Alternatives to Animal Experiments in Research and Regulatory Testing

7

Shikha Yadav and Vijay Pal Singh

Abstract

The term "alternatives" is used to describe any method resulting in the replacement of animals or reduction in the numbers used in that procedure or refinement of techniques that may minimize pain and suffering in the animals. Every year, we use millions of experimental animals of various species in biomedical research and in testing to assess the safety and effectiveness of drugs, cosmetics, and chemicals. The pain and suffering that these animals experience during these scientific experiments have always been an issue of serious debate. Several scientists, organizations, and institutes across the globe are working for developing and validating alternative methods, and progress has certainly been made in replacing methods using animals that have been in use for several years now with alternate methods. Some of these alternate methods have become a standard practice due to being more ethical, safe, cost-effective, quick, and accurate. However, it is certainly time now that the scientific communities consider using the available alternative methods like computer models, cell and tissue cultures, microorganisms, invertebrates, lower vertebrates, human tissues, and volunteers wherever possible and prioritize the replacement of animals over the refinement and reduction strategies. One of the most effective ways to advance this vision would be by increasing awareness in the scientific community about the available alternative methods and by sharing knowledge through education and training.

S. Yadav (✉)
National Institute of Biologicals, Ministry of Health and Family Welfare, Government of India, Noida, UP, India
e-mail: syadav@nib.gov.in

V. P. Singh
FSSAI, Ministry of Health and Family Welfare, Government of India, New Delhi, India
e-mail: vp.singh@igib.res.in

© The Author(s), under exclusive license to Springer Nature Singapore Pte Ltd. 2021

P. Nagarajan et al. (eds.), *Essentials of Laboratory Animal Science: Principles and Practices*, https://doi.org/10.1007/978-981-16-0987-9_7

The purpose of this chapter is to make the readers aware of the available alternative methods that they may use to replace, reduce, or refine the use of animals and to provide guidance on some of the available resources where they can look for these methods. We can certainly be hopeful that with the new, stricter regulations and smarter and scientifically validated alternatives being developed by scientists, the number of animals that are currently being used in research, testing, and education will continue to decline in the future.

Keywords

Alternative methods · In silico methods · Prediction of toxicity · Tissue cultures · Organ-on-a-chip models · Microorganisms · *S. cerevisiae* · *Salmonella* assay · *Drosophila melanogaster* · *Caenorhabditis elegans* · Zebrafish · Human volunteers

7.1 Introduction

The term "alternatives" is used to describe any method which results in the replacement of animals or reduction in the numbers used in that procedure or refinement of techniques that may minimize pain and suffering in the animals. This term was first introduced by David Henry Smyth in his book *Alternatives to Animal Experiments* in 1978, and it can be seen that Smyth's term "alternatives" is synonymous with the term "three Rs," i.e., replacement, reduction, and refinement which was given by W.M.S. Russell and R.L. Burch in their book *The Principles of Humane Experimental Technique* in 1959. Every year, we use millions of experimental animals of various species like mice, rats, hamsters, rabbits, guinea pigs, birds, dogs, nonhuman primates, etc., in biomedical research and in testing to assess the safety and effectiveness of drugs, cosmetics, and chemicals. The use of these laboratory animals in biomedical and behavioral research has helped to significantly increase our scientific knowledge and has contributed enormously to the betterment of human health by helping prevent, cure, and treat a large number of diseases. The laboratory animals also help us as tools in understanding the effects of several medical procedures and surgical experiments. However, the pain and suffering experienced by these animals during these experiments have always been an issue of serious debate. Animal rights activists and anti-vivisectionist groups find animal experimentation to be very cruel and unnecessary and call for the total abolition of all animal research regardless of its purpose or benefit to mankind. This approach too would certainly have some very severe consequences on the advancement of scientific research. And, it is equally important to mention here that scientists themselves do not want to use or cause unnecessary pain and suffering to the animals and that is why they readily accept all the regulatory and ethical controls over the use of animals in education, research, and testing. The scientific community is striving continuously to develop suitable alternatives. It includes "absolute replacement," where animals are not required at all, or "relative replacement," where a less sentient animal species is used. The purpose of this chapter is also to make the readers aware of the available alternative

methods that they may use to replace, reduce, or refine the use of animals and to provide guidance on some of the available resources where they can look for these methods. In the last few decades, a large number of promising non-animal methods have been developed such as complex cell cultures, computational models, organ-on-a-chip, etc., but they have not been very successful in replacing the animals completely. This is because they have not been able to mimic the diversity of different cell types and tissues present in a living organism; the complex interactions that occur between various cells, tissues, and organs in various locations in the body; and the influence of the tissue organization on the cellular environment. Although cell culture and tissue culture models may answer many questions about the molecular, cellular, tissue, and even organ functions, animal models are certainly required to investigate how the tissues and organ systems interact with each other.

7.2 3Rs: Centers, Institutes, and Databases

Although researchers are constantly working on ways to develop alternatives, it may not be possible to eliminate animal experiments in the near future. The actual shift from animal models to alternatives would depend on how well they reflect the complex human biology. But presently, it is certainly important for scientists to seriously consider using the available validated alternative methods and start prioritizing replacement over the refinement and reduction strategies. One of the most effective ways to advance this vision would be by increasing awareness among the scientific community about the available alternative methods and by sharing knowledge through education and training.

Several organizations and institutes across the globe are working dedicatedly for the effective implementation of the 3Rs, specifically for developing and validating alternative methods that can replace the use of animals in education, research, and regulatory testing. They also provide a lot of useful information about the 3Rs and available alternatives on their websites. Information on these institutes and their aims and missions are provided in Table 7.1, which may be helpful to the readers to obtain appropriate and updated information about the available alternatives as well as about the ones which are under the process of validation.

