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Vulnerable windows for developmental ethanol toxicity in the Japanese medaka fish (*Oryzias latipes*)

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Abstract

Ethanol (EtOH) is a well-known developmental toxicant that produces a range of abnormal phenotypes in mammalian systems including craniofacial abnormalities, cognitive deficits and growth retardation. While the toxic potential of developmental EtOH exposure is well characterized clinically, the effect of timing on the extent of toxicity remains unknown. Fish models such as the Japanese medaka, *Oryzias latipes*, provide a convenient system for investigating the effects of developmental EtOH exposure *in vivo*. In this study, medaka embryo toxicity tests were used to assess temporal variations in developmental EtOH toxicity. Fertilized eggs were collected and incubated during early, middle or late egg development (e.g., 0–3, 3–6 or 6–9 days post-fertilization) with various sub-lethal concentrations of EtOH [0.1% (17.2 mM), 0.5% (86.0 mM) or 1% (172 mM)]. Uptake of EtOH by the embryo was 60–68% of the solution concentration across all windows. Time to hatch, head width, total body length and whole embryo caspase activity were used to assess toxicity. Hatching delays were noted only at the highest concentration of EtOH. Head width was affected at all ethanol levels, regardless of the window of exposure. EtOH-induced decreases in body length, however, appeared to be most pronounced when exposure occurred either during the first or last window. The effect on caspase-3/7 activity also depended on the window of exposure, with increases in caspase noted in embryos treated on days 1 or 2 (first window) and decreases seen in embryos treated on day 6 (second window) or day 8 (third window). In general, these data suggest that critical periods for heightened sensitivity to developmental EtOH exposure may vary according to the specific endpoint used to assess toxicity.
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Keywords: Ethanol; FAS; Developmental toxicity; Embryo; Vulnerable windows; Window of sensitivity; Fish

1. Introduction

Women who consume large amounts of ethanol during pregnancy often give birth to children exhibiting phenotypic abnormalities collectively referred to as the Fetal Alcohol Syndrome (FAS). These anomalies include growth deficiency, cognitive impairment and distinctive craniofacial features (reviewed in Coles, 1993). Ethanol is a well-known developmental toxicant in humans and laboratory animals. The mechanism for the toxic actions of ethanol on the developing organism is, however, unknown (for a recent review, see Goodlett et al., 2005). Many hypotheses exist, including interaction with neurotrophins (Kentroti, 1997), cell-adhesion molecules (Bearer, 2001), or specific receptors (e.g., Costa and Guizzetti, 2002); increased apoptosis (Olney et al., 2002a,b); or increased oxidative stress (Cohen-Kerem and Koren, 2003). Not only are the mechanisms as yet unknown (or multifunctional), but the issue of sensitive periods remains unresolved. That is, it is unclear if certain stage(s) of development are more sensitive to ethanol exposure.

Very few studies have systematically examined differences in the vulnerability of the entire central nervous system at distinct periods throughout brain development. There are primate studies, however, that compared short periods of ethanol

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exposure early in development with more protracted exposures, which included the early window. In most cases the short, early exposure was as detrimental as the longer, more comprehensive exposure (Clarren et al., 1992; Schneider et al., 2001). A different pattern was noted in a rat study (Tran et al., 2000) that compared various windows of ethanol exposure (gestation days 1–10, 11–22 or postnatal days 2–10) using brain weight, brain:body weight ratio, motor activity and exploratory behavior as measures of toxicity. The authors were also careful to equate peak blood ethanol concentrations across the three exposure periods. In this study, developing rats exposed to ethanol later in development experienced more adverse effects than those exposed during the earliest period. As yet, the issue of critical windows for ethanol toxicity remains unresolved.

Fish models offer several advantages over traditional mammalian model systems for the study of developmental timing. The *ex utero* development eliminates the confounder of maternal toxicity and allows for accurate and precise timing of developmental exposures in a cohort. Fish are susceptible to developmental toxicity, including that caused by ethanol. Studies of ethanol-induced toxicity in zebrafish have shown that ethanol is a teratogen that produces developmental delay (Reimers et al., 2004); craniofacial abnormalities (Bilotta et al., 2004; Blader and Strähle, 1998; Carvan et al., 2004; Loucks and Carvan, 2004); skeletal and cardiovascular toxicity (Wang et al., 2006a); alterations in eye development (Bilotta et al., 2002, 2004); cell death (Carvan et al., 2004; Loucks and Carvan, 2004) and behavior (Carvan et al., 2004). Because fish are vulnerable to ethanol toxicity and develop *ex utero*, they are a convenient, alternative model to explore the temporal variations in ethanol sensitivity during development.

We have examined this interaction between toxicity and the timing of ethanol exposure using the Japanese medaka fish (*Oryzias latipes*). The Japanese medaka, a small, egg laying freshwater fish native to Japan, Korea, and eastern China, has been widely used in developmental, genetic, and toxicological studies (Gogal et al., 1999; Kashiwada et al., 2002; Kelly et al., 1998; Law, 2003; Mullins et al., 1994; Wittbrodt et al., 2002). Medaka are ideal for developmental studies of the interaction between timing and toxicity when compared to zebrafish because the maturation of the egg spans 3 days in zebrafish days as compared to 9 days in medaka. This protracted development of the egg allows for more accurate delivery of compounds during discrete periods of development. In the present studies, hatching delays, growth inhibition, caspase-3/7 activity (marker of apoptosis) and embryonic ethanol concentrations were used to compare differences in ethanol toxicity following sublethal ethanol exposures during early [0–3 days post-fertilization (dpf)], middle (3–6 dpf) and late (6–9 dpf) stages of development.

