Published in final edited form as:

Comp Biochem Physiol C Toxicol Pharmacol. 2009 November ; 150(4): 495–502. doi:10.1016/j.cbpc.2009.07.007.

Ethanol disrupts chondrification of the neurocranial cartilages in medaka embryos without affecting aldehyde dehydrogenase 1A2 (*Aldh1A2*) promoter methylation

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Abstract

Medaka (*Oryzias latipes*) embryos at different developmental stages were exposed to ethanol for 48 h, then allowed to hatch. Teratogenic effects were evaluated in hatchlings after examining chondrocranial cartilage deformities. Ethanol disrupted cartilage development in medaka in a dose and developmental stage-specific manner. Compared to controls, the linear length of the neurocranium and other cartilages were reduced in ethanol-treated groups. Moreover, the chondrification in cartilages, specifically trabeculae and polar cartilages, were inhibited by ethanol. To understand the mechanism of ethanol teratogenesis, NAD⁺: NADH status during embryogenesis and the methylation pattern of *Aldh1A2* promoter in whole embryos and adult tissues (brain, eye, heart and liver) were analyzed. Embryos 6 dpf had higher NAD⁺ than embryos 0 or 2 dpf. Ethanol (200 or 400 mM) was able to reduce NAD⁺ content in 2 and 6 dpf embryos. However, in both cases reductions were not significantly different from the controls. Moreover, no significant difference in either NADH content or in NAD⁺: NADH status of the ethanol-treated embryos, with regard to controls, was observed. The promoter of *Aldh1A2* contains 31 CpG dinucleotides (-705 to +154, ATG = +1); none of which were methylated. Compared to controls, embryonic ethanol exposure (100 and 400 mM) was unable to alter *Aldh1A2* promoter methylation in embryos or in the tissues of adults (breeding) developmentally exposed to ethanol (300 mM, 48 hpf). From these data we conclude that ethanol teratogenesis in medaka does not induce alteration in the methylation pattern of *Aldh1A2* promoter, but does change cartilage development.

Keywords

Fetal alcohol spectrum disorder; Alcohol; medaka; neurocranium; *Aldh1A2* promoter; epigenetics

Introduction

Fetal alcohol syndrome (FAS) is a pattern of developmental anomalies found in children born to alcohol abusing mothers who consumed alcohol during pregnancy. This was first noticed in France (Lemoine et al., 1968), and in the USA, Jones and Smith (1973) correlated FAS phenotypes with maternal alcohol ingestion. Since then many studies have been done to

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understand the mechanisms of FAS, however, very limited success has been achieved. The problem is complicated because of the pleiotropic nature of ethanol action at the cellular level during embryogenesis (Dunty et al., 2001; Goodlett et al., 2005; Sant'Anna and Tosello, 2006). Due to ethical reasons, human studies of FAS are very limited, and our current knowledge of FAS is mainly based on studies in many mammalian and non-mammalian animal models. However, the selection of an animal model is very important in FAS studies because every model system has its own distinctiveness. Also, the effects of ethanol are very specific to the dose, duration, and the developmental status of the embryo (Cudd, 2005). Fish models, mainly zebrafish (*Danio rerio*) and Japanese medaka (*Oryzias latipes*), are currently used for FAS studies (Carvan et al., 2004; Reimers et al., 2004; Wang et al., 2006; Oxendine et al., 2006; Hu et al., 2008; Wu et al., 2008; Loucks and Ahlgren, 2009; Mao et al., 2009). Optical clarity of the embryo, short generation time, devoid of parental influences during development, and ease of maintenance in the laboratory, are some of the advantages of using these models in evaluating ethanol teratogenesis.

