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5. Zebrafish as a model for developmental toxicity assessment

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Abstract. The zebrafish embryo has emerged as promising alternative model for traditional in vivo developmental toxicological screening due to their advantageous characteristics as their small size and transparency. In this paper, we reviewed the applicability of the zebrafish embryo model in some relevant areas to human toxicology as developmental toxicity, cardiovascular toxicity and neurotoxicity (behavioral assessment). Despite the promising results, further optimization and testing of more substances as well as a harmonized methodology is needed to streamline the methods and make the assay conducive to medium-throughput.

Introduction

The potential toxicity of a chemical has traditionally been carried out through in vivo mammalian screening approaches. Traditional guideline methods are laborious, costly, require large number of animals and attract increasingly ethical concerns [1]. Moreover, animal testing will increase

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dramatically over next decade as a consequence of implementation of EU initiative for the Registration, Evaluation and Authorization of Chemicals, REACH [2]. Reproductive and developmental toxicity studies will use by far the most animals and resources within REACH (61% of animals and 54% of resources). Thus, the use of alternative methods in hazard assessment is encouraged and animal experiments should only be used as a last resort [3]. In addition to the REACH initiatives, it has been estimated that out of 10,000 new drug entities that a pharmaceutical may start with, only one or two are finally approved by the European Medicines Agency (EMA) at an estimated cost of €1,172 million [4]. A large proportion of this cost is due to animal testing. The use of alternative methods at multiple stages in the drug discovery process would potentially reduce the attrition rate and costs by reducing the use of mammals and unnecessary clinical trials. The use of screening assays before clinical phases to select the best candidates is also one strategy to identify potential new active pharmaceuticals ingredients.

Fish have been used for years to assess the ecotoxicity of xenobiotics, but during the last years assays using embryonic stages of the zebrafish (*Danio rerio*) have attracted the attention of toxicologists due to their several advantages. In particular, in compliance with international animal welfare regulations, the fish embryo provides an ethically acceptable small-scale analysis system with complexity of a complete organism [5-7]

Most advanced and promising in the field of human hazard prediction is the use of the zebrafish embryo test (ZFET) to screen for developmental disorders as an indicator of teratogenic effects. Modifications of the ZFET protocol allow the use of this model also for the detection of specific organ toxicity such as cardiotoxicity, neurotoxicity, hepatotoxicity or nephrotoxicity [8].

In the following sections, the applications of the zebrafish embryo test in toxicology are reviewed as a screening level tool and as a system to predict mammalian developmental toxicity. Considering the current research activity on this topic, and based in our own research experience, we have selected some examples of the use of the zebrafish embryo model in toxicology. Additionally, we have included some of our last results in the field.

1. The zebrafish embryo as a model organism

Zebrafish is a tropical freshwater teleost fish native to the rivers of India and South Asia [9]. Adult fishes are simple and inexpensive to raise and maintain. One pair of adults routinely lays hundreds of fertilized eggs in a single cross. Large-scale breeding chambers allow the efficient and reliable generation of thousands of embryos each day (Fig. 1).

Figure 1. Custom made breeding chamber. A grid is located in the bottom in order to avoid predation of the eggs by the parent fishes. Plastic plants and marbles can be placed in the tank in order to stimulate the spawning. The shape of the tank, as a funnel on the bottom, allows simple release of the newly spawned eggs.

The development of zebrafish is very similar to the embryogenesis in higher vertebrates, but, unlike mammals, the zebrafish egg develops outside the mother. Moreover, the embryos themselves are transparent during the first few days of their lives allowing an easy visualization of internal organ-tissues that facilitates developmental and organ toxicity studies. Also its development is very fast and has been well characterized, including morphological, biochemical and physiological information at all stages of early development [10].

During the first 24 hours after fertilization, all major organs are formed and within 3 days the fish hatches. After 3-4 months zebrafish are sexually mature and can generate new offspring.

Besides this, the small size of the embryo allows the ability of culture large number of zebrafish embryos in small volumes of media facilitating

rapid toxicity testing of compounds, while using a minimal amount of compound [11]. All the above mentioned properties of the zebrafish model represent a reduction on the experimental time and cost.

