

Ethanol- and acetaldehyde-mediated developmental toxicity in zebrafish

Mark J. Reimers, Amanda R. Flockton, Robert L. Tanguay*

Department of Environmental and Molecular Toxicology, Oregon State University, 1007 Agriculture and Life Sciences, Corvallis, OR 97331, United States

Available online 27 July 2004

Abstract

Ethanol is a well-established developmental toxicant; however, the mechanism(s) of this toxicity remains unclear. Zebrafish are becoming an important model system for the evaluation of chemical and drug toxicity. In this study, zebrafish embryos were utilized to compare the developmental toxicity resulting from either ethanol or acetaldehyde exposure. Embryos were exposed to waterborne ethanol concentrations for various lengths of time but encompassed the earliest stages of embryogenesis. The waterborne ethanol concentration that causes 50% mortality (LC₅₀) following a 45-h ethanol exposure was approximately 340 mM (1.98% v/v). A number of reproducible endpoints resulted from ethanol exposure and included pericardial edema, yolk sac edema, axial malformations, otolith defects, delayed development, and axial blistering. When the exposure period was reduced, similar signs of toxicity were produced at nearly identical ethanol concentrations. To estimate the embryonic dose following a given waterborne ethanol concentration, a kinetic alcohol dehydrogenase (ADH) assay was adapted. The average embryonic ethanol dose was calculated to be a fraction of the waterborne concentration. Embryos exposed to waterborne acetaldehyde resulted in similar, but not identical, endpoints as those induced by ethanol. Embryos were however, almost three orders of magnitude more sensitive to acetaldehyde than to ethanol. Ethanol and acetaldehyde both negatively impact embryonic development; however, ethanol is more teratogenic based on teratogenic indices (TIs). These results demonstrate that the zebrafish model will provide an opportunity to further evaluate the mechanism of action of ethanol on vertebrate development.

© 2004 Elsevier Inc. All rights reserved.

Keywords: Acetaldehyde; Alcohol; Aquatic toxicology; Embryos; Ethanol; Ethyl alcohol; Development; Developmental toxicity; Fetal alcohol syndrome; Larvae; Teratogen; Teratogenicity; Toxicity; Zebrafish

1. Introduction

The developmental toxicity associated with ethanol was first documented in human fetuses in 1968 [19]. Fetal Alcohol Syndrome (FAS) was described in children born to women who drank alcohol during pregnancy in 1973 [17]. FAS is characterized as a delay in development, cardiac abnormalities [17], central nervous system abnormalities, abnormal craniofacial features, and intellectual delays [15,17]. Historically, the term Fetal Alcohol Effects (FAE)

was used to describe an affected individual with incomplete characteristics of FAS [11]. FAE has been subdivided into Alcohol-Related Birth Defects (ARBD) and Alcohol-Related Neurodevelopmental Disorder (ARND) to more accurately describe the effects. ARBD is defined as congenital anomalies, such as malformations and dysplasias of various organ systems. ARND is characterized by reduced cranial size or brain structural abnormalities at birth and behavioral or cognitive abnormalities [11]. In the general population, the incidence of FAS has been estimated to be approximately 10–20 per 10,000 live births [21]. The incidence of FAE is more difficult to assess, but has been estimated to range between 17 and 900 per 10,000 live births in the general population [21]. While the teratogenic properties of ethanol have been firmly established, the underlying mechanism(s) of toxicity remains unclear. Two molecular mechanisms have been postulated,

Abbreviations: dpf, days postfertilization; FAS, fetal alcohol syndrome; hpf, hours postfertilization; S.E.M., standard error of the mean; S.D., standard deviation; TI, teratogenic index.

* Corresponding author. Tel.: +1 541 737 6514; fax: +1 541 737 7966.

E-mail address: Robert.Tanguay@oregonstate.edu (R.L. Tanguay).

which include direct ethanol effects and the indirect effects associated with ethanol metabolism, such as acetaldehyde formation and oxidation stress [26]. Ultimately, both pathways could lead to central nervous dysfunction (reviewed in Ref. [26]).

The ability of ethanol to cause developmental anomalies has been demonstrated across taxa ranging from mammals to insects [3,8,23,24]. Mice have been extensively utilized to investigate the teratogenic signs of ethanol exposure. Fetal malformations were observed in mice exposed to ethanol between 2 and 6 g/kg during either preorganogenic, organogenic, and postorganogenic periods (reviewed in Ref. [3]). Rabbit embryo cultures exposed to 154 mM of ethanol at Gestational Day 9 for 48 h displayed an increase in the occurrence of facial and brain abnormalities and reduction in embryonic growth [22]. *Drosophila* larvae reared in ethanol containing media up to 14% w/v (3.0 M) resulted in an increase in the frequency of abnormalities [23,24]. It is probable that the molecular mechanism underlying these ethanol-dependent responses across species is conserved; therefore, the use of model systems to further our understanding of ethanol developmental toxicity is reasonable.

Zebrafish provide an excellent vertebrate model to study developmental toxicity because they share many cellular and physiological characteristics with higher vertebrates. The embryos rapidly develop externally and are transparent. Organogenesis is completed within the first 48 h of development. Because zebrafish embryos develop externally, changes in development may be observed in detail without sacrificing the maternal component and removes the complication of maternal/placental/fetal interaction, which greatly facilitates developmental time course studies. In addition, large clutches allow for the use of high-throughput screening of potentially developmentally toxic compounds. Because zebrafish development has been well characterized, results from zebrafish are easily compared to mammalian developmental toxicity studies. Finally, the practical advantages of this model allows for saturation mutagenesis screens and knockdown approaches that can be used to identify genes involved in toxic responses.

Previous studies in zebrafish demonstrated that ethanol leads to craniofacial abnormalities, cardiac and structural malformations, and developmental delays [2,18]. Zebrafish embryos exposed to 1.5% v/v ethanol during development had impaired visual function [4]. In three adult zebrafish strains, ethanol caused different behavioral responses; thus, genetic differences may result in a range of effects in response to ethanol [12]. The role that ethanol metabolism may play in these ethanol-dependent endpoints in zebrafish remains unknown. This study was undertaken to systematically compare the developmental toxicity following ethanol and acetaldehyde exposure in zebrafish. We report that the signs of toxicity are similar in animals exposed to ethanol and acetaldehyde and

suggest that zebrafish is a suitable model to study the molecular mechanisms of ethanol-mediated developmental toxicity.

2. Materials and methods

2.1. Materials

Alcohol dehydrogenase from *Saccharomyces cerevisiae* and β -nicotinamide adenine dinucleotide (NAD) was purchased through Sigma (St. Louis, MO). Absolute ethyl alcohol USP, 200 proof, was purchased from AAPER Alcohol and Chemical (Shelbyville, KY). Acetaldehyde (>99% purity) was acquired from EMD Chemicals (Gibbstown, NJ). Glass exposure vials with Teflon-lined lids were purchased from Fisher Scientific.

2.2. Fish care and husbandry

Adult AB strain zebrafish (*Danio rerio*) were raised and housed according to Institutional Animal Care and Use Committee protocols. Zebrafish were reared in 2.0-l polycarbonate tanks on a recirculating system in which the water was maintained at 28 ± 1 °C and a pH of 7.0 ± 0.2 . The fish were fed twice daily with either crushed TetraMin Tropical Flake (Blacksburg, VA) or live artemia from INVE (Grantsville, UT). Newly fertilized eggs were collected and embryos were rinsed several times in water prior to their use. Normally dividing and spherical embryos at the 256 cell stage [2.5 hours postfertilization (hpf)] through to the oblong stage (3.7 hpf), were selected and utilized for all of the described studies [30].

2.3. Embryo exposure

All animals were waterborne exposed using a static method in which 25 embryos composed each exposure group. Each group was considered a single replicate. Embryos were exposed to either ethanol or acetaldehyde in 20-ml glass vials sealed with Teflon-lined lids (VWR International, West Chester, PA) to prevent losses by volatilization. The embryos were exposed to ethanol for two different exposure periods, from 3 through 48 hpf and from 3 through 24 hpf. For the acetaldehyde studies, embryos were exposed from 3 through 24 hpf. All exposure solutions were replaced with fresh solutions at 24 hpf. At the end of the exposure period, the embryos were washed several times with water and allowed to develop until 120 hpf.

