CHAPTER

11

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Mechanism-based models in reproductive and developmental toxicology

David Pamies, Carmen Estevan, Miguel A. Sogorb, and Eugenio Vilanova

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INTRODUCTION

p0010 The term reproductive toxicology is defined as the adverse effect either on fertility of parental generation or on the development of the progeny. The term developmental toxicology is defined as the adverse effects on the developing organism from the moment of conception to the time of sexual maturation and therefore developmental toxicology can be considered as part of reproductive toxicology. The term embryotoxicity is defined as the toxic effects in progeny in the first period of pregnancy between conception and the fetal stage and therefore is included within developmental toxicology and by extension also within reproductive toxicology. Finally, the term teratogenicity is defined as the structural malformations or defects in offspring and after the period of embryogenesis and is considered as a developmental toxicology effect.

p0015 It seems obvious that the complexity of the reproductive process cannot be studied with a single *in vitro* model and therefore it is necessary to split the whole process into certain steps (maturation of gamete, fertilization, implantation, embryogenesis, fetogenesis, etc.). This chapter will be mainly focused on the study of the currently available models for testing developmental toxicity (embryotoxicity and teratogenicity). Other parts of the processes such as infertility, endocrine disruption, mutagenicity of germinal cells, etc. are already covered in other chapters of this book.

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THE NECESSITY OF ALTERNATIVE MODELS FOR TESTING REPRODUCTIVE AND DEVELOPMENTAL TOXICOLOGY

p0020 The OECD has several validated guidelines for *in vivo* studies of reproductive toxicology (covering in the same assay therefore fertility and developmental issues) and for *in vivo* studies of developmental toxicology (covering teratogenicity, since the exposure starts after embryogenesis). These guidelines are discussed in detail in the previous chapter of this book. However, it needs to be mentioned that there are no guidelines for testing only *in vivo* embryotoxicity. This is a relevant gap because a guideline for this purpose would allow the detection of developmental toxins in early stages of development without waiting for teratogenicity.

p0025 Regulations in all developed countries require *in vivo* studies regarding the toxicity to reproduction in order to perform the necessary risk assessment before registration and authorization of the use of chemicals with medium and high volume of production. Regulations also concern the use of a large number of animals with the corresponding ethical, logistical and economic implications. Höfer and coworkers (2004) have estimated the number of animals needed to perform a basic set of assays for testing toxicity to reproduction. This number would include a total of 3,910 vertebrates according to the following scheme: 150 animals (either rats or rabbits) for developmental studies (OECD Guideline 414); 560 animals (rats) for reproduction/developmental toxicity screening test (OECD Guideline 421); and 3,200 animals (rats) for the two-generation reproduction toxicity study (OECD Guideline 416). According to data published by Fleischer (2004), obtained from a survey performed among laboratories in the European Union and Switzerland, the cost of this set of assays would reach €446,000.00. Rovida and Hartung (2009) have estimated that the area of reproductive toxicology would demand 90% of animals and 70% of the economical cost of assays for registration.

p0030 Taking into consideration the figures outlined in the above paragraph it is easy to understand that the use of fast, safe and reliable alternative models for testing reproductive toxicology would be highly appreciated by the industry. These models might be especially relevant for the process of massive high throughput screening performed in the early stages of developing molecules as biocides, cosmetics, food additives, etc. Also, other potential applications of these alternative models would be (Spielman, 2005): (1) to compare the developmental toxicity potential of a new chemical that is only a slight modification of an existing chemical that has

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already been tested *in vivo*; and (2) evaluating compounds for which testing is not routinely performed, usually since the anticipated exposure is very low.

p0035 The next section describes the main alternative models for testing developmental toxicity. These models are divided between validated models (whole-embryo culture test (WEC), micromass test (MM) and embryonic stem cell test (EST)) and those that are not currently validated (although have proven scientific validity) as is the case of zebrafish, frog embryo teratogenesis assay (FETAX), *in silico* models for predicting embryotoxicity, *in vitro* cellular models different from the EST method, and methods using fragments of embryos.

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VALIDATED ALTERNATIVE MODELS FOR TESTING DEVELOPMENTAL TOXICOLOGY

p0040 Between 1996 and 2000 the European Centre for Validation of Alternative Methods (ECVAM) sponsored prevalidation and validation studies of the WEC, MM and EST methods. These three methods reduce, refine and/or replace the use of animals and therefore fit within the philosophy of alternative methods. WEC uses whole rat embryos with 10 days of gestation that are further exposed *in vitro* to the tested chemical during 48 hours, while MM and EST are methods based on the determination of the effects of the chemical on differentiation of cells from two different sources: embryos (in the case of MM) or embryonic stem cells (in the case of EST). These three methods also share a common approach based on obtaining records for their respective endpoints and further analysis of these records with validated statistical functions that allow assignment of the tested chemicals to the following three categories: non-, weakly or strongly embryotoxic. The ECVAM Scientific Advisory Committee has endorsed the scientific validity of the three methods: EST and WEC were considered to be scientifically validated for distinguishing among non-, weak and strong embryotoxins, whereas MM was considered scientifically validated for identifying only strongly embryotoxic chemicals (ESAC, 2002).

Whole-embryo culture test (WEC)

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p0045 The embryotoxicity testing in postimplantation rat or mouse whole-embryo culture (WEC) is intended to identify substances with the capability of inducing malformations resulting in embryotoxicity and it is proposed to be used within the context of OECD Guideline 414 for testing teratogenicity (ECVAM, 2006a).

p0050 The rationale of this study is based on the *in vitro* exposure of embryos during the time where major aspects of organogenesis occur, as in the case of heart development, closure of the neural tube, and the development of ear and eye, branchial bars and limb buds. Therefore, it is assumed that interferences during this period may lead to general retardation of growth and development or to specific malformations.