2. Materials and methods

2.1. Chemicals

Embryo rearing medium (ERM; 17.1 mM NaCl, 272 μM $CaCl_2{\cdot}2H_2O$, 402 μM KCl, 661 μM $MgSO_4{\cdot}7H_2O$, pH 6.3) was used as the culture medium for all exposures. Ethanol (95%), alcohol dehydrogenase and nicotinamide adenine dinucleotide were purchased from Sigma–Aldrich (St. Louis, MO).

2.2. Test animal

Japanese medaka embryos used in this study were collected from an orange-red stock (an outbred line) maintained under standard re-circulating aquaculture conditions. All procedures were approved by the Duke University Institutional Animal Care and Use Committee (IACUC) and the U.S. EPA National Health and Environmental Effects Laboratory IACUC.

2.3. Embryo collection and animal husbandry

Egg collection and broodstock maintenance followed the experimental procedures described by Marty et al. (1991). Medaka broodstock were fed Otohime B1 food (Ashby Aquatics, West Chester, PA) 5 times/day and supplemented with brine shrimp nauplii for 5 days each week under a 16:8 h light/dark cycle at 25 ± 1 °C. Brine shrimp nauplii were reared separately at 25 ± 1 °C using Instant Ocean (Marineland, Moorpark, CA) under continuous aeration. For supplemental feedings, hatched brine shrimp nauplii were taken from this separate container and aliquots were fed to the medaka broodstock. Fertilized eggs from at least 50 females were siphoned from aquaria within 6 h of fertilization and separated by gently rolling egg clusters by hand to disrupt attaching filaments. Eggs were pooled and washed briefly in 20 ppm Instant Ocean (Marineland, Moorpark, CA) to inhibit fungal growth. Fertilized eggs were then placed in ERM and staged according to Iwamatsu (1994). Embryos of the early high blastula stage (stage 10; Fig. 1) were selected for study. Embryonic development was examined non-invasively through the transparent chorion (i.e., egg shell membrane) using a Nikon 1500 stereo dissecting microscope (Nikon, Melville, NY). To prevent sample bias, embryos from collected clutches were pooled and individual stage 10 embryos were randomly assigned to individual wells of microtiter plates [Multiscreen™ (40 μm nylon mesh) microtiter plates (catalog no. MANMN4050, Millipore Corp., Bedford, MA) (Oxendine et al., 2006)]. Treated and control embryos were assessed daily for mortality and hatching delays. Medaka embryos were incubated at 25 ± 1 °C under a 16:8 h light:dark cycle until hatching and all solutions were renewed daily. For daily solution changes, the sample tray with embryos was removed, briefly blotted with a paper towel, then lowered into a second buffer tray containing new aliquots of ethanol or ERM stock solutions.

Death was defined as an opaque embryonated egg (early) or an absence of cardiac contraction (after day 3 post-fertilization). Only those embryos that had successfully completed hatching were used to determine hatching success; embryos that were partially hatched or were unable to exit the chorion fully were scored as ‘not hatched’. To determine the time to hatch, embryos were checked once daily, and if fully hatched, that date was recorded as the day of hatch (the day of fertilization was considered day 0).