Range-finding experiments were conducted initially in order to estimate the lethal concentration to cause 50% mortality (LC_{50}) in the zebrafish embryos. In subsequent experiments, the concentrations were adjusted to more accurately calculate the LC_{50} and effective concentrations to cause 50% malformations (EC_{50}) with at least three

independent experiments. Developmental parameters were monitored and documented daily between 48 and 120 hpf. Individual malformations and abnormalities, such as yolk sac edema, pericardial edema, axial edema/blistering, axial malformations (crooked/clubbed), and developmental delays were tabulated. Embryos with delays in development were compared to the control embryos. All embryos were staged as previously described [30], using the pectoral fin, yolk sac, anal pore, and swim bladder as indicators of developmental stage.

2.4. Embryonic ethanol dose determination

Embryonic ethanol dose determination was based on an enzymatic assay using yeast alcohol dehydrogenase (ADH), NAD, and the ethanol contained within whole embryo homogenates. Embryos were collected following ethanol exposure and transferred into 1.5-ml microcentrifuge tubes on ice. The ethanol solution was removed and the embryos (with intact chorions) were quickly washed twice with cold 3.5% v/v perchloric acid to remove residual ethanol. A final aliquot of 3.5% perchloric acid was added to the rinsed embryos, which were then homogenized with a pestle in the microcentrifuge tube. The samples were centrifuged at 4 °C for 10 min at 2000×g. The samples were stored at 4 °C in sealed tubes until all time course study samples were collected. The assay to determine the embryonic dose of ethanol was performed with the SpectraMax 90, 96-well plate spectrophotometer (Molecular Devices, Sunnyvale, CA) at 340 nm to measure NADH production in a 200- μ l reaction volume. Two replicates for each sample and standard were analyzed. Ethanol standards were diluted to generate a standard curve. The NAD solution was dissolved in a 0.5-M Tris buffer, pH 8.8 at a final concentration of 1 mg/ml. The yeast ADH was dissolved in water at 4 °C for a final concentration of 0.75 mg/ml. The NAD solution (174 μ l) and 8.7 μ l of either a sample or standard was added to each well. To initiate the reaction, 17.3 μ l of a 0.75 mg/ml ADH solution was added. The reaction was incubated for 10 min at 37 °C. After the 10-min incubation, the production of NADH was measured. A blank reaction with no ethanol was run simultaneously without the addition of substrate to correct for any substrate-independent generation of NADH. The embryonic dose of ethanol was calculated from standard curves.

2.5. Statistical analysis

Data are illustrated as the mean with either standard deviation (S.D.) or standard error of the mean (S.E.M.). Sigmoidal regression analysis was completed using Sigma-Plot 2001 for Windows (SPSS, Chicago, IL). ANOVA statistical analysis was applied to calculate statistical significance followed by Bonferroni's analysis as a post hoc test to independently compare each exposure group to

the control group using a *P* value of 0.05 for significance. This was tabulated with SigmaStat Version 2.03 for Windows software (SPSS).

3. Results

3.1. Zebrafish embryos exposed to ethanol

Initially, zebrafish embryos were exposed to various ethanol waterborne concentrations from 3 to 48 hpf (45 h exposure) and were monitored daily for mortality until 120 hpf. The calculated lethal concentration to cause 50% mortality (LC_{50}) in embryos was 338.5 mM or 1.98% v/v of ethanol (Fig. 1A). Embryo survival was severely impacted at concentrations greater than 300 mM with 100% mortality occurring at concentrations greater than 500 mM. To determine if a shorter period of ethanol exposure would also produce similar effects, a series of experiments was conducted in which ethanol was present from 3 to 24 hpf (21 h exposure). The LC_{50} of embryos exposed to ethanol during this period was calculated at 380.5 mM (2.22% v/v; Fig. 1B). In this 21-h developmental window, ethanol concentrations greater than 400 mM had serious consequences on embryonic survival.

A number of ethanol-dependent endpoints were also measured during the two exposure paradigms. Initially, zebrafish embryos were exposed to various waterborne concentrations of ethanol and were monitored for their ability to hatch over 120 hpf (data not shown). Concentrations less than 150 mM did not significantly impact hatching. However, concentrations of ethanol between 200 and 1000 mM significantly reduced hatching rates. The embryonic malformations observed following the 45- and 21-h ethanol exposures were visually identical. The endpoints monitored included development delay, axial edema or axial blistering, axial malformations (crooked/clubbed), otolith defects, pericardial edema, and yolk sac edema. Individual endpoints were scored daily and a cumulative graph was plotted to calculate respective effective concentration that resulted in 50% malformations (EC_{50} ; summarized in Table 1). All observed individual malformations displayed an increase in incidence with increasing ethanol concentrations. Embryos that were exposed to ethanol from 3 to 24 hpf displayed one or more of a number of reproducible abnormalities at 120 hpf (Fig. 2). Embryos exposed to ethanol concentrations greater than 150 mM exhibited a reduction in retinal eye diameter and craniofacial dysmorphogenesis, pericardial and yolk sac edema were apparent in embryos exposed to ethanol concentrations greater than 150 mM (Fig. 2). Embryos subjected to 300 mM ethanol, also, had axial malformations (Fig. 2), while those exposed to 400 mM ethanol had axial blistering, axial malformations, pericardial and yolk sac edemas, and facial defects (Fig. 2). One exceptional abnormality, more appreciable from the ventral

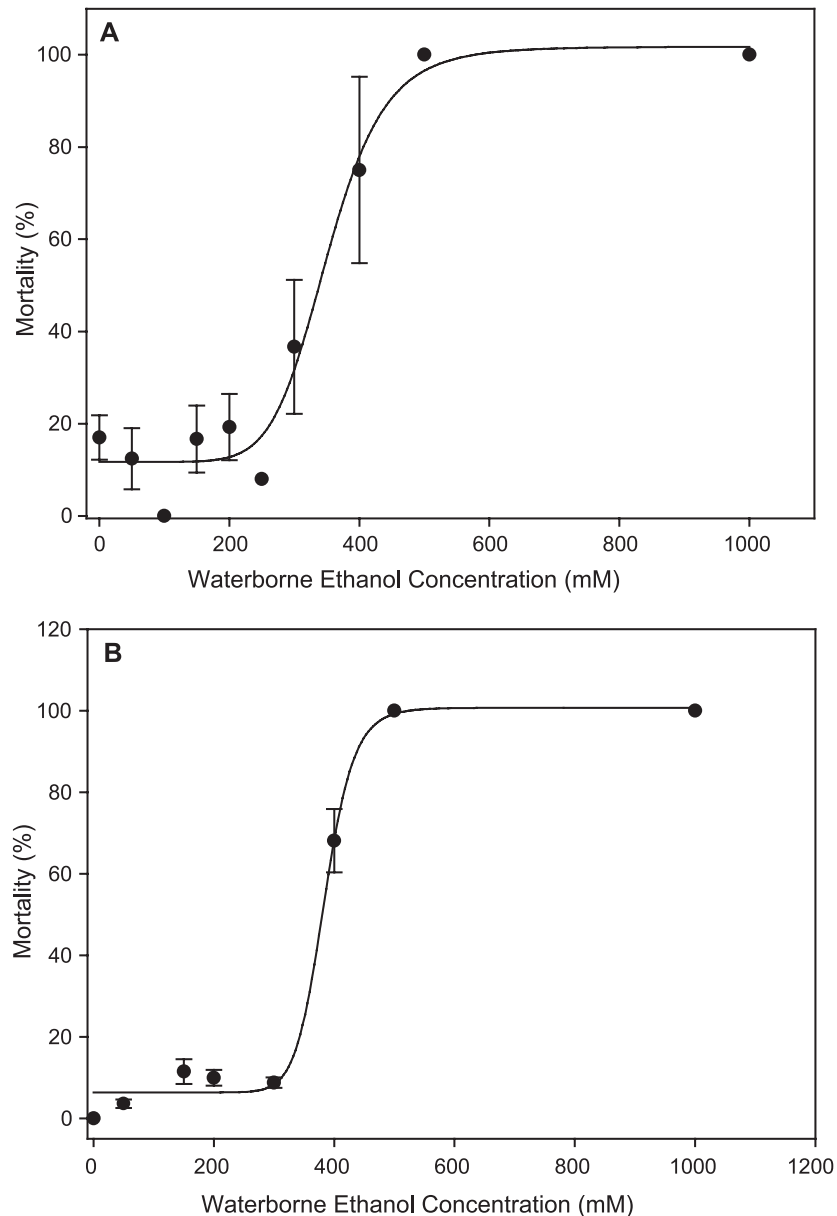


Fig. 1. Ethanol-mediated mortality in zebrafish embryos. Groups of zebrafish embryos were exposed to graded concentrations of ethanol and the effect on mortality were scored at 120 hpf. (A) Lethality curve from embryos exposed to ethanol between 3 and 48 hpf. From the curve, the LC_{50} was calculated to be 338.5 mM with an r^2 of .958. (B) Lethality curve from embryos exposed to ethanol between 3 and 24 hpf. The calculated LC_{50} is 280.5 mM with an r^2 of .989. ●, Mean mortality percentage \pm S.E.M.; $n=3$.

view, was ethanol-mediated cyclopia in the 400 mM embryo (Fig. 2B).