FAS phenotypes in human are physically identified by examining the facial features, like the smooth philtrum, thin vermilion border of the upper lip, and short palpebral fissures. In addition to these soft tissues, the facial dysmorphology by ethanol can be observed in facial bones with a small head circumference (microcephaly), apparent flattening of the nasal bridge and midface, micrognathia (small jaw), facial asymmetry, and cleft palate (Su et al., 2001; Naidoo et al., 2005). In animal models, such as rat, chicken and zebrafish, prenatal ethanol exposure also produced a pattern of abnormal craniofacial defects (Guerrero, 1990; Su et al., 2001; Ahlgren et al., 2002; Carvan et al., 2004). The molecular mechanism of these defects has been proposed to be due to the induction of apoptosis in the neural crest (NC) cells (the precursor of many craniofacial cartilages and bones) during development. In medaka, we have observed that the embryos exposed to ethanol during embryogenesis developed precocious FAS features in craniofacial, skeletal and cardiovascular organs (Wang et al., 2006) which are analogous to human FAS phenotypes. Also, the macromolecular constituents (Protein, RNA and DNA) and the alcohol metabolizing enzyme mRNA contents were reduced in medaka embryos by ethanol in a dose and developmental stage-specific manner (Wu et al., 2008; Hu et al., 2008). Although the mechanism of ethanol teratogenesis in medaka is yet to be identified, we hypothesize that the oxidative stress generated due to ethanol metabolism is a possible cause of ethanol teratogenesis. Like many other model organisms, medaka embryos express alcohol dehydrogenase (Adh) and aldehyde dehydrogenase (Aldh) mRNAs during development (Dasmahapatra et al., 2005; Wang et al., 2006, 2007; Hu et al., 2008) which suggests that embryonic ethanol metabolism in medaka occurs through the ADH/ALDH pathway. ADH requires NAD^+ to metabolize ethanol and to produce acetaldehyde and NADH. Further oxidation of acetaldehyde by ALDH also requires NAD^+ . Therefore, ethanol metabolism during medaka embryogenesis is hypothesized to alter NAD^+ : NADH status and induce oxidative stress which may disrupt the migration of NC cells from the developing central nervous system (CNS) resulting in a malformed head skeleton. Moreover, ADH and ALDH are also responsible for retinoic acid (RA) synthesis; a key molecule which plays a significant role in patterning and morphogenesis during embryo development. Insufficient or excessive RA levels induce abnormalities in craniofacial and skeletal organs (Clagett-Dame and DeLuca, 2002). ALDH1A2 (previously known as retinaldehyde dehydrogenase 2), which catalyzes the synthesis of RA from retinol, is considered as the predominant enzyme responsible for the production of nearly all embryonic RA (Niederreither et al., 1999, 2002, Mic et al., 2002). In medaka, RA- induced developmental disorders are reported (Hayashida et al., 2004). We cloned Aldh1A2 cDNA in medaka and observed that ethanol was able to reduce the expression of Aldh1A2 mRNA in precirculating medaka embryo (Hu et al. 2008). We expect that oxidative stress generated during ethanol metabolism may be able to induce epigenetic errors in *Aldh1A2* gene specifically increased methylation, and that change is associated with decreased Aldh1A2 mRNA expression during medaka embryogenesis. In the present communication we

have analyzed craniofacial cartilage anomalies in hatchlings, NAD^+ : NADH status of the embryos and the methylation pattern of the promoter region of *Aldh1A2* gene of the embryos and adults of medaka developmentally exposed to ethanol. Our results show that ethanol is able to reduce the linear length of neurocranium and other cartilages of the head skeleton in a dose and developmental stage-specific manner. Moreover, NAD^+ content appears to be reduced by ethanol treatment, but no significant reduction was established in NADH content, or the NAD^+ : NADH. Finally, the methylation pattern of *Aldh1A2* gene promoter in embryos or in adult tissues of medaka remained unaltered after embryonic ethanol exposure.

