Research Article**Evaluation of teratogenicity of ethanol and DMSO in Zebrafish****Tuse Trupti*, Bhise Satish***Department of Pharmacology,**Smt. Kashibai Navale college of Pharmacy, Kondhwa (Bk), Pune, Maharashtra, India*

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Abstract

Objective: The zebrafish has been widely used in toxicological studies to evaluate either a single compound or small panels of compounds. This study is aimed at standardizing and validating the zebrafish model with ethanol and DMSO and to have a quick testing method to screen synthetic compounds for primary evaluation of teratogenicity. **Material and methods:** In this study various concentrations of each solvent to evaluate the teratogenicity in zebrafish were used. The phenotype changes as pericardial edema and yolk sac edema at 96 hpf were observed with ethanol and DMSO. **Results and conclusion:** Dose dependent phenotype changes as pericardial edema and yolk sac edema were observed with these solvents. Thus, it can be concluded that the zebrafish model could provide a useful tool in the screening of new drugs for treating human diseases. Some solvents act as carrier solvents for water insoluble drugs hence a controlled study of carrier solvents is needed as these solvents alone can have teratogenic potential.

Keywords: Ethanol, DMSO, Zebrafish embryo, solvents, phenotype

Introduction

In the last few years assays using embryonic stages of the vertebrate zebrafish have paid the attention of toxicologists due to their various advantages. The zebrafish has been widely used in toxicological studies to evaluate either a single compound or small panels of compounds. The zebrafish embryo is a potential alternative model in some fields of biomedical research, such as drug screening, safety pharmacology, and assessment of developmental toxicity (Barros, 2008; Spitsbergen, 2007; Lieschke, 2007; Rubinstein, 2003; McGrath, 2008). This whole animal model may be useful as a rapid, high throughput and low cost assay in the early stages of the drug development (Redfern, 2008).

Zebrafish maintenance is easy and it produces large numbers of embryos which develops outside the mother. Transparency of zebrafish embryos permits the scoring of teratological and embryotoxic effects easily. Also, the development is fast and well characterized which includes morphological and

physiological information at all stages of early development (Hill, 2005). The development process is highly conserved among vertebrates and the zebrafish genome is completely characterized. Hence, zebrafish embryos represent an attractive model allowing reduction and refinement of animal use in research (Yang, 2009). Various studies have been reported which explores the capacity of zebrafish assays for the assessment of the teratogenic potential of chemicals showing a good concordance with in vivo results in mammals (Hermesen, 2011; Selderslaghs, 2009; Teixidó, 2012; Carlsson, 2011). Developmental delay is usually considered as a reversible and unspecific effect. However, it might lead to persistent delays or deficits in function (Daston, 2010).

Ethanol is used as an important solvent in pharmacology and chemistry, and is commonly used to dissolve substances proposed for human consumption or human contact (including not only drugs, but also scents, food colors, and flavorings), it is also a psycho active substance and a teratogen. Ethanol has showed well characterized teratogenic malformations in multiple species. In zebrafish, ethanol was previously found to show various defects depending upon the developmental stage at which embryos or larvae are exposed. Zebrafish embryos, treated for a short time with ethanol at early gastrula stages, developed cyclopia due in part to defects in the migration of the prechordal plate

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(Blader, 1998). Earlier exposure of ethanol in development led to incomplete epiboly, whereas the acute treatment of larvae directed to a dose dependent locomotor response, with intermediate doses resulting in hyperactivity, and high doses causing hypoactivity and sedation (Lockwood, 2004).

DMSO i.e. dimethyl sulfoxide, a polar solvent most commonly used solvent for the delivery of compounds and extracts in both cell and zebrafish-based bioassays. Although DMSO is commonly used as a solvent for screening of compounds, both in zebrafish and in cell-based assays, this solvent does not dissolve all compounds. Although initial toxicological data have been obtained in zebrafish for DMSO and ethanol in particular, most other solvents and carriers have not yet been tested in zebrafish (Maes, 2012). One of the studies has reported the teratogenicity of ethanol and DMSO (Ali, 2011). Hence, the present study was under taken to evaluate and validate the teratogenicity of ethanol and DMSO in zebrafish and to have a quick testing method to screen synthetic compounds for primary evaluation of teratogenicity. The compounds were added to water in which the embryos develop hence this study focused on water soluble compounds.

Material and methods

Ethics statement

The guidelines for zebrafish maintenance and experimentation, as per the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Govt. of India, were followed. The animal care and experimental protocols were sanctioned by Institute Animal Ethics Committee (Approval No. SKNCOP/IAEC-110-16).

Experimental animals

In this study zebrafish (*Danio rerio*), wild-type were used for the experiment. All experiments were performed on zebrafish larvae at 24-96 hpf (hour post fertilization). All the embryos were incubated at 28.5°C in E3 medium. Fishes were maintained on a 14-h light: 10-h dark cycle. The fishes were fed with dry flake food and live brine shrimp. The day before eggs were required, males and females were placed in breeding tanks. The breeding tank with mesh egg trap used to prevent the eggs from being eaten by fishes. On the next morning, the eggs were collected 30 min after the light had been turned on. Eggs were transferred into plastic Petri dishes containing fresh E₃ medium. Eggs were washed repeatedly to remove debris. Unfertilized and dead embryos were identified under a stereomicroscope and removed.