One of the most sensitive biomarkers for ethanol exposure in zebrafish was the prevalence of pericardial edema, indicated by the EC_{50} values of 148.2 and 148.5 mM for the 45- and 21-h exposure regimens, respectively (Table 1). The degree of pericardial edema varied from mild to severe in exposure groups (Fig. 2). The incidence of pericardial edema was statistically significant in ethanol concentrations greater than 150 mM in both the 45- and 21-h exposure studies (Fig. 3A). Another indicator of ethanol-mediated effects was the occurrence of yolk sac edema with an EC_{50} of 240.9 and 248.9 mM in the 45- and 21-h ethanol

exposure groups, respectively (Table 1). The prevalence of yolk sac edema increased in a concentration-dependent manner (Fig. 3B). The prevalence of yolk edema was statistically different at >200 mM in the 45-h exposed embryos. Embryos exposed to ethanol concentrations of >300 mM had a significant increased incidence of yolk edemas in the 45- and 21-h exposure regimens.

Axial malformations and otolith defects also increased with increasing ethanol concentrations. Axial malformations were defined as developmental abnormalities associated with the longitudinal axis, including hooked, curved, curled, or clubbed axis (Fig. 3C, Table 1). Axial malformations were significant at concentrations >200 mM in the

Table 1
Summary of ethanol and acetaldehyde dose responsive endpoints

	48-h Ethanol exposure (mM)	24-h Ethanol exposure (mM)	24-h Acetaldehyde exposure (mM)
Mortality (LC ₅₀)	338.5	380.5	0.541
Individual malformations (EC ₅₀)			
Axial blistering	313.0	373.75	0.775
Axial malformations (curved/clubbed)	250.1	251.2	0.490
Otoliths defects	275.0	284.7	0.507
Pericardial edema	148.2	148.5	0.354
Yolk sac edema	240.9	248.9	0.495
Developmental delay	291.5	158.6	0.369
Total malformations (EC ₅₀)	137.9	133.5	0.346

45-h exposure groups and >300 mM in the 21-h exposure groups and are illustrated in Fig. 2A. Zebrafish normally possess two otoliths per otic vesicle [30]. The otoliths are dark round bones within the otic vesicle that are positioned over the sensory macula. The otic vesicle contains the auditory sensory hair cells [30]. The otolith defects observed included one to multiple otoliths per vesicle and abnormally shaped otoliths. Control embryos have two round otoliths whereas the embryos exposed to 400 mM ethanol frequently had malformed otoliths. Ethanol concentrations greater than 300 mM led to a significant increase in otolith anomalies for both the 45- and 21-h treatment groups (Fig. 3D, Table 1).

Ethanol treatment also resulted in developmental delays. The stage of embryogenesis was measured by daily monitoring pectoral fin development, swim bladder inflation, and yolk sac as previously described [30]. Ethanol-mediated developmental delay was ethanol concentration dependent. For the 45-h exposure window, ethanol concentrations greater than 200 mM led to statistically significant differences between exposed and control embryos (Fig. 3E). For the 21-h exposure window, ethanol concentrations ≥ 150 mM significantly delayed development (Fig. 3E).

Based on calculated EC₅₀ values, the least sensitive parameter monitored was axial blistering (Table 1). Axial blistering was defined as blebs along the posterior axis (Fig. 3E). The predominance of embryonic axial blisters was statistically significant only in the 300 and 400 mM exposure groups (Fig. 3F). Embryos exposed for 21 h notably have lower incidence of axial blistering than the 45-h-treated embryos.

The EC₅₀ values suggest that the individual malformations that exhibit the greatest sensitivity occur in the following ranked order for the 45-h studies: pericardial edema \gg yolk sac edema \gg axial malformations \gg otolith defects \gg developmental delay \gg axial blistering. For the 21-h exposure regiments, the rank was pericardial edema \gg

developmental delay \gg yolk sac edema \gg axial malformations \gg otolith defects \gg axial blistering (summarized in Table 1). With the documentation of individual dysmorphogenesis, a cumulative malformation graph was constructed to calculate a composite malformation EC₅₀ value. To generate this composite malformation curve, an embryo either having one or multiple malformations was counted as one malformation. The effective waterborne ethanol concentration to induce malformations (EC₅₀) in 50% of the embryos was calculated at 137.9 mM or 0.80% v/v ethanol for the 45-h ethanol exposure experiments (Fig. 4A and Table 1) and EC₅₀ for the 21-h exposure studies was

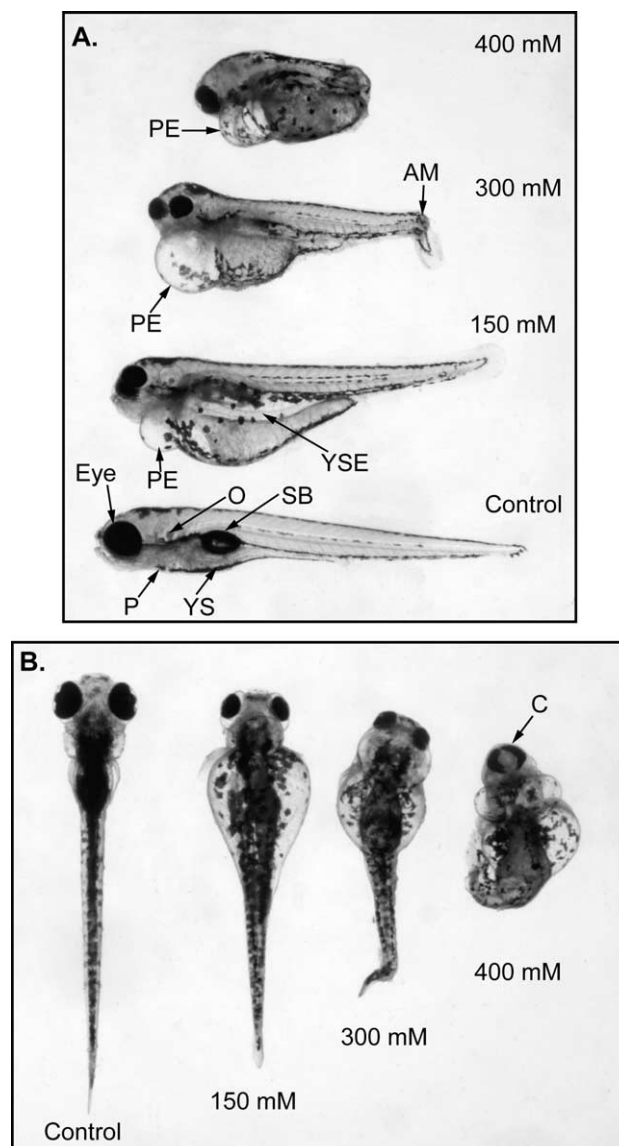


Fig. 2. Ethanol-dependent developmental toxicity endpoints in zebrafish. Embryos were exposed to either water or the indicated ethanol concentrations between 3 and 24 hpf. (A) Lateral view of 120 hpf embryos revealing numerous malformations. (B) Ventral view of the embryos from (A). Axial malformation, AM; cyclopia, C; otolith, O; pericardium, P; pericardial edema, PE; swim bladder, SB; yolk sac, YS; yolk sac edema, YSE.

