies and pharmaceutics to avoid wastage of time on several harmful substances. Currently, embryonic stem cells derived from human cardiac tissue are successfully used for toxicological testing of disease. Researches are going on the development of stem cells models of the liver (Bremer and Hartung, 2004). There are certain disadvantages of using stem cells as an alternative such as inside the organism uncontrolled growth and formation of teratoma can occurs. Furthermore, stem cell are unable to anticipate the effect of a drug's subsequent metabolite inside the whole living body as it throw back response of a single organ inside the petridish.

Non- invasive imaging techniques

Imaging techniques such as Magnetic Resonance Imaging (MRI), functional MRI (fMRI), single-photon emission computed tomography, positron emission tomography, event-related optical signals, magnetoencephalography and transcranial magnetic stimulation allow to see the areas inside the body especially the brain giving us the information about the structure and function of brain that is impossible to be studied in animals (Balls, 1994). Certain limitations of imaging techniques are that it provides invaluable information and due to low resolution whole part of brain having different types of brain cells is seen rather than of individual cells.

Epidemiologic data on humans

Epidemiological survey on humans is a useful alternative to animal testing. In this, existing data or data of previously exposed species is studied for finding the correlation in lifestyle situation in populations. In the 18th century, these studies were used for detection of cancer in humans. Scrotal cancer in chimney sweeps, smoking leading to cancer, heart disease by high cholesterol, spina bifida in pregnancy by folic acid deficiency were detected by epidemiological data. Recently it has been reported that alcohol consumption leads to risk of glioblastoma (Baglietto et al., 2011). Disadvantage of epidemiological studies is that, a disease which develops after a longer period of time, human exposure can occur before the detection of toxic effect and it is also more or less expensive to perform.

Microfluidics chips

Microfluidic chips are just 2 cm wide and have tissue samples from different body parts into a series of tiny chambers. These chambers are connected by micro-channels through which substitute of blood flows that mimic the pathways and processes occurring in the whole body parts on a micro scale (Bunney *et al*., 2003). The drug to be tested is mixed in the blood substitutes and circulated around the device. Chip has a sensor which signals feedback information for computer analysis. These are used for study of biological and disease processes and metabolism of drug. The limitation of micro-fluiding chip is that it provides less information than whole body testing.

Micro-dosing

Micro-dosing is a more recent and excellent method for study of drug metabolism through the administration of doses of drugs too small to produce a cellular or pharmacological effect in human volunteers without producing adverse effect in the whole body system of human (Jenkins *et al*., 2002). Use of micro-dosing relies on accelerator mass spectrometry which is highly sensitive and detects pg/ml concentration of substances present in blood and plasma. This also detects individual molecules which are radiolabeled with carbon-12. Micro-dosing lowers the discrepancy between human and animal reaction to a drug. Screening out of drugs is faster and cheaper in micro-dosing. Limitation of micro-dosing is that it considers only phase 0 clinical trial of drug and full dose testing is required for safety, efficacy and approval of drug. Micro-dosing also only test small dose of drugs but unable to predict the pharmacological effect of higher dose of drug (Garner and Lappin, 2006).

DNA chips

DNA chips are made up of glass slides in which DNA fragments or array of genes are fixed. These are used to study the pharmacogenetics of drugs so that personalized treatment can be done. DNA sample present in chips are tagged with a fluorescent dye, made in contact to a new drug, after which washing is done over the chip. When the gene present on chip get match with sample DNA, sticking occurs and formation of colors in a pattern of light appears. This reveals that experimental drug has either activated or suppressed the genes. Drug designing for a particular individual can be done by this technique (Nuwaysir *et al*., 1999).

Analysis of plants

Success of plant substitution is limited in animal research. Moreover studies had been done to demonstrate the effect on certain plants due to exposure of a substance which are closely related to humans. Recently, effect of environmental contaminants such as pharmaceuticals residues on Brassica juncea is studied. It was found that oxidative stress detoxification mechanism activated and drug-induced defense response generated (Bartha *et al*., 2010).

Physico-chemical techniques

These techniques are done in vitamin and drug researches and help to find human responses with respect to chemical and biological substances (Balls, 1994). The complex substances are separated by Gas chromatography and identified and measured by mass spectrometry. Chitosan films are used as an alternative for animal and human cadaver epidermal sheets and are also used for study of preliminary in vitro permeation of both polar and nonpolar drugs (Rana *et al*., 2004). Currently, the antimicrobial activity of certain plants extracts (Thymus vulgaris, Matricaria chamomilla, Croton lecheleri, Calendula officinalis) is tested against the periodontal pathogens (Porphyromonas gingivalis and Aggregatibacter actinomycetemocomitans) by using chitosan films as local delivery systems (Rodriguez-Garcia, 2010).

