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An integrated approach for detecting embryotoxicity and developmental toxicity of environmental contaminants using *in vitro* alternative methods

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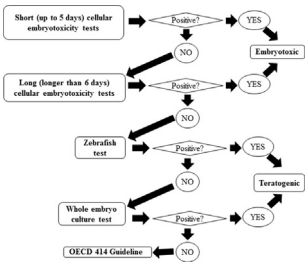
HIGHLIGHTS

- Stem cells under differentiation are useful model for testing embryotoxicity *in vitro*.
- Zebra fish and WEC are widely used for testing teratogenicity *in vitro*.
- Omic approaches contributed to enhance predictivity of developmental toxicity.
- A single test would not show enough predictivity for screening developmental toxicity.
- A tiered approach strategy would reduce bioethical concerns in developmental toxicity.

GRAPHICAL ABSTRACT



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ABSTRACT

The main available alternatives for testing embryotoxicity are cellular tests with stem cells and *in vitro-ex vivo* tests with embryos. In cellular tests, the most developed alternative is the embryonic stem cell test, while the most developed tests involving embryos are the zebrafish and whole embryo culture test. They are technically more complex than cellular tests, but offer the advantage of determining the expectable phenotypic alteration caused by the exposure. Many efforts are currently being made, basically through proteomic and genomic approaches, in order to obtain improvements in predictivity of these tests. Development is a very complex process, and it is highly unlikely that a single alternative test can yield satisfactory performance with all types of chemicals. We propose a step-wise approach where model complexity, and consequently technical skills and economical costs, gradually increase if needed. The first level would be run short cellular assays to detect effects in early differentiation stages. The second level would involve longer cellular embryotoxicity tests to search embryotoxicants that have an effect on late differentiation stages. The third stage would consider tests with embryos because they allow the determination of hazards based on molecular and morphological alterations, and not only on differentiating cells.

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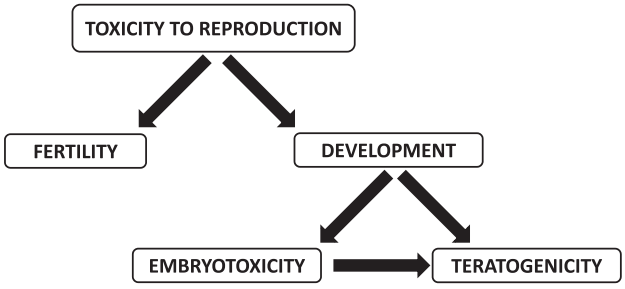

Fig. 1. Relationships among toxicity to reproduction, developmental toxicity, embryotoxicity and teratogenicity.

1. Introduction

According to their definitions, developmental toxicity is adverse effects on the growing organism from the embryonal state to the time of an individual's sexual maturation, embryotoxicity is the toxic effects in the progeny between conception and the foetal stage, while teratogenicity is structural malformations or defects in offspring after the embryogenesis period. Thus, embryotoxicity and teratogenicity are both considered to be toxic effects on development. Toxicity to reproduction is a term that encompasses alterations in fertility and development caused by chemicals. Fig. 1 outlines the relationships among these different toxicity classes included under the term "toxicity to reproduction". The developmental toxicity of environmental contaminants is an issue that causes much concern for society, and toxicologists must possess safe and reliable procedures to test it. The main protocols normalised by the Organization for Economic Co-operation and Development (OECD)[1] to test developmental toxicity include the following guidelines: 414, for testing teratogenicity; 416, for testing toxicity to reproduction (fertility plus development) in two generations; 421, a screening study for testing developmental toxicity; 422, for testing toxicity to reproduction simultaneously with repeated dose toxicity; 426, for testing neurodevelopmental toxicity (Estevan et al., 2011). OCDE guideline 443, for testing reproductive toxicity in one-extended generation, was added to the list in 2012.

