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Disruption of Circulation by Ethanol Promotes Fetal Alcohol Spectrum Disorder (FASD) in Medaka (*Oryzias latipes*) Embryogenesis

Yuhui Hu^a, Ikhlas A. Khan^a, and Asok K. Dasmahapatra^{a,b*}

^aNational Center for Natural Product Research, Environmental Toxicology Research Program, Research Institute of Pharmaceutical Sciences, University of Mississippi, University, MS 38677, USA

^bDepartment of Pharmacology, School of Pharmacy, University of Mississippi, University, MS 38677, USA

Abstract

Japanese medaka (*Oryzias latipes*) embryos exposed to ethanol have developed craniofacial, cardiovascular and skeletal defects which can be compared with the phenotypic features of fetal alcohol spectrum disorder (FASD) observed in human. The present experiment was designed to show that the disruption in circulation by ethanol during embryogenesis is a potential cause of FASD. Fertilized eggs were exposed to ethanol (0, 100 and/or 400 mM) for 24 or 48 h at various developmental stages (Iwamatsu stages 4–30) and were analyzed at 6 day post fertilization (dpf). It was observed that controls and the embryos exposed to 100 mM ethanol were in circulating state; however, a significant number of embryos of stages 4–24 exposed to 400 mM ethanol had disrupted circulation. Compared to controls, protein and RNA contents were significantly reduced in non-circulating embryos. Lipid peroxidation (LPO) analysis was made at 3, 6, 24, 48, 96 and 144 hour post fertilization (hpf). LPO was increased with the advancement of morphogenesis; however, ethanol or the circulation status had no effect. We further analyzed alcohol dehydrogenase (Adh 5 and adh8) and aldehyde dehydrogenase (Aldh9A and Aldh1A2) enzyme mRNAs in the embryos exposed to 400 mM ethanol for 24h. A developmental stage specific reduction in these enzyme mRNAs by ethanol was observed. We conclude that ethanol-induced disruption in circulation during embryogenesis is a potential cause of the development of FASD features in medaka.

Keywords

Alcohol; Japanese medaka; Development; RNA; protein; Lipid peroxidation

1. Introduction

Alcohol is a teratogen and exposure to alcohol in utero is toxic to the developing fetus. Fetal alcohol spectrum disorder (FASD), an umbrella term, is now used to describe the irreversible array of developmental anomalies associated with embryonic alcohol exposure (Riley and McGee, 2005). Alcohol consumption during pregnancy is the prime cause of the development

*Corresponding Author: Asok K. Dasmahapatra, National Center for Natural Product Research, Environmental Toxicology Research Program, RIPS, School of Pharmacy, 313 Faser Hall, P.O. Box 1848, University, MS 38677, USA, Voice: 662-915-7077 ; FAX: 662-915-5148, e.mail: asok@olemiss.edu.

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of FASD; however, the mechanism by which alcohol induces the birth defects is unknown. Moreover, the time and the dose of ethanol required for the development of FASD is still under investigation. Although, human studies of FASD are very limited due to ethical constraints, several non-human vertebrate and invertebrate animal models have been successfully utilized to understand the mechanism of FASD (Sulik, 2005; Cudd, 2005).

Fish models, primarily zebrafish (*Danio rerio*) and Japanese medaka (*Oryzias latipes*), are currently emerging as alternative non-mammalian vertebrate models for the study of gene function (Wittbrodt et al., 2002; Furutani-Seiki, 2004). These models have several advantages over mammalian models with regard to fertilization, development and transparency of the egg chorion. The effects of ethanol on zebrafish embryos have been studied by a number of investigators (Bilota et al., 2004; Carvan et al., 2004; Lockwood et al., 2004; Reimers et al., 2004; Arenzana et al., 2006; Li et al., 2007; Tanguay and Reimers, 2008), establishing that the fish can be used as a model organism to study FASD. Compared to zebrafish, medaka are less utilized in studying ethanol-induced toxicity. We are developing Japanese medaka embryogenesis as an alternative/ complementary model to the zebrafish to study the molecular mechanism of ethanol toxicity (Wang et al., 2006, 2007a, 2007b; Wu et al., 2008). We have demonstrated that medaka embryos exposed to ethanol for 48 hour post fertilization (hpf) have developed several phenotypic features in cardiovascular, craniofacial and skeletal systems which are comparable to the FASD features observed in human (Wang et al., 2006). Moreover, the effects at the cellular level have shown that ethanol is able to reduce total protein, RNA and DNA contents of the embryos in a dose-and time-dependent manner (Wu et al., 2008). Several other morphological and biochemical features including reduction in total body length, head width and alteration in caspase 3/7 activity by ethanol during medaka embryogenesis have also been reported (Oxendine et al., 2006). However, the developmental stage-specific effects of ethanol on medaka embryogenesis are not clearly understood from these studies (Oxendine et al., 2006). We hypothesized that the retarded growth in FASD phenotype is due to the reduced synthesis of macromolecules in the embryo and can be recovered by preventing disruption in cardiovascular development.