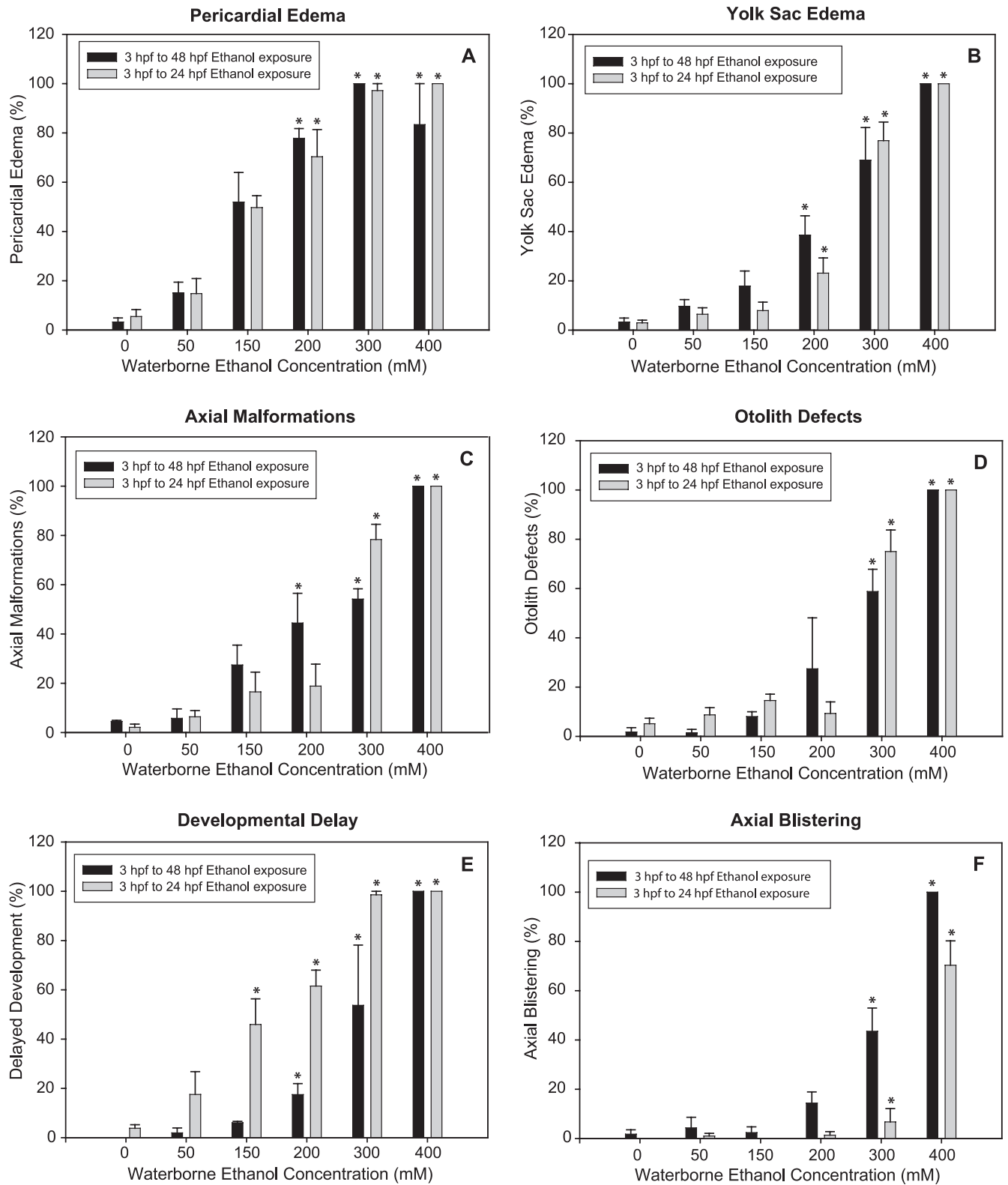


Fig. 3. Cumulative 120 hpf summary of individual morphologic abnormalities occurring in zebrafish exposed to ethanol between 3 and 48 hpf (black bars) or between 3 and 24 hpf (gray shading). (A) Pericardial edema, (B) yolk sac edema, (C) axial malformations, (D) otolith defects, (E) developmental delay, and (F) axial blistering. *Indicates a statistical difference between control and the ethanol groups. Level of significance, $P < 0.05$. Mean percentage \pm S.E.M.; $n=3$.

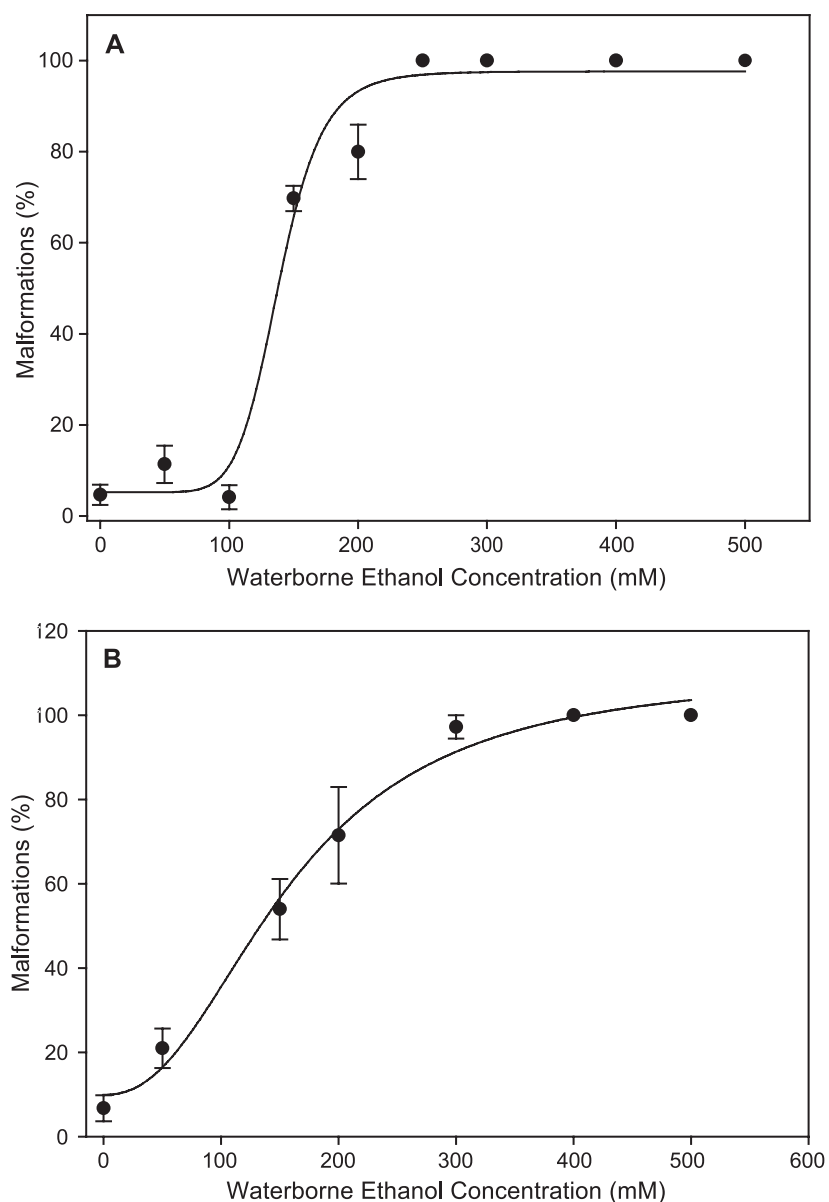


Fig. 4. Ethanol-mediated malformations in zebrafish. Groups of zebrafish embryos were exposed to graded concentrations of ethanol and the cumulative malformations were scored at 120 hpf. (A) Malformation curve from embryos exposed to ethanol between 3 and 48 hpf resulted in a calculated EC_{50} of 137.9 mM with r^2 of 0.968. (B) Malformation curve resulting from embryos exposed to ethanol between 3 and 24 hpf resulted in an EC_{50} of 133.5 mM having a r^2 of 0.981 ●, Mean mortality percentage \pm S.E.M.; $n=3$.

calculated at 133.5 mM (0.78% v/v; Fig. 4B and Table 1). The teratogenic index (TI) for the 48- and 21-h ethanol exposures were 2.5 and 2.9, respectively. The LC_{50} and EC_{50} values suggest that the developmental defects, which were monitored in these studies, are largely the result of exposure during the first 21 h of embryonic development. This developmental window includes midblastula, gastrulation, and organogenesis.