The main inconveniences of these OECD guidelines are that they are expensive, time-consuming and use a large number of animals, plus the associated bioethical and social concerns. Table 1 shows the economic cost and the minimum number of animals requested for applying OECD guidelines to test toxicity to reproduction. Indeed, the estimated economic cost ranges between €54,600 for guideline 421 and €1,100,000 for guideline 426 (Rovida and Hartung, 2009). Moreover, the number of animals required ranges between 412 for guideline 422 and 3,200 for guideline 416 (Rovida and Hartung, 2009). It is remarkable that testing embryotoxicity alone is not considered among the various OECD guidelines. This part of developmental toxicity must be assayed necessarily in the long general test of toxicity to reproduction, where fertility, embryotoxicity, teratogenicity and development are assessed in the same test.

All these data suggest that cheap and reliable methods for testing developmental hazards of environmental contaminants and the subsequent risk assessments would be welcomed. In this scenario, alternative and *in vitro* methods for testing developmental toxicity might play a relevant role. The term alternative method is assigned to those methods used to study toxicity that Reduce, Refine or Replace (3Rs) animal experimentation (Russell and Burch, 1959).

[1] An integrated approach for detecting embryotoxicity and developmental toxicity of environmental contaminants using in vitro alternative methods.

Table 1
Economical cost and number of animals needed to apply the OECD Guidelines for testing reproductive toxicology (data taken from Rovida and Hartung, 2009).

OECD guideline	Purpose	Animals	Estimated cost (€)
414	Teratogenicity	784	63,100 (rats) 92,500 (rabbits)
416	Reproductive toxicity in two generations	3,200[a]	328,000
421	Screening test for reproductive and developmental toxicity	560	54,600
422	Combined repeated dose toxicity study with the reproduction/developmental toxicity screening test	412	92,000
426	Neurodevelopmental toxicity study	1,400[a]	1,100

[a] Considering all the discarded pups.

The OECD guidelines for testing chemicals include 20 different *in vitro* methods to test various aspects of toxicity, such as dermal and ocular impairments, genotoxicity and endocrine disruption. Yet none is devoted to toxicity to reproduction, developmental toxicity or embryotoxicity.

Nevertheless, several *in vitro* alternative methods have been developed for testing embryotoxicity and teratogenicity. They all offer high predictivity and concordance with *in vivo* test results, meet the 3Rs requirements, and are cheaper than *in vivo* tests. The main alternative tests for assessing embryotoxicity are based on the *in vitro* study of alterations in cellular differentiation, while alternative methods for testing teratogenicity are based on the *in vitro* or *ex vivo* exposure of whole embryos, with further analyses of the alterations caused by the chemical being assessed (Table 2). Whole Embryo Culture (WEC), micromass (MM) and Embryonic Stem cell Test (EST) have been validated in blind studies (Genschow et al., 2002) and a study is currently under way with zebrafish (Gustafson et al., 2012). These studies have been carried out under the typical perspective of verifying reproducibility and relevance. However, mechanistic validation has been recently proposed as a tool to proceed in order to generate valuable information for decision-making on the basis of *in vitro* results (Hartung et al., 2013a).

This review describes the main alternative *in vitro* tests available for determining embryotoxicity and teratogenicity of environmental contaminants and proposes an integrated approach with a step-wise strategy that would allow the assessment of developmental toxicity on the basis of these robust *in vitro*-alternative tests. The proposed integrated approach is outlined in Fig. 2. In this step-wise approach, model complexity and, consequently technical skills and economical costs, can be gradually increased if required. The first level would be to run short (up to 5 days) cellular assays to detect embryotoxicants that exert effects on early differentiation stages. The second level would entail longer (more than 6 days) cellular embryotoxicity tests to search for embryotoxicants that have effects on late differentiation stages. If positive effects are proven at either of these two levels, the environmental contaminant may be considered an embryotoxicant. If negative

Table 2
Main alternative methods available for assessing developmental toxicity.

Embryotoxicity	Teratogenicity
Embryonic Stem cell Test (EST)	Micromass test (MM)
Mouse embryonic stem cell adherent cell differentiation and cytotoxicity (ACDC) test	Whole embryo culture test (WEC)
Assays with human embryonic stem cells	Zebrafish embryonic development test
Rat cerebellar granule cells	