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REVIEW ARTICLE

3R Principle and Alternative Toxicity Testing Methods

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Summary

Toxicity tests are essential for evaluating the safety or hazards of several substances. Many of the current toxicity testing methods include the use of animals, especially rodents. However, in the last three decades, there has been an inclination towards using less animals and performing alternative methods that reduce, refine and replace animal use (3R principle). The viewpoint of "reduction" is to decrease the number of animals required for a test method, while remaining consistent with scientific practices that are necessary to aquire valid results. The standpoint of "refinement" is using procedures that bring less pain or distress in animals. The aspect of "replacement" is to use non-animal systems instead of animals, or to use a phylogenetically lower species of live animals. Among all approaches, the use of alternative techniques replacing animals has a potential for the future research. The alternative test methods include in vitro models like cell cultures, and tissue/organ assays and in silico testing. Cell cultures have several advantages and scientist expect that in vitro testing will succeed against in vivo testing in the forthcoming years. This review will focus on the 3R principle and alternative test methods.

Key Words: *3R principle, reduction, refinement, replacement, alternative test methods, cell lines, in silico*

3R İlkesi ve Alternatif Toksisite İnceleme Yöntemleri

Özet

Toksisite yöntemleri, birçok maddenin tehlike ve güvenlik değerlendirmelerinde gereklidir. Günümüzde kullanılan toksisite yöntemlerinin çoğunda özellikle kemiriciler olmak üzere deney hayvanları kullanılmaktadır. Ancak son 30 yıldır daha az hayvan kullanımı ve hayvan deneylerini azaltma, iyileştirme ve hayvan kullanımı yerine başka yöntemleri uygulamayı içeren (3R ilkesi) alternatif yöntemlerin kullanılması eğilimi bulunmaktadır. 'Azaltma ' kavramı, geçerli sonuçları almak için gerekli bilimsel uygulamalardan uzaklaşmadan bir yöntem için istenen hayvan sayısının azaltılmasını kapsamaktadır 'Iyileştirme', hayvanlarda daha az acı ve baskıya neden olabilecek işlemleri kullanmayı içermektedir.'Yerine Koyma', yöntemlerde hayvan yerine filogenetik olarak daha düşük türlerin yada hayvan dışı sistemlerin kullanımını ifade eder. Gelecekteki araştırmalarda hayvanların yerine geçen yaklaşımların kullanımı önem kazanacaktır. Alternatif yöntemler hücre kültürleri, doku/organ yöntemleri gibi in vitro yöntemler ve in siliko incelemeleri içermektedir. Hücre kültürlerinin pekcok üstünlüğü bulunmaktadır, araştırıcılar ileri yıllarda in vitro incelemelerin in vivo deneylere karşı daha başarılı sonuçlar vereceğine inanmaktadır. Bu derleme 3R ilkesi ve alternatif inceleme yöntemleri üzerine yoğunlaşmıştır.

Anahtar Kelimeler: *3R ilkesi, azaltma, iyileştirme, yerine koyma ,alternatif inceleme yöntemleri, hücre serileri, in siliko*

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INTRODUCTION

Toxicity testing is performed to assess the safety or hazards of several substances such as industrial chemicals, pharmaceuticals and consumer care products. Many of the current toxicity testing methods include the use of laboratory animals (e.g. mice, rats, rabbits) (1). However, in the last three decades, there has been a tendency towards using less animals and performing new methods that can serve as new options in biological sciences. Alternative tests are methods that reduce, refine and replace animal use (1). Reduction, refinement, and replacement are commonly referred to as "the 3Rs of alternatives" and they were first described by **William Russell and Rex Burch (1959)** in the *"Principles of Humane Experimental Technique"* (2). In recent years, another "R" has been added to the Rs of alternatives: "Rehabilitation". The concept is now sometimes referred as "4Rs" (3).

In the European Union (EU), all industry sectors, including pharmaceutical, chemical, cosmetic, agrochemical and food manufacturers, have already been obliged to apply available methods to replace, reduce, and refine animal use (3Rs) in safety and efficacy evaluations under the existing animal protection legislation. Directive 86/609/EEC, Article 7.2 states that "An experiment shall not be performed if another scientifically satisfactory method of obtaining the result sought, not entailing the use of an animal, is reasonably and practicably available". Article 23 states that the Commission and the Member States should encourage research in the development and validation of alternative techniques. As a response to Articles 7.2 and 23, the Commission announced the establishment of European Centre for the Validation of Alternative Methods (ECVAM) in a communication to the Council and the Parliament in October 1991 (4, 5).

The Directive 86/609/EEC was revised and was replaced by the Directive 2010/63/EU that required member states to ensure a scientifically satisfactory method or testing strategy, not entailing the use of live animals, wherever possible (5). On the other hand, "Declaration of Bologna" which was adopted in 1999 by the "The Third World Congress on Alternatives and Animal Use in the Life Sciences" strongly endorsed and reaffirmed the "3R Principle" (6).