7.3 Alternative Methods: Historical Perspective

Continuous efforts are being made by the scientific community to replace animal tests since the 1960s. Earlier, to confirm pregnancy in a woman, her urine was injected into immature rabbits, and the human chorionic gonadotropin (hCG) in the sample of pregnant women would induce ovulation, which was detected only after the rabbit was killed. This test was replaced as gonadotropin could be detected directly using chemical tests to confirm pregnancy. Polio vaccine was used to be produced in primary monkey kidney cells for which a large number of monkeys were killed every year. By the 1970s, use of long-lived human or monkey cell

Table 7.1 Organizations working for alternatives to animal experiments

Organization	Main objectives and activities
Fund for the Replacement of Animals in Medical Experiments (FRAME) (established in London in 1969) https://frame.org.uk/	Promotes 3Rs and in particular "replacement" by validated reliable and reproducible alternative methods Works for eliminating the need to use laboratory animals in any kind of medical or scientific procedures
The Johns Hopkins Center for Alternatives to Animal Testing (CAAT) (established in the USA in 1981) https://caat.jhsph.edu/about/index.html	Promotes development of in vitro and other alternative techniques Facilitates acceptance and implementation of alternative methods Provides reliable information on alternatives to academia, government, industry, and the general public Educates and trains in the application of alternatives Website "Altweb" devoted to 3Rs and alternatives news and information Publishes open-access articles on alternatives to animal experimentation through *ALTEX* journal
European Centre for the Validation of Alternative Methods (ECVAM) (established in EU in 1991) https://ec.europa.eu/jrc/en/eurl/ecvam	Works for development, validation, and acceptance of methods to replace, reduce, or refine the use of animals in laboratories Developed publicly accessible Database on Alternative Methods to Animal Experimentation (DB-ALM) that provides summaries and protocols on alternative methods Developed Tracking System for Alternative Methods toward Regulatory Acceptance (TSAR) which tracks the progress of alternative methods for testing chemicals or biologicals like vaccines from time of submission to validation and final regulatory acceptance as a recognized test method. https://tsar.jrc.ec.europa.eu/test-methods?
The Interagency Coordinating Committee on the Validation Of Alternative Methods (ICCVAM) (established in the USA in 2000) https://ntp.niehs.nih.gov	Facilitates collaborations to promote development, regulatory acceptance, and use of alternative test methods Provides guidance to scientists developing alternative test methods Evaluates recommendations from expert peer reviews of alternative toxicological test methods and makes recommendations to federal agencies on their use The website provides details of alternative methods for chemical safety testing that have been accepted by the US and international regulatory authorities

(continued)

Table 7.1 (continued)

Organization	Main objectives and activities
Japanese Centre for the Validation of Alternative Methods (JaCVAM) (established in Japan in 2005) https://www.jacvam.jp/en/index.html	Promotes the 3Rs in animal experiments for the evaluation of chemical substance safety in Japan Collaborates internationally to establish guidelines for new alternative methods
Norwegian Reference Centre for Laboratory Animal Science and Alternatives (NORECOPA) (established in Norway in 2007) https://norecopa.no/alternatives/alternatives-to-animal-research-and-testing	National Consensus Platform of Norway for the advancement of "the 3Rs" in animal experiments Maintains databases like the following: Text Base that provides information on textbooks available in the field of laboratory animal science https://norecopa.no/textbase-database 3R Guide that contains 3R resources for those planning or conducting animal experiments https://norecopa.no/3r-guide-database NORINA database having information on more than 3000 audiovisual aids which can be used as alternatives in education and training https://norecopa.no/norina-database Inventory of 3Rs, Education and Training Courses and Resources that was produced by EU https://norecopa.no/european-commission-inventory-of-3rs-education-training-resources
Korean Centre for the Validation of Alternative Methods (KoCVAM) (established in Korea in 2009) http://www.nifds.go.kr/kocvamen/	Formulates and promotes policies concerning the development and approval of alternatives to animal testing Provides information regarding alternative test methods and educational programs for industry, academia, and research institutions Coordinates the process of validating and peer-reviewing alternative test methods and proposing validated test methods to the OECD for guidelines
Brazilian Centre for the Validation of Alternative Methods (BraCVAM) (established in Brazil in 2013)	Plays an important role in the development and validation of alternative methods, through the active participation of the National Network on Alternative Methods (ReNaMA) Prepares final recommendations on the validated test method and sends to the National Council for the Control of Animal Experimentation (CONCEA) which then is in charge of the regulatory adoption of all validated test methods in Brazil
Canadian Centre for the Validation of Alternative Methods (CaCVAM) (established in Canada in 2017) https://www.uwindsor.ca/ccaam/	Works with the Canadian regulators to expedite the development, validation, and regulatory acceptance of the new alternative methods
AltTox (http://alttox.org/)	A comprehensive resource for scientists and policymakers to promote the use of alternatives

(continued)

Table 7.1 (continued)

Organization	Main objectives and activities
	primarily for animal-based toxicity testing Its website AltTox.org is focused on providing information on the development, validation, and international acceptance of nonanimal toxicity test methods and by the national and regional regulatory authorities as replacements for many of the animal toxicity test methods which are currently required for regulatory submissions A section on toxicity testing, i.e., Methods, Approaches, Programs & Policies (MAPP), provides information on alternative methods for toxicity testing including the in vitro, ex vivo, in chemico, and in silico methods along with those in vivo methods which may still help in reducing or refining the animal use Provides details of agencies that have endorsed the scientific validity of these methods and the status of the regulatory acceptance at international (OECD) or at national or regional level (European Pharmacopeia, FDA, REACH, etc.) http://alttox.org/mapp/table-of-validated-and-accepted-alternative-methods/

cultures not only resulted in saving monkeys but also made the vaccine safer by eliminating the risk of contamination with animal viruses [1]. In the same year, the potency test of the yellow fever vaccine that was used to be performed on animals in lethal dose tests was replaced by the cell culture-based plaque reduction neutralization test [2]. Insulin that is used in diabetic patients was earlier extracted from the pancreas of cows and pigs, but obtained from bacterial cultures by using recombinant DNA technology. The mouse convulsion test that was used to test insulin required 600 mice per batch. The number of animals going into convulsions after the injection was used to measure the strength of insulin. However, as science progressed and the analytical techniques improved, animals were no longer used, and insulin was directly assayed by chromatographic techniques [3]. Earlier, all the new cosmetics were tested on animals such as rabbits to test their potential for skin irritation. However, in the past two decades, multilayered human epidermal cell culture models like EpiDerm™ by Mattec Corporation and EpiSkin™ by SkinEthic have been shown to provide accurate results for the irritation potential of any product that is to be used on human skin and have completely replaced the need for testing in animals. It is realized that in the near future, it might not be possible to replace the in vivo Draize eye test in rabbits with any one single in vitro eye irritation test which would predict the full range of irritation for all the classes of chemicals. However, careful combinations of several alternative test methods in a tiered testing strategy have been able to replace this in vivo test to some extent. The Bovine Corneal

