Reproductive Toxicology 28 (2009) 308–320

Contents lists available at ScienceDirect

Reproductive Toxicology

journal homepage: www.elsevier.com/locate/reprotox

# Development of a screening assay to identify teratogenic and embryotoxic chemicals using the zebrafish embryo

Ingrid W.T. Selderslaghs[a,b,*], An R. Van Rompay[a], Wim De Coen[b], Hilda E. Witters[a]

[a] VITO NV, Flemish Institute for Technological Research, Unit of Environmental Risk and Health, Toxicology, Boeretang 200, 2400 Mol, Belgium
[b] UA, University of Antwerp, Department of Biology, Laboratory for Ecophysiology, Biochemistry and Toxicology, Groenenborgerlaan 171, 2020 Antwerp, Belgium

ARTICLE INFO

*Article history:*
Received 28 January 2009
Received in revised form 7 April 2009
Accepted 6 May 2009
Available online 15 May 2009

*Keywords:*
Zebrafish embryo
Screening
Chemicals
Teratogenicity
Embryotoxicity

ABSTRACT

We developed and optimized a screening procedure, in which zebrafish embryos were explored as a model for the evaluation of the specific embryotoxic and teratogenic potential of chemicals. A selection of known positive (retinoic acid, valproic acid, caffeine, lithium chloride) and negative (glucose, saccharin) compounds for developmental toxicity were used to evaluate this method. We exposed embryos and evaluated embryotoxicity and morphological characteristics of the embryos at 24, 48, 72 and 144 h post fertilization. After evaluation of the induced effects, concentration–response curves were created for both embryotoxicity and teratogenic effects. Values for teratogenic indices (TI) were calculated as the ratio $LC_{50}/EC_{50}$. The results obtained were compared to existing data from studies with laboratory animals and humans. We demonstrated that our classification of the compounds, based on TI values, allows to distinguish teratogens from non-teratogens and supports the application of zebrafish embryos as an alternative method for developmental toxicity studies to predict effects in mammals.

© 2009 Elsevier Inc. All rights reserved.

## 1. Introduction

Embryogenesis is highly sensitive to toxicant exposure, yet many chemicals currently registered for commercial use lack comprehensive safety data [1]. Developmental toxicity testing according to current international guidelines include a prenatal developmental toxicity study (OECD Test Guideline TG 414) [2], a two-generation reproduction toxicity study (OECD Test Guideline TG 416) [3], a reproductive/developmental toxicity screening test (OECD Test Guideline TG 421) [4] and a repeated dose toxicity study combined with the reproductive/developmental toxicity screening test (OECD Test Guideline TG 422) [5]. These *in vivo* test methods, which involve exposure of pregnant animals and subsequent assessment of toxic effects in dams and their fetuses, require large numbers of animals and are time-consuming and expensive [6].

Recent estimations by the European Chemicals Bureau show that two-generation reproductive toxicity studies (38% of animals, 30% of resources) and developmental toxicity studies (23% of animals, 24% of resources) will use by far the most animals and resources within REACH (Registration, Evaluation and Authorization of Chemicals), which is aimed at completing a minimal toxicity database for thousands of existing chemicals during the coming decade [7]. Considering the number of compounds that need testing, novel methods are required to expedite reliable hazard identification and reduce ethical concerns which accompany the fore mentioned animal studies [1]. In the field of developmental toxicity testing, a wealth of alternative methods have been developed over the last two decades which vary greatly in complexity and biological endpoints employed [6].

Examples of alternative methods for testing developmental activity of chemicals include the embryonal stem cell test (EST) [8,9], the mammalian micromass (MM) test [10,11], a cell culture based assay [12] and the whole-embryo culture (WEC) test [13]. These assays are useful for screening putative toxicants but do not necessarily incorporate the range of complexity of mammalian development and chemical susceptibility. Ideally, one would supplement *in vitro* testing with *in vivo* assays to provide a more complete and relevant assessment of embryotoxic and teratogenic potential following developmental exposure to a given chemical [1]. Therefore, alternative methods which use non-mammalian species or invertebrates could be valuable for testing because, as whole organisms, they more fully represent the complexity of early development. Candidate species include chicken [14], fruit flies [15], fish [16] and frogs [17] and others.

The zebrafish, *Danio rerio*, offers several advantages for toxicity testing including economic husbandry requirements, high fecundity and rapid *ex utero* development [18]. Zebrafish development has been well characterized [19]. The eggs remain transparent from fertilization up to and beyond pharyngulation when the tissues become dense and pigmentation is initiated. This allows

* Corresponding author at: VITO NV, Flemish Institute for Technological Research, Unit of Environmental Risk and Health, Toxicology, Boeretang 200, 2400 Mol, Belgium. Tel.: +32 14 335261; fax: +32 14 582657.
*E-mail address:* ingrid.selderslaghs@vito.be (I.W.T. Selderslaghs).

0890-6238/$ – see front matter © 2009 Elsevier Inc. All rights reserved.
doi:10.1016/j.reprotox.2009.05.004

unobstructed observation of the main morphological changes during earlier developmental stages. Furthermore, zebrafish embryos that are malformed, lack organs, or display organ dysfunction can usually survive well beyond the time at which those organs normally start to function in healthy individuals [18]. In addition, fish are sensitive to chemical exposure during early development [20]. These characteristics make the zebrafish an attractive candidate for screening of toxicants and the elucidation of mechanisms thereof [18].

Zebrafish has already been used as a model organism in numerous studies that assess toxicity endpoints of compounds or their mechanisms of action [21–25]. In addition to all the described advantages, the zebrafish is also listed as a recommended test species in the "Fish, Early-life Stage Toxicity Test" (OECD Test Guideline TG 210) [26] and the "Fish, Short-term Toxicity Test on Embryo and Sac-fry Stages" (OECD Test Guideline TG 212) [27] for the determination of lethal and sub-lethal effects of chemicals.

In this study, we investigated the potential of zebrafish embryos and larvae as candidates for developmental toxicity screens for an extended period of 144 h to cover more of the essential developmental stages. Other studies performed are short-term [26,28,29] and limited to specific mechanism, e.g. angiogenesis [22], heart development [30], and cell cycle inhibition [31]. First, we developed and optimized a screening procedure for the evaluation of the embryotoxic (lethal for embryo) and teratogenic potential of chemicals. Second, we employed this method to evaluate a number of positive and negative compounds for developmental toxicity and compared the results to available zebrafish, mammalian and human data. It was the final purpose to assess whether this test with zebrafish embryo can be integrated in screening programs for the hazard identification of chemicals and support the 3Rs (refinement, reduction, replacement) principle for animal testing [32].

## 2. Materials and methods

### 2.1. Test animals

Adult, wild type, zebrafish were obtained from a commercial supplier (Aquaria Antwerp, Aartselaar, Belgium) and kept at the VITO laboratory facility at least 3 weeks prior to the first intended spawning. The adult fish were maintained in large 60–70 l aquaria with a maximum density of 1 g fish/l tap water at $25 \pm 2\,^{\circ}C$ with a constant light–dark (14–10 h) cycle. The water was continuous aerated and renewal of the water occurred in a semi-static manner. Fish were regularly provided with a varied diet consisting of commercial dry flake food, young daphnids and live brine shrimp. The health condition of the fish was checked regularly (daily except weekends). VITO acquired the approval of the animal use protocol (approval nr. 06-004) from the local ethical committee.

### 2.2. Collection of eggs

Prior to spawning, males and females were housed separately for a minimum of 5 days. The day before eggs were required, males and females were placed in breeding tanks with a 6:4 male–female ratio. The breeding tanks were equipped with a spawning tray, which consists of a fine net with an appropriate mesh size for eggs to fall through, close to the bottom of the tank. The fish were left undisturbed overnight and eggs could be collected 1 h after the light had been turned on the next morning. Eggs were rinsed in 0.0002% methylene blue (CAS #7220-79-3, Sigma–Aldrich) diluted in medium (see below) and placed into large Petri dishes.

### 2.3. Chemicals and test media

Fish water, similar to the reconstituted water described in OECD 203, annex 2 (measured ranges; pH 7.5–8.0, conductivity 632–676 $\mu S/cm^2$, hardness 217–235 mg/l $CaCO_3$, oxygen 92–98%) was used as the medium for all solutions during the experiments. The pH was checked for all solutions and adjusted to 6.8–8 when necessary by adding sodium hydroxide or hydrogen chloride solution (stock solutions 1 M) and oxygen levels of the solutions were always higher than 80%. We selected four chemicals known to be teratogenic for mammalians, all-trans retinoic acid (CAS #302-79-4, Sigma–Aldrich), valproic acid sodium salt (CAS #1069-66-5, Sigma–Aldrich), caffeine (CAS #58-08-2, PharmInnova) and lithium chloride (CAS #7447-41-8, Sigma–Aldrich), and as negative compounds, glucose (CAS #50-99-7, Merck) and saccharin (CAS #81-07-2, Sigma–Aldrich). For retinoic acid, valproic acid, caffeine and lithium chloride, the concentration range was determined in preliminary experiments. Presence of a 0 and 100% effect level (for both malformation and mortality) was used as the criterion for the selection of the concentration range. Concentration ranges for glucose and saccharin were chosen arbitrarily, starting from a highest level (55 mM).

### 2.4. Optimization of test conditions

Initially, embryo development was observed in fish water and compared to observations of normal embryonic development [19] in embryo medium [33], in order to make sure that fish water could be used as a medium which allows normal development of the embryos up to 144 h post fertilization (hpf). As the exposure period in our teratogenic assay was extended to 144 h to include more developmental stages, issues with respect to test recipient and safe levels of solvent were investigated. For high-throughput screening, several multi-well plates, including 24-, 48- and 96-well plates, were used as recipients and filled with 2, 1 and 0.2 ml fish water per well respectively, in order to determine the optimal conditions for future exposure studies. To compare the different multi-well plates, morphological characteristics with special emphasis on the presence of skeletal deformities of control zebrafish larvae were evaluated after hatching (55, 72 and 144 hpf). Finally, the effects of the selected solvent, DMSO, on normal development were characterized to determine the no observed effect concentration (NOEC). Therefore, DMSO was added to fish water and embryos were placed in diluted DMSO solutions varying from 0.014 to 0.56 mM, corresponding to 0.1–4% (v/v), within 2 hpf in 24-well plates, and observed up to 144 hpf. In this way a safe concentration of DMSO to study development could be selected and used as a final solvent concentration. The results of preliminary tests should be incorporated in the development of the standard protocol for the evaluation of the embryotoxic and teratogenic potency of chemicals.

### 2.5. Testing of chemicals

For all test compounds at least one range-finding test and three final tests were performed. The tested concentrations were selected based on the presence of a 0 and 100% effect level (for both malformation and mortality) as derived from the range-finding test. We defined 0 hpf as 1 h after the light had been turned on. Immediately after egg collection the embryos were exposed within 2 hpf to test media containing different concentrations of the test compound. For each concentration of the compounds tested, along with a negative (test medium only) and/or solvent control, 15–20 embryos were randomly distributed into wells of a 6-well culture plate containing 8 ml solution. The embryos were placed at $28.5 \pm 0.5\,^{\circ}C$ in a temperature and light controlled incubator with a constant light–dark (14–10 h) cycle which was set at the same circadian rhythm as the adult zebrafish. At 4 hpf, fertilization success was determined and 12 fertilized eggs for each concentration were transferred individually to a well of a 24-well plate containing 2 ml solution, one embryo per well. Each 24-well plate held two different concentrations of the test compound. Embryos in the control and/or solvent control group were placed on a separate plate. The exposure was static and continuous throughout 144 hpf, solutions were not renewed during the overall experiment. This set-up allows for high-throughput testing with minimum manipulation of the embryos.