Zebrafish has been used predominantly in fundamental research (developmental biology and molecular genetics), so a lot of resources are available (genetic tools, mutant lines…) which the community can get the most benefit. In addition, zebrafish shares a high degree of homology with the human genome (about a 70%) [12], as well as structural similarities (about 86% of orthologs of human drug targets) [13]. All these unique advantages make using zebrafish an attractive alternative that also represents an advance towards the aim of reducing and refining animal use in research [14].

2. Zebrafish embryo assays in toxicology

Teratogenesis

The assessment of potential developmental toxicity is an integral part of European (and international) regulation for the risk assessment of pharmaceuticals, industrial chemicals, food additives, biocides and plant protection products. The assessment is performed based on OECD guidelines to allow international harmonization. Particularly, in teratogenesis tests, pregnant laboratories animals of two species, typically rats and rabbits, are exposed to the investigated chemical during the period of major organogenesis and offspring is monitored for endpoints such as death, growth changes, and morphological abnormalities. The use of mammalian models is laborious, time-consuming and results in the suffering of animals.

Ever since the thalidomide tragedy, there has been a presumed need to routinely use two species for developmental toxicity testing. Next to ethical concerns, animal experiments have been also criticized because of the partially weak reliability for the prediction of human teratogenicity [15]. Further problems have arisen due to the new EU cosmetics directive, which excludes any animal testing for cosmetic ingredients. Therefore, there is an increasing demand to develop alternative *in vitro* methods.

Up to date a few alternative approaches have been proposed for teratogenicity testing of chemicals and drugs. These approaches require either cultured cell lines [16], dissociated cells of embryonic buds or the midbrain of rat embryos [17], or the culture of whole embryo rodents [18] and lower vertebrates (e.g. Xenopus and zebrafish)[19].

Results from numerous small-scale pilot studies with zebrafish embryos have been shown to correctly classify mammalian teratogens and non-teratogens with an overall concordance of 72-92% (Table 1). These assays are focused on three manifestations of deviant development: death, malformation and growth retardation [20]. However, they applied different experimental protocols and the number and the variety of assayed substances were limited. Currently there is no consensus about the optimal procedure in some basic features as the specific endpoints and scoring systems to use, the time of exposure and the stage of embryonic/larval development to do the observations. There is an increasing common interest to harmonize zebrafish developmental toxicity assays in order to ensure high concordance with mammalian data and increase cross-laboratory reliability [21].

Table 1. List of the published zebrafish teratogenicity assays with its overall concordance with mammalian data. Exposure was done during different windows of development and the teratogenic potential of chemicals was based on different criteria.

Reference	Chemicals tested	Exposure window (hpf)	Mammalian concordance (%)	Teratogenic potential
Nagel, [22]	41	1 to 48	88	LC_{50}/EC_{50}
Chapin et al., [23] [a]	12	24 to 96	83	LD_{50}/LOAEL
Eimon and Rubinstein, [11] [b]	18	1 to 24/48	72	n/d
Eimon and Rubinstein, [11] [c]	24	4-6 to 120	92	NOAEL/LC_{25}
Brannen et al., [24]	31	4-6 to 120	87	NOAEL/LC_{25}
Selderslaghs et al., [25]	27	2 to 144	81	LC_{50}/EC_{50}
Hermsen et al., [26]	14	1 to 72	n/d	BMC_{GMS}
Van den Bulck et al., [27]	15	4 to 96	75	Teratogenic endpoint and body burden
Gustafson et al., [21] [d]	40	4-6 to 120	85	NOAEL/LC_{25}

Note: a, collaborative pilot study of Phylonix, Inc. with Bristol-Myers Squibb. b, developed by DanioLabs Ltd and evaluated in a pilot study run by Pfizer, Inc. and ECVAM. c, results from Bristol-Myers Squibb group. d, compounds tested in the ring test-2. n/d, not defined. Hours post-fertilization (hpf).

In this regard, our group focused on achieving a better characterization of growth retardation in the zebrafish embryo test [28]. In this study the suitability of a biochemical endpoint, the measurement of acetylcholinesterase activity (AChE), as a marker of developmental delay in zebrafish embryos was explored. The expression of AChE starts early and increases with age along the embryo development. It is an easy, robust and sensitive endpoint that could be liable to automation and higher throughput than morphological endpoints like the measurement of head-trunk angle. Evidently, any substance with some specific action on AChE expression or activity, as AChE inhibitors, could produce results unrelated to developmental age. These substances can be easily discarded by means of an *in vitro* AChE activity assay.