Opacity and Permeability (BCOP) test method is an organotypic model that enables scientists to maintain the normal physiological and biochemical functions of the bovine cornea obtained from freshly slaughtered animals in vitro and has been adopted by OECD in July 2013 (TG 437) for identifying chemicals inducing serious eye damage and for chemicals not requiring classification for eye irritation or serious eye damage, thus resulting in a considerable reduction in the number of animals being used in Draize test. Regulations also make it mandatory to ensure that each batch of drug products that are intended for parenteral administration is free from pyrogens, and historically, the rabbit pyrogen test (RPT) was the required test. However, with the advancement in technologies, this animal-based test has gradually been replaced by alternative tests like bacterial endotoxin test (BET), monocyte activation test (MAT), and recombinant factor C (rFC) test in most of the cases, although the suitability of these alternatives has to be demonstrated in a product-specific validation to ensure quality control of parenteral drugs. Therefore, it can be seen that significant progress has been made in replacing animals completely or in reducing their use considerably, and these alternative methods that have been in use for several years now have become a standard practice and proved to be more ethical, cost-effective, quick, and accurate.

7.4 Available Alternative Methods and Recent Developments

7.4.1 Computer Models

Several in silico computer simulation software are available that help predict the various possible biological or toxic effects of a potential drug/chemical without the use of animals so that only the most promising molecules are then taken for in vivo experimentation, thus helping reduce the number of animals enormously. These software are available in the public domain and can be accessed either free of cost or on a fee-based service.

7.4.2 Prediction of Toxicity

Computer-aided drug design (CADD) software helps to screen the chemicals for potential biological activity in animals, by identifying the receptor binding site for the new potential drug candidate. Such software programs also help to tailor-make new drug molecules for specific binding sites, thus requiring testing in animals only in the final stage to confirm the results [4], thus helping in an enormous reduction in the total number of animals used in such studies.

Several software related to structure-activity relationship (SAR) such as Deductive Estimation of Risk-based on Existing Knowledge (DEREK), Toxtree, Ecostar, and OECD QSAR Toolbox have been developed that help predict multiple toxicity endpoints and species for new drug candidates. SAR programs are also available that help to predict the biological activity of potential drug molecules based on the

presence of certain chemical moieties found attached to the parent compounds. Quantitative structure-activity relationship (QSAR) mathematically describes the relationship between physicochemical properties and biological activity of the drug molecules. When compared to animal testing, this software is a faster and inexpensive way to predict several activities like carcinogenicity and mutagenicity of potential drug candidates [5]. The QSAR Toolbox developed by OECD [6] is a free software application used for filling gaps of (eco) toxicity data needed to assess the potential hazards of substances. It helps in providing reproducible and transparent chemical hazard assessment. Some of the other online toxicity prediction tools available are as follows:

(a) **Toxtree**—free stand-alone software that applies a decision tree approach to estimate toxic hazards.
(b) **PredSkin**—helps to predict the skin sensitivity potential of a chemical.
(c) **Endocrine Disruptome**—helps to predict the endocrine action of any molecule against 18 structures belonging to 14 nuclear receptors.
(d) **ProTox-II**—a web server which is used for the prediction of toxicity of chemicals.
(e) **eMolTox**—another webserver useful for toxicity prediction and drug safety analysis.

Besides, several in silico methods have been developed that are in use in the pharmaceutical industry to study and predict the physicochemical, pharmacokinetic, and pharmacodynamic parameters of drugs.

Pharmacodynamics/Target Prediction

(a) **iDrug-Target**—helps to predict drug-target interaction.
(b) **SEA-2D**—is used for similarity-based approach.
(c) **SwissTarget**—helps to predict drug-target based on 2D and 3D similarity approach.
(d) **PASS Online—**helps to predict over 4000 different kinds of biological activities like the mechanisms of action, pharmacological as well as toxic effects, interaction with metabolic enzymes and transporters, influence on gene expression, etc.

Pharmacokinetics (ADME)

(a) **SwissADME**—is used for computation of both physiochemical properties and pharmacokinetic parameters.
(b) **SOM Prediction**—helps to predict the potential metabolic sites of a molecule and its metabolic products.
(c) **admetSAR 2.0**—a free comprehensive tool for evaluating chemical ADMET properties,
(d) **pkCSM**—predicts small-molecule PK properties using graph-based signatures.

7.4.3 Adverse Drug Reactions

Owing to the huge global health burden and failure of drugs, predicting adverse drug reactions (ADRs) in the preclinical stages has become very important to reduce drug failures as well as the time and cost of development and also to provide efficient and safe therapeutic to the patients. In recent years, many computational methods for in silico ADR prediction [7] have been put forward, but it remains a challenge for the drug developers, as many deaths still occur every year due to ADRs that are not apparent until the particular drug has been marketed and used by the patients. Though computer models for identification of ADRs have limitations mainly owing to lack of complete human metabolomics data, they certainly can be a valuable tool in drug development as the early detection of ADRs would not only help to reduce costs but also help to considerably reduce the number of animals used in preclinical trials.

In the recent years, the use of these in silico methods is on the rise mainly due to the requirements for reducing animal testing according to the REACH (Registration, Evaluation, Authorization and Restriction of Chemicals) legislation of the European Union and the implementation of similar laws and regulations globally, rapid technological progress leading to the development of new reliable alternative methods, and also the economic incentives on using alternatives [6]. In recent years, there has been a rapid evolution in the implementation of these in silico methods into regulatory use, and these may gradually replace many of the classical in vivo tests.