### 2.6. Evaluation of developmental effects

At selected time points, namely 24, 48, 72 and 144 hpf, embryotoxicity (= mortality) and morphological characteristics of the embryos were evaluated using an inverted microscope (IX 81, Olympus, Belgium) or a stereomicroscope (Stemi 2000-C, Zeiss, Germany). The embryos were evaluated for the presence and morphological development (as appropriate) of somites, tail detachment, otic vesicle and otoliths, eyes, heart beat and blood circulation. After hatching, the aforementioned characteristics were no longer scored; instead, larvae were evaluated for skeletal deformities, body position (normally absence of a sideways position) and their ability to swim (after stimulation if necessary). All individuals were evaluated at all time points. The time points at which these characteristics were evaluated and considered in the overall malformation score (see below), are directly related to the evolution of normal development at that time (Table 1), which corresponds to stages described in literature [19]. Scores were given for each characteristic in a binominal way, normal (0) or abnormal (1). Observations were recorded in an excel template form (Microsoft Excel 2002).

### 2.7. Data evaluation

The excel template forms were designed in a way that, based on the scores given for each characteristic, a score for overall effect was generated for each individual in the experiment. An embryo was considered either normal (all scores = 0), dead, or malformed as for surviving animals (score = 1 for one or more morphological characteristics evaluated). Furthermore, effects were considered as a function of time. When increased mortality occurred at later time points, incidences for malformations were calculated as the sum of the incidence at the previous time point for dead embryos/larvae and the incidence for living embryos/larvae at that time. Thus, each individual in the experiment did get scores for both mortality and malformation

**Table 1**
Morphological characteristics evaluated as measures for the teratogenic potency of the test compound at the designated times points (×).

| Life stage | Embryotoxicity | Developmental endpoints to be evaluated | Time point for observation of normal development (normal = score 0, abnormal = score 1) | | | |
|---|---|---|---|---|---|---|
| | | | 24 h | 48 h | 72 h | 144 h |
| Zebrafish egg | Egg coagulation | | × | × | × | × |
| | | Somites | × | × | × | × |
| | | Tail detachment | × | × | × | × |
| | | Otolith | | × | × | × |
| | | Eyes | | × | × | × |
| | | Heart beat | | × | × | × |
| | | Blood circulation | | × | × | × |
| Hatching | | | | | × | × |
| Zebrafish larvae | Larvae alive | | | | × | × |
| | | Skeletal deformities | | | × | × |
| | | Side-wise position | | | | × |
| | | Active swimming (eventually upon tail stimulation) | | | | × |
| Embryotoxic effect/time point | % of organisms with score 1 for mortality at each time point of observation | | | | | |
| Teratogenic effect/time point | % of organisms with score 1 for any of the developmental endpoints at each time point of observation | | | | | |

at selected time points. This allowed effect percentages to be calculated for each concentration at each time point. The percentage of embryotoxicity was calculated as the ratio of dead embryos and/or larvae over the number of embryos (generally 12 fertilized eggs) at the start of exposure. On the other hand the percentage of malformation for 24, 48, 72 and 144 hpf was calculated as the ratio of malformed embryos and/or larvae over the number of embryos that were alive at 24 hpf. The resulting output then consisted of the cumulative percentage for each time point of observation of individuals that were malformed or dead. Detailed records with observations on each morphological characteristic were also available and were used to compare developmental effects in zebrafish embryos to mammalian studies.

The resulting data, from minimum 3 independent experiments ($n = 3$) each with 12 replicates (one embryo per well) per concentration, from the excel template form were imported into Graphpad Prism (Graphpad Prism, version 2.01) to create concentration–response curves for mortality and malformation for each time point. The sigmoidal curves had a variable slope that adequately fitted the data. The top and bottom of the curve were set to 100 and 0 respectively with the prerequisite that percentages close to 0 and 100 for effects were present in the selected concentration range. These concentration–response curves were required to determine $EC_{50}$ (teratogenic effects) and $LC_{50}$ (lethal/embryotoxic effects) values. The $EC_{50}$ and $LC_{50}$ were derived from a 4-parameter equation describing the curve as follows:

$$Y = \text{Bottom} + \left( \frac{\text{Top} - \text{Bottom}}{1 + 10\ \exp(\log \text{EC}_{50} - X) \times \text{Hill Slope}} \right)$$

where $Y$ = response (percentage of death or malformed individuals), $X$ = the logarithm of the concentration of the test substance.

Based on $LC_{50}$ and $EC_{50}$ values, a teratogenic index (TI) was calculated as the ratio $LC_{50}/EC_{50}$ for each time point. The higher the TI, the more specific teratogenic effects of the chemical can be expected compared to overall embryotoxicity, as measured by mortality of organisms.

### 2.8. Statistical analysis

Statistical analysis was performed in Statistica (StatSoft, Inc. 2007, STATISTICA data analysis software system, version 8.0, www.statsoft.com). The statistical significance of the results was tested using a Kruskal–Wallis ANOVA to determine the effect of concentration, followed by a Mann–Whitney *U*-test between groups (control versus exposed).

## 3. Results

### 3.1. Optimal test conditions

Zebrafish embryos, kept in Petri dishes filled with fish water, showed a survival rate of 90% or higher (data not shown) and normal development up to 144 hpf relative to published landmarks [19]. Based on these results we concluded that fish water can be used as medium for the exposure studies. On the other hand, development of embryos kept in different multi-well plates over a period of 144 h showed malformations in some plates. Skeletal deformities such as tail kinks (Fig. 1) were more often present in larvae kept in smaller wells, as those of a 96-well plate in comparison to 24- and 48-well plates. Furthermore, skeletal deformities were more common at 144 hpf in comparison to 55 and 72 hpf. This result is most pronounced for the 96-well plate. As the water quality in the 96-wells might deteriorate and cause teratogenic effects, the test medium was changed in the 96-well plate, but results of skeletal deformities gave the same outcome (data not shown). Based on the results from the experiments with the different multi-well plates, the 24-well plate was used in all further exposure experiments as a recipient for the embryos.

DMSO was tested in the range from 0.014 to 0.56 mM (0.1–4% (v/v)) in 3 independent experiments ($n = 3$) and showed adverse effects on the development of the zebrafish embryos, as illustrated in Fig. 2 for 144 hpf (24, 48, 72 hpf; data not shown). At 144 hpf, kinks in the tail were mostly present, even at a concentration as low as 0.07 mM (0.5% (v/v)) DMSO (mean% larvae with a kink in their tail >10). The NOEC of DMSO was determined to be 0.035 mM (0.25% (v/v)) (mean% larvae affected <10; not significantly different from controls), but we preferred a standard final DMSO concentration of 0.014 mM, corresponding to 0.1% (v/v), for the exposure experiments.

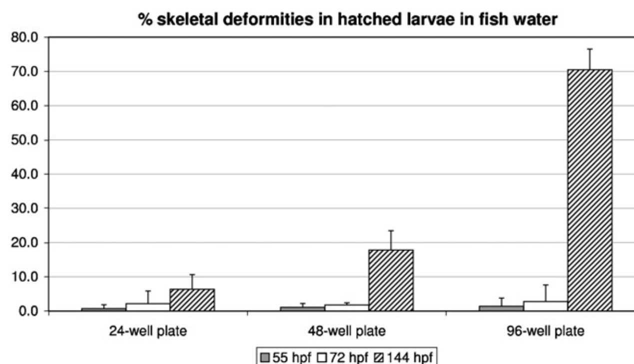


**Fig. 1.** Mean percentages and standard deviation ($n = 3$ experiments) of hatched larvae in fish water with skeletal deformities, placed in 24-, 48- and 96-well plates at 55, 72 and 144 hpf.

**Table 2**
Effect percentages (4 independent experiments) for abnormal morphological characteristics evaluated in the zebrafish teratogenicity assay after exposure to retinoic acid.

