Review Article

Animal Models Alternatives: Desideratum during COVID-19 Pandemic

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ABSTRACT

Aim: The present work aimed at the utilization of alternative animal models in research. **Background:** Animal models from a long time played an important role in drug and vaccine development. However, due to the limitation like logistic, scientific and regulatory the need of alternative animal models became the need of the hour. **Materials and Methods:** Alternative animal models are strategies which basically substitute the live models. During the COVID-19 ongoing pandemic the fast, safe and effective drug and vaccine development became very crucial. However, due to the animal model limitations, the traditional animal model methods seem to become point of hindrance in the development of COVID-19 drugs and vaccines. **Conclusion:** Therefore, there is the need of alternative animal models during this pandemic for enhancing the frequency of clearance of clinical trials of corona vaccines. Hence, in the present article the authors briefly discussed about the animal testing, its limitation, various animal models alternatives and their usefulness during COVID-19.

Keywords: *In-silico*, Non-animal models, Clinical trials, Alternative models, Toxicological studies.

INTRODUCTION

Alternatives, substitutes, or non-animal approaches are strategies that substitute techniques that use live animals, or methods of measuring substances without the use of live animals. Some people use the term advanced technologies because they often rely on more advanced technology and are more human-relevant than the animal tests they substitute.[1] Every year, it is estimated that at least 115 million animals are used for research purposes around the world.[2] Animals are widely used to assess whether an action will affect humans or other animals of the same or different species, or whether it will perform, in the case of effectiveness testing. Testing contaminants (such as cosmetics, synthetic materials, medicines, pesticides, food additives, and biocides); medical devices; surgical techniques; environmental changes; or other methods of modifying the physiology and/or behaviour of a live animal are all examples of interventions. Safety testing is strictly controlled and is often performed after effectiveness testing, if possible, to ensure that an intervention is safe for humans and/or other animals. Efficacy research is less formalized and happens often in universities, where theories are evaluated in live animals as a "proof of concept" before developing actual methods to benefit humans or other animals.[3]

Alternatives to animal research have the same goals as whole animals in terms of maintaining and enhancing human wellbeing and comfort. Biomedical and biochemical science are largely responsible for the innovations that underpin the alternatives.[4] However, it is not intended to fully eliminate the use of animals in science, as this may jeopardize biological research, testing, and production of new medicines, vaccines, and surgical methods.[5-6]

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Increased interest and regular developments within the scientific community have led to the creation of alternative approaches in an effort to minimize, substitute, and optimize the number of animals used in laboratory studies, thus decreasing the costs associated with the procurement of research animals.[7] Currently, efforts are being made toward validating alternative tests. A reassessment of the use of animals in laboratory experiments is a worldwide trend, as evidenced in several countries by the founding of various institutions aimed at developing and validating new methods, regulations, and means by which to implement alternative tests in an attempt to legalize and standardize their use.[8-11]

Since, they allow for a decrease in the number of animals used in laboratory research, changes in toxicological procedures that are less painful or unpleasant for the animals undergoing studies, or the replacement of animal tests with *in vitro* and *ex vivo* tests or *in silico* systems, the use of these alternative methods has become important.[12]

Even though, the establishment of programmes with the aim of developing and implementing alternatives to animals in research has accelerated in recent years. The technological hurdles of designing and validating new technologies, however, are not the only obstacles to these tests' adoption. Many regulatory schemes and product liability laws require testing, and validation is essentially based on science, regulatory, and legal acceptance. However, Public concern about animal testing tends to be rising in lockstep with public concern about product and drug safety. Surprisingly, the public's growing concern about safety can lead to increased testing. It does, however, encourage the development of new techniques, especially those that promise to be less expensive and faster than current whole-animal methods. Another irony is that creating and validating alternatives often necessitates the use of animals.[4]

Limitations associated with the animal models, different alternative methods, regulatory limitations and recent advancement in alternative methods and their need in this pandemic era is discussed in the article.