3.2. Embryonic ethanol dose determination

Because the ethanol concentrations were based on waterborne exposures, it was vital to determine the ethanol dose in zebrafish embryos following a given waterborne

concentration. Embryos with intact chorions were exposed to waterborne concentrations of ethanol (100 and 200 mM) between 3 and 48 hpf; and four sampling time points were selected: 8 hpf (4 h exposure), 12 hpf (approximately 10 h exposure), 24 hpf (21 h exposure), and 48 hpf (45 h exposure) to calculate the resulting embryonic ethanol dose. After the 4-h ethanol exposure, the embryonic ethanol dose was approximately 30 and 60 mM, for the 100 and 200 mM waterborne ethanol concentrations, respectively (Table 2 and Fig. 5). This equates to approximately 30% of waterborne concentrations. There was a significant decrease in embryonic ethanol concentration after the 10-h exposure time point for both waterborne concentrations (Fig. 5). At the end of the 45-

Table 2
Embryonic ethanol dose determination

Water ethanol concentration (mM)	Exposure time (h)	Embryo stage at sampling (hpf)	Mean embryonic ethanol dose (mM)	Standard deviation (mM)	Percentage of waterborne ethanol concentration
100	4	8	29.21	6.52	29.21
	10	12	14.34	1.25	14.34
	24	24	17.10	6.27	17.10
	48	48	31.23	5.97	31.23
200	4	8	59.78	8.94	29.89
	10	12	31.93	5.29	15.96
	24	24	57.36	3.93	28.68
	48	48	71.70	6.27	35.85

h exposure point, the embryonic ethanol dose was 31.2 and 71.7 mM for 100 and 200 mM waterborne ethanol concentrations, respectively (Fig. 5).

3.3. Zebrafish embryos exposed to acetaldehyde

Because acetaldehyde is the major metabolite of ethanol metabolism, and it has been postulated that in vivo production of acetaldehyde may play a role in the developmental toxicity of ethanol in some target tissues, zebrafish were exposed to acetaldehyde to determine the embryonic response. Zebrafish embryos exposed between 3 and 24 hpf to graded waterborne concentrations of acetaldehyde resulted in an LC_{50} of 0.54 mM (Fig. 6). Overall, the endpoints induced by acetaldehyde were similar to those observed in the ethanol studies. Pericardial edema (EC_{50} =0.354 mM) was the most sensitive biomarker of morphological abnormalities based on the EC_{50} values (Table 1). The severity of pericardial edema is quite evident in acetaldehyde-treated embryos and increases with acetal-

dehyde concentration (Fig. 7). However, pericardial edema was significantly elevated in embryos exposed to ≥ 0.50 mM acetaldehyde concentrations (Fig. 8A).

Delayed development was also observed in an acetaldehyde concentration-dependent manner, as evidenced by a decrease in axial length (Fig. 7), which was significantly different at acetaldehyde concentrations of ≥ 0.50 mM (Fig. 8B). One notable difference between ethanol and acetaldehyde exposures was that all embryos exposed to 1.0 mM acetaldehyde were drastically delayed in development. For instance, at the end of the exposure period (24 hpf), the acetaldehyde-exposed animals had only progressed to the 90% epiboly stage which is a equivalent to 9 hpf (data not shown). Following acetaldehyde removal, structures in these animals continued to develop despite the presence of dramatic malformations (Fig. 7). For instance, the body failed to extend, however, some cranial structures were formed and pigmentation was induced, indicating at least partial developmental progression.

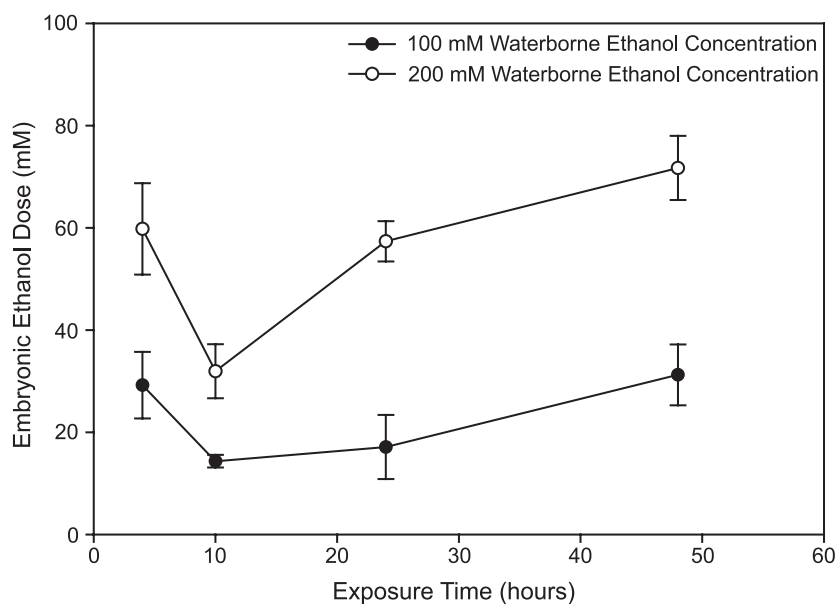


Fig. 5. Embryonic ethanol dose determination. The embryonic dose following a 100-mM (●) or 200-mM (○) waterborne ethanol concentration was estimated using an ADH-dependent kinetic assay at the several developmental stages. Estimated embryonic ethanol concentration \pm S.D.; $n=3$.

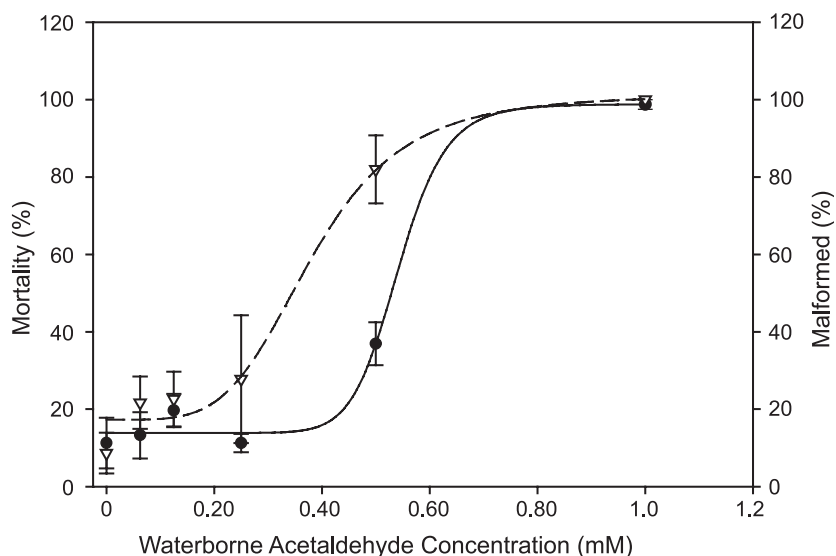


Fig. 6. Acetaldehyde-mediated mortality in zebrafish embryos. Groups of zebrafish embryos were exposed to graded concentrations of acetaldehyde between 3 and 24 hpf and the effect on mortality and malformations were scored at 120 hpf. The acetaldehyde LC_{50} was 0.541 mM, interpolated from sigmoidal regression analysis of the curve ($r^2=0.980$) and the malformation EC_{50} was calculated to be 0.346 mM with an r^2 of 0.958. ●, Mean mortality percentage \pm S.E.M.; $n=3$; ▽, mean malformation percentage \pm S.E.M.; $n=3$.

The prevalence of acetaldehyde-induced embryonic axial malformations in the 0.50- and 1.0-mM groups were significant when compared to control embryos (Fig. 8C) with an EC_{50} of 0.490 mM (Table 1). Slight aberrations, including arched axis, were common and are observable in the 0.125-mM acetaldehyde-exposed embryo (Fig. 7) and more severe arched axial malformations occurred at higher acetaldehyde concentrations (not shown). Acetaldehyde treatment also led to an increase in yolk sac edema. The acetaldehyde EC_{50} for yolk sac edema was 0.495 mM (Table 1). Morphologically, acetaldehyde- and ethanol-mediated yolk sac edemas were similar. Embryos exposed to 0.50 mM acetaldehyde have large edema with most of the yolk sac separated from the ventral part of the embryo (Fig. 7). Acetaldehyde-mediated yolk sac edema in ≥ 0.50 mM exposure groups were statistically different from that observed in the control embryos (Fig. 8D).