| Retinoic acid concentration (mM) | Embryotoxicity[a] | | Otoliths[b] | | Eyes[b] | | Somites[b] | | Tail detachment[b] | | Heart beat[b] | | Blood circulation[b] | | Hatching | | Kink in the tail[c] | | Sidewise position[c] | | Active swimming[c] | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Mean | SD | Mean | SD | Mean | SD | Mean | SD | Mean | SD | Mean | SD | Mean | SD | Mean | SD | Mean | SD | Mean | SD | Mean | SD |
| **24 hpf** | | | | | | | | | | | | | | | | | | | | | | |
| Control | 2.1 | 4.2 | | | | | 0.0 | 0.0 | 0.0 | 0.0 | | | | | | | | | | | | |
| Solvent | 13.9 | 12.7 | | | | | 0.0 | 0.0 | 0.0 | 0.0 | | | | | | | | | | | | |
| $8.52 \times 10^{-9}$ | 12.5 | 8.3 | | | | | 0.0 | 0.0 | 0.0 | 0.0 | | | | | | | | | | | | |
| $4.26 \times 10^{-8}$ | 8.3 | 9.6 | | | | | 0.0 | 0.0 | 0.0 | 0.0 | | | | | | | | | | | | |
| $2.13 \times 10^{-7}$ | 8.3 | 11.8 | | | | | 0.0 | 0.0 | 0.0 | 0.0 | | | | | | | | | | | | |
| $1.07 \times 10^{-6}$ | 14.6 | 8.0 | | | | | 0.0 | 0.0 | 0.0 | 0.0 | | | | | | | | | | | | |
| $5.33 \times 10^{-6}$ | 12.5 | 10.8 | | | | | 0.0 | 0.0 | 0.0 | 0.0 | | | | | | | | | | | | |
| $2.66 \times 10^{-5}$ | 18.8 | 10.5 | | | | | 0.0 | 0.0 | 2.5 | 5.0 | | | | | | | | | | | | |
| $1.33 \times 10^{-4}$ | 20.8 | 4.8 | | | | | **7.5** | 15.0 | **34.2** | 28.6 | | | | | | | | | | | | |
| $6.66 \times 10^{-4}$ | 27.1 | 21.9 | | | | | **56.7**[**] | 41.6 | **69.4**[**] | 47.5 | | | | | | | | | | | | |
| $3.33 \times 10^{-3}$ | 43.8 | 31.5 | | | | | **100.0**[*] | / | **100.0**[*] | / | | | | | | | | | | | | |
| $1.66 \times 10^{-2}$ | 97.9 | 4.2 | | | | | **100.0**[*] | / | **100.0**[*] | / | | | | | | | | | | | | |
| **48 hpf** | | | | | | | | | | | | | | | | | | | | | | |
| Control | 2.1 | 4.2 | 2.1 | 4.2 | 2.1 | 4.2 | 0.0 | 0.0 | 0.0 | 0.0 | 2.1 | 4.2 | 2.1 | 4.2 | | | | | | | | |
| Solvent | 13.9 | 12.7 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | | | | | | | | |
| $8.52 \times 10^{-9}$ | 12.5 | 8.3 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | | | | | | | | |
| $4.26 \times 10^{-8}$ | 8.3 | 9.6 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 2.1 | 4.2 | 2.1 | 4.2 | | | | | | | | |
| $2.13 \times 10^{-7}$ | 8.3 | 11.8 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 2.1 | 4.2 | | | | | | | | |
| $1.07 \times 10^{-6}$ | 14.6 | 8.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 13.6 | 27.3 | | | | | | | | |
| $5.33 \times 10^{-6}$ | 12.5 | 10.8 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 5.0 | 10.0 | 32.2 | 28.2 | | | | | | | | |
| $2.66 \times 10^{-5}$ | 18.8 | 10.5 | **56.2**[**] | 32.2 | **90.7**[**] | 12.9 | 0.0 | 0.0 | 7.5 | 9.6 | **100.0**[**] | 0.0 | **100.0**[**] | 0.0 | | | | | | | | |
| $1.33 \times 10^{-4}$ | 20.8 | 4.8 | **100.0**[**] | 0.0 | **100.0**[**] | 0.0 | **31.7** | 27.3 | **33.6** | 40.1 | **100.0**[**] | 0.0 | **100.0**[**] | 0.0 | | | | | | | | |
| $6.66 \times 10^{-4}$ | 27.1 | 21.9 | **100.0**[**] | 0.0 | **100.0**[**] | 0.0 | **87.2**[**] | 9.5 | **97.5**[**] | 5.0 | **100.0**[**] | 0.0 | **100.0**[**] | 0.0 | | | | | | | | |
| $3.33 \times 10^{-3}$ | 66.7 | 23.6 | **100.0**[**] | 0.0 | **100.0**[**] | 0.0 | **83.3**[**] | 28.9 | **100.0**[**] | 0.0 | **100.0**[**] | 0.0 | **100.0**[**] | 0.0 | | | | | | | | |
| $1.66 \times 10^{-2}$ | 100.0 | 0.0 | – | – | – | – | – | – | – | – | – | – | – | – | | | | | | | | |
| **72 hpf** | | | | | | | | | | | | | | | | | | | | | | |
| Control | 2.1 | 4.2 | **16.7** | 28.9 | **16.7** | 28.9 | **16.7** | 28.9 | 0.0 | 0.0 | **16.7** | 28.9 | **16.7** | 28.9 | **13.1** | 11.7 | 0.0 | 0.0 | | | | |
| Solvent | 13.9 | 12.7 | 0.0 | / | 0.0 | / | 0.0 | / | 0.0 | / | 0.0 | / | 0.0 | / | 4.2 | 8.3 | 0.0 | 0.0 | | | | |
| $8.52 \times 10^{-9}$ | 12.5 | 8.3 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | **14.2** | 5.0 | 5.6 | 6.6 | | | | |
| $4.26 \times 10^{-8}$ | 8.3 | 9.6 | **12.5** | 25.0 | **12.5** | 25.0 | **12.5** | 25.0 | 0.0 | 0.0 | **12.5** | 25.0 | **12.5** | 25.0 | **13.8** | 5.5 | **12.2** | 17.7 | | | | |
| $2.13 \times 10^{-7}$ | 8.3 | 11.8 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 8.5 | 11.8 | **26.3**[**] | 21.8 | | | | |
| $1.07 \times 10^{-6}$ | 14.6 | 8.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 8.3 | 16.7 | **25.0** | 50.0 | **21.9** | 3.9 | **37.0**[**] | 31.3 | | | | |
| $5.33 \times 10^{-6}$ | 12.5 | 10.8 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | **66.7** | 47.1 | **12.5** | 16.0 | **75.1**[**] | 12.2 | | | | |
| $2.66 \times 10^{-5}$ | 18.8 | 10.5 | **88.9**[**] | 22.2 | **91.9**[**] | 10.6 | **5.3** | 6.1 | **15.3**[**] | 17.2 | **97.5**[**] | 5.0 | **100.0**[**] | 0.0 | **92.3**[**] | 9.2 | **100.0** | 0.0 | | | | |
| $1.33 \times 10^{-4}$ | 22.9 | 8.0 | **100.0**[**] | 0.0 | **100.0**[**] | 0.0 | **67.5**[**] | 32.8 | **45.6**[**] | 33.3 | **100.0**[**] | 0.0 | **100.0**[**] | 0.0 | **97.5**[**] | 5.0 | **100.0**[*] | / | | | | |
| $6.66 \times 10^{-4}$ | 41.7 | 15.2 | **100.0**[**] | 0.0 | **100.0**[**] | 0.0 | **100.0**[**] | 0.0 | **95.8**[**] | 8.3 | **100.0**[**] | 0.0 | **100.0**[**] | 0.0 | **100.0**[**] | 0.0 | | | | | | |
| $3.33 \times 10^{-3}$ | 93.8 | 8.0 | **100.0**[**] | 0.0 | **100.0**[**] | 0.0 | **75.0**[**] | 35.4 | **75.0**[**] | 35.4 | **100.0**[**] | 0.0 | **100.0**[**] | 0.0 | **100.0** | 0.0 | | | | | | |
| $1.66 \times 10^{-2}$ | 100.0 | 0.0 | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | | | | |
| **144 hpf** | | | | | | | | | | | | | | | | | | | | | | |
| Control | 4.2 | 4.8 | **100.0**[#] | / | **100.0**[#] | / | **100.0**[#] | / | 0.0 | / | **100.0**[#] | / | **100.0**[#] | / | 2.1 | 4.2 | 4.4 | 5.0 | 4.4 | 5.0 | 8.8 | 0.4 |
| Solvent | 16.7 | 8.3 | | | | | | | | | | | | | 0.0 | 0.0 | **13.7** | 9.7 | 5.8 | 7.1 | **17.2** | 12.3 |
| $8.52 \times 10^{-9}$ | 12.5 | 8.3 | | | | | | | | | | | | | 0.0 | 0.0 | **22.5** | 15.0 | **12.5** | 9.6 | **22.5** | 15.0 |
| $4.26 \times 10^{-8}$ | 10.4 | 8.0 | | | | | | | | | | | | | 0.0 | 0.0 | **31.2**[**] | 25.9 | **11.7** | 8.8 | **26.2**[**] | 15.9 |
| $2.13 \times 10^{-7}$ | 8.3 | 11.8 | | | | | | | | | | | | | 0.0 | 0.0 | **43.8**[**] | 22.4 | **39.0**[**] | 26.5 | **35.5**[**] | 22.4 |
| $1.07 \times 10^{-6}$ | 18.8 | 8.0 | | | | | | | | | | | | | 0.0 | 0.0 | **59.6**[**] | 37.8 | **29.8** | 29.4 | **41.4** | 45.6 |
| $5.33 \times 10^{-6}$ | 16.7 | 6.8 | | | | | | | | | | | | | 0.0 | 0.0 | **92.7**[**] | 9.5 | **65.0**[**] | 23.4 | **97.7**[**] | 4.5 |

Table 2 (*Continued*)

| Retinoic acid concentration (mM) | Embryotoxicity[a] | | Otoliths[b] | | Eyes[b] | | Somites[b] | | Tail detachment[b] | | Heart beat[b] | | Blood circulation[b] | | Hatching | | Kink in the tail[c] | | Sidewise position[c] | | Active swimming[c] | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Mean | SD | Mean | SD | Mean | SD | Mean | SD | Mean | SD | Mean | SD | Mean | SD | Mean | SD | Mean | SD | Mean | SD | Mean | SD |
| $2.66 \times 10^{-5}$ | 58.3 | *11.8* | **75.0** | *50.0* | **75.0** | *50.0* | **42.9** | *50.8* | **24.4**** | *21.9* | **100.0**** | *0.0* | **100.0**** | *0.0* | **60.0**** | *33.9* | **100.0**** | *0.0* | **88.9**** | *19.2* | **88.9**** | *19.2* |
| $1.33 \times 10^{-4}$ | 68.8 | *17.2* | **75.0** | *50.0* | **100.0**** | *0.0* | **87.5**** | *25.0* | **41.7**** | *28.9* | **100.0**** | *0.0* | **100.0**** | *0.0* | **95.0**** | *10.0* | **100.0** | / | **100.0** | / | **100.0** | / |
| $6.66 \times 10^{-4}$ | 97.9 | *4.2* | **100.0** | / | **100.0** | / | **100.0** | / | **100.0*** | / | **100.0** | / | **100.0** | / | **100.0** | / | | | | | | |
| $3.33 \times 10^{-3}$ | 100.0 | *0.0* | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – |
| $1.66 \times 10^{-2}$ | 100.0 | *0.0* | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – |

*Notes*: Incidences of morphological effects >10% are shown in bold, cells have been left blank when no observations were made.
Abbreviations used: (hpf) hours post fertilization; (–) no data available due to 100% mortality; (/) no SD could be calculated since data originated from 1 experimental run.
[a] Mean percentage ± SD of mortality based on all eggs.
[b] Mean percentage ± SD of teratogenic effect based on surviving, pre-hatch embryos.
[c] Mean percentage ± SD of teratogenic effect based on surviving, post-hatch embryos.
[*] At $p < 0.05$ there was a significant effect of concentration as determined by a Kruskal–Wallis ANOVA.
[**] At $p < 0.05$ there was a significant effect of concentration as determined by a Kruskal–Wallis ANOVA and between groups as determined by a Mann–Whitney *U*-test.
[#] Data represent 1 individual out of 46 controls, alive at 144 hpf, that did not hatch and was malformed.

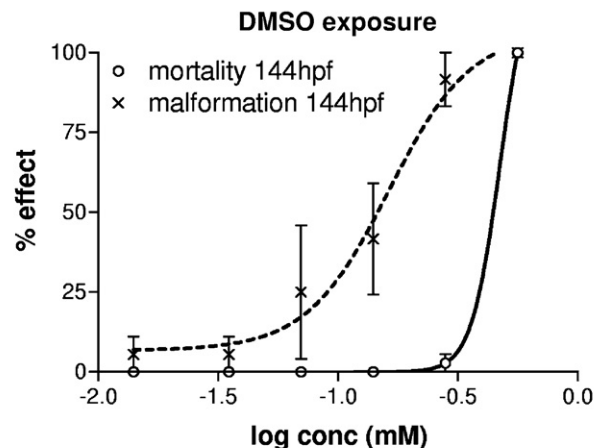


**Fig. 2.** Concentration–response curve for malformation and mortality for DMSO exposure ($n = 3$ experiments) at 144 hpf. %Effect (mean ± SD) is shown versus the logarithm of the concentrations tested (log mM) (range 0.014–0.56 mM ∼ 0.1–4% (v/v)).

### *3.2. Data obtained for selected test compounds*

All-trans retinoic acid was tested in the range between $8.52 \times 10^{-9}$ and $1.66 \times 10^{-2}$ mM, selected after a range-finding test, with a final DMSO concentration of 0.1%. Results are shown in Table 2. We observed malformation of eyes (Fig. 3A), otoliths (Fig. 3A), somites, and cardiovascular defects (Fig. 3B) in the early developmental stages (24–48 hpf). At later developmental stages (72–144 hpf) skeletal deformities (Fig. 3D) and lack of swimming behavior were most prominent. Effects (>10%) were seen already at the lowest concentration of $8.52 \times 10^{-9}$ mM and a concentration dependent increase of malformations is seen at higher concentrations. Significant effects were apparent at $4.26 \times 10^{-8}$ mM and higher.

Valproic acid was tested in the range from 0.04 to 12.0 mM as derived from a range-finding test. Overall effects seen with exposure to valproic acid were similar to those for retinoic acid, although effect concentrations were higher (Table 3). Effects (>10%) were seen at a valproic acid concentration as low as 0.09 mM, which were significantly different from controls at 0.38 mM and 144 hpf.

For caffeine, concentrations tested were between 0.05 and 12.9 mM. At 48 hpf, blood circulation was absent at a concentration of 1.61 mM and higher and starting from 72 hpf, mostly kinks in the tail were observed when embryos are exposed to at least 0.39 mM caffeine (Table 4). Down to 0.19 mM caffeine, significant differences were shown compared to control.

The concentrations of lithium chloride tested, were between 0.05 and 235.9 mM. Results are shown in Table 5. At 24 and 48 hpf, mortality is observed at the highest concentration tested, while no other teratogenic effects were obvious. Although not an endpoint in the above described zebrafish teratogenic assay, kinks in the chorda were observed occasionally (Fig. 3C). This malformation was not observed in a systematic manner at different concentrations. Apparently lithium chloride exerts its major teratogenic potential at later time points. At 72 hpf, a slight retardation in development could be observed, manifested as a delay in hatching after exposure to 58.9 mM lithium chloride. At 144 hpf skeletal deformities and reduced swimming behavior are most prominent.