7.5 Cell and Tissue Cultures

Scientists have been growing different types of animal and human cells in laboratories. The traditional two-dimensional (2D) cell cultures have been crucial in improving our understanding of cell biology and mechanisms of several diseases and also in the drug discovery and development process. They have successfully provided an alternative to the animal model in several types of studies like preclinical research of drugs, cancer research, studies on gene function, etc., thus contributing greatly to reducing the use of animals. These 2D cell cultures have the advantage of being simple and having a low maintenance cost. However, they have certain limitations also as the adherent cultures growing as monolayers on flat flasks do not mimic the natural structures and microenvironments of the cells and tissues or tumors, thus affecting the cell-cell and cell-extracellular environment interactions which in turn might affect several cellular functions. Moreover, the adherent cultures allow the researchers to study only one type of cell at a time. Therefore, scientists had been making efforts to develop better in vitro cell culture models that closely resemble the in vivo conditions.

Of late, three-dimensional (3D) cultures have been developed that provide a more realistic way to study diseases and test new therapies. The organ-on-chip models utilize a dynamic 3D environment very similar to the human body, providing good

opportunities for understanding the pathogenesis of several human diseases and also providing a better model to screen novel drug molecules. These models have been developed for all the major organs of the body including the heart, lung, and kidney to test the effect of any new drug candidate on these individual organs on a small, microfluidic scale simulating the biology as well as the physiology of the human organs. These miniature 3D cell culture-based "organ-on-chip" models have been designed to mimic all the processes of their regular-sized counterparts including electrophysiological responses, exchange of gases, and fluid filtration. This also allows the researchers to use a variety of imaging techniques to study the processes in the organs, which is a major advantage over the typical 2D and 3D cell cultures. Scientists can arrange single types of cells opposite to one another along a porous membrane, thus enabling the exchange of the cellular products as well [8]. The use of microfluidics technology offers the advantage of performing tissue culture in controlled environments and adjusted to optimize the pH, temperature, supply of nutrients, and disposal of waste [9]. Researchers have also integrated these organ-on-chip systems with various sensors and actuators so that the key parameters in the human body can be monitored and controlled more accurately [10]. These chips can contribute extensively to the discovery and development of new drugs as they can model the complex and dynamic processes of absorption, distribution, metabolism, and excretion (ADME) of drugs, which form an efficacy benchmark for any new drug molecule. These chips can be used to study various disease states, particularly those diseases that are specific to humans where the animal models are not able to provide answers such as those involving the brain or immune system. They are also useful for testing the efficacy of various drugs and vaccines to know how they may function when administered in vivo. In recent years, scientists have been attempting to develop innovative microfluidic designs so that these chips can closely simulate the actual organs. A group of scientists has developed a biomimetic microsystem that not only mimics the functional alveolar-capillary interface of the human lung but also reproduces the complex integrated organ-level responses, on the introduction of bacteria and inflammatory cytokines into the alveolar space [11]. In 2012, an organ-on-a-chip was developed to model pulmonary edema of humans which mimics the lung function in response to mechanical strain and cytokine IL-2 and also accurately predicted the activity of new drug candidates [12]. In 2015, a heart-on-chip system using microfluidics was developed to assess the effects of the cardiovascular drug which had the advantage of precisely regulating some conditions like flow rate, pulsatile flow, and shear stress in addition to having high-throughput capabilities [13]. A disease model of cardiomyopathy of BTHS based on heart-on-chip technology and iPSC-derived cardiomyocytes (CMs) from iPSCs of Barth syndrome (BTHS) patients was developed [14]. This model has been used successfully to test new treatment options involving pharmacology and genetic modification and also for identifying new potential therapeutic targets for BTHS [15, 16]. The two main factors that influence the time for which the drugs stay on the cornea are blinking and tear flow, and based on this, a corneal epithelium-on-a-chip model has been developed which mimics the cellular environment and the tear flow associated with the eye-blinking mechanism [17]. Drug transport and their permeability across

the rate-limiting barrier can also be analyzed. As the biology of the corneal epithelium on the chip is quite similar to that of humans, it can be used for understanding ocular pharmacokinetics and physiology and thus can also support the ophthalmic drug test method. A 3D tissue microfabrication construct has been developed comprising heart, lung, and liver cells in separate but interconnected chambers which has demonstrated the highly critical interorgan response to drug administration [18]. Bleomycin was shown to be cardiotoxic as determined by altered kinetics of heart cell beat followed by a complete cessation in this multi-organ system, while it was not cardiotoxic in the "heart-only" construct. This could be attributed to bleomycin-induced secretion of IL-1β in the lung tissue which is known to be cardiotoxic [19]. Thus, the multi-organ system paves way for analysis much closer to the "in vivo" techniques.

Models of human-on-a-chip are also being developed that would move closer to mimicking the whole human response and enable scientists to study the systemic effect of drugs on the human body. This includes individual vital organs on chips that are further connected by a microfluid circulation system in a microfabrication bioreactor. These models closely mimic the interactions between multiple tissues, realistic size ratios, and physiological fluid flow conditions of a human body [20]. They also enable the scientists to study the effects of various drugs on human cells that are organized in a similar anatomical way, which certainly offers a distinct advantage over the animal models, as these human cells in culture behave in almost the same way as they would behave in the body, thus reducing or replacing the need for animal testing.

Although these organ-on-a-chip and human-on-a-chip models have some limitations, the new emerging technologies have certainly improved their capability for translational research, high-throughput analysis, and precision medicine and thus can contribute enormously as alternative methods in the preclinical drug development studies and in estimating the toxicity of environmental contaminants.

7.6 Microorganisms

7.6.1 *Saccharomyces cerevisiae*

Saccharomyces cerevisiae (brewing yeast) is one of the most common eukaryotic microorganisms used for biological studies. Its cellular architecture is similar to multicellular eukaryotes, and the genome is very well characterized and studied. Besides, rapid growth, short generation time, ease of replica plating, and mutant isolation with a highly versatile DNA transformation system provide distinct advantages. The various membrane-bound organelles present in the yeast-like nucleus, peroxisome, mitochondria, and organelles of the secretory pathway are similar to the functions of mammalian cells [21]. *S. cerevisiae* has also been studied as a model to replace the traditional LD50 test invertebrates, and the LD50 tests in yeast model were found to correlate well with the customary LD50 test in mice, rats, and other laboratory animals for nearly 160 common drugs and other chemicals

[22]. It is also used as a model to study programmed cell death and cancer [23]. Its utility in studying the mechanisms of aging and longevity of multicellular organisms, however, has been limited [24]. Engineered yeast models have been developed to study the endogenous or heterologous proteins that lie at the root of complex human diseases and have proven to be powerful tools for understanding the molecular mechanisms of neurodegenerative diseases such as Parkinson's, Alzheimer's, and Huntington's disease [25, 26]. The yeast screening assays have also been greatly useful in the first-line high-throughput screening of potentially active compounds and help to greatly reduce the number of animals used in the discovery of new therapeutic agents.

