Review Article

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Toxicity testing strategies – current innovations and future outlook

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ABSTRACT

Humans are often exposed to chemicals in the form of pharmaceutical substances, household products, pollutants and it is vital to screen these chemicals for their toxicity so that it can be used safely without subjecting human lives at risk. Scientific advancements in the field of toxicology paved a better platform for evaluating these chemicals. A plethora of techniques are used for this purpose which includes toxicity test using animals, *in vitro* testing methods for enhanced toxicity testing (cell culture systems, stem cells, toxicogenomics, *in silico* techniques - Toxicity database, QSARs, Human knowledge-based methods) etc. These techniques vary in their scope, use, reliability, limitations so a search is needed to find out a testing method which fulfils all aspects of a testing method. This review highlights the general toxicity testing approaches, current developments in the field of toxicology, basics and significance of alternative testing methods in toxicological research for enhanced toxicity testing.

KEY WORDS: *in vitro*, toxicogenomics, *in silico*, database, QSARs.

INTRODUCTION

Toxicology is the study of how chemical substances interact with living systems and affect normal processes and the use of this information to predict safe exposure levels. Toxicologists are involved in evaluations of household products, medicines and the effects of incidental and occupational exposure to natural and manufactured substances. Determination of effects of toxicants also helps us to develop the best treatment in the event that accidental overexposure occurs. The US Food and Drug Administration (FDA) state that it is essential to screen new molecules for pharmacological activity and toxicity potential in animals[1].

Objectives of toxicity studies

The basic objectives of a toxicity testing are

1. Identification of potential toxic hazards
2. Determination of qualitative or quantitative risk for adverse effects in target populations to establish safe level of exposure.
3. Selection of least toxic candidate compound in product development
4. Understanding the mechanisms of toxicity
5. Designing therapeutic strategies and implementing it during accidental exposure to toxicants.

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Components of a toxicity test

An *in vitro* test consists of three components namely biological model, endpoint measurement and interpretation of the results. The biological model consists of biological system to be utilized and the appropriate culture conditions .The biological models is selected on the basis of the toxicological studies to be carried out. For an example cells of hepatic origin are more relevant to studies of hepatotoxicity than neurotoxicity and vice versa. The end point measurement is an analytical determination of the test chemical on the biological system. Finally, interpretation of data and conclusion of the study[2].

Types of toxicity testing

Animal toxicity studies

In order to evaluate the safety of a chemical or drug, various toxicity studies are carried out in animals such as mice, rats, guinea pig, dogs and monkeys under varying conditions of a drug administration.

The tests include:

- **Systemic toxicity studies**
- **Local toxicity studies**
- **Specialized toxicity studies**

I Systemic toxicity studies

(a) Single dose toxicity studies.

Single dose study involves the determination of minimum lethal dose (MLD), maximum tolerated dose (MTD) and if possible, **the target organ of toxicity**. In this the effect of drug is tested with a single dose in 2 rodent species using the same route as that intended for humans. Animals are observed for mortality

for up to 14 days. Detailed observations are made regarding effects of the drug on important physiological functions and body weight.

(b) Repeated dose toxicity studies.

These studies involve initial determination of MTD and final systemic toxicity that are carried out in two species out of which one is non-rodent with three doses i.e., the highest dose having observable toxicity, the mid-dose causing some symptoms but no gross toxicity and the lowest free of toxicity. The recommended duration of the repeated dose toxicity studies is usually related to the duration, therapeutic indication, and scale of the proposed clinical trial. In principle, the duration of the animal toxicity studies should be equal to or exceed the duration of the human clinical trials up to the maximum recommended duration of the repeated dose toxicity studies.

II Local toxicity studies: It deals with the study of local effects caused by the chemical substance that are applied to the appropriate site, e.g. skin, vagina or cornea to determinate local effects in suitable species.

III Specialized toxicity studies:

(a) **Male fertility studies** detect effects of a drug or chemical substance on structure and functions of male reproductive organs.

(b) **Female reproduction and fetal developmental toxicology studies** includes observations on the mating behavior, progress of gestation, parturition, health during pregnancy and in post-partum period. Ability of the drug to induce fetal malformations and/or death in utero (i.e. **teratogenicity**) when given throughout organogenesis is observed. These studies are carried out in one rodent species and one non rodent species.

(c) **Allerginicity/ hypersensitivity tests** are carried out in guinea pigs to determine the minimum irritant dose and the effect of a challenge after sensitization.

(d) **Genotoxicity tests**: These are *in vivo* and *in vitro* tests conducted to detect genetic damage, if any. Because certain drugs and toxicants are known to produce genetic abnormalities. As genes are bearers of hereditary information, abnormal genes may produce various types of overt and convert abnormalities in the subsequent generations.