Basic procedure

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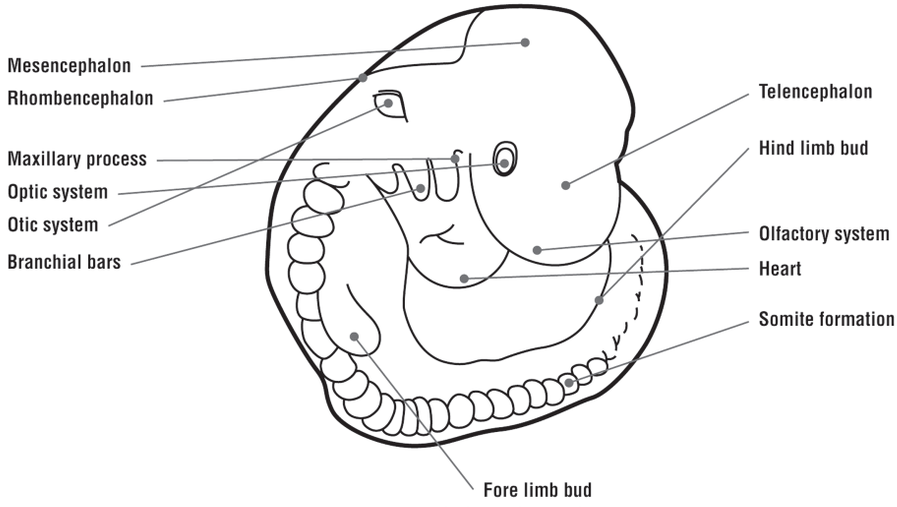
p0055 Rat or mouse embryos are *in vitro* cultured for 48 hours starting on day 9.5 of gestation in the presence of the tested substance. After 48 hours each embryo is transferred to a Petri dish (Figure 11.1) and scored according to the parameters described in Table 11.1. These parameters include records about growth (yolk sac diameter, crown–rump length and head length), function (heartbeat, yolk sac circulation and allantoic circulation), morphology (final minus initial somite number) and malformations (as indicated in Table 11.1 from A through R). The assay is performed in two steps. The first one is a range finding test carried out using three embryos per concentration and using ten-fold concentration intervals. In the second step the highest ineffective concentration and a concentration which results in at least a 50% reduction of control total morphological scores (estimated as is indicated in Table 11.1), as well as two intermediate concentrations, are tested to a total of seven embryos per concentration. The assay is considered valid when it meets the following criteria: (1) a maximum rate of malformed embryos in the control group of 15% (1 out of 7); (2) a positive control of 5-fluoracil (0.03, 0.1, and 0.3 mg(ml)) and a negative control of penicillin G (1 mg/ml) are run together with the main assay.



f0010 **FIGURE 11.1** Morphology of rat embryo.

p0060 The WEC can be considered an alternative model because the number of animals needed is lower than its *in vivo* equivalent OECD Guideline 414 (therefore causing a reduction in the number of needed animals) and refines the animal suffering since the exposure is performed *in vitro*.

s0035 Endpoints of the assay and prediction model

p0065 The results of a prevalidation study performed with six chemicals of well-known *in vivo* embryotoxic potential and four independent laboratories were analyzed in order to create a biostatistically based prediction model (PM) to identify the embryotoxic potential (non-, weakly and strongly embryotoxic) of the tested chemicals.

p0070 Two PMs (PM1 and PM2) were created using linear discriminant analysis (Genschow *et al.*, 2000). PM1 was developed during the prevalidation study and showed poorer results than the EST test (see detailed explanation about EST test in another section of this chapter). Because PM1 took into consideration parameters exclusively focused on differentiation and development but no measures of cytotoxicity, the PM2 was further developed with the aim of improving the performance of PM1 by including cytotoxicity data with 3T3 fibroblast coming from the EST test (Genschow *et al.*, 2002).

p0075 The PMs of the WEC test are displayed in Table 11.2 and include the following endpoints: (1) $IC_{NOEC\ TMS}$, which describes the lowest assayed concentration that has no effect on the total morphological score (TMS) estimated as shown in Table 11.1; (2) $IC_{50\ MAL}$, which is the concentration at which 50% of all tested embryos are malformed; (3) IC_{MAX}, obtained as the lowest assayed concentration at which a maximum malformation rate is obtained; and (4) $IC_{50\ 3T3}$, which corresponds with the concentration which causes 50% of reduction in viability of 3T3 mouse fibroblasts after 10 days of exposure in conditions described for EST assay.

p0080 The analysis of these endpoints allowed the creation of three lineal discrimination functions for each of the PMs. The relationships among these three functions allow for the

t0010 **TABLE 11.1** Parameters to score after exposure of rat or mouse embryos in the WEC assay

Growth parameters		**Malformations (0 for normal/1 for malformed)**
Yolk sac diameter (mm)		Yolk sac vessel defect
Crown–rump length (mm)		Allantois not fused with ectoplacental cone
Head length (mm)		Allantois large size
Functional parameters (1 for normal/0 for abnormal)		Flexion deficient
		Pericardiac sac wide, filled with fluid
Yolk sac circulation		Heart ventrally turned
Allantois circulation		Posterior neuropore open
Heartbeat		Dorsal midline irregular
Somite development		Prosencephalon open
Final somite number		Rhomboencephalon narrow
Final–initial somite number		Cranial neural folds suture line irregular
Morphological scores		Head small and bent backwards
A	Yolk sac blood vessels	Craniofacial appearance abnormal
B	Allantois	Neural tube haemorrhagic
C	Flexion	Rhombencephalon large and transparent
D	Heart	Rhombencephalon narrow
E	Caudal neural tube	Otic vesicles deformed
F	Hind brain	Optic vesicles deformed
G	Mid brain	Branchial bars deformed
H	Fore brain	Maxillary process swollen
J	Otic system	Mandibular processes unapproached
K	Optic system	Mandibular process deformed
L	Olfactory system	Somites small
M	Branchial bars	Somites irregular
N	Maxillary process	Tail kinked
P	Mandibular process	Rail short and thickened
Q	Fore limb	Subcutaneous blisters
R	Hind limb	Haemorrhages
Total Morphological Score (A + B + C + … + R)		Other

Taken from official validated protocol available in the European Centre for Validation of Alternative Methods (ECVAM)

t0015 **TABLE 11.2** Linear discriminant functions for the prediction of embryotoxicity in the three validated methods

(a) Prediction model of the embryonic stem cell test (EST)

$$\text{Function I} = 5.92 \log (IC_{50\ 3T3}) + 3.50 \log (IC_{50\ D3}) - 5.31 \frac{IC_{50\ 3T3} - ID_{50}}{IC_{50\ 3T3}} - 15.7$$

$$\text{Function II} = 3.65 \log (IC_{50\ 3T3}) + 2.39 \log (IC_{50\ D3}) - 2.03 \frac{IC_{50\ 3T3} - ID_{50}}{IC_{50\ 3T3}} - 6.85$$

$$\text{Function III} = -0.125 \log (IC_{50\ 3T3}) - 1.92 \log (IC_{50\ D3}) + 1.50 \frac{IC_{50\ 3T3} - ID_{50}}{IC_{50\ 3T3}} - 2.67$$

(b) Prediction model number 1 of the whole embryonic test (WEC)

$$\text{Function I} = 18.08 \log (IC_{50\ Mal}) - 11.56 \log (IC_{NOEC\ TMS}) - 10.19$$

$$\text{Function II} = 21.55 \log (IC_{50\ Mal}) - 15.31 \log (IC_{NOEC\ TMS}) - 10.65$$

$$\text{Function III} = 8.70 \log (IC_{50\ Mal}) - 8.53 \log (IC_{NOEC\ TMS}) - 2.53$$