Limitation of Animal Testing

Logistical limitations

Thousands of animals have been used in toxicity monitoring and scientific experiments around the world in the hopes of finding treatments for human diseases. Taylor and colleagues (2008) reported that 58.3 million living nonhuman vertebrates were subjected to fundamental or medically applied biomedical research, toxicity monitoring, or educational usage in 179 countries in 2005, based on publication rates. When animals killed for experimental tissues, used to preserve proven genetically modified strains, or bred for laboratory use but killed as surplus to requirements were included, the total number of non-human vertebrates worldwide was estimated to be about 127 million. However, due to a variety of factors, these Figures were deemed to be exceedingly conservative.[13]

Furthermore, significant increases in laboratory animal usage have recently been suggested as part of several chemical research programmes aimed at filling information gaps about the toxicity of chemicals manufactured or imported in large quantities into Europe or the United States, or that otherwise raise special concerns.[14,15] These initiatives are in response to rising public, political, and regulatory concern about the toxicity of a number of environmental, occupational, and consumer chemicals.[13]

The 2003 Commission of the European Communities (EC) proposal for the Registration, Evaluation, and Authorization of Chemicals (REACH), which went into effect on June 1, 2007, is one of the most prominent of these programmes. REACH aims to evaluate the toxicity of over 30,000 chemicals manufactured or imported into the European Union (EU) each year in excess of one metric ton.[16-20]

REACH's testing criteria are one-of-a-kind. For all substances produced or imported in excess of 10 metric tonnes annually, for example, reproductive and developmental toxicity data are needed. Traditional whole animal testing has been estimated to require the use of approximately 22 million vertebrate animals to fulfil data criteria, at a cost of up to several hundred thousand dollars per registered drug – though total animal numbers may be reduced to 10 million or lower if patented in-house toxicity data and suitable non-animal testing are used instead.[21]

According to Bremer and colleagues (2007), requiring *in vivo* testing for any adverse effect in these high-throughput chemical testing programmes would exceed the ability of available scientific facilities and expertise, result in unacceptable high false positive rates, and, most likely, jeopardize the programmers' performance.[13]

Alternative research methods have sparked a lot of interest as a result of these factors. This piqued interest even further when deadlines for ending animal testing of cosmetics and marketing bans on cosmetics tested *in vivo* were imposed within the EU under the seventh Amending Directive 2003/15/EC to Cosmetics Directive 76/768/EEC.[22,23]

Scientific Limitations

The scientific limitations imposed by using animals to model humans in basic or clinically applied research and toxicity testing are important, wide-ranging, and increasingly recognized. Differences between organisms and sex, for example, can have an effect on toxico- and pharmacokinetics, as well as pharmacodynamics. The use of unrealistic doses and exposure durations; the loss of biological diversity or predictability due to the use of in-bred strains, young animals, gender limitation, and insufficient group sizes; the absence of co-morbidities or other human risk factors; and stress-related physiological or immunological distortions are all common examples.[24-26]

The P450 based monooxygenase of the xenobiotic family of enzymes is a good example of interspecies variations.[27] P450 main job is to oxidize foreign substances like drugs and toxins, resulting in toxin-free blood-soluble compounds that make kidney function easier. Differences in species cause anomalies or differences in metabolic pathway rates, which reduce efficacy. This is the most serious flaw in animal research.[28] As a result, after demonstrating safety in animal tests, only 8% of all drugs going forward to human trials obtain FDA approval.[13] Another example of animal studies that have failed completely is the study of HIV drugs. Anti-HIV drugs have so far only been tested on chimps, which have a metabolism that differs from humans.[29]

Regulatory Limitations

The public, as well as governing bodies have become more concerned about large-scale animal killings. When non-animal alternative models are available, certain authorities ban laboratory animal use. The use of animal models must also be justified, according to authorities. Animal Rights Act, 1966 is one such statute in the United States. Mice, birds, and rats are safeguarded under this act. Furthermore, tests should be conducted after analgesics or anaesthetics have been administered, and researchers should avoid repeating previous experiments. The Japanese Animal Protection Law went into effect in 2005, and it focuses on alternative models in 2006. Since only Japan consumed 1.1 million living vertebrates in 2004 for toxicity testing, educational purposes, or biomedical research, the legislation was implemented. Animal use must be supervised by an Animal Ethical Committee, according to Japanese law.[30]

Novel Animal Free Models in COVID-19

The scientific world is facing a significant challenge as the COVID-19 pandemic spreads. The goal is to not just identify appropriate vaccinations and/or treatments, but to do so as quickly as feasible. Unlike many other diseases, there is not just a medical need, but also significant political and economic pressure. Ursula von der Leyen, the President of the European Commission (EC), for example, expressed optimism that a vaccine would be available by autumn 2020.[31] In light of these and other comments, it's worth considering what tools and regulatory processes might be available to help us overcome this unprecedented health problem. Even without such time constraints, viral infections are the prototypic species-specific diseases, making animal experimentation difficult. Their duration and expenses, particularly when genetically modified strains sensitive to the disease must be developed, do not support such lofty ambitions, whereas current bioengineered human (multiple) organ models are well-suited to antiviral medication research.[32,33] The Innovative Medicines Initiative (IMI), a public–private partnership, has also contributed significantly to the development of treatments and diagnostics to combat present and future coronavirus outbreaks.