The new Directive 2010/63/EU has further enforced the role of ECVAM and its duties and tasks are defined as follows (7):

- Coordinating and promoting the development and use of alternatives to procedures including in the areas of basic and applied research and regulatory testing
- Coordinating the validation of alternative approaches at EU level
- Acting as a focal point for the exchange of information on the development of alternative approaches
- Setting up, maintaining and managing public databases and information systems on alternative approaches and their state of development
- Promoting dialogue between legislators, regulators, and all relevant stakeholders, in particular, industry, biomedical scientists, consumer organizations and animal-welfare groups, with a view to the development, validation, regulatory acceptance, international recognition, and application of alternative approaches
- Maintaining database on validated alternative methods (Tracking System for Alternative test methods, TSAR) and database on *in vitro* test protocols (INVITTOX)
- Maintaining bibliographic database (Database Service on Alternative Test Methods to Animal Experimentation, DBALM)

According to the "Fourth Report on the Statistics on the Number of Animals Used for Experimental and other Scientific Purposes in the Member States of the EU", the total number of animals used in 2002 was 10.7 million. The European Commission's recent proposal on the Registration, Evaluation and Authorisation of Chemicals (REACH) has served to highlight stakeholders and general public concerns regarding the continued need for animal testing as a mean to protect human and animal health and the environment. At the same time, it should be recognized that there is considerable pressure from the public and the regulators to better understand risks to human and the environment from chemicals and to increase assurance in product safety. In the absence of validated alternative methods, the current

legislative paradigm requires animal use (8).

In the United States, Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) is an interagency committee of the U.S. Government, with members representing fifteen U.S. federal regulatory and research agencies that use, generate, or disseminate toxicological information used to determine the safety or potential adverse health effects of chemicals and products to which workers and consumers may be exposed to (9). ICCVAM was formally established by law with signing of the ICCVAM Authorization Act of 2000 (10). The committee was originally organized by NIEHS in 1997 in collaboration with the other 14 federal agencies. ICCVAM coordinates interagency technical reviews of the new and revised safety testing methods with regulatory applicability, including alternative test methods that may reduce, refine, or replace the use of animals in order to advance animal welfare while ensuring human health and safety. ICCVAM also coordinates cross-agency issues relating to development, validation, acceptance, and national and international harmonization of new, modified, and alternative toxicological test methods. Since 1998, ICCVAM has contributed to the national and international regulatory acceptance of 51 alternative safety-testing methods, including 33 that do not use live animals (9). The National Toxicology Program (NTP) Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM) administers ICCVAM and provides scientific and operational support for ICCVAM-related activities. NICEATM works closely with ICCVAM to carry out test method evaluations, organize peer reviews and workshops, and communicate with ICCVAM stakeholders. NICEATM also conducts independent validation studies for new proposed safety testing methods to characterize their usefulness and limitations for regulatory applications (9).

What does test method validation mean?

Test method validation is a process based on scientifically sound principles by which "reliability" and "relevance" of a particular test, approach, method or process are established for a scientific purpose. "Reliability" can be summarized as the extent of reproducibility of results from a test within and among laboratories overtime, when performed using the same standardized protocol. "Relevance" of a test method is the relationship between the test and the effect in the target species, and whether the test method is meaningful and useful for a defined purpose, with the limitations identified. Predictive ability and reliability of a toxicity test is judged by its sensitivity, specificity, predictability, accuracy, and reproducibility (within a laboratory and between laboratories). Since, 1980s, the validation criteria for new toxicological test methods have been developed as a collaborative effort of the leading scientists in this field, under the auspice of Organisation for Economic Cooperation and Development (OECD), ECVAM and ICCVAM (11).

3R PRINCIPLE

A. REDUCTION

The viewpoint of "reduction" alternative is to decrease the number of animals required for a test method, while remaining consistent with scientific practices necessary to obtain valid results. This may be achieved by reducing the number of variables through good experimental design, by using genetically homogeneous animals or by ensuring that the conditions of the experiment are rigorously controlled. The need of reducing the number of animals was first published in 1943 by **Deichmann and LeBlanc** (12).

Using the "Lethal Dose 50 (LD_{50})" test was the center of controversy for many decades. LD_{50} test was first introduced in **1927** by **Trevan** for testing substances intended for human use. The test aimed to find the single lethal dose of a substance that kills half of the animals in a test group (13). By the 1970s, it got generally accepted as a basis of comparing and classifying the toxicities of chemicals, and gradually became a required test for various regulatory bodies concerned with new drugs, food additives, cosmetic ingredients, household products, industrial chemicals and pesticides. The test required up to 100 animals, sometimes for each of the two species (normally the rat but also the mouse when a second species was needed) for each substance tested (14).

OECD incorporated the LD_{50} test into its new Test Guidelines in 1981. However, by this time, it was commonly accepted that the statistical precision of the LD_{50} value and its confidence intervals as well as the slope of the dose–mortality curve were not needed for normal hazard and risk assessment purposes. Hence, the 1981 guideline for acute oral toxicity required the use of only five animals per sex per dose group, with three dose groups per test which were chosen, from sighting studies or from historical data, to span the LD_{50} value. An upper dose level limit of 5000 mg/kg was also introduced. (15). The concept of a "Limit Test" was included for essentially non-toxic substances. Similar guidelines were also published for acute dermal (16) and inhalation (OECD 403, 1981. OECD Guidelines for the Testing of Chemicals, OECD 403. Acute Inhalation Toxicity. , Organisation for Economic Cooperation and Development, Paris (1981).17) toxicity. By the end of 2002, the LD_{50} test was finally repealed after many years of controversy and debate.