Otolith defects were also induced by acetaldehyde exposures. The otolith defect EC_{50} for the 21-h acetaldehyde was calculated to be 0.507 mM (Table 1). There was a low occurrence of otolith defects in 0.0625, 0.125, and 0.250 mM treatment groups, which was not statistically significant when compared to the controls (Fig. 8E). However, there was a significant increase in otolith malformations at acetaldehyde concentrations greater than 0.5 mM. Axial blistering was the least sensitive observed malformation with an EC_{50} value of 0.775 mM. A representative sample of posterior blistering can be observed in the 0.5 mM-treated embryo (Fig. 7). Numerous blisters can be seen on the ventral side of the embryo with one large blister on the dorsal side (Fig. 7). Axial blistering in the 0.50 and 1.0 mM acetaldehyde exposure groups was statistically significant when compared to the control group (Fig. 8E).

Based on the individual EC_{50} malformation values, the rank order of sensitivity would be as follows: pericardial edema > delayed development > axial malformations > yolk sac edema > otolith defects \geq axial blisters (Table 1). The composite malformation EC_{50} was calculated to be 0.346 mM or 345.5 μ M (Fig. 7 and Table 1) with a TI of 1.6.

4. Discussion

We have conducted a series of exposure studies in zebrafish to determine the dose–response relationship for mortality and developmental toxic endpoints associated with either ethanol or acetaldehyde exposure. From the two ethanol exposure paradigms, we have determined that exposure to ethanol during the first 24 h of embryogenesis is sufficient to induce a number of developmental malformations. Of the ethanol-dependent endpoints scored, pericardial edema was the most sensitive malformation observed and occurred in both the long and short ethanol exposure studies (Table 1). Prior to this study, there were no attempts to calculate the embryonic dose resulting from a given water concentration in zebrafish. These data are essential to determine if the zebrafish embryonic ethanol loads were relevant to the levels achieved in mammalian ethanol developmental toxicity studies. We modified a kinetic assay and determined that embryonic ethanol dose was approximately 32% of the waterborne ethanol concentration during the exposure period (Table 2). If the EC_{50} values are adjusted to reflect the unequal ethanol distribution, the 21-h waterborne ethanol EC_{50} value of 133.5 mM would be reduced to 42.7 mM, a value very similar to mammalian doses.

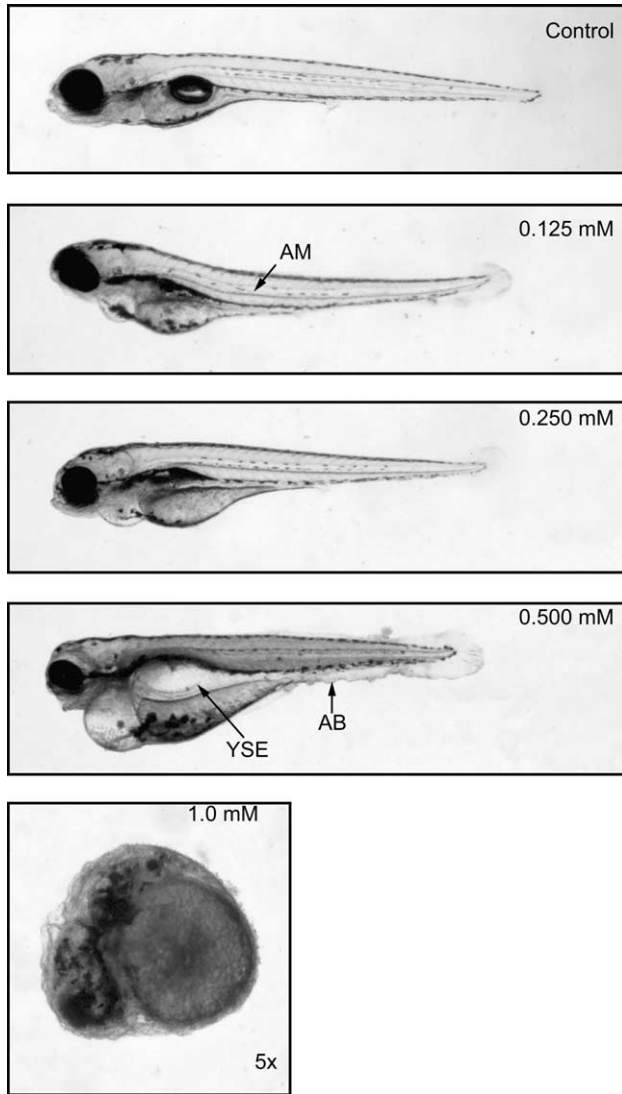


Fig. 7. Acetaldehyde-dependent developmental toxicity endpoints in zebrafish. Embryos were exposed to either water or the indicated acetaldehyde concentrations between 3 and 24 hpf. Axial malformation, AM; swim bladder, SB; yolk sac edema, YSE.

In pregnant CD-1 mice administered intraperitoneally with 6 g/kg of ethanol, the resulting embryonic dose was 65.1 mM [6]. Rabbit embryo cultures exposed for 48 h to 154 mM ethanol beginning at Gestational Day 9 resulted in a significant increase in occurrence of morphologic malformations, such as brain, facial, and cardiac abnormalities [22]. In a number of other studies in mice, embryos were exposed to ethanol concentrations between 60 and 152 mM from Gestational Day 6 to 16; this resulted in a wide range of malformations, including craniofacial defects, brain abnormalities, and neural tube defects (reviewed in Ref. [3]). These stages of development are similar to the development windows covered in our studies and include preorganogenic, organogenic, and postorganogenic periods. A study conducted by Blader and Strahle [5] illustrated that cyclopia was induced in zebrafish embryos exposed to 2.4% v/v (approximately 425 mM) ethanol for a short period

during the gastrula stage. This was also accompanied with the loss of gene expression (sonic hedgehog, *shh*; winged helix transcription factor, *axial*; and homeobox gene, *nk2.2*) in the ventral fore- and midbrain of the embryos [5].

The ability of an embryo or fetus to metabolize ethanol during ethanol sensitive periods of development remains unclear. Therefore, it is critical to understand the temporal expression of the ethanol-metabolizing enzymes during early life stages. Mammalian studies demonstrate that ethanol can be oxidized to acetaldehyde by ADHs, cytochrome *P*-450 2E1 (CYP2E1), and catalase [20]. ADH was detected in human fetal tissues at a mean gestational age of 11 weeks [28]. Estonius et al. [13] were able to detect Class 1 ADH as early as 18 weeks gestation in human fetal lung, liver, and kidney, but not in the brain. We have identified and characterized an ethanol-metabolizing ADH in zebrafish (ADH8A). ADH8A is expressed as early as 36 hpf but is not detected at 24 hpf by reverse-transcriptase coupled polymerase chain reaction (RT-PCR) [25].

CYP2E1 is primarily expressed in the liver; however, the data concerning human CYP2E1 developmental expression is somewhat controversial. Two laboratories were unable to detect CYP2E1 by immunoblotting in five fetal liver samples less than 12 weeks of gestational age [31] and between 11 and 13 weeks of gestational age in three fetal hepatic samples [27]. Furthermore, CYP2E1 mRNA was not detected in 16 fetal liver samples staged between gestation ages of 11 and 24 weeks using RT-PCR [14]. These studies conflict with several others that illustrate that CYP2E1 is expressed early in the fetus. CYP2E1 was detected in 11 fetal liver samples by immunoblotting between 16 and 24 weeks gestational age [9]. Furthermore, CYP2E1 mRNA transcripts were detected at 19, 23, and 25 weeks gestation [9]. However, this same group was unable to detect CYP2E1 mRNA in two fetal samples less than 10 weeks of gestation. In a recent study, CYP2E1 was detected by immunoblotting from human hepatic microsomes in 18 out of 49 second trimester samples, corresponding to 93–186 days of gestation, and in 12 of 15 third trimester (>186 days) fetal samples [16]. We have recently identified a zebrafish cytochrome *P*-450 enzyme, tentatively named CYP2E. The zebrafish CYP2E mRNA was first detected at 36 hpf by RT-PCR. (R.L. Tanguay, unpublished data). Taken together with our ADH mRNA expression data, it suggests that zebrafish may have a very limited capacity to oxidize ethanol within the first 24 h of development, the period found to be particularly sensitive to ethanol insult. We cannot conclude from our data that acetaldehyde is not produced during the ethanol exposure period as we did not directly measure acetaldehyde levels. For instance, in vivo cellular-specific ethanol oxidation, although difficult to detect, could result in local toxic levels of acetaldehyde. Similarly, temporal specific expression of ethanol-metabolizing enzymes could also affect the embryonic response. For example, we found that the embryonic ethanol