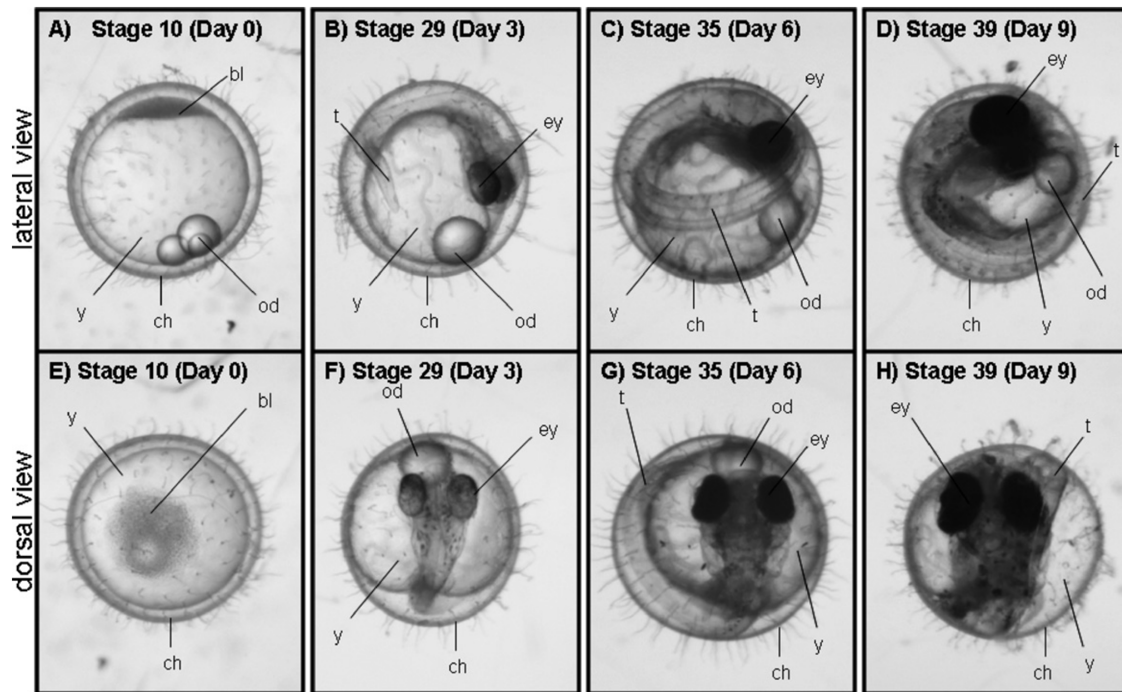

Fig. 1. Dorsolateral series of figures showing medaka embryonic development. (A) Multi-cell stage embryo at the early high blastula stage [∼6.5 h post-fertilization (hpf); day 0], the animal pole is five cell layers thick, consisting of approximately 1000 blastomeres. (B) Chorionated embryo at stage 29 (∼74 hpf; day 3). The embryo has completed axis formation and somite formation; the eyes are developing. (C) Chorionated embryo at stage 35 (day 6). (D) By the time embryos are ready to hatch (∼day 9), the tail extends well beyond the posterior head region. At this developmental stage, hatching enzyme released from the buccal cavity breaks down the inner chorion so that embryos may use their body movement to rip through the outer chorion and emerge tail first. bl: blastula, ch: chorion, ey: eye, od: oil droplet, t: tail, y: yolk.

2.4. Overt toxicity (mortality) study

Initial range finding studies were performed to determine an optimal ethanol dosing regimen for induction of developmental toxicity in the absence of embryo mortality. Embryos ($n = 16$) were exposed to ethanol (0, 0.1, 0.5, 1.0, 1.5, 2.0, or 3.0%, final concentration; 1% ethanol = 172 mM) for days 0–8 of egg development, followed by ERM only from days 9 to 15; if an embryo had not hatched by day 15 (99–100% of the controls hatch by day 15), then the embryo was scored as ‘not hatched’. Embryonic death was ascertained as described above. All animal husbandry practices and plate changing were as described in Section 2.3.

2.5. Assessment of the windows of vulnerability

Stage 10 embryos were exposed to various ethanol concentrations [0%, 0.1% (17.2 mM), 0.5% (86 mM) or 1.0% (172 mM)] during three distinct periods of development (0–3, 3–6, or 6–9 dpf) to investigate the effects of timing and dose on ethanol-induced embryo toxicity (Figs. 1 and 2). Embryos were assessed for treatment-related effects on growth, mortality and hatching. Morphometric criteria were evaluated on the day of hatching to determine how the timing of ethanol exposure affected medaka growth and development. ERM control replicates were also included as a temporal reference of normal embryonic development.

2.6. Morphometric assessment

Embryos were photographed on the day of hatching using a Nikon SZM 1500 stereomicroscope and coded for a blinded analysis of the effects of developmental ethanol exposure. Linear measures of head width (maximal distance from the lateral aspect of one eye to the lateral-most aspect of the other) and total body length (distance from the rostral-most portion of the head to the caudal-most portion of the tail fin) were used to determine whether developmental ethanol exposure induced a phenotype in fish that is qualitatively similar to human FAS.

2.7. Caspase activity

Embryos at each age (i.e., 1 dpf, 2 dpf, etc.) were treated with 1% ethanol for 24 h. At the end of that exposure period, groups of embryos were homogenized ($n = 6$ pools of 10 embryos at each age; 10 embryos/100 μl) in lysis buffer (10 mM HEPES, 42 mM KCl, 5 mM $MgCl_2$, 1 mM dithiothreotol, 0.1 mM EDTA, 0.1 mM EGTA, pH 7.4) and centrifuged at 14,000 × g for 20 min at 4 °C. Supernatants were aliquoted and stored at −80 °C until analysis. Caspase activation was measured as the release of aminoluciferin from a selective substrate for caspase-3/7 (Z-DEVD-AML) using the Caspase-Glo 3/7® Luminescence Assay kit (Promega, Madison, WI). Luminescence was determined using a FLUOstar Optima luminometer. The assay was optimized so that caspase-3/7 activity was linear with respect to

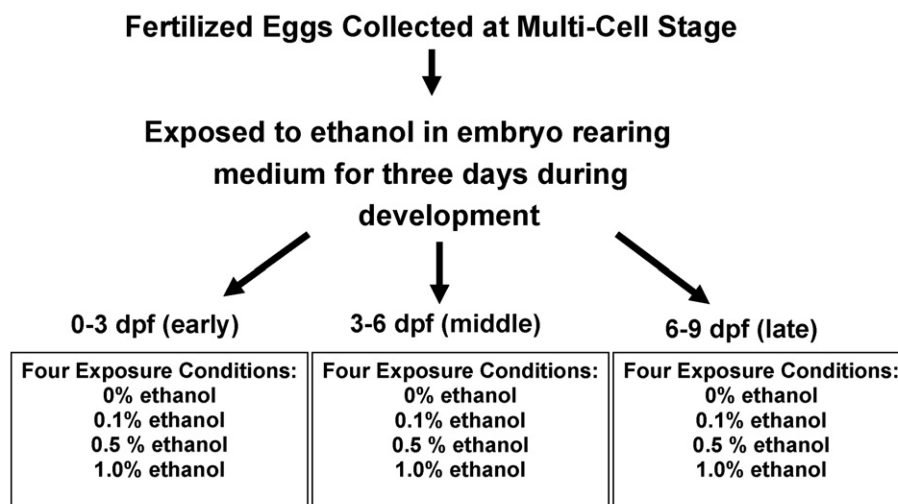

Fig. 2. Schematic representation of experimental design. To investigate the effects of timing and dose on ethanol-induced embryo toxicity, embryos ($n = 20$ per dose group/window) were exposed within 96-well microtiter plates to one of a range of ethanol concentrations (0, 0.1, 0.5 or 1.0%) in embryo rearing medium during three distinct periods of development (e.g., 0–3, 3–6 or 6–9 days post-fertilization).

