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Alternatives to *in vivo* tests for teratologic screening

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Summary. - During the last decade there has been a tremendous increase in publications describing methods for *in vitro* toxicological research and emphasizing their advantages, suitability and necessity, rather than the classical *in vivo* studies. In this review we shall look at and consider only a few short term tests, chosen on the basis of the particular relevance that they have in the evaluation of the substances: rodent whole embryo culture (for both pre- and post-implantation embryos), non-mammalian vertebrate embryo and invertebrate embryo cultures, organ culture, and at last cell culture. We have seen however that sometimes some of the developed methods suit neither the necessity of saving time, money, and animals, nor the consistency of the results in xenobiotic screening. The concomitant application of both *in vivo* and *in vitro* methodologies will improve the quality of teratological research, and therefore will contribute to a critical evaluation of developmental hazards.

Key words: alternative methods, teratology, developmental toxicity.

Riassunto *(Alternative ai test* in vivo *per screening di teratologia).* - In questo ultimo decennio si è assistito ad un'enorme proliferazione di pubblicazioni che descrivono nuovi metodi per studi di embriotossicità *in vitro*, e che sottolineano i vantaggi, la convenienza e la necessità di tali test alternativi ai classici studi *in vivo*. In questa rassegna vengono presi in esame e valutati solo alcuni test a breve termine, sulla base della particolare rilevanza che hanno avuto nella valutazione delle sostanze testate: coltura di embrioni di roditori in toto (sia pre- che post-impianto), coltura di embrioni di vertebrati non mammiferi, coltura di embrioni di invertebrati, coltura di organi ed infine colture cellulari. Si è visto tuttavia che spesso alcune di queste metodiche non si sono rivelate convenienti sia per quanto riguarda il risparmio di tempo, costi e animali, sia dal punto di vista dell'attendibilità dei risultati, quando utilizzate nello screening di xenobiotici. La contemporanea applicazione delle metodiche *in vivo* ed *in vitro,* comproverebbe la qualità dello studio teratologico ed inoltre contribuirebbe ad una valutazione critica dei rischi embriotossici.

Parole chiave: metodi alternativi, teratologia, tossicità dello sviluppo.

Introduction

During the last decade there has been a tremendous increase in embryotoxicity studies using alternative experimental models, rather than the classical *in vivo* studies.

There are many reasons why such a large number of academic and industrial researchers have changed to these alternative methods.

The main reasons for the choice are the needs for more rapid and cost effective testing without the consumption of animals, and for testing the toxic potential of both new and known chemicals. Each year, there are hundreds of new chemical compounds developed, and there are thousands of old compounds which have not been tested.

The pressure from the animal welfare groups, and their aim to reduce the consumption of animals in these studies, is often cited as the main reason for this change of direction in teratological assessment. However it seems to us that is a secondary matter. The strongest drive behind the development of alternative methods comes from the perceived needs of scientists throughout the world to quickly maximize the available knowledge in this biomedical research sector. A sector, which even today has more unknowns than certainties. A few considerations are sufficient to demonstrate the "state of the art" of teratology:

1) only in 30 to 35% of congenital malformation cases the causes of the malformation are known. Over 60% of the cases are noted as "spontaneous malformation", which means that the causes are not recognized;

2) the frequency of congenital malformations (c.m.) in western countries has been around 1.5% for many years. This fact, from one point of view, is considered to be positive, given that there has not been any rise in malformed births despite the tremendous increase in use, and of emissions of new chemical substances. From another viewpoint, it means that despite the great economic and scientific efforts made, there has been a failure to reduce the incidence of malformations;

3) thirty years have passed since the best known, and most dramatic case of an "epidemic" of malformations, due to the use of Thalidomide during pregnancy. Despite the large number of studies that have been made since, the mechanisms of action of this substance in the production of malformations, are completely unknown. The same is true for almost all the teratogenic agents affecting man. It is only recently that the mechanism of action of one very special teratogenic agent (retinoic acid), has begun to be understood.

In the face of these serious and frustrating knowledge deficiencies, the scientific world has revolutionized the experimental procedures, substituting simpler and more easily interpreted *in vitro* tests for the multi variable and difficult to interpret *in vivo* experiments.

When one speaks of alternative tests, the reference is not to *in vitro* testing exclusively. The term includes all experimental models which do not use pregnant mammals. The number and variety of these tests is very great, and is such that to examine them in detail would be beyond the scope of this review. The reader is referred to publications dealing specifically with this subject, such as Neubert and Merker [1] and Faustman [2].

In this review we shall look at only a few short term tests, chosen on the basis of the particular relevance that they have in the evaluation of the substances being examined; on the amount of work carried out using them; and their practicality in the context of their destined use.