(e) **Carcinogenicity studies** detect the ability of a drug to induce malignancy. Generation of pharmacokinetic data during the toxicity studies helps to relate doses and systemic exposures achieved with toxicological findings[3].

Animal models in toxicity testing

Toxicity testing of new compounds is essential for drug development process and for their approval of use in humans by the legal bodies. The preclinical toxicity testing on various biological systems reveals the information such as species-, organ-and dose-specific toxic effects of an investigational product. And also animal models are useful for perform specialized and specific toxicity studies that include study of effects of various agents on general reproduction, including developmental toxicology, and subsequently for assessing the potential impact of toxicants upon other species also. Although animal studies serve as useful experimental models, the administration of a biologically active agent to human beings is accompanied with an element of risk which cannot be predicted by even the most careful animal studies. Hence the product under study has to be carefully evaluated by human experiments for its safety and efficacy before it is accepted for therapeutic use.

Rats and mice have been used widely with extensive information on their normal development, growth, function and reproduction along with responses to many toxicants with relevance to humans is available. Because of species variability, no single type of animal is an ideal model for human beings. Consequently, multiple animal models are desirable to understand about the toxicity of the agents.

Rabbit - A model for reproductive and developmental toxicity studies

As a non-rodent model, the rabbit is the smallest laboratory animal that has been well characterized, and can be used to monitor the same endpoints used in rodent studies. The rabbit has an advantage in that semen can be obtained easily for monitoring many functions which is of particular value because of the importance of semen quality in assessing effects of exposure of subject to reproductive toxicants. The female rabbit is also amenable to studies of reproductive toxicology and developmental toxicity. The rabbit visceral yolk sac, a pre-placental organ, and extra-embryonic membranes more closely resemble those in humans than do rodents. Also the rabbit is large enough to collect fluids and tissues from the same animal repeatedly. The rabbit is the least expensive non-rodent laboratory animal that can be utilized to obtain innumerable of endpoints for testing agents of potential harm to people[4].The limitations in using rabbit as an animal model, that it is more expensive than rodents and use of rabbit is limited to certain area of toxicological studies such as reproductive and developmental toxicity studies.

Transgenic Animal Models

Transgenic animals are genetically designed animals created by the introduction of DNA coding for specific genes into the germline of an organism and that act as a tool to identify and characterize the expression of targeted genes. In the field of toxicology, transgenic animal models have primarily been used to screen for toxicity and to elucidate mechanisms of toxicity. For example, this technology facilitates the identification of chemically induced mutations and helps in determining the mechanism of such mutations.

Transgenic animal models are powerful tools for developing a more detailed understanding on the roles of specific genes in biological pathways and systems. These investigations will permit innovative approaches in understanding the mechanism of gene alteration by the agents at the genome level. Transgenic animal models are well utilized, especially for the screening of mutagenic and carcinogenic potential and for the characterization of mechanisms of action of toxicants[5].

Limitations

There are number of problems that need to be addressed relating to use of these models in the testing of chemicals. For all models the exogenous material may cause instability in the genome, resulting in altered responsiveness of the model. Assurance is required that when there is a negative response, the agent is truly non-carcinogenic and not that genetic instability has resulted in a lack of response.Tg.AC transgenic mice, a model for altered gene regulation show an increased incidence of spontaneous tumours at sites that are unique to rodents and/or are very specific to the model, such that their relevance to human risk assessment is questionable. A sequence alteration leading to a loss of responsiveness has been observed in some heterozygous Tg.AC mice[6].

***In vitro* testing strategies for enhanced toxicity testing- alternatives to animal testing**

No tests on animals, however meticulous and prolonged, can ever prove with absolute certainty the effects of a toxicant in man. Animal pharmacological studies would only indicate the probable toxic effects that may be expected during human studies. Correlation of experimental data obtained in animal and human studies are highly difficult because of differences in genomic profiles. Increased concerns with animal welfare have resulted in the need for alternative to whole animal testing. The term "alternatives" encompasses the 3Rs of Reduction, Refinement and Replacement. The concept of the 3Rs was first proposed by Russell[7] which has got acceptance all over the world. The 3Rs are 'reduction' -the use of the least number of animals in toxicological research that results in meaning full data, 'refinement'- the improvement of whole animal toxicological research in such a way as to reduce or eliminate pain and discomfort, and 'replacement'- the use of alternative toxicological testing methods that do not involve the intact animal. This concept of reducing, refining and replacing animals can help in resolving various legal and ethical issues associated to animal experiments and also to encounter the increasing needs of the society. However substitution of *in vitro* methods for *in vivo* techniques must be harmless to the humans[8].
In vitro toxicity testing is the scientific analysis of the effects of toxic chemical substances on cultured mammalian cells, -omics technology, computational toxicology. Due to regulatory constraints and ethical considerations, the pursuit for alternatives to animal testing has gained a new momentum. Most toxicologists believe that *in vitro* toxicity testing methods can be more useful and cost-effective than toxicology studies in animal models.