protein concentration and incubation time. On the day of assessment, all samples were thawed on ice and 10 μl of the sample lysate (~30 μg protein) was added to 90 μl of the Caspase-Glo substrate and incubated in the dark for 1 h at room temperature. Purified caspase-3 enzyme (Biomol no. SE-169, Plymouth Meeting, PA) was included as a positive control, and addition of the specific caspase-3 inhibitor, AC-DEVD-CHO (Calbiochem) was used to show that approximately 90% of the observed luminescence was due to caspase-3 activity (data not shown).

2.8. Determination of embryonic ethanol concentration

Ethanol exposed and control embryos were washed three times with ERM, incubated in 3.5% $HClO_4$ (30 embryos/90 μl solution) for 1 h at 4 °C, sonicated on ice and then centrifuged at $14{,}000 \times g$ for 10 min at room temperature. Supernatants were then collected for use in an enzymatic reaction between alcohol dehydrogenase and nicotinamide adenine dinucleotide. NADH accumulation was determined via the method of Reimers et al. (2004) with the following modifications to assure linearity with respect to ethanol concentration and incubation time: the concentration of NAD was increased four-fold, the concentration of alcohol dehydrogenase was decreased by 50%, and the assay was conducted at 30 °C instead of 37 °C. All assays were performed in 96-well microtiter plates and NADH accumulation was monitored spectrophotometrically using a ThermoMax microplate reader set at 340 nm (Molecular Devices, Sunnyvale, CA). Ethanol standards and sample lysates were read kinetically (at 10 s intervals) for 10 min at 30 °C; the slope of the absorbance at 340 nm was used to calculate the ethanol concentration.

2.9. Statistical analyses

All statistical analyses were conducted using StatView® (version 5.0.1, SAS Institute Inc.). The two, non-parametric dependent variables (lethality and hatching success) were analyzed using an unpaired, Mann–Whitney test. The parametric data (i.e., length of time the embryo remained in the egg) were analyzed using a global ANOVA using day of hatch as the dependent variable, and ethanol and dose as the independent variables. Any subsequent interactions at the $p \leq 0.05$ level were followed by step-down ANOVAs. When necessary, a Fisher's PLSD *post hoc* test was conducted to determine differences between ethanol dosage groups. The embryonic ethanol concentration data were analyzed with a repeated ANOVA using embryo ethanol concentration and solution ethanol concentration as the dependent variables, and window of exposure as the independent variable. The caspase data were analyzed using a global ANOVA with caspase activity as the dependent variable and age of the embryo and ethanol exposure as the independent variables. A Fisher's PLSD *post hoc* test was conducted to determine differences between the ethanol treated and control groups.

3. Results

3.1. Overt toxicity

An initial range-finding study was completed to determine the optimal ethanol dosing regimen for induction of developmental toxicity in the absence of embryo mortality. Ethanol concentrations at or above 2% were lethal to embryos (defined as an opaque egg or the absence of a heartbeat). Embryos exposed to 1% ethanol showed no increased lethality (data not shown). As the above exposures spanned a period of 8 days, a 3 day dosing regimen using 1% ethanol as the highest, non-lethal exposure concentration was adopted for subsequent exposures.

3.2. Vulnerable window studies

3.2.1. Length of egg development (time to hatch)

There was no interaction between window of exposure and ethanol treatment. There was, however, an effect of ethanol treat-

