DERLEME REVIEW

DOI: 10.5336/medsci.2018-59993

# Alternative Methods to Animal Experiments

## Hayvan Deneyleri için Alternatif Yöntemler

Saima MUSHTAQ,[a]
Yavuz Kürşad DAŞ,[a]
Abdurrahman AKSOY[a]

[a]Department of Pharmacology and Toxicology,
Ondokuz Mayıs University,
Faculty of Veterinary Medicine,
Samsun

*Received:* 31.01.2018
*Received in revised form:* 01.03.2018
*Accepted:* 01.03.2018
*Available online*: 04.06.2018

*Correspondence:*
Yavuz Kursad DAS
Ondokuz Mayıs University,
Faculty of Veterinary Medicine,
Department of Pharmacology and Toxicology, Samsun,
TURKEY/TÜRKİYE
ykdas@hotmail.com

**ABSTRACT** In research based sciences *"Alternatives to Animals"* can be stated as testing methods which can replace partial or absolute use of animals; and this field not merely rely on the replacement of tests but the development and implementation of those testing methods to avoid the use of live animals also comes under this section. There are two major alternatives to in-vivo animal testing: The first ones are in vitro cell culture techniques, the seconds ones are in silico computer simulations. Microdosing is one of the other alternative options to study the basic behaviour of drugs by using lower than expected doses to produce whole body effects in volunteer human beings. Microfluidic chips are getting key interest in alternatives; because of the provision of more complex information as compare to other *in vitro* tests. Imaging studies like computed tomography (CT), magnetic resonance imaging (MRI), functional magnetic resonance imaging (fMRI), positron emission tomography (PET) and single photon emission computed tomography (SPECT) are playing unmatchable role while studying some organ systems of the body. Non-Animal Method Database resources are gaining much more popularity since the concept of alternative to animals is culminating all around the world. Because of the advancement in molecular and cellular biology experiments; a lot of information is being generated and stored for in vitro and in silico experiments for better scientific understandings of experimental drugs on body systems. Three Rs (3Rs) Replacement, Reduction and Refinement of Russell and Burch are like guiding principles for more ethical use of animals in testing. In this review, main alternative methods to animal studies are summarized.

**Keywords:** Animal experiments; alternative methods; 3Rs; *in vitro* tests; *in silico* techniques; microdosing techniques; microfluidic chips; toxicological database

**ÖZET** Hayvanlar üzerinde yapılan bilimsel araştırmalarda, araştırmanın bir parçası ya da tamamının yerine geçebilecek test yöntemleri gittikçe önem kazanmaktadır. Bu alanda yalnızca hayvan deneyleri yerine geçebilecek yöntemler değil, ayrıca hayvanların bu araştırmalarda kullanımından kaçınmak için yöntemlerin geliştirilmesi ve uygulanması da yer almaktadır. In vivo hayvan deneyleri için başlıca iki alternatif vardır. Bunlardan ilki in vitro hücre kültürü teknikleri, ikincisi in silico bilgisayar simülasyonlarıdır. Mikro doz tekniği ilaçların temel özelliklerini gönüllü bireylerde vücuttaki meydana getirmiş olduğu etkileri düşük dozda ortaya koyan alternatif bir diğer yöntemdir. Mikro-akışkan çip tekniği diğer yöntemlere kıyasla daha detaylı veriler sunması nedeni ile giderek önem kazanan alternatif bir yöntemdir. Bilgisayarlı tomografi (BT), manyetik rezonans görüntüleme (MR), fonksiyonel manyetik rezonans (fMR), pozitron emisyon tomografi (PET), tek foton emisyon bilgisayarlı tomografi (SPECT) gibi görüntüleme yöntemleri vücutta bazı sistemlerin incelenmesinde eşsiz rol oynarlar. Hayvansal olmayan yöntem veri tabanları dünya çapında hayvan deneylerine alternatif olma açısından giderek popülerlik kazanmaktadır. Moleküler ve hücresel biyoloji deneylerindeki gelişmeler sayesinde, in vitro ve in silico deneyler ile vücut sistemlerinde ilaç denemelerinin bilimsel olarak daha iyi anlaşılması için daha fazla bilgi elde edilip, saklanmaktadır. Russel ve Burch tarafından sunulan 3R kuralı yerine koyma (replacement), azaltma (reduction) ve hayvan refahı (refinement) ilkeleri bilimsel amaçlı deneylerde hayvanların daha etik kullanımına ilişkin rehber niteliği taşımaktadır. Bu derlemede hayvan deneylerine alternatif başlıca metotlar sunulmuştur.

**Anahtar Kelimeler:** Hayvan deneyleri, alternatif yöntemler; 3R; in vitro testler; in siliko teknikler; mikro-akışkan çipler; toksikoloji veritabanları

History of human and animal relationship is as old as the human being itself. Animals were used for various purposes like transportation, food, sports and pets. Because of the advancement in technology and research based sciences especially in medical field; pushed scientists to use various animals like: guinea pigs, dogs, cats, mice, hamsters, rats, fish and rabbits etc. for experimental purposes.[1] Experiment on animals has played a central role in biomedical research throughout history, however from centuries it has also been an issue of heated public and philosophical debate.[2]

Mostly studies are being done to develop and test drugs and their toxicological effects. Beside these studies on medical procedures effects and surgical techniques on experimental basis are key indications for the use of animals in biomedical sciences. As comes to the industrial and business purpose; animals are being used to develop vaccines and certain antibiotics on mass scale.[3-5]