For all positive compounds tested, the number of individuals affected is concentration-dependent. Based on the percentage individuals affected (malformation for any of observed characteristics) for each concentration, concentration–response curves were created for each time point (Fig. 4). $LC_{50}$ (for embryotoxic effects or lethality) and $EC_{50}$ (for specific teratogenic effects) values were

**Table 3**
Effect percentages (4 independent experiments) for abnormal morphological characteristics evaluated in the zebrafish teratogenicity assay after exposure to valproic acid.

| Valproic acid concentration (mM) | Embryotoxicity[a] | | Otoliths[b] | | Eyes[b] | | Somites[b] | | Tail detachment[b] | | Heart beat[b] | | Blood circulation[b] | | Hatching | | Kink in the tail[c] | | Sidewise position[c] | | Active swimming[c] | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Mean | SD | Mean | SD | Mean | SD | Mean | SD | Mean | SD | Mean | SD | Mean | SD | Mean | SD | Mean | SD | Mean | SD | Mean | SD |
| **24 hpf** | | | | | | | | | | | | | | | | | | | | | | |
| Control | 0.0 | 0.0 | | | | | 0.0 | 0.0 | 0.0 | 0.0 | | | | | | | | | | | | |
| $4.70 \times 10^{-2}$ | 2.1 | 4.2 | | | | | 0.0 | | 0.0 | | | | | | | | | | | | | |
| $9.40 \times 10^{-2}$ | 2.1 | 4.2 | | | | | 0.0 | 0.0 | 0.0 | 0.0 | | | | | | | | | | | | |
| 0.19 | 0.0 | 0.0 | | | | | 0.0 | 0.0 | 0.0 | 0.0 | | | | | | | | | | | | |
| 0.38 | 4.2 | 4.8 | | | | | 0.0 | 0.0 | 0.0 | 0.0 | | | | | | | | | | | | |
| 0.75 | 2.1 | 4.2 | | | | | 0.0 | 0.0 | 2.1 | 4.2 | | | | | | | | | | | | |
| 1.5 | 18.8 | 26.7 | | | | | **12.7** | 19.8 | **20.0**[**] | 17.1 | | | | | | | | | | | | |
| 3.01 | 47.9 | 41.0 | | | | | **57.9**[**] | 41.7 | **90.5**[**] | 16.5 | | | | | | | | | | | | |
| 6.02 | 81.3 | 26.7 | | | | | **100.0**[**] | 0.0 | **100.0**[**] | 0.0 | | | | | | | | | | | | |
| 12.0 | 100.0 | 0.0 | | | | | – | – | – | – | | | | | | | | | | | | |
| **48 hpf** | | | | | | | | | | | | | | | | | | | | | | |
| Control | 0.0 | 0.0 | 0.0 | 0.0 | 2.1 | 4.2 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | | | | | | | | |
| $4.70 \times 10^{-2}$ | 2.1 | 4.2 | 0.0 | | 0.0 | | 0.0 | | 0.0 | | 0.0 | | 0.0 | | | | | | | | | |
| $9.40 \times 10^{-2}$ | 2.1 | 4.2 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | | | | | | | | |
| 0.19 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | | | | | | | | |
| 0.38 | 4.2 | 4.8 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 2.1 | 4.2 | 4.4 | 5.0 | | | | | | | | |
| 0.75 | 4.2 | 4.8 | 2.1 | 4.2 | 2.3 | 4.5 | 0.0 | 0.0 | 0.0 | 0.0 | 4.2 | 8.3 | **58.5**[**] | 31.0 | | | | | | | | |
| 1.5 | 39.6 | 40.5 | **33.3** | 40.8 | **70.8** | 47.9 | **10.4** | 20.8 | 8.3 | 16.7 | **33.3** | 47.1 | **100.0**[**] | 0.0 | | | | | | | | |
| 3.01 | 66.7 | 47.1 | **100.0**[**] | 0.0 | **100.0**[**] | 0.0 | **50.0** | 70.7 | **87.5**[**] | 17.7 | **100.0**[**] | 0.0 | **100.0**[**] | 0.0 | | | | | | | | |
| 6.02 | 85.4 | 29.2 | **100.0**[*] | | **100.0** | | **100.0**[**] | | **100.0**[*] | | **100.0**[*] | | **100.0**[*] | | | | | | | | | |
| 12.0 | 100.0 | 0.0 | – | – | – | – | – | – | – | – | – | – | – | – | | | | | | | | |
| **72 hpf** | | | | | | | | | | | | | | | | | | | | | | |
| Control | 2.1 | 4.2 | | | | | | | | | | | | | 0.0 | 0.0 | 4.2 | 8.3 | | | | |
| $4.70 \times 10^{-2}$ | 2.1 | 4.2 | | | | | | | | | | | | | 0.0 | | 9.1 | / | | | | |
| $9.40 \times 10^{-2}$ | 2.1 | 4.2 | | | | | | | | | | | | | 0.0 | 0.0 | 2.8 | 4.8 | | | | |
| 0.19 | 2.1 | 4.2 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | **23.2**[**] | 14.1 | 3.7 | 6.4 | | | | |
| 0.38 | 4.2 | 4.8 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 4.2 | 8.3 | 4.2 | 8.3 | **30.1**[**] | 13.8 | **11.1** | 15.7 | | | | |
| 0.75 | 4.2 | 4.8 | 0.0 | 0.0 | 4.2 | 8.3 | 0.0 | 0.0 | 0.0 | 0.0 | **24.0** | 28.9 | | | | | | | | | | |
| **69.8**[**] | 25.8 | **80.3**[**] | 14.5 | **41.7** | 52.0 | | | | | | | | | | | | | | | | | |
| 1.5 | 62.5 | 43.8 | **50.0**[*] | 70.7 | **50.0**[*] | 70.7 | **25.0**[*] | 35.4 | **12.5**[*] | 17.7 | **90.0**[**] | 14.1 | **100.0**[**] | 0.0 | **100.0**[**] | 0.0 | | | | | | |
| 3.01 | 93.8 | 8.0 | **100.0**[**] | 0.0 | **100.0**[**] | 0.0 | **50.0** | 70.7 | **100.0**[**] | 0.0 | **100.0**[**] | 0.0 | **100.0**[**] | 0.0 | **100.0**[**] | 0.0 | | | | | | |
| 6.02 | 93.8 | 12.5 | **100.0**[*] | / | **100.0**[*] | / | **100.0**[*] | / | **100.0**[*] | / | **100.0**[*] | / | **100.0**[*] | / | **100.0**[**] | / | | | | | | |
| 12.0 | 100.0 | 0.0 | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | | | | |
| **144 hpf** | | | | | | | | | | | | | | | | | | | | | | |
| Control | 2.1 | 4.2 | | | | | | | | | | | | | 0.0 | 0.0 | 5.6 | 4.8 | 2.8 | 4.8 | **14.1** | 17.2 |
| $4.70 \times 10^{-2}$ | 2.1 | 4.2 | | | | | | | | | | | | | 0.0 | / | 0.0 | / | 0.0 | / | 0.0 | |
| $9.40 \times 10^{-2}$ | 2.1 | 4.2 | | | | | | | | | | | | | 0.0 | 0.0 | **51.1** | 33.7 | **25.4** | 23.0 | **17.0** | 11.2 |
| 0.19 | 2.1 | 4.2 | | | | | | | | | | | | | 0.0 | 0.0 | **87.5** | 17.7 | **70.8** | 29.5 | **50.0** | 47.1 |
| 0.38 | 4.2 | 4.8 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | **33.3** | 57.7 | **66.7** | 57.73 | **11.4**[**] | 4.6 | **67.9** | 30.2 | **100.0** | 0 | **62.1** | 22.9 |
| 0.75 | 37.5 | 33.7 | **45.5** | 64.3 | **31.8** | 45.0 | **40.9** | 57.9 | **36.4** | 51.4 | **75.0** | 35.4 | **100.0** | 0.0 | **50.0** | 50.0 | **75.0** | 35.4 | **100.0** | 0.0 | **100.0** | 0.0 |
| 1.5 | 93.8 | 12.5 | **100.0** | / | **100.0** | / | **100.0** | / | **100.0** | / | **100.0** | / | **100.0** | / | **100.0** | / | | | | | | |
| 3.01 | 100.0 | 0.0 | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – |
| 6.02 | 100.0 | 0.0 | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – |
| 12.0 | 100.0 | 0.0 | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – |

*Notes*: Incidences of morphological effects >10% are shown in bold, cells have been left blank when no observations were made.
Abbreviations used: (hpf) hours post fertilization; (–) no data available due to 100% mortality; (/) no SD could be calculated since data originated from 1 experimental run.
[a] Mean percentage ± SD of mortality based on all eggs.
[b] Mean percentage ± SD of teratogenic effect based on surviving, pre-hatch embryos.
[c] Mean percentage ± SD of teratogenic based on surviving, post-hatch embryos.
[*] At $p < 0.05$ there was a significant effect of concentration as determined by a Kruskal–Wallis ANOVA.
[**] At $p < 0.05$ there was a significant effect of concentration as determined by a Kruskal–Wallis ANOVA and between groups as determined by a Mann–Whitney *U*-test.

**Table 4**
Effect percentages (3 independent experiments) for abnormal morphological characteristics evaluated in the zebrafish teratogenicity assay after exposure to caffeine.