Concept of 4R

Alternatives to animal testing have been suggested to address some of the disadvantages of animal testing while avoiding unethical practices. The 3Rs strategy is being implemented, which stands for reduction, refining, and replacement of animal use in laboratories.[30] The definition of "3R" was introduced to the world in 1957 by Charles Hume and William Russell at the Universities Federation of Animal Welfare.[11] This idea is extended to the use of laboratory animals in drug research. Various processes, models, and replacement animals were used to carry out this strategy. In recent years, the 4th R, i.e., responsibility, has been added.[30] The 4R's are discussed as follows (Figure 1):

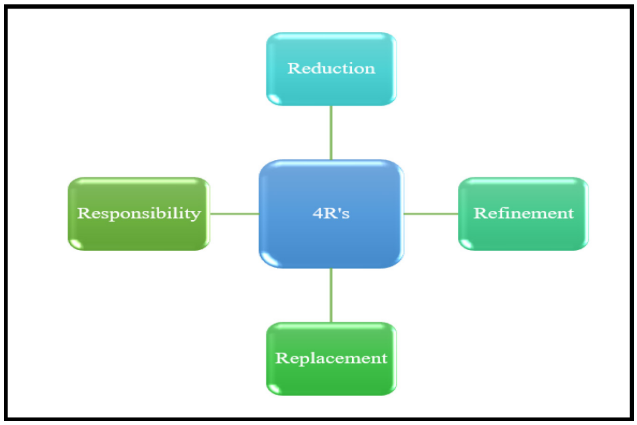

Figure 1: 4R's classification.

Reduction

The aim of this strategy is to reduce the number of laboratory or experimental animals used in research. Scientific findings can be achieved by the use of statistical evidence and the careful selection of study design. *In vitro* cell culture is available for early-stage compound screening. Human hepatocyte cultures are used in the analysis of drug metabolism and removal.[34] The embryo *in-vitro* embryonic stem cell culture test is used to differentiate toxic compounds from non-toxic compounds.[30]

Refinement

Animals dislike being confined in the same way that humans dislike being confined. Animals are stressed because they are held in cages. It makes the animals fearful and anxious. Animal hormone levels fluctuate as a result of pain, anxiety, and discomfort, resulting in errors in the results, either directly or indirectly. As a consequence, the number of animals needed grows. As a result, refinement is essential to enhance animal housing facilities and testing efficiency.[35]

Replacement

"Any scientific method employing non-sentient material that may replace the use of conscious living vertebrates in animal experimentation" is described as animal replacement. Non-living systems and computer simulations are used to replace living systems. Cultures, *in-vitro* methods, computer models, and other alternatives are used.[9,35]

Responsibility

"RESPONSIBILITY" is a new addition to the fourth R. This R denotes that humans bear the primary responsibility for animal welfare and cruelty prevention. As a result, a new era of performance-based management is gaining traction. The letter "R" denotes the importance of animal life in biomedical research.[30]

Animals in Drug Testing: Alternative Methods

Animals have been replaced in drug testing in clinical and toxicology studies using a variety of approaches. We'll go over a couple of them that will be useful (Figure 2).[30]

In silico Method

Drug development necessitates a significant amount of money and effort. Various procedures must be followed throughout the discovery process. Animal Research and Protection is an example of such a protocol. Animal research can be replaced with *in-silico* modelling approaches to minimize workload. *In silico* models are

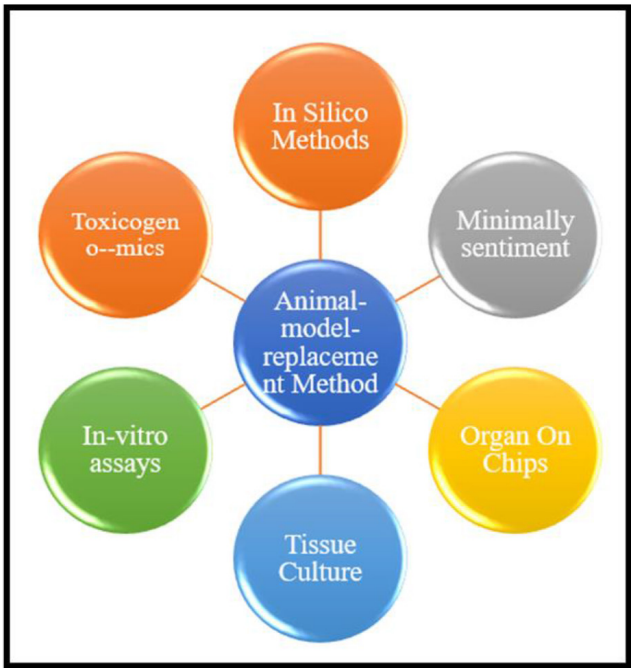

Figure 2: List of alternatives to using animals in drug testing.