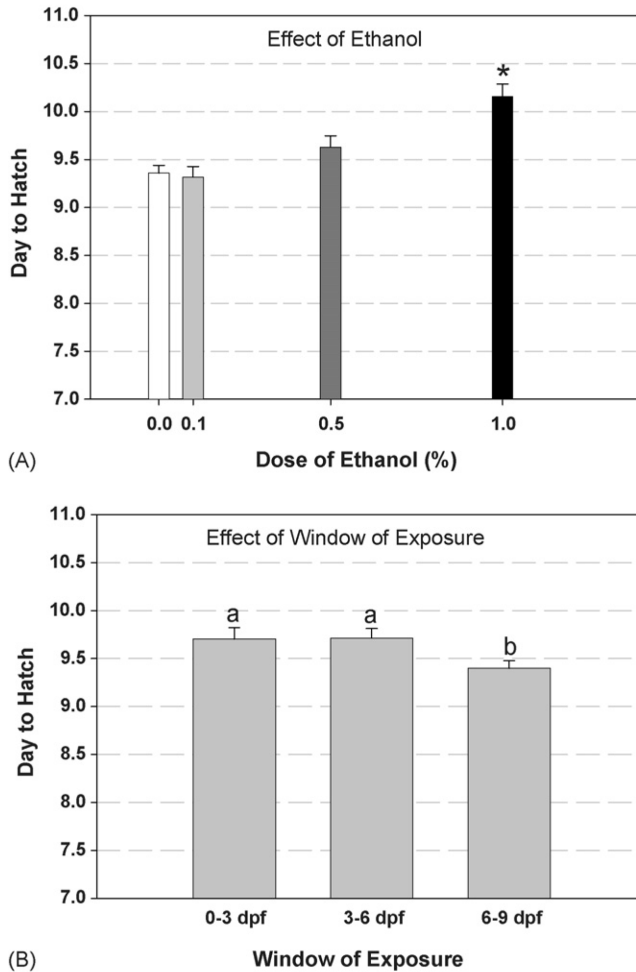

Fig. 3. Effects of developmental ethanol exposure on time required for hatching. Values represent the mean ± S.E.M. from two cohorts (total $n = 20$ per dose group/window). ANOVA analysis revealed that there was no interaction between ethanol dose and window of exposure. Panel A: these data are collapsed across windows of exposure: only the 1% ethanol exposure delayed hatching. Panel B: these data are collapsed across all dosage groups. If two bars have the same letter above it, then they are not different from one another. If, however, they have different letters above them, then they are different from one another. In this case, when embryos were treated during the first two windows, there was delayed hatching as compared to no delay if the embryos were treated during the last window. Data are presented as mean ± S.E.M.

ment (Fig. 3A) and an effect of window of treatment (Fig. 3B). Hatching was delayed by at least 1 day in embryos treated with the highest concentration of ethanol (1%) (Fig. 3A). This effect was most pronounced in embryos exposed either from 0 to 3 or 3 to 6 dpf when compared to embryos exposed from 6 to 9 dpf.

3.2.2. *Head width*

Again, there was no interaction between window of exposure and ethanol treatment. All concentrations of ethanol decreased head width compared to control, and this effect was not dependent upon the window of exposure (Fig. 4).

3.2.3. *Body length*

For body length, there was an interaction between ethanol effect and window of exposure (Fig. 5). All three ethanol doses

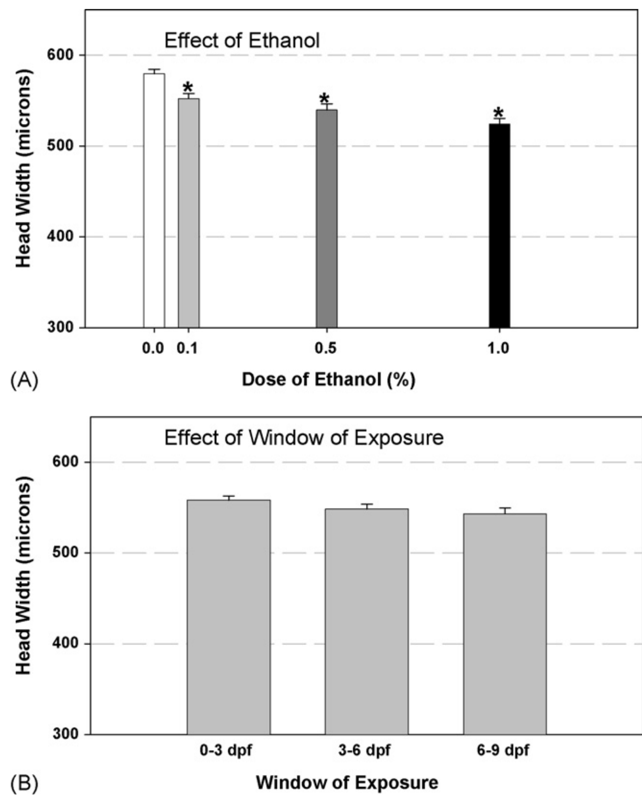

Fig. 4. Effects of developmental ethanol exposure on head width in the newly hatched medaka fry. Values represent the mean ± S.E.M. from two cohorts (total $n = 20$ per dose group/window). Ethanol exposure produced dose-related decreases in head width that were not correlated with the time of exposure. ANOVA analysis revealed that there was no interaction between ethanol dose and window of exposure. Panel A: these data are collapsed across windows of exposure: all three-dose levels of ethanol produced decreased head width. Panel B: these data are collapsed across all dosage groups. The window of exposure did not affect the decrease in head width. Data are presented as mean ± S.E.M.

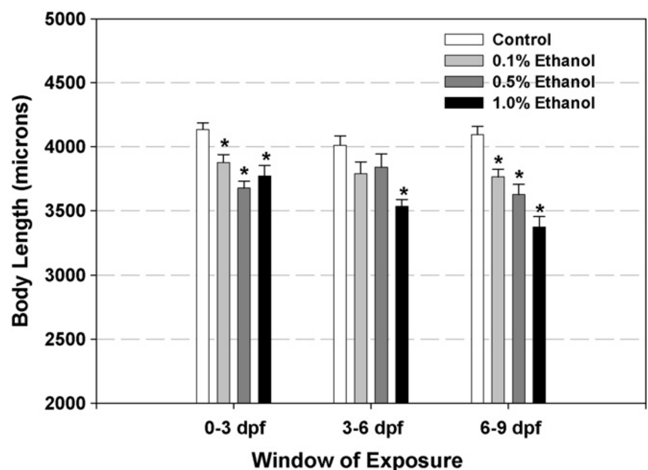

Fig. 5. Effects of developmental ethanol exposure on total body length of newly hatched medaka fry. Values represent the mean ± S.E.M. from two cohorts (total $n = 20$ per dose group/window). Ethanol exposure decreased total body length in a dose- and window-of-exposure-dependent fashion, i.e., there was an interaction between ethanol dose and window of exposure. The degree of ethanol-induced growth inhibition was most severe with high dose exposures that occurred late in development. The asterisks indicate a difference between the ethanol dosed group and the control for each respective window of exposure. Data are presented as mean ± S.E.M.