| Caffeine concentration (mM) | Embryotoxicity[a] | | Otoliths[b] | | Eyes[b] | | Somites[b] | | Tail detachment[b] | | Heart beat[b] | | Blood circulation[b] | | Hatching | | Kink in the tail[c] | | Sidewise position[c] | | Active swimming[c] | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Mean | SD | Mean | SD | Mean | SD | Mean | SD | Mean | SD | Mean | SD | Mean | SD | Mean | SD | Mean | SD | Mean | SD | Mean | SD |
| **24 hpf** | | | | | | | | | | | | | | | | | | | | | | |
| Control | 0.0 | *0.0* | | | | | 2.8 | *4.8* | 2.8 | *4.8* | | | | | | | | | | | | |
| 0.05 | 2.8 | *4.8* | | | | | 0.0 | *0.0* | 0.0 | *0.0* | | | | | | | | | | | | |
| 0.10 | 2.8 | *4.8* | | | | | 5.8 | *5.0* | 5.8 | *5.0* | | | | | | | | | | | | |
| 0.19 | 2.8 | *4.8* | | | | | 0.0 | *0.0* | 0.0 | *0.0* | | | | | | | | | | | | |
| 0.39 | 2.8 | *4.8* | | | | | 0.0 | *0.0* | 0.0 | *0.0* | | | | | | | | | | | | |
| 0.77 | 2.8 | *4.8* | | | | | 0.0 | *0.0* | 0.0 | *0.0* | | | | | | | | | | | | |
| 1.61 | 5.6 | *4.8* | | | | | 0.0 | *0.0* | 0.0 | *0.0* | | | | | | | | | | | | |
| 3.22 | 25.0 | *22.0* | | | | | 4.2 | *7.2* | 4.2 | *7.2* | | | | | | | | | | | | |
| 6.44 | 87.5 | *17.7* | | | | | **100.0** | / | **100.0** | / | | | | | | | | | | | | |
| 12.9 | 100.0 | *0.0* | | | | | – | – | – | – | | | | | | | | | | | | |
| **48 hpf** | | | | | | | | | | | | | | | | | | | | | | |
| Control | 2.8 | *4.8* | 0.0 | *0.0* | 0.0 | *0.0* | 0.0 | *0.0* | 0.0 | *0.0* | 0.0 | *0.0* | 0.0 | *0.0* | | | | | | | | |
| 0.05 | 2.8 | *4.8* | 0.0 | *0.0* | 0.0 | *0.0* | 0.0 | *0.0* | 0.0 | *0.0* | 0.0 | *0.0* | 0.0 | *0.0* | | | | | | | | |
| 0.10 | 2.8 | *4.8* | 2.8 | *4.8* | 5.8 | *5.0* | 5.8 | *5.0* | 5.8 | *5.0* | 2.8 | *4.8* | 5.8 | *5.0* | | | | | | | | |
| 0.19 | 2.8 | *4.8* | 0.0 | *0.0* | 0.0 | *0.0* | 0.0 | *0.0* | 0.0 | *0.0* | 0.0 | *0.0* | 0.0 | *0.0* | | | | | | | | |
| 0.39 | 2.8 | *4.8* | 0.0 | *0.0* | 3.0 | *5.2* | 0.0 | *0.0* | 0.0 | *0.0* | 0.0 | *0.0* | 0.0 | *0.0* | | | | | | | | |
| 0.77 | 2.8 | *4.8* | 0.0 | *0.0* | 0.0 | *0.0* | 0.0 | *0.0* | 0.0 | *0.0* | 0.0 | *0.0* | 2.8 | *4.8* | | | | | | | | |
| 1.61 | 5.6 | *4.8* | 0.0 | *0.0* | 0.0 | *0.0* | 0.0 | *0.0* | 0.0 | *0.0* | 0.0 | *0.0* | **70.7**[**] | *9.7* | | | | | | | | |
| 3.22 | 25.0 | *22.0* | **19.8** | *21.6* | **32.5**[**] | *36.9* | **22.6** | *21.5* | 0.0 | *0.0* | **24.0**[**] | *16.5* | **100.0**[**] | *0.0* | | | | | | | | |
| 6.44 | 100.0 | *0.0* | – | – | – | – | – | – | – | – | – | – | – | – | | | | | | | | |
| 12.9 | 100.0 | *0.0* | – | – | – | – | – | – | – | – | – | – | – | – | | | | | | | | |
| **72 hpf** | | | | | | | | | | | | | | | | | | | | | | |
| Control | 5.6 | *4.8* | 0.0 | / | 0.0 | / | 0.0 | / | 0.0 | / | 0.0 | / | 0.0 | / | 3.0 | *5.2* | 3.3 | *5.2* | | | | |
| 0.05 | 2.8 | *4.8* | | | | | | | | | | | | | 0.0 | *0.0* | 6.1 | *10.5* | | | | |
| 0.10 | 5.6 | *4.8* | 0.0 | / | 0.0 | / | **50.0** | / | **50.0** | / | **50.0** | / | **50.0** | / | 6.1 | *10.5* | 7.4 | *10.5* | | | | |
| 0.19 | 2.8 | *4.8* | | | | | | | | | | | | | 0.0 | *0.0* | 2.8 | *4.8* | | | | |
| 0.39 | 2.8 | *4.8* | | | | | | | | | | | | | 0.0 | *0.0* | **45.5**[**] | *7.9* | | | | |
| 0.77 | 2.8 | *4.8* | | | | | | | | | | | | | 0.0 | *0.0* | **100.0**[**] | *17.6* | | | | |
| 1.61 | 5.6 | *4.8* | **33.3** | *57.7* | 0.0 | *0.0* | **16.7** | *28.9* | 0.0 | *0.0* | 0.0 | *0.0* | **77.8**[**] | *38.5* | **29.5**[**] | *14.2* | **100.0**[**] | *14.2* | | | | |
| 3.22 | 27.8 | *25.5* | 5.6 | *9.6* | **11.1** | *19.2* | **41.4** | *46.0* | 0.0 | *0.0* | **23.9**[**] | *22.7* | **100.0**[**] | *0.0* | **97.2**[**] | *4.8* | **100.0** | *4.8* | | | | |
| 6.44 | 100.0 | *0.0* | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | | | | |
| 12.9 | 100.0 | *0.0* | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | | | | |
| **144 hpf** | | | | | | | | | | | | | | | | | | | | | | |
| Control | 5.6 | *4.8* | | | | | | | | | | | | | 0.0 | *0.0* | 5.6 | *9.6* | 0.0 | *0.0* | 0.0 | *0.0* |
| 0.05 | 2.8 | *4.8* | | | | | | | | | | | | | 0.0 | *0.0* | **14.9** | *19.1* | 3.0 | *5.2* | 6.1 | *10.5* |
| 0.10 | 8.3 | *8.3* | | | | | | | | | | | | | 0.0 | *0.0* | **15.8** | *12.3* | 0.0 | *0.0* | 2.8 | *4.8* |
| 0.19 | 8.3 | *8.3* | | | | | | | | | | | | | 0.0 | *0.0* | **49.2**[**] | *23.9* | 3.3 | *5.8* | **30.9** | *37.6* |
| 0.39 | 2.8 | *4.8* | | | | | | | | | | | | | 0.0 | *0.0* | **85.9**[**] | *4.4* | **22.2** | *31.5* | **41.4**[**] | *42.9* |
| 0.77 | 2.8 | *4.8* | | | | | | | | | | | | | 0.0 | *0.0* | **97.2**[**] | *4.8* | **38.4**[**] | *37.6* | **59.6**[**] | *27.8* |
| 1.61 | 61.1 | *39.4* | 0.0 | / | 0.0 | / | 0.0 | / | 0.0 | / | **100.0** | / | **100.0** | / | 3.3 | *5.8* | **100.0**[**] | *0.0* | **100.0**[**] | *0.0* | **100.0**[**] | *0.0* |
| 3.22 | 97.2 | *4.8* | 0.0 | / | 0.0 | / | 0.0 | / | 0.0 | / | **100.0** | / | **100.0** | / | **100.0** | | | | | | | |
| 6.44 | 100.0 | *0.0* | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – |
| 12.9 | 100.0 | *0.0* | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – |

*Notes*: Incidences of morphological effects >10% are shown in bold, cells have been left blank when no observations were made.
Abbreviations used: (hpf) hours post fertilization (–); no data available due to 100% mortality (/); no SD could be calculated since data originated from 1 experimental run.

[a] Mean percentage ± SD of mortality based on all eggs.

[b] Mean percentage ± SD of teratogenic effect based on surviving, pre-hatch embryos.

[c] Mean percentage ± SD of teratogenic effect based on surviving, post-hatch embryos.

[**] At $p < 0.05$ there was a significant effect of concentration as determined by a Kruskal–Wallis ANOVA and between groups as determined by a Mann–Whitney *U*-test.

**Table 5**
Effect percentages (3 independent experiments) for abnormal morphological characteristics evaluated in the zebrafish teratogenicity assay after exposure to lithium chloride.

| Lithium Cl concentration (mM) | Embryotoxicity[a] | | Otoliths[b] | | Eyes[b] | | Somites[b] | | Tail detachment[b] | | Heart beat[b] | | Blood circulation[b] | | Hatching | | Kink in the tail[c] | | Sidewise position[c] | | Active swimming[c] | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Mean | SD | Mean | SD | Mean | SD | Mean | SD | Mean | SD | Mean | SD | Mean | SD | Mean | SD | Mean | SD | Mean | SD | Mean | SD |
| **24 hpf** | | | | | | | | | | | | | | | | | | | | | | |
| Control | 2.8 | 4.8 | | | | | 0.0 | 0.0 | 0.0 | 0.0 | | | | | | | | | | | | |
| $5.76 \times 10^{-2}$ | 2.8 | 4.8 | | | | | 0.0 | 0.0 | 0.0 | 0.0 | | | | | | | | | | | | |
| 0.23 | 0.0 | 0.0 | | | | | 0.0 | 0.0 | 0.0 | 0.0 | | | | | | | | | | | | |
| 0.92 | 2.8 | 4.8 | | | | | 0.0 | 0.0 | 0.0 | 0.0 | | | | | | | | | | | | |
| 3.69 | 0.0 | 0.0 | | | | | 0.0 | 0.0 | 0.0 | 0.0 | | | | | | | | | | | | |
| 14.7 | 0.0 | 0.0 | | | | | 0.0 | 0.0 | 0.0 | 0.0 | | | | | | | | | | | | |
| 58.9 | 0.0 | 0.0 | | | | | 2.8 | 4.8 | 0.0 | 0.0 | | | | | | | | | | | | |
| 235.9 | 100.0 | 0.0 | | | | | – | – | – | – | | | | | | | | | | | | |
| **48 hpf** | | | | | | | | | | | | | | | | | | | | | | |
| Control | 2.8 | 4.8 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | | | | | | | | |
| $5.76 \times 10^{-2}$ | 2.8 | 4.8 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 2.8 | 5.9 | 2.8 | 5.9 | | | | | | | | |
| 0.23 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | | | | | | | | |
| 0.92 | 2.8 | 4.8 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | | | | | | | | |
| 3.69 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | | | | | | | | |
| 14.7 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | | | | | | | | |
| 58.9 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 2.8 | 4.8 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | | | | | | | | |
| 235.9 | 100.0 | 0.0 | – | – | – | – | – | – | – | – | – | – | – | – | | | | | | | | |
| **72 hpf** | | | | | | | | | | | | | | | | | | | | | | |
| Control | 2.8 | 4.8 | 0.0 | / | 0.0 | / | 0.0 | / | 0.0 | / | 0.0 | / | 0.0 | / | 2.8 | 5.9 | 6.1 | 12.9 | | | | |
| $5.76 \times 10^{-2}$ | 5.6 | 4.8 | | | | | | | | | | | | | 0.0 | 0.0 | 0.0 | 0.0 | | | | |
| 0.23 | 0.0 | 0.0 | | | | | | | | | | | | | 0.0 | 0.0 | 0.0 | 0.0 | | | | |
| 0.92 | 2.8 | 4.8 | 0.0 | / | 0.0 | / | 0.0 | / | 0.0 | / | 0.0 | / | 0.0 | / | 2.8 | 4.8 | 0.0 | 4.8 | | | | |
| 3.69 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | **33.3** | 70.7 | **11.1**[**] | 4.8 | 0.0 | 0.0 | | | | |
| 14.7 | 0.0 | 0.0 | 0.0 | / | 0.0 | / | 0.0 | / | 0.0 | / | 0.0 | / | 0.0 | / | 5.6 | 0.0 | 8.3 | 14.4 | | | | |
| 58.9 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 6.7 | 14.1 | 0.0 | 0.0 | 0.0 | 0.0 | 4.8 | 0.0 | **50.0**[**] | 26.8 | **38.4**[**] | 33.4 | | | | |
| 235.9 | 100.0 | 0.0 | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | | | | |
| **144 hpf** | | | | | | | | | | | | | | | | | | | | | | |
| Control | 2.8 | 4.8 | 0.0 | / | 0.0 | / | 0.0 | / | 0.0 | / | 0.0 | / | 100.0 | / | 2.8 | 5.9 | 0.0 | 0.0 | 2.8 | 5.9 | 2.8 | 5.9 |
| $5.76 \times 10^{-2}$ | 5.6 | 4.8 | | | | | | | | | | | | | 0.0 | 0.0 | 5.8 | 6.4 | 5.8 | 6.4 | 0.0 | 0.0 |
| 0.23 | 0.0 | 0.0 | | | | | | | | | | | | | 0.0 | 0.0 | **11.1** | 11.8 | 0.0 | 0.0 | **11.1** | 23.6 |
| 0.92 | 2.8 | 4.8 | | | | | | | | | | | | | 0.0 | 0.0 | 2.8 | 5.9 | 2.8 | 5.9 | 2.8 | 5.9 |
| 3.69 | 0.0 | 0.0 | 0.0 | / | 0.0 | / | 0.0 | / | 0.0 | / | 0.0 | / | 0.0 | / | 2.8 | 5.9 | **20.2** | 4.3 | 0.0 | 0.0 | **11.1** | 23.6 |
| 14.7 | 0.0 | 0.0 | | | | | | | | | | | | | 0.0 | 0.0 | **86.1**[**] | 5.9 | **91.7**[**] | 17.7 | **66.7**[**] | 11.8 |
| 58.9 | 75.0 | 8.3 | 0.0 | 0.0 | 0.0 | 0.0 | **25.0** | 35.4 | 0.0 | 0.0 | **100.0** | 0.0 | **100.0** | 0.0 | **55.6** | 23.6 | **100.0** | / | **100.0** | / | **100.0** | / |
| 235.9 | 100.0 | 0.0 | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – |

*Notes*: Incidences of morphological effects >10% are shown in bold, cell have been left blank when no observations could be made.
Abbreviations used: (hpf) hours post fertilization (–); no data available due to 100% mortality; (/) no SD could be calculated since data originated from 1 experimental run.
[a] Mean percentage ± SD of mortality based on all eggs.
[b] Mean percentage ± SD of teratogenic effect based on surviving, pre-hatch embryos.
[c] Mean percentage ± SD of teratogenic effect based on surviving, post-hatch embryos.
[**] At $p < 0.05$ there was a significant effect of concentration as determined by a Kruskal–Wallis ANOVA and between groups as determined by a Mann–Whitney *U*-test.