Materials and methods

Experimental Procedure

Methods of animal maintenance, egg collection and embryo culture conditions were previously described (Hu et al., 2008). Ethanol (100 - 400 mM) was added to the culture medium at five different time points of development (Table 1) and discontinued after 48 h, following a one time renewal of ethanol at 24 h. Embryos were examined daily for routine developmental changes (cardiovasculature, blood clots, active circulation) under a phase contrast microscope (AO Scientific Instruments) with a 50 % static renewal of the medium (when alcohol was no longer present in the medium). Embryonic development was classified after Iwamatsu (2004). In the present experimental conditions (28 ± 1 °C, 16L: 8D) the embryos started to hatch at 8 dpf. However, as the development of the ethanol-treated embryos are comparatively slower than the controls, we allowed them to hatch ~ 15 dpf (day of fertilization was considered as 0 dpf). In order to avoid post hatch growth, hatchlings were preserved in 4% paraformaldehyde within 24 h of hatching and used for cartilage staining by Alcian Blue (Wang et al., 2006). Embryos which had still not hatched at 15 dpf were excluded from the experiments. For methylation studies, embryos were sacrificed 6 dpf (~144 hpf) for genomic DNA extraction after examining the circulation status of the embryos. Some of the embryos exposed to 300 mM of ethanol for 48 hpf were raised to adults and at approximately three months of age (the onset of breeding, Iwamatsu stage 44) were sacrificed. The sex of the adult fish was examined by observing the anal fin and fin rays (Yamamoto, 1958) before sacrifice and by gonads (testis or ovary) after sacrifice. The brain, eye, heart, and liver tissues were collected for genomic DNA analysis. For NAD^+ and NADH detection, embryos were exposed to ethanol (200 and 400 mM) 48 hpf and harvested for NAD^+ and NADH assay, or maintained in hatching solution for 6 dpf, then used for the coenzyme (NAD^+ and NADH) assay. To determine the basal level of these coenzymes, fertilized eggs (~ 1 hpf) were used for analysis immediately after collection.

Morphometric analysis

The hatchlings were stained with alcian blue (Wang et al., 2006), and examined microscopically for structural deformities of the neurocranial and splanchnocranial cartilages, and were used for morphometric analysis. Photomicrographs of the entire neurocranium or the stained cartilages were taken using an Olympus B-MAX 40 microscope at fixed magnification. The linear length of neurocranium and other cartilages were determined by using image analysis software (Media Cybernetics, Silver Springs, MD, USA). After staining with alcian blue, the ethmoid plate (EP) with lamina orbitonasalis, paired trabeculae (TB), anterior orbital cartilage (AOC), posterior orbital cartilage (POC), epiphyseal bar (EB), basilar plate (BP) with anterior (ABC) and posterior (PBC) basicranial commissures, hypophyseal plate (HP) with paired polar cartilages (PC), and auditory capsules in the neurocranium are clearly visible. In the splanchnocranium, Meckel's cartilage (MC), pterygoid processes (PT), quadrate (QU), hyosymplectic (HYS), hyoid (HYO), basihyal (BH), ceratohyal (CH), 4 basibranchials (BB), 3 pair hypobranchials (HBR), 5 pair ceratobranchial (CBR), 4th epibranchial (EBR) and 4th hypobranchial (HBR) are also identifiable (Figures 1A-D). The nomenclature of the cartilages

used in this communications was obtained from Langille and Hall (1987). In the present experimental conditions, generally embryos of groups A and B exposed to 400 mM ethanol and above, were unable to hatch even after 15 dpf. In groups C, D and E, a few embryos exposed to 500 mM were able to hatch. Therefore, we were unable to present any data in hatchlings of A and B group exposed to 400 and 500 mM ethanol. In groups C, D, and E data on 500 mM ethanol are not presented. During morphometrical analysis the length of the entire neurocranium, EP, AOC, and EB are linear. However, in ABC, the linear length was measured from the anterior end of the notochord to the head of the ABC (anterior end) on both sides and then averaged. In TC and PC, because in ethanol-treated samples the length of left and right sides are unequal in most samples (Figures 2B-D), therefore, they were measured individually and an average calculated. In some samples, the cartilages were completely absent. In those cases the length of TC or PC was considered as zero. In the splanchnocranium, the linear lengths of QU, BH, CH, BB (1-4) CBR (1-4) were measured directly. In case of BB, the first three cartilages are not visualized separately, therefore, the lengths of first three BB are the cumulative; the 4th one was visible separately, therefore measured individually. In 5th CBR an imaginary line was drawn joining the pharyngeal teeth of the left and right CBR (5th) and the distance was measured from the center. The bones of lower jaw (MC) are also curved. So, the height of the lower jaw was determined as the distance between the meeting points of two MC and the mid-point of an imaginary line drawn between the two ends of MC. The POC, auditory capsule, PBC in neurocranium and PT, HYS, HYO in the splanchnocranium were not considered for morphometric analysis because of their irregular shape. At least 8 hatchlings per group were used for morphometric analysis.

