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Ethanol induced attenuation of oxidative stress is unable to alter mRNA expression pattern of catalase, glutathione reductase, glutathione-S- transferase (GST1A), and superoxide dismutase (SOD3) enzymes in Japanese rice fish (*Oryzias latipes*) embryogenesis

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Abstract

Although the mechanism of ethanol toxicity during embryogenesis is unknown, our earlier studies on Japanese rice fish (*Oryzias latipes*) embryos indicated that the effects might be mediated through oxidative stress. In this study we have determined the oxidative stress and the mRNA content of four antioxidant enzymes (catalase, glutathione reductase, glutathione-S-transferase, and superoxide dismutase) during Japanese rice fish embryogenesis (from 0 day post-fertilization to hatching) and after exposing the embryos to ethanol (100 and 300 mM) for 48 h at three stages (0–2, 1–3 and 4–6 day post fertilization, dpf) of organogenesis. We observed that oxidative stress was minimal in blastula, gastrula or neurula stages, increased gradually with the advancement of morphogenesis and reached its maximum level in hatchlings. The antioxidant enzyme mRNAs were constitutively expressed throughout development; however, the expression pattern was not identical among the enzymes. Catalase and superoxide dismutase (SOD) mRNAs were minimal in the fertilized eggs, but increased significantly in 1 dpf and then either sharply dropped (SOD) or maintained a steady-state (catalase). Glutathione S-transferase (GST) was very high in fertilized eggs and sharply dropped 1 dpf and then gradually increased thereafter. Glutathione reductase (GR) maintained a steady-state throughout the development. Ethanol was able to attenuate oxidative stress in embryos exposed only to 300 mM 1–3 dpf; no significant difference with controls was observed in other ethanol-treated groups. The antioxidant enzyme mRNAs also remained unaltered after ethanol treatment. From these data we conclude that the attenuation of oxidative stress by ethanol is probably due to the inhibition of normal growth of the embryos rather than by inhibiting catalase, GST, GR or SOD- dependent activities.

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Keywords

Alcohol; Japanese rice fish; oxidative stress; Development; Antioxidant enzymes

1. Introduction

Fetal alcohol spectrum disorder (FASD) is an important clinical problem resulting from prenatal exposure to alcohol which can cause serious central nervous system (CNS), cardiovascular, and craniofacial defects affecting the entire lifetime of an individual (Abel et al., 1995; Streissguth and O, Mally, 2000; Autti-Ramo et al., 2006, Manning and Eugene, 2007). There are also high costs to society, both socially and medically, associated with the disorder (Thanh and Jonsson, 2009). Significant efforts have been made to educate women about the dangers of alcohol intake during pregnancy; however, the incidence has remained unchanged (Floyd et al., 2009). The precise mechanism by which alcohol is able to induce FASD is also unknown, but a number of potential pathways have been proposed (Goodlett et al., 2005). One of the mechanisms proposes that because ethanol promotes systemic oxidative stress by generating reactive oxygen species (ROS), the cellular damages mediated by oxidative stress and ROS are able to cause FASD phenotypes (Cohen-Kerem and Koren, 2003). Another hypothesis suggests that ethanol-induced vasoconstriction in the umbilical vessels results in impaired oxygenation and nutrient delivery to the fetus, which ultimately transforms a normal embryo into a FASD phenotype (West 1994; Siler-Khodr et al., 2000; Signore et al., 2008). However, all of these mechanisms may be different in different models and need further studies.