based on biological concepts. New drug development is aided by specially developed computer models and software. It predicts potential drug and receptor binding sites, reducing or eliminating the number of animals used. This is made possible by the use of computer software called Computer Aided Drug Discovery (CADD), which predicts a drug's likely therapeutic and pharmacological action, as well as its ability to attach receptors. CADD aids in the development of new drugs that bind to a particular receptor. This would eliminate the use of non-bioactive chemicals. As a result, the total number of animals required is reduced, allowing us to meet the 4R strategy's target.[36,37]

Quantitative Structure Activity Relationship is another approach in use (QSAR). It is a theoretical tool for predicting a compound's therapeutic action based on chemical groups present on the parent compound. A mathematical expression of the relationship between a drug's physicochemical and biological properties is known as QSAR. QSAR estimates the carcinogenicity and mutagenicity of a drug candidate in a short amount of time. These computer models are more precise, less costly, and provide faster performance.[38] Apart from QSAR, another essential component of the toolbox of silico method is the target prediction models method which predicts protein targets for chemicals. Protein goal predictions have gotten a lot of press in recent years, but not always in a toxicological sense.[39-41] Protein target prediction approaches can be divided into three categories: There are three types of methods:

1) ligand-based methods, 2) protein structure-based methods, and 3) methods that combine both ligand and protein structure knowledge.[42] Hence, to find the drug for the treatment of COVID-19, the *in-silico* tools are extensively used. *In-silico* tools are utilized not only in case of drug repurposing but also in searching the new drug molecule.[42,43]

Minimally Sentient

Several regulatory authorities must be complied with throughout the manufacture of a drug or medication. Animal Safety and Regulatory Bodies are one such authority. According to the Authority's guidelines, only the smallest amount of live vertebrate animals can be used. Furthermore, the Animal Ethical Committee has many restrictions against studying higher animals like guinea pigs, monkeys, and so on. As a result, lower-phylogenetic animal orders must be used as a replacement for higher animal orders. In 2007, Cosson *et al.*, proposed that protozoans be used instead of rodents for bacterial infection assays. The explanation for this is that the bacteria Pseudomonas aeruginosa uses the same defense mechanism in both unicellular amoeba and multicellular mammalian cells. This makes it possible to research drugs in pre-clinical trials using lower vertebrates and invertebrates.[44]

Lower vertebrates

They're often used because their genetic makeup is close to that of vertebrates. In addition, less legal standards must be followed. Danio rerio, also known as zebra fish, is a freshwater fish that grows to a length of 2-4 cm. The translucent body of the zebra fish assists in the observation of its internal organs. This openness enables simple screening, direct observation of developmental stages, toxicity testing, and many other applications. Danio reria has been used for a variety of purposes from childhood to adulthood. Zebra fish are ideal for laboratory use due to their short life cycle, small size, and high fecundity.[45] The zebra fish needs a small amount of habitat, little upkeep, and little manpower. In petri-dishes, the embryos and larvae can be easily produced and tested. Since the entire genome sequence of the zebra fish is readily accessible, it is the perfect option for molecular and genetic research. Currently, zebra fish are used in cancer research, neurological disorders, organ cell mutations caused by chemicals, behaviour research, and a variety of other applications.[46]

Invertebrates

Invertebrates, like lower vertebrate species, have a wide variety of laboratory applications in the study of diseases such as Parkinson's disease, cell ageing, and diabetes

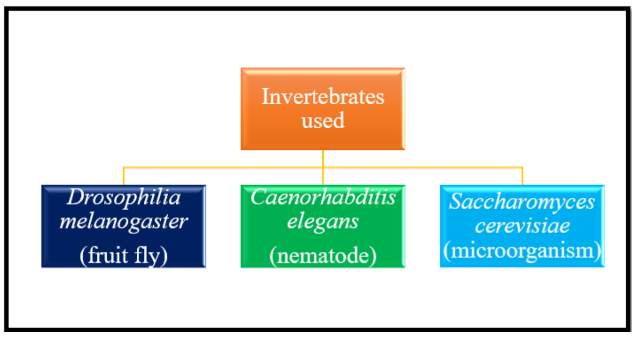

Figure 3: Invertebrates used as alternative of animal testing.