Potential components for *in vitro* testing systems

1. Cell culture system
2. Stem cells
3. Toxicogenomics
4. *In silico* toxicology
   a) Toxicity database
   b) QSARs
   c) Human knowledge-based methods

1. Cell culture system

Presently many of the toxicity screening processes count on results from early-stage *in vitro* cell based assays expected to authentically represent essential facets of *in vivo* pharmacology and toxicology. Several *in vitro* designs are designed and optimized for high throughput to benefit screening efficiencies, allowing the most of potential pharmacologically relevant or possible toxin molecules to be screened for different types of cell signals relevant to tissue damage or to therapeutic goals.

In vitro toxicology analysis using cell cultures has been developed into an important alternative for early toxicity assessment. There are several *in vitro* cell culture models which is gaining paramount importance in toxicology such a) organ/explant culture, b) organotypic culture, c) dissociated cell culture- primary cells, immortalized (transformed) cell lines, d) three-dimensional cell cultures and e) whole embryo culture assay.

a) Organ/ explant culture

Organ culture preserves whole histological architecture of a surgically removed organ, allowing study of *in vivo* processes ex vivo. Organ explant slices or precision-cut tissues slices (PCTS) are also extremely popular in developmental and toxicological studies. Similar to organ culture, PCTS can be maintained *in vitro* while maintaining local histology, representing the majority of different cell types and cellular interactions.The tissue slice system has the advantage of its suitability for assay that may require visual analysis or scoring, immunochemistry or live imaging.

Limitations

The main limitations of using organ culture and tissue Slice system are careful and laborious preparation, lack of procedure that guarantee high viability of the organ culture and short term survival in culture. Due to these issues, both cultures have low applicability for HTS drug screening techniques.

The tactic of preserving the innate microenvironment in combination with tissue engineering techniques as significant methods in creating *in vivo*-relevant models can be extended to screening innovations, thereby developing organ culture techniques for toxicological screening and HTS.

b) Organotypic culture

Organotypic culture is an *in vitro* technique using multiple different cell types to reiterate *in vivo* cell heterogeneity. Organotypic cultures can incorporate different aspects of other models, for example they can use supporting matrices to mimic organ cultures or use 3-D scaffolds to produce *in vivo* like tissue architectures and morphologies. Skin models have been used for the last two decades in a variety of pharmacological and pharmacokinetic studies. Skin models are the most well characterized organotypic system and are suitable example for a system with native tissue architecture and biochemistry that yield *in vivo*-relevant characteristics.

c) Dissociated cell culture

Use of dissociated cell culture in the form of primary culture and

immortalized cell lines expanded on 2-D tissue culture treated plastic surfaces is the primary model used for cell biology and testing research.

Primary cells

Primary cells are obtained from fresh tissue sources and the protocol involves methods for disaggregation from host tissues using mechanical, enzymatic or chemical dispersion methods followed by subsequent plating on to tissue culture appropriate surfaces.

Primary cells are often desired due to their wild type, unadulterated nature, which translates to *in vitro* cultures with cells preserving better structural and biochemical complexity found *in vivo*.

Disadvantages

(a) Since diverse of cells found together in most tissues, most primary cells are hard to extract from tissue as a homogenous population.
(b) Also many primary cell harvests are expected to be contaminated by other cells of distinct origins and phenotypes from the targeted cell type.
(c) Additionally, isolated primary cells begin to differentiate within hours to days when cultured on 2-D surfaces in a process that is difficult to control, requiring repeated host tissue isolation.
(d) Primary cells are very sensitive to passaging, resulting in altered phenotypes, slow proliferation rates, metabolic capacities and early senescence after only a few expansions, so handling of primary cell require more sophisticated and tissue culture training.