We have been evaluating Japanese rice fish or medaka (*Oryzias latipes*) as a non-mammalian vertebrate model to identify the molecular mechanisms involved in FASD (Dasmahapatra et al., 2005; Wang et al., 2006, 2007a, b; Wu et al., 2008; Hu et al., 2008, 2009). We observed that Japanese rice fish embryos exposed to ethanol developmentally have precocious craniofacial, skeletal and cardiovascular features (Wang et al., 2006; Hu et al., 2008) which were analogous to human FASD phenotypes (Figure 1). Moreover, the macromolecular constituents (Protein, RNA and DNA) and the alcohol metabolizing enzyme mRNAs were reduced in a dose and developmental stage-specific manner (Wu et al., 2008; Hu et al., 2008). We hypothesize that the detrimental effects of ethanol on Japanese rice fish development were probably mediated through the generation of reactive oxygen species (ROS) and thus oxidative stress. To elucidate the mechanisms further we have measured lipid peroxidation and NAD⁺/NADH status in Japanese rice fish embryos developmentally exposed to ethanol (Hu et al., 2008, 2009). Surprisingly, we were unable to establish any significant alteration in these parameters in this fish model. Therefore we are searching for other parameters which can be used as an indicator of oxidative stress that show sensitivity to ethanol treatment. In the present communication we have measured oxidative stress as hydrogen peroxide concentration in post-fertilized Japanese rice fish embryos (~1hpf–6 dpf) and hatchlings (9 and 10 dpf) during normal development and after exposing the embryos to ethanol for 48 h at specific developmental stages. Moreover, increasing evidence indicated that oxidative stress alters the expression of antioxidant enzymes. We therefore measured the mRNA contents of four antioxidant enzymes (catalase, glutathione reductase, glutathione-s- transferase, and super oxidative dismutase) under identical conditions. Our data showed that both oxidative stress and antioxidant enzyme mRNAs maintained distinct developmental patterns during Japanese rice fish embryogenesis; however, ethanol exposure (300 mM) is able to attenuate oxidative stress in a few occasions without altering the mRNA expression pattern of catalase, GR, GST and SOD.

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 and embryo culture conditions were performed as previously described (Hu et al., 2008, 2009). In brief, fertilized eggs of Japanese rice fish (*Oryzias latipes*, medaka) after collection were maintained in hatching solution (17 mM NaCl, 0.4 mM KCl, 0.6 mM MgSO₄, 0.36 mM CaCl₂, with required amount of NaHCO₃ to maintain the pH 7.4 and 0.0002% methylene blue to reduce fungal infection) in a Precision high performance incubator (Thermo Fisher Scientific, Waltham, MA, USA) at 26±1 °C with 16L: 8D light cycle. Ethanol (100–300 mM) was added to the medium after transferring the viable embryos at respective developmental stages to 2 mL tubes (1 egg/tube in 1 mL medium) and the tubes were tightly capped to stop ethanol loss due to evaporation. Alcohol treatment was given to the embryos at three different time points of development (0 dpf, Iwamatsu stages ~ 4–10; 1 dpf, Iwamatsu stages ~17–20; 4 dpf, Iwamatsu stages ~30–32) and discontinued after 48 h, following a one time renewal of ethanol after 24 h. Embryos were examined for routine developmental changes (cardiovasculature, blood clots, active circulation) under a phase contrast microscope (AO Scientific Instruments) and were classified after Iwamatsu (2004) before sacrifice. To observe FASD phenotypes in hatchlings some of the control and ethanol-treated embryos were maintained in clean hatching solution and allowed to hatch. After successful hatching the hatchlings were either transferred to balance salt solution (BSS, 17 mM NaCl, 0.4 mM KCl, 0.6 mM MgSO₄, 0.3 mM CaCl₂, pH 7.4) or maintained in hatching solution and fed with TetraMin Flake (Fish pet, Lancaster, PA, USA) and live brine shrimp (*Artemia salina*) nauplii. The photographs (Figure 1) of the hatchlings were taken on desired dates by an Olympus B-max 40 microscope at constant magnification (Wang et al., 2006).

2.2 Analysis of oxidative stress

The oxidative stress (hydrogen peroxide concentration) was analyzed as described by Ozawa et al (2004) with some modifications. In brief 6–8 medaka embryos or hatchling at different periods of post fertilization or after required period of ethanol treatment were homogenized in 100 µL extraction buffer (130 mM KCl, 5 mM MgCl₂, 20 mM NaH₂PO₄, 20 mM Tris-HCl, 30 mM glucose, pH 7.4) and centrifuged at 10,000 g for 15 min at 4 °C. Fifty µL of the supernatant was transferred to the wells of a 96 well black microtiter plate (BD Falcon, BD Biosciences, San Jose, CA, USA) and 5 mM of 2', 7'-dichlorodihydro-fluorescein diacetate (DCHFDA; Molecular probes, Eugene, OR, USA) in DMSO was added to each well. The final reaction volume was adjusted to 200 µL by extraction buffer. Reaction blank was made by adding 200 µL of extraction buffer with 5 mM DCHFDA. The microplate was incubated in dark at 37 °C for 60 min. Following incubation the fluorescence intensity in dark was monitored for 60 min after an excitation at 485 nM and emission at 535 nM using spectra Max M5 (Molecular Devices, Sunnyvale, CA, USA). The protein concentration of the supernatant was determined using Bio-Rad DC protein assay technique (Bio-Rad laboratories, Hercules, CA, USA) as described previously (Wu et al., 2008). The ROS level was expressed as relative fluorescence intensity units/h/mg protein.