(Figure 3). The advantages of using invertebrates include a short life span, small size, low maintenance, and lower housing costs. The only drawback is that they lack an adaptive immune system, which results in certain differences in human diseases.[47] Some invertebrates used are discussed as follows,

Drosophilia melanogaster

These invertebrates have a broad range of applications. The genome of the fruit fly has been extensively studied, which assists in the analysis of molecular processes in human diseases. 75% of genes have roles that are similar to those of human genes. Fruit fly produces fast results due to its short life cycle. The phases of the life cycle are the egg, larva, pupa, and adult stages. Fruit flies are called multiple model organisms since each stage is used for testing.[47,48] Organogenesis, cell fate determination, and axon path finding are all studied at the embryo level. Foraging is one of the physiological and development mechanisms that larvae are used for. Adulthood is a very dynamic period in the life of a person. As a result, it's useful for a variety of structural studies, including the heart, gut, kidneys, and lungs.[48] Fruit flies have a special function in that they behave similarly to central nervous system drugs in mammals. The brain is remarkable because it contains over 1,00,000 neurons that form a network that regulates a variety of complex behaviors such as sleep, eating, learning, and flight navigation. As a result, in diseases such as Alzheimer's, Parkinson's, and Huntington's and to study human genetics fruit flies are used.[49,50] Also in covid-19 drug development it can prove to be a promising candidate.[51]

Caenorhabditis elegans

The eukaryotic nematode *Caenorhabditis elegans* is a type of nematode. This one-millimeter multicellular organism has a limited life span (around 2 to 3 weeks). It is translucent and has a basic cellular structure, similar to zebrafish. The life cycle includes stages such as embryogenesis, morphogenesis, and adulthood. It's

used to study disorders such as Parkinson's, Alzheimer's, cancer, and diabetes.[52-55]

Saccharomyces cerevisiae

Brewing yeast is the common name of this microorganism *Saccharomyces cerevisiae*. It is the most common and important model due to its rapid development, well-defined genetic system, and highly flexible DNA transformation system. Both solid and liquid culture media can be used to grow them. It is simple to develop and evaluate a large population on cultural media due to the short generation span.[56] *Saccharomyces cerevisiae* has approximately 16 chromosomes with a total of 13 million base pairs, as well as an extra nuclear genome in the mitochondria. The number of genes and their size are both tiny. Saccharomyces cerevisiae is the best candidate for drug testing because of its multicellular organism-like cellular structure and life cycle. Since many of Saccharomyces cerevisiae's cell-bound organelles resemble the roles of mammalian cells, it's used to study apoptosis and how it's regulated, which is useful in cancer research.[57] In the diseases like Alzheimer's, Parkinson's, and Huntington's disease *Saccharomyces cerevisiae* may help researchers better understand how they develop cellularly.[58,59]

Organ- on- chips

Organs are complex in terms of their specialized structure, cells, and tissue, and they each perform one or more distinct functions (FIgure 4). Because of the complexity of the situation, there are no valid models that can show exact or near-exact functions. *In-vitro* testing of human cells or *in-vivo* testing in animals are the only choices available to scientists and researchers. Both solutions have their own set of disadvantages.[60] Due to constant variation among human cells, obtaining exact human cells from a homogeneous population to study is a difficult task. Even if these cells are collected, the issue is that they are highly susceptible to passaging, altered phenotype, and evolving metabolic activities due to variation. Another issue that researchers face is that advanced technologies are needed for organ culture and use. The use of immortalized cells is favored to solve these issues. However, in order to achieve immortality, those cellular behaviours had to be disrupted, and the anomalies or inconsistencies from the original or primary cell activity had not yet been thoroughly investigated.[61] "Microfluidic techniques" also arisen to overcome all of these obstacles. Significant advancements in the field of microfluidics have been made in recent decades, with an emphasis on "organ on chips." The term microfluidics is made up of two words: micro, which means thin, and fluidics, which refers to a collection of liquid or gas movements. As a result of the combination of tissue engineering and micro-engineering, the effect of the growing environment can be studied in both human cell cultures and animal models that promote the growth of human cells in physiologically relevant conditions.[62] Recently in the drug repurposing study for the covid-19 the organ-on-chips method was utilized and in specific lungs on chip was used to study the effect of repurposed drugs. Hence, by this study it was suggested that in biothreat situations induced by pandemic viruses, human Organ Chip technology could be utilized in concert with existing quick cell-based screening tests to explore human disease pathophysiology and speed drug repurposing.[62]