It is indeed a puzzle that ethanol, a single compound, is able to produce such a diverse effect in embryonic tissues during development. However, in morphogenesis, heart is the first organ to start function prior to the development of other organs. Therefore, proper development and function of cardiovascular system is critical during embryogenesis. Although the anatomy of fish and human heart is significantly different, heart development, vasculogenesis and hematopoiesis are precisely guided by conserved genetic programs in vertebrates (Lambrechts and Carmeliet, 2004). Moreover, the main advantage of using zebrafish or medaka as model to study cardiovascular defects is that the embryo of these fish, due to small size and low metabolism, can survive for several days with a compromised cardiac function, while a similar phenotype in mammals results early lethality (Lambrechts and Carmeliet, 2004). Among these two fish species (zebrafish and medaka), the cardiovascular development in medaka embryos followed the most common embryonic circulatory pattern observed in other vertebrates including human, while in zebrafish the pattern is different (Fujita et al., 2006). In this communication, we have reported that alteration in circulation status of the medaka embryos by ethanol affects cellular growth which is specific to the developmental status of the embryo.

2. Materials and Methods

The Institutional Animal Care and Use Committee (IACUC) of the University of Mississippi (UM) approved all the experimental protocols.

2.1. Experimental Procedure

Methods of animal maintenance, egg collection, RNA, and protein preparation and purification, semi-quantitative relative (rRT-PCR) and quantitative real-time PCR (qRT-PCR) techniques were previously described (Dasmahapatra et al., 2005; Wang et al., 2006, 2007b; Wu et al., 2008). The major modification made in the present experimental protocol was in the maintenance of medaka embryos during ethanol treatment. In all of our previous experiments we maintained embryos in one mL hatching solution (17 mM NaCl, 0.4 mM KCl, 0.36 mM CaCl₂, 0.6mM MgSO₄, NaHCO₃) with or without ethanol in a covered 48 well culture plate. In the present experiments embryos were maintained in 1.5 or 2 mL tightly capped microcentrifuge tubes in one mL hatching solution (one embryo/tube) with or without ethanol (control). The precaution was adapted to prevent the loss of ethanol from the media due to evaporation. After the desired period of ethanol treatment (24 or 48 h), viable embryos were transferred to 48 well culture plates (one egg/well/mL hatching solution). The hatching solution contains 0.0002% methylene blue (MB) to prevent fungal growth. Although MB is a mutagen, the concentration (0.0002%) we are using in our experiments was found to be ineffective in inducing any adverse effects in medaka development (data not shown). Ethanol (100 and 400 mM) was added to the culture medium at five different time points of development (Table 1) and discontinued either after 48h (groups A, B, C, D and E) or 24 h (F,G and H) following one time change of ethanol at 24h (groups A, B, C, D and E). The embryos were examined daily for routine developmental changes (cardiovasculature, blood clots, active circulation) under a phase contrast microscope (AO Scientific Instruments) with 50 % static renewal of the medium (when alcohol is no longer present in the medium). The embryonic development was classified after Iwamatsu (2004). The circulation status of the embryos was examined under a microscope and the flowing of blood through any of the circulating vessels was considered as the embryos were in circulating state. When blood was not flowing even through a single vessel the embryos were considered as in non-circulating state. The embryos were sacrificed at 6 (~144 hpf) day post fertilization (dpf) after determining the circulation status of the embryos. RNA was extracted from the intact embryo (embryos with yolk) by Trizol reagent (Wang et al., 2006). Total protein was extracted from embryos (after the yolk was removed) by 3.5% perchloric acid precipitation (Wu et al., 2008). The primers used for rRT-PCR and qRT-PCR of alcohol dehydrogenase (Adh5 and Adh8) and aldehyde dehydrogenase (Aldh1A2 and Aldh9A) enzyme mRNAs are in Table 2. The rRT-PCR and qRT-PCR techniques were published previously (Wu et al., 2008). For lipid peroxidation (LPO) experiments described below, embryos were exposed to 100 and 400 mM of ethanol 3, 6, 24 and 48 hpf and used for LPO assay immediately after alcohol removal. Controls (no ethanol) were maintained in hatching solution. Embryos exposed to ethanol (0, 100 and 400 mM) 48 hpf (group A) were also used for lipid peroxidation (LPO) analysis on 4 and 6 dpf.