Every year millions of animals are being used for research and experimental purposes because of the long lasting and fast race in the developmental sports of the biomedical sciences. It is extremely hard job to come up with an exact and accurate number of experiments conducted on animals and total number of used animals but in one of the publication conducted on a large scale, authors came up with a close estimate of 115.3 million animals used in 179 countries of the world. Despite a wide range of authors were still not satisfied and concluded to be an underestimate of the total.[6]

Globally, there are two small countries know to have banned on animal experiments; one is the European Principality of Liechtenstein in 1989 and the other one is Republic of San Marino in 2007.[7] As an European Union (EU) member state Malta had declared no animals were used in experiments until 2008, however it reported that 690 animals were used for scientific purposes in 2008.[8]

From later mentioned sources United Kingdom has used about 3.71 million animals in 2011 for research purpose and 3.94 million procedures were conducted on living animals for experimental purposes in year 2016.[9] In USA greater than 820.812 animals used in 2016 for research purposes (except mice and rats).[10] In USA, 16.430.368 is estimated total number of vertebrate (rats, mice, birds, fish, etc.) used in year 2011.[11] In 2015 in Germany there was 17% decreased as compared to 2014. The data published by the German Federal Ministry of Food and Agriculture, (BMEL) shows that 2.753.062 animals were used for research purposes in Germany in 2015 and in 2014 this number was 3.313.898 so the usage of animals decreased by 17 percent.[12]

In Turkey a total of 148,957animals were used in experiments in Turkish universities and government institutions in 2008 of which 42,965 were fish, 34,096 were mice, 30,300 were birds and 26,347 were rats.[13]

Teaching and research institutes like universities and breeding centers are the "*class A*" dealers to fulfil the requirements of such type of experimental animals. Beside this "*class B*" dealers are like brokers and they arrange animals from various legal and illegal sources like some animals can be included from wild life also depends on the requirement of the tested drug or experiment.[14] That's why monkeys and birds are also indicated in few research studies for experimental purposes. Hence, assessment of the animal welfare is being done and monitored nowadays on scientific basis especially for the laboratory animals in terms of their supply, breeding and rearing conditions.[15]

Now major concern is the way these animals are being used, sometimes the requirement of the experiment is tissue or organ so animals are being euthanized by the set method by the control department in a country where it is practiced and target organ or tissue is taken for experimental purpose.[14] Sometimes whole animal is used as a tool regardless of their natural instinct and euthanized at the end of a clinical procedure to reduce the sufferings or pain.[16] In clinical testing and dose calculation; sometimes animals die also or might be they can face a severe type of sufferings or pain which is the major area of debate and argument in this subject since longer period of time. Major argument from defence group is the life. Animals also have life and they can feel pain and distress so they have all the rights to live a distress free life and this type of unethical use of animals should be stopped.[17]

Presently, there are huge numbers of societies and organizations working on this issue to stop unethical use of animals and prevent cruelty to them. Various laws and acts have been passed in different countries and unions to control the unethical use of animals and also to minimize the pain and distress to animals during experimentation. One of the most pioneer organizations named Royal Society for the Prevention of Cruelty to Animals (SPCA) was founded in 1824. In United Kingdom first act to prevent cruelty to animals was constituted in 1876.[18] Later on in 1960's this act was established in India, France and United States also.[14]

Currently there is a long list of rules and acts followed by and being implemented on international level to be supervised by government agencies and private organizations like: International Conference on Harmonization of technical requirements for registration of pharmaceuticals for human use (ICH), Indian Committee for Purpose of Control and Supervision on Experiments on Animal (CPCSEA), UK Society for the Prevention of Cruelty to Animals (SPCA), U.S National Institute of Health (NIH), Organization for Economic Cooperation and Development (OECD), People for the Ethical Treatment of Animals (PETA) American, European Society of Dog and Animal Welfare (ESDAW). These organizations and agencies play a vital role in providing and monitoring implementation of guidelines for humane animal use especially for scientific purposes and make sure to minimize pain and distress of experimental animals.[17] Moreover manpower, skilled staff and highly uneconomical protocols are some of the extra drawbacks of animals being used for research base purposes in biomedical sciences.[14,18]

Studies shown that there is <50% chance of success of experiments performed on animals which correlate the desired and accurate results for the target species like human beings.[19] Most of the time because of the physical and physiological and even biochemical changes of the animals as compare to human beings can lead the research to another direction which is usually undesirable, so all of these reasons compelling scientists to look for alternative options by keeping in mind of animal welfare, accuracy economics of the testing method in drug research.[20]

## THREE BASIC RS AND THE ADDITION OF 4th R

The uncontrolled use of animals has been contempt in literature by far since 1780 by an English Barrister "Jeremy Bentham", who questioned the lack of moral regard towards animals. Since then there has been a growing awareness and public believes against the inhuman or cruel use of animals for experimental purposes.[21]

In 1959 Russell and Burch first time described the humane ways for ethical use of animals in scientific research testing which were named as 3Rs "*Replacement, Reduction* and *Refinement"* and followed worldwide in the establishment of many scientific tests. Later on in some studies introduction of the 4th R termed as "*Responsibility*" was also considered as essential part of the guiding principles for the ethical use of animals.[19,22,23] This fourth R is sometimes termed as "*Rehabilitation*" also in literature.[21,24]

### REPLACEMENT

Replacement section of the guidelines refers to the achievement of similar level of results with preferred use of non-animal methods over animal use methods.[25] Among experimental animals we must use animals with advance nervous system or the animals that are less sensitive to pain.[26] *In vitro* models, cell cultures, imaging and computer models are some of the famous techniques being investigated as alternatives to animals.[14] Extraction of insulin from the bacterial cultures is one of the famous example of replacement. Moreover this extracted insulin is checked for its purity, efficacy and dose by the use of chromatography techniques which is also a replacement technique over animals.[27]