Different organ on chips are discussed as follows,

Skin-on-chips

The skin, which serves as a protective barrier between the human body and the environment, is often referred to as the "largest organ." For the application of safe topical products such as creams, lotions, powder, and cosmetics, a thorough understanding of the physiology of the skin and its layers is needed. The epidermis, which is the top layer of skin, is easily accessible in an *in-vitro* static analysis, but the deeper layers are not.[63] Human fibrinoblast cultures were inoculated in collagen type-I medium matrix to create a full thickness skin model for *in-vitro* use. The dermis layer grew and developed as a result of this. The dermis layer is seeded with melanocytes and keratinocytes after it has completely developed in order to acquire hair follicles. Human Skin Equivalent reflects the maximum growth production

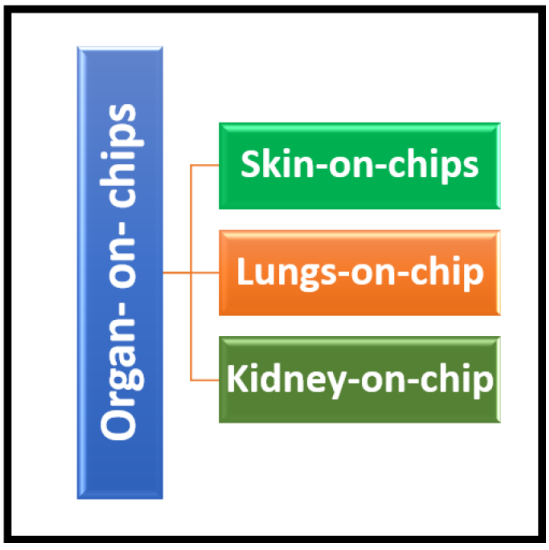

Figure 4: Different organ-on-chips used as alternative method.

(HSE). The newly developed model portrays three to four layers of keratinized cells, as well as a distinct distinction between the dermis and epidermis layers.[64]

It has recently been discovered that it is possible to investigate the effects of shear stress on skin development. Two fluidic chambers are included in the newly advanced chip model design. In the liquid-air interface chamber, skin biopsies are grown over *ex-vivo* subcutaneous tissue such as adipose cells and macrophages. Follicular hair extracts are immersed in the second chamber. The mixing of subcutaneous tissue resulted in a huge success in the chip device, while these layers were shattered in static cultures.[65] Wagner *et al.* attempted to show organ crosstalk between the skin and the liver. The first chamber was left unchanged, but the second chamber now contains hepatocytes (liver cells). Hepatocytes contain albumin, which is ingested by the skin, according to the experiment. However, the distinguishing feature is that when the liver reaches equilibrium, it does not generate excessive albumin. This demonstrates organ communication between the skin and the liver, which could pave the way for a "whole body-on-a-chip system" in the future.[66]

Lungs-on-chip

The pulmonary system's main role is gas exchange between air and blood. As a result, simulating the pulmonary system *in vitro* was a difficult challenge. In the lungs, gases are exchanged at alveoli. Infections, inhaled spores, and complications from various diseases expose alveolar epithelium cells to a combination of chemical and mechanical stimuli.[67] The *in-vitro* model was discovered using Trans wells to simulate the air-liquid interface of lung barrier tissues. Nowadays, microfluidic devices made of two layers of PDMS are used. The top chamber, or upper layer, is air-filled and serves to allow gas exchange. The vascular chamber layer which is present below, mimics a capillary network, enables perfusion of the underlying cultured tissues. A porous membrane separates the liquid-air interface, which serves as a site for cell seeding and development.[13] The immortalized lung epithelial cells obtained from a mouse were demonstrated by Fritsch *et al.* These cells are cultured on a surface of polystyrene micro carrier beads before being injected into the parenchymal chamber (top chamber) for even distribution.[68] The inflammatory reactions of primary bronchial epithelial cells to pollen dust particles were studied by Blume *et al.* using the same template. The findings were unmistakable and previously impossible to achieve using static cultures. As a result, the chip system was more susceptible to environmental influences.[69]

Kidney-on-chip

The kidneys' two most essential functions are drug metabolism and removal. The medication and other substances circulating in the body are filtered by kidney cells. The filtration of drugs by the kidney often results in nephrotoxicity, which is a common problem in drug safety testing. As a result, research into renal barriers is needed for drug development in order to increase blood resistance time and ensure safe compound removal after impact. More water, salt, carbohydrates, and proteins are reabsorption by the epithelial cell layer of the kidney. Some drugs that are not excreted correctly or left behind cause unintended toxic effects as the eliminated compounds transfer from urine precursor.[70,71]