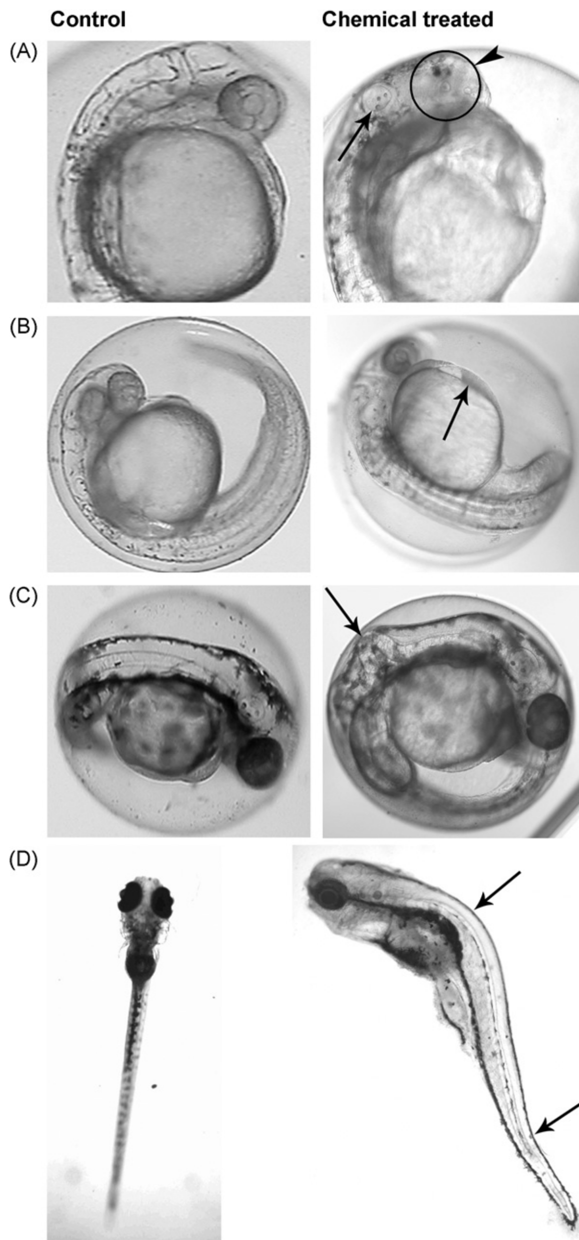


**Fig. 3.** Photographs of embryos and larvae at different time points. Exposure conditions and malformations are indicated, respective controls for all individuals are shown on the left. (A) $1.33 \times 10^{-4}$ mM retinoic acid, malformation of the eye (arrowhead) and otoliths (arrow), 24 h post fertilization (hpf); (B) $2.66 \times 10^{-5}$ mM retinoic acid, accumulation of red blood cells (arrow), 24 hpf; (C) 58.97 mM lithium chloride, kink in the chorda (arrow), 48 hpf; (D) $2.66 \times 10^{-}5$ mM retinoic acid, kinks in the tail (arrows) and sidewise position, 72 hpf.

derived from the concentration–response curves for all time points evaluated, based on a minimum of three independent experiments (Table 6). The distance between the concentration–response curves at the 50% effect concentration for embryotoxicity and malformation (Fig. 4) is considered a measure of the specific teratogenic, non-embryotoxic potential of the compound. This is also demonstrated by the TI-values, which are calculated as the ratio $LC_{50}/EC_{50}$ (Table 6). Furthermore, effects of malformation shift to the left (lower concentrations) as a function of time (Fig. 4).

Glucose and saccharin, used as negative compounds, were tested between 0.09 and 55 mM. As expected, glucose showed neither lethal nor teratogenic effects to the zebrafish embryos in the range tested for the whole duration of the experiments ($n = 3$)

**Table 6**
$LC_{50}$, $EC_{50}$ (mean values of '$n$'-independent experiments) and TI values as derived from the concentration–response curves for retinoic acid, valproic acid, caffeine and lithium chloride.

| | Retinoic acid ($n = 4$) | | | Valproic acid ($n = 4$) | | | Caffeine ($n = 3$) | | | Lithium chloride ($n = 3$) | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | $LC_{50}$ (mM) | $EC_{50}$ (mM) | TI ($LC_{50}/EC_{50}$) | $LC_{50}$ (mM) | $EC_{50}$ (mM) | TI ($LC_{50}/EC_{50}$) | $LC_{50}$ (mM) | $EC_{50}$ (mM) | TI ($LC_{50}/EC_{50}$) | $LC_{50}$ (mM) | $EC_{50}$ (mM) | TI ($LC_{50}/EC_{50}$) |
| 24 hpf | $1.61 \times 10^{-3}$ | $9.43 \times 10^{-5}$ | 17.07 | 3.06 | 1.61 | 1.90 | 4.13 | 3.81 | 1.08 | 115.2 | / | / |
| 48 hpf | $1.26 \times 10^{-3}$ | $5.78 \times 10^{-6}$ | 217.99 | 2.09 | 0.72 | 2.90 | 3.67 | 1.39 | 2.64 | 115.1 | / | / |
| 72 hpf | $0.56 \times 10^{-3}$ | $9.32 \times 10^{-7}$ | 600.86 | 1.36 | 0.48 | 2.83 | 3.64 | 0.4 | 9.10 | 115.3 | 38.49 | 2.99 |
| 144 hpf | $2.58 \times 10^{-5}$ | $3.44 \times 10^{-7}$ | 75.00 | 0.84 | 0.09 | 9.33 | 1.48 | 0.19 | 7.79 | 53.43 | 10.71 | 4.99 |

*Notes*: Abbreviation used: (hpf) hours post fertilization; (/) cannot be calculated.

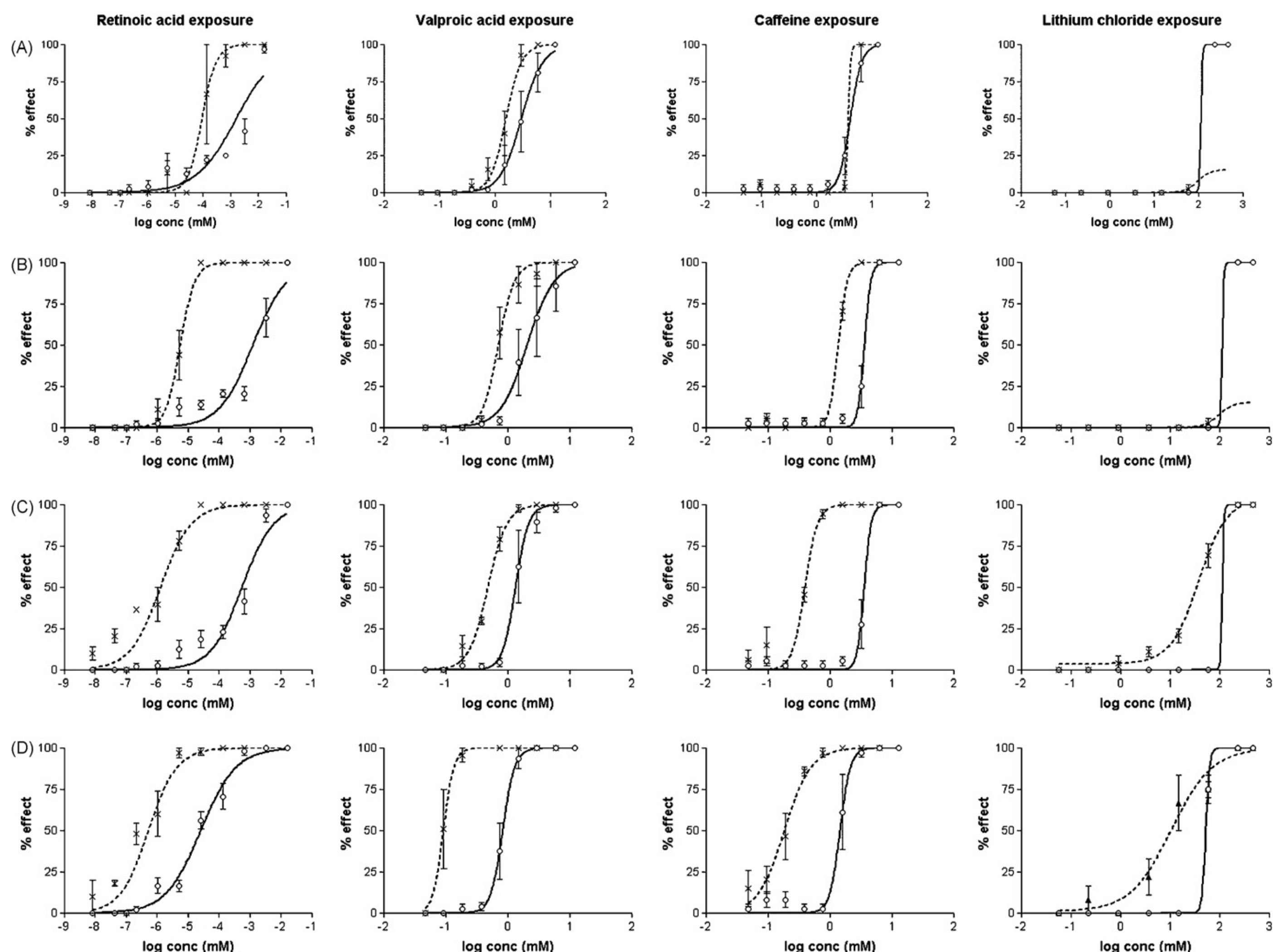


**Fig. 4.** Concentration–response curves for malformation (×, dotted line) and mortality (o, full line) for retinoic acid ($n$ = 4) valproic acid ($n$ = 4) caffeine ($n$ = 3) and lithium chloride ($n$ = 3) at (A) 24 hpf; (B) 48 hpf; (C) 72 hpf and (D) 144 hpf. %Effect (mean ± SD) is shown versus the logarithm of the concentrations tested (log mM).

(data not shown). Saccharin induced mortality (91.66%) as soon as 24 hpf when embryos were exposed to 55 mM but furthermore, at later time points and lower concentrations there were no effects observed ($n$ = 3) (data not shown). Due to the lack of 50% effect levels for glucose and saccharin, in the range tested, no concentration–response curves could be generated or TI values calculated. Therefore, we can only state a no observed effect concentration for these compounds. A concentration up to 27.9 mM glucose or saccharin had no effect on zebrafish development.

## 4. Discussion

In this study, we optimized and standardized a screening procedure to identify the specific teratogenic potential of chemicals compared to overall embryotoxicity by use of the zebrafish embryo model. We determined the optimal conditions to monitor embryo development to 144 hpf. Experiments with different multi-well plates showed that embryos placed in 96-well plates are more likely to have skeletal deformities in comparison to embryos placed in 24- or 48-well plates. Although other high-throughput studies have been performed in 96- or even 384-well plates, exposure times in these studies did not exceed 72 hpf [34,35] or solutions were renewed daily [36]. It may be possible that the quality of the medium in which the embryos resided diminished over time due to build up of toxic waste. Renewal of solutions after hatching was tested and did not affect the outcome in our set-up. The cause of spontaneous malformations remains unknown but may be attributed to limited space at the onset of swimming. In order to maintain a high-throughput experimental set-up that allows for minimal manipulations, 24-well plates were selected as the recipient in which further experiments should be conducted, while control groups showed <10% effect.