Determination of NAD and NADH

We used the fluorescent NAD/NADH detection kit (Cell Technology, Mountain View, CA, USA) and followed their protocol for determination of NAD⁺: NADH. In this procedure, separate samples were used for the extraction of NAD⁺ and NADH and the extracted NAD⁺ is converted to NADH before assay. Ten to 16 embryos, after requisite period of treatment, were homogenized in 50 μ L of either NAD⁺ or NADH extraction buffer containing 50 μ L of NAD/NADH lysis buffer. The homogenate was heated at 60 °C for 15 min and 25 μ L of the reaction buffer was added to the cooled samples. The samples were vortexed and 50 μ L of opposite extraction buffer (NAD⁺ buffer for NADH samples and NADH buffer for NAD⁺ samples) was added to neutralize the homogenate. The mixture was vortexed and centrifuged at 8,000 g for 5 min. The clear supernatant was carefully collected and used for NADH assay. Fifty μ L of the sample or NADH standard (0-3000 nM) in duplicate was transferred to the wells of a black 96 well plate, to which 100 μ L of the reaction cocktail was added. The mixture was incubated in dark at room temperature for 1-1.5 h, after which the fluorescence was detected on a spectra Max M5 (Molecular Devices, Sunnyvale, CA, USA) with an excitation peak at 570 nm and emission peak at 600 nm. The protein concentrations of the extracts were determined using Biorad DC protein assay technique (Biorad laboratories, Hercules, CA, USA) as described previously (Wu et al., 2008). The NADH concentration of the samples was calculated from the standard curve and the results were expressed as nMol NADH/mg protein. The NAD⁺: NADH in control and ethanol- treated embryos was calculated manually.

Methylation analysis of Aldh1A2 promoter region

Methylation of the promoter region of *Aldh1A2* gene of medaka was determined after Contractor et al (2004) with some modifications. Genomic DNA was isolated either from pooled embryos (6-8 embryos on 6 dpf) or from the brain, eye, heart and liver tissue of control or ethanol-treated (fertilized eggs were exposed to 300 mM of ethanol 48 hpf and then raised to adults) individual fish at breeding stage (~3 months of age; Iwamatsu stage 44). Genomic DNA (10 μ g) was isolated as described previously (Wu et al., 2008) and ~10 μ g of DNA was denatured with 3 M sodium hydroxide. For deamination, denatured DNA was incubated with

3.6 M freshly prepared sodium bisulfite for 16 h at 50 °C and purified using Qiagen Purification kit. The purified DNA was again denatured with 3 M sodium hydroxide, neutralized with 10 M ammonium acetate, and purified using Qiagen Purification kit (Qiagen). Modified DNA was resuspended in 30 µL sterile water and stored at -20 °C until use. Primers were designed by using Methyl Primer Express software v 1.0 (Applied Biosystems, Foster City, CA, USA). Bisulfite treated DNA from control fish was also amplified with primers for the wild type sequences in order to verify the integrity of the deaminated DNA. The PCR mixture contained bisulfite modified or unmodified DNA (~10-25 µg), primers (50 pM, Qiagen, Valencia, CA, USA or IDT, Coralville, IA, USA) and PCR master mix (Qiagen, Valencia, CA) in a total volume of 20 µL. Amplification was carried out in MJR PCR system (Opticon2, MJ Research,). The PCR product was separated on 1% agarose gel electrophoresis, stained with ethidium bromide and visualized under UV light. Bands were cut out and DNA was extracted using Qiagen QIAEXII Gel extraction kit. For cloning and sequencing, extracted DNA was ligated into pGEM-T easy vector system and transformed into JM 109 competent cells (Promega, Madison, WI, USA). DNA from colonies (three colonies per sample) was purified using a mini prep. Clones containing the predicted insert (identified by *Eco* RI digest) was quantified and sequenced. Homology search was carried out either manually (compared to CG dinucleotides in wild type) or by using Vector NTI software (Invitrogen, Carlsberg, CA, USA).

Statistics

Data were analyzed by one way ANOVA followed by Post-hoc Tukey's multiple comparison test. The results were expressed as mean $\pm$ SEM with $p < 0.05$ considered as significant.