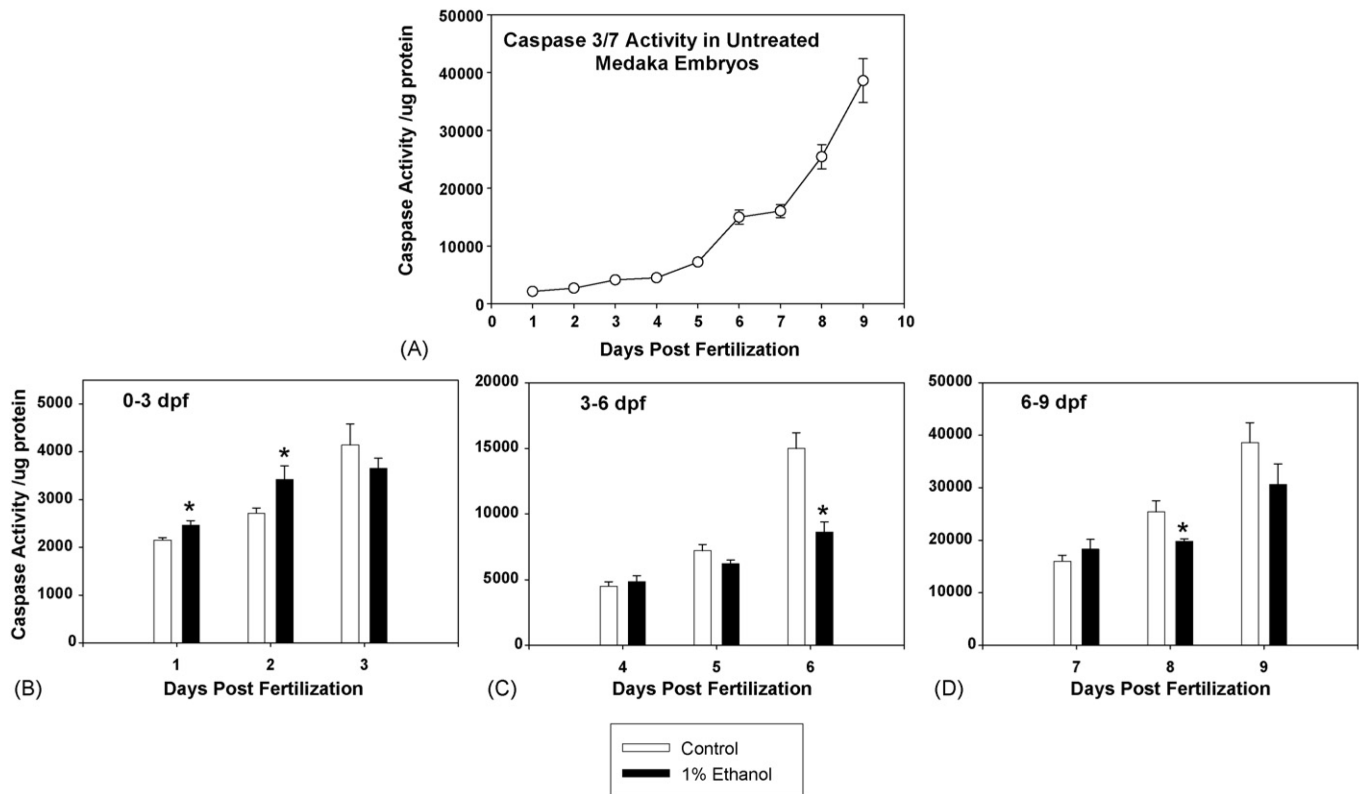

Fig. 6. Caspase-3/7 activity in the developing medaka embryo (panel A) and the effects of developmental ethanol exposure on caspase-3/7 activity (panels B, C and D; note that the y axes are different on the lower three panels). Values represent the mean response from three cohorts (total $n = 6$; each observation was a pool of 10 embryos). A single 24 h exposure to 1% ethanol-induced reciprocal effects on caspase-3/7 activity depending upon the timing of exposure. Early developmental ethanol exposure increased and later developmental ethanol exposure decreased caspase-3/7 activity. Data are presented as mean ± S.E.M. An overall ANOVA indicated that there was a main effect of ethanol treatment and a main effect of age of the embryos and an interaction between the two. Step-down ANOVAs comparing the caspase-3/7 activity for each age embryo revealed differences on 1, 2, 6 and 8 dpf. The asterisks indicate that the ethanol treated group is different from the age-matched control group ($p \leq 0.05$). The control data in the panel A and panels B, C, and D are identical.

decreased body length if embryos were exposed either early (0–3 dpf) or late (6–9 dpf) in development, but this effect was only apparent at the highest (1%) dose when ethanol exposure occurred during the middle window of development (3–6 dpf).