The measurement of AChE activity allowed us to detect substances with a clear effect on developmental delay (valproic acid, methoxyacetic acid and boric acid) at non-teratogenic concentrations, thus increasing the sensitivity of the assay. As figure 2 shows, these substances presented a strong correlation between AChE activity and head-trunk angle with a slope similar to that obtained in normally developmental embryos at different developmental stages. However, our results do not allow concluding about

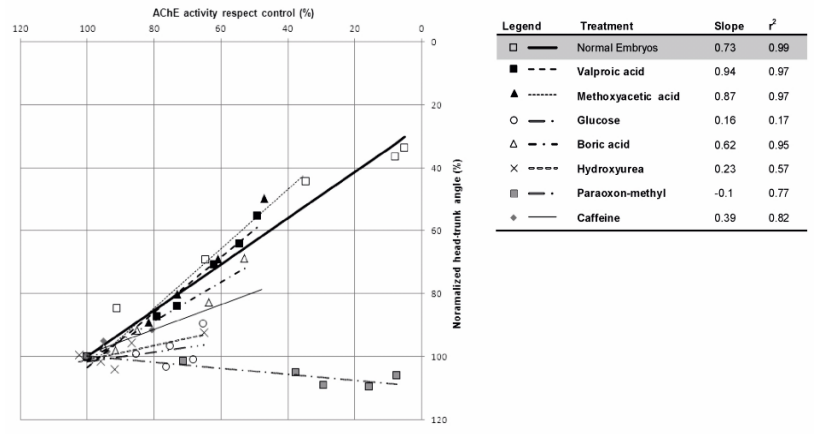


Figure 2. Correlation analysis between AChE activity values versus normalized head-trunk angle values [(180°- head-trunk angle)*100/(180°- control head-trunk angle)] in each compound that produced a significant change in AChE activity. Slope and coefficient of correlation of each simple linear regression. The correlation analysis of the values of normally developed embryos was obtained from the evaluation of the age-dependent AChE activity and head-trunk angle.

the discriminating efficiency of developmental retardation endpoints in the zebrafish assay to predict teratogenic potential in mammals. Probably, for a major part of substance, developmental delay is an unspecific effect unrelated or secondary to teratogenic activity.

Cardiovascular toxicity

Cardiotoxicity is a highly relevant area of toxicity assessment as drug candidates were frequently found to have cardiac adverse effects being a leading cause of drug withdraws [29]. Current available *in vivo* assays such as the Langendott-perfused rabbit heart model are laborious, costly and time-consuming [30]. Moreover, *in vitro* cardiotoxicity screenings such as the patch clam assay focusing on assessing the effects of compounds on potassium, sodium and calcium ion channels are limited by biological simplicity and an inability to detect drug–drug interactions [31]. Due to the limitations of both traditional mammalian models and *in vitro* approaches, researchers are showing increasing interest in zebrafish-based assays to assess cardiovascular safety and toxicity.

The cardiovascular system is the first major system to function within the embryo. Unlike the double circulation in mammals, the fish heart is two-chambered, consisting of an atrium and a ventricle separated by an atrioventricular valve. Zebrafish and mammalian heart exhibit a closed cardiovascular system, but organs and tissues of zebrafish embryos do not depend on the cardiac output for oxygen delivery. Embryos rely on oxygen diffusion through the skin from the swimming medium up to 14 days post-fertilization (dpf) [32]. Therefore, this feature permits embryos with severe cardiovascular defects to survive during the initial phase of embryonic development. By contrast, avian and mammalian embryos would die rapidly in the absence of a functional cardiovascular system.

Effects on cardiovascular system can be visually assessed in living zebrafish using a stereomicroscope and a microscope. Different cardiac and vascular abnormalities from developmental exposure to zebrafish have been reported (Table 2) being edema and heart rate change the most commonly assessed [33].

Several methods have been employed successfully in the larval fish to study heart function, for example, electrocardiogram [34], laser Doppler microscope technique [35] and laser confocal scanning microscopy [36]. However, these tools are labor-intensive, require special instrumentation and are not scalable for high-throughput screening. Therefore, we propose the use of a non-invasive method using simple light microscopy and a fast digital camera. Recent development in digital image analysis tools makes

Table 2. Cardiac and vascular abnormalities reported from developmental exposure to zebrafish (Reviewed by [33]).