2.2. Lipid Peroxidation

The thiobarbituric acid reactive species (TBARS) assay was used as an index of lipid peroxidation in embryo homogenates based on the formation of lipid peroxidation products as previously described by Oakes and Van Der Kraak (2003). In brief, 6–8 embryos were homogenized in 100 µL of 1.15% KCl containing 35 µM butylated hydroxytoluene (BHT). Fifty µL of the homogenate was mixed with equal volume of 8.1% sodium dodecyl sulfate (SDS), 375 µL of 20% acetic acid (pH 3.5), 375 µL of 0.8% thiobarbituric acid (Sigma-Aldrich, St. Louis, MO) and 150 µL of distilled water. An additional 50 µL of 67 mM BHT in ethanol was added to the mixture and the samples were heated at 95 °C for 1h. After cooling on ice, 250 µL distilled water and 1.25 mL n-butanol: pyridine (15:1) mixture was added to the samples and vortexed. After centrifugation at 2500 g for 20 min, the upper layer was removed carefully and the fluorescence was measured on a Spectra Max M5 (Molecular Devices, Sunnyvale, CA, USA) by setting excitation peak at 515 nm and emission peak at 553 nm. A stock solution of tetramethoxypropane (Sigma-Aldrich) in 40 % ethanol was prepared and used

for the preparation of standard curve which runs parallel in each assay. The protein concentration of the embryo homogenates was determined after Wu et al. (2008). The concentration of lipid peroxides generated during reaction was calculated as nM TBARS per mg protein.

2.3. Statistics

The data were analyzed by one way ANOVA followed by post-hoc Tukey's multiple comparison test. The results were expressed as mean $\pm$ SD of 3–8 independent experiments and $p < 0.05$ considered as significant.

3. Results

3.1. Ethanol Disrupts Embryonic Circulation in a Dose and Time-Dependent Manner

The effect of ethanol on cardiovascular system development of medaka embryos as a result of disruption in blood circulation is presented in Figure 1 and Figure 2. In normal development, medaka embryos initiate circulation by 50 hpf (Iwamatsu Stage 25). We exposed the embryos to ethanol (100 and 400 mM) for two consecutive days (with one-time change of ethanol after 24 h) before (group A, B and C; Iwamatsu stage 4–24) and after (group D and E, Iwamatsu stage 25–30) the onset of circulation. It was observed that control embryos (groups A–H) in the present experimental conditions were able to maintain the normal circulation in 6 dpf without any cardiovascular developmental deformities. Moreover, the embryos exposed to 100 mM ethanol (groups A–E) were also not affected by ethanol treatment. In contrast, a significant number of embryos of groups A, B and C treated with 400 mM of ethanol were unable to initiate normal circulation on 6 dpf (compared to control and 100 mM) and majority of them were developed blood clots (one to many) in different parts of the circulating vessels (Figure 1). Their heart either remained straight (tube heart) or unable to loop completely. Further analysis of the data showed that the number of embryos with disrupted circulation was significantly higher in group A than group C (Figure 2A). The embryos of groups D and E treated with equal ethanol concentration (400 mM) were able to maintain their normal circulation status as in controls and 100 mM ethanol-treated embryos (Figure 2A). In groups F, G and H, all of these embryos were in a non-circulating stage at the beginning of the ethanol treatment (Iwamatsu stages 4–24). These embryos were exposed to 400 mM of ethanol only for 24 h and examined on 6 dpf. It was observed that the number of embryos with circulation in groups F and G were significantly increased in comparison with the embryos treated with two consecutive days ethanol (Group A, B or C), however, those with disrupted circulation have blood clots and cardiac deformities. All embryos of group H (Iwamatsu stage 23–24; no circulation) treated with 400 mM ethanol (2 dpf–3 dpf) were able to initiate circulation in 6 dpf as in controls (Figure 2B).

