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Review Article

ADVANCES ON ALTERNATIVE TO ANIMAL EXPERIMENTATION: A REVIEW

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ABSTRACT

Animals are increasingly being used in experimental to test the safety and effectiveness of medications as medical research and development advances. Millions of animals are utilized annually for experimental experimentation, enduring agony before being ultimately killed. In addition to bio ethical concerns, there are a number of drawbacks to animal experiments, including high expense, the need for trained labor, approval, and time commitment. As a result, scientists have tried to create a variety of substitute techniques that might avoid using animals in study. In addition to providing precise data, these techniques have the potential to save millions of animals' lives each year. In order to find innovative ways to replace animal testing, research approaches such as computer and robotics as well as molecular biology techniques are used. In this review, several alternative approaches are addressed. Some of these techniques are just as accurate as in-vivo animal models at predicting are just as accurate as in-vivo animal models at predicting how pharmaceuticals would behave. These substitute techniques also have a number of benefits over using animals in experiments. To replace experimental animals, new, precise, and trustworthy techniques must yet be found and developed.

Keywords: Cell culture, Substitute organisms, Refinement, Replacement, Vertebrates.

INTRODUCTION

Dangerous microorganisms, which are known to cause numerous serious illnesses, are continuously present in human environments. Humanity has worked from the beginning of time to find and create novel chemical compounds that could be utilized to successfully diagnose, prevent, and treat both communicable and Non-Communicable Illnesses (NCDs). However, before a medication molecule is used in people, it must first undergo a number of experimental animal tests to confirm its safety and efficacy. The use of animals has significantly increased in recent years as a result of the development of study and experimentation in a variety of medical sectors. In academia, experimental animals are used as illness or testing models to teach students, but in research, animal's experiments aid in examining the pharmacokinetics, safety, and effectiveness of medication candidates. The investigation is being conducted using guinea pigs, dogs, hamsters, fish, birds, mice, rats, rabbits, and fish. They serve as a tool for comprehending the effects of novel chemicals, medications, and biologics, including vaccinations, medicinal treatments, and surgical tools (Lili. W, *et al.,* 2008; FP. Gru., T. Har; 2004). Animals are used in drugs testing and toxicological screening for both infections and non infectious disorders. To assess in-vivo therapeutic efficacy and toxicity studies for a range of illnesses, such as cancer, diabetes, and acquired immunodeficiency syndrome (AIDS), numerous animal models have been created. Experimental animals, particularly when determining the Lethal Dose 50 (LD_{50}). Animals undergo pain, suffering, and death during scientific experiments, which has become a contentious topic. Millions of experimental animals are tortured and killed worldwide in order to carry out scientific experiments for study and teaching (SE Wil-San., 2011; MJ. Wolf., HA. Roc, 2008). All of these, however, are carried out and governed by reputable ethical committees, including institutional ones, which uphold the ethics of using animal models in scientific study (SK. Dok., SC. Dha, 2015). The use of animals in experiments is opposed by certain social activists and animal lovers who feel that it is unethical and excessively harmful to the animals. Over

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the past few decades, numerous worldwide laws, rules regulations, and recommendations have been created regarding the use of animals in studies. Institutional (UG. Comm, 2014; MF. Fes., DG.Alt ,2002). Ethical review committees only authorized experiments with animals that cause the least amount of pain and are consistent with the goals of science. A non-animal, alternative, or replacement method is a test or procedure that substitutes other testing for live animals; improves an animal experiment. Along with the use of expert systems, which falls under non-testing methods, it includes "testing methods" such as ex vivo, *In vitro*, *In silico*, or chemico reduced/refined procedures. There are numerous scientifically proven techniques that support animal's welfare that may be able to decrease, eliminate, or improve the use of experimental animals. These techniques have been promoted as important substitutes for carrying out various biological studies and tests. (SK. Dok. SC. Dha ,2015) New treatments can be developed thanks to computer-based drug design, docking, simulation research, and discoveries. Therefore, we have looked at approaches to lessen, replace, or improve the use of experimental animals in various biological research and experiments (W. Yu., AD. Mac, 2017).

ALTERNATIVE METHODS TO EXPERIMENTAL ANIMALS

Alternative techniques, typically based on the 3Rs principles (Replacement, Reduction, and Refinement), are continually being developed to replace the usage of animals. In 1959, (WMS. Rus., RL. Bur ,1959). Russell and Burch initially noted that 3Rs sever as a supervisory frame work for many ethical animal procedures. (WMS. Rus., RL. Bur.,1959; D. BerMel., 2013; J. Tan, *et al*., 2015).