(c) Prediction model number 2 of the whole embryonic test (WEC)

$$\text{Function I} = 21 \frac{IC_{50\ 3T3} - IC_{NOEC\ TMS}}{IC_{50\ 3T3}} + 15.37 \log (IC_{max}) - 23.58$$

$$\text{Function II} = 27 \frac{IC_{50\ 3T3} - IC_{NOEC\ TMS}}{IC_{50\ 3T3}} + 17.71 \log (IC_{max}) - 32.37$$

$$\text{Function III} = 9.3 \frac{IC_{50\ 3T3} - IC_{NOEC\ TMS}}{IC_{50\ 3T3}} + 4.21 \log (IC_{max}) - 4.23$$

(d) Prediction model of the micromass test (MM)

$$\text{Function I} = 6.65 \times \log (ID_{50}) - 9.49$$

$$\text{Function II} = 6.16 \times \log (ID_{50}) - 8.29$$

$$\text{Function I} = -1.31 \times \log (ID_{50}) - 1.42$$

Abbreviations: $IC_{50\ 3T3}$ = Concentration that reduces viability of 3T3 cells to 50% after exposure according to the protocol; $IC_{50\ D3}$ = Concentration that reduces viability of D3 cells to 50% after exposure according to the protocol; ID_{50} = Concentration that reduces to 50% the differentiation of either D3 cells to cardiomyocytes (EST) or primary culture of limb bud cells to cartilage (MM) after exposure according to the respective EST and MM protocols; $IC_{50\ Mal}$ = Concentration at which 50% of exposed embryos display malformations; $IC_{NOEC\ TMS}$ = The lowest concentration without observed effect on the total morphological score (see Table 11.1); IC_{max} = The lowest concentration that causes the maximum malformation rate.

classification of the tested chemical as non-, weakly or strongly embryotoxic according to criteria showed in Table 11.3.

Performance of the WEC test

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p0085 After the prevalidation study a blind validation study was boarded by the participating laboratories adding 14 new compounds (thus for a total of 20 compounds) to the list of tested chemicals. Because the test chemicals were assigned to only three categories of embryotoxicity, 33% of correct classifications might be expected by chance. In consideration of this the criteria for evaluating the results of the validation study were established as stated in Table 11.4.

p0090 All strongly embryotoxic chemicals could be identified (precision) and correctly predicted (predictivity) with PM2 (Table 11.4). The predictivity for non-embryotoxic and weakly embryotoxic chemicals ranged between 56 and 76% for both PM1 and PM2, while these same records for precision ranged between 45 and 80% (Table 11.4). Applying the two PMs to the results obtained in the WEC test provided a maximal overall accuracy (the proportion of correct outcomes of the method independently of the category of the tested chemical) of 80% correct prediction of embryotoxic potential *in vivo* (Table 11.4).

p0095 Based on the successful outcome of the validation study the ECVAM Scientific Advisory Committee has endorsed the WEC as a scientifically validated test since it yields reproducible results, the correlation between *in vivo* and *in vitro* data was good and the test proved applicable to testing a diverse group of chemicals of different embryotoxic potentials (ESAC, 2002). Therefore, the WEC test is ready to be considered for regulatory purposes.

t0020 **TABLE 11.3** The embryotoxic potential classification criteria according to prediction models displayed in Table 11.2 for EST, WEC and MM methods

Classification	Requirements
Strong embryotoxicity	Function III > Function II and Function III > Function I
Weak embryotoxicity	Function II > Function III and Function II > Function I
No embryotoxicity	Function I > Function III and Function I > Function II

The micromass test (MM)

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p0100 This method uses rat micromass cultures of limb bud and detects the inhibition of cell differentiation and growth, which are parameters suitable for testing teratogenicity. This is because this method is also intended to be used within the context of OECD Guideline 414 (ECAVM, 2006b).

p0105 This method is based on the capability of the primary culture of limb bud cells of mammalian origin to reproduce cartilage histogenesis, a fundamental step in the morphogenesis of the skeleton, cell proliferation and differentiation, cell to cell communication and cell to extracellular matrix interactions. Therefore, interference in these basic cell developmental functions may result in teratogenic consequences.

Basic procedure

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p0110 Embryos are obtained from Wistar rats on day 14 of gestation and the limb buds (Figure 11.1) are isolated. A primary culture of these cells is generated with tripsin and the cells are next seeded in 96-well plates. The cells are further exposed to the tested compound during 5 days and finally the number of differentiated cells is determined with alcian blue (a cartilage-specific proteoglycan stain). The test is performed in two steps. Initially, a range-finding study is performed using as highest concentration the limit of solubility of the tested chemical (or alternatively a maximum concentration of 1 mg/ml) plus seven additional concentrations separated by a dilution factor of 10. This range-finding experiment allows selection of the relevant concentration range and a final experiment must be further performed using eight different dilutions (with a maximum dilution factor of 1.5). The quality criteria of the experiments require that there must be at least three concentrations within the range of 90 to 10% of control differentiation values and a positive (5-fluoracil) and a negative (penicillin-G) control must be [AQ1]

t0025 **TABLE 11.4** Results of the validation study of EST, MM and WEC protocols

	WEC PM1	WEC PM2	MM	EST
Predictivity for non-embryotoxic (%)	56 (insufficient)	70 (sufficient)	57 (insufficient)	72 (sufficient)
Predictivity for weakly embryotoxic (%)	75 (good)	76 (good)	71 (sufficient)	70 (sufficient)
Predictivity for strongly embryotoxic (%)	79 (good)	100 (excellent)	100 (excellent)	100 (excellent)
Precision for non-embryotoxic (%)	70 (sufficient)	80 (good)	80 (good)	70 (sufficient)
Precision for weakly embryotoxic (%)	45 (insufficient)	65 (sufficient)	60 (insufficient)	83 (good)
Precision for strongly embryotoxic (%)	94 (excellent)	100 (excellent)	69 (sufficient)	81 (good)
Total accuracy (%)	**68 (good)**	**80 (good)**	**70 (sufficient)**	**78 (good)**

Assessment	Rate of correct classifications
By chance	=33%
Insufficient	<65%
Sufficient	≥65%
Good	≥75%
Excellent	≥85%

Taken from Glenschow *et al*. (2002)

included. For each chemical two independent experiments meeting these quality controls are required, although these two experiments do not necessarily have to use exactly the same concentrations.