3.2.4. Caspase-3/7 activity

Under normal conditions, caspase-3/7 activity in the medaka embryo increases dramatically within the first 7 days of embryonic development, exhibiting an 18-fold increase in activity between days 1 and 9 post-fertilization (Fig. 6A). Interestingly, there are two plateaus in caspase-3/7 activity that occur between 3 and 4 dpf and then again between 6 and 7 dpf (Fig. 6A). During the first 3 days of development, caspase-3/7 activity increases approximately two-fold, and then increases more than three-fold between days 4 and 6 post-fertilization. This is followed by a 2.5-fold increase in caspase-3/7 activity that occurs during the last 3 days of development (6–9 dpf) (Fig. 6A). Treatment with 1% ethanol for 24 h produced differential effects on caspase-3/7 activity depending upon the timing of exposure; data are presented in blocks of 3 days due to differences in the magnitude (scale) of caspase activity. For example, if embryos were treated with ethanol on days 1 or 2 post-fertilization, there was an increase in caspase-3/7 activity (Fig. 6B); if the embryos were treated with 1% ethanol on day 6 after fertilization, there was a substantial decrease in caspase-3/7 activity (Fig. 6C), and if the embryos were treated on day 8, there was also a decrease in caspase-3/7 activity (Fig. 6D).

3.3. Embryonic concentration of ethanol during development

The above data outline the toxicodynamic effects of ethanol during three windows of development in the medaka fish. In order to determine the concentration of ethanol in the embryo during these same windows of exposure, embryonic ethanol levels were assessed after exposure to 1% ethanol. It was found that the embryo took up approximately the same amount of ethanol regardless of developmental stage (Fig. 7). The amount of ethanol that enters the embryo after 3 days of exposure to 1% ethanol (changing solutions every day) is approximately 60–68% (grey bars, Fig. 7) of the nominal concentration of the solution in the well. Moreover, the nominal concentration present in the solution surrounding the embryo did not decrease from the original amount (1%), which was added to the well 24 h before this sample was taken (black bars in Fig. 7).

4. Discussion

Most FAS studies focus on isolated events that occur within one specific stage of development and/or to one specific group

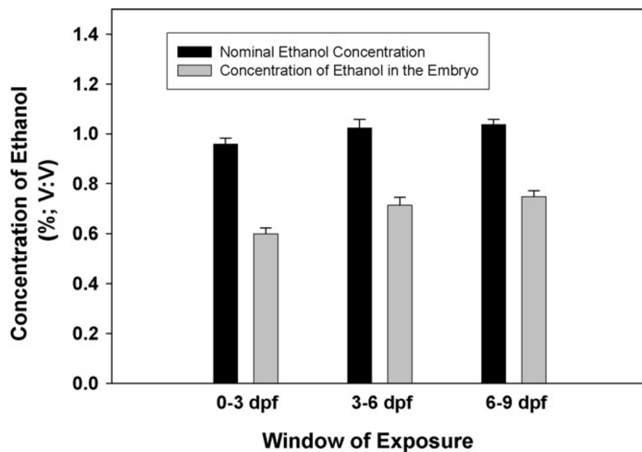

Fig. 7. Determination of ethanol dose. The embryonic ethanol dose was assessed after a 3-day exposure to 1% ethanol in embryo rearing medium during early, middle and late development; the solutions were renewed daily. Nominal and embryonic ethanol concentrations were determined ($n = 3$ pools of 30 embryos per exposure window). Data are presented as the mean ± S.E.M. Although ANOVA analysis revealed the nominal and embryonic ethanol concentrations to be different from one another, the amount of ethanol taken up by the embryo at each exposure window was not different depending on the age of the embryo.

of cells or neurons. Few investigators have examined the global effects on development obtained when ethanol administration is strictly partitioned within the confines of specific stages of development. The literature seems to indicate multiple effects on multiple systems with no clear indication of whether early or late gestational exposure to ethanol is more deleterious to the developing embryo.

The present studies used the extended duration of medaka embryonic development to assess systematically temporal variations in developmental ethanol toxicity. Table 1 shows that, in general, no window of development appears to be the least or most sensitive, rather the pattern of effects depends on the endpoint examined. If exposure occurs early in development (0–3 dpf), the embryo is extremely sensitive to reduced head width, body length and increased caspase-3/7 activity (presumably an increase in embryonic apoptosis). A similar pattern is observed if the embryos are exposed late in development (6–9 dpf) except that a decrease, rather than an increase, in caspase-3/7 activity is noted (discussed below). Embryos exposed to ethanol during the middle interval of development (3–6 dpf), however, appear less responsive to the ethanol-induced decreases in body length, equally responsive to the decreases in head width, and again show decreased caspase-3/7 activity. Some measures, like head width, are changed at all doses and all windows of exposure, whereas other endpoints like caspase-3/7 activity are very sensitive to the window of exposure, both with regard to direction of change and degree of alteration.

To put these changes in perspective, one can compare the results in Table 1 with known effects of ethanol on mammalian development. In humans, ethanol exposure during gestation produces persistent decreases in height and head circumference (Habbick et al., 1998; Sokol and Clarren, 1989) and these parameters are generally considered to be the most sensitive diagnostics for ethanol-induced intrauterine growth restriction (Streissguth et al., 1991). These effects are also seen in the present data, which show ethanol-induced deficits in body length and head width even at the lowest ethanol concentration (0.1%).

