Review Article

Teratogen Screening: State of the Art

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Received: 17 Jun 2010
Accepted: 9 Aug 2010

Abstract

Due to the number of new substances coming into use every year and the increasing amounts of chemicals, which are introduced into the environment, there is a high demand for a rapid, reliable and cost-effective method for detection of developmental toxicity. To meet this challenge various *in vitro* techniques have been established additional to *in vivo* animal testing. This review introduces the techniques in existence at the moment. Requirements on an ideal *in vitro* teratogenicity test system are stated, and the advantages and disadvantages of the present methods are discussed.

Avicenna J Med Biotech 2010; 2(3): 115-121

Keywords: Animal testing alternatives, Cell culture techniques, Teratogens

Introduction

The need for efficient methods to screen new chemicals, drugs and environmental pollutants for their toxicity is obvious. However, from of the thousands of chemicals in commercial use, only a small fraction has been tested for their teratogenic activity so far [(1)]. Moreover, the underlying mechanisms behind the developmental toxicity of compounds known to have a teratogenic potential has solely partially elucidated if any [(1)].

In context of pharmaceutically relevant substances the evaluation of the embryo toxic potential is of special importance. Very early in the course of the development of drugs a newly synthesized compound has to be tested for its acute and chronic as well as for its developmental toxicity.

This preclinical evaluation of the long-term safety of a drug implies high throughput screenings of possible cytotoxic, mutagenic, embryo toxic and teratogenic effects.

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This review introduces the techniques in existence at the moment. In this, *in vitro* systems are brought into the focus of attention. Requirements on an ideal *in vitro* teratogenicity test system are stated, and the advantages and disadvantages of the present methods are discussed.

Animal Test Systems

The preclinical evaluation of drugs traditionally involves large numbers of animals to predict possible drug side-effects.

Various animal-based test systems established for screening a potential teratogenic activity are conducted on pregnant laboratory

animals, usually mammals such as mice, rats, rabbits and nonhuman primates. This way, the test compound is administered daily during the period of organogenesis of the fetus. Near-term the fetus is examined for skeletal, visceral and external anomalies [2, 3].

Although this screening procedure has been of value especially until the eighties in the last century, however there are certain drawbacks to consider. Generally, the *in vivo* assays are very time consuming, laborious and expensive, thus contravening with the current need for rapid testing of potential drugs [2, 3]. Moreover, due to differences in maternal metabolism, transport and maternal-fetal membrane relationships animal studies often indicate substantial differences of toxicity between species. A compound that is demonstrably teratogenic in animals may not be so in man or vice versa [2, 3].

To complicate the situation numerous additional factors as the nutritional state of the dam, the variability in the developmental age of embryos from litter to litter or within the same litter as well as placental functions must to be taken into account at interpretation of data [2, 3]. It can be stated, that pregnant animal testing alone is not qualified to predict the teratogenic potential of new compounds. Furthermore, there is an increased political and public demand to reduce the use of laboratory animals due to objections to experiments on living animals.

In vitro Techniques

Many of the variables of *in vivo* test systems *e.g.* species differences can be eliminated or at least controlled by *in vitro* techniques. Besides, *in vitro* tests are simple and cost-efficient. However, unlike other toxicity testing systems, *in vitro* analysis for teratogenicity presents certain special circumstances since the target in this case is a rapidly growing embryo, whose tissues are simultaneously embarked on divergent differentiation pathways. Thus, the test system has to be designed in a way that the *in vitro* data can be interpreted in terms of a possible *in vivo* outcome.

Main characteristics of an ideal *in vitro* teratogenicity screening system (Table 1) include its relevance to mechanisms of teratogenesis as well as the involvement of developmental events in addition to desirable features common to other test systems [4].

In the majority of cases the evaluation of the developmental toxicity of a component proceeds in at least two distinct phases: (i) exploratory analysis of the fundamental biological properties of a substance by a series of individual tests (test batteries) and (ii) detailed analysis in more sophisticated versions of the test [5]. Several *in vitro* model systems based on a vast range of cells and tissues have been developed for detecting the embryo toxic hazard of chemicals. This includes whole embryo culture tests, organ culture teratogen assays (e.g. micromass teratogen test), as well as eukaryotic cell culture systems (e.g. embryonic stem cell test and *Dictyostelium discoideum*). In addition, immunological as well as molecular approaches have been used to establish new and more predictive toxicological endpoints.

Table 1. Main characteristics of an ideal *in vitro* teratogenicity screening system adapted from Wilson (1978) [4]

	Features of an ideal *in vitro* teratogenicity test system
I	Simple, easy to perform, yield of interpretable results
II	Rapid, usage of large numbers of samples
III	Giving few false negative
IV	Having relevance to mechanisms of teratogenesis
V	Involving some aspects of progressive development
VI	Usable with various types of agents
VII	Usage of intact organisms capable to absorbe, circulate and excrete chemicals

Whole Embryo Culture Test

Culturing of whole embryos at an early stage of organogenesis, and exposing of these to a potential teratogen, allows for the valuation of a relative index of teratogenicity of the test substance [2]. Both mammalian embryos, namely from the rat or the mouse (rodent embryo culture) [2, 3], and embryos of the South African clawed frog *Xenopus laevis* (frog embryo teratogenesis assay-*Xenopus*, FETAX) [6, 7] are in use in teratogen screening.