7.6.2 *Salmonella* Species

The *Salmonella* assay also known as the Ames test has played an invaluable role in identifying rodent and human carcinogens [27, 28]. Over the years, this assay has undergone several modifications which have enabled the use of the very minute number of samples in semi-throughput modes [29] and testing of body fluids like urine [30], feces [31], cervical mucus [32], breast milk [33], and breast nipple aspirates [34]. Approximately 30,000 chemicals that are produced in an amount exceeding 1 ton/year are required to be tested by the *Salmonella* assay under the European Union's REACH legislation [35]. The flexibility of this assay has made it useful for almost every type of environmental and molecular epidemiology study, and the experience of the scientists with the *Salmonella* assay can be used for developing new approaches for predicting and understanding the toxicology of substances [36].

Several other prokaryotes, protists, and fungi can also be used as alternatives. *Bacillus subtilis* is a bacterial model that has been used for studying cellular differentiation. *Escherichia coli*, *Dictyostelium discoideum*, and *Schizosaccharomyces pombe* have been used as a model for molecular and genetic studies and *Neurospora crassa* as a model for genetics as well as for circadian rhythm and metabolic regulation studies [37].

7.7 Invertebrates

Invertebrates have been used as models in teaching and research since the eighteenth century. Due to the public and ethical concerns about the use of vertebrate animals in research, interest in establishing and using invertebrate models like nematodes, insects, crustaceans, mollusks, etc., has increased considerably in the last few decades. The most commonly used invertebrate models are *Drosophila melanogaster* and *Caenorhabditis elegans*.

7.7.1 *Drosophila melanogaster*

Drosophila melanogaster, commonly known as fruit fly, is one of the most widely used invertebrate models in research because of the various advantages that it offers over the other vertebrate models, especially mammals. It is easy to grow in laboratories and has an extremely low maintenance cost. It has a very short life cycle consisting of four stages, i.e., embryo, larva, pupa and adult, each of which is used to study various scientific concepts. The embryo is used as a model to study organogenesis and neuronal development, while the larva is useful for studying the physiological and developmental processes. In an adult fly, functions of various organs like the heart, lungs, gut, reproductive tract, and kidney are found to be similar to that in mammals [38]. *Drosophila* genome is 60% homologous to that of humans, its complete genome has been sequenced, and almost 75% of the genes that are found to be involved in human diseases have a functional homolog in this fly [39, 40]. The heart of the fly is an ideal model for studying cardiac development and cardiac diseases and also for understanding the underlying cellular and molecular mechanisms in morphogenesis because of the conservation of key genes and close similarity with the vertebrate cardiogenesis [41]. *Drosophila* is also used for studying the blood cell development of vertebrates because of having similarities with the mammalian mechanisms of hematopoiesis [42]. Researchers have developed diabetic, obese, genetically "lean," as well as hypoglycemic phenotypes of these flies which are excellent models to study the pathogenesis of important metabolic diseases like obesity and diabetes [43]. The insulin signaling in these flies is very similar to that in humans, making it a very good model to study the mechanisms by which insulin regulates metabolism [44].

Drosophila is a good model for studying human genetics due to several similarities in their development and behavior [45] and has also been used to study human diseases for comparing the resulting pathologic conditions by expressing the specific protein products found in human disease. It is also an excellent model to investigate neurodegenerative diseases like Parkinson's, Alzheimer's, and Huntington's diseases [46, 47]. The molecular mechanisms driving wound healing in *Drosophila* was found to resemble those involved in the tissue fusion events during animal development making it a good model for studying wound healing [48]. The excretory system of the fly has been very useful in understanding the development and differentiation of the renal system across species [49]. This fly has been used extensively to understand the molecular mechanisms regulating stem cell activity in all animals. This model was used to demonstrate apoptosis in response to damage or stress and the molecular signals that initiate tissue regeneration by activating the proliferation of the stem cells [50, 51]. *Drosophila* can also be used as a tool in the clinical drug discovery process as an initial, fast, and high-throughput screening alternative. An added advantage is that the genetic background of this fly can be manipulated very easily to mimic a diseased state which can then be used as a model to test the efficacy of potential drugs for that particular disease.

7.7.2 *Caenorhabditis elegans*

Caenorhabditis elegans is a eukaryotic, multicellular, transparent nematode with a length of 1 mm, which is a highly prolific breeder having a very short generation time. It has a 2–3-week-long life cycle in which embryogenesis takes place in 12 hours and an adult develops within 2.5 days. It can be easily grown in laboratories and is relatively inexpensive to maintain. *C. elegans* has almost the same number of genes as humans and has several similarities at genetic and molecular levels. It has been used as a model to understand the molecular mechanisms involved in many human diseases such as Parkinson's disease, Alzheimer's disease, cancer, diabetes, and some immune disorders [52–54]. It has also been used in the development and testing of new drug molecules for the treatment of these diseases [55]. *C. elegans* can be used as a model for forward and reverse genetics as their transgenic, mutant, and knockouts can be developed very easily and those expressing the green fluorescent protein (GFP) offer an added advantage of allowing the scientists to observe the cellular and metabolic processes in vivo [56, 57].

7.8 Lower Vertebrates

In cases where complete replacement of animals is not possible, efforts can be made to see if at least the higher animals can be "replaced" with lower vertebrates or invertebrates. The zebrafish is one such alternative that has become quite popular in recent years.