p0115 As in the case of WEC, the MM also can be considered an alternative model because the number of animals needed is lower than its *in vivo* equivalent OECD Guideline 414 (reduction) and reduces animal suffering since the exposure is performed in primary cultures and not in the whole animal.

s0055 Endpoints of the assay and prediction model

p0120 During the prevalidation study performed with six chemicals a discriminate PM was developed using two endpoints, the cytotoxicity and the inhibition of the differentiation of the MM cultures. A further refinement of the PM determined that the concentration that inhibited 50% of cell differentiation to cartilage (ID_{50}) was enough to discriminate among the three categories of embryotoxic potential on the basis of the three lineal functions displayed in Table 11.2 according to the criteria showed in Table 11.3 (Genschow *et al.*, 2000).

s0060 Performance of the MM test

p0125 The predictivity of MM for strongly embryotoxic chemicals was 100%; however, predictivity was insufficient (only 57%) and good (71%) for non- and weakly embryotoxic, respectively (Table 11.4). The precision of the MM ranged between 60 and 80% for weakly and non-embryotoxic chemicals, being the accuracy of the method (for all chemicals) of 70% (Table 11.4).

p0130 Based on these results the ECVAM Scientific Advisory Committee agreed with the conclusion that the MM test is scientifically validated for identifying strongly embryotoxic chemicals and that it is ready to be considered for regulatory purposes (ESAC, 2002). Therefore, the main difference with the WEC test is that the MM test is suitable for testing only strongly embryotoxic chemicals, while WEC can be used for testing all three categories

s0065 The embryonic stem cell test (EST)

p0135 In this method the embryotoxic potential of chemicals is determined by the evaluation of the inhibition of the differentiation of mouse embryonic stem cells belonging to the D3 line and the inhibition of growth of these D3 cells and also of mouse fibroblast belonging to the 3T3 line. As in the case of WEC and MM the EST test is also proposed to be used within the context of the OECD Guideline 414 for testing developmental toxicity (ECVAM, 2006c).

p0140 Two permanent cell lines are used in the EST test; D3 cells represent embryonic tissues while adult tissues are represented by 3T3 cells. EST is the only validated embryotoxicity test that totally eliminates the use of animals. D3 cells can be maintained in the undifferentiated stage in the presence of the cytokine leukemia inhibition factor. When released from this leukemia inhibition factor the embryonic stem cells form embryonic bodies and differentiate into the major embryonic cell lineages. This test is based on the determination of the inhibition of the differentiation of the embryonic stem cells and on the differences in the sensitivity between embryonic and adult cells against a cytotoxic insult.

Basic procedure for the differentiation assay s0070

p0145 Seven to eight concentrations with a 1.2–3-fold dilution factor covering the relevant range of cytotoxicity must be tested in each experiment. On day 0 a suspension of 37,500 D3 cells/ml is prepared in culture medium (in absence of leukemia inhibition factor and containing the appropriate concentration of the chemical) and 20 µl of this suspension (thus containing 750 D3 cells) is dispensed on the inner side of a 100 mm Petri dish. At least 24 drops per tested concentration must be prepared. The lid is carefully turned into its regular position and put on the top of the Petri dish filled with 5 ml of phosphate buffer saline. These hanging drops are incubated until day 3 when they are gently transferred to a 60 mm Petri dish with 5 ml of culture medium containing freshly prepared tested chemical. On day 5, the embryonic bodies are transferred to a 24-well plate (one embryonic body per well containing 1 ml of fresh medium with the tested chemical in each well). Finally, on day 10 the embryonic bodies should be differentiated into contractile cardiomyocytes and the number of beating embryonic bodies is determined under light microscopy. The assay is acceptable when at least 21 of the 24 control embryonic bodies are beating after the differentiation period; the negative control (1 mg penicillin/ml) has been differentiated in the same proportion as the controls; and a concentration of 5-fluoracil (the positive control) between 48 and 60 ng/ml is able to inhibit 50% of the differentiation. At least two independent experiments meeting these quality criteria must be run to validate the results.

Basic procedure for the cytotoxicity assay s0075

p0150 The cytotoxicity of D3 and 3T3 cells must be initially assayed in a range-finding study covering from the highest soluble concentration plus a series of eight dilutions each with a factor of 10. The main experiment is performed with seven concentrations covering the relevant range of doses determined in the range-finding experiment. The experiment starts seeding 500 D3 or 3T3 cells on 96-well plates with medium without leukemia inhibition factor and with the appropriate concentration of the tested chemical. The seeded cells are incubated during 10 days with changing of medium at the same points that the differentiation test (days 3 and 5). The viability of the exposed cells is tested on day 10 [AQ2] with the MTT (thiazolyl blue formazan) assay. MTT is based on the colorimetric determination of formazan formed in the mitochondria using MTT as substrate. The amount of formed formazan directly relates with the amount of viable cells there is in the medium, which is a reflection of both mitochondrial integrity and the level of functionality of the mitochondrial dehydrogenases (Borenfreud *et al.*, 1988). At least two independent assays might be performed meeting the following quality criteria: (1) concentration of 5-fluoracil (positive control) exhibits a capability to cause 50% of cytotoxicity ranging between 48–86 ng/ml and 120–500 ng/ml for D3 and 3T3 cells, respectively; (2) the negative control (1 mg penicillin-G/ml) does not affect the viability of the cells (neither D3 nor 3T3).

Endpoints of the assay and prediction model

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p0155 Three different endpoints were needed to build the three different discriminating functions shown in Table 11.2. These endpoints are (Genschow *et al.*, 2000): ID_{50} as the concentration with the capability to inhibit 50% of the differentiation of D3 cells into beating cardiomyocytes; and $IC_{50\,D3}$ and $IC_{50\,3T3}$ as the concentrations with the capability to reduce to 50% the viability of D3 and 3T3 cells in MTT assay, respectively. These endpoints allow the discrimination of the embryotoxic potential of the tested chemical according to criteria shown in Table 11.3.

Performance of the EST test

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p0160 The testing of the 20 test chemicals employed in the EST validation study provided 78% accuracy (correct classifications) (Table 11.4) (Genschow *et al.*, 2004). The highest precisions were detected for weak and strong embryotoxic chemicals, which were correctly detected in 83% and 81% of the cases, respectively (Table 11.4). The poorest precision was recorded for detection of non-embryotoxic compounds (70%) (Table 11.4). Finally, the predictivity for strongly embryotoxic chemicals was, as for MM and WEC, 100%, while predictivity for non- and weakly embryotoxic compounds was similar (70 and 72%, respectively) (Table 11.4).

p0165 The ECVAM Scientific Advisory Committee agreed that EST is a scientifically validated test applicable to testing a diverse group of chemicals of different embryotoxic potentials (ESAC, 2002). EST is also ready to be considered for regulatory purposes since the results obtained in the validation study were highly reproducible and the correlations between *in vivo* and *in vitro* data were good (ESAC, 2002).