3.2. Effects at the Cellular Level

3.2.1. Macromolecules—Total protein and RNA content of the medaka embryos developmentally exposed to ethanol are presented in Figure 3. The cellular constituents (protein and RNA) taken from fish embryos exposed to ethanol for two consecutive days (groups A–E) were pooled irrespective of their circulation status. Those exposed for one day (groups F, G and H) were separated into non-circulating embryos (F- and G-) and circulating embryos (F+, G+, H+) prior to be being pooled. It was observed that ethanol at 400 mM concentration significantly reduced the total protein and RNA contents of the embryos from groups A, B and C at 6 dpf in comparison with the controls and 100 mM groups. However, no significant differences in these macromolecular components (total protein and RNA) were observed in groups D and E embryos (Figures 3A and 3C) exposed to 0 (control), 100 or 400 mM ethanol. In groups F, G and H, embryonic protein content was significantly lower in the ethanol-treated embryos. Further, embryos without circulation (F- and G-) had significantly less protein than

the embryos with circulation (F+ and G+) (Figure 3B). In the case of RNA, the results were slightly different. Those embryos who had failed to initiate circulation on 6 dpf (F- and G-) due to ethanol treatment (400 mM for 24 h) had significantly less amount of RNA than the embryos with circulation (controls or F+, G+ or H+ groups). No statistical difference on 6 dpf embryos was observed in the RNA contents of the control and the ethanol-treated embryos with circulation (F+, G+ and H+) (Figure 3D).

3.2.2. Lipid peroxidation—The alterations in lipid peroxidation status of medaka embryos during development with or without ethanol are presented in Figure 4. It was observed that lipid peroxidation status of medaka embryos was normally increased during development. Embryos at 6 dpf (~144 hpf) had significantly higher thiobarbituric acid reactive species (TBARS) than the embryos at 3 hpf (Figure 4). Ethanol (both 100 and 400 mM) was unable to alter significantly the TBARS content of the embryos at any stages of development with regard to the corresponding controls. Moreover, circulation status had no effect on lipid peroxidation.

3.2.3. Alcohol metabolizing enzyme mRNAs—The expression of alcohol metabolizing enzyme mRNAs (Adh5, Adh8, Aldh9A and Aldh1A2) during medaka embryogenesis with (400 mM, 24 h exposures) or without ethanol is presented in Figure 5 and Figure 6. We used rRT-PCR for Adh5, Adh8 and Aldh9A mRNAs (Figure 5) and qRT-PCR for Adh5, Adh8, Aldh9A and Aldh1A2 mRNA (Figure 6) for analysis. The data obtained by rRT-PCR showed that the embryos treated with ethanol and have circulation (group F+, G+ and H+) were able to maintain equal amount of Adh and Aldh mRNAs on 6dpf as in controls. However, embryos without circulation (F- and G-) showed developmental stage specific response (reduction). Embryos of group F- (embryos at Iwamatsu stages 4–10 exposed to 400 mM of ethanol for 24h and without circulation on 6 dpf) showed significant reduction in these mRNA (Adh5, Adh8 and Aldh9) levels with respect to controls, however, embryos of G-group (Iwamatsu stages 17–20, exposed to 400 mM ethanol 24h and without circulation on 6 dpf) had the same level of the mRNAs mentioned above as in controls (Figures 5A, B, and C). In qRT-PCR analysis, Adh5, Adh8 and Aldh1A2 mRNA expression (Figures 6A, 6B and 6D) showed similar nature of changes (decreased in F- and remained at the same level in F+, G+, G- and H+) as in rRT-PCR, however, Aldh9A (Figure 6C) mRNA level in F, G and H groups were remained at the same level as in controls regardless of the circulation status of the embryo.

4. Discussion

As a continuation of our previous studies of the effects of ethanol during medaka embryogenesis, the present experimental data indicates that ethanol at early stages of development (Iwamatsu stages 4–24; embryos without circulation) induces abnormalities in cardiovascular development (no circulation) followed by reduction in protein and RNA content (Figure 1, Figure 2 and Figure 3). LPO increases with the advancement of morphogenesis; however, ethanol is unable to induce any significant changes in LPO status of the embryos (Figure 4). Further analysis of Adh and Aldh mRNAs also indicates that embryos at early stages of development are more sensitive to ethanol than the embryos at late stages (Figure 5 and Figure 6). Our previous studies showed that exposure to ethanol disrupted the circulation status of the embryos in a dose-dependent manner (Wang et al., 2006). Other morphological parameters like craniofacial, cardiovascular and skeletal deformities by ethanol were also found to be dose-specific in medaka (Wang et al., 2006). Recently, we have demonstrated that medaka embryos exposed to ethanol for 48 hpf have reduced cellular macromolecular constituents (Protein, RNA and DNA) which might have a direct effect on the embryonic growth during morphogenesis (Wu et al., 2008). However, we were unable to demonstrate any significant alteration in alcohol metabolizing enzymes (Adh5, Adh8, and Aldh9A) and transcription factors (emx2, en2, iro3, otx2, shh, wnt1, zic5) mRNAs in medaka embryos in