Immortalized cell lines

Immortalized cells have their geneses from primary cells that have been purposefully genetically modified to overcome the tedious problem of their primary cell counterparts. Immortalization results from oncogene introduction in to the cell's genome to enable rapid proliferation in culture, resistance to de-differentiation, improved passaging and greater resilience in culture. Immortalized cell lines has the advantages of easy to maintain and propagate in culture in dilute serum or serum-free media, can be expanded and stored as frozen stocks, exhibit reproducible results when thawed and re-seeded and are stable for up to 25-50 passages.

d) Three-dimensional cell culture

Culture in 3-D has its origins in tissue engineering and regeneration studies, but techniques and innovation adopted are increasingly as cellular techniques for disease modelling, toxicological and molecular target identification work. 3-D cell culture models can utilize cells, healthy or diseased tissue biopsies or complete organ as their living components.

3-D cell culture systems are usually made by suspending cells in compatible hydrogels that can be gently cross-linked *in vitro* to suspend cells within these materials in the presence of excess water or by seeding cells on solid support matrices, typically spongy, high porosity polymer or ceramic solid foams or growing cells on the surface of a non-polar support material.

Cellular models have a proven record as powerful tools for toxicity assessments. But these models are only as good as their ability to recapitulate explicit *in vivo* physiologic and pathologic processes and cell properties specific to the context under study[9].

e) Whole embryo culture assay

Embryos are particularly sensitive to teratogenic agents during organogenesis. Many of the malformations induced have been similar to those obtained after administration of the same agents *in vivo* and have demonstrated a direct teratogenic effect on the embryo independent of the maternal metabolism. It is suggested that culture methods may provide a valuable additional screening procedure for new drugs and other potentially embryopathic agents.

Rat whole embryo culture assay

The rat whole embryo culture (rWEC) assay is based on the culture of rat embryos between gestation days 10 and 12[10].

Strengths

(a) During this period of embryogenesis a major part of the essential morphogenetic process of organogenesis takes place. The development of each of these structures can be monitored in culture.
(b) Embryo develops almost at the same rate as *in vivo* and no differences were observed.
(c) Development of each of the organ anlagen can be morphologically scored, and the effects of chemicals can be assessed.
(d) The embryo developing separately from the mother offers advantages as only direct effects are studied.
(e) The compound concentration in the culture medium can be more easily related to the dose at the target in *in vivo* embryo toxicity studies.
(f) Differential gene expression assessment is now employed as a supplemental tool to study embryo toxicity in a more detailed and mechanistic way possibly enhancing the predictive capacity of the method[11].

Zebrafish (*Danio rerio*) embryonic culture

Zebrafish has been a significant and prominent vertebrate model in a variety of biological disciplines. Extensive information gathered from developmental biology research and genetic research, together with near-completion of the zebrafish genome project, has placed zebrafish in an attractive position for use as a toxicological model.

Initially toxicological research using the zebrafish model concentrated on ecotoxicology and the determination of acute toxicities. In recent years, the use of embryos of the zebrafish has gained acceptance as an established model in the area of teratology[12]**.** The capability of early embryos of zebra fish to perform phase I metabolism and transform proteratogens to active metabolites made it as significant model to assess the toxicity of teratogens**.** Zebrafish have become a popular

testing model because of gene conservation with humans and similarities of some disease processes in the two species[11]**.** The use of zebra fish embryos is particularly significant for several reasons: (i) fish embryos are considered an alternative to animal testing and their use is not restricted by regulations for animal welfare. (ii) The transparent embryo provides an array of applications for the detection of morphological disorders, functional genetics and gene characterization (iii) the easy availability of embryos offers the potential to design high throughput screening (HTS) approaches for toxicants. Since the assessment of toxicology is essential for the development and registrations of drugs and chemicals, the zebrafish embryo model exhibits a high potential for applied research and testing[13, 14].

Limitations of zebra fish use in toxicology

Anatomical features of zebrafish vary with respect to human anatomy due to lack of certain organs which found in human beings. While the experimental benefits of the zebrafish have firmly established its role as a model in mechanistic toxicological studies, it is still struggling for recognition by regulators and industry as screening tool in drug development and toxicological testing of chemicals other than water quality assessment. Although available experimental data are promising, they are not sufficient to develop a comprehensive depiction with relevance to humans. Therefore, the reliability of the zebrafish as model for the prediction of toxic effect of chemicals on the humans needs to be established rigorously by further systematic assessment of the activity of pharmacological and toxicological relevant substances[15]**.**

Vast number of available literatures shows that several researches have been done in other cell culture systems such as *in vitro* blood-brain barrier model for high throughput toxicological screening. The 12d/6w *in vitro* BBB model developed by Dehouck et al. in 1990, consisting of a co-culture of bovine brain capillary endothelial with rat primary glial cells is currently considered as one of the most powerful alternatives to *in vivo* studies of the BBB that can be used routinely in industrial drug discovery and development programs[16]. Another example for *in vitro* cell culture model that has significant place in toxicity assessment of certain chemicals is use of human primary renal cells. Weiwei li et al. in 2005 used human primary renal cells as a model for toxicity assessment of chemotherapeutic drugs used in cancer treatment.