Rodent whole embryo culture

This is the *in vitro* system which has been, and is most used as it allows the experimentation on the whole embryo, without the complications due to the maternal body. There are systems available for both pre- and post-implantation embryos, with end points and applications which differ widely.

Pre-implantation embryos

Mammalian embryos (mouse embryos have been mainly used) can be flushed from the oviducts at the 2 to 8 cell stage [3, 4].

Several experimental techniques are possible, for example, treatment *in vivo* followed by culture; culture in treated animal serum; treatment *in vitro* followed by reimplantation in pseudopregnant females.

Up to a short while ago, it was thought that the pre-implantation period was not involved in the induction of full term malformations, as the interference with such a young embryo was thought to lead to its death, or complete recovery.

However, our knowledge of the effects of exogenous factors on pre-implantation embryos is now more extensive.

Several studies have shown that the exposure of pre-implantation embryos to genotoxic agents can lead at term not only to an increase of perinatal mortality, but also to retarded development or morphological alterations.

In particular, Spielmann *et al.* [5], using cyclophosphamide and other embryotoxic agents, demonstrated that mouse blastocyst cells gave a graduated reaction to the substances to which they were exposed, and could therefore be used to show the effect of different levels of toxicants. This contrasted completely with the theory of "all or nothing" in terms of pre-implantation response.

These authors' results, support those obtained by others both *in vivo* and *in vitro*, and show that mouse and rabbit embryos, before implantation, are able to metabolize xenobiotics.

In the light of this, it seems important that in routine testing for development toxicity, there should be coverage of this delicate stage of embryonic development.

For an evaluation of the teratogenic effects on pre-implantation embryos, *in vitro* culture has several advantages over *in vivo* techniques. It requires few embryos and is quicker, and more precise due to the separation of maternal and embryonic factors.

However, the *in vitro* techniques only permit the evaluation of the direct effects of teratogenic substances on the embryo, up to the early egg cylinder stage, and only permit an indirect assessment of the disturbance to maternal physiology. The detection of maternal effects of early treatment require transplant experiments. The transplantation of treated embryos into pseudopregnant females is also necessary for the verification of the full term congenital malformations.

Post-implantation embryos

The technique for the cultivation of post implantation embryos was developed by New and his collaborators [6-8], and is now widely utilized. The laboratories that use the technique have tended to develop variations, in line probably with perceived advantages, so there end to be variations of culture conditions, of embryo age at extraction, of culture media, and of the timing and concentration of the gases used, the duration of the culture, and the type of assessment carried out at the end of the culture period.

As a rule, mice or rat embryos are taken from the maternal uterus at the 8.5th or the 9.5th day of gestation respectively, and are transferred to the culture medium. They are then maintained for a variable period between 24 and 72 hours, which allows embryo development from a stage of 0 to 2 somites, up to a maximum of around 30 somites.

The impossibility of prolonging the culture beyond this limit is probably due to an insufficiency of nutrients and oxygen in a developmental stage in which, *in vivo*, the allantoic placenta starts to fully perform its functions.

However, even if the culture period is limited, it covers a fundamental time interval for morphogenesis and organogenesis in the cultivated embryo.

End points that are routinely monitored in these cultures include viability, gross and histological abnormalities, growth and macromolecular content. Several attempts [9-11] have been made to standardize as far as possible the reporting on the degree of development of the embryos in this *in vitro* system, with a view to being able to discern the difference between a general embryotoxic effect and a specific teratogenic effect. The latest analysis seems to indicate that the scoring system proposed by Brown and Fabro [9] is the one with widest application.

The clearest advantage of this system is that it is able to verify the embryotoxicity of an agent that directly affects the embryo in the absence of biotransformation, and also that of chemicals that require metabolic activation.

In fact, it is possible to add a metabolizing system (post-mitochondrial supernatant of microsomes) that is able to activate all those agents that need a biotransformation to be able to carry out their dysmorphogenic actions.

The system allows the clarification of the action mechanism of some substances, and to identify an eventual organ specificity of the dysmorphogenetic action.

In vitro cultures are promoted on the basis that they save time, materials, and therefore reduce costs. We are skeptical on the animal savings, as the preparation of rat serum for culture media requires the sacrifice of large numbers of animals.

Over 150 substances have been tested with whole embryo culture, and the results show a high degree of correlation with results obtained *in vivo*. This leads us to conclude that *in vitro* embryo culture is a valuable tool for the selection process in new product development.

Non-mammalian embryos

These tests are more rapid and are easier to carry out than the culture of mammalian embryos. They permit the testing of a large number of substances, using full and therefore statistically significant sampling.

Invertebrate embryos

With the exception of the type of test which involves the use of insect embryos at the metamorphosis stage [12, 13], the others all use artificial embryonic systems.