Alternative organisms
Several model organisms are also used as an alternative of higher model vertebrates like rats, mice, dogs, guinea pig etc. due to ethical issues on these animals.

Lower vertebrates
These can be an attractive option due to less ethical issues and genetic relatedness of lower vertebrates with higher vertebrates. Danio rerio commonly known as zebra fish are used to detect the toxicological effects of various chemicals and pharmaceuticals, cancer detection, diseases of heart, neurological dysfunctioning, behavioral problems and investigation of mutations and malfunctioning in development of organ (Peterson, 2008). The uses of lower vertebrates as alternative animals also reduces the space requirement for performing the experiment, cost, test chemicals, manpower etc. (Hill, 2005).

Invertebrates
Invertebrates due to their small size, simplified anatomy, brief life cycle, less cost on housing in comparison of animals are widely preferred as an alternative of animals. Thousands of flies can be kept at a place where only a small number of mice are accommodated (Wilson-Sanders, 2011).These are used in the study of Parkinson's disease, muscular dystrophy, wound healing, dysfunctioning of endocrine and memory, cell aging, apoptosis, biological study of retrovirus, diabetes and toxicological studies (Lagadic and Caquet, 1998). Drosophila melanogaster, commonly known as fruit fly, have 75% of functional homology with human genes and lower cost is required for its maintenance, propagation and screening in comparison to other models of mammals (Baglietto, 2011; Reiter, 2001; Gilbert, 2008). Each of four stages of fruit fly has been considered as a multiple model organism for studying different concepts (Pandey and Nichols, 2011). Embryo is frequently used for the study of cell fate determination, development of neuron, organogenesis etc. and larva is generally used for the study of developmental and physiological processes. Several structure such as heart, lungs, gut, kidneys and reproductive tract of fruit fly have functional similarity to mammals (Rothenfluh and Heberlein, 2002). Fruit fly is also used to study the human genetics, diseases and investigation of many neurodegenerative diseases such as Alzheimers, Huntingson's and Parkinson's disease (Iijima and Iijima-Ando, 2008). Caenorhabditis elegans, a eukaryotic nematode, is commonly referred as a model or organism for research purposes due to its characteristics of simple cellular structure, transparency and genetically persuasion (Strange, 2007). It is used to study certain neurological disorders, immune disorders, cancer and diabetes (Artal-Sanz *et al*., 2006; Pujol *et al*., 2008). Some limitations are also associated with the use of invertebrates such as underdeveloped organ systems which do not possess an adaptive immune system.

Microorganisms
Microorganisms are mostly used for mutagenicity, carcinogenicity, autopsy, cell death regulators and toxicological studies (Madeo *et al*., 2002). Saccharomyces cerevesiae is the most commonly used microorganisms due to its property of rapid growth, dispersed cells, clearly explained genetic makeup, ease in isolation of mutant cells, inconstant system of DNA transformation and easy to analyze after its growth in large population (Mell and Burgess, 2002). Yeast is also used to study the endogenous or heterologous proteins so that the fundamental aspect of neurodegenerative diseases such as Alzheimer's, Parkinson's and Huntingston's diseases can be known (Siggers and Lesser, 2008). Fungus such as Cunninghamella elegans have the ability to metabolize different variety of drug so used for study of drug metabolism (Sharma *et al*., 2011). The fungus is also used to test vast variety of drugs such as diuretics, anticonvulsants, anti-coagulants and haemorheologic agents. Recently a bacteria named as Vibrio vulnificus was used to study the toxic RtxA 1 (RtxA 1 is a gene that encode multifunctional-auto processing RTX, a toxin produced by Vibrio vulnificus) modulation responsible for causing acute toxicity and mostly used for treatment of infectious diseases now a days (Kim *et al*., 2010). Benefit of using microorganism as an alternative are easy to handle, non-mammalian, mostly predictable (revealing how the development of drug is taking place), reduce the use of number of animals, but cannot purely replace them (Zurlo *et al*., 1983).