Advantages of *in vitro* techniques[8]

(a) Number of animals used in tests is reduced because the cells from a single animal can be used to conduct a complete experiment or set of experiments.
(b) In terms of cost savings, reduced maintenance costs in housing and feeding of animals.
(c) Cells used in experiments, often are of a uniform population, being derived from a single animal
(d) *In vitro* system can be utilized to rapidly identify metabolites of test agents in a matter of hours instead of days using whole animals.
(e) *In vitro* systems also have added advantage of being capable to assess toxicity of compounds of limited quantity because doses used in study are in the range of micrograms, or less, instead of milligrams per kilogram body weight.

General Limitations of *in vitro* testing systems

Because cells are usually derived from the digestion of a particular organ, these isolated cells have been removed from their normal homogeneous cellular milieu. The loss of organ architecture and cell orientation may result in cell responding differently to chemical or drug exposure than those observed *in vivo*. In addition, the digestion of a particular organ may result in alteration in cell membrane lipids and receptors of the isolated cell type, which can lead to erroneous conclusions in toxicity studies using these cells. Furthermore, these cells have been removed from their physiologic relationships with the intact animal's circulatory system. Therefore, these factors such as chemical or drug absorption, distribution, and elimination cannot be assessed *in vitro*. Cellular functions may become unstable over time in culture and the cells from different species may respond differently to a product under examination. Also, cultured cell lines are often altered in many aspects comparing with their tissues of origin. The sensitivity to chemical agents in these cells may not recapitulate the sensitivity of the original normal tissues[8, 17-19].

2.Stem cells

During the last decade, stem cells have been the subject of increasing scientific interest because of their utility in numerous bio medical applications. stem cells are able of renewing themselves, they can be continuously cultured in an undifferentiated state or giving rise to more specialized cells of the human body such blood, bone marrow, liver, heart, nerve cells etc. Therefore, stem cells are an important tool for developing *in vitro* model systems from all species including man to test xenobiotic and improve the prevalence of toxicity in human.

Stem cells are defined by two essential abilities: (i) they are able to generate identical copies of themselves, or self-renew, and (ii) they give rise to different cell types. Differentiation is the process whereby cells acquire new morphological and functional characteristics.

Stem cells can be classified into two major categories, according to their developmental status: embryonic and non-embryonic or adult stem cells.

Embryonic stem cells (ES)

Embryonic stem (ES) cells are pluripotent cells, isolated from the inner cell mass of the blastocyst-stage mammalian embryo. Pluripotent cells are capable of giving rise to most tissues of the organism, including the germ like during development. In the mouse, these cells have been the most important instruments for understanding mammalian gene function by means of genetic manipulation[20].

The whole embryo culture in rats, an embryonic method that found a broader development was used for several decades as an academic tool. Later, its hidden ability of predicting the developmental toxicity of a wide variety of chemical structures was identified and evaluated.

A mouse embryonic stem cell method was developed in the 90s, with the endpoint being the differentiation of pluripotent stem cells into beating cardiomyocytes after 10–11 days. The mouse embryonic stem cell test has already undergone a validation according to ECVAM criteria in 2001 and the pros and cons of using mEST in predicting toxicity of embryotoxicants.

Human embryonic stem (hES) cell test, an effective *in vitro* approach was developed few years back. A hES cell derived *in vitro* model of human developmental toxicity, may offer the best *in vitro* solution for concordance with human response and prediction. Pioneering researches were made using hES and presented data on a hES cell based *in vitro* assay, devTOX, for human developmental toxicity. This unique model offers an opportunity to understand human development and the impact of pharmaceuticals and chemicals encountered in the environment on the developing human embryo in a possible way[11, 21].

Embryonic stem cell & toxicogenomics in the field of developmental toxicity assessment

Embryonic stem cell test is the most extensively studied *in vitro* alternative testing method for identification of developmental toxicity. Genomics technologies have already provided a proof of principle of their value in identification of toxicants such as carcinogenic compounds. The advent of technical developments in molecular biology have resulted in key tools that are capable of measuring thousands of endpoints in one single assay, such as transcriptomics that evaluates genome-wide gene expression. Also transcriptomics profiling can be used for the identification of biomarkers that may improve the prediction of specific toxic effects. In addition, identified biomarkers may be used for improved discrimination of compound classes, because exposures that affect similar biological processes will likely share comparable gene expression changes. Additionally genomic technologies have the advantage that toxic responses can be studied on the biological pathway level which may provide a useful approach in modern toxicity prediction. Also within the EST, gene expression profiling has shown its value in the identification of developmental toxicity and in the evaluation of factors critical for risk assessment, such as dose and time responses. It is expected that implementation of genomics in to the EST will provide a more detailed end point evaluation as compared to the classical morphological scoring of differentiation cultures. Therefore, genomics may contribute to improvement of the embryonic stem cell test (EST), both in terms of its applicability domain as well as its predictive capacity[22].