Cardiac and vascular abnormalities
Heart function
Tachycardia (increased heart rate)
Bradycardia (slow heart rate)
Arrhythmia
Pericardial edema
Defective heart morphology
Altered heart size
Apoptosis in heart region/pericardial epithelium
Circulatory system
Aberrant vascular patterning in trunk/common cardinal vein
Abnormal/disorganized cranial vessels
Smaller blood vessels
Altered blood flow
Blood accumulation in yolk extension
Hemorrhage
Trunk edema
Hematopoietic system
Altered number of circulating erythrocytes
Abnormal erythrocytes

analysis of cardiovascular function, such as cardiac output, traveling speed of the blood cells, blood cell count, visualization and analysis of blood cells distribution in transparent zebrafish larvae easier [37].

The zebrafish is also used as a preclinical model in order to study drug-induced cardiac arrhythmias such as QT prolongation [38]. Drug-induced prolongation of the QT interval in the electrocardiogram usually results from concentration dependent inhibition of the hERG (the human Ether-à-go-go-Related Gene) potassium channel. Moreover, drugs blocking this potassium current either as an intended pharmacologic effect (eg. antiarrhythmics dofetilide and almokalant) or as an unwanted side-effect (eg. antihistamine drugs, antidepressive drugs and macrolide antibiotics) are potential human teratogens [39].

Numerous groups have screened pharmacologically relevant collections of compounds in zebrafish using bradycardia or/and atrioventricular dissociation as indicators of QT prolongation [30, 40, 41]. These assays were able to detect known QT prolonging drugs that block hERG such as terfenadine or cisapride, as well as compounds that block L-type calcium channels, not affecting the hERG channel.

In our case, we established the methodology for screening cardiovascular drugs using two reference compounds, terfenadine and isoprenaline (Fig. 3 and Fig. 4). Embryos exposed to terfenadine (10 µM) displayed a 2:1 atrioventricular block and bradychardia with a reduced cardiac output. In contrast, isoprenaline exposure increased the heart rate and cardiac output without causing an atrioventricular block.

Cardiovascular performance can also be assessed by recording the blood flow through the dorsal aorta. Scan lines obtained parallel to the flow of blood within the dorsal aorta were used to measure the cellular velocity and other cardiac indices. Figure 5 shows as an example the measured changes in blood flow velocity and diameter of the blood vessel in zebrafish embryos exposed to terfenadine and isoprenaline.

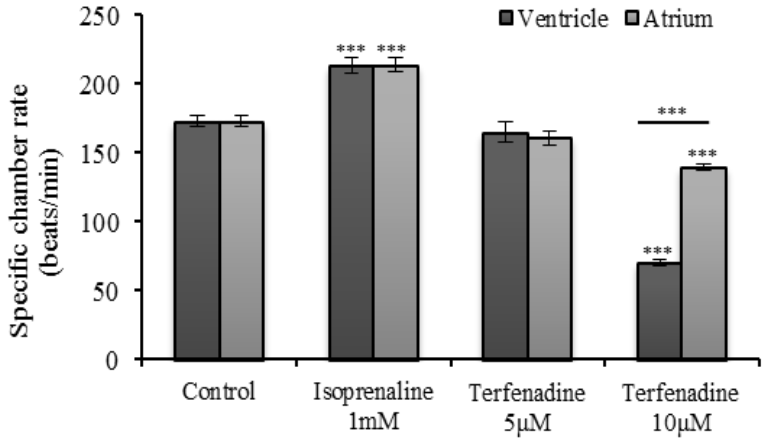
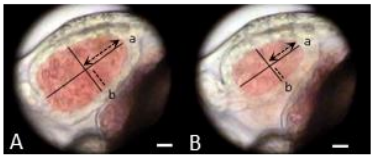


Figure 3. Atrial and ventricular rates of embryos exposed to terfenadine and isoprenaline for 3 h at 72 hpf. Values are mean ± SEM of two independent test (n= 6). Asterisks indicate significant difference at ***p<0.001, t-student test.