Dummies or stimulators
These are mainly used to teach the surgical draping, incision making on animals, sterile techniques, suturing of different layers, assessment of jugular vein etc.

Rubber koken rat
These are used to train the person for handling, collection of blood from intravenous and lateral vein and oral gavage.

Slaughter house material
Organ or tissue samples obtained from slaughter houses can be used for study different physiological and pharmacological effects of drugs.

CONCLUSION

The animal ethics are an important issue related to human welfare and efforts are needed to replace the use of animals in experimentation. Various animal alternatives presently available are in urge of being implemented in effective manner and this will lead to less involvement of animals in scientific studies. Resources available in the organization play pivotal role to speed up the strategies for development of use of animal alternatives.

Possible appropriate alternatives can be used instead of animals for testing a new drug before its use in patients. Use of alternative methods help to minimize the number of animals needed for research of drug, but they will not completely remove the animal use from preclinical studies. Thus an approach should be made in practice so that alternative of animals can help in minimizing the use of animals related to animal experimentation in future.

REFERENCES

1. **Arora T., Mehta A. K., Joshi V., Mehta K. D., Rathor N., Mediratta P. K. and Sharma K.K.** (2011). Substitute of Animals in Drug Research: An Approach towards Fulfillment of 4R's. *Indian J. Pharm. Sci*. 73(1): 1–6.
2. **Artal-Sanz M., de Jong L. and Tavernarakis N.** (2006). Caenorhabditis elegans: a versatile platform for drug discovery. *Biotech. J.* 1 (12): 1405–1418.
3. **Baglietto L., Giles G.G., English D.R., Karahalios A., Hopper J.L. and Severi G.** (2011). Alcohol consumption and risk of glioblastoma; evidence from the Melbourne collaborative cohort study. *Int. J. Cancer*. 128 (8): 1929–1934.
4. **Balls M.** (1994). Replacement of animal procedures: alternatives in research, education and testing. *Lab. Anim*. 28 (3): 193–211.
5. **Bartha B., Huber C., Harpaintner R. and Schröder P.** (2010). Effects of acetaminophen in Brassica juncea L. Czern.: Investigation of uptake, translocation, detoxification, and the induced defense pathways. *Environ. Sci. Pollut. Res. Int*. 17 (9): 1553–1562.
6. **Baumans V.** (2005). Science-based assessment of animal welfare: laboratory animals. *Revue Scientifique et Technique*. 24 (2): 503-513.
7. **Borenfreund E. and Puerner J. A.** (1985). Toxicity determined in vitro by morphological alterations and neutral red absorption. *Toxicology Lett*. 24 (2-3): 119-124.
8. **Bremer S. and Hartung T.** (2004). The use of embryonic stem cells for regulatory developmental toxicity testing in vitro--the current status of test development. *Curr. Pharm. Des*. 10 (22): 2733–2747.
9. **Bronaugh R.L. and Maibach H.I.** (1991). *In vitro* Percutaneous Absorption: Principles, Fundamentals and Applications. CRC Press, Boca Raton.
10. **Brunner D., Jürgen F., Helmut A., Harald S., Walter P. and Gstraunthaler G.** (2010). Serum-free Cell Culture: The Serum-free Media Interactive Online Database. *Altex*. 27 (1): 53–62.
11. **Bunney W.E., Bunney B.G., Vawter M.P., Tomita H. Li. J., Evans S. J., Choudary P.V., Myers R. M., Jones E.G., Watson S.J. and Akil H.** (2003). Microarray technology: A review of new strategies to discover candidate vulnerability genes in psychiatric disorders. *Am. J. Psychiatry*. 160 (4): 657–666.
12. **Burton A.B.G., York M. and Lawrence R. S.** (1981). "The In Vitro Assessment of Severe Eye Irritants, "Food Cosmet. *Toxicol*. 19 (4): 471-480.
13. **Garner R.C. and Lappin G.** (2006). The phase 0 microdosing concept. *Br. J. Clin. Pharmacol*. 61(4): 367-370.
14. **Giacomotto J. and Segalat L.** (2010). High-throughput screening and small animal models, where are we? *Br. J. Pharmacol*. 160 (2): 204–216.
15. **Gilbert L.I.** (2008). Drosophila is an inclusive model for human diseases, growth and development. *Mol. Cell Endocrinol*. 293 (1-2): 25–31.
16. **Hendriksen C.F.** (2007). Three Rs achievements in vaccinology. *AATEX*. 14 (special issue): 575–579.
17. **Hendriksen C.F.** (2009). Replacement, reduction and refinement alternatives to animal use in vaccine potency measurement. *Expert Rev. Vaccines*. 8 (3): 313–322.
18. **Hill A.J., Teraoka H., Heideman W. and Peterson R.E.** (2005). Zebra fish as a model vertebrate for investigating chemical toxicity. *Toxicol. Sci*. 86 (1): 6–19.
19. **Iijima K. and Iijima-Ando K.** (2008). Drosophila models of Alzheimer's amyloidosis: The challenge of dissecting the complex mechanisms of toxicity of amyloid-beta 42. *J. Alzheimers Dis*. 15 (4): 523–540.
20. **Jenkins E.S. Broadhead C. and Combes R.D.** (2002). The implications of microarray technology for animal use in scientific research. *Altern. Lab. Anim*. 30 (4): 459–465.
21. **Jorgensen W.L.** (2010). Drug discovery: Pulled from a protein's embrace. *Nature*. 466 (7302): 42–43.
22. **Kalyaanamoorthy S. and Chen Y.P.** (2011). Structure-based drug design to augment hit discovery. *Drug Discov. Today*. 16 (17-18): 831–839.
23. **Kim J.R., Cha M.H., Oh D.R., Oh W.K., Rhee J.H. and Kim Y.R.** (2010). Resveratrol modulates RTX toxin-induced cytotoxicity through interference in adhesion and toxin production. *Eur. J. Pharmacol*. 642 (1-3): 163–168.
24. **Knight A., Bailey J. and Balcombe J.** (2006). Animal carcinogenicity studies: 3 alternatives to the bioassay. *Altern Lab Anim*. 34 (1): 39-48.
25. **Kore P.P., Mutha M.M., Antre R.V., Oswal R.J. and Kshirsagar S.S.** (2012). Computer-Aided Drug Design: An Innovative Tool for Modeling. *Open J. Med. Chem*. 2 (4): 139-148.
26. **Lagadic L. and Caquet T.** (1998). Invertebrates in testing of environmental chemicals: are they alternatives? *Environ. Health Perspect*. 106 (suppl 2): 593-611.
27. **Madeo F., Engelhardt S., Herker E., Lehmann N., Maldener C., Proksch A. and Frohlich K.U.** (2002). Apoptosis in yeast: a new model system with applications in cell biology and medicine. *Curr. Genet*. 41 (4): 208–216.
28. **Mell J.C. and Burgess S.M.** (2002). Yeast as a model genetic organism. In: Encyclopedia of Life Sciences. Macmillan Publishers Ltd, Nature Publishing Group. 1-8.
29. **Nardone R. M. and Bradlaw J.** (1983). Toxicity Testing With In Vitro Systems: I. Ocular Tissue Culture. *Journal of Toxicology: Cutaneous and Ocular Toxicology*. 2(2-3): 81-98.