Renal toxicity is one of the most commonly mentioned drug assessment criteria, and it's also one of the most frequently diagnosed in clinical trials recently. Via protein analysis and gene analysis, experiments on Madin-Darley Canine Kidney (MDCK) cells, proximal, and distal tubule cells revealed that shear stress played a critical role in renal barrier functions. They showed an increase in regulation of sodium, calcium, and phosphate homeostasis, as well as genes involved in H+ transport and urine pH regulation, in the fluidic medium in which they were grown. MDCK cells also showed increased control of phase I and II enzymes, multi-drug resistance genes, and phase-III transporters, among other things. As a result, they resemble kidney cells *in vivo*.[72]

Tissue culture

In-vitro cultivations of tissue, cells, tissues, and embryos are all examples of tissue cultures. Proteolytic enzymes are used to separate primary cell cultures from animal tissues. These cells have tissue-specific roles and biotransformation potential. The only disadvantage is that cellular isolation can harm the cell membrane, as well as cause the loss or damage of cellular contents and membrane receptors. Unfavorable changes occur inside cultures when they are held for a long time, necessitating the isolation or removal of new cells for each new experiment. The use of "cell lines" is a solution to these problems. However, as time passes, the metabolic ability of these cell lines decreases, and cellular function is altered.[73] *In-vitro*, immortalized cell lines have the ability to spread and, in certain cases, indefinitely. They're made by injecting viral oncogenes like SV40, big T, and EIA into primary cells and then treating them with calcium phosphate. Rabbit kidney cells, rat hepatocytes, and osteoblasts have also been immortalized.[74,75] The process of immortalization typically alters the structure and functions of the entity. Adult progenitor cells and mammalian blastocysts

are also origins of stem cell lines. In the presence of feeder and Leukotriene Inhibitory Factor, stem cells remain undifferentiated (LIF). Human embryonic stem cells now pose a slew of ethical and sociological questions. The main distinction between stem cells and immortalized cells is that stem cells have the ability to self-renew, while immortalized cells do not. As a result, stem cells are favored over immortalized cells for this purpose.[76,77] In the case of degenerative diseases, stem cells are mostly used in research centers to replace lost tissue and functional cells. Stem cells are also involved in the development of blood cells (hemopoiesis) and some hematopoietic cytokines, which have recently been used in cancer treatment alongside chemotherapy and radiation therapy.[78] Also in covid-19 the *in-vitro* evaluation can play an important role in drug and vaccine discovery. As different cell lines and organoids has been used extensively to study the covid-19.[78,79]

In-vitro assays

In-vitro assays using bacteria, yeast, human cell cultures, and mammalian cells are used to verify the toxicity of eye corrosion, skin corrosion, and carcinogenicity. The benefit is that some of them have been scientifically tested, while the others are in the early stages of development (FIgure 5).[80] Some *in-vitro* assays discussed are as follows,

Perfused cultures

Tissue culture is usually done in one of two ways: static or perfused with culture media through pumps. Perfusion cultures demonstrate effective monitoring of cytotoxins as cells are exposed to toxins. Biomarkers present in the perfusion are used to identify and detect cytotoxins. The benefit is that important parameters such as onset of toxic action, toxicity scale, and period time of toxic action can be calculated in perfusion cultures, which were previously difficult to quantify in static cultures or *in-vivo* study.[81]

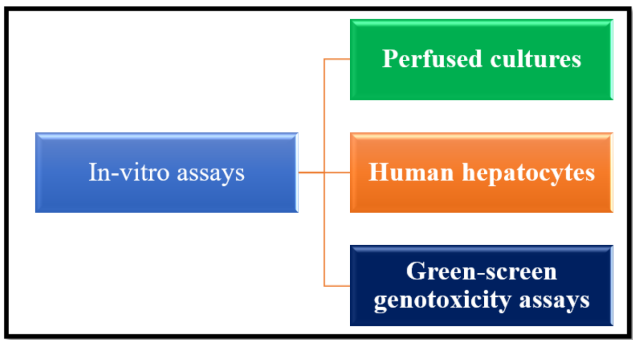

Figure 5: *In-vitro* assays used in alternative methods.