### REDUCTION

Reduction is the method through which researcher utilize better statistical analysis and best quality study design with least number of animals in order to obtain maximum information.[19,25] Using small number of animals and gaining more knowledge is proving the positive side of this method.[26] In one study model; human hepatocyte culture was proposed to get the information about how a drug would be metabolized and eliminated from the body in preliminary stages of the experiment. So,

Inclusion of such types of innovations in a study design helps to eliminate unsuitable compounds in preliminary stages of the study and minimizes the use of animals in further testing.[28]

### REFINEMENT

Refinement of animal procedures refers to improvement of scientific techniques which implicate animals to minimize pain and suffering over the lifetime of the animal.[29] Try to decrease the pain to maximum possible extent during experiment and if end of the study euthanasia is needed choose the most appropriate method for it.[26] Moreover these type of conditions cause an imbalance in hormonal level of animals leading to fluctuations in the results under the stress and discomfort.[30] For example in one study of the genetically modified mice for Huntington's disease showed remarkable changes in results when one group of mice was provided with more comfortable and near to their natural environment as compare to the other group which was caged barren.[14]

### RESPONSIBILITY

Because of the advancement and extremely fast developments in biomedical sciences produced a need and platform by the animal welfare group to push scientists in the 4th R of responsibility. *Responsibility* "R" was added to the basic 3Rs of Russell and Burch. Basically this section implies the addition of responsibility to follow and implement the basic three Rs with full integrity and honesty for the proper and reasonable use of laboratory animals. This section makes us sure that animal life is required and necessary for biomedical research advancement.[19]

Sometimes in literature this 4th R is termed as "*Rehabilitation*" because of the moral obligation of the researcher to take responsibility of the animal in post experiment phase regardless of the outcome, especially after-care is the main-stay of this point.[21,24]

## ALTERNATIVE TO ANIMALS

Because of the advancement in research base studies and biomedical sciences many alternatives have been developed up to some extent for reasonable testing of drugs and chemicals. Advantages associated with these alternatives are reduction of manpower, economics and time consuming. Most common alternatives are described in detail as follows:

### IN VITRO TESTING (CELL CULTURE)

*In vitro* testing is the use of artificially grown cells or tissues under laboratory conditions as alternative to animals to study drugs and chemicals effects. These experimental tissues or cells are obtained from different parts of the body of the animals and preserved in a suitable medium from days to years. The mechanism of the growth of cells or tissues is pretty much similar like bacterial growth on growth medium. So, monolayer of cells is isolated from the target organ and grown on plates or in flasks in the relevant growth medium for a particular type of tissue.[31]

Major advantages associated with this technique are time saving, repeatable, conducive, economical and easy to carry on and follow up. The most important thing which is associated with this type of testing is the preliminary screening which can lead the scientist to a pathway whether it will be beneficial to carry on further in-depth testing or he/she need to stop it here. So, the efficacy and toxicity associated with a drug or chemical can be determined at the preliminary stage of the testing.[32,33]

Currently all cosmetics, especially topical drugs and chemicals are tested for their efficacy and toxicity by the use of these tests. Famous example is the eye irritancy test. Previously Draize test was used particularly on rabbit eyes, nowadays; it has been almost completely replaced by *in vitro* cultured bovine cornea. As damaging a rabbit eye was more distress and inhumane test to check the toxicity and efficacy of the drug. To obtain this type of corneal tissue; bovine cornea is cultured in laboratory up to three weeks, finally to evaluate the irritancy, the toxicological effect or efficacy of drugs.[34]

Even though cell or tissue culture methods may reduce the number of animals being consumed in an experiment but still maintenance of the cells or tissue cultures require animal derived-serum. Basically, the animal use is still there but at least this testing method is somehow a relative replacement in terms of reducing the number of animals, overall distress phase will be shortened or may be less to no distress at all and there will less to no use of protected animals, which is really important.[21]

As concerned with the supply of animal-derived serum, it is difficult to obtain exact figures but the estimated figures shows that in one year at least one million foetal cows are sacrificed to obtain the world's supply of foetal bovine serum, used to grow cell culture.[35] Furthermore *in vitro* techniques are limited to cellular level so they cannot be replaced easily on whole body testing. Because drug efficacy and safety will be determined in a more accurate and applicable way in whole body instead of a cell layer or small tissue.[36]

## MICROFLUIDIC CHIP TESTING

A more complex and improved testing method as compare to *in vitro* testing; to study biological disease process and drug efficacy and safety is microfluidic chip testing. Structurally microfluidic chips are just 2 cm wide and contain an organized series of tiny chambers, each chamber contains a sample of tissue from different parts of the body.[19] These chambers which are known as compartments also are linked by microchannels through which a blood substitute flows on the rule of physics explaining microscale behaviour of fluids flow is different from macrofluidic behaviour.[37,38] Finally the tested substance is added to the fluid which is going to circulate in microchannels and nourish those compartmental tissues and sensors in the chip collect information and send it to a linked computer for analysis.[39] Hence it can give us a better idea of microscale information being derived from a particular tissue.[19,40]

Furthermore, this technique also has limitations as the processes are being screened and analysed at the cellular or tissue levels, but multiple organs participation can be there which cannot replace the whole body. Still conclusive trials need to be conducted on animals.[39]

## ISOLATED TISSUE/ORGAN FOR TISSUE OR ORGAN BATH SYSTEM

The *in vivo* absorption of the drugs can be studied on isolated tissue/organ system which is one of the most common method and being used over decades. For example, rat gut which was preserved in possible close to the physiological conditions can be mounted on the organ bath system and absorption of the drug can be studied on it.[19]