After the deletion of LD_{50}, three alternative animal tests, the **"Fixed Dose Procedure (FDP)"**, the **"Acute Toxic Class Method (ATC)"** and the **"Up and Down Procedure (UDP)"** were introduced. These tests provided significant improvements in animal welfare. They have recently undergone revision both for improving their scientific performance and increasing their regulatory acceptance. They can now be used within a strategy for acute toxicity testing for all types of test substances. Along with these particular tests, i*n vitro* cytotoxicity tests could be used to improve the dose level selection and thus enable reductions in the number of animals to be used. However, the total replacement of animal tests with *in vitro* tests will at least take 10 years as new and validated *in vitro* tests should be developed for relaying the *in vivo* tests (14).

The three tests approved by OECD are discussed below in detail, summarized in Table 1 and described below:

a. Fixed Dose Procedure (OECD 420)

The "Fixed Dose Procedure (FDP)" test was first proposed in 1984 by the British Toxicology Society, as an acute oral toxicity test. In comparison to the older

Table 1. The principles of the three alternati

	Fixed Dose Procedure (FDP, OECD 420)	Acute Toxic Class Method (ATC, OECD 423)	Up and down Procedure (UDP, OECD 425)
Animals	Young adult rats (one sex)	Young adult rats (one sex)	Young adult rats (one sex)
Dosing Type	Single bolus dose	Single bolus dose	Single bolus dose
Dosing route	Oral gavage with constant volume and concentration	Oral gavage with constant volume and concentration	Oral gavage with constant volume and concentration
Observations	Clinical observations, body weight follow-up	Clinical observations, body weight follow-up	Clinical observations, body weight follow-up
Sighting Study	Yes	No	No
Methodology	Fixed doses of 5, 50, 500, and 2000 mg/kg	Fixed doses of 5, 50, 500, and 2000 mg/kg	Starting at estimation for LD50 (or with 175 mg/kg dose), using a dose
Aim	Determination of lowest fixed dose that causes evident toxicity	Determination of lowest fixed dose that causes mortality	Estimation of LD50
Estimation	Range estimation of LD50	Range estimation of LD50	Point estimation of LD50 with confidence intervals
Necropsy	Yes	Yes	Yes
Endpoint	Sign(s) of acute toxicity in target organ(s)	Sign(s) of acute toxicity in target organ(s)	Sign(s) of acute toxicity in target organ(s)

LD_{50} test, this procedure produces similar results, while using fewer animals and causing less pain and suffering. It was published in 1990 after both national and international validation studies (18). This new test reduced the number of animals required from 30 to 20. As a result, in 1992 this test was proposed as an alternative to the LD_{50} test by the OECD under OECD Test Guideline 420 (19).

In this procedure, the test substance is given orally at one of the four fixed-dose levels (5, 50, 500, and 2000 mg/kg) to five male and five female rats. The objective is to identify the lowest dose that produces clear signs of toxicity but no mortality. Outputs are range estimation of LD_{50}, predicting signs of acute toxicity and predicting target organs. Depending on the results of the first test, either no further testing is needed or a higher or a lower dose is applied: If mortality occurs, retesting at a lower dose level is necessary (except if the original dose chosen is 5 mg/kg). If no signs of toxicity occur at the initial dose, it is necessary to retest at a higher dose level. The results are thus interpreted in relation to animal survival and evident toxicity. It becomes possible to assign the chemical to one of the OECD classification categories (20). FDP is able to provide results that enable substances to be ranked according to the EU system of classification in a way which is broadly compatible with how they would have been allocated by LD_{50} values derived from classical acute oral toxicity tests (21).

b. Acute Toxic Class Method (OECD 423)

The aim to develop the oral ATC method was to replace the oral LD_{50} test with the use of significantly fewer animals on the basis of a sequential testing procedure and the use of three animals of one sex per step (22, 23).

For the first time ever, the principle of this new toxicological test method was evaluated by biometric calculations, before animal experiments were initiated. The biometric calculations of the ATC method were carried out, not only for the classification categories of EU, but also for the classification criteria of various countries and organizations, currently in use. The oral ATC method was validated by animal experiments and biometric evaluations (24) in a national (25) and an international (26) ring study. An excellent correlation between biometric evaluations and the results of animal experiments were documented and up to 70% fewer animals in comparison to the LD_{50} test were used. Depending on the mortality rate three but never more than six animals are used per dose level. In 1996, OECD accepted ATC as an alternative to LD_{50} and published the guideline in 2001 (27). The ATC method has been successfully used in Germany and in 2003 more than 85% of all tests on acute oral toxicity testing were conducted as oral ATC tests. In member states of EU, the ATC method is used in the range of 50% of all tests conducted (26).