Vehicle (DMSO) toxicity was also evaluated. Consistent with [37], DMSO at levels below 1.5% can be used up to 96 hpf but at 1.5% did induce adverse effects at 144 hpf. Thus, the final DMSO concentration has been set to 0.1% for this experimental set-up. With regards to the positive and negative test compounds, we assume a teratogenic effect for TI = 1 or higher. All positive controls were classified as teratogenic, although lithium chloride only showed teratogenic effects at later developmental time points (72 and 144 hpf). Negative controls failed to return computable TI values at concentrations tested here, up to 55 mM. For D-glucose, no teratogenicity or embryotoxicity was observed. No abnormalities were observed in embryos exposed to the highest concentration of saccharin tested; hence, saccharin was classified as a non-teratogen; however, higher concentrations of saccharin were embryotoxic.

TI values can be used to rank teratogenic compounds, e.g. the higher the TI value the greater the teratogenic potential a

compound will demonstrate. Retinoic acid was the most teratogenic compound of compounds tested here, and furthermore the concentration levels with significant effects were the lowest for retinoic acid—a strong teratogen across species and a common reference compound for validating developmental toxicity [38–40]. The teratogenic mechanism of retinoic acid is well known [41]. Exogenous vitamin A is teratogenic in mice, rats, guinea pigs, hamsters, rabbits, dogs, pigs, chicks and monkeys [42], and zebrafish as well. Common malformations induced by retinoic acid in rats exposed gestational days 2–4 until day 16, include exencephaly, cleft palate, spina bifida, eye defects, hydrocephaly and shortening of the mandible and maxilla [43]. Other teratogenic targets include the heart, skeleton and limbs [41]. We observed similar malformations in zebrafish—eyes, otoliths and somites, cardiovascular defects and edema at the early developmental stages (24–48 hpf) and skeletal deformities (72–144 hpf). Despite the fact that all trans-retinoic acid is readily oxidized and/or isomerized, especially in the presence of oxidants including air, light and excessive heat [44], we did not refresh the solutions during the whole duration of the experiments for purposes of high-throughput screening. Adverse effects observed however, showed a clear time-dependent course with lower concentrations that showed no effects and lower $EC_{50}$ values at later time points of evaluation. Furthermore, another study [45] showed that a short pulse (0.5, 1 or 2 h) of treatment with all trans-retinoic acid is almost as effective as a longer treatment (22 h). Thus, all trans-retinoic acid is taken up readily by the zebrafish embryos and that it remains stored, possibly, within the lipophilic yolk [45].

Sodium valproate produced an increase in congenital anomalies when tested in mice, rats, rabbits and monkeys (in high doses) [46]. Case reports of valproic acid induced teratogenic effects in humans began to appear in 1980. The risk of neural tube defects after prenatal exposure to valproic acid has been estimated to be 1–2%, which is 10–20 times higher than the background rate. Limb anomalies are also increased. Numerous reports [47–49] also described a constellation of minor terata that are associated with *in utero* exposure to valproic acid. These features are now collectively referred to as Fetal Valproate Syndrome and include a consistent presentation of craniofacial abnormalities [50]. Terata induced by valproic acid suggest interference with pattern formation similar to retinoic acid. In zebrafish, we could also observe malformations induced by valproic acid exposure that were very similar to those seen when embryos were exposed to retinoic acid.

Although the association between coffee and/or caffeine uptake and the incidence of teratogenic effects in human fetuses seems very weak or absent and thus remaining controversial, caffeine can induce malformations in different animal species (rat, mice) [51]. A possible explanation for this discrepancy is that human consumption of coffee occurs in multiple distinct moments of intake during the day while in animal studies, high doses of caffeine are administered at once. In healthy adults, caffeine is absorbed very rapidly and its half-life ranges between 2.5 and 6 h [51]. It seems that there are relatively few types of malformations induced by caffeine. Those most frequently observed are malformations of the limbs and digits, craniofacial malformations and delays in ossification of limbs, jaw and sternum [52–57]. Caffeine has teratogenic effects on the fetal cardiovascular system [58]. In this study with zebrafish, the absence of blood circulation and the presence of skeletal deformities were observed. Although a relatively small number of characteristics were evaluated for this study, in comparison to animal studies, the teratogenic effects that were present in zebrafish, are again in agreement with observations in mammals.

The results of various studies and a small number of case reports indicate that lithium is a “weak” human teratogen. The main effects attributable to lithium are cardiac malformations and babies with increased birth weight [59]. In two cohort studies, risk ratios of 3.0 (95% confidence interval (CI), 1.2–7.7) and 1.5 (95% CI, 0.4–6.8) for all congenital anomalies have been observed [60]. Lithium treatment protocols designed to produce human therapeutic serum levels have no adverse developmental effects on rats, mice, rabbits or monkeys; however, in rats and mice, higher doses of lithium are teratogenic causing a wide spectrum of birth defects including cleft palate, exencephaly and skeletal defects [59]. Other anomalies observed are species-, strain- or time-dependent. Treatment of Sprague–Dawley rats with lithium chloride from days one through 16 resulted in defects of the eye, ear and cleft palate [61]. On the other hand, an increased frequency of exencephaly, kinked spinal cord and dilation of the fourth ventricle was reported in JBT/Jd mice after treatment with lithium carbonate during the critical period of organogenesis [62]. In zebrafish, skeletal deformities are most frequently observed although kinked chorda were also present in some cases. These findings are in agreement with what was observed most frequently in rats and mice. Klein and Melton compared developmental effects in *Xenopus laevis* after exposure to lithium chloride or lithium acetate and showed that they were mediated specifically by the lithium ion and not by other monovalent cations or by chloride [63]. This indicates that the results from studies with different lithium salts can be compared.

For the compounds that were used as negative control compounds in this study, D-glucose and saccharin, no evidence was found for potential teratogenic effects in animals or humans [64,65].

Experimental results, supported by literature, demonstrate that the teratogenic assay with zebrafish has provided accurate identification for the compounds tested. We tested a total of 6 compounds in this study, which would require at least 560 animals if OECD tests (OECD TG 414, 416, 421 and 422) [2–5] have to be performed, with a minimum duration of 341 days. By comparison, the zebrafish assay could provide results within a few weeks and no adult fish have to be exposed. Beside zebrafish, a number of other alternative methods have been proposed. And even though three alternative methods, namely the EST, the MM and the WEC test have been involved in a validation study by ECVAM (European Centre for Validation of Alternative Methods) and it was shown that these methods were able to discriminate teratogens from non-teratogens [38,39,66], some concerns related to these methods remain. For instance, the classification of compounds in the EST is based on cytotoxicity, a non-specific endpoint, and the differentiation of stem cells into cardiac muscle cells is only one of so many mechanistic endpoints in a developing organism. These arguments also apply to the MM test where growth of limb bud cells and differentiation into chondrocytes is assessed. Furthermore, for MM the test system still involves the sacrifice of pregnant mammals. WEC also requires the sacrifice of pregnant mammals for harvesting early embryos.

The use of zebrafish as a model species offers practical advantages over other systems. Zebrafish embryos, until free-living larval stages, are a whole embryo model but not subject to the European Council Directive 86/609/EEC regarding the protection of animals used for experimental and other scientific purposes to enable complex mechanisms in teratogenesis. Due to rapid development the assay requires short incubations and is cost-effective for medium- to high-throughput screening of chemicals. A number of groups have reported exploratory studies with the zebrafish [35,67,68]. In these studies, development of the embryos was monitored for maximal 3 days post fertilization and the number of endpoints evaluated were limited. Based on the experience with lithium chloride it is important to note that valuable information becomes available if the zebrafish embryo assay is extended to time points post-hatching. Early observations within 48 h of development [67,68] may not be sufficient to classify this compound (lithium chloride) as teratogenic. For retinoic acid, valproic acid and caffeine, the no observed effect concentration for teratogenic effects is shifted to lower concentrations as a function of observations at 72 and 144 hpf.

Despite the advantages noted above there are shortcomings with the model that require further research. Extrapolation from data acquired with the zebrafish to humans remains an issue, as it does for other methods [69]. We cannot define the exact dose of compound that reaches the embryo itself since the chorion of the embryo can act as a biological protective barrier [70,71] or the actual uptake of a compound by the embryo. Another issue remains the evaluation of compounds that are metabolized, although it is known that Cyp genes are present in zebrafish [72–74]. A possibility to overcome the metabolism is the use of an exogenous mammalian metabolic activation system [67].

In conclusion, we demonstrated that the method described can offer a valid alternative for developmental toxicity testing. Further research on these specific issues and tests for an extended list of compounds, will provide scientific information for this assay to be integrated into screening programs for the hazard identification of chemicals. In this way it can also contribute to a reduction of the use of mammalian organisms for developmental toxicity assessment.

## Conflict of interest

The authors declare that there are no conflicts of interest.

## Acknowledgements

Ingrid Selderslaghs was supported by a VITO fellowship. This work was supported by grants from the John Hopkins Center for Alternatives to Animal testing (CAAT 2006-28 and 2007-22) and Henkel-Phenion. For technical assistance the authors are grateful to Francis Boonen and Guy Geukens.