2.3. Analysis of antioxidant enzyme mRNAs

We have used a previously described (Wang et al., 2006) semi-quantitative reverse transcriptase polymerase chain reaction (rRT-PCR) and quantitative real-time reverse transcriptase polymerase chain reaction (qPCR) method for the quantitative estimation of the 4 antioxidative enzyme mRNAs in medaka embryos and hatchlings. In brief, the gene-

specific primers (Table 1) were designed from the nucleotide sequences reported in ensembl (www.ensembl.org) or in the GenBank (<http://www.ncbi.nlm.nih.gov>) and the nucleotide sequences of the amplified PCR products were analyzed by using a CEQ Dye terminator cycle sequencing quick start kit (Beckman Coulter, Fullerton, CA, USA) in a Beckman-Coulter CEQ 8000 Genetic Analysis System (Beckman Coulter) and verified with the sequences reported in ensembl or GenBank. After verification it was observed that the amplified SOD is matched with SOD3 and GST with GST1A. Other two sequences (catalase and GR) were identical with the sequences reported in the medaka genome database (ENSORLT00000002176 for catalase and ENSORLT00000000771 for GR). Total RNA was extracted from 6–8 medaka embryos and hatchlings at different developmental stages or after required period of ethanol treatment. Extracted RNA was used for cDNA synthesis after removing genomic DNA contamination (Wang et al., 2006). The cDNA (1 μ L) was amplified in an Opticon 2 thermal cycler (MJ research) for 30 cycles containing required primers (target and internal control) and buffer (Qiagen) in a 20 μ L reaction volume (94 °C, 2 min, 1 cycle followed by 30 cycles of 94 °C 30 s, 60 °C 1 min, 72 °C 2 min). A final extension for 7 min at 72 °C had done. Five μ L of the amplified products were separated in 2% agarose gel electrophoresis containing 0.001% ethidium bromide. The band intensity was analyzed in a versadoc image analyzer (Bio-Rad) and the results were expressed relative to internal control (β -actin).

For qPCR analysis, 1 μ L of cDNA was added to a reaction mixture (2X buffer from Qiagen: 10 μ L; gene specific sense and antisense primers 50 pM each; 400X diluted SYBR Green I from Sigma-Aldrich: 0.2 μ L; the final volume was adjusted to 20 μ L by nuclease-free water). The primers used for the amplification of target genes are listed in Table 1. The gene-specific standards for each target genes (catalase, GST1, GR, and SOD3) were prepared by RT-PCR as cDNA, using the same sense and antisense primers (Table 1) used in qPCR analysis; the RNA prepared from normal hatchlings (10 dpf) was used as template. The amplified PCR product was purified electrophoretically in 1% agarose gel containing ethidium bromide (0.001%), eluted and quantified spectrophotometrically (Nanodrop 2000c, Thermo Fisher Scientific). The standards were aliquoted out in separate tubes and stored at –80 °C. During qPCR analysis (DNA Engine Opticon 2) the standards were diluted to requisite concentration (copy numbers) with nuclease free water and used for the preparation of a standard curve. The reaction conditions were initial denaturation at 95 °C for 3 min, one cycle, followed by 40 cycles of denaturation at 94 °C for 30 s, annealing for 1 min at 60 °C, extension at 72 °C for 2 min, fluorescence data collection for 1 s. After the end of each cycle the samples were incubated again for 1 s at 78 °C and a second set of fluorescence data collection was made to prevent error due to the formation of primer dimers, if any. A final extension of one cycle at 72 °C for 10 min was made. The melting curve was constructed by plotting fluorescence data (1 s) against temperature (65 °C to 95 °C with an interval of 0.2 °C and a holding time is 0.5 s). Each reaction was performed in duplicate. The cycle threshold or C(t) line was set at the point where the signals surpass background noise and began to increase. This threshold was applied to all wells for consistent analysis of individual samples and standards (R^2 ranged from 0.8–0.9 for standard curves) for the experiment. Standards were run each time in every set of qPCR analysis. The copy numbers of mRNA in each tube was determined by using a software program (MJ Opticon Monitor Analysis software Version 3.1, Bio-Rad laboratories, Hercules, CA, USA). The results were expressed as copy number of target gene mRNA/ng of total RNA.