Improvements for the EST test

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p0170 Various studies have proposed improvements for the performance of the EST method. Some of these proposals are: (1) the use of *in vitro* data combined with pharmacokinetic studies (Verwei *et al.*, 2006); (2) the optimization of the culture protocols for D3 cells (De Smedt *et al.*, 2008; Marx-Stoelting *et al.*, 2009); (3) the use of automated image processing systems (Paparella *et al.*, 2002; Peters *et al.*, 2008); (4) the quantification of the differentiation by the expression of the actin and heavy myosin chain genes (Seiler *et al.*, 2004); (5) to split cellular differentiation from cellular proliferation (van Dartel *et al.*, 2009); (6) the quantification of endothelial cell-induced differentiation by means of specific marker genes of this particular lineage (Festag *et al.*, 2007); (7) the quantification of cell differentiation using flux cytometry (Buesen *et al.*, 2009); (8) the substitution of D3 cells for other mouse embryonic stem cells (Marx-Stoelting *et al.*, 2009); (9) substitution of MTT test for other viability tests (Marx-Stoelting *et al.*, 2009); (10) generation of embryonic bodies either by horizontal shaker or in suspension instead of hanging drops (Marx-Stoelting *et al.*, 2009); (11) to reduce to 2 (yes/no) the embryotoxicity categories (Marx-Stoelting *et al.*, 2009); (12) to employ a protocol of sequential differentiation of embryonic stem cells starting in the heart and passing through neurons, bone and finishing in cartilage (Marx-Stoelting *et al.*, 2009); (13) use of transcriptomics (van Dartel *et al.*, 2010a); (14) a reduction of the exposure time combined with the use of transcriptomics (van Dartel *et al.*, 2010b). Finally, the adaptation of the protocol to human embryonic stem cell lines has been also proposed (Adler *et al.*, 2008; Stummann *et al.*, 2009).

Main advantages and disadvantages of the three validated methods

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p0175 Table 11.5 displays a comparison among the main characteristics of EST, MM and WEC. The two main limitations common to all three methods are the absence of metabolic competence and their incapability to detect teratogenic chemicals with mechanisms acting beyond the initial embryo differentiation steps. The three methods require different levels of technical difficulties, being WEC, the most demanding method, since it manages the highest number of endpoints and requires staff trained in the identification and quantification of the specific embryo malformations displayed in Table 11.1. These technical requirements make the WEC test the least appropriate for massive high throughput screening processes. EST is the method with an intermediate level of technical difficulty because it requires two endpoints and cultures of two different cell lines, while MM requires a single endpoint with only one cell primary culture. It is also remarkable that EST is the only method that does not need the sacrifice of pregnant animals, and WEC is the only method which yields information about the morphological alterations caused by the teratogen. [AQ3]

Optimization of the predictivity of the validated tests

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p0180 ECVAM has issued certain recommendations in order to overcome some of the above listed limitations. These recommendations include (Spielman *et al.*, 2006):

o0010 1. To develop a metabolically competent *in vitro* system to be integrated into the three methods;

o0015 2. To integrate protocols for the differentiation of ESC into specific lineages because the current approach

t0030 **TABLE 11.5** Characteristics of the main embryotoxicity–teratogenicity assays

Assay	Biological tissue	Type of assay	Time of exposure (days)	Technical difficulty	Biotransformation	Animal (mammals) sacrifice	Morphological alterations determination	Throughput capability
EST	Mouse	Cellular assay	10	Medium	NO	NO	NO	High
WEC	Mouse or rat	Whole embryo	2	High	NO	YES	YES	Poor
MM	Rats	Fragment of embryo (cellular assay)	5	Low	NO	YES	NO	Very high
FETAX	Frog	Whole embryo	4	Medium	YES	NO	YES	Medium
Zebrafish	Fish	Whole embryo	2–5	Medium	YES	NO	YES	Medium

(differentiation to beating cardiomyocytes coming from the mesoderm) might not detect embryotoxicity exerted in other cellular lineages as endoderm or ectoderm;

o0020 3. To develop additional PM for specific purposes or compound classes;

o0025 4. More quantitative endpoints for the EST as the use of tissue-specific gene expression markers, immunohistochemical methods or flow cytometry (these approaches would improve the quantification of the alterations in the differentiation regarding the current method based on the simple examination of the beating cardiomyocytes);

[AQ4]

o0030 5. To create mathematic and pharmacokinetics models to correlate the effective concentrations of test chemicals in the *in vitro* test with the effective concentrations in maternal serum; and

o0035 6. To develop and integrate an *in vitro* model for considering the role of the placenta.

s0105 Possible future uses for validated embryotoxicity test

p0215 On the one hand, the successful improvement of the methods could allow them to be used for regulatory purposes and then a positive result would allow a chemical to be classified as toxic to reproduction without the necessity of animal assays. On the other hand, a negative result with the same method would not rule out the necessity of assays with animals, but would allow these assays to be more directed and therefore would reduce the number of animals employed in the assay.

s0110 NON-VALIDATED ALTERNATIVE MODELS FOR TESTING DEVELOPMENTAL TOXICOLOGY

s0115 Frog Embryo Teratogenesis Assay (FETAX)

p0220 Organogenesis is a process highly conserved in the phylogenetic scale and therefore amphibians can be used as models for testing this process in mammalians. In addition to that, amphibian embryos are very sensitive to chemicals, easily handled in the laboratory and the availability of embryos is not seasonal because ovulation can be induced with chorionic gonadotropin. All these reasons make frog embryos ideal models for testing alterations in the development of vertebrates. Specifically, the first 96 hours of embryonic development in *Xenopus laevis* parallel many of the major processes of human organogenesis (NICEATM, 2000). Nevertheless, other authors suggest that other species of *Xenopus* as *Xenopus tropicalis* can also be effectively used (Fort *et al.*, 2004).

p0225 The endpoints for the FETAX assay are (NICEATM, 2000): (1) mortality, expressed as the concentration that causes 50% mortality (LC_{50}); (2) malformations, evaluated and recorded according to the *Atlas of Abnormalities* (Bantle *et al.*, 1998) and expressed as the concentration that causes malformations in 50% of embryos (EC_{50}); (3) grown, estimated as the distance between head and tail; (4) teratogenic index, estimated as the ratio between LC_{50} and EC_{50}; and (5) minimum concentration to inhibit growth. A chemical ranked with a teratogenic index greater than 1.5 is an ideal candidate to be teratogenic in the absence of significant mortality. In the same way, teratogenic hazard is considered to be present when either growth is significantly inhibited at concentrations below 30% of LC_{50} or when the ratio between minimum concentration to inhibit growth and LC_{50} is lower than 0.30.