similar conditions (Wang et al., 2006, 2007b; Wu et al., 2008). The critical period of teratogenic action of alcohol during embryonic development of medaka was not known to us. We therefore followed a strategy to determine the critical phase of ethanol toxicity in medaka embryogenesis and used circulation status as morphological and macromolecular contents (RNA and Protein) as biochemical markers of ethanol teratogenesis. In our culture conditions (16 h light and 8 h dark, temperature 25 °C) control embryos were hatched ~ 175 hpf (Wang et al., 2006). We therefore modified the treatment conditions [exposed the embryos with ethanol at different stages of development (Iwamatsu stages 4–30) and continued the treatment either for 24 or 48 h] and all viable embryos were examined and harvested on 6 dpf to avoid hatching.

The cardiovascular development in medaka embryo has been studied by several investigators (Iwamatsu, 2004; Fujita et al., 2006). The heart becomes visible under a microscope ~38 hpf (Iwamatsu stage 22). The blood vessels (Cuvierian ducts and vitello-caudal vein or marginal vein) and the intermediate cell mass (ICM) or blood island are pronounced in Iwamatsu stage 23 (~41 hpf). The heart starts beating at ~ 44 hpf (Iwamatsu stage 24) with very low heart rate (32–64 beats/min); the embryonic erythrocytes and hemangioblasts are aggregated in ICM during this period. During ~50 hpf (Iwamatsu stage 25) the heart rate increased (70–80 beats/min) and the globular blood cells come out from the ICM (onset of circulation). At ~64 hpf (Iwamatsu stage 28) the blood cells appear to be flatten (Iwamatsu, 2004). The differentiation of embryonic heart into atrium, ventricle, sinus venosus and bulbous aorta begins ~74 hpf (Iwamatsu stage 29). Branching of arteries to supply blood to trunk muscles, gills and brain starts at ~ 82 hpf (Iwamatsu stage 30) and the circulation to the pectoral fins occurs at ~ 121 hpf (Iwamatsu stage 34). The looping of heart is complete at ~144 hpf (Iwamatsu stage 36).

Our experimental design can be justified in terms of cardiovascular development in medaka. The embryos belonging to group A were exposed to ethanol from very early stages of development (early to late blastula) to the stage prior to the onset of blood circulation (0–48 hpf). In group B, ethanol treatment started at early neurula stage and continued to the stage of heart chamber formation (24–72 hpf); in group C, embryos prior were exposed to ethanol prior to the onset of circulation and the treatment was continued to the beginning of the blood supply to the organs (48–96 hpf). In group D (72–120 hpf) and E (96–144 hpf), embryos were exposed to ethanol when the circulation was already started. With these treatment conditions we observed that embryos exposed to 100 mM of ethanol were able to initiate circulation as in controls; however, significant numbers of embryos exposed to 400 mM of ethanol in groups A, B and C were unable to initiate circulation on 6 dpf. If the embryos were exposed to ethanol after the onset of circulation, no alteration in the circulation status of the embryos was observed on 6 dpf (groups D and E). We therefore conclude that onset of circulation can be used as a hallmark of ethanol toxicity in medaka embryogenesis. We have also observed that some of the ethanol-treated embryos were able to establish circulation in later stages of development and were survived with FAS features like craniofacial disorders (Wang et al., 2006) after hatching. However, the embryos that were unable to establish circulation due to ethanol treatment, majority of them (specifically group A) were died prior to or after hatching. To find the critical phase of ethanol toxicity we have reduced the alcohol exposure window to 24h. Embryos of group A, B and C treated with 400 mM ethanol were removed from ethanol after 24 h (group F, ethanol: 0–24hpf, no sign of cardiovascular development; group G, ethanol: 24–48hpf, heart and blood vessel development phase; group H, ethanol: 48–72hpf, onset of circulation phase). It was observed that more embryos were able to initiate circulation on 6 dpf in groups F, G and H compared to the corresponding A, B and C groups. Moreover, in group H, all embryos were able to recover from ethanol stress (all stresses were located within the circulatory system). It is therefore further concluded that the effects of ethanol on cardiovascular development of medaka are specific to the dose, duration, and developmental status of the embryos. Ethanol at 100 mM concentration (48 h duration) had no effect in altering circulation status of the embryos, however, those exposed to 400 mM ethanol either for 48 h