7.8.1 *Danio rerio* (Zebrafish)

Zebrafish is an important vertebrate model that offers several advantages over the other vertebrate models. They require a simple habitat and can be easily cost-effectively maintained in a laboratory. The short generation time of 3–5 months and large clutch size of around 250–300 help in providing sufficient animals for research. Another unique advantage of the zebrafish is that they have external fertilization which in turn facilitates observation of the development pattern as well as an experimental manipulation of the embryos. Moreover, the transparent embryos also allow the use of noninvasive imaging techniques for visualization of fluorescently labeled individual genes throughout the developmental process [58, 59]. Scientists have constructed 3D imaging in live zebrafish by using fluorescent probes, thus enabling high-throughput imaging with good reproducibility of results [60]. This model can also be used to study the pathogenesis of several human diseases [61]. The zebrafish embryos have been shown to exhibit similar responses as other mammals to drugs for cardiovascular diseases, cancer [62], and neurological drug discovery [63, 64]. The zebrafish possesses a molecular, structural, and functional similarity of the blood-brain barrier (BBB) with those of the higher vertebrates [65]. The small size and optical clarity of the embryos and larvae enable the use of

this model in high-throughput drug discovery programs [66]. Zebrafish is a powerful and validated vertebrate model for studying human neurodegenerative diseases like Alzheimer's disease [67] because of the close resemblance in the neuroanatomic and neurochemical pathways of zebrafish and the human brain. Emotional, physiological, and social-behavioral pattern similarities with that of higher animals have also been observed in zebrafish. Zebrafish age gradually like humans and also show similar aging-related changes across both cognitive and neurobiological spectra making it a good model to study the neurobiological mechanisms that underlie aging-related cognitive decline [68].

7.9 Human Tissues and Volunteers

Animals used in research have undoubtedly played a vital role in scientific and medical advances and have enhanced the lives of millions of people. With advances in technology and scientific processes, now it is certainly possible to use human tissues to replace some use of animals in research. All the tissues and organs donated by human donors or those collected at the time of surgeries or autopsy are useful for biomedical research and contribute to advancing the fundamental understanding of human biology, improve our knowledge of disease pathways and mechanisms, and help in developing better diagnostic techniques and in the discovery of better cures and treatments for all the diseases that occur in humans. By creating greater awareness about donating samples, it is possible to increase the availability of human tissues and organs for research, which not only allows the scientists to study diseases in tissues that are infected but also helps to reduce the number of animals used in scientific and medical testing.

Another way to prevent the use of animals is by safely using available human volunteers. Micro dosing is one method that utilizes the technological advances in analytical techniques and helps to assess the basic behavior of a new drug by injecting the novel molecule in human volunteers at doses which are much lesser than those that are expected to produce whole-body effects so that even the potentially harmful substances do not pose a threat [69]. It is also possible for the scientists to use sophisticated scanning technologies like CT, PET, and MRI to see abnormalities and track the progress of the treatment in the brains of the patients suffering from Alzheimer's disease, Parkinson's diseases, schizophrenia, epilepsy, and brain injury. Similarly, the recent advancements in brain imaging technologies have enabled the researchers to measure the human brain activity noninvasively and with high precision, thus creating hope of soon replacing the highly invasive and painful procedures that are presently done in monkeys [70].

7.10 Barriers to the Implementation of Alternatives and the Way Forward

The efforts made toward the use of alternatives have certainly helped in reducing the numbers of animals that are being used and in refining the experimental procedures to minimize their pain and suffering. Despite all these efforts, the actual success in eliminating the use of animals in research and regulatory testing has been minimal. Some of the factors that contribute to the delay in the acceptance and implementation of alternative are as below:

- The possibility of replacing animals with alternatives depends on the nature and requirements of each experiment. While isolated cell and tissue cultures are not able to mimic a complicated living system, it is not possible to use human volunteers in potentially harmful experiments. Therefore, it is very important to understand the scientific barriers to replacement in specific types of experiments so that scientists can focus on overcoming these barriers.
- The medical and veterinary colleges and some specific areas of biological research continue to depend on the use of animals.
- Often scientists have their preferred ways of performing experiments, and a lot of persuasion and training in alternative methods would be required to convince them to shift to the alternatives.
- Developing countries are not able to prioritize developing or implementing alternative methods due to a lack of funding and resources.
- Over the years, the increasing standard of ethical approval and control has certainly improved the implementation of 3Rs. However, the members of ethical committees need to be trained and sensitized about the available validated alternatives and then encouraged to perform very critical ethical review so that animal use could be avoided wherever possible.
- Developing alternative methods in product safety testing of medicines or chemicals is very challenging and time-consuming because of the existing laws and regulations in several countries. The period between the successful validation of the alternative methods and their regulatory acceptance and actual implementation is long. The processes of multinational agreements required for revision of the pharmacopoeial monographs and regulatory guidelines delays the process. The production of the new reference material for the new test systems in sufficient quantities is time-consuming and expensive.
- Although there are many successful alternative methods available in basic research, their use is mostly limited to the laboratories where they are developed. Publications with detailed methodologies, commercialization, and technology transfer of these methods are required to bring about their widespread use in the scientific community. Stricter enforcement of regulations to ensure the use of the alternatives where available would expedite the replacement of animals that are being used in research.

7.11 Conclusion

The scientific community certainly wants to replace all experiments that cause suffering in animals with humane alternatives. While several options are already available and can be used, some are not sophisticated enough to eradicate animal testing. However, it is certainly time now that the scientific community considers using the available validated alternative methods and prioritizes the replacement of animals, over the refinement and reduction strategies. With new, stricter regulations and smarter and scientifically validated alternatives being developed by scientists, we can certainly be hopeful that the number of animals used in research, testing, and education will continue to decline in the future.