Human hepatocytes

The liver is the primary organ responsible for drug metabolism and delivery in the body. Via portal circulation, the liver receives a bolus of concentrated ingested drug. Thus, when a foreign material is ingested, liver cells are the first to react and respond. P450 dependent monooxygenase, for example, is an enzyme involved in metabolism. Human hepatocyte cultures can be used to study liver cells in a lab setting. They are often used in academic and research laboratories for drug-drug interaction, drug potential, and drug toxicity. Membranes are protected from damage during preserving techniques thanks to the invention of crypto-preservation and the use of reagents and protocols.[30]

Green-screen genotoxicity assays

Genetically engineered *Saccharomyces cerevisiae* with green fluorescent protein fused to the RAD54 yeast promoter to detect genotoxic test compounds was used by Lichtenberg- Frate and colleagues. This is referred to as "green-screen genotoxicity assays." According to an analysis of 75 chemicals, this technique is used to verify the number of genotoxins and non-genotoxins with a high degree of precision.[82]

Toxicogenomics

Toxicogenomics (TGx) is based on the simple premise that compounds with similar toxicity mechanisms and outcomes should perturb the transcriptome in similar ways, and these perturbations could be used as more efficient and/or predictive biomarkers of downstream toxicity outcome. A number of groundbreaking studies, such as the one reviewed in, demonstrated strong correlations between histopathology, clinical chemistry, and gene expression when various hepatocellular injuries were induced by chemical agents.[36,83,84]

Toxicogenomics is an important tool in toxicologists' toolbox for a variety of reasons, including:

1. It enables genome-wide studies of a toxicant's effects, overcoming the limitation of being tied to a single endpoint.
2. Because it is a relatively high-throughput technique, toxicological risk assessment should be accelerated.
3. Other techniques, such as histopathology, are unable to detect more subtle changes.
4. It can be used to find biomarkers that are specific to certain toxic effects.
5. It allows researchers to conduct mechanistic toxicology studies.[85]

As a result, toxicogenomics has a lot of promise in terms of predictive toxicology.[11] Toxicogenomics has two targets:

1. To comprehend the toxicity's underlying mechanism
2. To clarify the connection between environmental or chemical stress and disease in humans.[8,-87]

Only in the last decade has toxicogenomics become routine, due to the development of microarrays that can calculate thousands of transcripts at once. The use of toxicogenomics data is still limited by a number of major issues, such as probe annotation and data comparison between various experiments and array platforms.[88-90]

A standard toxicogenomics experiment involves a multiple dose *in vivo* or *in vitro* experiment followed by gene expression profiling on a biological sample using a microarray chip. It is then possible to differentiate and recognize the genes that are associated with toxicological effects caused by the test drug using data mining methods, comparative study, and a systematic classification of toxicological effects.[91-93] Despite these advancements, the problems associated with this method remain unsolved, with some posing major challenges such as a lack of information about particular molecular targets, classification of toxicological effects, data integration, and database creation.[94-96]

CONCLUSION

For a long time, animal models are used for drug testing clinical trials, and toxicological studies or in other words animals are sacrificed for the sake of human beings which somewhere seems unethical practice apart from that requirement of a large no. of animal models and the genetic difference between animal and humans, or in stress and fear condition show different result these all limitations are termed as social, logistic and scientific respectively drive us for the more reliable methods called as alternative methods. The alternative methods can overcome these limitations and can be more reliable. At present, different alternative methods like the *in-silico* method, minimal sentient, *in-vitro, in-vivo*, toxicogenomics, organ-on-chips have shown their efficacy and effectiveness during different testing and toxicological studies. Hence, it can be concluded that alternative methods in the future can replace the traditional animal testing methods to a great extent.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ABBREVIATIONS

TGx: Toxicogenomics; **LIF:** Leukotriene Inhibitory Factor; **MDCK:** Madin-Darley Canine Kidney; **HSE:** Human Skin Equivalent; **QSAR:** Quantitative Structure Activity Relationship; **CADD:** Computer Aided Drug Discovery; **EC:** European Commission; **IMI:** Innovative Medicines Initiative; **REACH:** Registration, Evaluation, and Authorization of Chemicals.

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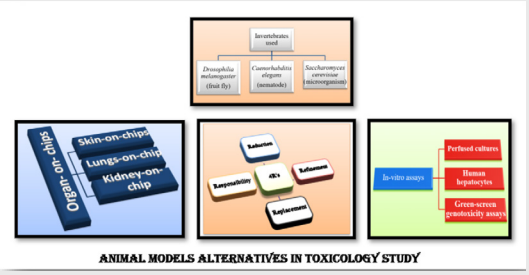
PICTORIAL ABSTRACT



SUMMARY

Animals are sacrificed for the sake of human beings which somewhere seems unethical practice. The alternative methods can overcome these limitations and can be more reliable. Alternatives to animal research have the same goals as whole animals in terms of maintaining and enhancing human wellbeing and comfort. At present, different alternative methods like the *in-silico* method, minimal sentient, *in-vitro, in-vivo*, toxicogenomics, organ-on-chips have shown their efficacy and effectiveness during different testing and toxicological studies.

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