Results

Morphometric analysis of the craniofacial cartilages of Japanese medaka developmentally exposed to ethanol

The chondrification of the neurocranium of medaka embryo begins *in ovo* on 5 dpf (Iwamatsu stage 33) and ends prior to or after hatching (Iwamatsu stage 40). A typical medaka embryo immediately after hatching (hatchling) has a chondrocranium with auditory and nasal capsules, a well-developed neurocranium and splanchnocranium and a minimal amount of chondral braincase elements (Figures 1A-D). Most of the cartilages in the NRC of hatchlings remained intact after developmental ethanol exposure, except TC and PC which were either deformed or absent in the hatchlings in groups A-D treated with ethanol during embryogenesis (Figure 2). In the E group, all hatchlings either control or treated with ethanol (100-400 mM), have TC associated with EP. Further, it was observed that the average length of the neurocranium compared to control was reduced in ethanol-treated embryos (100-400 mM) in a dose- and developmental stage-specific manner, however, the data are not significantly different in all cases. Ethanol at 300 mM concentration or above showed significant reduction in all groups; lower doses in groups A (both 100 and 200 mM) and E (100 mM) were unable to reduce neurocranial length significantly when compared with the controls (Table 2). In individual cartilages, such as EP, TC, EB, AOC, ABC, and PC, like NRC, the lengths were reduced with the increase of ethanol concentration in the medium, however, the data when compared with the controls are not significantly different in all cases, especially in lower doses (100 and 200 mM). In the splanchnocranium no cartilage loss was observed, however, ethanol treatment is able to reduce the linear lengths of cartilages considered for analysis (MC, QU, BH, CH, BB1-4, CBR1-5). As in the neurocranium, the reduction was dose-dependent and specific to the developmental stages of the embryos; however, the data particularly in lower ethanol doses (100-200 mM) were not significantly different from the corresponding controls (Table 3). Moreover, in BH, BB4 and CB5, ethanol at 100-300 mM concentration was found to be ineffective in embryos of A and B groups (Table 3).

Effect of ethanol on NAD and NADH content of medaka embryo

Alcohol is metabolized by alcohol dehydrogenase and generates acetaldehyde. During the reaction one molecule of NAD^+ is reduced to NADH, the coenzyme ratio is altered, and induction of oxidative stress occurs. We exposed the embryos (0 dpf) to 200 and 400 mM of ethanol 48 hpf and measured the coenzyme concentrations (NAD^+ and NADH) immediately after alcohol removal (48 hpf), or maintained the viable embryos (both control and ethanol-treated) in hatching solution and measured the coenzymes on 6 dpf. Some of the fertilized eggs immediately after collections were also analyzed. The results were expressed as nM NADH/mg protein (Figures 3A and 3B). It was observed that NAD^+ concentration in medaka embryos was more than that in NADH all throughout the development (0d/2d/6d) and the concentrations of both coenzymes were apparently increased with the advancement of morphogenesis. Ethanol treatment was able to alter the concentrations of both NAD and NADH compared to the corresponding controls; however, the decrease was not significantly different. Accordingly, NAD^+ : NADH remained unaltered (Figure 3C).

Aldh1A2 promoter analysis

The information about the nucleotide (nt) sequences of 5' untranslated region (UTR) and first exon of *Aldh1A2* (-705 to +154, ATG = +1) of Japanese medaka were obtained from Ensembl (http://ensembl.org/Oryzias_latipes) and were verified by PCR amplification (859 bp) followed by cloning and sequencing (Figure 4). After bisulfite treatment of the genomic DNA, we used three sets of primers (Table 4) to amplify a fragment of 753 nt (-636 to +117 nt) which contained 26 CpG dinucleotides. None of the CpG doublets were found to be methylated in control animals. Medaka embryos exposed to ethanol (100 and 400 mM) for 48 hours at different stages of development (Table 1) showed no alteration in the number of unmethylated CpG dinucleotides in the promoter region of *Aldh1A2* gene. Some of the ethanol treated embryos (300 mM, 48 hpf) were raised to adults (breeding) and *Aldh1A2* promoter methylation was analyzed in brain, eye, heart and liver tissues. The methylation pattern of *Aldh1A2* is remained unaltered in these organs compared to control animals (Table 1).