Treatment	Cardiac output (nL/min)
Control	40.2 ± 1.3
Terfenadine 5 µM	38.8 ± 3.6
Terfenadine 10 µM	20.8 ± 1.3***
Isoprenaline 1 mM	64.1 ± 5.3***

Figure 4. Representative end-diastolic (A) and end-systolic (B) images of a heart ventricle of zebrafish larva at 3 dpf. Calculation of ventricle volume during systole and diastole was based on the formula for the volume of a prolate spheroid: $4/3\pi ab^2$ where a represents the major axe radius and b of the minor axe radius of the ventricle image. Cardiac output (nl/min) = Stroke volume (end-diastolic volume – end-systolic volume) x heart rate. (Scale bar= 20µm).Table at the right shows the cardiac output of zebrafish embryos exposed to terfenadine and isoprenaline (n= 6). Asterisks indicate significant difference respect the control group at ***p<0.001, t-student test.

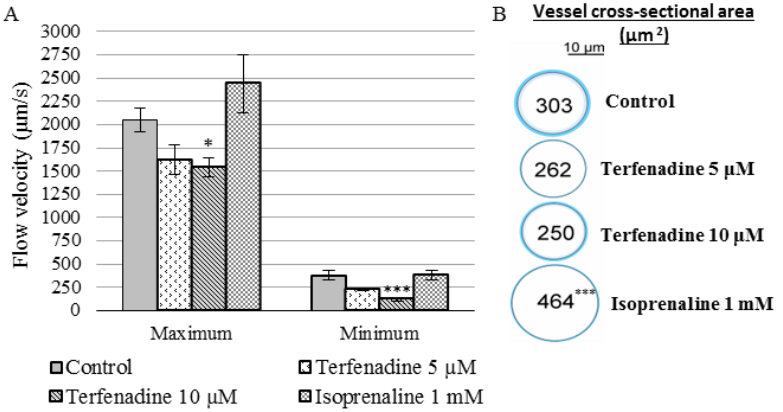


Figure 5. (A) Maximum and minimum blood cell velocity, measured from embryos treated with terfenadine and isoprenaline. Data are presented as mean ± SEM (n=6). (B) Mean vessel cross section represented as a black outline, while the surrounding color shading indicates the standard deviation of the mean. Numerical values indicate the corresponding vessel cross-sectional area (µm2). Asterisks indicate significant difference at *p<0.05 and ***p<0.001, t-student test.

In addition, several transgenic lines with heart tissue and vascular fluorescent reporters have been generated [42, 43] and can be really useful for detecting changes in specific tissues, to visualize cardiomyocytes and vascular endothelium as well as precursor cells.

These tools can contribute to a better understanding of the key cardiotoxic mechanisms in a whole-organism, cost-effective and medium high-throughput manner.

Behavioral assessment of neurotoxic effects

Current in vivo methods for the assessment of developmental neurotoxic compounds [44] are designed to screen for adverse effects of pre and postnatal exposures on the development and function of the nervous system. However, these guidelines are unsuitable for screening large number of chemicals for many reasons including low throughput, high cost, and questions regarding reliability [45]. Therefore, new, reliable, and efficient screening and assessment tools are needed for better identification, prioritization, and evaluation of chemicals with the potential to induce developmental neurotoxicity.

Larval zebrafish nervous system exhibits developmental, structural and pharmacological conservation with the mammalian nervous system [46]. Locomotor patterns that develop early in the larva [47] (see Fig. 6) can be monitored for systematic screening of the genes, pharmaceuticals and environmental toxicants that can influence behavior [48, 49]. Moreover,

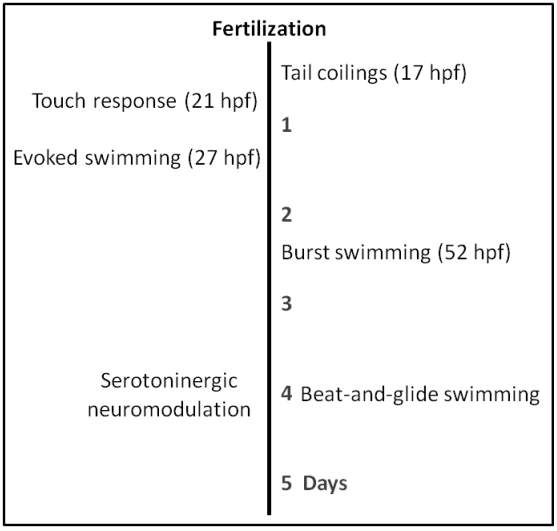


Figure 6. Chronological sequence of appearance of motility patterns during development of the zebrafish.

several studies suggest that zebrafish larvae are sensitive to neuroactive drugs and that their locomotor response is similar to that of mammals [50, 51].