Recently drug studies and experiments have been moved towards human tissue samples for more improved preclinical pharmaceutical research and assessment of the safety parameters of the drugs related to these experiments which is being considered as a good alternative to animals. The idea is more emphasized on the surgically resected tissues which are being discarded most of the time after pathological examination. So, it is stressed that these tissues can be a great contribution to alternative to animals if they are being stored, preserved and produced after that for research purposes in a more ethical and legally approved manner.[41]

In one study isolated chicken ileum was introduced as an alternative to animals. Although still, the research part being studied is coming from the chicken or chick which has been slaughtered for commercial sale purpose. Hence, intestine will be the waste product for the slaughter house and researchers do not need to distress any other laboratory animals for tissue sample.[42]

## MICRODOSING

To test efficacy and safety of drugs at earlier stages of trials with most economical way, this microdosing test was developed. In this method metabolism data of the human body is obtained which is used to analyse the drug being tested or under trial. Microdosing test depend on the ultra-sensitivity of accelerator mass spectrometry which is a very sensitive device. 40% of drugs fail in Phase I clinical trials as per studies.This phase trial need time period of 18 months and cost about £3-5 million. Hence, Microdosing can screen out drugs destined to be effective earlier, rapid and cheaper. This test takes only 4 to 6 months and costs about £0.25 million per drug. Its accuracy of predicting human metabolism is excellent.[19,43]

The bottom line is that in microdosing human volunteers are substituting animals for drug tests. Drugs are introduced into body in a high enough but minimum quantity to cause some cellular effects and then metabolism data helps to study the

efficacy and safety of drugs. But the drawback is that still we need animals for full dose testing to apply final recommendations for human beings.[44]

## IMAGING STUDIES

Because of the advancement in radiological devices it is possible now to study most of the inner body disease processes, structure and function of the different parts of the body especially complicated organs like brain and whole nervous system. Also these gadgets are helpful to study the function of drugs and change in a biological system after administration of these drugs.[45] Some of the most common imaging techniques are as follow: Magnetoencephalography (MEG), magnetic resonance imaging (MRI), functional magnetic resonance imaging (fMRI), magnetic resonance spectroscopy (MRS), positron emission tomography (PET), single photon emission computed tomography (SPECT), event-related optical signals (EROS), transcranial magnetic stimulation (TMS).[46]

These techniques offer a great and user friendly inner view of the human body particularly the brain that cannot be gained by studying animals only.[19] The only drawback is that these imaging techniques can not reveal all the information for all types of drugs still we need animals studies and these devices rely on data obtained from animal testing. But these techniques can be applicable directly on human studies.[47]

## IN SILICO TECHNIQUES (COMPUTER MODELS AND SIMULATIONS)

These types of tests are using computer software and sometimes mixture of mathematical equations also to generate imitation of the operation of the real world process like creating human body organ's structure, function and metabolism. So, in this aspect; computers technology and mathematical equations are helpful to understand the various basic principles of biology. The ultimate goal is designing of new medicines and their verification on specialized computer models and software programs. Basically these simulations are being used to predict the possibility of biological and toxic effect of an experimental drug or chemical without any animal abused.[48]

In computer modelling and simulation studies biological effect of a body system can be represented in an equation and logarithmic form; than these models are more helpful even more accurate in virtual human organs than real time body organs of experimented animals. The best and most successful example of this statement is the designing of the protease inhibitors for HIV patients. Because of the severity of this condition an urgent and most successful treatment protocol was required so protease inhibitors were designed by computers and tested on human tissue cultures and computer organs. Hence it was a success in terms of bypassing animals for experimental purposes while looking on the efficacy of this drug.[19,48]

In one of the pharmacological screening study computer aided drug design (CADD) was considered as a successful software to predict the receptor binding site for a drug molecule to be experimented.[49] Once the primary screening result would have appeared more satisfactory than *in vivo* studies can be performed with high confidence and results usually come more promising. Finally, with the help of such software programs we can easily make a new drug for a specific binding site and then in final stage animal testing can be done to obtain final results which can be confirmatory.[50]

Furthermore, some more software like Structure Activity Relationship (SAR) are being used to predict biological activity of a drug based on the presence of chemical moieties attached to the parent compound. Quantitative Structure Activity Relationship (QSAR) is the mathematical description of the relationship between physico-chemical properties of a drug molecule and its biological activity.[48,51]

Computer database are used to predict the potential drug candidate type of activities like carcinogenicity and mutagenicity. QSAR software which is more sensitive because of its updated and advance database system shows more accurate and appreciated results while predicting the carcinogenicity of any molecule. Advantages of computer models when compared with conventional animal models shows speedy and relatively inexpensive procedures and a reliable substitute to animals.[52]

Dewhurst et al. (1994) also assessed the effectiveness of computer models over the traditional laboratory practices resulted in better problem solving attitude of the group experimenting on computer assisted learning (CAL) protocols as compare to the traditional wet-lab technique group as knowledge gain was the ultimate goal by both groups to be assessed by test, questionnaires, calculations and interpretations. Although *in silico* is quite advance technique to substitute animals to some extent but sometimes resulted findings from computer models or simulations require confirmation on whole animal, so it is still not hundred percent replacement over animals.[53]

In one study computer simulation models were considered as satisfactory inclusion in existing pharmacological teaching schedules for its user friendly and adaptable quality. Hence, students graded them good to excellent teaching programs.[54]