The tests are able to evaluate single compounds or their joint action as well as environmental mixtures. However, the question as to what minimal change in a developmental parameter would display the presence of a potential teratogen is still challenging. There are numerous parameters which may indicate a deviation from normal (Table 2) [2]. Regrettably, at present no general agreement exists on the extent of correlation between many of these events and teratogenicity. So, the three endpoints generally used are mortality, malformation and growth inhibition [6].

Whole embryo culture fulfills most of the requirements of Wilson`s ideal teratogenicity screen. The advantages and disadvantages of the technique are listed in table 3 [3]. However, teratogen test systems employing embryo culture are unlikely to be adopted as sole predictors of teratogenic potential in humans. The validity of data derived remains uncertain. In particular, variations due to technician biases in terms of judging malformation and in selecting test concentrations narrow the significance of gained results [7].

Despite these limitations, the potential of whole embryo culture systems to mimic human teratogen metabolism, coupled with their assessment of developmentally relevant endpoints may secure their place in a battery of teratogen screens.

Micromass Teratogen Test

The micromass teratogen test is an *in vitro* system that can detect the interference of substances with some of the normal processes of cell differentiation observed in the developing embryo. The test is based on chick, mouse and accordingly rat embryo midbrain or limb cells, which were exposed to test compounds for varying times and concentrations [8]. *In vitro* culturing of embryo limb or rather central neural cells in small volumes at high density results in a differentiation of the cells into chondrocytes or neurons starting from numerous small aggregates or foci of cells [9].

Hence, several aspects of cell behavior critical for normal embryogenesis can be observed in micromass cultures including cell adhesion, movement, communication, division and differentiation involving the new synthesis of tissue specific patterns of enzymes and structural proteins [8].

The technique is ideal for preparing large numbers of homogeneously responding cultures from small amounts of embryo tissue (performance in 96-well microtiter plates [10]).

Exposure of the cells to the test compound can be either directly in culture or transplacentally prior to culture in *ex vivo* experiments. By this way, the test system can

Table 2. Various parameters which may indicate teratogenic activity of a compound in whole embryo culture test systems adapted from Kochar (1980) [2]

Parameters indicating teratogenicity of a substance in whole embryo culture tests	
I	Changes in macromolecule synthesis as DNA, RNA or proteins
II	Mitotic inhibition, cell cycle chances
III	Cytotoxicity
IV	Changes in cell behavior as cell to cell aggregation, cell adhesion, locomotion
V	Block in cell differentiation
VI	Altered organ morphology or cell shape
VII	Malformed embryo

Table 3. Advantages and disadvantages of whole embryo culture test systems adapted from Fantel (1982) [3]

Advantages and disadvantages of whole embryo culture testing	
Advantages	
I	Rapidity (24-48h for rodent embryo culture; 96h for FETAX)
II	Precise control over embryonic exposure
III	Use of small quantities of expensive materials
IV	Removal of potentially confounding maternal variables
V	Elimination of resorption losses
VI	Possibility to definite the role of maternal metabolism by adding a metabolic activation system
Disadvantages	
I	Laborious
II	Routes of administration of teratogens are not representative to the *in vivo* situation
III	Impossibility to derive adult and developmental toxicity relationships
IV	Relationship between embryonic defects seen following exposure *in vitro* and anomalies of the fetus and newborn often remains unclear

include a phase of *in vivo* embryo exposure that will act as a control for the effects of drug metabolism and pharmacokinetics [11].

Furthermore, via using cells from different organs and species the micromass test is applicable to reproduce the *in vivo* sensitivity of particular embryonic tissues or species to teratogenic agents [8].

Studies comparing chemicals drawn from a variety of classes suggest that the percentage of teratogens detected may vary between 60 and 90%, and the percentage of non-teratogens detected may vary between 89 and 100% depending on the test configuration selected, the choice of compounds and the length of exposure to test agent [12 - 14].

Interlaboratory variability has been reported to be small. Thus, the micromass teratogen test is a robust *in vitro* system for studies of potential teratogens. However, even if an *in vitro* test is considered to be well validated, limited *in vivo* data should be generated to establish a correlation between *in vitro* and *in vivo*.

Embryonic Stem Cell Test

Permanent lines of pluripotent stem cells, which are characterized by a nearly unlimited self-renewal capacity, have been shown to develop into differentiated cells of all three primary germ layers *in vitro* [15]. In this, embryonic stem cells recapitulate cellular developmental processes and gene expression patterns of early embryogenesis [16]. Based on the resemblance between early embryonic stages and the differentiation of embryonic stem cells *in vitro* the cell lines are used to identify cytotoxic, mutagenic, embryo toxic and teratogenic effects of chemical compounds.