REPLACEMENT

The process of replacing larger animals with smaller ones. Warm-blooded creatures, for example, can be replaced with a variety of microbes, plants, eggs, reptiles, and invertebrates. (D. Huh, *et al.*,2011). Cell culture, *In-Vitro* models, and computer models are examples of new analytical approaches that can be utilized as animals in many research processes. (PC. Arc, 2019).

REDUCTION

It simply refers to reducing the quantity of animals used in a study (WMS.Rus., RL. Bur ;1959). A small number of animals can be used with appropriate findings because to the advancement of modular experimentation design and sophisticated statistical approaches. The quantity of living embryos is decreased with the use of In-Vitro embryonic stem cell culture experiments. Findings such as the properties of the test drug's excipients can be shared, along with the results obtained from each animal and from multiple experiments conducted at the same time, to avoid the need for animal research (J. Tan, *et al*., 2015).

REFINEMENT

It seeks to lessen the suffering and anguish experienced by animals in research procedures. Scientists should improve the animal's facility to lessen the pain, suffering, and misery of animals and experimental operations throughout their lives. Because animals experiencing stress and discomfort have different hormone levels, the results can vary (WMS.Rus., RL.Bur., 1959). The experiments, which involve a large number of animals, must be repeated. Another R, which highlights the 3Rs notion in alternative ways for animals in research, has been introduced in addition to the 3Rs system. It explains how future research on animals' reproductive studies pertaining to immune response may help us comprehend the pathophysiology of numerous pregnancy- related illnesses in humans. Many efforts have been made to investigate substitutes for research animals, such as tissue and tissues generated from bio banks, Computational techniques, organ -on-a -chip technology, or engineering strategies (PC. Arc, 2019).

ALTERNATIVES TO ANIMAL MODEL: COMMON MODEL ORGANISMS

Due to ethical concerns, limitations have been placed on the use of higher vertebrate models in the lab, including rats, guinea pigs, dogs, monkeys, etc. Therefore, using different animals has been investigated. In order to study metastasis, various cancer models have recently been employed, Including Cell culture, non - mammal reptiles such as the loggerhead sea turtle, Plymouth rock chicken, soft-shell clam, saltwater crocodile, African black -footed penguin, Corn snake, Sulfur-crested cockatoo cockatiel, Embryonic stem cells, northern leopard frog,eastern diamondback (B. Abu-Hel., L.van der Wey, 2019).

LOWER VERTEBRATES

Because of their genetic resemblance, lower vertebrates are the best substitute for mammals (higher vertebrate). Additionally, there are fewer ethical issues when using lower vertebrates in experiments. The life cycle of lower vertebrates is brief; therefore, they are extensively researched. Another name for Danio rerio is Zebra fish. D. rerio is a small freshwater fish that ranges in length from 2to 4 cm. Its transparent body during the early stages of development makes it easy to see the inside anatomy. The expense of working area for the experimental chemical solutions needed for the test, as well as the people involved, are reduced when D. rerio is used instead of animals.

EXAMPLE

Danio rerio referred to as a Zebra fish, It is a little freshwater fish that is around 1.5 inches long. In its early stages of development, its bid is nearly translucent, which facilitates the visual observation of internal anatomy. Precise observation of embryonic stages, mutagenesis phenotypic detection, straight forward sampling, toxicity result test determination, and direct monitoring of gene expression using light microscopy are all made possible by optical clarity. High fertility, short life cycles, and small

volumes all aid in the utilization of the laboratory. The operating room, lab solution costs, chemical experiment costs, and labor costs are all decreased by choosing option D. Its eggs and progeny can be produced and processed in petridishes and cell culture plates. Because entire genome sequences are accessible, Zebras are a desirable option for molecular and genetic study. This is specifically utilized to identify a wide range of chemical and pharmaceutical toxicity investigations among an abundance of early-to-early year requests. Additionally, flaws and organ growth abnormalities linked to exposure to research compounds in cancer, heart illnesses, neurological malfunctions, and behavioral dysfunction are frequently observed and studied using this method. It may be possible to ameliorate the phenotypic of some human diseases and organs development defects by modelling them in zebra fish. The *Danio rerio* model was widely used in human and animal health research as well as, more recently, in aquaculture. Although its rodents are by far the most widely utilized model to follow, the *Danio rerio* technique has had a substantial growth in popularity in the scientific regulatory organization as needed. When compared to the models of these more well-documented creatures, the Zebra fish model reduces efforts and expense. This compares well with the in vitro data and offers a recent update and predictive capability. This model might therefore be used to eliminate and replace the use of mammals in research. Drastically reducing the issues related to the welfare of those animals alone (CH. Ste, *et al*., 2021).