2.4. Statistics

All the data were analyzed by using one-way ANOVA followed by post-hoc Tukey's multiple comparison test. The oxidative stress and semi-quantitative RT-PCR data were log

transformed before analysis. The results were expressed as mean $\pm$ SEM of 3–6 independent experiments and $p < 0.05$ considered as significant.

3. Results

3.1. Oxidative stress in medaka embryogenesis is developmentally regulated

The oxidative stress in Japanese rice fish embryogenesis was determined by measuring the amount of hydrogen peroxide (H_2O_2) generated during development. It was observed that measurable amount of H_2O_2 was detected when the embryos were in let blastula (Iwamatsu stage ~11; 8–10 hpf) and early gastrula stages (Iwamatsu stage ~12; 10–12 hpf). The activity was gradually increased with the advancement of development and reached maximum level in hatchlings (Figure 2A). In our culture conditions (16L:8D and 26 ± 1 °C) the embryos generally initiated hatching 7+ dpf with maximum hatching (more than 50%) in 10 dpf. Therefore, in order to see any influence of oxidative stress in hatching processes, we have determined oxidative stress in 9 and 10 dpf embryos both in *ovo* (unhatched) and in hatched conditions. The data appear to be identical between these two groups (hatched versus unhatched) and were not significantly different either in day 9 or day in 10 of development (Figure 2A).

To determine the effect of ethanol in the oxidative stress status of the embryos, fertilized Japanese rice fish eggs (0 dpf, Iwamatsu stages ~4–10), neurula stages (1 dpf, Iwamatsu stages ~17–20), and circulating embryos (4 dpf, Iwamatsu stages ~30–32) were exposed to ethanol (100 and 300 mM) for 48 h and then used for oxidative stress analysis (Figure 2B). We observed that all the embryos were in circulating state at the time of assay; however, embryos exposed to 300 mM 0 dpf were unable to initiate circulation 3 dpf. The oxidative stress was increased in embryos with the advancement of morphogenesis and ethanol at 300 mM concentration was able to alter (reduce) the status of oxidative stress in embryos at 3 and 6 dpf (significantly lower in 3 dpf embryos exposed to 300 mM ethanol compared to controls). Embryos exposed to 100 mM ethanol did not have significantly different levels of oxidative stress compared to controls.

3.2. Antioxidant enzyme (catalase, GR, GST, and SOD) mRNAs maintained distinct developmental patterns and remained unaltered after ethanol exposure

Developmental expression of four antioxidant enzyme mRNAs (catalase, GR, GST, and SOD) of Japanese medaka embryos was analyzed by both rRT-PCR (Figure 3) and qPCR (Figure 4) techniques. It was observed that all 4 antioxidant enzyme mRNAs were constitutively expressed in fertilized embryos (0 dpf = ~2 hpf), however, the expression during embryogenesis were different among mRNAs and also not exactly identical between the two techniques (rRT-PCR and qPCR) used in this study. In rRT-PCR, catalase mRNA content was significantly increased in 1 dpf embryos compared to 0 dpf and then maintained a steady-state until hatching (Figure 3A). GR was very high in 0 dpf embryos and maintained a steady-state all throughout the development (Figure 3B). GST dramatically dropped on 1 dpf compared to 0 dpf and then increased gradually until hatching (the values of 4 and 6 dpf embryos and 10 dpf hatchlings were significantly increased from the values of 1 and 2 dpf; moreover, hatchlings 10 dpf were also significantly higher than that of the 3 dpf) (Figure 3C). In the case of SOD, a sharp increase was observed in embryos 1 dpf compared to zero dpf and then significant reduction was observed from 2 dpf which was maintained even after hatching (Figure 3D). In qPCR analysis it was observed that catalase (Figure 4A) and GR (Figure 4B) compared to 0 dpf remained unaltered throughout the development; GST (Figure 4C) was more or less identical with rRT-PCR (drop in 1 dpf and then gradual increase until hatching) but the data were not significantly different from each other. In the case of SOD, like rRT-PCR analysis, a dramatic increase was observed in 1 dpf

embryos compared to zero dpf and then the values were sharply decreased in 2 dpf and the same status was maintained in the rest of the developmental period. The values for embryos at 2 dpf to hatchling stages were also significantly higher compared with the zero dpf embryos (Figure 4B).