Embryonic stem cells were isolated from blastomeres of the early mouse embryo from the 8-cell up to the blastocyst stage [15, 16]. During *in vitro* cultivation the stem cells differentiate spontaneously at this forming cell aggregates, the so called embryoid bodies [16]. Since spatially controlled signals are lacking, morphogenetic development does not occur within the embryoid bodies [16].

However, genes coding for tissue-specific proteins have been shown to be expressed in a developmentally regulated and time-dependent manner closely resembling the patterns observed during *in vivo* embryogenesis [16]. Thus, the embryonic stem cell test is suitable to analyze developmental processes on a cellular level.

The evaluation of cell differentiation is performed both morphological and via mo-

lecular techniques (analysis of tissue-specific genes via reporter gene expression; automated high-through put screening for changes in gene and protein expression patterns using microchip arrays for transcriptome and proteome analysis) [16]. However, the identification of predictive marker genes for the major target tissues during organogenesis is crucial to get precise information on the teratogenic action of a test compound.

Dictyostelium discoideum

A simple biological test system for the rapid screening for potential developmental toxicity of compounds is based on the cellular slime mold *Dictyostelium discoideum* (*D. discoideum*) [17 - 19]. The single-cell, eukaryotic microorganism requires relatively simple culture conditions and can be produced in large cell numbers. One outstanding property of *D. discoideum* is that it is competent to undergo both vegetative growth and development (fruiting body formation). The synchronous differentiation of a single-cell population into multicellular organisms can be induced by removing the food source [17 - 19]. The mechanism of this relatively simple cell differentiation program leading to the formation of stalk and spore cells has been shown to be similar, to some extent, to the development of mammals [20 - 22].

So far, four transgenic *D. discoideum* strains have been constructed [17 - 19]. Each of these strains expresses the reporter gene β-galactosidase under the control of a distinct developmentally regulated *D. discoideum* promoter. The promoters in use are (i) cprB: active in both stalk and spore, but not in undifferentiated vegetatively growing *D. discoideum* cells, (ii) pspA: active in spore cells, (iii) ecmA: active in prestalk cells and (iv) ecmB: active in stalk cells [17 - 19]. Hence, the reporter genes serve as highly specific indicators for the developmental fate of a certain cell at a given time point.

Compared to mammalian cell cultures or animal teratogenicity models *D. discoideum* cell culture is easier to handle and less expensive. Furthermore, the system is suitable for automated screening. Thus, the *Dictyostelium*-based assay may be qualified for rapid large-scale screenings of chemicals, even though the test system may not securely predict the teratogenic potential of these compounds in humans.

Most Recent Techniques on Teratogen Study and Assay

Actually, focus is set on changes in gene expression of cells cultured *in vitro* with known or potential teratogens [23 - 25]. The correlation of the transcriptome with traditional toxicological endpoints may reveal so far unknown molecular targets and biomarkers of developmental toxicity. Beyond that, assessment of dose-response relationships between environmental exposures and the disruption of specific processes essential during early embryonic development is expected to provide information beneficial for determining the mechanisms that underlie teratogenicity.

The current technologies in use for detecting relative mRNA expression levels are (i) microarray analysis and (ii) quantitative real-time reverse transcription PCR [24]. Both techniques enable the detection of genes susceptible to deregulation by teratogens. So far, gene expression responses identified to be related to *in vivo* effects of teratogenic substances include histone deacetylase inhibition, G1 phase cell cycle arrest and induction of apoptosis [26]. Thus, studying toxicogenomic responses to short-time (≤6 *hr*) exposures of a teratogenic compound *in vitro* could be a useful component in mechanistic studies and screening tests for developmental toxicity.

Additional computer-based techniques have been developed for the prediction of the teratogenic potential of a given compound covering the computer simulation of normal and abnormal development of cells and tissues [27] as well as the analysis of structure-activity relationships and hydrogen-bond formation of chemicals [28].

Additional Remarks

To reduce the number of experimental animals, various *in vitro* alternatives have been developed. However, all *in vitro* tests share the possibility of generating false negative or positive data. Teratogenicity of a specific compound may be based on the biochemical modification of the substance *in vivo* which is not realized by the *in vitro* test system.

A further critical factor is the pharmacokinetic. The embryo *in vivo* may not be exposed to concentrations of a chemical that adversely affects the development of cells *in vitro*. Even small differences in the molecular structure can affect the pharmacokinetic of the substance, independently of the absolute sensitivity of the embryo *in vivo* or of the cultured cells *in vitro*. Thus, pharmacokinetic data must be taken into account for valuation the risk of developmental toxicity of a compound.

Generally, the *in vitro* test system used has to be selected with a view to obtain optimal and relevant information. At present, we know relatively little about the mechanisms of developmental toxicity. However, the sensitivity of a certain *in vitro* test to a teratogenic agent may depend on the underlying mechanism of action of the compound. Often several types of test systems are required to securely identify different classes of teratogenic agents (test batteries).

To sum up, it seems to be unlikely that *in vitro* testing will replace animal testing entirely. However, it has the potential to reduce the number of animals used in screening chemicals, drugs and environmental pollutants for their developmental toxicity.

Conflict of Interest

The author declares no conflict of interest.

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