(groups A, B and C) or for 24 h (groups F and G) have a significantly disrupted circulation status depending upon the stage of ethanol treatment (Figure 1A). Embryos of group D and E were non-responsive to ethanol with regard to their circulation status. Analysis of protein and RNA content of the embryo further support our hypothesis that alterations in circulation status during embryogenesis were reflected in the reduction of cellular protein and RNA content in the embryonic body (Figure 3) which ultimately can cause growth retardation in embryo, a major phenotypical feature observed in FASD babies. The yolk is the only source of nutrients during embryo development in medaka, and supply of nutrients from the yolk to the embryo occurs through the circulation. Therefore, disruption in circulation/ circulatory system by ethanol inhibited the normal growth of the embryo. Present data are in agreement with our previous observation that medaka embryos exposed to 400 mM ethanol 48 hpf have reduced protein and RNA content on 4 and 6 dpf (Wu et al., 2008). Moreover, from the present data it may also be concluded that medaka embryos are more sensitive to ethanol in early stages of development before the onset of circulation (Iwamatsu stages 4–24) than the embryos in late stages with circulation (stages 25–30).

One of the potential mechanisms of ethanol toxicity is the production of oxidative stress due to ethanol metabolism by ADH and ALDH enzymes (Dennery, 2007). The oxidative stress can generate either free radicals or highly reactive aldehydes that damage macromolecules (Reimers et al., 2004). Moreover, oxidative stress and redox signaling are essential for normal developmental processes (Dennery, 2007). In chick embryo, ethanol altered the early vascular development in area vasculosa in a concentration-dependent manner (Tufan and Satioglu-Tufan, 2003). The mechanisms proposed for the disruption were the generation of reactive oxygen species, perturbation of retinoic acid signaling, and alteration in the expression of growth regulatory vasculogenic factors and their receptors (Tufan and Satioglu-Tufan, 2003). Reduction in cellular protein and RNA contents in medaka embryogenesis by ethanol (an index of oxidative stress) thus prompted us to extend our investigation to determine the status of oxidative stress and alcohol metabolizing enzyme mRNA levels in medaka embryos. Quantification of oxidative stress is very difficult due to short half-lives of free radicals and many products initially produced from polyunsaturated fatty acids (Esterbauer, 1996). For this reason, the detection of oxidative stress is mainly based on the quantification of malondialdehyde (MDA) which is formed as a result of peroxidation of polyunsaturated fatty acids (Rio et al., 2005). The reaction of MDA with 2-thiobarbituric acid produced 2-thiobarbituric acid reactive substances (TBARS) which is widely used in oxidative stress assay (Liu et al., 1997). However, this assay is not very specific; many other substances can participate in TBARS formation. Our data (Figure 4) have documented an increase in LPO in medaka embryos with the advancement of morphogenesis, however, embryos (Iwamatsu stages 4–10) exposed to ethanol (100 mM or 400 mM) at 3, 6, 24 and 48 hpf were unable to show any significant difference with respect to controls. Moreover, group A embryos (embryos were exposed to ethanol for 48 hpf and then maintained in hatching solution) were analyzed at 96 and 144 hpf also showed no change after ethanol treatment. Enhancement of LPO during development was also reported in other species (Freitas et al., 2007). However, ethanol has been shown to induce LPO in fetal rat hepatocytes (Henderson et al., 1999) which are not in agreement with the results obtained here. More studies with different LPO analysis techniques are needed before accepting the view that ethanol is unable to induce LPO in medaka embryogenesis.

Previously, we were unable to demonstrate any statistically significant difference in Adh5, Adh8 and Aldh9A mRNA contents in the embryos on 6 dpf exposed to ethanol 48 hpf with controls (Wang et al., 2006, 2007b). However, the expression of these mRNA was developmentally regulated (Dasmahapatra et al., 2005; Wang et al., 2007a,b). In our current experiment the Aldh9 mRNA data obtained by rRT-PCR and qRT-PCR were not identical, but we were still able to demonstrate a significant dose- and developmental stage-specific reduction

in these mRNA level by ethanol during medaka embryogenesis. The reduction may be related to the growth retardation rather than inhibition of the synthesis of these enzymes during development. Previously we have reported that the expression of Adh8, Aldh9A, and Aldh1A2 was developmentally regulated and the level of mRNAs was increased with the advancement of morphogenesis (Wang et al., 2007a). Therefore, the reduced level of Adh and Aldh mRNAs in medaka embryogenesis by ethanol is probably due to the inhibition in morphogenesis.