Human embryonic stem cells & Metabolomics

Teratogens may cause fetal abnormalities during development and are responsible for a significant number of birth defects. Animal models used to predict teratogenicity often do not faithfully correlate to human response. Recently, a more predictive developmental toxicity model based on an *in vitro* method that utilizes both human embryonic stem cell and metabolomics to discover biomarkers of developmental toxicity have been developed. In this approach, hES cells were dosed with product under study, then LC-MS analysis was performed to measure changes in abundance levels of small molecules in response to dosing. Statistical analysis is employed to select for specific mass features that can provide a prediction of the developmental toxicity of a substance. These molecules can serve as biomarkers of developmental toxicity, leading to better prediction of teratogenicity. This platform is a robust alternative to animal and other *in vitro* models for the prediction of the developmental toxicity of chemicals that may also provide invaluable information about the underlying biochemical pathways[23].

Adult stem cells

Adult stem cells (ASCs), also known as mesenchymal stem cells (MSCs) or multipotent adult progenitor cells (MAPCs) are specialized cells found within many tissues of the body where they function in tissue homeostasis and repair. Multipotent cells are precursor cells capable of differentiation into several different cell types but not all cell types in the organism like pluripotent cells. Multipotent ASCs can be harvested from organs, grown in culture as a homogeneous adherent population of fibroblast-like cells, and induced to differentiate into multiple cell types. Maintaining ASCs undifferentiated in cultures is dependent upon culture conditions.

Adult stem cells have been used to carry out genotoxicity including mutagenesis, teratogenesis, carcinogenesis and epigenesis, but the experimental data and its conclusions obtained in these studies are far apart from those obtained from the *in vivo* studies. So understanding the mechanism by which physical/chemical mutagenic, cytotoxic or epigenetic toxicants lead to death, teratogenesis, carcinogenesis, atherogenesis, reproductive immunotoxicities, as well as premature aging and the diseases of aging, requires knowledge of the homeostatic regulation of cell proliferation, differentiation, apoptosis and senescence of cells[20,24].

Many researches have been conducted to test the effect of toxicants using stem cells. Cardiomycytes derived from human stem cells provide a new method to screen new chemical entities for potential cardiotoxicants[25]. Furthermore growing number of publications implies that primary hepatocytes and some transformed cell lines remain the most suitable *in vitro* liver cell models for xenobiotic metabolism and toxicity studies[26].

Limitations of stem cell use in toxicological testing

About 300 human embryonic cell lines have been developed around the world[27]. However most of the lines have limited lifespan, even cell lines which are considered to be able to proliferate indefinitely die out. Considerable work is still required to characterize the mechanisms that regulate differentiation of stem cells[28]. Furthermore a detailed analysis about human ES, EG and EC is the current need of the hour in order to select the most suitable cell lines for development of different *in vitro* toxicological test systems.

3.Toxicogenomics

Genomic, proteomic and metabolomic methods are being employed by all sectors of industry, academia, and regulatory agencies at an unprecedented rate. Toxicogenomics is concerned with the identification of potential human and environmental toxicants, and their pu-

tative mechanisms of action, through the use of genomics resources. The field of Toxicology uses numerous *in vivo* model systems, including the rat, mouse, and rabbit, to assess potential toxicity testing. However in the past several decades, a numerous of *in vitro* techniques have been developed to measure toxicant induced DNA damage. Gene expression is a unique way of characterizing how cells and organisms adapt to changes in the external environment. The measurements of gene expression levels upon exposure to a chemical can be used both to provide information about the mechanism of action of the toxicant and to form a sort of "genetic signature" for the identification of toxic products[29]**.** One valuable resource which has gained importance in recent years is DNA microarrays or chips, which allow the monitoring the expression levels of thousands of genes simultaneously and other examples of *in vitro* system include the Ames test, the Syrian hamster embryo cell transformation assay, micronucleus assays and many others. Fundamental concepts that are common to all of these methods are the fact that toxicity is often preceded by, and results in, alternations in gene expression. In many cases, these changes in gene expression are a far more sensitive, characteristic, and measurable end point than the toxicity itself.