References

1. Bookchin D, Schumacher J (2005) The virus and the vaccine: contaminated vaccine, deadly cancers, and government neglect. St Martin's Press, New York
2. World Health Organization (WHO) (2007) guidelines for plague reduction neutralisation testing of human antibodies to dengue viruses. WHO/IVB/o707
3. Underhill LA, Dabbah R, Grady LT, Rhodes CT (1994) Alternatives to animal testing in the US P-NF : present and future. Drug Dev Ind Pharm 20:165–216
4. Vedani (1991) Computer-aided drug design: an alternative to animal testing in the pharmacological screening. ALTEX 8(1991):39
5. Matthews EJ, Contrera JF (1998) A new highly specific method for predicting the carcinogenic potential of pharmaceuticals in rodents using enhanced MCASE QSAR-ES software. Regul Toxicol Pharmacol 28:242–264
6. Raunio H (2011) In silico toxicology—non-testing methods. Front Pharmacol 2. Article 33:1–8
7. Kuang Q, Wang MQ, Li R, Dong YC, Li Y, Li M (2014) A systematic investigation of computation models for predicting adverse drug reactions (ADRs). PLoS One 9(9):e105889
8. Zhu J (2018) Application of organ-on-chip in drug discovery. J Biosci Med 2020(8):119–134
9. Bhatia SN, Ingber DE (2014) Microfluidic organs-on-chips. Nat Biotechnol 32:760–772. https://doi.org/10.1038/nbt.2989
10. Yu F, Hunziker W, Choudhury D (2019) Engineering microfluidic organoid-on-a-chip platforms. Micromachines 10:165. https://doi.org/10.3390/mi10030165
11. Huh D, Matthews BD, Mammoto A, Montoya-Zavala M, Hsin HY, Ingber DE (2010) Reconstituting organ-level lung functions on a chip. Science 328:1662–1668
12. Huh D, Leslie DC, Matthews BD, Fraser JP, Jurek S, Hamilton GA et al (2012) A human disease model of drug toxicity-induced pulmonary edema in a lung-on-a-chip microdevice. Science Translational Medicine 4:159ra147
13. Skommer J, Wlodkowic D (2015) Successes and future outlook for microfluidics- based cardiovascular drug discovery. Expert Opin Drug Discovery 10:231–244. https://doi.org/10.1517/17460441.2015.1001736
14. Wang G, Mccain ML, Yang L, He A, Pu WT (2014) Modeling the mitochondrial cardiomyopathy of Barth syndrome with iPSC and heart-on-chip technologies. Nat Med 20:616–623
15. Martins AM, Vunjak-Novakovic G, Reis RL (2014) The current status of iPS cells in cardiac research and their potential for tissue engineering and regenerative medicine. Stem Cell Rev Rep 10:177–190
16. Ribas J, Sadeghi H, Manbachi A, Leijten J, Khademhosseini A (2016) Cardiovascular organ-on-a-Chip platforms for drug discovery and development. Appl Vitro Toxicol 2:82–96

17. Bennet D, Estlack Z, Reid T, Kim J (2018) A microengineered human corneal epithelium-on-a-chip for eye drops mass transport evaluation. Lab Chip 18:1539–1551. https://doi.org/10.1039/C8LC00158H
18. Skardal A, Murphy SV, Devarasetty M, Mead I, Kang HW, Seol YJ, Atala A et al (2017) Multi-Tissue Interactions in an Integrated Three-Tissue Organ-on a- Chip Platform. Scientific Reports 7:8837
19. Didagelos M, Boutis A, Diamantopoulos N, Sotiriadou M, Fotiou C (2013) Bleomycin cardiotoxicity during chemotherapy for an ovarian germ cell tumor. Hippokratia 17(2):187–188
20. Esch MB, King TL, Shuler ML (2011) The role of body-on-a-chip devices in drug and toxicity studies. Annu Rev Biomed Eng 13:55–72
21. Mell JC, Burgess SM (2002) Yeast as a model genetic organism encyclopedia of life sciences. Mcmillan Publishers Ltd.
22. Koch HP, Hofeneder M, Bohne B (1993) The yeast test: an alternative method for the testing of acute toxicity of drug substances and environmental chemicals. Methods Find Exp Clin Pharmacol 15(3):141–152
23. Madeo F, Engelhardt S, Herker E, Lehmann N, Maldener C, Proksch A, Frohlich KU (2002) Apoptosis in yeast: a new model system with applications in cell biology and medicine. Curr Genet 41:208–216
24. Bilinski T, Bylak A, Zadrag-Tecza R (2017) The budding yeast Saccharomyces cerevisiae as a model organism: possible implications for gerontological studies. Biogerontology 18 (4):631–640
25. Pereira C, Bessa C, Soares J, Leão M, Saraiva L (2012) Contribution of yeast models to neurodegeneration research. J Biomed Biotechnol 2012:941232
26. Outeiro TF, Lindquist S (2003) Yeast cells provide insight into alpha-Synuclein biology and pathobiology. Science 302(5651):1772–1775
27. Kirkland D, Aardema M, Henderson L, Muller L (2005) Evaluation of the ability of a battery of three in vitro genotoxicity tests to discriminate rodent carcinogens and non-carcinogens I. sensitivity, specificity and relative predictivity. Mutat Res 584:1–256
28. Morita T, Asano N, Awogi T, Sasaki YF, Sato S, Shimada H et al (1997) Evaluation of the rodent micronucleus assay in the screening of IARC carcinogens (Groups 1, 2A and 2B): the summary report of the 6th collaborative study by CSGMT/JEMS MMS. Mutat Res 389:3–122
29. Kamber M, Flückiger-Isler S, Engelhardt G, Jaeckh R, Zeiger E (2009) Comparison of the Ames II and traditional Ames test responses with respect to mutagenicity, strain specificities, need for metabolism and correlation with rodent carcinogenicity. Mutagenesis 24:359–366
30. Cerná M, Pastorková A (2002) Bacterial urinary mutagenicity test for monitoring of exposure to genotoxic compounds: a review. Cent Eur J Public Health 10:124–129
31. De Kok TM, Van Maanen JM (2000) Evaluation of fecal mutagenicity and colorectal cancer risk. Mutat Res 463:53–101
32. Holly EA, Cress RD, Ahn DK, Aston DA, Kristiansen JJ, Wu R et al (1993) Detection of mutagens in cervical mucus in smokers and nonsmokers. Cancer Epidemiol Biomark Prev 2:223–228
33. Phillips DH, Martin FL, Williams JA, Wheat LM, Nolan L, Cole KJ et al (2002) Mutagens in human breast lipid and milk: the search for environmental agents that initiate breast cancer. Environ Mol Mutagen 39:143–149
34. Klein P, Glaser E, Grogan L, Keane M, Lipkowitz S, Soballe P et al (2001) Biomarker assays in nipple aspirate fluid. Breast J 7:378–387
35. Poth A, Jaeger M (2002) Alternative testing—the intelligent way to REACH compliance. AATEX 14:799–803
36. Claxton LD, Umbuzeiro GA, DeMarini DM (2010) The salmonella mutagenicity assay: the stethoscope of genetic toxicology for the 21st century. Environ Health Perspect 118 (11):1515–1522
37. Doke SK, Dhawale SC (2015) Alternatives to animal testing: a review. Saudi Pharm J 23 (3):223–229