The expression of all these antioxidant enzyme mRNAs were analyzed in embryos developmentally exposed to ethanol by using both qRT-PCR (Figure 5) and qPCR (Figure 6) techniques. It was observed that ethanol treatment (100 or 300 mM) was unable to alter the mRNA expression pattern of any of these antioxidant enzymes (catalase, GR, GST, or SOD) in 2, 3 or 6 dpf embryos compared to that of the corresponding controls (Figures 5 and 6).

4. Discussion

While searching the molecular mechanisms of FASD in Japanese rice fish embryogenesis in this study we have investigated the status of oxidative stress and the expression pattern of four antioxidant enzyme mRNAs during development and their modulation by ethanol. The experimental strategies we have followed in the present investigations were previously standardized in our laboratory (Wang et al., 2007; Hu et al., 2008, 2009; Wu et al., 2008). Embryos are exposed to ethanol (100 and 300 mM) in three periods of development (0–2 dpf, 1–3 dpf, and 4–6 dpf), because the major ethanol-sensitive organs (brain, heart, notochord) in Japanese rice fish are formed and began functioning during these periods of development (Iwamatsu, 2004). Moreover, we have restricted our investigations to two doses of ethanol (100 and 300 mM) and the exposure period is only 48 h because our previous and present studies indicate that in this model generally 100 mM ethanol is found to be ineffective while 300 mM is effective to induce FASD phenotypes (Figure 1). Furthermore, in this condition (48 h exposure) embryonic ethanol concentration is only 15–20% of waterborne ethanol (Wang et al., 2006). Therefore the calculated amount of ethanol available to the Japanese rice fish embryo (45–60 mM) to induce FASD is equivalent to the concentration observed in mice (65.1 mM; Blakley and Scott, 1984).

It is evident from the data that the embryos maintain a distinct developmental regulatory pattern of oxidative stress (Figure 2A) as well as antioxidant enzyme (catalase, GR, GST, and SOD) mRNAs (Figures 3 and 4). Moreover, there is no significant difference in oxidative stress between hatched and unhatched embryos in a particular day of development (9 or 10 dpf) which indicates that the hatching process has no effect on oxidative stress status of this animal (Figure 2A). Ethanol (300 mM) is able to attenuate oxidative stress in 3 and 6 dpf embryos (Figure 1B; although not at statistically significant levels at 6 dpf), however, it is unable to alter these antioxidant enzyme (catalase, GR, GST, and SOD) mRNA contents (Figures 5 and 6). Our data further indicate that oxidative stress is necessary in medaka embryogenesis and deregulation of this process by ethanol can induce developmental abnormalities. Reactive oxygen species (ROS) such as H_2O_2 and hydroxyl radicals are formed by a variety of physiological and pathophysiological reactions. Moreover, ROS formation can be enhanced by many agents including xenobiotics, drugs, and environmental chemicals (Wells et al., 2009). Reactive oxygen species are short lived, and participate in signal transduction, but in excess can contribute to the deregulation of signal transduction, and oxidative damage to cellular macromolecules (Dennery, 2007). Previously we have observed that macromolecules, such as total protein, RNA and DNA contents of Japanese rice fish embryos were reduced by developmental ethanol exposure; however, lipid peroxidation and NAD: NADH status of embryos remained unaltered (Wu et al., 2008; Hu et al., 2008, 2009). Present data also indicate that ethanol (300 mM) is able to inhibit the oxidative stress/ H_2O_2 production in medaka embryos which is not in agreement with others (Dong et al., 2010; Yan et al., 2010) who have observed that ethanol is able to