The organisms of choice for these methods are the Hydra attenuata [14] and the planaria [15]. These are low invertebrates with a marked capacity for regeneration, following disaggregation in the first case, and following head lesions in the case of planaria.

The more sensitive method is that using the Hydra. The regeneration of an adult organism from a pellet of basal material is based on mechanisms of reaggregation and differentiation, fundamental activities also in the development of the embryos of higher organisms. We are referring particularly to the phenomena of adhesion, of reciprocal induction between the cells, of differentiation and intercellular coordination.

The teratogenic potential is assessed as the ratio between the toxic dose for the adult and the dose which inhibits reaggregative development (the greater the value of the ratio, the more probable that there is dysmorphogenetic activity).

Whilst these tests have been validated for many substances, their use in the prediction of teratologic risks for man is open to question. These methods are unable to identify dysmorphogenetic mechanisms in complex embryos, nor are they able to determine eventual target organs.

Their use could however be relevant in the preliminary screening of xenobiotics of interest.

Non-mammalian vertebrate embryos

The validation of methods using embryos of non-mammalian vertebrates, is based on substantial ontogenetic uniformity in all vertebrates, in the first stages of development. A historical cornerstone of experimental teratology is the experiment that calls for the use of the chick embryo *in ovo* (Dareste [16], readapted today by Jelinek [17]). This experimental model is currently thought not to be sound, due to the known hypersensitivity of the chick embryo to xenobiotics and due to its incapability of actuating metabolizing systems. The alternative method which is enjoying great success in the teratological field is the so called FETAX (frog embryo teratogenesis assay: Xenopus) [18]. Like the tests that use fish embryos [19], the most direct advantage of this method is the ability to follow the normal morphogenetic development through every phase, though under very rigorous experimental conditions. The FETAX experimental model, has been shown to be very sensitive during validation, and from the data collected to date it seems to be reasonably predictive for teratogenetic phenomena in mammals.

The main advantages of this method lie in the possibility of selecting embryos, so that experiments can be conducted with embryos at exactly the same stage of development; in the possibility of analyzing the developmental deviations of embryos of a species whose morphogenetic processes have been well known for many years; in the possibility of having as large a sample as desired; and finally the low cost. Particularly relevant is the possibility to introduce metabolizing systems which can mimic the bio-transformations of the human organism.

The limit of this method however, lies in the high percentage of malformed individuals in the control groups, and above all in the problem of extrapolating data from the development of an amphibian into a teratological risk analysis for the vastly more complex human organism.

Organ culture

Cultures of fetal organs may be suitable for studying the interplay of specific development mechanisms leading to organogenesis.

Organ culture is when the embryonic bud, or a representative part of the organ (a lobe of liver, or lung for example) is cultivated *in vitro*, and as well as differentiating morphogenetically, grows as well. Numerous organs (limb buds, palatal shelves, bones, digits, lung, lens, liver, pancreas, kidney, skin, tooth, etc.) can be cultured, and some of these systems have been proposed for the investigation of prenatal toxicology, even though the larger part of the cultures carried out are primarily done for the evaluation of the pathogenesis associated with congenital malformations, with cancer, with transplacental carcinogenesis, or in studies on the structure and function of the particular organ that is in course of differentiation.

For organ culture two different techniques are used:

Trowell method (static). - In this method the explant is kept on a filter or other suitable support, and differentiation takes place at the culture medium/gas interface [20].

Suspension method (non-static). - In which the organ explant is submerged in the culture medium, and shaking or rotation ensures gas exchange [21].

Limb bud

The embryonic limb bud seems particularly adapted for a system of screening, as during its development it experiences many steps known to be important in normal organogenesis, such as localized cell death, differential growth, tissue interaction, cell proliferation and differentiation, and morphogenetic cell movements.

As a rule, rodent limbs are excised from the embryo at around 10 to 11 days (33-45 somites, in the mouse). At this stage the limb bud is still almost undifferentiated, and has only the blasteme covered by the epithelium. During the 7 to 9 days of maintenance *in vitro*, the limb bud develops cartilaginous structures representing the scapula, humerus, ulna, radius, and the bones of the hand, and even if this differentiation develops more slowly than *in vivo*, it is reproducible and consistent. This system of organ culture therefore, offers a wide range of end points, which can be monitored when potentially teratogenic agents are introduced into the system, e.g. cellular proliferation, differential growth, nucleic acid and protein content, size and shape of limb parts, chondrogenesis, and collagen or proteoglycan biosynthesis.

It has been demonstrated that adult hepatic subcellular fractions (S-9 and microsomes) are compatible with limb bud culture, and it is therefore possible to test substances which require biotransformation [22]. Neubert [21], perfected a score system for the morphogenetic differentiation of limbs. This assigns points for every trace of bone, according to the grade of development achieved, and this allows the establishment of eventual relationships between dose and effect in induced morphogenetic alterations.