p0230 Seven different concentrations must be assessed in each assay. For each dose group, two dishes containing 25 embryos in 10 ml of test solution are used. Control condition is assayed with four dishes of 25 embryos. The exposure takes place at 24 ± 2°C during 96 h (or until 90% of control embryos reach stage 46 of development). Frog embryos lack metabolic competence and in order to cover this gap the assays are run in two conditions, in absence and in presence of a metabolic activation system (rat liver microsomes and NADPH-generating system). The positive control without metabolic activation is run with 6-aminonicotinamide, which should yield a teratogenic index of around 446; while the positive control with metabolic activation is run with cyclophosphamide, which should cause 100% mortality of embryos after 96 h of exposure to 4 mg/ml. One of the main quality requirements to consider in a valid assay is that mortality and mean of malformations in control embryos should be both lower than 10%.

p0235 Several studies of validation for FETAX assays have been run with promising results (Bantle *et al.*, 1996, 1999; Fort *et al.*, 1998, 2000). Nevertheless, the US Environmental Protection Agency (US EPA) asked the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) to evaluate the FETAX test. In 2000, an expert scientific panel concluded that FETAX is not sufficiently validated or optimized for regulatory use. Nevertheless and despite this consideration, FETAX is an assay of proven scientific validity and is widely used for identification of hazards to human and environmental health (for recent examples see Bacchetta *et al.*, 2008; Longo *et al.*, 2008; Bosisio *et al.*, 2009).

Developmental toxicity assays with zebrafish s0120

p0240 The teleost zebrafish (*Danio rerio*) is a well-known organism frequently used in general and developmental toxicology (Froehlicher *et al.*, 2009), neurotoxicology (Anichtchik *et al.*, 2004; Bretaud *et al.*, 2004; Linney *et al.*, 2004) and also in other basic sciences such as embryology (Ticho *et al.*, 1996). The small size, cheap maintenance, easy conditions for breeding, high nativity rate (a single female can lay up to 400 eggs per week (Laale, 1977)), the spawn throughout the year under laboratory conditions, the transparency of its embryos and the fact they develop outside the mother make zebrafish an excellent model for research (Yang *et al.*, 2009).

p0245 The zebrafish was widely introduced into laboratories as a model to study development (Maves and Kimmel, 2005) and neurobiology (Froehlicher *et al.*, 2009) between the end of the 1980s and the start of the 1990s. In a short period of time, the increase of the genetic techniques along with the advantages of this model placed the zebrafish in an ideal position as a model organism for drug target discovery, target validation, drug-finding strategies and toxicological studies (Langheinrich, 2003).

p0250 The stages of embryonic development of zebrafish were described in detail more than 30 years ago. These stages highlight the changes of the major developmental processes

that occur faster (during the first 3 days after fertilization) in zebrafish than in mammalians. The knowledge regarding zebrafish embryonic development stages together with the availability of its genetic sequence and of a large number of mutants and transgenic lines provides this model with a number of experimental possibilities (Yang *et al.*, 2009).

Zebrafish embryonic development

s0125

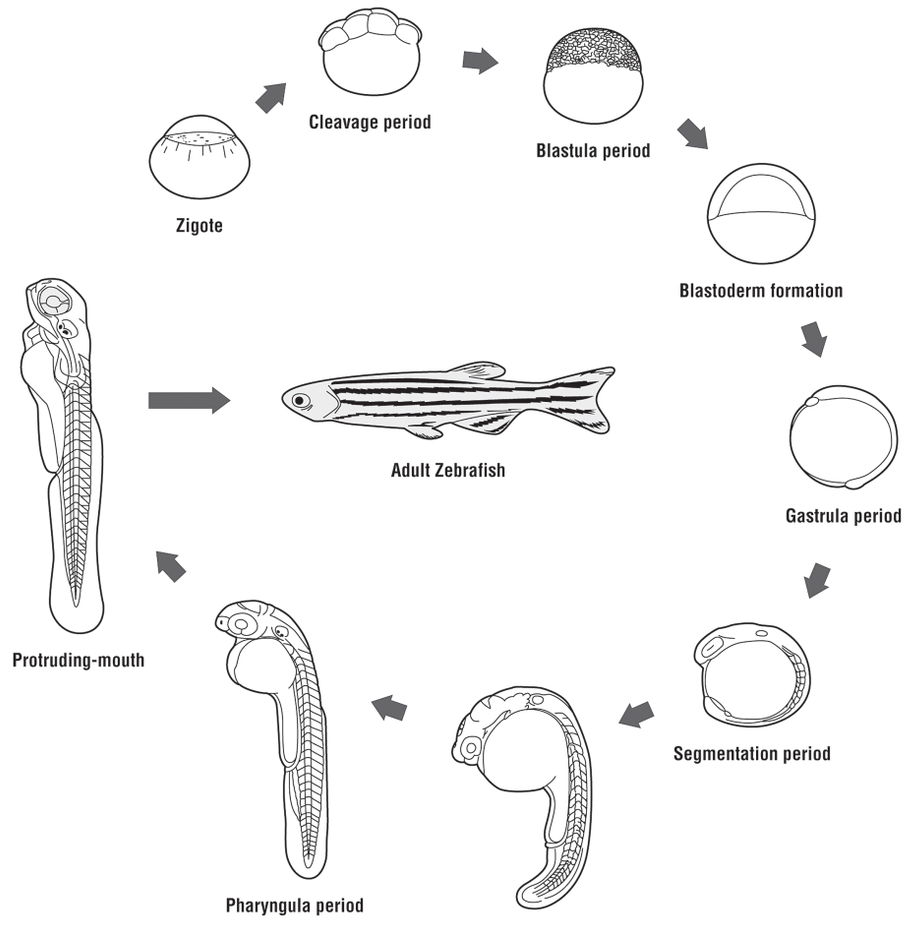
p0255 The development of the embryo in zebrafish is particularly fast. The stages of embryonic development of *Danio rerio* are divided in seven periods (Figure 11.2): the zygote, cleavage, blastula, gastrula, segmentation, pharyngula and hatching periods. The zygote is formed immediately after union of female with male gametes. Cleavage is produced between 45 minutes and 2 hours and consists of a series of mitotic cell differentiation that produces the blastula. The formation of the blastula is produced between 2 and 4 hours and is a hollow structure consisting of a single layer of cells. Gastrula is produced around 10 hours after fertilization and consists of the migration of the cells forming different structures that result in the formation of the three primary germ layers, ectoderm, endoderm and mesoderm. Segmentation is a morphogenetic process where the somites develop and start to be patent the rudiments of the primary organs, the tail [AQ5] bud becomes more prominent and the embryo of zebrafish elongates. The formation of pharyngula starts at 24 hours after fertilization and takes another 24 hours. In this stage all vertebrate embryos show important similitude. However, at this moment the notochord and post-anal tail are developed, along with rapid cerebellar morphogenesis of the metencephalon. Hatching is a period between 48 and 72 hours after fertilization and consists of the formation of the primary organ system, rudiments of the pectoral fins, the jaws and the gills (Kimmel *et al.*, 1995).

p0260 The effect produced for the exposition of different chemicals or drugs can be divided into six different phenomena (angiogenesis, hemostasis, apoptosis and proliferation, lipid metabolism, inflammation and neural tolerance) (Langheinrich, 2003).