increase H₂O₂ level and thus oxidative stress. Reactive oxygen species (ROS) is a generic name given to a variety of molecules that are formed as unavoidable by-products of aerobic respiration and various other catabolic and anabolic processes (Halliwell, 1991; Covarrubias et al., 2008). It is now well accepted that ROS are signaling molecules and transduce messages from the extracellular milieu to generate a specific cellular response. Due to high reactivity of some ROS, the location where they are produced is critical for their biological effects, including their role in FASD (Heaton et al., 2003). Moreover, levels of ROS are not only determined by production, but also by the rate of ROS degradation or inactivation. Because oxidative stress is the balance between the production and degradation of ROS that maintains the cellular homeostasis (Covarrubias et al., 2008) we also examined the mRNA content of four antioxidant enzymes that play a significant role during the maintenance of oxidative stress. The ultimate effects of antioxidants either enzymatic or non-enzymatic are to decrease the amount of active ROS. The common non-enzymatic antioxidants are glutathione (GSH) and thioredoxin (Wu et al., 2004; Holmgren et al., 2005) and the enzymatic antioxidants include SOD, catalase, GST, GR, and many other enzymes (Yan et al., 2010). Antioxidant enzymes act in concert to remove various ROS produced by free radical reactions. Once GSH is oxidized (GSSG), the reduced form can be regenerated by the GR. Superoxide dismutases (SOD) scavenge the superoxide radical converting it into hydrogen peroxide and oxygen. Catalase converts hydrogen peroxide into water (O'Brien, 2000; Krikman and Gaetani, 2007). Therefore we have investigated the effects of ethanol in the expression pattern of these enzyme mRNAs after exposing the embryos to ethanol at a specific phase of development (0–2, 1–3 and 4–6 dpf).

We followed two RT-PCR methods: rRT-PCR which is semiquantitative and qPCR which is highly sensitive and quantitative. In rRT-PCR we have used β -actin as internal control and all of the data were expressed are relative to the expression level of β -actin. However, β -actin is not universally accepted as a unique house keeping gene and studies in Japanese rice fish also raises question about the feasibility of the use of this gene (β -actin) as a reference gene (Zhang and Hu, 2007). From our experience we believe that the expression of β -actin is also variable during development (seems to be low in blastula and gastrula stages compared to later stages of development). But the technique (rRT-PCR) is very straightforward and in most cases supports the data obtained by qPCR. The internal standards used in qPCR techniques in this study are prepared in the laboratory by RT-PCR techniques by using the same forward and reverse primers of the target genes. A serial dilution of the respective standards as copy numbers was made and used during the assay. Therefore, the data obtained by qPCR is absolute (mRNA copy number of target genes/ng of total RNA). We followed both techniques to justify our hypothesis.

Our data indicate that all the antioxidant enzymes investigated in this study maintained a distinct developmental pattern (Figures 3 and 4). This is the first report that the expression of these antioxidant enzymes (catalase, GR, GST and SOD) in Japanese rice fish is developmentally regulated. These observations also support the concept that proper maintenance of oxidative stress is a prerequisite for the normal development of an embryo (Dennery, 2007). To correlate the oxidative stress with the mRNA expression pattern of the antioxidant enzymes we have observed that the gradual increase of oxidative stress started 2 dpf onwards, and catalase, GR and SOD were maintained almost a steady-state during this period of development; GST tended to be increased. The measured GST in this study is GSTA1 which is an alpha class of GST. This class is most abundantly expressed in liver, playing a significant role in bilirubin metabolism and protects the cells from ROS. In marine fish plaice (*Pleuronectes platessa*) it was observed that GSTA1 mRNA was expressed in a wide range of tissues and induced in the gill and liver by perfluoro-octanoic acid (a man-made chemical that does not occur naturally in the environment) and in spleen and intestine by β -naphthoflavone (Leaver et al., 1997). Moreover, there are many other GST genes

reported in the Japanese rice fish genome database and their role during development and thus oxidative stress has not been studied. Several other reports showed that GST was found to be increased in Japanese rice fish in postnatal life with the advancement of age (DeKoven et al., 1992). Moreover, adult fish (Japanese rice fish) exposed to diethylnitrosamine (DEN) (McCarthy et al., 1991), and ethylene dibromide (EDB) (Hawkins et al., 1998) increased the activity of GST. Therefore the enhancement of GST during embryogenesis as we have observed in our studies (more pronounced in rRT-PCR) is probably due to the maintenance of appropriate balance in oxidative stress necessary for normal development.