INVERTEBRATES

Invertebrates have also been frequently publicized as an alternative to animal testing. Numerous illnesses, including memory loss, endocrine Parkinson's disease, cell aging, diabetes, and toxicological tests, have been investigated utilizing them. Their short lifespan, simple structure, and small size have made them a favorite choice among researchers (D. Ber Mel, 2013). Another name for the fruit fly is Drosophila melanogaster. It is among the invertebrates that are most frequently researched in research. It takes less money for screening, propagation, and upkeep than the other mammal-based models. It's a useful technique for studying neurodegenerative illnesses including Alzheimer's Parkinson's, and Huntington's (B. Abu-Hel., L.van der Wey ,2019). The multicellular eukaryotic nematode *Caenorhabditis elegans* (C. elegans) is around 1 mm long and has an extremely quick generation time. Numerous neurological conditions, including Parkinson's disease, Huntington's disease, different immunological disorders, cancer, diabetes, etc., are studied using this organism (N. Ran., I. Kup ,2012).

EXAMPLE

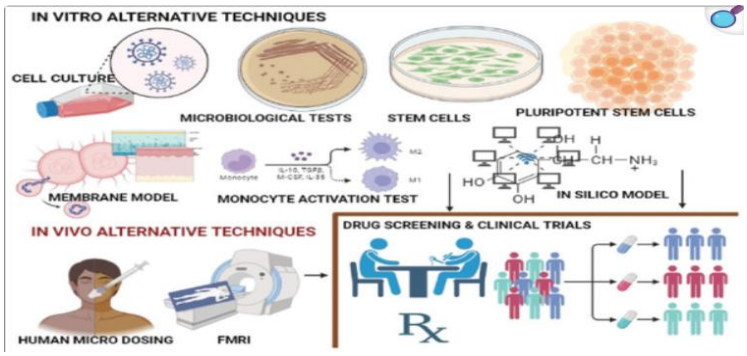
Drosophila melanogaster, an arthyopod, belongs to the Drosophila family. This bug utilized as a biological model about a century ago, and it was critical to the growth of genetics and related studies. Therefore, mentioned fly, which has historically been employed as a model for genotoxicity, has only lately been used as a model for systemic toxicology research or as a substitute model for toxicology trails. Ed Lewis was awarded the Nobel Prize in physiology and Medicine in 1894 for her revolutionary study on the idea of fly genetics. Melanogaster attained ECVAM in research trails using animal models.

MICROORGANISMS

A common substitute for animal experiments is microbes. Numerous biological illnesses and their impact on life have been investigated. The most important and admired model organism is the brewing yeast (CF.Hen, 2009) *Saccharomyces Cerevisiae.* Numerous neurological illnesses, including Huntington's, Parkinson's and Alzheimer's, (N. Ran., I. Kup,2012; CF.Hen.,2009; DM. Ibr ,2006) are studied using S. cerevisiae. Microbes such as *Escherichia coli* (bacterium), *Schizosaccharomyces pombe* (fungus), and *Dictyostelium discoideum* (protista) are excellent models for molecular and genetic studies, although cellular differentiation can be studied with the help of Bacillus.

TYPES OF ALTERNATIVES TECHNIQUES

Because of advancements in tissue engineering and micro-fabrication technology, researchers are particularly interested in developing novel In-Vitro disease models Figure 1.



*Adapted from internet

Among the *In-Vitro* and *In-Vivo* substitutes for animals are the following:

Because of advancements in tissue engineering and micro-fabrication technology, researchers are particularly interested in developing novel In-Vitro disease models. Among the *In-vitro* and *In-vivo* substitutes for animals are the following: Animals testing in the medication research, approval, and screening processes can be replaced with *In-vitro* and *In-vivo* methods. The potential to improve, decrease, and eventually eliminate animal testing is indicated by the expanding range of these methods for replacing and reducing experimental animals. (SK. Dok., SC. Dha ;2015).