DNA Microarrays

DNA microarrays are now commonly used to characterize genetic diversity, predict biological functions of genes, define biochemical pathways, diagnose disease, characterize drug responses, identify new drug targets, and assess the toxicological properties of chemicals. Many researches have demonstrated and reviewed the application domain of the microarray technique[30-33].

DNA microarrays are an organized by arrangement of multiple DNA probes that has the origin of cDNAs, PCR products or cloned DNA fixed on an immobilized surface such as nylon filters, glass slides or silicon chips. Thousands of DNA spots, usually ranging from 70 to 100 μm in diameter, can be spotted onto a single microscope slide or membrane, with the potential of each spot to represent a unique gene or DNA sequence that are chosen to cover specific endpoints or pathways or may include genes which cover a wide range of biological processes. The differences in gene expression can be then determined with the help of a fluorescently or radioactively labelled probes hybridized to the oligomer carrying arrays. Then the surface of the array is scanned for fluorescent signal or radioactivity at each spot using phosphorimager analysis or autoradiography. By this technique, the expression profile of thousands of genes can be analysed on a single array and the relative changes in gene expression between two or more biological samples can be measured[19, 34].

DNA microarrays can be categorized in to two general types: i) oligonucleotide arrays and ii) PCR-amplicon arrays.

i) Oligonucleotide arrays

Oligonucleotide arrays are comprised of short immobilized DNA sequences of approximately 20–70 DNA bases long, designed as complimentary probes to specific gene sequences. Oligonucleotide arrays have the general advantage of greater specificity for target sequences compared to PCR-amplicon arrays, and are the ideal tools for discriminating between closely related gene sequences.

Disadvantages

Relative high cost associated with manufacturing the DNA oligonucleotides, dependency on DNA sequence data for designing probes, and the difficulty of controlling stringency of mass hybridization of multiple target DNAs.

ii) PCR-amplicon arrays

PCR-amplicon arrays consist of PCR-amplified DNA products obtained from tissues or cloned libraries and subsequently spotted onto an array. In addition, expressed sequence tags (ESTs) or previously uncharacterized gene sequences may be used as targets on PCR-amplicon arrays. The disadvantage of PCR-amplicon arrays is that gene sequences with high similarity can display a significant level of cross hybridization.

DNA microarrays combine the ability to analyse the expression of a large number of genes with parallel data acquisition. This parallel-processing power allows experimental designs that are much less costly, time-consuming, and produce significantly more data than conventional molecular methods, yet produce similar results[34].

Issues to be resolved

Successful integration of genomic data with toxicology faces several challenges and issues.

1. One challenge is the sources of variability in gene expression levels due to different physiological stages, sex, age, and natural genetic polymorphisms in populations. These variations may results in diverse gene expression, lending microarray data more complex that is difficult to explain. Therefore, careful selection of experimental individuals and the straightforward data treatment are of paramount importance for the derivation of reliable biological meaning.
2. Another challenge of microarray technology is the initial cost and time-consumption in both the development of the technology and data analyses. The availability of genomic resources for experimentation is limited for most species, developing of microarrays is impossible in a short span of time. Fortunately, studies suggest cross-species microarray hybridization has proved to be feasible among aquatic species as well as among mammals[35].
3. These technologies are at the budding stage of maturity and they not yet standardized, and many protocols are being developed for use in different laboratories. Many laboratories are developing their own custom microarrays, which are typically prepared with specific sets of genes of interest to the investigator. So there is a lag in evaluation of inter-laboratory reproducibility and establishing a common standardized format.
4. Another major issue with array technology is quality control and characterization of analytical performance. Further,

the arrays need to be manufactured to a standard that yields reproducible results. Reproducibility, sensitivity, and robustness must be determined for the arrays and the biological meaning of alterations in specific expression patterns must also be determined. The strengths and limitations of these new tools must be evaluated through carefully conducted studies in multiple laboratories.

5. Another challenge associated with this new technology is that of treating the generated data in a meaningful way such as acquiring, storing, and analysing the extensive amount of data that is generated by these type of studies. There is a need for valid sophisticated methods of storing and analyzing data, as well as standardized analysis approaches and algorithms that facilitate comparison of data among laboratories[36].

The full potential of microarray platform in toxicology research can be realized only when these limitations are resolved. This can be achieved by efforts to standardize established methods with help of experience in the form of large data sets of toxicant exposures.

Despite of these issues; it is clear that new advanced microarray hybridization technique will have a tremendous impact on toxicology research. In near future, data generated from microarray experiments will form the footing for an improved alternative method to evaluate the impact of chemicals on human and environmental health.