30. **Nuwaysir E.F., Bittner M., Trent J., Barrett J.C. and Afshari C.A.** (1999). Microarrays and toxicology: the advent of toxicogenomics. *Mol. Carcinog*. 24(3): 153-159.

31. **Pandey U.B. and Nichols C.D.** (2011). Human disease models in Drosophila melanogaster and the role of the fly in therapeutic drug discovery. *Pharmacol. Rev*. 63 (2): 411–436.

32. **Peterson R.T., Nass R., Boyd W.A., Freedman J.H., Dong K. and Narahashi T.** (2008). Use of non-mammalian alternative models for neurotoxicological study. *Neurotoxicology*. 29(3): 546–555.

33. **Pujol N., Cypowyj S., Ziegler K., Millet A., Astrain A., Goncharov A., Jin Y., Chisholm A.D. and Ewbank J.J.** (2008). Distinct innate immune responses to infection and wounding in the *C. elegans* epidermis. *Curr. Biol*. 18 (7): 481–489.

34. **Rana V., Babita K., Goyal D., Gorea R and Tiwary A.** (2004). Optimization of chitosan film as a substitute for animal and human epidermal sheets for in vitro permeation of polar and nonpolar drugs. *Acta Pharm*. 54 (4): 287–299.

35. **Ranganatha N. and Kuppast I. J.** (2012). A review on alternatives to animal testing methods in drug development. *Int. J. Pharm. Pharm. Sci*. 4 (suppl. 5): 28-32.