The ATC also uses the concept of fixed dose levels but retains "mortality" as a principal endpoint. The oral ATC method is a sequential testing procedure with the use of three animals of one sex per step. During the development of the new study protocol, the starting doses (25, 200 or 2000 mg/kg b.w.) have been chosen mainly from the class limits for classification of the EU at that time (25, 28) and modified at a later stage to 5, 50, 300 or 2000 mg/kg b.w. based on the class limits of the Globally Harmonized System for Classification and Labeling of Chemicals (GHS) (27, 29). The result of each step will determine, if (29Commission, 2004 Commission of the European Communities, 2004. Commission Directive 67/59/EEC of April 30, 2004 adapting to technical progress for the 29th time Council Directive 67/548/EEC on the approximation of laws, regulation and administrative provision relating to the classification, packaging and labelling of dangerous substances. Off. J. Eur. Comm. L152/173.);

- no further testing is needed
- dosing of three additional animals, with the same dose is required
- dosing of three additional animals at the next higher or the next lower dose level is needed

c. Up and Down Procedure (OECD 425)

"Up and Down Procedure (UDP)" is another alternative method for LD_{50} acute toxicity test (30). Compared to the "classical LD_{50} procedure", the UDP permits to reduce the number of animals substantially

(almost three-fold), required for determining LD_{50} values as well as ED_{50} values of a variety of other indices (31).

The concept of the up-and-down testing approach was first described by Dixon and Mood (31, 32). In 1985, Bruce proposed to use UDP for the determination of acute toxicity of chemicals (33). There are several variations of the UDP experimental design for estimating an LD_{50}. This guideline is based on the procedure Bruce (1985) as adopted by American Society for Testing and Materials (ASTM) in 1987 (34) and revised in 1990. In this procedure, animals are dosed once. If an animal survives, the dose for the next animal is increased; if it dies, the dose is decreased (33). A survey of 48 acute toxicity tests on rats showed that the great majority of the animals that ultimately died within 1 or 2 days. Therefore, in this procedure, each animal should be observed for 1 or 2 days before the next animal is dosed. Besides, it is recommended that surviving animals be monitored for delayed death for a total of 7 days. As females are generally more sensitive in the survey studies, testing in females alone is recommended but selective follow-up in males may sometimes be needed. The method cannot be recommended for testing materials where deaths are observed in longer periods of time (33).

The "effective dose 50 (ED_{50})" values derived through the UDP were virtually identical to those obtained from dose–response experiments using a traditional least squares linear regression method of analysis. The primary benefit of the UDP is that it substantially reduces the number of animals required to determine ED_{50} values. In addition to this animal welfare concern, substantial savings in animal, labor, and supply costs may be achieved, especially when surgical procedures are required. The UDP can be extended to determine ED_{50} values especially for analgesics with great accuracy while using significantly fewer animals than those required in full dose–response studies (35).

B. REFINEMENT

"Refinement" has been the least striking of the "Three Rs" because it does not produce marked changes in the number of animals used unlike reduction and replacement, which are vigorously controlled. The standpoint of "refinement" alternative is to use procedures that bring less pain or distress to animals (36). "Refinement" alternative requires reducing animal suffering as a whole in animal testing and it is defined as "any approach which avoids or minimizes the actual or potential pain, distress and other adverse effects experienced at any time during the life of the involved animals, and which enhances their well-being" (37). Although scientific community generally accepts that animal tests are required for taking forward our knowledge of health and medicine, they still inform about the need for alternatives to refine animals while testing. Pain or distress eliminations as well as the establishment of early endpoints for intervention in a study that has the potential to cause pain or distress are fine examples of refinement. Refinement will enable less variability and improvement in the outcome of results (36).

There are several methods employed to reduce the pain and distress in animals:

a. Providing Suitable Environment and Reducing Psychological Distress

Minimization of psychological distress is another aim of refinement. A distressed animal will not provide usable laboratory data, so researchers should minimize stress for both the animal's sake and for the interests of successful experimentation. One way to achieve this is to reward animals for cooperating with the procedures. Not only does this minimize any potential stress caused to the animals, but it also improves the quality of data obtained, because the animals are less stressed, as indicated by changes in blood pressure and heart rate (42).

Another way to improve animal welfare is to provide an environment that meets the animals' specific needs (43). It is really difficult to guess what actually matters to the animals. Providing animals with larger cages and toys can make a significant difference in the animal's emotional well-being. Different animals have different behavioral requirements (e.g. mice like to make a nest, primates like wooden perches)

and it is important for their well-being in captivity that they can express natural behaviors. If behavioral needs are not met, the animals can suffer from mental and physical stress which may affect the outcome of the experiments performed (38).

The general well-being of all animals must be checked at least once daily. Special care must be taken to ensure adequate monitoring of animals housed above head height and in the lower tiers of cage racks (44). Rats were used to be kept in barren cages with grid floors that were easy to clean. In recent years, they are more often kept in cages with solid floors containing places to hide. These refinements are supported by scientific experiments in which, for example, rats are taught to push against a weighted door to gain access to cages with a solid floor or extra space. How much the rats will lift indicates how important these resources are to the rats. Rats are able to lift 83% of their body weight to gain access to a cage with a solid floor. Another important refinement under investigation concerns the frequency of cage cleaning for rodents. In the past, the emphasis has been on keeping cages clean and animals were transferred on a regular basis to fresh cages with clean bedding; but animals such as mice use odors in their urine to maintain social hierarchies and mark territories. Therefore, frequent cage cleaning is likely to disrupt these odor cues and this may be stressful for the mice if they regularly have to re-establish hierarchies and territories (38).