## TOXICOLOGICAL DATABASES

Non-Animal Method Database resources are gaining much more popularity since the concept of alternative to animals is culminating all around the world. Because of the advancement in molecular and cellular biology experiments; a lot of information is being generated and stored for *in vitro* and *in silico* experiments for better scientific understandings of experimental drugs on body systems by establishing database services.[55] Usage of existing database to obtain new information and vision in biomedical research may be a major underused resource if the paucity of published results is any criteria.[56] In toxicological field, new data and information is created at a remarkable pace and is being published at an exponential rate. To deal with this growing body of literature and make it easy and accessible to users these databases have been created.[57]

Currently there are different highly specialized institutes (e.g. ECVAM: "European Centre for the Validation of Alternative Methods") worldwide to validate alternative methods to animal testing and generate data regarding experimental compound to provide to public database service in connection to current and future experiments in terms of alternative methods to animal experimentation.[58] These public database services provide information relevant to the development and app= lication of alternative techniques, including methodology, project type, compound and test results, authors and institutions, and references. Currently, this database not only being applied in one particular type of subject or study but it is being applied widely in different biomedical sciences, pharmacological and toxicological disciplines.[55] For example presently, the public has access to a variety of databases containing mutagenicity and carcinogenicity data. A key to quick advancement in the field of chemical toxicity databases is that of combining information technology with the chemical structure as identifier of the molecules. This allows an extended range of operations (e.g. retri= eving chemicals or chemical classes, describing the content of databases, finding similar chemicals, crossing biological and chemical inter= rogations, etc.) that are not allowed by other classical databases.[59]

A considerable work has been done to determine the appropriateness, ease of use and quality of contents available on the recognized websites linked by validating authorities of the testing methods which are alternative to animals.[60] All databases offered by the National Library of Medicine (NLM) are delivered on a non-fee basis through Medical Literature Analysis and Retrieval System (MEDLARS). MEDLARS is a constellation of databases associated with PubMed; others fall within the TOXNET series such as The Hazardous Substances Databank (HSDB), TOXLINE, The Integrated Risk Information System (IRIS), The Chemical Carcinogenesis Research Information System (CCRIS), ChemIDplus, GENE-TOX and others.[61] Furthermore, Laamanen et al. (2008) came up with Table 1 (given below) of most appropriate and reliable 21 sources of toxicological database which have fulfilled their inclusion criteria out of 822 results.[60]

A brief overview on the complexity of cosmetic database work was exemplified by Kim and Kim (2016). Where focus was on database (toxicological mechanisms and safety information) construction, integration and usage in cosmetic industry, as animal testing began to be banned and alternative to animals methods are being introduced which are still in the developmental phase and

some are being validated in final stages. In this study research data is stressed to be related to test substances is critical for the development of novel alternative tests. For information related to the safety of cosmetic authors developed the CAMSEC database (Consortium of Alternative Methods for Safety Evaluation of Cosmetics).[55] Earlier then this CAMSEC database Comiskey et al. (2015) worked on a novel database for exposure to fragrance ingredients and personal care and cosmetic products.[62] Similarly Goldsmith et al. (2014) developed DockScreen, a database of *in silico* biomolecular interactions designed to enable Rational molecular toxicological insight within a computational toxicology framework. This database is composed of chemical/target (receptor and enzyme) binding scores calculated by molecular docking of more than 1000 chemicals into 150 protein targets and contains nearly 135 thousand unique ligand/target binding scores.[63]

## VALIDATION OF ALTERNATIVE METHODS

Basically validation of alternatives is defined in many ways in validation studies but purpose remained same for example Balls et al. (1990) stated validation as a process by which the relevance of a procedure and reliability are established for a specific purpose.[64] Despite the fact that validation is an important part of the development and eventual regulatory acceptance of an alternative method, many problems have arisen with the proper conduct of validation studies.[65] Balls et al. (1995) compiled a long list of pronounced general deficits in validation studies which later on being cited as major causes of failure in their adaptability, relevancy and reproducibility. A lot of work is being done in this era to improve the validation process.[66]

The experiments have been designed in past (since the beginning of the alternatives to animal testing movement) to evaluate results from an *in vitro* assay as compare to an *in vivo* but recently this type of exercise has been subjected to attempt at formalization and standardization.[58] These relatively recent efforts have been made by the need to evaluate objectively those *in vitro* tests that could be used to reduce, refine, or replace animals

**TABLE 1:** The available toxicological databases.[60]

| No | Name | Country/Org | Web page URL |
|---|---|---|---|
| 1 | ATSDR-HazDat database | USA | http://www.atsdr.cdc.gov/hazdat.html |
| 2 | Chemical Sampling Information (CSI) | USA | http://www.osha.gov/dts/chemicalsampling/toc/toc_chemsamp.html |
| 3 | ESIS – European Chemical Substances Information System | EU | http://ecb.jrc.it/esis/ |
| 4 | EXTOXNET | USA | http://extoxnet.orst.edu/ghindex.html |
| 5 | GESTIS-database on hazardous substances | GER | http://www.hvbg.de/e/bia/gestis/stoffdb/index.html |
| 6 | Haz-Map | USA | http://hazmap.nlm.nih.govhttp://hazmap.nlm.nih.gov/ |
| 7 | High Production Volume Information System (HPVIS) | USA | http://www.epa.gov/hpvis/ |
| 8 | Hazardous Substances Data Bank (HSDB) | USA | http://toxnet.nlm.nih.gov/cgi-bin/sis/htmlgen?HSDB |
| 9 | Hazardous Substances Information System (HSIS) | AUS | http://hsis.ascc.gov.au/SearchHS.aspx |
| 10 | IARC Monographs | UN | http://monographs.iarc.fr/ENG/Monographs/PDFs/ index.php |
| 11 | IPCS INCHEM | UN | http://www.inchem.org/ |
| 12 | International Toxicity Estimates for Risk Database | USA | http://www.tera.org/iter/ |
| 13 | IRIS database for risk assessment | USA | http://www.epa.gov/iris/index.html |
| 14 | MSDS Database – (Material Safety Data Sheet db) | CAN | http://www.ohsah.bc.ca/533/1402/ |
| 15 | NIOSH Pocket Guide to Chemical Hazards | USA | http://www.cdc.gov/niosh/npg/search.html |
| 16 | PAN Pesticides Database | USA | http://www.pesticideinfo.org/index.html |
| 17 | Scorecard | USA | http://www.scorecard.org/chemical-profiles/ |
| 18 | Screening Information Data Set (SIDS) for High Volume Chemicals | UN | http://www.chem.unep.ch/irptc/sids/OECDSIDS/ sidspub.html |
| 19 | SOLV-DB | USA | http://solvdb.ncms.org/solvdb.htm |
| 20 | The chemical database | USA | http://ull.chemistry.uakron.edu/erd/ |
| 21 | TOXNET | USA | http://toxnet.nlm.nih.gov/ |