36. **Reiter L.T., Potocki L., Chien S., Gribskov M. and Bier E.** (2001). A systematic analysis of human disease-associated gene sequences in Drosophila melanogaster. *Genome Res*. 11 (6):1114–1125.

37. **Rodriguez-Garcia A., Galan-Wong L.J. and Arevalo-Niño K.** (2010). Development and in vitro evaluation of biopolymers as a delivery system against periodontopathogen microorganisms. *Acta. Odontol Latinoam*. 23 (2): 158–163.

38. **Roncaglioni A. and Benfenati E.** (2008). In silico-aided prediction of biological properties of chemicals: Oestrogen receptor-mediated effects. *Chem. Soc. Rev*. 37 (3): 441–450.

39. **Rothenfluh A. and Heberlein U.** (2002). Drugs, flies, and videotape: the effects of ethanol and cocaine on Drosophila locomotion. *Curr. Opin. Neurobiol*. 12 (6): 639–645.

40. **Rowan A. N. and Goldberg A. M.** (1985). Perspectives on Alternative to Current Animal Testing Techniques in Preclinical Toxicology. *Ann. Rev. Pharmacol. Toxico*. 25: 225-247.

41. **Rusche B.** (2003). The 3 Rs and animal welfare-conflict or the way forward. *ALTEX*. 20 (suppl. 1): 63–76.

42. **Schäfer-Korting M., Bock U., Diembeck W., Düsing H.J., Gamer A., Haltner-Ukomadu E., Hoffmann C., Kaca M., Kamp H., Kersen S., Kietzmann M., Korting H.C., Krächter H.U., Lehr C.M., Liebsch M., Mehling A., Müller-Goymann C., Netzlaff F., Niedorf F., Rübbelke M.K., Schäfer U., Schmidt E., Schreiber S., Spielmann H., Vuia A. and Weimer M.** (2008). "The use of reconstructed human epidermis for skin absorption testing: Results of the validation study". *Altern Lab Anim*. 36 (2): 161–87.

43. **Sharma K.K., Mehta T., Joshi V., Mehta N., Rathor A.K., Mediratta K.D. and Sharma P.K.** (2011). Substitute of animals in drug research: An approach towards fulfillment of 4R's. *Indian J. Pharm. Sci*. 73(1): 1–6.

44. **Shay J.W. and Wright W.E.** (2000). The use of telomerized cells for tissue engineering. *Nat. Biotech*. 18 (1): 22–23.

45. **Shopsis C., Borenfreund E., Walberg J.** *et al*., (1984). "In Vitro Cytotoxicity Assays as Potential Alternatives to the Draize Ocular Irritancy Test," Alternative Methods in Toxicology Alternative Approaches, A.M. Goldberg (cd.) (New York: Mary Ann Leibert, Inc.

46. **Siggers K.A. and Lesser C.F.** (2008). The yeast Saccharomyces cerevisiae: a versatile model system for the identification and characterization of bacterial virulence proteins. *Cell Host Microbe*. 4 (1): 8–15.

47. **Słoczyńska K., Powroźnik B., Pękala E. and Waszkielewicz A.M.** (2014). Antimutagenic compounds and their possible mechanisms of action. *Journal of Applied Genetics*. 55(2): 273-285.

48. **Steinhoff G., Stock U., Karim N., Mertschin H., Timke A., Meliss R.R. and Bader A.** (2000). Tissue engineering of pulmonary heart valves on allogenic acellular matrix conduits in vivo restoration of valve tissue. *Circulation*. 102 (19 suppl.3): 50–55.

49. **Strange K.** (2007). Revisiting the Krogh principle in the post-genome era: Caenorhabditis elegans as a model system for integrative physiology research. *J. Exp. Biol*. 210 (Pt.9): 1622–1631.

50. **Wilson-Sanders S.E.** (2011). Invertebrate models for biomedical research, testing, and education. *ILAR. J*. 52 (2): 126–152.

51. **Xu K.P., Li, X.F. and Fu-Shin X.Y.** (2000). Corneal organ culture model for assessing epithelial responses to surfactants. *Toxicol. Sci*. 58 (2): 306–314.

52. **Zhang S.** (2011). Computer-aided drug discovery and development. *Methods Mol. Biol*. 716: 23–38.

53. **Zurlo J., Rudacille D. and Goldberg A.M.** (1983). Animals and alternatives in toxicity testing. London: Academic Press. 502p.