The confirmation of the validity of this system, in the preliminary screening of xenobiotics, comes from the number of publications in which agents identified through *in vivo* studies as potential teratogens, have induced an abnormal development of the limb bud, *in vitro*.

Cell culture

These methodologies, using isolated cells, avoid those phenomena which occur at cellular level following complex interactions between cells and tissues. Cell culture may be the culture of stabilized cell lines, or the culture of primary cells.

Stabilized cell lines

These are not normally used for studies of toxicity in development, as they do not show the typical reactions that occur during embryonic development. Furthermore, the cells lose their particular characteristics through the succession of generations, and assume the morphology and functions typical of cultured cells.

Primary cultures of embryonal cells

Cells taken from embryos continue to undergo a number of developmental processes while being cultured. These may include growth, hysto-differentiation, appearance of specific gene products, etc.. Therefore these cells can be used to screen substances thought to alter cell development.

The culture method *in vitro* of cells dispersed from embryonic organs and grown at high density, is known by the name of "spot", or "micromass".

Limb bud micromass. - Numerous studies of the toxicity of development have been carried out using cells derived from the limb buds of rodent embryos, cultivated using the micromass method [23-25]. During the 6 days of the cultivation they multiply, and

differentiate into chondrocytes. The cellular aggregation, the cell density, and the differentiation into cartilaginous tissue, are verified [21]. General cytotoxicity can be observed as the suppression of fibroblastic and mesenchymal-like cell outgrowths. Growth and differentiation can be assessed spectrophotometrically using alycan blue (cartilage specific proteoglycan stain), or radiochemically. Guntakatta *et al.* [23] used this method to verify the cytotoxicity of 27 compounds (22 teratogens and 5 non-teratogens in the *in vivo* tests). They reported an overall agreement of 89%, with a false negative rate of 15%.

Neural tissue cell cultures. - The micromass culture method for neural cells, for short term screening, was developed and perfected by Flint [26, 27].

Neural cells are taken from the mesencephalus of rat embryos at 12 days, they are treated with trypsin, and are prepared as a high density suspension. During the 5 days of culture, biochemical and morphological differentiation of neuronal cell foci occurs.

The degree of differentiation achieved can be determined by fixing and staining the cells with hematoxylin-eosin, and assessing the number of foci formed. An inhibition of the differentiation (reduction of the number of foci) is taken as an indication of a potential teratogenic effect. Flint and Orton [27] tested 46 compounds using this method. Of these, 27 were teratogenic and 19 non-teratogenic in the *in vivo* tests, and they reported a 91% agreement with the *in vivo* results.

Even though the results of these comparisons between micromass and *in vivo* tests seem to be very encouraging, it must be borne in mind that the cell culture techniques have the disadvantage of not allowing the study of typical morphogenetic differentiation, but only of differentiation at the cellular level (chondroblasts differentiate chondrocites, neuroblasts differentiate neurons, etc.). At the end of the culture period one only finds groups of cells more or less differentiated, but not definitely organized in morphogenetic structures.

Conclusions

In vitro testing for teratogenic screening began as a need to find simpler means than *in vivo* tests which would be easy to use and to interpret, and which would reduce costs and consumption of animals. We have seen however, that at times, the various methods developed do not satisfy these needs, nor are they always used in the most fitting way. Some *in vitro* tests were developed as means for the study of specific teratogenic mechanisms, and were then used to evaluate the teratogenicity of xenobiotics. It must also be stated that none of these short term tests alone is sufficient to provide a full evaluation of dysmorphogenetic risks. The tests are either conducted over limited time periods during gestation, and/or limited organ system processes are studied. A predictive analysis of these risks would only be possible through a hypothetical system active throughout all the phases of the embryo-fetal period. In this light it appears to us that from all the tests proposed, those using whole rodent embryos are the most indicated for teratogenetic screening. They take into account all the complex morphogenetic and organogenetic phenomena that take place during the embryonic development, and therefore are better equivalents to the *in vivo* situation. Each of the methods proposed and examined can make a contribution towards the determination of possible negative effects of xenobiotics on developing organisms, even if limited to some specific aspect. The studies on low level vertebrates and on invertebrates, which do not seem to be credible as predictive tests in man due to the enormous phylogenetic differences, have a more suitable application in the assessment of the ecological impact of chemical substances.

Finally, there is a need to standardize the procedures for the short term tests, both in regard to culture conditions, and the assessment of data obtained. This is necessary for a clearer understanding of the real embryotoxicities in play, and to allow meaningful comparisons of the results from different laboratories.

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