In Japanese rice fish there is only one catalase and three SOD genes (*SOD1*, *SOD2*, and *SOD3*) reported in the database (www.ensembl.gov). In this study the investigated SOD is SOD3 which, like SOD1, has Cu and Zn at its catalytic center; however, in mammals it is localized on extracellular elements (Zelko et al., 2002). Our data analyzed by both rRT-PCR and qPCR indicate that SOD mRNA is constitutively expressed in 0 dpf (blastula) embryos, sharply increased in 1 dpf (neurula), and then decreased and maintained at a steady-state (Figures 2D and 3D). In mouse and rat lung SOD3 mRNA was undetectable until late gestation (Zelko et al., 2002) which is not identical with our studies in whole embryos of Japanese rice fish. Catalase like SOD is also constitutively expressed and significantly enhanced in neurula stage (1 dpf) and then it maintained steady-state (rRT-PCR data; Figure 2A). Although oxidative stress is found to be increased during medaka embryogenesis and SODs and catalase are the key enzymes to catalyze the dismutation of superoxide anions, at present we are unable to explain the exact reason of steady-state SOD3 and catalase mRNA maintenance after 2 dpf of development when oxidative stress increases. Further studies with other SODs (SOD1 and SOD2) are needed.

Our data also indicate that Japanese rice fish embryos exposed to ethanol at three stages of development were unable to alter the expression pattern of these enzyme mRNAs (Figures 5 and 6) while the oxidative stress in the embryos exposed to 300 mM was less compared to controls in 3 (significantly different) and 6 dpf. These data are not exactly identical with the studies observed in mouse embryos where 2.9 g/kg ethanol for 6 hours was able to increase oxidative stress and mRNA expression pattern of SOD3 and GR but not catalase (Dong et al., 2008). Moreover, SOD has been shown to diminish ethanol-induced superoxide anion generation, lipid peroxidation, and cell death, as well as the incidence of neural tube defects in cultured mouse embryos (Kotch et al., 1995). *In vivo* studies have also shown that, in mice, maternal treatment with an SOD and catalase mimetic, EUK-134, reduces ethanol-induced apoptosis in selected cell populations in the developing limb buds (Chen et al., 2004). Therefore, further studies are needed to explain the exact mechanisms involved in the teratogenic effects of ethanol during Japanese rice fish embryogenesis. We have previously observed that ethanol is able to disrupt cardiovascular development and circulation in medaka embryos (Wang et al., 2006). Moreover, embryonic protein, RNA and DNA contents were reduced after ethanol treatment which indicates a developmental delay induced by ethanol during medaka embryogenesis (Wu et al., 2008). After observing oxidative stress status (which was found to be less in ethanol exposed embryos) we conclude that ethanol disrupts the supply of nutrients to the developing embryos by blocking normal circulation. The embryos of these fish unlike mammal can survive in a compromised condition of circulation for several days. Therefore the teratogenic effects in Japanese rice fish as we have observed previously are primarily due to disruption in circulation and thus nutrient and oxygen supply.

In summary, our results indicate that oxidative stress is essential in normal embryogenesis of Japanese rice fish and attenuation of this process by ethanol is able to induce FASD phenotypes. The antioxidant enzymes investigated in this study is developmentally regulated

and remained unaltered after ethanol treatment which suggests a different mechanism of FASD induction occurs in this model.

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Figure 1. Representative Figures showing ethanol-induced FASD phenotypes in Japanese rice fish

Fertilized Japanese rice fish embryos at 1 dpf of development (Iwamatsu stage 17–18) were exposed to ethanol (0, 100, and 300 mM). After 48 h of treatment ethanol was removed from the medium (3 dpf) and the embryos were maintained in clean hatching solution and allowed to hatch. After successful hatching they were fed with TetraMin Flake and brine shrimp naupli and maintained in hatching solution. The photographs were taken at constant magnification in an Olympus B-max 40 microscope on 15 dpf. A= control (no ethanol), B= ethanol 100 mM; 1–3 dpf, C= ethanol 300 mM; 1–3 dpf. The developmental deformity in vertebral column in hatchlings treated with 300 mM ethanol developmentally was noticed.

Figure 2. Developmental regulation (A) and effect of ethanol (B) on the oxidative stress status of Japanese rice fish embryos during development

The embryos were collected within ~2 hpf and maintained in the laboratory in hatching solution with 16L: 8D light cycle and 26 ± 1 °C. Some of the embryos were exposed to ethanol (0, 100 and 300 mM) in the respective days (0, 1 and 4 dpf) for 48 h and analyzed on 2, 3 and 6 dpf, respectively. 6–8 embryos were used for oxidative stress analysis. Each bar is the mean $\pm$ SEM of 4–8 observations. The data (FU= Fluorescent unit) were log transformed and analyzed by one way ANOVA followed by post-hoc Tukey's multiple comparison test; $p < 0.05$ was considered as significant. Bar head with pound (#) symbol indicates that the data are significantly different from 0 dpf embryos (A) or from the corresponding control group (B). The lower case letter "h" used in 9-h and 10-h data points in x-axis (Figure A) indicates that the measured oxidative stress is on hatched embryos either at 9 dpf (9-h) or in 10 dpf (10-h).