in biomedical experimentation in order to promote regulatory acceptance of them.[65]

Validation of an alternative is quite lengthy and cost effective process especially with respect to its reproducibility and finally its predictivity. For example; reproducibility factor is correlated with the results not only in one laboratory but also should be comparable in different laboratories as well with the ease of flexibility in its methodology.[36] For example, the validation study conducted by ECVAM on rodent post-implantation embryo culture permitted the use of different rat strains, different culture apparatus and different culture media. Even so, reproducibility of results was quite high between laboratories.[67] As concerned with the predictivity of a test it is defined by comparison of *in vitro* results by existing *in vivo* data. For example, in developmental toxicity testing; one can be more interested in predicting data available for human beings but limitations in the database from existing studies is a major constraint; furthermore existing animal related data is not optimum to predict same pattern in human beings.[36]

Finally, Kandarova and Letasiova (2011) enlisted a number of validated and pre-validated alternatives which can be partially or completely replaced by animal testing and their results are comparable to animal involved studies. Furthermore, it was concluded from the same study that several international validation studies proven helpful and fruitful to replace the animals or reduce the number of tests on animals partially or completely.[58]

## CONCLUSION

Animal welfare issue is as important as human welfare. Not all but various common alternatives have been proposed in this article which can be adopted to uplift the implementation of 3Rs and reduce the number of animals required for drug research. Although there is no way so far that they can completely eliminate the need for animals in preclinical studies. Hence intact animal does provide a better model of the complex interaction of the physiological process than does an alternative technique.

### *Source of Finance*

*During this study, no financial or spiritual support was received neither from any pharmaceutical company that has a direct connection with the research subject, nor from a company that provides or produces medical instruments and materials which may negatively affect the evaluation process of this study.*

### *Conflict of Interest*

*No conflicts of interest between the authors and / or family members of the scientific and medical committee members or members of the potential conflicts of interest, counseling, expertise, working conditions, share holding and similar situations in any firm.*

### *Authorship Contributions*

***Idea/Concept:*** *Abdurrahman Aksoy, Yavuz Kürşad Daş;* ***Design:*** *Abdurrahman Aksoy, Yavuz Kürşad Daş;* ***Control/Supervision:*** *Abdurrahman Aksoy, Yavuz Kürşad Daş;* ***Data Collection and/or Processing:*** *Abdurrahman Aksoy, Yavuz Kürşad Daş, Saima Mushtaq;* ***Analysis and/or Interpretation:*** *Abdurrahman Aksoy, Yavuz Kürşad Daş;* ***Literature Review:*** *Abdurrahman Aksoy, Yavuz Kürşad Daş, Saima Mushtaq;* ***Writing the Article:*** *Abdurrahman Aksoy, Yavuz Kürşad Daş, Saima Mushtaq;* ***Critical Review:*** *Abdurrahman Aksoy, Yavuz Kürşad Daş.*

## REFERENCES

1. CULABBR. Use of laboratory animals in biomedical and behavioral research. Committee on Use of Laboratory Animals in Biomedical and Behavioral Research. National Research Council and Institute of Medicine. 1st ed. Washington, DC: National Academy Press; 1998. p.12-5.
2. Franco NH. Animal experiments in biomedical research: a historical perspective. Animals (Basel) 2013;3(1):238-73.
3. Giacomotto J, Ségalat L. High-throughput screening and small animal models, where are we? Br J Pharmacol 2010;160(2):204-16.
4. Hendriksen CF. Three Rs achievements in vaccinology. AATEX 2007;14:575-9.
5. Hendriksen CF. Replacement, reduction and refinement alternatives to animal use in vaccine potency measurement. Expert Rev Vaccines 2009;8(3):313-22.
6. Taylor K, Gordon N, Langley G, Higgins W. Estimates for worldwide laboratory animal use in 2005. Altern Lab Anim 2008;36(3):327-42.
7. Knight A. The beginning of the end for chimpanzee experiments? Philos Ethics Humanit Med 2008;3(1):16.
8. EC. Report from the commission to the council and the european parliament. Sixth Report on the Statistics on the Number of Animals used for Experimental and other Scientific Purposes in the Member States of the European Union SEC 1107. Brussels; 2010. 8.12.2010.
9. Great Britain Home Office. Annual Statistics of Scientific Procedures on Living Animals Great Britain 2016. p.57. https://www.gov.uk/ government/uploads/system/uploads/attachment_data/file/627284/annual-statistics-scientific-procedures-living-animals-2016.pdf. Accessed: November 21, 2017.