38. Rothenfluh A, Heberlein U (2002) Drugs, flies, and videotape: the effects of ethanol and cocaine on drosophila locomotion. Curr Opin Neurobiol 12:639–645
39. Reiter LT, Potocki L, Chien S, Gribskov M, Bier E (2001) A systematic analysis of human disease-associated gene sequences in Drosophila melanogaster. Genome Res 11:1114–1125
40. Wilson-Sanders SE (2011) Invertebrate models for biomedical research, testing, and education. ILAR J 52:126–152
41. Medioni C, Senatore S, Salmand PA, Lalevee N, Perrin L, Semeriva M (2009) The fabulous destiny of the drosophila heart. Curr Opin Genet Dev 19:518–525
42. Crozatier M, Meister M (2007) Drosophila haematopoiesis. Cell Microbiol 9:1117–1126
43. Bharucha KN (2008) The epicurean Fly: using Drosophila Melanogaster to study metabolism. Pediatr Res 65(2):132–137
44. Teleman AA (2009) Molecular mechanisms of metabolic regulation by insulin in drosophila. Biochem J 425:13–26
45. Beckingham KM, Armstrong JD, Texada MJ, Munjaal R, Baker DA (2005) Drosophila melanogaster: the model organism of choice for the complex biology of multi-cellular organisms. Gravit Space Biol Bull 18:17–29
46. Bonini NM, Fortini ME (2003) Human neurodegenerative disease modeling using drosophila. Ann Rev Neurosci 26:627–656
47. Iijima K, 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 15:523–540
48. Agnès F, andNoselli S (1999) Dorsal closure in drosophila. A genetic model for wound healing? C R Acad Sci III 322(1):5–13
49. Denholm B, Helen Skaer H (2009) Bringing together components of the fly renal system. Curr Opin Genet Dev 19(5–3):526–532
50. Ryoo HD, Gorenc T, Steller H (2004) Apoptotic cells can induce compensatory cell proliferation through the JNK and the wingless signaling pathways. Dev Cell 7:491–501
51. Jennings BH (2011) Drosophila—a versatile model in biology & medicine. Mater Today 14 (5):190–195
52. Link CD, Johnson CJ, Fonte V, Paupard M, Hall DH, Styren S, Mathis CA, Klunk WE (2001) Visualization of fibrillar amyloid deposits in living, transgenic Caenorhabditis elegans animals using the sensitive amyloid dye, X-34. Neurobiol Aging 22:217–226
53. Nass R, Merchant KM, Ryan T (2008) Caenorhabditis elegans in Parkinson's disease drug discovery: addressing an unmet medical need. Mol Intervention 8:284–293
54. Pujol N, Cypowyj S, Ziegler K, Millet A, Astrain A, Goncharov A, Jin Y, Chisholm AD, Ewbank JJ (2008) Distinct innate immune responses to infection and wounding in the C. elegans epidermis. Curr Biol 18:481–489
55. Artal-Sanz M, de Jong L, Tavernarakis N (2006) Caenorhabditis elegans: a versatile platform for drug discovery. Biotechnol J 1(12):1405–1418
56. Wicks SR, Yeh RT, Gish WR, Waterston RH, Plasterk RH (2001) Rapid gene mapping in Caenorhabditis elegans using a high density polymorphism map. Nat Genet 28:160–164
57. Jorgensen EM, Mango SE (2002) The art and design of genetic screens: Caenorhabditis elegans. Nat Rev Genet 3:356–369
58. Cooper MS, D'Amico LA, Henry CA (1999) Confocal microscopic analysis of morphogenetic movements. Methods Cell Biol 59:179–204
59. Luengo-Oroz MA, Ledesma-Carbayo MJ, Peyrieras N, Santos A (2011) Image analysis for understanding embryo development: a bridge from microscopy to biological insights. Curr Opin Genet Dev 21:630–637
60. Hammers MD, Taormina MJ, Cerda MM, Montoya LA, Seidenkranz DT, Parthasarathy R, Pluth MD (2015) A bright fluorescent probe for H2S enables analyte-responsive, 3D imaging in live zebrafish using light sheet fluorescence microscopy. J Am Chem Soc 137:10216–10223
61. Lieschke GJ, Currie PD (2007) Animal models of human disease: zebrafish swim into view. Nat Rev Genet 8:353–367

62. Hill AJ, Teraoka H, Heideman W, Peterson RE (2005) Zebrafish as a model vertebrate for investigating chemical toxicity. Toxicol Sci 86(1):6–19
63. Scalzo FM, Levin ED (2004) The use of zebrafish (Danio rerio) as a model system in neurobehavioral toxicology. Neurotoxicol Teratol 26:707–708
64. Guo S (2009) Using zebrafish to assess the impact of drugs on neural development and function. Expert Opin Drug Discov 4:715–726
65. Fleming A, Diekmann H, Goldsmith P (2013) Functional characterisation of the maturation of the blood-brain barrier in larval zebrafish. PLoS One 8:e77548
66. Lessman CA (2011) The developing zebrafish (Danio rerio): a vertebrate model for high-throughput screening of chemical libraries. Birth Defects Res C Embryo Today Rev 93:268–280
67. Saleem S, Kannan RR (2018) Zebrafish: an emerging real-time model system to study Alzheimer's disease and neurospecific drug discovery. Cell Death Discovery 4:45
68. Adams MM, Kafaligonul H (2018) Zebrafish—a model organism for studying the neurobiological mechanisms underlying cognitive brain aging and use of potential interventions. Front Cell Dev Biol 6:135
69. Lappin G (2015) The expanding utility of microdosing. Clin Pharmacol Drug Dev 4 (6):401–406
70. Bailey J, Taylor K (2016) Non-human primates in neuroscience research: the case against its scientific necessity. ATLA 44:43–69