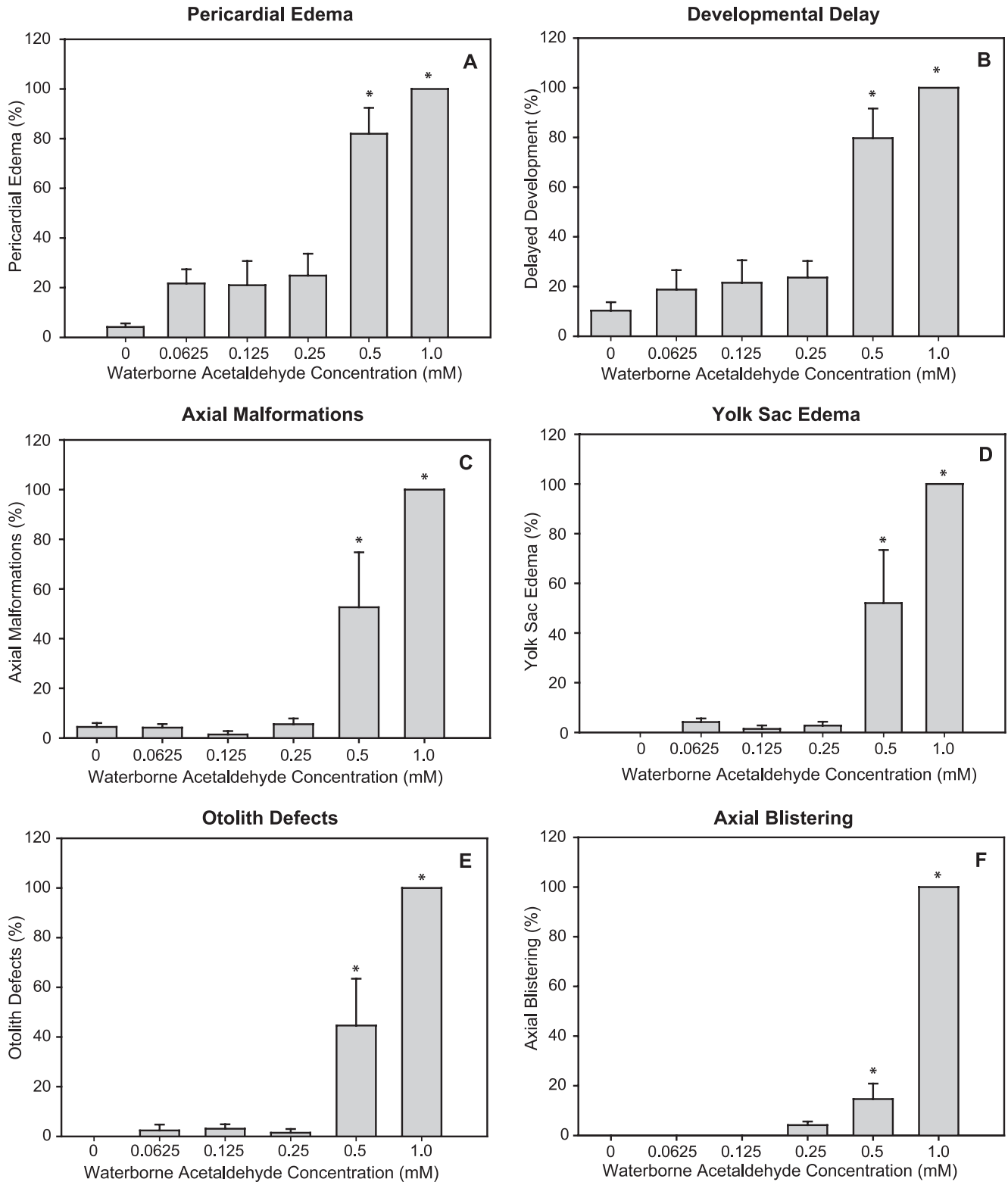


Fig. 8. Cumulative 120 hpf summary of individual morphologic abnormalities occurring in zebrafish exposed to acetaldehyde between 3 and 24 hpf. (A) Pericardial edema, (B) delayed development, (C) axial malformations, (D) yolk sac edema, (E) otolith defects, and (F) axial blistering. *Indicates statistical difference between acetaldehyde exposure and control groups. Level of significance $P < 0.05$. Mean percentage \pm S.E.M.; $n=3$.

concentration expressed as a fraction of the waterborne concentration varied throughout the first 48 h of development (Fig. 6). At 12 hpf, the ethanol concentration was

approximately 15% of the waterborne concentration, whereas at 8 hpf, it was approximately 29%. It remains a possibility that at around the 12 hpf time point, there is a

transient expression of yet to be identified enzymes capable of efficient ethanol metabolism. It will be important to thoroughly characterize the ethanol-metabolizing enzyme levels and activities during the earliest stages of zebrafish development to make predictions regarding their roles in mediating ethanol effects in vivo.

To determine if acetaldehyde is developmentally toxic to zebrafish, embryos were exposed to graded waterborne concentrations of acetaldehyde. This is the first report characterizing the effects of acetaldehyde on zebrafish development. The 21-h LC₅₀ was 0.541 mM with an EC₅₀ of 0.346 mM (Table 1). Again, the observation of pericardial edema was the most sensitive endpoint. Acetaldehyde was clearly more embryonic lethal than ethanol based on LC₅₀ values. However, the acetaldehyde mortality and malformation curves nearly overlapped, indicating that the acetaldehyde effects were so severe that lethality may have masked some of the teratogenic outcomes. For instance, Webster et al. [29] demonstrated that acetaldehyde caused only mild teratogenic affects in C57BL/6J mice at doses approaching the LD₅₀ values.

Several studies support the hypothesis that ethanol is the direct teratogen in vivo and that ethanol metabolism is not required to elicit the characteristic developmental responses. Dose-dependent increases in lethality and malformations in mice exposed to ethanol were enhanced when animals were coadministered with 4-methyl pyrazole, an inhibitor of ADH [7]. Furthermore, acetaldehyde treatment alone did not result in a significant increase in the percentage of resorptions and malformed fetuses [7]. Rats coadministered with 4-methyl pyrazole and ethanol developed an increase in microencephaly as compared to control groups [10]. Finally, in *Drosophila melanogaster*, there are three congenic *Adh* fly strains with high, medium, and low ADH activity. Ethanol teratogenicity in these strains is inversely related to ADH activity. Flies with the lowest ADH activity had twice the incidence of malformations compared to the flies with high ADH activity [24]. We have demonstrated that zebrafish embryos lack significant expression of ADH and CYP2E mRNA between 3 and 24 hpf, yet ethanol was teratogenic during this time period, suggesting that in vivo production of acetaldehyde in zebrafish early life stages may not be significant. We have not measured acetaldehyde levels directly in ethanol-exposed animals to conclusively demonstrate that lack of ethanol oxidation during the first 24 h of development. Considering that zebrafish respond to micromolar concentrations of acetaldehyde with signs of toxicity similar to those produced by millimolar ethanol concentrations, at least evokes the possibility that in vivo production of acetaldehyde could potentially underlie the effects of ethanol exposure.

The TI is a useful parameter calculated from the comparisons between LC₅₀ and EC₅₀ values that allow teratogenic comparisons between different chemicals [1]. This is particularly useful because it is a unit-less value. A

greater TI value is associated with a toxic agent that produces wide separations between the malformation and lethality dose–response curves. It is possible to have a toxic agent that causes severe malformations but not mortality; conversely, a potentially developmentally toxic chemical can be so lethal that malformations are not observed. In zebrafish, the calculated TI values were 2.7 and 1.6 for ethanol and acetaldehyde, respectively, indicating that ethanol is significantly more teratogenic than acetaldehyde (Table 1). However, it must remain clear that zebrafish are approximately three orders of magnitude more sensitive to waterborne acetaldehyde concentrations than to ethanol.