b. Application of Pain Relievers

Pain is fundamentally a subjective experience. Therefore, animal technicians and other relevant staff must be trained to detect discomfort or pain in animals. Although it is essential for the technicians to rely on the animal's behaviors and clinical signs of pain, it is still a difficult task to properly assess this issue. It is generally postulated that if the procedure would cause pain in humans, it will cause as much pain or more in animals. Thus, the goal for the researchers should be preventing pain from occurring through the use of drugs whenever possible. Appropriate use of anesthetics, analgesics and other therapeutic measures are very important refinement measures in invasive studies. Close observation of animals when monitoring for adverse effects can reveal subtle changes; modern anesthetic drugs not only produce better anesthesia (e.g. better muscle relaxation, predictable duration of action, less irritation, fewer physiological side effects) but are safer, resulting in loss of fewer animals (40). Tranquilizers can also calm and relax animals during an experiment (40). However, it is important to note that a pain-relieving drug may interfere with the drug being tested and cause inaccurate results. Thus, it is usually in the best interests of research to reduce or eliminate pain whenever possible (36).

c. Using Technology

Using less invasive technologies is also considered as one of the refinement techniques. Various advanced scans and less invasive equipment that still provide comparable information, are all refinement techniques, because they minimize physical and psychological discomfort in the animal. For instance, ultrasound and Magnetic Resonance Imaging (MRI) can both be considered refinement techniques (41).

d. Husbandry

In the past, animals were often singly housed in cages that provided very little substrate or space for normal behavioral activities. Young rabbits kept in small cages developed skeletal abnormalities because they were unable to hop and run when their muscles and bones were maturing. Most research animals are social in behavior and isolation may be stressful (42). Most animals may be kept in social groups in complex environments that allow them to behave in a normal manner. There are many reports documenting the beneficial effects of this type of husbandry. For example, rats living in a socially and physically complex environment develop a thicker cerebral cortex, with more dendritic connections compared with those that are kept in isolation (42, 43).

e. Alternatives to Previously Used Blood Sampling Techniques

Although the retro-orbital sinus of some small species (particularly rodents) was a convenient site from which to collect fairly large samples of blood, the procedure had risks (e.g., the eye could be damaged, especially if samples were taken repeatedly), and

was painful for the animal. Several alternatives have been developed, including blood sampling from the tail vein, the saphenous vein and the jugular vein. Although some skill is required to perform these efficiently, the risk of causing severe damage to the animal is greatly reduced (44).

e. Humane Killing

Humane killing of animals before death can produce more meaningful scientific results (45).

C. REPLACEMENT

The aspect of "replacement" alternative is to use non-animal systems instead of animals, or to use a phylogenetically lower species of live animal. Among all approaches, the use of alternative techniques replacing animals has potential for the future research. Replacement can be full, partial or relative. In full replacement, there is no need for the use of animals and the *in vivo* method is completely replaced by an *in vitro* method. In partial replacement, after the *in vitro* testing, at least one of the confirmation studies must be performed on animals. Relative replacement is humane killing of animals to obtain *ex vivo* tissues/organs for *in vitro* studies (46). Replacement of higher animals by lower species or cell cultures/tissues can be currently used in hazard identification of chemicals (local toxicity, genotoxicity, various screening tests), in cosmetic raw material and products testing, in quality control and potency testing of vaccines and in the production of monoclonal antibodies (46).

The "replacement" principle can be applied by the following points mentioned below:

a. Replacement with Lower Animals/Species

It is recognized that many of the more fundamental processes are common to a wide range of organisms including invertebrates. One alternative to replace vertebrates and higher animals may be the use of lower, less sentient animals. Invertebrates are particularly considered to be an acceptable means of replacing higher animals as research subjects. For instance, the nematode, *Caenorhabditis elegans*, is widely used to study basic neuronal function. This organism has 302 neurons in its nervous system, so it is reasonable to study the function of each neuron and its interaction with other neurons (47). In a similar vein, genetic researchers have used fruit flies for many years (48). There are other important replacement alternatives in research: one of the most common and useful one is the replacement of rodent-based methods by *in vitro* methods for monoclonal antibody production (49).

b. Replacement in Education and Training

Animals have also been used in education and training, other than research. Learning skills, from simple techniques such as blood sampling to complicated surgical procedures such as laparoscopic surgery, are an important part of the training of medical and veterinary personnel. However, some skills such as suturing techniques may be developed without using animals. Discarded placentas may be used to practice microsurgery techniques. On the other hand, for practical skill training, there are now inanimate models that can be used to practice procedures. The acceptance of these inanimate objects for training comes when the touch and feel of the training is similar to that experienced when using a living organism (36). The "Koken rat model" is an important model for training and practice. In this model, larynx, trachea, stomach, and the tail vein are anatomically correct and replicated inside the model of the rat. To achieve a texture similar to that of a live rat, original silicone and soft vinyl chloride are used. When placed in position, the posture is the same as that of a live rat. The dosage in the stomach can be confirmed because part of the abdomen is transparent. The insertion of a needle into the tail vein can be confirmed by the reflux of the imitation blood. This model will allow a student to practice tail vein injections many times before it is attempted on a live animal. By Koken rat, retention, dosage, tail intravenous dosage and blood collection and endotracheal intubation techniques can be performed (50). On the other hand, computer-based technologies are available to allow surgeons to practice laparoscopic surgeries.