IN -VITRO CELL/ TISSUSE CULTURE TECHNIQUES

CELL CULTURE

Several animal experiments can be substituted with cell culture. Different organ culture, tissue cultures, callus cultures, and cell cultures are utilized for different kinds of study (K. Duv, *et al*., 2017). The immortalized primary cell culture is typically used to assess the toxicity of in vitro-cultured cells. (SR. Bra, *et al*., 2010; S. Liu, et *al*., 2017). Additionally, this discipline holds promise for gene therapy as an alternative to animal models, cell-based bio-assays, recombinant DNA technology, and drug screening (UG. Comm, 2014).

IN-VITRO PYROGEN TEST

This is predicated on the release of inflammatory mediators and the leukocytes' reaction to pyrogen contamination. The following assays are used in place of the pyrogen test, which is conducted on rabbits: The aqueous extract of horseshoe crab or *Limulus Polyphemus* blood cells is known as Limulus Amoebocytes Lysate (LAL). Pharmaceutical testing and devises for testing blood or cerebrospinal fluid are both done with LAL (M. Mar *et al*., 2019). The Monocyte Activation Test (MAT), which use cryopreserved human whole blood, detects pyrogens by eliciting an interleukin-1β response. It is better than the rabbit pyrogen and LAL tests (OECD Guidelines.,2023; Lili. W *et al*., 2008).

NEUTRAL RED UPTAKE TEST

The Draize Rabbit eye test can be replaced by this one. In this assay, neutral red enters the cell membrane and accumulates intracellularly in lysosomes (Lipinski C. Lip., A. Hop., 2004; T. Aro *et al.*, 2011).

CELL TRANSFORMATION ASSAYS

Assays for cell transformation are quicker, more costly, and require fewer animals. It serves as a substitute for both the transgenic mouse model for the carcinogenicity test and the rodent bio-assay. The Syrian hamster embryo (SHE) and the Balb/ c3T3 test are two examples (T. Aro, *et al*., 2011; M. Cos JE.Sut ., 2001).

STEM CELL MODELS

Stem cell models are utilized in place of animals in experiments for the In-Vitro toxicological assessment of illnesses (X. Wei.et *al.*, 2013., P.Val.. *et.al*., 1992; PR. West, *et al*., 2022).

MICROBIOLOGICAL TESTS

The Ames test is a microbiological-based assay that can identify between 80 and 90 percent of all carcinogenic substance. It is mostly employed as a screening mechanism. It may also be used as a substitute for animal testing after through validation. (M.Cos ., JE.Sut ., 2001).

MEMBRANE MODEL

The hen's egg test (HET), which uses chorioallantoic membrane and /or vessels to vessels to conduct the experiment, is an alternative to the Draize test for assessing irritation response. This test, which is based on lusis, coagulation, and bleeding, is also known as the HET-chorioallantoic membrane (HET-CAM) test.

ORGAN MODEL

An experimental model for assessing irrational eye toxicity can be the cornea of a freshly killed animal, such as a dead rabbit or chicken. (FA.Bari.,2010). To asses in vitro eye irritation, (N. Alé, *et al.*, 2013) CTT HCETM is a commercial product made from primary human corneal cells (Bio solution Co., Seoul, South, South Korea). Skin - derived epidermal Keratinocytes from humans. As a substitute model for assessing skin irritation, (N. Alé, *et al*., 2013) *t*hese are employed commercially to create the epithelial model Epi Ocular TM. The neutral red uptake (NRU) assay has also been used to assess human keratinocyte vitality in order to assess in-vivo animal irritation. (L. San, *et al*.,2006; R. Osb., Perkins MA. Per., 1991).

IN SILICO METHODS

The required calculations are carried out utilizing specifically created computer programs. There is a wide range of computer -based simulation software that that is now being utilized for educational purposes all around the world, replacing the use of animal models. (SE. Wils-San., 2011; Henkle., 2009; M. Bau, *et al.*,2008; VR. Kum *et al*.,2016). Furthermore, using such software programs, a novel medicine can be changed and created for a specific binding site, and the findings are then obtained through animal testing.

INVIVO ALTERNATIVE TECHNIQUES MICRODOSING

Up to 100µg, micro dosing is defined as less than one hundredth of the pharmacological dose. There is no need

for government animal testing data on the safety of an experimental medicine and the metabolism process that will occur in humans prior to extensive human trails because this method helps screen compounds that won't function in humans.