Comparison of the present data on ethanol toxicity in medaka with previously published studies of ethanol toxicity in fish shows some similarities and some differences. The dose of ethanol producing overt toxicity (i.e., 2% ethanol) is very similar to that noted by other studies. In medaka embryos, Wang et al. (2006a) estimated the 48 h LC_{50} for ethanol to be 3.2%, which is comparable to the present lethal dose calculation of 2% for 8 days. The zebrafish literature indicates that sensitivity to ethanol lethality is strain dependent (Loucks and Carvan, 2004), with the 3-day (i.e., embryonic) LC_{50} hovering around 1.7–2.0% (Loucks and Carvan, 2004; Reimers et al., 2004). There is disagreement, however, regarding how much ethanol enters the embryo. Although, Blader and Strähle (1998) assumed that 100% of the nominal (i.e., media) concentration of ethanol reached the zebrafish embryo because the rate of cyclopia was the same in chorionated and dechorionated embryos, later assessment of the intra-embryonic ethanol dose was estimated at only 30% of the nominal concentration in zebrafish (Reimers et al., 2004) or 20% of the nominal concentration in medaka (Wang et al., 2006a). This is in contrast to the present data which indicate that approximately 60–70% of the waterborne ethanol enters the embryo. These differences may be due to the longer exposure interval (3 days) used in the present study or to our optimization of the assay for measuring the ethanol concentration. In sum, most zebrafish and medaka studies find that concentrations above 2% ethanol are lethal to the embryo, but there is disagreement regarding the amount of ethanol that enters the embryo during exposure.

Central nervous system structures are heterogeneous, with individual neurons developing on different timescales that vary across brain regions and neuronal populations. If the mechanism of ethanol toxicity is not specific for the type of neuron

Table 1
Summary of results

Endpoint	Window of exposure		
	0–3 dpf	3–6 dpf	6–9 dpf
Length of egg development (time to hatching)	↑ only at highest ethanol dose	↑ only at highest ethanol dose	No effect
Head width	↓ at all doses	↓ at all doses	↓ at all doses
Body length	↓ at all doses	↓ only at highest ethanol dose	↓ at all doses
Caspase-3/7 activity (only assessed at 1% ethanol)	↑ 14% on day 1, ↑ 26% on day 2	↓ 42% on day 6	↓ 22% on day 8
Embryonic (ethanol)	60% of nominal	66% of nominal	69% of nominal

affected, but rather dependent upon the stage of neuronal development, this may explain the lack of specificity observed with ethanol related developmental effects. The more homogenous an experimental system is, the more likely a critical window for ethanol toxicity may be identified, but as the system becomes more heterogeneous (as in the entire brain), the temporal effects of ethanol are likely to appear multifaceted and diffuse (Miller, 1995).

No single mechanism can account for all of the adverse effects observed in FAS, but animal models have identified several possibilities, many of which precipitate cell death by apoptosis or necrosis. These include ischemia, oxidative stress and subsequent mitochondrial damage, interference with growth factor and second messenger function, decreased protein synthesis, insufficient nutrient utilization, abnormal glucose metabolism, altered prostaglandin homeostasis, depletion of energy stores and aberrant cell cycle kinetics (Abel and Hannigan, 1995; Guerri et al., 1984; Maier et al., 1996; Phillips and Cragg, 1982; Santillano et al., 2005; Schenker et al., 1990; West et al., 1994). Our data support changes in apoptosis as a potential mechanism of developmental ethanol toxicity. All animals exposed to 1% ethanol during development showed changes in caspase-3/7 activity regardless of the window of exposure. The earlier exposure increased caspase-3/7 activity, while exposure during mid to late egg development decreased caspase-3/7 activity. It is well accepted that an increase in caspase-3/7 activity signifies an increase in apoptosis, but it has become increasingly apparent in the last 8 years that this view may be too simplistic. Caspase-3 may possess non-apoptotic functions as well, including effects on cellular differentiation (Ishizaki et al., 1998; for recent reviews, see Abraham and Shaham, 2004; Launay et al., 2005; Sehra and Dent, 2006). In fact, our results are consistent with a recent paper exploring changes in caspase-3 activity in the pancreas of rats following ethanol exposure, where a substantial decrease in caspase-3 activity (Wang et al., 2006b) was noted. Although changes in caspase-3 activity are often viewed in terms of apoptotic potential, an alternative explanation may be deregulation of the developmental differentiation profile of the embryo. The developmental effects of ethanol exposure include changes in differentiation and cell cycle kinetics (Marcussen et al., 1994; Santillano et al., 2005). Because these processes are highly integrated, one could speculate that ethanol interference with caspase signaling is mediated by the inherent variability in the extent of programmed cell death that occurs naturally during different developmental stages. The present results, however, may not be solely related to the nervous system, as the caspase activity measured here is whole embryo caspase activity and not just confined to the nervous system. With an axis of development, however, it is likely that some, if not a major portion, of the caspase activity is related to the central or peripheral nervous system.

In his comprehensive review of the critical windows of developmental ethanol toxicity, West (1987) concluded that the last trimester human equivalent appeared to be most sensitive to ethanol toxicity. That conclusion was confounded by a lack of experiments that compared all three trimesters with one another, and also a lack of standardization with regard to blood ethanol concentrations. In fact, West posited that the third trimester equivalent may just appear more sensitive to ethanol toxicity because in general the blood ethanol concentrations tended to be higher. In the present study, we have addressed many of these issues by conducting a side-by-side comparison of different developmental windows while maintaining a comparable ethanol body burden in the embryo. Our general conclusion echoes the conclusions of West (1987) and Tran et al. (2000), that ethanol is toxic to all windows of development, but if one window must be chosen, the later windows appear to be more sensitive.

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