10. USDA. Annual Report Animal Usage by Fiscal Year: 2016. United State Department of Agriculture. Animal and Plant Health Inspection Service; 2017. p.10. June 27, 2017. https://www.aphis.usda.gov/animal_welfare/downloads/reports/Annual-Report-Animal-Usage-by-FY2016.pdf. Accessed: December 13, 2017.
11. Leary SA. The exclusion of mice, rats, and birds. The Animal Welfare Act At 50; 2017. p.2. http://aavs.org/assets/uploads/2017/ 08/2017-1_av-magazine_exclusion-mice-rats-birds.pdf?x82509. Accessed: January 15, 2018.
12. Max-Planck-Gesellschaft. Animal research in Germany. https://www.mpg.de/animal-studies- portal/animal-research Accessed: December 13, 2017.
13. Izmirli S, Aldavood SJ, Yasar A, Phillips CJ. Introducing ethical evaluation of the use of animals in experiments in the Near East. Altern Lab Anim 2010;38(4):331-6.
14. Doke SK, Dhawale SC. Alternatives to animal testing: a review. Saudi Pharm J 2015;23(3): 223-9.
15. Baumans V. Science-based assessment of animal welfare: laboratory animals. Rev Sci Tech 2005;24(2):503-13.
16. Rusche B. The 3Rs and animal welfare-conflict or the way forward? ALTEX 2003; 20(Suppl 1):63-76.
17. Rollin BE. Toxicology and new social ethics for animals. Toxicol Pathol 2003;31 Suppl: 128-31.
18. Balls M. Replacement of animal procedures: alternatives in research, education and testing. Lab Anim 1994;28(3):193-211.
19. Arora T, Mehta AK, Joshi V, Mehta KD, Rathor N, Mediratta PK, et al. Substitute of animals in drug research: an approach towards fulfilment of 4R's. Indian J Pharm Sci 2011;73(1):1-6.
20. Flecknell PA. Refinement of animal use--assessment and alleviation of pain and distress. Lab Anim 1994;28(3):222-31.
21. Sharma S, Bhatt M, Dahiya N, Gupta AK, Brashier DBS, Sharma AK. Alternatives to animal experiments: an overview. Asian J Pharm Toxicol 2015;3(7):1-6.
22. Russell WMS, Burch RL. The Principles of Humane Experimental Technique. 1st ed. London: Methuen; 1959. p.238.
23. Banks RE. The 4th R of research. Contemp Top Lab Anim Sci 1995;34(1):50-1.
24. Pereira S, Tettamanti M. Ahimsa and alternatives--the concept of the 4th R. The CPCSEA in India. ALTEX 2005;22(1):3-6.
25. Schechtman LM. Implementation of the 3Rs (refinement, reduction, and replacement): validation and regulatory acceptance considerations for alternative toxicological test methods. ILAR J 2002;43 Suppl 1:S85-94.
26. Melikoglu Golcu B, Aksoy A. [An overview of legislation on the use of experimental animals in the european union and Turkey]. Turkiye Klinikleri J Lab Anim 2017;1(1):56-62.
27. Foreman DM, Pancholi S, Jarvis-Evans J, McLeod D, Boulton ME. A simple organ culture model for assessing the effects of growth factor on corneal re-epithelialization. Exp Eye Res 1996;62(5):555-64.
28. Kimber I, Pichowski JS, Betts CJ, Cumberbatch M, Basketter DA, Dearman RJ. Alternative approaches to the identification and characterization of chemical allergens. Toxicol In Vitro 2001;15(4-5):307-12.
29. Emerson M. Refinement, reduction and replacement approaches to in vivo cardiovascular research. Br J Pharmacol 2010;161(4): 749-54.
30. Saka VP, Srinivasababu P, Himaja V, Bhagawathi V, Prasannanjaneyulu P, Rao YV, et al. Review on alternative to testing animals. Int J Advan Pharm Sci 2016;7(2):3050-3.
31. Pizzoferrato A, Ciapetti G, Stea S, Cenni E, Arciola CR, Granchi D, et al. Cell culture methods for testing biocompatibility. Clin Mater 1994;15(3):173-90.
32. Shay JW, Wright WE. The use of telomerized cells for tissue engineering. Nat Biotechnol 2000;18(1):22-3.
33. Steinhoff G, Stock U, Karim N, Mertsching H, Timke A, Meliss RR, et al. Tissue engineering of pulmonary heart valves on allogenic acellular matrix conduits in vivo restoration of valve tissue. Circulation 2000;102(19 Suppl 3):III50-5.
34. Xu KP, Li XF, Yu FS. Corneal organ culture model for assessing epithelial responses to surfactants. Toxicol Sci 2000;58(2):306-14.
35. Brunner D, Frank J, Appl H, Schöffl H, Pfaller W, Gstraunthaler G. Serum-free cell culture: the serum-free media interactive online database. ALTEX 2010;27(1):53-62.
36. Piersma AH. Alternative methods for developmental toxicity testing. Basic Clin Pharmacol Toxicol 2006;98(5):427-31.
37. Chokkalingam V, Weidenhof B, Krämer M, Maier WF, Herminghaus S, Seemann R. Optimized droplet-based microfluidics scheme for sol-gel reactions. Lab Chip 2010;10(13):1700-5.
38. Kirby BJ. Nanofluidics: fluid and current flow in molecular-scale and thick-EDL systems. In: Kirby BJ, ed. Micro- and Nanoscale Fluid Mechanics: Transport in Microfluidic Devices. 1st ed. Cambridge: Cambridge University Press; 2010. p.336-44.
39. Prot JM, Leclerc E. The current status of alternatives to animal testing and predictive toxicology methods using liver microfluidic biochips. Ann Biomed Eng 2012;40(6):1228-43.