ADVANCEMENTS IN ALTERNATIVE METHODS TECHNOLOGY

For decades, scientists have used cadavers, animal models, or two- dimensional culture to gather useful information on the etiology of disease, drug testing, and safety assessments; nevertheless, there is disagreement regarding the accuracy, applicability, and reproducibility of human translation. Thus, throughout the past few decades, the concept of developing predictive and reliable systems for drug testing and disease modelling, as well as tissue/ organ and in vitro models, including or ganoids, multi physiological system, and organs -on -chips, are now available to researchers. (B. Med., A.Pra .,1996; PETA., 2022; Khalil AS. Kha.,2020). Numerous in vitro models are comparable to or better than animal models.

HUMAN - PATIENT STIMULATORS

Physiology and Pharmacology are taught to students using computerized human patient simulators that can breathe, bleed, shake, talk, and even have life. Animals' mutilation is inferior to these simulators. (D. Hom.,1986; GM. Cra., DJ. New., 2009; S. Cha., K. Nag., 2013; AD. Kin, *et al.*,2003; S. Moh., 2006).

ORGAN - ON -A CHIP AND BIOLOGICAL CHIP

Human cells are cultivated in a cutting-edge system on a chip to replicate the composition and functionally of a human organ and organ system. Instead of utilizing animals in medication testing, illness research, or toxicity investigations, these chips can be employed. It is very possible that the day of drug testing without the use of animals is closer than we think because of the recent advancements in organoids, computer simulations, and other technologies that mimic drug interactions. (N. Clap, *et al.*,2021).

ORGANOIDS

Organoids were developed as a substitute for 2D cell culture in order to study cell behavior in an environment similar to the human body. These in vitro simplified and miniature organ model systems. Have garnered significant attention for use in tissue formation and disease modelling, drug screening, cell therapy, and personalized medicine. (Hofer M., MP.Lut. 2021). *The* pharmaceutical industry has placed a large bet on the results of studies in this area to significantly reduce animal testing and save money. (I. Hut, *et al.*,2022).

THREE DIMENTIONAL (3D) BIOPRINTING

3D bio-printing is a promising method for creating personalized in vitro models with scientific and medicinal value, offering an alternative to using animal models. Instead of employing animal models, 3D bio-printing advances research by cutting costs, speeding up research development, and boosting the possibility of novel treatments' success.

3D HUMAN DERICED MODELS

One notable achievement is the recent creation of 3D human-derived model that are translated into non-animal technology. By optimizing these human-derived models using omics and in silico methodologies, researchers should be able to gather more relevant human data that can be used for clinical trails design and improving effective clinical outcomes.

ARTIFICAL INTELLIGENCE (AI): BIG DATA AND MACHINE LEARNING (ML) IN 3RS

Big data mining sources have the potential to drastically change how animals are utilized in the future, as demonstrated here. To demonstrate this, the researchers developed an ML model called "Simple RASAR" that uses logistic regression to predict risks based on similarities for every molecule. Simple RASAR (read - across structure activity relationship) and Data Fusion RASAR can predict hazards with an accuracy of 80 - 90% through cross-validation. The reports' second and third main outcomes are test reproducibility in regard to OECD recommendations, simple RASAR, and data fusion RASAR modes, respectively. In terms of chemical specificity and precision, the reproducibility accuracy performed brilliantly (more than 90% accurate). In other words, it is similar to experiments on animals. Computational techniques have advanced significantly in the last ten years, and they can now recognize patterns in data that humans have never seen before. This is especially true in computational toxicology, where algorithms that use chemical structure comparisons to forecast toxicity outperform repeatable animal experiments. With the use of reliable datasets and computer vision, AI models can save the lives of millions of animals. (P Béd , *et al.*,2020; AG Kur, *et al*., 2022). Numerous investigations and discoveries have been made possible by the development of quantum computing. (P Béd ,*et al*., 2020; AG. Kur, *et al.*, 2022). Sophisticated analytical techniques and bio informatics are needed for data curation and understanding. It is unrealistic to anticipate the existence of "click and play" technology in the future. (Z. Lin., Cho. WC. 2022).