4. In Silico toxicology

The term *in silico* toxicology generally refers to a computational experiment, mathematical calculation, or scientific analysis of substances and organization of substance related data through a computer-based analysis[37]**.** The advantages of these methods compared with *in vitro* and especially *in vivo* approaches includes higher throughput, less expensive, less time consuming, constant optimization possible, have higher reproducibility if the same model is used, have low compound synthesis requirements, have potential to reduce the use of animals[39].

In silico toxicology tools

Toxicology-based computational approaches are typically aimed at building toxicity databases, QSAR modelling, Expert Systems, descriptor-based methods, and ligand and target-based methods. Also data visualization tools, a hybrid containing toxicology prediction tools like QSARs and systems biology and pharmacology pathway analysis are also available.

a) Toxicity database

Toxicity database is a valued set of electronic information (i.e., data) that can be related to the toxicity of substances of which the information is accessible by computer, organized by software, and utilized for safety and risk analysis of chemicals, product discovery and development, or academic research for settings in the biomedical and toxicological sciences.

The fundamental concept in developing chemical toxicity database is the ability to harness data of acceptable scientific quality from previous toxicity studies in order to build an electronic repository that may be searched, modelled, and used to derive relationship among structurally related compounds. Toxicity databases such as AERS, ACToR, BDSM, CCRIS, DSSTox, ECOTOX, Gene-Tox normally serve as a resource of data rich with authenticated structures, experiments on substance-induced toxicity or other similar scientific-based evidence, hierarchical data relationships and should include controlled vocabulary and capability for relational "read across" chemo informatics[37].

b) QSARs

Toxicologically based QSARs are mathematical equations used as a prognostic technique to estimate toxicity of new chemicals based upon a model of a training set of chemicals with known activity and a defined chemical space[37]. The QSAR quantifies features of the new chemical structure so that overall toxic properties of the compound can be predicted based on the relationship between structure and activity computed using the knowledge of toxicity derived from the training set. Numerous QSARs database have been established so far, few examples are MCASE, lazar, PASS, TOPKAT etc[38].

c) Expert Systems

Expert Systems attempt to formalize the knowledge of human experts, who assess the toxicity of a new compound, in a computer program. This approach is naturally appealing to most users, because it promises easy access to toxicological knowledge, and many of the most successful Predictive Toxicology software tools are in fact Expert Systems. Examples of expert system software tools are HazardExpert, Oncologic and DEREK[38].

Limitations

Limitations includes quality and transparency of training set experimental data, transparency of the program, descriptors sometimes confusing, applicability domain sometimes not clear[39].

Validation and regulatory acceptance of alternatives testing

Scientists and toxicological researchers are facing several difficulties and numerous hurdles in obtaining regulatory acceptance for the alternative methods. The main issue is achieving *in vivo* data of sufficient quality for use in evaluating the predictive value of the results obtained in *in-vitro* tests. However, several years are needed to validate and gain regulatory acceptance for alternative methods. There is still a lack of 3R alternatives for systemic and long-term toxicity. There is a great urge for the scientists in developing, validating and obtaining regulatory acceptance of alternative techniques since a ban has been called upon for most animal testing for the various chemical substances by statutory bodies[7].

CONCLUSION

This review is an attempt to make the reader to acquaint about some new conceptual approaches to toxicology testing. It is increasingly obvious that the development and incorporation of stepwise testing strategies, combining experimental data from a range of alternative methods (Cell culture system, Embryonic stem cell together with toxicogenomics and metabolomics, Toxicity database, QSARs, Ex-

pert systems and other *in vitro* tests) provide the most advanced and enhanced way to predict toxicity, at the same time eliminating the legal and ethical issues in using laboratory animals for testing purposes. Also there has been a significant progress in the development and validation of alternative methods. These improvements will allow faster, reliable, more efficient and objective assessment of alternative methods in future.

Future outlook

It is anticipated that in the future more and more emphasis will be placed on *in vitro* assays to study toxicity. For this it will be necessary to develop, improve, evaluate and validate the systems. The 3Rs approach will gain further paramount importance in forth coming years. Currently the priority research need to be fulfilled for the development of alternative methods is to ascertain more completely defined evaluating strategies/models for predicting the effects of toxicants on humans as well as other species. It is no doubt that increasing number of molecular biological techniques will have further impact upon the development, predictive performance and applicability domain of alternative testing systems. Applying molecular techniques seems to be significant because often the outputs obtained in terms of results are more human like response. The progresses achieved in the successful integration of -omics technologies within toxicology and effective implementation of these technologies may provide a better future in adequate human risk assessments.

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