40. Bunney WE, Bunney BG, Vawter MP, Tomita H, Li J, Evans SJ, et al. Microarray technology: a review of new strategies to discover candidate vulnerability genes in psychiatric disorders. Am J Psychiatry 2003;160(4):657-66.
41. Thasler WE, Schlott T, Kalkuhl A, Plän T, Irrgang B, Jauch KW, et al. Human tissue for in vitro research as an alternative to animal experiments: a charitable "honest broker" model to fulfil ethical and legal regulations and to protect research participants. Altern Lab Anim 2006;34(4):387-92.
42. Undale VR, Jagtap PN, Yadav AV, Sangamnerkar SK, Upasani CD, Bhosale AV. An isolated chicken ileum: alternative to laboratory animals for isolated tissue experimentation. IOSR J Pharm 2012;2(5):39-45.
43. Zanni GR, Wick JY. Microdosing: the new pharmacokinetic paradigm? Consult Pharm 2006;21(10):757-76.
44. Tewari T, Mukherjee S. Microdosing: concept, application and relevance. Perspect Clin Res 2010;1(2):61-3.
45. Hudson M. The welfare and scientific advantages of non-invasive imaging of animals used in biomedical research. Anim Welfare 2005;14(4):303-17.
46. Davis MA. Bioimaging of laboratory animals: the visual translation of molecular insights. ILAR J 2008;49(1):1-3.
47. Balaban RS, Hampshire VA. Challenges in small animal noninvasive imaging. ILAR J 2001;42(3):248-62.
48. Roncaglioni A, Benfenati E. In silico-aided prediction of biological properties of chemicals: oestrogen receptor-mediated effects. Chem Soc Rev 2008;37(3):441-50.
49. Vedani A. [Computer-aided drug design: an alternative to animal testing in the pharmacological screening]. ALTEX 1991;8(1):39-60.
50. Reddy RN, Mutyala R, Aparoy P, Reddanna P, Reddy MR. Computer aided drug design approaches to develop cyclooxygenase based novel anti-inflammatory and anti-cancer drugs. Curr Pharm Des 2007;13(34):3505-17.
51. Knight A, Bailey J, Balcombe J. Animal carcinogenicity studies: 3. alternatives to the bioassay. Altern Lab Anim 2006;34(1):39-48.
52. Matthews EJ, Contrera JF. A new highly specific method for predicting the carcinogenic potential of pharmaceuticals in rodents using enhanced MCASE QSAR-ES software. Regul Toxicol Pharmacol 1998;28(3): 242-64.
53. Dewhurst DG, Hardcastle J, Hardcastle PT, Stuart E. Comparison of a computer simulation program and a traditional laboratory practical class for teaching the principles of intestinal absorption. Am J Physiol 1994;267(6 Pt 3):S95-104.
54. Badyal DK, Modgill V, Kaur J. Computer simulation models are implementable as replacements for animal experiments. Altern Lab Anim 2009;37(2):191-5.
55. Kim SW, Kim BH. A web-based alternative non-animal method database for safety cosmetic evaluations. Toxicol Res 2016;32(3): 259-67.
56. U.S. Congress, Office of Technology Assessment. Alternatives to Animal Use in Research, Testing, and Education. OTA-BA-273. Washington, DC: U.S. Government Printing Office; 1986. p.433.
57. Voigt K, Brüggemann R. Toxicology databases in the metadatabank of online databases. Toxicology 1995;100(1-3):225-40.
58. Kandárová H, Letašiová S. Alternative methods in toxicology: pre-validated and validated methods. Interdiscip Toxicol 2011;4(3):107-13.
59. Benigni R, Battistelli CL, Bossa C, Tcheremenskaia O, Crettaz P. New perspectives in toxicological information management, and the role of ISSTOX databases in assessing chemical mutagenicity and carcinogenicity. Mutagenesis 2013;28(4):401-9.
60. Laamanen I, Verbeek J, Franco G, Lehtola M, Luotamo M. Finding toxicological information: an approach for occupational health professionals. J Occup Med Toxicol 2008;3(1):18.
61. Wright LL. Searching fee and non-fee toxicology information resources: an overview of selected databases. Toxicology 2001;157(1-2): 89-110.
62. Comiskey D, Api AM, Barratt C, Daly EJ, Ellis G, McNamara C, et al. Novel database for exposure to fragrance ingredients in cosmetics and personal care products. Regul Toxicol Pharmacol 2015;72(3):660-72.
63. Goldsmith MR, Grulke CM, Chang DT, Transue TR, Little SB, Rabinowitz JR, et al. Dockscreen: a database in of silico biomolecular interactions to support computational toxicology. Dataset Pap Sci 2014;Article ID 421693.
64. Balls M, Blaauboer B, Brusick D, Frazier J, Lamb D, Pemberton M, et al. Report and recommendations of the CAAT/ERGATT workshop on the validation of toxicity test procedures. Altern Lab Anim 1990;18:313-37.
65. Huggins J. Alternatives to animal testing: research, trends, validation, regulatory acceptance. ALTEX 2003;20(Suppl 1):3-61.
66. Balls M, Blaauboer BJ, Fentem JH, Bruner L, Combes RD, Ekwall B, et al. Practical aspects of the validation of toxicity test procedures. The report and recommendations of ECVAM Workshop 5. Altern Lab Anim 1995;23:129-47.
67. Piersma AH, Genschow E, Verhoef A, Spanjersberg MQ, Brown NA, Brady M, et al. Validation of the postimplantation rat whole-embryo culture test in the international ECVAM validation study on three in vitro embryotoxicity tests. Altern Lab Anim 2004;32(3):275-307.