Advantages of zebrafish endpoints

s0130

p0265 Zebrafish eggs have the property to remain transparent until 72 hours after fertilization; this allows a better study of embryonic development until the moment when the tissues become denser and pigmentation starts. There are techniques that eliminate some of this pigment, for example use of phenylthiourea or beaching after fixation (Hill *et al.*, 2005). Another major advantage of zebrafish embryos is that they have very high survivability, and are able to survive long periods despite their lack of certain organs, severe



f0015

FIGURE 11.2 Embryological stages of zebrafish.

dysfunction or some other kind of abnormality, which allows embryologic changes to be observed at very high concentrations without death of the embryo. Another advantage of this model is that it has been demonstrated that zebrafish possess more than 80% of orthologs of human drug targets (Gunnarsson *et al.*, 2008).

s0135 *Dario rerio* embryotoxicity test (DarT)

p0270 DarT is based on the study of the effects on embryos as a consequence of their exposure to the tested chemical for 48 h. DartT is considered an *in vitro* test and is accepted as an alternative method to animal experimentation. Twenty embryos per concentration are incubated during 48 hours after fertilization in a 24-well plate with a 12 hour light–dark cycle. After 48 hours of exposure different parameters are analyzed: size of the eggs, position of the eye and the sacculi/otoliths, pigmentation, the tail not detached and the frequency of spontaneous movements (Busquet *et al.*, 2008). A positive control of 3,4-dichloroaniline is run in parallel with the test compound since the effect of this compound on embryo zebrafish development is well known (Nagel *et al.*, 1991).

s0140 Variants about the DarT

p0275 Certain studies show that teratogenic effects are due to the biotransformation of the chemical in the mother instead of the parental chemical itself (Fantel, 1982; Webster *et al.*, 1997). Therefore, the use of a metabolic activation system, such as S9-mix, microsomes, hepatocytes, etc., has been proposed coupled with whole embryo systems (Fantel, 1982). Certain studies try to employ this exogenous metabolic activation with DarT (Busquet *et al.*, 2008; Weigt *et al.*, 2008).

p0280 One different approach to DarT is called Gene-*Dar*T (gene expression *Danio rerio* embryo test). This method allows the identification of the teratogenic mechanisms through the analysis of changes in expression of different genes using a 14k zebrafish oligonucleotide microarray.

p0285 The model MolDart uses the detection of changes in the expression of specific target genes after 120 hours of exposure (Liedtke *et al.*, 2008). This test system uses developing zebrafish and detects changes in mRNA abundance of selected target genes after exposure for 5 days. The aim of this test system is to allow the detection of multiple effects using biomarker analysis. Feasibility of this assay for detection of estrogenic effects by vitellogenin 1 mRNA induction has been demonstrated (Muncke and Eggen, 2006; Muncke *et al.*, 2007).

p0290 The mechanosensory lateral line zebrafish test is normally used in neurotoxicity and yields a very clear idea about the effects of the exposure to chemicals in embryonic development. This method consists of the study of mechanoreceptors found in an interconnected network between head and body. In recent years a large number of genes related to these sensory cells have been reported (Li *et al.*, 2010), which allows the early detection of dysfunctions and problems using molecular approaches. This method may serve as a test for detecting chemicals with effects in the development of neurosensory function, and detection of variations in gene expression can further be used to discern different mechanisms of action of toxic compounds.

Validated versus non-validated models: a comparison s0145

Table 11.5 displays a comparison among the main characteristics of the three validated models (EST, MM and WEC) and non-validated models (FETAX and zebrafish). FETAX and zebrafish are methods that use, as is the case with WEC, a whole embryo and therefore also both allow the identification of the specific malformation caused by exposure to the tested chemical. However, animal models used in FETAX and zebrafish are not mammals and, as in the case of EST, these methods can be considered to suppress the use of superior animals. In close and inverse relationship with the complexity of the animal model is the fact that the number of endpoints to analyze and score (and consequently also the technical difficulty) is lower in FETAX and zebrafish assays than in WEC. FETAX and zebrafish include the possibility of being coupled with an exogenous metabolization source, which is not considered in EST, MM and WEC. p0295

Developmental toxicity assays with cell lines s0150

The cell lines are easy to manipulate in the laboratory, can be stored for long periods of time, and the economical costs of assays performed with cells are lower than those performed with animals. This favors the possibility of developing a battery of tests with different cell lines to mimic the different stages of vertebrate development and testing the effect of chemicals in each of these stages. p0300

Developmental toxicity assays with stem cells s0155

Developmental toxicity assays with stem cells display a prominent position within the available battery of cellular assays since some studies have demonstrated that stem cells can be used to illuminate the processes underlying organogenesis, as has been shown in the case of the heart (Miller *et al.*, 2008). Therefore, the study of the interferences in stem cell differentiation caused by chemicals can be used to detect potential developmental toxicants. An additional advantage of these models is that the genetic molecular approaches allow for an exhaustive analysis of which genes are affected by each substance and subsequently research into the mechanisms of action underlying the teratogenic or embryotoxic effects caused by the assessed chemical. p0305

The Adherent Cell Differentiation and Cytotoxicity (ACDC) assay is a test that establishes a model system that would evaluate the chemical effect using a single cell culture (instead of two as the EST) in order to improve the feasibilities for throughput assays (Barrier *et al.*, 2010). ACDC assay uses quantitative markers for differentiation degree and for cell proliferation. In this assay, pluripotent J1 mouse embryonic stem cells are plated in a 96 multiwell plate and further cultured in differentiation medium for 9 days. After that, each well is assessed for cell number and differentiation to cardiomyocytes (using quantitative in-cell Western analysis for myosin heavy chain protein normalized with cell number). This method has already proved its suitability testing the effects of haloacetic acids and their major metabolites (Jeffay *et al.*, 2010). p0310

p0315 The most developed method for testing embryotoxicity using stem cells is the EST method, which was presented and discussed in detail together with a number of proposals for improving its performance elsewhere in this chapter and therefore is not further discussed here.