Discussion

As a continuation of our previous studies of the effects of ethanol during medaka embryogenesis, the present experimental data indicate that ethanol (100–400 mM) is able to reduce the linear length of neurocranium and several other chondrocranial cartilages, and induces a phenomenon of microcephaly. Moreover, inhibition of chondrification, specifically in TC and PC by ethanol, also suggests that mediolateral (ML) polarity of the neurocranium is disrupted by embryonic ethanol exposure. The effects are found to be dose- and developmental stage -dependent.

Previously we have observed that medaka embryos required very high concentrations (300–400 mM) of ethanol to produce significant phenotypical or biochemical changes (Wang et al., 2006; 2007a, b; Wu et al., 2008; Hu et al, 2008). However, the embryonic ethanol concentration in our culture conditions as we had determined in earlier studies was ~15–20% of waterborne ethanol (Wang et al., 2006). Therefore, the calculated embryonic ethanol concentration is equivalent to 15–80 mM which is in good agreement with the mammalian models (65.1 mM in mice; Blakley and Scott, 1984). In the present study, it was observed that ethanol at 100–200 mM concentrations is able to reduce the linear length of several neurocranial and splanchnocranial cartilages (Tables 2 and 3) which is also specific to the developmental stage of medaka. Ethanol exposure of the embryos during prenatal development disrupts craniofacial and neurocranial cartilage development in other species including human, mouse, rat, chick, Xenopus and zebrafish (Nakatsuji, 1983; Sulik et al., 1988; Webster and Ritchie, 1991; Smith 1997; Roda-Moreno et al., 2000; Ahlgren et al., 2002; Loucks and Carvan, 2004). The primary

cause of this defect is the induction of apoptosis in NC cells which are destined to give rise to facial structure (Smith 1997). In our model we have also observed a phenomenon of microcephaly due to the reduction of linear length of many chondrocranial cartilages. Moreover, TC and PC are the major cartilages where the disruptions are very prominent, and in many ethanol-treated (200-400 mM) embryos of groups A-D, complete absence of TC and PC in the neurocranium have been observed (Figures 2A-D). During embryogenesis, TC in medaka arises as two C-shaped rods at the anterolateral border of the head, and curved backward and inward to lie adjacent to each other along the midline (Langille and Hall, 1987). Later they fuse anteriorly to form EP and remain separated posteriorly. The PC arises as paired rods, flanking the hypophyseal fenestrae, fusing with the BP posteriorly and separating from each other anteriorly. The anterior ends of medaka PC, unlike zebrafish, do not fuse with the caudal ends of TC (Figure 1A). There is another diamond shaped membrane bone, the parasphenoid (remained unstained by alcian blue), which remains within TC and PC underlies the hypophyseal fenestrae with its flattened portion and the top and bottom corners elongated into thin rods. The anterior rod lies between the TC but does not reach the rostrum, the posterior rod extends below the BP, ending before the notochord (Langille and Hall, 1987).

The majority of the chondrocranial cartilages of medaka examined in the present investigations are formed after the migration of NC cells from the developing CNS (Langille and Hall, 1988). Neural crestectomies in mesencephalon and preotic rhombencephalon are able to disrupt EP and TC formation; however, formation of PC is not affected by NC extirpations (Langille and Hall, 1988). Therefore, from the present data it is also evident that the neurocranial cartilages, which are not affected by neural crestectomy (PC), are also disrupted by ethanol. Ethanol metabolism generates oxidative stress which is cytotoxic as well as apoptotic. Complete or partial loss of TC or PC in ethanol-treated embryos further indicate that oxidative stress may induce abnormalities in the migration pattern of NC cells or target the expression of a gene which is specifically expressed only in TC and PC precursor cells. Alternatively, oxidative stress induces apoptosis which may disrupt gene expression in the precursor cells of TC and PC due to some unknown specificity. As a result, ML polarity in the neurocranium of medaka embryo is also affected after ethanol exposure. Studies in other models indicate that the members of common signaling pathways such as sonic hedgehog (shh), fibroblast growth factor (fgf), wingless (wnt) and bone morphogenic protein (bmp) have significant impact in NC survival, proliferation, differentiation and thus craniofacial development (Tapadia et al., 2005). Therefore, we think that in medaka ethanol like other vertebrates may be able to alter the functions of these signaling molecules and induces dysmorphogenesis in neurocranial and splanchnocranial cartilage development.