c. Replacement in Safety and Efficacy Tests

The use of animals for safety and efficacy testing of new products has increased greatly over the past forty years. Medical products make up the greatest

bulk. Public concerns for safety of products drove the need for increased testing, and however there is need to seek alternatives. One of the major challenges for the proponents of alternative methodologies for testing new compounds has been to prove that they are as effective as the animal based tests they are intended to replace. ECVAM and ICCVAM are trying to validate new proposed alternatives to animals in testing. Although regulatory agencies throughout the world have been cautious about accepting these alternatives, progress continues to be made (36, 50).

d. Replacement Using New Technologies and New Models

A number of new emerging fields and techniques are contributing major new insights for replacing sentient animal use within biomedical research and toxicity testing. These can be classified as follows:

i. Computerized Modeling: The value of bioinformatics, *in silico* technologies and systems biology in analyzing data, identifying new pathways and predicting toxicity is inarguable. *In silico* is an expression used to mean "performed on computer or *via* computer simulation". *In silico* research in medicine is thought to have the potential to speed the rate of discovery while reducing the need for expensive lab work and clinical trials. However, the quality of the data used for *in silico* model is extremely important. If low quality data is used, the system is designed to fail. When designing *in silico* methods using *in vivo* data, it is vital to have data from well-designed experiments that indicate the time course of toxicity and data that correlates pathology with molecular and mechanistic endpoints. Moreover, if data from in *vitro* experiments should be used in *in silico* models, extreme attention must be paid. For example, if data from tumor cell lines is used for *in silico* modeling, this data will not help to discover the perfect outcome in normal cells as the morphology and the signaling pathways of cancer cells is already altered (52).

Producing and screening drug candidates more effectively is one of the advantages that *in silico* technologies offer. More research is needed in this field to estimate the quantitative *in vitro*- to-*in vivo* (QIVIVE) extrapolations to predict the systemic toxicity for chemicals and drugs. Computerized quantitative structure-property (QSPR) and computerized modeling based on quantitative structure–activity relationship models (QSAR models) is needed to create a biochemical model. By using QIVIVE *in vivo* human toxicity estimations may be made in the future, and these *in silico* techniques may replace some of the animal tests at least (53).

ii. Pharmacokinetic and Toxicokinetic Modeling: This kind of modeling predicts the disposition of xenobiotics and includes (54):

- ADME (Absorpsion-Distribution-Metabolism-Excretion) parameter predictors
- metabolic fate predictors
- metabolic stability predictors
- cytochrome p450 substrate predictors
- physiology-based pharmacokinetic (PBPK) or biokinetic (PBBK) modeling software

iii. Microarray Technology: A microarray can be defined as a "multiplex lab-on-a-chip". It is a 2D array on a solid substrate (a glass slide or silicon thin-film cell) that assays large amounts of biological material using high-throughput screening methods. DNA microarrays (gene chip, DNA chip, or biochip) are collections of microscopic DNA spots attached to a solid surface and are used to measure the expression levels of large numbers of genes simultaneously or to genotype multiple regions of a genome. Each DNA spot contains picomoles of a specific DNA sequence, known as "*probes*" (or "*reporters*"). These can be a short section of a gene or other DNA element that is used to hybridize a cDNA or cRNA sample ("*target*") under high-stringency conditions. Probe-target hybridization is usually detected and quantified by detection of fluorophore-, silver-, or chemiluminescence-labeled targets to determine relative abundance of nucleic acid sequences in the target (55). Micro arrays are used in gene expression profiling, comparative genomic hybridization, chromation immunoprecipitation, single nucleotide polymorphism (SNP) detection, alternative splicing detection, detection of fusion genes and for genome taling (55).

iv. Omics Technology: The aim of each "omics" technology is to extract information that has mechanistic and predictive value. These include:

- **Genomics** is a discipline in genetics concerned with the study of the genomes of organisms. The field includes efforts to determine the entire DNA sequence of organisms and fine-scale genetic mapping and studies of intragenomic phenomena such as heterosis, epistasis, pleiotropy and other interactions between loci and alleles within the genome. Genomics includes the scientific study of complex diseases such as heart disease, asthma, diabetes, and cancer, because these diseases are typically caused more by a combination of genetic and environmental factors than by individual genes. Genomics is offering new possibilities for therapies and treatments for some complex diseases, as well as new diagnostic methods (56).
- **Proteomics** is the large-scale study of proteins, particularly their structures and functions. (57).
- **Metabonomics/Metabolomics** is the scientific study of chemical processes involving metabolites. Specifically, metabolomics is the "systematic study of the unique chemical fingerprints that specific cellular processes leave behind", the study of their small-molecule metabolite profiles (58 59). The metabolome represents the collection of all metabolites in a biological cell, tissue, organ or organism, which are the end products of cellular processes (60). Thus, while mRNA gene expression data and proteomic analyses do not tell the whole story of what might be happening in a cell, metabolic profiling can give an instantaneous snapshot of the physiology. The distinction between metabonomics and metabolomics is mainly philosophical, rather than technical. Metabonomics broadly aims to measure the global, dynamic metabolic response of living systems to biological stimuli or genetic manipulation. T he focus is on understanding systemic change through time in complex multicellular systems. Metabolomics seeks an analytical description of complex biological samples, and aims to characterize and quantify all the small molecules in such a sample. In practice, the terms are often used interchangeably, and the analytical and modelling procedures are the same (58).
- **Transcriptomics:** A transcriptome is a collection of all mRNA molecules in a population of cells. Along with DNA, these RNA molecules help create proteins. Transcriptomics provides tools that help researchers gain a better understanding of how genes and pathways are involved in biological processes (61).
- **Glycomics** is the comprehensive study of glycomes (the entire complement of sugars, whether free or present in more complex molecules of an organism), including genetic, physiologic, pathologic, and other aspects (62).
- **Lipidomics** may be defined as the large-scale study of pathways and networks of cellular lipids in biological systems. The word "lipidome" is used to describe the complete lipid profile within a cell, tissue or organism (63). Lipidomics is a relatively recent research field that has been driven by rapid advances in technologies such as mass spectrometry (MS), nuclear magnetic resonance (NMR) spectroscopy, fluorescence spectroscopy, dual polarisation interferometry and computational methods, coupled with the recognition of the role of lipids in many metabolic diseases such as obesity, atherosclerosis, stroke, hypertension and diabetes (64).

iv. *In Vitro* Models

In vitro models are dynamic systems designed to assess the effect of several drugs and environmental chemicals by mimicking *in vivo* conditions. In vitro models provide controlled testing conditions and high level of standardization. However, neither systemic nor chronic effects can be fully evaluated by these models. Cell cultures or target organ/tissue assays are used as *in vitro* models depending on the objective of the study (65).

a. Cell Cultures

Cell cultures comprise a very important test tool for toxicity testing. Cell cultures can be primary cultures, finite cell lines, or continuous cell lines. A remarkable research is performed in the field of development of new replacement alternatives for animals with *in vitro* systems for drug activity and xenobiotic toxicity studies. The advantages of culture studies can be listed as below (66, 67):

1. It is possible to examine cells under the microscope and to investigate the changes quantitatively, qualitatively, and simultaneously.
2. It is feasible to report each change that takes place by changing the environmental conditions. For example, researchers can change pH, temperature, amino acid and vitamin concentration of the medium and can clarify the effects of such conditions.
3. It is possible to obtain higher growth and development especially by cell lines and this enables more work with less time consumption.
4. It is likely to obtain similar results with 100 cell culture flasks to 100 rat or human.
5. It is possible to choose the appropriate cell line for the chosen endpoint. For example, for drug metabolism studies hepatic cell cultures, for excretion studies renal cell cultures or for drug accumulation studies muscle cell cultures can be used.

The disadvantages of culture studies to the researcher can be listed as below (66, 67):

1. Some cell cultures have low proliferation capacity and high phenotipical changing capability. It is not possible that *in vitro* tests may represent *in vivo* conditions under such circumstances.
2. Some primary cell cultures cannot show colonial growth and show loss of viability in short periods of time. Such cell lines cannot be used for chronic toxicity studies.
3. Animal cell cultures do not always represent similar results with human cell cultures because of the interspecies differences. It is difficult and costly to use human cell cultures.
4. There are not definitive and precise test procedures for *in vitro* toxicity tests given by regulatory authorities.

The advantages and disadvantages of different *in vitro* models are summarized in Table 2.

b. Target Organ/Tissue assays

Target organ or tissues are mostly used to predict toxicity of xenobiotics on eye or skin. Skin tests include skin irritation, skin corrosion, skin sensitization, skin penetration and photo-toxicity tests, which are performed by several approved assays.

The widely used **ocular toxicity assays** are **Bovine Corneal Opacity and Permeability (BCOP) Test (OECD TG 437)** (68)**, Isolated Rabbit Eye Test (IRE) (OECD TG 438)** (69) **and Chicken Enucleated Eye Test (CEET)** (70). In BCOP test, test materials are applied to the epithelial surface of the living bovine corneas, isolated from slaughtered animals (68). The end points to be tested are the changes in opacity

Table 2. The advantages and disadvantages of different *in vitro* models

Models	Advantages	Disadvantages
Subcellular preparations	Enables molecular level studies Toxic metabolite formation and covalent binding in macromolecules can be assessed	Only quantitative information available
Isolated cells (primary cultures, cocultures)	Retain original capabilities and properties Mimic in vivo response	Loss of influences such as hormones and immunity
Multicellular tissue (slices, cubes, aggregates, explants)	Retention of 3D structure Cell-cell interaction	Poor retention of viability Oxygen and chemicals cannot penetrate
Isolated organs	High reproducibility	Short period of viability
Stem cells	Pluripotent	Can form tumors
Cell lines	Can be maintained for a prolonged period	Prolongation results in decreased metabolic capacity and altered cellular function
Immortalized cell lines	Capable of extended and indefinite growth in vitro	Immortalization alters their characteristics and functions

and permeability which are measured by optical devices. In IRE test, instead of using isolated corneas, live rabbits are used and test substances are applied topically to the eyes of test rabbits and the effects are observed with slit lamp biomicroscope. With IRE, extremely potent irritants are evaluated (68). In CEET, after the chickens were sacrificed eyes were isolated and test material is directly applied to the isolated eyes and eye irritation potential of the test compound is evaluated as the primary end point (70).