Many assays have been developed in zebrafish larvae by taking advantage of its locomotor repertoire and inherent visual reflexes (Table 3). Among these is the visual motor response test that consists in brief (10-30 min) alternating periods of light and dark and is characterized by low (basal) locomotor activity under light exposure and transient but robust behavioral hyperactivity on sudden transition to dark [52].

Table 3. Different types of behavioral patterns exhibited by zebrafish larvae that can be measured as indicators of toxicant exposure (modified from [53]).

Behavior	Time	Stimulus	Selected chemical (reference)
Coiling	21-25hpf	None	Clorpyrifos [49]
Touch-induced escape response	>26hpf	Touch	Cadmium [55]
Evoked swimming	27hpf until hatching	Touch	Fluoxetine [51]
Photomotor response	30hpf	Light intensity	Neuroactive drugs [56]
Visual motor response	>4dpf	Alternating light-dark periods	Ethanol [50]
Optokinetic response (eye movements)	>73hpf	Moving objects	Ethanol [57]
Optomotor response (Whole-body movements)	>4dpf	Moving objects	Atropin [30]
Startle response	>5dpf	Acoustic	Ethanol [58]
Turning behavior	6-9 dpf	Touch, approaching object, sudden change of light conditions, sound	Clorpyrifos [49]
Prey capture	>5dpf	Prey	Methylmercury [59]

Locomotor activity endpoints included swimming speed, distances swum, time spent in the different sections of the tank, time spent immobile, erratic movements, turning rate, etc. For an accurate, objective, and efficient measurement of these parameters, the use of automated video tracking systems is recommended [53]. In these systems, movement in the vertical plane is usually ignored or minimized by reducing water depth of the vessels [54] and currently, several commercial setups are available on the market (e.g. Viewpoint ZebraLab videotrack system).

Figure 7 shows as example the assessment of locomotor activity of zebrafish after being exposed to increasing concentrations of d-amphetamine. The experimental conditions and data processing implemented in our laboratory permitted to reproduce the effects of d-amphetamine being the results similar to those reported by Irons et al. [50]. Our results demonstrated that d-amphetamine produced an evident biphasic "inverted U" concentration-response pattern with a highly consistent behavioral pattern between light and dark periods. The lowest concentration

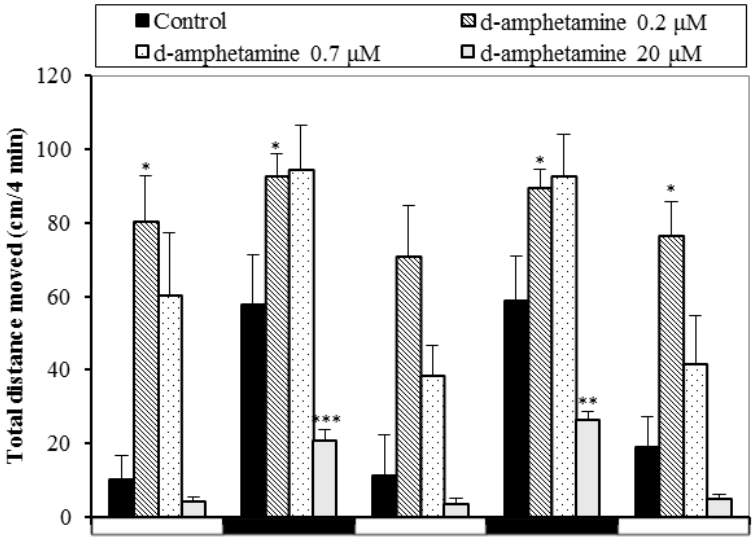


Figure 7. Effects of d-amphetamine on larval locomotion. Values are mean + SEM of the total distance moved in 4 min interval Asterisks indicate significant difference at *p<0.05, **p<0.01 respect control. Black and white bars at the bottom signify dark and light conditions, respectively.

of d-amphetamine produced hyperactivity and the highest concentration tested caused hypoactivity, mainly in dark periods. The effects reflect those found in mammalian models, proving the usefulness of the zebrafish embryo model for neurobehavioral studies. In addition, it is likely that can be used to unveil abnormal nervous system developmental/maturation due to developmental neurotoxicity [50].