Taken together, the data obtained from present experiments and from our previous studies, we can conclude that medaka embryo with regard to cardiovascular development can be used as a unique non-mammalian vertebrate model to study ethanol teratogenesis and thus FASD. The embryos were very sensitive to ethanol before the onset of circulation than in late phases. Medaka embryos from Iwamatsu stage 25 onwards can be likened to a fetus. These embryos (stage 25 and onwards) have well developed heart with circulation, several organs including brain (organogenesis), pectoral fin buds, which can be compared with the features found in a human fetus. Therefore, in medaka, embryos are more ethanol-sensitive than in the fetal-like stages and the critical ethanol- sensitive phase was the embryonic phase.

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Figure 1. Representative photomicrographs of medaka embryos showing blood clots in the circulating vessels

Group F embryos (0–24 hpf treatment) were photographed at 96 hpf. A= control (no ethanol); B= 400 mM ethanol (0–24 hpf). Arrow (→) represent the area near to the blood island where the first sign of blood clots seen (B) absent in control (A). NC indicates the notochord and T indicates the tail of the embryo.

Figure 2. Effect of ethanol on circulation status of medaka embryos during development
 Embryos were exposed to 100 or 400 mM ethanol for 48h in groups A, B, C, D and E, and 400 mM ethanol for 24h in groups F, G and H. Parallel groups with no ethanol served as controls. For group details see Table 1. Circulation status of the embryos was examined and analyzed on 6 dpf. Each group contained 6–8 embryos per treatment. Each bar is the mean $\pm$ SEM of 15 to 20 separate treatment repetitions. Bar head with pound symbol (#) indicates that the data are significantly different ($p < 0.05$) from corresponding controls (no ethanol) and asterisks (*) indicates the difference ($p < 0.05$) with group A. Panel marked A= Embryos were exposed to ethanol (100 or 400 mM) for 48h, and marked B= embryos exposed to ethanol (400 mM) for

24h. The values of A, B and C in panel A are significantly different from the corresponding values of F, G, and H of panel B.

Figure 3. Effect of ethanol on protein and RNA contents of medaka embryos during development Embryos were exposed to 100 or 400 mM ethanol for 48h in groups A, B, C, D, and E, and 400 mM ethanol for 24h in groups F, G and H. Parallel groups with no ethanol serve as control. For group details see Table 1. Viable embryos were sacrificed on 6 dpf and were used for protein (panels A and B) and RNA (panels C and D) analysis. The circulating embryos from the same group were separated from the non-circulating ones before protein and RNA assay in groups F and G. Each group consists of 6–8 embryos per treatment. Each bar is the mean $\pm$ SEM of 4–6 separate treatment repetitions. Bar head with pound symbol (#) indicates the data are significantly different ($p < 0.05$) from the corresponding control and with asterisk (*) indicate that the data are different from the circulating embryos. Panels A and B= Protein; Panels C and D= RNA. In panels B and D, the group (F, G and H) marked '+' indicate that the embryos were in circulation on 6 dpf and marked '-' indicate that the embryos were unable to initiate circulation.

Figure 4. Effect of ethanol on lipid peroxidation status of medaka embryo during development Embryos were exposed to 100 and 400 mM of ethanol for various time points and used for lipid per oxidation (LPO) assay. Embryos exposed to ethanol for 3, 6, 24 and 48 h and were used for LPO assay immediately after alcohol removal. Embryos used in 96 and 144 hpf were exposed to 100 and 400 mM of ethanol for 48 hpf and then maintained in hatching solution. Parallel groups with no ethanol served as control. Each group consists of 6–8 embryos per treatment. Each bar is the mean $\pm$ SEM of 4–6 separate treatment repetitions. Bar head with pound symbol (#) indicates that the control data are significantly different ($p < 0.05$) from the 3h control groups.