Figure 3. Developmental regulation of antioxidant enzymes of Japanese rice fish embryos during development analyzed by semiquantitative RT-PCR. A= catalase, B= glutathione reductase (GR), C=glutathione-s-transferase (GST), D= super oxide dismutase (SOD)

The embryos were collected within ~2 hpf and maintained in the laboratory in hatching solution with 16L: 8D light cycle and 26 ± 1 °C. Each bar is the mean $\pm$ SEM of 4 observations. The data were log transformed and analyzed by one way ANOVA followed by post-hoc Tukey's multiple comparison test; $p < 0.05$ was considered as significant. Bar head with pound (#) symbol or lower case letter "a" indicate that the data are significantly different from 0 dpf (#) or 1 dpf (a) embryos.

Figure 4. Developmental regulation of antioxidant enzymes of Japanese rice fish embryos during development analyzed by qPCR. A= catalase, B= glutathione reductase (GR), C=glutathione-s-transferase (GST), D= super oxide dismutase (SOD)

The embryos were collected within ~2 hpf and maintained in the laboratory in hatching solution with 16L: 8D light cycle and 26 ± 1 °C. Each bar is the mean $\pm$ SEM of 4 observations. The data were analyzed by one way ANOVA followed by post-hoc Tukey's multiple comparison test; $p < 0.05$ was considered as significant. Bar head with pound (#) symbol or lower case letter "a" indicates that the data are significantly different from 0 dpf (#) or 1 dpf (a) embryos

Figure 5. Effect of developmental ethanol exposure on the antioxidant enzyme mRNA content of Japanese rice fish embryo analyzed by semiquantitative RT-PCR

Fertilized Japanese rice fish eggs at specific days of development (0, 1, and 4 dpf) were exposed to ethanol (0, 100 and 300 mM) for 48 h and analyzed on 2, 3, and 6 dpf, respectively. After the removal of ethanol from the medium the embryos were used for analysis. Each bar is the mean $\pm$ SEM of 4 observations. The data were log transformed and analyzed by one way ANOVA followed by post-hoc Tukey's multiple comparison test. No significant difference between the control and different ethanol-treated groups was noticed.

Figure 6. Effect of developmental ethanol exposure on the antioxidant enzyme mRNA content of Japanese rice fish embryo analyzed by qPCR

Fertilized Japanese rice fish eggs at specific days of development (0, 1, and 4 dpf) were exposed to ethanol (0, 100 and 300 mM) for 48 h. After the removal of ethanol from the medium (2, 3, and 6 dpf) the embryos were used for analysis. Each bar is the mean $\pm$ SEM of 4 observations. The data were analyzed by one way ANOVA followed by post-hoc Tukey's multiple comparison test. No significant difference between the control and different ethanol-treated groups were noticed.

Table 1

Primers Used in semi-quantitative RT-PCR (rRT-PCR) and the qPCR amplifications of the antioxidant enzyme mRNAs of *Oryzias latipes* embryos

mRNA	Sense (5'-3')	Antisense (5'-3')	Product size (bp)	Target/internal standard	Ensembl number/GenBank Accession
SOD	GGCACTCTGTATGCCGGCTGC	GTTACCAAAAGTCTCCCGGATG	276	target	ENSORLG000000019434
catalase	GCGGTACAAACAGCGCAGATGAAG	GGATGGACGGCCTTCAAGTTC	171	target	ENSORLT000000002176
GST	GAACTCTGCAAGGGCTACAAAC	GGCCCTCAAAACATGCGTTGG	241	target	GenBank X95200
GR	GGACTACTCTGCAATCCACACAG	CATTGACTCTTCTGCGTGTGATG	165	target	ENSORLT000000000771
β-actin	CCTGACCCCTGAAAGTATCCCA	AGCGACAAGATGAGCTGGTT	542	Internal standard	ENSORLT0000000017152
GPX	CATCATCACCAACGTGGCCTC	CTTACTGAACAGATCAAAATG	204	target	GenBank DQ660323