Skin irritation is the reversible damage of the skin. Irritating reactions are scored from 0 to 4 for erythema and oedema formation and in these tests only non-corrosive chemicals are tested. Typical exposure is 4 h. Liquid substances tested are not diluted and solid substances are dissolved in minimal amount of water. Skin irritation tests widely used are **Episkin (Reconstructed Human Epidermis Test, OECD TG 439)** (71)**, Epiderm, pig ear,** and **PREDISKIN**. Episkin is a 3D model of human skin with reconstructed epidermis and a functional stratum corneum. Test material is applied to this layer. Toxicity is assessed by using 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (72, 73). The primary end point is cell viability. Epiderm was developed and validated by ECVAM for *in vitro* skin irritation testing of chemicals, including cosmetic and pharmaceutical ingredients. This test incorporates normal human keratinocytes cultured on permeable Milipore membranes. The primary end point is half maximal inhibitory concentration (IC_{50}) (74). In pig ear test, non-perfused pig ear is the test material for 4 h, to distinguish between irritants and non-irritants. The end point is increase in trans-epidermal loss (73). In PREDISKIN test, human skin cultures are exposed to test material and toxicity is assessed by MTT assay and the primary end point is percentage of cell viability (73).

Skin corrosion is the irreversible damage of the skin. A severe skin corrosion can be caused by strong acids, strong alkalis, some organic solvents, defatting agents or some salts. Corrosive reactions are typified by ulcers, bleeding, bloody scabs, discoloration, alopecia and scars. The skin corrosion tests approved by OECD are **Corrositex (Membrane Barrier Test, OECD TG 435)** (75)**, Transcutaneus electrical resistance (TER) (OECD TG 430)** (76) **and SkinEthic (Reconstituted Human Epidermal Model, OECD TG 431)** (77). Corrositex is a protein membrane, and it can measure the penetrating rate of a chemical in the simulated skin barrier and the primary end point is the color-change in the membrane (78). In TER assay, corrosive materials are identified by their ability to produce a loss of integrity and the end point is the reduction in TER of layers of skin to an applied current (79). SKINETHIC is the assay that involves the estimating of the loss of viability of human epidermal keratinocyte cultures overtime when substances are applied topically. The primary end point is the loss in cell viability and the release of cytokines (80).

There is only one alternative skin sensitization test widely used and it is the **Murine Local Lymph Node (LLNA) Assay (OECD TG 429)** (81). In this test live animals are used and this test measures sensitization of a chemical in mice as function of proliferative activity induced in lymph nodes draining the site of exposure to the test chemical. The end point is the increase in thymidine (3H-TdR) incorporation (81).

There is also one photo-toxicity test approved by OECD: **3T3 NRU Photo-toxicity Test (OECD TG 432)** (82). This test is easy to perform and high throughput testing is possible. However the system lacks barrier properties, and with this test, it is difficult to test substances that are poorly water soluble. The test is performed on mouse fibroblast 3T3 cell line. The test compares the cytotoxic effect of the test chemical in the presence and absence of a non-cytotoxic dose of UVA/Visible light. Cytotoxicity is evaluated using neutral red uptake (NRU) assay. On the other hand, **ECVAM Pre-validated EpiDerm Phototoxicity Test** is also used for testing phototoxicity. This test uses reconstructed human skin model. The test compares the cytotoxic effect of the test chemical in the presence and absence of a noncytotoxic dose of UVA/Visible light. Cytotoxicity is evaluated by using MTT viability assay (83, 84).

There is also one skin penetration test approved by OECD **(OECD TG 428)** (85). This test measures

diffusion of chemicals into and across excised skin. In this test, *ex vivo* human or pig skins or reconstructed skin models are used, with radio-labeled or non-radio-labeled test material. This test is required for the safety assessment studies of cosmetics and topically applied pharmaceutical formulations.

CONCLUSION

Alternative approaches have been advanced over the last two decades, providing one-by-one replacements of animal tests. Moreover, validation tests have produced evidence that these tests do not decrease safety standards and international acceptance has been widely achieved. After the success of skin irritation, sensitization and corrosion tests, scientists with regulatory authorities are trying to develop new tests for carcinogenicity and reproductive toxicity, in the last decade.

"Do we really need animals for our study?" is the first question of a scientist to ask himself before starting tests with higher organisms. If there are other choices like lower organisms or like cell lines, these tests must be chosen at first sight. Besides, *in silico* technologies may be good choices for structure–activity relationship models as well as for ADME studies. If there are no other options, the number of the animals that should be used must be kept as low as possible, while remaining consistent with sound scientific practices necessary to obtain valid results. Moreover, the refining process must be kept in mind as it might also affect the outcomes of the study. In conclusion, all researchers should continue their commitment to reduce animal usage and suffering. Scientists are hopeful that the new *in vitro* test procedures that are being developed will succeed the *in vivo* tests and animals will not be used in science other than in mandatory experiments in the future.

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