s0160 Developmental toxicity assays with other (non-stem) cell lines

p0320 Other cell lines have been used to assess the effect of chemicals on development. Cell lines such as the embryonic carcinoma cells have been used for detecting chemicals that affect embryonic development of specific processes such as neural tube development (Jergil *et al.*, 2009). Other lines such as mouse fibroblastoid L929 have also been used for detecting changes in morphology and proliferation (Walmod *et al.*, 2004).

s0165 Developmental toxicity assays with embryo fragments

p0325 The use of whole embryos has been already commented on (see sections devoted to the use of rat (WEC), frog (FETAX) and zebrafish (DarT) embryos for testing developmental toxicity) in former sections of this chapter. Another approach to the use of embryos is the use of only certain parts of these embryos. The main inconvenience of these methods is that they do not totally avoid the use of animals and that the technical skills needed for staff involved in the assys should be higher than for methods employing cell cultures. Various *in vitro* systems have been developed using parts of embryos, as is the case of cells derived from embryo rodent midbrain and limb buds (see MM method in another section) (Cicurel and Schmid, 1988; Flint and Orton, 1984). Other methods related to the development of fetal maxillary have shown good capacity to detect changes in normal development of the rodent maxillary fetus region cultured *in vitro* (Kosazuma *et al.*, 2004; Mino *et al.*, 1994; Shiota *et al.*, 1990). Although most studies on this topic are performed on rats, there are other approaches in other animals, such as rabbits (Carney *et al.*, 2008) and mice (Hunter *et al.*, 2006).

s0170 *In silico* methods for testing developmental toxicity

p0330 The complexity of the reproductive process makes delicate the development of reliable *in silico* methods for predicting [AQ6] toxicity to development. Despite these considerations, several methods based on mathematical approaches have been proposed as alternatives to animal experimentation for testing developmental toxicity (Hewitt *et al.*, 2009).

p0335 With exception of quantitative structure activity relationships (QSAR) for the variables related to endocrine disruption, and in particular with the estrogen and androgen receptor (Cronin and Worth, 2008), the number of available *in silico* methods for testing toxicity to development is lower than for testing other areas of toxicology. There are a number of reasons for the lack of progress in the development of QSAR for reproductive and developmental toxicology. These include: (1) a perceived lack of high quality toxicity data needed for modeling; (2) the lack of knowledge of modes and mechanisms of action that is required for modeling; (3) the appreciation that reproductive toxicity is a composite effect comprising a number of endpoints, some of them with very specific mechanisms; (4) a perceived difficulty in modeling reproductive toxicology due to a combination of the previous three points; and (5) the QSAR and modeling community has possibly not viewed reproductive toxicity as an area of concern or interest because there are no readily available databases for modeling such as there are in other areas of human and environmental toxicology (Cronin and Worth, 2008). In the next paragraphs we summarize some of the available *in silico* methods for predicting toxicity to reproduction.

The CAESAR developmental toxicity model s0175

p0340 The CAESAR (Computer Assisted Evaluation of industrial chemical Substances According to Regulations) developmental toxicity model is based on the next four points: skin sensitization, mutagenicity, carcinogenicity and toxicity to development. The developmental toxicity CAESAR model uses a QSAR model which includes 292 substances classified according to risk factors for the FDA with 13 different descriptors (Benfenati *et al.*, 2009; Kirchmair *et al.*, 2007; Novic and Vracko, 2010). This model is available in a Java-based web application found at http://www.caesar-project.eu

Super-endpoint reproductive toxicity s0180

p0345 This is the set of knowledge generated for over 15 years by numerous scientists working on this project (Marchant *et al.*, 2008; White *et al.*, 2003). This system is designed to predict carcinogenicity, mutagenicity, genotoxicity, skin sensitization, teratogenicity, irritation, respiratory sensitization, hepatotoxicity, chromosome damage and ocular toxicity. This predictive system is based on the analysis of the molecular structures of substances and uses a series of algorithms to correlate the structure and the hypothetical mechanism of action of each substance to study. More information about this system is available at https://www.lhasalimited.org/derek/general_information

Toxmatch s0185

p0350 Toxmatch is a model designed to find similarities between substances according to chemical structure, and is based on the codification of the substances according to a series of indices of similarity. Some of the parameters used by Toxmatch are the octanol–water partition coefficient, molecular weight, ionization potential, maximum diameter, minimum diameter, molecular surface area, etc. Toxmatch has been developed by the European Commission and is available free at http://ecb.jrc.ec.europa.eu/qsar/qsar-tools/index.php?c=TOXMATCH

COREPA s0190

p0355 COREPA software has been developed and commercially distributed by the Laboratory of Mathematical Chemistry of the Bourgas University (Bulgaria). The discrimination parameters used by this method are placed in groups that are

considered to have endpoints or similar activities. A detailed Bayesian tree is further used to classify the substances according to their toxic potential (Mekenyan *et al.*, 2003; Serafimova *et al.*, 2007).

s0195 CONCLUDING REMARKS AND FUTURE DIRECTIONS

p0360 Embryonic development is a very complex process which includes a number of coordinated complex processes in several stages. The alteration of whatever of these processes due to the action of chemicals might potentially suppose an [AQ7] embryotoxic–teratogenic effect. Due to the complexity of the embryonic development the whole process cannot be covered with a single alternative *in vitro* model and therefore the toxicity to development must be studied with a battery of assays covering each of the stages of embryonic development.

p0365 To date only three *in vitro* methods (MM, EST and WEC) have been validated by an international agency (ECVAM) in order to be used for testing the embryotoxicity potential of chemicals, although other models such as FETAX and zebrafish have also proved their validity for this purpose. Methods based on the employment of embryos allow the specific malformation expected after exposure to the chemical to be determined, while methods based on cellular systems are more relevant in order to determine the mechanism underlying the adverse observed effect and still display a wide field for improving their prediction capability. *In silico* methods for testing developmental toxicity need further development and improvement although the information obtained through these methods might be used to support other information obtained using embryos or cellular systems.

p0370 In conclusion, the analysis and integration of all information collected with this battery of embryotoxicity–teratogenicity assays might be very relevant for risk assessment of chemicals and for their classification and labeling with a strong reduction and refinement in the number of vertebrate animals employed for these purposes in the corresponding *in vivo* assays, although strong efforts are still needed to improve the prediction capability of these testing models.

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