There are several conditions which need to be controlled in order to get consistent and reproducible outcomes especially involving locomotor activity (Table 4). For example, MacPhail et al. [60] found that zebrafish larvae are more active in the morning than in the afternoon, so locomotor activity depends on the time of day. It must be noted that, although behavior is the ultimate result of neuronal development and signaling, not all behavioral modifications are of neurological origin. Malformed limbs or other morphological based conditions may be associated with behaviors not apparent in normal individuals. For instance, a reduced visual sensitivity or higher visual threshold could delay the transition in activity when darkness is switched to light [60]. Therefore, if our final objective is to detect neurotoxic effects, embryos with malformations should be not used in the locomotor activity assay [61].

Table 4. Variables that influence larval zebrafish behavior.

Factors that influence zebrafish behavior	Reference
Developmental stage	[61]
Time of day	[60]
Density of rearing zebrafish embryos	[62]
Intensity of light or light conditions	[61]
Lighting conditions during development	[63]
Acclimatization-time before testing	[60]
Containing vessels during test	[61]
Developmental malformations	[61]
Carrier solvents	[64]

Hurdles to the acceptance of zebrafish assays

Despite the promising zebrafish studies described above, there are still significant issues to be addressed before the zebrafish is accepted as a toxicological model.

Absorption, distribution, metabolism and excretion are crucial factors affecting the toxicity of chemicals. Most of the zebrafish assays relay on aqueous exposure and compound uptake predominantly by diffusion through the skin. Compound uptake may be not linear and it is dependent upon a number of physicochemical variables [65]. Internal concentration analysis is therefore needed in order to correlate the toxic phenotype observed with the actual concentration of the compound within the larvae. It is also necessary in order to identify false-negative results attributable to poor compound absorption and to link effect concentrations between mammals and fish embryos [66].

One of the alleged weaknesses of the zebrafish embryo as a model for teratogenicity in mammals is the difference in metabolic activity towards exogenous substances. This is especially relevant in the case of xenobiotics that need bioactivation. Zebrafish embryos and larvae have the ability to perform both phase I (oxidation, n-demethylation, o-demethylation and n-dealkylation) and phase II (sulfation and glucuronidation) metabolism reactions [65]. In particular, zebrafish have a total of 94 CYP genes, distributed among 18 gene families, most of which are direct orthologs of human CYPs. Most of these CYPs are expressed in embryos during various time courses along the first 48 hours after fertilization. Indeed, some maternally-derived CYPs RNA transcripts are present in the unfertilized egg [67]. Some studies have been shown the capacity of zebrafish embryos to metabolize different drugs, some of that known human proteratogenic substances [68-70]. However, differences to mammalian metabolic pathways have been identified. Cisapride, for example, was mainly metabolized to cisapride N-sulfate in zebrafish larvae, which is only a minor metabolite in mammals. Therefore, more extensive studies are required to evaluate the similarities and differences in metabolic pathways between human and zebrafish.

Conclusions

We have shown various examples of the applicability of the zebrafish embryo model in toxicology, focusing on the developmental toxicity evaluation of chemicals. The zebrafish embryo model is still struggling for recognition by regulators and industry as a screening tool in drug development and toxicological testing of chemicals other than water quality assessment. The first steps are promising [13, 71] and demonstrate the reliability of the zebrafish embryo model in human risk assessment. Further systematic testing of toxicologically concerning substances in all applicable areas relevant to humans will definitely provide a significant picture of the

predictive power of the zebrafish embryo assay. However, embryonic development is a very complex biological process so a single *in vitro* test capable of covering this whole process with satisfactory predictivity cannot be expected. An integrated testing approach with different test species should help minimize risk in the animal-human extrapolation [72].

In order to accurately predict and relate chemical impacts across species, it is necessary to have a mechanistic understanding of the effects of pathway perturbation. Recently, the use of the concept of Adverse Outcome pathways (AOPs) provides a framework in which data and knowledge are collected at many levels of biological organization and can be synthesized in a way that is useful to risk assessors and toxicologists that support this activity [73]. All this new developments in hazard assessment will contribute to have a more human-relevant and more predictive alternatives to traditional testing.

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