CREATION AND APPLICATION OF NON-ANIMAL TECHNOLOGY

Significant steps have not yet been done in India to build many of the new infrastructures and technology. over the past few years, there have been few or no laboratories dedicated to developing these non- animal technologies in both the public and private sectors, despite the fact that regulatory acceptance of alternative is growing due to the Prohibition of animals testing for cosmetics. Prohibition of

the Draize test in the testing of drugs and other chemicals. Amendment in the safety testing requirement for pesticides. When translating PK-PD studies conducted in animals to humans, it is important to consider the diversity of Indian communities. The Indian council of Medical Research (ICMR), New Delhi, formed an expert group in 2017 and conducted the brainstorming with all of the aforementioned concerns in mind. With this endeavour, it is hoped that the nation will look forward to a new era in which drug testing and basic science research would not only be more relevant to human ailments but also be humanitarian, economical, and time -efficient. Although the agricultural, insecticides, and cosmetic industries will also profit from sophisticated non-animal technology, this article concentrates on DD&D as a significant beneficiary. (Vasantha G, *et al*., 2024).

THE STATUS OF THE ART TODAY

Classical in vitro cell-base assays have the drawback of being static models that lack the niche, cell-to cell, and organ -to-organ connections that occur in vivo settings. The two signification developments are Organs and organ systems using multi-channel 3D-microfluidic cell culture chips. Disease -in-a dish models, in which patient-derived cells are grown into organoids for the purpose of predicting individual drug response organs (animals, successful phase III trials outcomes, and potentially making personalized medicine a reality in the future. In this model, a tandem connection to other organs using flow dynamics can be used to create more relvent in vivo systems. Organs cultures that exhibit the mechanical and functional characteristics of human organs have been made possible by the capacity to cultivate cell populations in three dimensions under regulated conditions. (Vasantha G, *et al*.,2024).

Because they fairly more accurately mimic human physiology, illnesses, and medication reactions, these organoids are helpful instruments for drug testing, disease research, and regenerative medicine. Compared to expensive experimental animals testing, 3D- culture systems offer more accurate transnational models and more afforadable *In vivo* data. Additionally, although organ -on-a-chip and disease models using microfluidics only need a few hundred grams of medication material, GLP animal testing requires a significant amount. The chemotherapeutic response of cells, tumor modelling, cellular adaptation, differentiation, biochemical events related to cell functional activities, tissue remodeling/ engineering, and co-culture response are all thought to be more closely related to human physiology in fluid dynamic models that incorporate human - derived organoid model . Currently, many nations have made the production of organoids a top priority in order to facilitate drug discovery. In fact, there are institutions in several nations specifically for this purpose. Given the vast diversity of Indian topics, it is imperative that the Indian government invest in organoid creation specifically for medication development.

PREDICTIVE TOXICOLOGY AND PHARMACOLOGY USING BIG DATA ANALYTICS OF OMICS, MACHINE LEARNING, AND ARTIFICAL INTELLIGENCE

Thousands of gigabytes of data are produced daily worldwide due to the development of -omics and other systems biology research. It is humanly hard to evaluate such large amounts of data and make well-informed decisions for upcoming medical applications. A large portion of this data is retrieved via cloud storage. Adverse outcome pathways (AOPs), which connect a molecular -level disruption of a biological system to an adverse outcome, are also utilized as a framework for gathering, organizing, and assessing the current knowledge derived from high throughput, -omics, guideline studies, clinical, epidemiology, and Eco-field studies. These are intended to provide a clear mechanistic depiction of the crucial detrimental (disease/toxicological) effects that penetrate different levels of the biological structure. An AOP explains how biological disturbance move from lower to higher levels of biological organization, culminating in a negative consequence with regulatory significance. If the data is applied and interpreted pragmatically, this helps the safety evaluation process without using non-human animals. (Vasantha G, *et al*.,2024).

CONCLUSION

Numerous biological experiments have been successfully conducted using a number of non-animal testing techniques. These techniques preserve animals' lives in addition to producing precise results. Researchers have an obligation to develop improve models and techniques for the benefit of both people and animals. Our knowledge of numerous diseases, diagnostics, therapies, and the mechanism of novel drug molecules will all be improved with the anticipated discovery of several fresh, innovation alternatives to animal testing.

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CONFLICT OF INTERESTS

The authors declare no conflict of interest

ETHICS APPROVAL

Not applicable

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AI TOOL DECLARATION

The authors declares that no AI and related tools are used to write the scientific content of this manuscript.

DATA AVAILABILITY

Data will be available on request

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