## References

[1] Oxendine SL, Cowden J, Hinton DE, Padilla S. Adapting the medaka embryo assay to a high-throughput approach for developmental toxicity testing. Neurotoxicology 2006;27(5):840–5.
[2] OECD. OECD guidelines for the testing of chemicals. No. 414: prenatal developmental toxicity study. Paris, France: Organisation for Economic Cooperation and Development; 2001. 6 pp.
[3] OECD. OECD guidelines for the testing of chemicals. No. 416: two-generation reproduction toxicity study. Paris, France: Organisation for Economic Cooperation and Development; 2001. 8 pp.
[4] OECD. OECD guidelines for the testing of chemicals. No. 421: reproduction/developmental toxicity screening test. Paris, France: Organisation for Economic Cooperation and Development; 1995. 10 pp.
[5] OECD. OECD guidelines for the testing of chemicals. No. 422: combined repeated dose toxicity study with the reproduction/developmental toxicity screening test. Paris, France: Organisation for Economic Cooperation and Development; 1996. 14 pp.
[6] Piersma AH. Validation of alternative methods for developmental toxicity testing. Toxicol Lett 2004;149(1–3):147–53.
[7] Piersma AH. Alternative methods for developmental toxicity testing. Basic Clin Pharmacol Toxicol 2006;98(5):427–31.
[8] Seiler A, Visan A, Buesen R, Genschow E, Spielmann H. Improvement of an in vitro stem cell assay for developmental toxicity: the use of molecular endpoints in the embryonic stem cell test. Reprod Toxicol 2004;18(2):231–40.
[9] Spielmann H, Pohl I, Döring B, Liebsch M, Moldenhauer F. The embryonic stem cell test, an in vitro embryotoxicity test using two permanent mouse cell lines: 3T3 fibroblast and embryonic stem cells. In vitro Toxicol 1997;10(1):119–27.
[10] Flint OP. In vitro tests for teratogens: desirable endpoints, test batteries and current status of the micromass teratogen test. Reprod Toxicol 1993;7(Suppl. 1):103–11.
[11] Kistler A, Tsuchiya T, Tsuchiya M, Klaus M. Teratogenicity of arotinoids (retinoids) in vivo and in vitro. Arch Toxicol 1990;64(8):616–22.
[12] Walmod PS, Berezin A, Gallagher HC, Gravemann U, Lepekhin EA, Belman V, et al. Automated in vitro screening of teratogens. Toxicol Appl Pharmacol 2002;181(1):1–15.
[13] Brown NA, Fabro S. Quantitation of rat embryonic development in vitro: a morphological scoring system. Teratology 1981;24(1):65–78.
[14] Jelinek R, Peterka M, Rychter Z. Chick embryotoxicity screening test—130 substances tested. Int J Exp Biol 1985;23:588–95.
[15] Collins TF. Teratological research using in vitro systems. V. Nonmammalian model systems. Environ Health Perspect 1987;72:237–49.
[16] Law JM. Issues related to the use of fish models in toxicologic pathology: session introduction. Toxicol Pathol 2003;31(Suppl.):49–52.
[17] Dumont J, Schultz T, Epler R. The response of the FETAX model to mammalian teratogens. Teratology 1983;27:39A–40A.
[18] Hill AJ, Teraoka H, Heideman W, Peterson RE. Zebrafish as a model vertebrate for investigating chemical toxicity. Toxicol Sci 2005;86(1):6–19.
[19] Kimmel CB, Ballard WW, Kimmel SR, Ullmann B, Schilling TF. Stages of embryonic development of the zebrafish. Dev Dyn 1995;203(3):253–310.
[20] Peterson RE, Theobald HM, Kimmel GL. Developmental and reproductive toxicity of dioxins and related compounds: cross-species comparisons. Crit Rev Toxicol 1993;23(3):283–335.
[21] Chiu LL, Cunningham LL, Raible DW, Rubel EW, Ou HC. Using the zebrafish lateral line to screen for ototoxicity. J Assoc Res Otolaryngol 2008;9(2):178–90.
[22] Tran TC, Sneed B, Haider J, Blavo D, White A, Aiyejorun T, et al. Automated, quantitative screening assay for antiangiogenic compounds using transgenic zebrafish. Cancer Res 2007;67(23):11386–92.
[23] Richards FM, Alderton WK, Kimber GM, Liu Z, Strang I, Redfern WS, et al. Validation of the use of zebrafish larvae in visual safety assessment. J Pharmacol Toxicol Methods 2008;58(1):50–8.
[24] Berghmans S, Hunt J, Roach A, Goldsmith P. Zebrafish offer the potential for a primary screen to identify a wide variety of potential anticonvulsants. Epilepsy Res 2007;75(1):18–28.
[25] Winter MJ, Redfern WS, Hayfield AJ, Owen SF, Valentin JP, Hutchinson TH. Validation of a larval zebrafish locomotor assay for assessing the seizure liability of early-stage development drugs. J Pharmacol Toxicol Methods 2008;57(3):176–87.
[26] OECD. OECD guidelines for the testing of chemicals. No. 210: Fish, early-life stage toxicity test. Paris, France: Organisation for Economic Cooperation and Development; 1992. 18 pp.
[27] OECD. OECD guidelines for the testing of chemicals. No. 212: Fish, short-term toxicity test on embryo and sac-fry stages. Paris, France: Organisation for Economic Cooperation and Development; 1998. 9 pp.
[28] ISO. ISO 15088:2007 "Water quality—Determination of acute toxicity of waste water to zebrafish eggs (*Danio rerio*)". International Organisation for Standardization; 2007.
[29] Lammer E, Carr GJ, Wendler K, Rawlings JM, Belanger SE, Braunbeck T. Is the fish embryo toxicity test (FET) with the zebrafish (*Danio rerio*) a potential alternative for the fish acute toxicity test? Comp Biochem Physiol C Toxicol Pharmacol 2009;149(2):196–209.
[30] Burns CG, Milan DJ, Grande EJ, Rottbauer W, MacRae CA, Fishman MC. High-throughput assay for small molecules that modulate zebrafish embryonic heart rate. Nat Chem Biol 2005;1(5):263–4.
[31] Murphey RD, Stern HM, Straub CT, Zon LI. A chemical genetic screen for cell cycle inhibitors in zebrafish embryos. Chem Biol Drug Des 2006;68(4):213–9.
[32] Balls M. The three rs: looking back ... and forward. Altex 2006;23(Suppl.): 29–32.
[33] Westerfield M. The zebrafish book. A guide for the laboratory use of zebrafish (*Danio rerio*). 4th ed. Eugene: University of Oregon Press; 2000.
[34] MacRae CA, Peterson RT. Zebrafish-based small molecule discovery. Chem Biol 2003;10(10):901–8.
[35] Peterson RT, Link BA, Dowling JE, Schreiber SL. Small molecule developmental screens reveal the logic and timing of vertebrate development. Proc Natl Acad Sci USA 2000;97(24):12965–9.
[36] MacPhail RC, Brooks J, Hunter DL, Padnos B, Irons TD, Padilla S. Locomotion in larval zebrafish: influence of time of day, lighting and ethanol. NeuroToxicology 2009;30(1):52–8.
[37] Hallare A, Nagel K, Kohler H-R, Triebskorn R. Comparative embryotoxicity and proteotoxicity of three carrier solvents to zebrafish (*Danio rerio*) embryos. Ecotoxicol Environ Saf 2005;63(3):378–88.
[38] Genschow E, Spielmann H, Scholz G, Pohl I, Seiler A, Clemann N, et al. Validation of the embryonic stem cell test in the international ECVAM validation study on three in vitro embryotoxicity tests. Altern Lab Anim 2004;32(3):209–44.
[39] Spielmann H, Genschow E, Brown NA, Piersma AH, Verhoef A, Spanjersberg MQ, et al. Validation of the rat limb bud micromass test in the international ECVAM validation study on three in vitro embryotoxicity tests. Altern Lab Anim 2004;32(3):245–74.
[40] Walmod PS, Gravemann U, Nau H, Berezin V, Bock E. Discriminative power of an assay for automated in vitro screening of teratogens. Toxicol In Vitro 2004;18(4):511–25.
[41] Ross SA, McCaffery PJ, Drager UC, De Luca LM. Retinoids in embryonal development. Physiol Rev 2000;80(3):1021–54.
[42] Geelen JA. Hypervitaminosis A induced teratogenesis. CRC Crit Rev Toxicol 1979;6(4):351–75.
[43] Collins MD, Mao GE. Teratology of retinoids. Annu Rev Pharmacol Toxicol 1999;39:399–430.
[44] Barua AB, Furr HC. Properties of retinoids. Structure, handling, and preparation. Mol Biotechnol 1998;10(2):167–82.
[45] Herrmann K. Teratogenic effects of retinoic acid and related substances on the early development of the zebrafish (*Brachydanio rerio*) as assessed by a novel scoring system. Toxicology In Vitro 1995;9(3):267–83.
[46] Ornoy A. Neuroteratogens in man: an overview with special emphasis on the teratogenicity of antiepileptic drugs in pregnancy. Reprod Toxicol 2006;22(2):214–26.
[47] Ardinger HH, Atkin JF, Blackston RD, Elsas LJ, Clarren SK, Livingstone S, et al. Verification of the fetal valproate syndrome phenotype. Am J Med Genet 1988;29(1):171–85.
[48] DiLiberti JH, Farndon PA, Dennis NR, Curry CJ. The fetal valproate syndrome. Am J Med Genet 1984;19(3):473–81.

[49] Wyszynski DF, Nambisan M, Surve T, Alsdorf RM, Smith CR, Holmes LB. Increased rate of major malformations in offspring exposed to valproate during pregnancy. Neurology 2005;64(6):961–5.
[50] Stodgell CJ, Ingram JL, O'Bara M, Tisdale BK, Nau H, Rodier PM. Induction of the homeotic gene Hoxa1 through valproic acid's teratogenic mechanism of action. Neurotoxicol Teratol 2006;28(5):617–24.
[51] Nehlig A, Debry G. Potential teratogenic and neurodevelopmental consequences of coffee and caffeine exposure: a review on human and animal data. Neurotoxicol Teratol 1994;16(6):531–43.
[52] Aliverti V, Bonanomi L, Giavini E, Leone VG, Mariani L. The extent of fetal ossification as an index of delayed development in teratogenic studies on the rat. Teratology 1979;20(2):237–42.
[53] Collins TF, Welsh JJ, Black TN, Ruggles DI. A study of the teratogenic potential of caffeine ingested in drinking-water. Food Chem Toxicol 1983;21(6):763–77.
[54] Elmazar MM, McElhatton PR, Sullivan FM. Studies on the teratogenic effects of different oral preparations of caffeine in mice. Toxicology 1982;23(1):57–71.
[55] Fujii T, Nishimura H. Teratogenic actions of some methylated xanthines in mice. Okajimas Folia Anat Jpn 1969;46(4):167–75.
[56] Fujii T, Sasaki H, Nishimura H. Teratogenicity of caffeine in mice related to its mode of administration. Jpn J Pharmacol 1969;19(1):134–8.
[57] Smith SE, McElhatton PR, Sullivan FM. Effects of administering caffeine to pregnant rats either as a single daily dose or as divided doses four times a day. Food Chem Toxicol 1987;25(2):125–33.
[58] Matsuoka R, Uno H, Tanaka H, Kerr CS, Nakazawa K, Nadal-Ginard B. Caffeine induces cardiac and other malformations in the rat. Am J Med Genet Suppl 1987;3:433–43.
[59] Giles JJ, Bannigan JG. Teratogenic and developmental effects of lithium. Curr Pharm Des 2006;12(12):1531–41.
[60] Cohen LS, Friedman JM, Jefferson JW, Johnson EM, Weiner ML. A reevaluation of risk of in utero exposure to lithium. JAMA 1994;271(2):146–50.
[61] Wright T, Hoffman L, Davies J. Teratogenic effects of lithium in rats. Teratology 1971;4:151–6.
[62] Jurand A. Teratogenic activity of lithium carbonate: an experimental update. Teratology 1988;38(2):101–11.
[63] Klein PS, Melton DA. A molecular mechanism for the effect of lithium on development. Proc Natl Acad Sci USA 1996;93(16):8455–9.
[64] Buchanan TA, Denno KM, Sipos GF, Sadler TW. Diabetic teratogenesis. In vitro evidence for a multifactorial etiology with little contribution from glucose per se. Diabetes 1994;43(5):656–60.
[65] Dropkin RH, Salo DF, Tucci SM, Kaye GI. Effects on mouse embryos of in utero exposure to saccharin: teratogenic and chromosome effects. Arch Toxicol 1985;56(4):283–7.
[66] Piersma AH, Genschow E, Verhoef A, Spanjersberg MQ, Brown NA, Brady M, et al. Validation of the postimplantation rat whole-embryo culture test in the international ECVAM validation study on three in vitro embryotoxicity tests. Altern Lab Anim 2004;32(3):275–307.
[67] Busquet F, Nagel R, von Landenberg F, Mueller SO, Huebler N, Broschard TH. Development of a new screening assay to identify proteratogenic substances using zebrafish *Danio rerio* embryo combined with an exogenous mammalian metabolic activation system (mDarT). Toxicol Sci 2008;104(1):177–88.
[68] Nagel R, Dar T. The embryo test with the Zebrafish *Danio rerio*—a general model in ecotoxicology and toxicology. Altex 2002;19(Suppl. 1):38–48.
[69] Piersma AH, Janer G, Wolterink G, Bessems JG, Hakkert BC, Slob W. Quantitative extrapolation of in vitro whole embryo culture embryotoxicity data to developmental toxicity in vivo using the benchmark dose approach. Toxicol Sci 2008;101(1):91–100.
[70] Gellert G, Heinrichsdorff J. Effect of age on the susceptibility of zebrafish eggs to industrial wastewater. Water Res 2001;35(15):3754–7.
[71] Hagedorn M, Kleinhans FW, Artemov D, Pilatus U. Characterization of a major permeability barrier in the zebrafish embryo. Biol Reprod 1998;59(5):1240–50.
[72] Corley-Smith GE, Su HT, Wang-Buhler JL, Tseng HP, Hu CH, Hoang T, et al. CYP3C1, the first member of a new cytochrome P450 subfamily found in zebrafish (*Danio rerio*). Biochem Biophys Res Commun 2006;340(4):1039–46.
[73] Tseng HP, Hseu TH, Buhler DR, Wang WD, Hu CH. Constitutive and xenobiotics-induced expression of a novel CYP3A gene from zebrafish larva. Toxicol Appl Pharmacol 2005;205(3):247–58.
[74] Wang-Buhler JL, Lee SJ, Chung WG, Stevens JF, Tseng HP, Hseu TH, et al. CYP2K6 from zebrafish (*Danio rerio*): cloning, mapping, developmental/tissue expression, and aflatoxin B1 activation by baculovirus expressed enzyme. Comp Biochem Physiol C Toxicol Pharmacol 2005;140(2):207–19.