Geometric series and LC₅₀

The selection of doses for organic solvents was done according to geometric series and LC₅₀ reported in previous studies for ethanol and DMSO (Ali, 2011).

Treatment of embryos

Zebrafish embryos of 24 hpf were transferred from the Petri dish using a sterile plastic pipette into 96-well plates. We placed single embryo in each well, so that dead embryos would not interfere with others embryos, and also to observe proper growth of individual embryos for the whole duration of the experiment. We used various concentrations of each solvent as per Ali et al., 2014. We used E₃ medium containing NaCl (5 mM), KCl (170 µM), CaCl₂ (330 µM) or MgCl₂ (330 µM), and methylene blue (0.6 µM) as a vehicle to prepare all the concentrations. We added 250 µL of either freshly prepared test compound, or vehicle as control in each well. We used 10 embryos for each concentration and 10 embryos as vehicle controls for each compound. Experiment with each concentration of each solvent was repeated 3 times ($n=30$ embryos per concentration of solvents and 30 embryos for vehicle).

Statistical analysis

Statistical analysis was performed using Graph Pad Prism for Windows, to see the impact of compounds on zebrafish larvae development. We used one-way analysis of variance and Dunnett's multiple-comparison test with a probability level of 5% as the minimal criterion of significance.

Results and discussion

In this study the effects of differing concentrations of ethanol and DMSO on zebrafish development were assessed. Embryos were exposed continuously from 24 to 96 hpf. They were then assessed for malformations at 96 hpf. All the compounds showed concentration dependent malformation. Analysis of larval morphology was carried out using stereomicroscope. The results of morphological analysis of larvae are summarized in table 1. The phenotypic effects that can be seen are illustrated in figure 1.

The zebrafish early life stage test has become a tool widely used to assess toxic effects of chemicals. In the present study percent morphological changes as, pericardial edema and yolk sac edema with different concentrations of ethanol and DMSO were recorded. Endpoints used for assessing the effects for all the solvents were at 96 hpf. Ethanol at 8000mg/L showed mild pericardial edema and showed yolk sac edema at 16000mg/L. We observed the same phenotype changes with DMSO with the same concentrations. Ethanol and DMSO showed greater sensitivity as evidenced by the occurrence of teratogenic malformations that were concentration dependent. The developmental defects observed in the present study represent generalized responses of zebrafish embryos to toxicants. Previous study has reported pericardial edema and dispersed pigment cells

Table 1. Percent morphological abnormalities per compound concentrations used in Zebrafish larvae assay

Compounds	Concentration (mg/L)	Pericardial edema	Yolk sac edema
Ethanol	0	0.00	0.00
	1000	0.00	0.00
	2000	0.00	0.00
	4000	0.00	0.00
	8000	39.88±9.354 ^b	35.11±5.285 ^a
	16000	53.17±7.054 ^a	57.93±4.826 ^a
DMSO	0	0.00	0.00
	2000	0.00	0.00
	4000	0.00	0.00
	8000	30.35±3.717 ^c	34.52±2.977 ^b
	16000	52.90±9.270 ^a	49.20±7.937 ^a

The values given are the mean percentage morphological abnormalities from three replicates. Significance levels of all phenotype effects for each concentration were calculated from three independent experiments. Dose dependent phenotype changes were seen. Statistical analysis with one-way analysis of variance followed by Dunnett's multiple comparison test, ^a $p < 0.0001$, ^b $p < 0.001$, ^c $p < 0.01$ compared with 0mg/L (control) concentration (N=30).

Ethanol 0mg/L **Ethanol 8000 mg/L** **Ethanol 16000 mg/L** **DMSO 0 mg/L** **DMSO 8000 mg/L** **DMSO 16000 mg/L**

Figure 1. Phenotype changes observed with ethanol and DMSO at 96hpf. Where, PE-Pericardial edema, YSE- Yolk sac edema

with ethanol, pericardial edema and uninflated swim bladder with DMSO (Ali, 2011). One of the studies in zebrafish demonstrated that ethanol leads to craniofacial abnormalities, cardiac and structural malformations, and developmental delays (Reimers, 2004). Reduction in heart rate and/or increased cardiac disease have been previously reported after exposure to ethanol and DMSO (Hallare, 2006).

This study was designed to evaluate and validate the zebrafish model with ethanol and DMSO. With the consideration of number of compounds that need testing, novel methods are required to accelerate consistent hazard identification. Our findings show that teratogenicity assessment on zebrafish larvae can provide a sensitive evaluation of the teratogenicity of a wide range of toxicants. However, future work in the validation of the zebrafish larval assay must include a wider range of compounds, including those that are known teratogens in humans. The zebrafish model could provide a useful tool in the screening of new drugs for treating human diseases. And it can be a quick testing model to evaluate teratogenicity of various synthetic drugs and compounds.

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Conflicts of interest

Authors are hereby declared that there is no conflicts of interest.

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