The use of zebrafish as a model system to study the mechanism of teratogen action offers exceptional promise. There remains a major gap in our understanding of precisely how early life stage ethanol exposure leads to the characteristic morphological and behavioral outcomes in vertebrates. The results of our studies firmly establish the embryonic zebrafish as an excellent research model to elucidate the molecular mechanism(s) of ethanol-induced developmental toxicity. It is likely that diverse consequences of ethanol exposure reported here involve the actions of multiple gene products. Molecular and genetic approaches will allow the identification of these factors, which underlie each ethanol-dependent endpoint.

Acknowledgement

We would like to thank Dr. Dennis Petersen for his assistance with these studies, Dr. Richard Radcliffe for his assistance with the ethanol dose determination assays and Drs. Eric Andreasen and Melissa Haendel for technical support. This work was supported in part by NIH/NIAAA grant #AA12783 and NIH/NIEHS grants #ES00210, and #ES03850.

References

- [1] J.A. Bantle, D.J. Fort, J.R. Rayburn, D.J. DeYoung, S.J. Bush, Further validation of FETAX: Evaluation of the developmental toxicity of five known mammalian teratogens and non-teratogens, *Drug and Chemical Toxicology* 13 (4) (1990) 267–282.
- [2] M. Baumann, K. Sander, Bipartite axiation follows incomplete epiboly in zebrafish embryos treated with chemical teratogens, *Journal of Experimental Zoology* 230 (3) (1984) 363–376.
- [3] H.C. Becker, J.L. Diaz-Granados, C.L. Randall, Teratogenic actions of ethanol in the mouse: A minireview, *Pharmacology, Biochemistry and Behavior* 55 (4) (1996) 501–513.
- [4] J. Bilotta, S. Saszik, C.M. Givin, H.R. Hardesty, S.E. Sutherland, Effects of embryonic exposure to ethanol on zebrafish visual function, *Neurotoxicology and Teratology* 24 (6) (2002) 759–766.
- [5] P. Blader, U. Strahle, Ethanol impairs migration of the prechordal plate in the zebrafish embryo, *Developments in Biologicals* 201 (2) (1998) 185–201.
- [6] P.M. Blakley, W.J. Scott Jr., Determination of the proximate teratogen of the mouse fetal alcohol syndrome: 2. Pharmacokinetics of the

- placental transfer of ethanol and acetaldehyde, *Toxicology and Applied Pharmacology* 72 (2) (1984) 364–371.
- [7] P.M. Blakley, W.J. Scott Jr., Determination of the proximate teratogen of the mouse fetal alcohol syndrome: 1. Teratogenicity of ethanol and acetaldehyde, *Toxicology and Applied Pharmacology* 72 (2) (1984) 355–363.
- [8] S.R. BuppBecker, I.A. Shibley Jr., Teratogenicity of ethanol in different chicken strains, *Alcohol and Alcoholism* 33 (5) (1998) 457–464.
- [9] S.P. Carpenter, J.M. Lasker, J.L. Raucy, Expression, induction, and catalytic activity of the ethanol-inducible cytochrome *P450* (CYP2E1) in human fetal liver and hepatocytes, *Molecular Pharmacology* 49 (2) (1996) 260–268.
- [10] W.J. Chen, R.E. McAlhany Jr., J.R. West, 4-Methylpyrazole, an alcohol dehydrogenase inhibitor, exacerbates alcohol-induced microencephaly during the brain growth spurt, *Alcohol* 12 (4) (1995) 351–355.
- [11] Committee to Study Fetal Alcohol Syndrome. D.o.B.S.a.M.D., Institute of Medicine, in: K. Stratton, C. Howe, F. Battaglia (Eds.), *Fetal alcohol syndrome: Diagnosis, epidemiology, prevention, and treatment*, National Academy Press, Washington, DC, 1996, pp. 63–81. Chapter 4.
- [12] C.A. Dlugos, R.A. Rabin, Ethanol effects on three strains of zebrafish: Model system for genetic investigations, *Pharmacology, Biochemistry and Behavior* 74 (2) (2003) 471–480.
- [13] M. Estonius, S. Svensson, J.O. Hoog, Alcohol dehydrogenase in human tissues: Localisation of transcripts coding for five classes of the enzyme, *FEBS Letters* 397 (2–3) (1996) 338–342.
- [14] J. Hakkola, M. Pasanen, R. Purkunen, S. Saarikoski, O. Pelkonen, J. Maenpaa, A. Rane, H. Raunio, Expression of xenobiotic-metabolizing cytochrome *P450* forms in human adult and fetal liver, *Biochemical Pharmacology* 48 (1) (1994) 59–64.
- [15] G.I. Henderson, J.J. Chen, S. Schenker, Ethanol, oxidative stress, reactive aldehydes, and the fetus, *Frontiers in Bioscience* 4 (1999) D541–D550.
- [16] E.K. Johnsrud, S.B. Koukouritaki, K. Divakaran, L.L. Brunengraber, R.N. Hines, D.G. McCarver, Human hepatic CYP2E1 expression during development, *Journal of Pharmacology and Experimental Therapeutics* 307 (1) (2003) 402–407.
- [17] K.L. Jones, D.W. Smith, Recognition of the fetal alcohol syndrome in early infancy, *Lancet* 2 (1973) 999–1001.
- [18] H.W. Laale, Ethanol induced notochord and spinal cord duplications in the embryo of the zebrafish, *Brachydanio rerio*, *Journal of Experimental Zoology* 177 (1) (1971) 51–64.
- [19] P. Lemoine, H. Harousseau, J.P. Borteyru, Les enfants de parents alcooliques: Anomalies observees a propos de 127 cas, *Ouest Medical* 21 (1968) 476–482.
- [20] C.S. Lieber, Alcohol and the liver: 1994 update, *Gastroenterology* 106 (4) (1994) 1085–1105.
- [21] MMWR, Trends in fetal alcohol syndrome—United States, 1979–1993, *MMWR* 44 (1995) 249–251.
- [22] J.A. Pitt, E.W. Carney, Evaluation of various toxicants in rabbit whole-embryo culture using a new morphologically-based evaluation system, *Teratology* 59 (2) (1999) 102–109.
- [23] S. Ranganathan, D.G. Davis, R.D. Hood, Developmental toxicity of ethanol in *Drosophila melanogaster*, *Teratology* 36 (1) (1987) 45–49.
- [24] S. Ranganathan, D.G. Davis, J.D. Leeper, R.D. Hood, Effects of differential alcohol dehydrogenase activity on the developmental toxicity of ethanol in *Drosophila melanogaster*, *Teratology* 36 (3) (1987) 329–334.
- [25] Reimers, M.J., Hahn, M.E., & Tanguay, R.L., Two zebrafish alcohol dehydrogenases sharing common ancestry and functional characteristics with mammalian class I, II, III, IV, and V ADH genes. *J. Biol. Chem.*, In Press.
- [26] J.D. Reynolds, J.F. Brien, Ethanol neurobehavioural teratogenesis and the role of L-glutamate in the fetal hippocampus, *Canadian Journal of Physiology and Pharmacology* 73 (9) (1995) 1209–1223.
- [27] T. Shimada, H. Yamazaki, M. Mimura, N. Wakamiya, Y.F. Ueng, F.P. Guengerich, Y. Inui, Characterization of microsomal cytochrome *P450* enzymes involved in the oxidation of xenobiotic chemicals in human fetal liver and adult lungs, *Drug Metabolism and Disposition* 24 (5) (1996) 515–522.
- [28] M. Smith, D.A. Hopkinson, H. Harris, Developmental changes and polymorphism in human alcohol dehydrogenase, *Annals of Human Genetics* 34 (3) (1971) 251–271.
- [29] W.S. Webster, D.A. Walsh, S.E. McEwen, A.H. Lipson, Some teratogenic properties of ethanol and acetaldehyde in C57BL/6J mice: Implications for the study of the fetal alcohol syndrome, *Teratology* 27 (2) (1983) 231–243.
- [30] M. Westerfield, *The Zebrafish book*, University of Oregon Press, Eugene, OR, 1995.
- [31] S.A. Wrighton, D.T. Molowa, P.S. Guzelian, Identification of a cytochrome *P-450* in human fetal liver related to glucocorticoid-inducible cytochrome *P-450H1p* in the adult, *Biochemical Pharmacology* 37 (15) (1988) 3053–3055.