Figure 5. Effect of ethanol on Adh 5, Adh8 and Aldh9A mRNA expression in medaka embryos developmentally exposed to ethanol

Total RNA was prepared from 6–8 pooled medaka embryos on 6 dpf, reverse transcribed and analyzed by rRT-PCR using β-actin primers (1 or 2) as internal control. For Adh5 internal control primer is β-actin (1) and for Adh8 and Aldh9A β-actin (2) was used. The results were expressed as the ratio of relative band intensity of target gene: β-actin. The data obtained were log transformed and used for one way ANOVA followed by post-hoc Tukey's multiple comparison test. Each entry is the mean $\pm$ S.E.M. of 4–6 separate experiments and $p < 0.05$ considered as significant. F, G and H represents the groups of medaka embryos exposed to ethanol for a specific development period [F= 0–24 hpf, G= 24–48 hpf, H=48–72 hpf]; '+'

indicates that the embryos were in circulation and ‘-’ indicates the embryos were unable to initiate circulation on 6 dpf. A= Adh5, B=Adh8, C= Aldh9A. Bar head with pound symbol (#) indicates that the mean data is significantly different from the controls. Representative gel pictures of the rRT-PCR analysis are presented on the right side of the panel A (Adh5), B (Adh8) and C (Aldh9A) in which ‘M’ indicates 100 bp ladder and ‘C’ for controls (no ethanol).

Figure 6. Effect of ethanol on Adh 5, Adh8 and Aldh9A mRNA expression in medaka embryos developmentally exposed to ethanol

Total RNA was prepared from 6–8 pooled medaka embryos on 6 dpf, reverse transcribed and analyzed by qRT-PCR. For each sample, the threshold cycle for internal standard (eif1a) amplification (Ct, eif1a) was subtracted from the threshold cycle of the corresponding enzyme mRNA amplification (Ct, enzyme) to yield ΔCt . For each treatment group, the data are the mean of ΔCt of control samples was subtracted from each individual samples to yield individual $\Delta\Delta Ct$. Fold induction relative to control samples was calculated with $2^{-\Delta\Delta Ct}$. The data obtained were log transformed and used for one way ANOVA followed by post-hoc Tukey's multiple comparison test; $p < 0.05$ was considered as significant. Each data are the mean $\pm$ S.E.M. of 3–4 separate experiments. F, G and H represents the groups of medaka embryos exposed to ethanol for a specific development period [F= 0–24 hpf, G= 24–48 hpf, H=48–72 hpf]. Group with '+' indicates that the embryos were in circulation and '-' indicates the embryos were unable to initiate circulation on 6 dpf. A= Adh5, B=Adh8, C= Aldh9A, D=Aldh1A2. Bar head with pound symbol (#) indicates that the mean data is significantly different from the controls.

Table 1

Group classification of medaka embryos considered during ethanol treatment

Groups	Alcohol treatment (hour post fertilization)	Developmental Stages at the beginning of ethanol treatment (Iwamatsu, 2004)	Observations after alcohol removal (h)
A	0–48	4–10	~96
B	24–72	17–20	~72
C	48–96	23–24	~48
D	72–120	28–29	~24
E	96–144	30–32	~1
F	0–24	4–10	~120
G	24–48	17–20	~96
H	48–72	23–24	~72

Table 2

List of primers were used in semi-quantitative RT-PCR (rRT-PCR) and quantitative real-time RT-PCR (qRT-PCR) amplifications of the alcohol metabolizing enzyme mRNAs of Japanese medaka embryo

mRNA	Sense (5'-3')	Antisense (5'-3')	Target/internal standard	Product (bp)	GenBank Accession
Adh5 ^a	GTCACACAGA TGCCTACACTC	GCCCCGGCAA CTTTGCAGCCC	Target	514	AY512892
Adh8 ^a	CATTGCTGGA CGGACCTGGAAG	GTCGGGAAAC ACTCAGGACTG	Target	206	AY682722
Aldh1A2 ^b	CATGACTTCCAGT AAGATCGAG	GATTTGTCCACA AACTCCAATAG	Target	550	DQ897366
Aldh9A ^a	TGCTTGCATCCCG AACGACATG	CTTGCCATTGT TGATCACTTC	Target	355	DQ535181
β-actin ^c (1)	TTCACAGCCCT GCCATGTA	GCAGCTCATAGCT CTTCTCCAGGGAG	Internal Standard	359	S74868
β-Actin ^c (2)	CCTGACCCTG AAGTATCCA	GAGCTATGAG CTGCCCTGACG	Internal Standard	542	S74868
Erf1a ^b	ACGACAAAGAT GAGCTGGTT	GGGCACAGCTT CTGGTAAAG	Internal Standard	300	NM_001104662

Superscript 'a' indicates that the primers were used for both rRT-PCR and qRT-PCR; 'b' indicates the primers used only for qRT-PCR, and 'c' indicates the primers used only for rRT-PCR. Medaka has only one β-actin gene reported in GenBank (GenBank Accession **S74868**). In the present experiment two sets of primers were designed in two different regions of the same β-actin gene.