Ethanol is metabolized through the ADH/ALDH pathway in medaka embryogenesis. Metabolism of ethanol through ADH requires NAD^+ and generates acetaldehyde. Further oxidation of acetaldehyde, which is mediated predominantly by the mitochondrial ALDH, also needs NAD^+ . Therefore, the rate-limiting factor in ethanol metabolism in a tissue is the amount of initial NAD^+ reserve. We have analyzed NAD^+ and NADH status of medaka embryo after exposing them with or without ethanol and propose to use the ratio of the coenzymes ($\text{NAD}^+:\text{NADH}$) as an index of oxidative stress. Our data indicate that medaka embryos have significant amounts of both NAD^+ and NADH in the yolk prior to or during the initiation of morphogenesis (Figure 3) and these coenzyme reserves are coming from the parental resources during oogenesis and/or during fertilization. Our results further indicate that both NAD^+ and NADH contents of the embryos are increased during the advancement of morphogenesis (Figures 3A and 3B). NAD^+ content tended to decrease in ethanol treated embryos compared to the corresponding controls both in 2 and 6 dpf embryos (Figure 3A), and NADH concentration in 400 mM groups of 6 dpf embryos show a downward tendency (Figure 3B). These results indicate that medaka embryos are able to metabolize ethanol during

embryogenesis through ADH/ALDH pathway. Although our data are not significantly different from the corresponding controls, there is an indication that alcohol is able to reduce the coenzyme concentration compared to controls and thus affects the NAD^+ . We are unable to measure the coenzyme activity separately in yolk and embryo, therefore the discrepancy in the result arises. We think that ethanol metabolism is restricted to the embryonic body and not in the yolk. Therefore, assay of the coenzymes separately in embryo and yolk may be able to establish an alteration in the coenzyme status.

Oxidative stress is also able to induce epigenetic changes in DNA. Because medaka embryos express *Aldh1A2* mRNA during embryogenesis and the expression of this mRNA was inhibited by ethanol in early stages of development (Hu et al., 2008), we hypothesized this gene could be epigenetically modified after ethanol treatment. We analyzed the methylation pattern of the promoter region of *Aldh1A2* gene; the product of this gene is an enzyme that catalyzes the synthesis of retinoic acid (RA) from retinaldehyde. The information about the nucleotide sequences were obtained from Ensembl. The first exon, a part of the first intron at the 5' end and the 5' UTR (total 859 bp), were considered for methylation analysis. There were 31 CpG dinucleotides found in this region (Figure 4) and bisulfite treatment identified that none contained methylated cytosines. Pooled genomic DNA extracted from the embryos treated with or without ethanol at various stages of embryonic development (Table 1) indicated that ethanol was unable to induce any hypermethylation in *Aldh1A2* promoter. Embryos that were raised to adult after ethanol treatment (300 mM, 48hpf) and the genomic DNA prepared from brain, eye, heart and liver tissues also showed an unchanged *Aldh1A2* promoter methylation. Although we have restricted our analysis to only 859 bp, based on results, the teratogenic effect of ethanol in medaka is not mediated through *Aldh1A2* promoter methylation. In medaka embryos, the majority of the genomic DNA is methylated at CCGG sites (i.e. *Hpa* II sites) and the global genomic 5-methyl cytosine methylation at these sites remains almost unchanged during embryogenesis (Walter et al., 2002). However, adult medaka exposed to 17 α -ethinylestradiol had altered methylation patterns in the promoters of estrogen receptor and aromatase genes in a tissue and gender-specific manner (Contractor et al., 2004). The promoter of *Aldh1A2* we have analyzed contains only three CCGG sites and these sites also remained unmethylated during embryogenesis, in adult stages, as well as after alcohol treatment.

In summary, our results indicate that developmental ethanol exposure of Japanese medaka embryos induces microcephaly with reduced neurocranial length and a dose-dependent disruption in head skeleton chondrification. The oxidative stress generated during ethanol metabolism due to the alteration of NAD^+ and NADH concentration is unable to induce any alteration in the methylation pattern of the *Aldh1A2* promoter, a gene, which was found previously to be ethanol sensitive during the early phase of medaka embryogenesis.

Acknowledgments

This study was supported partially by the Office of Research and Sponsored Program, National Center for Natural Product Research, Environmental Toxicology Research Program of the University of Mississippi and the National Institute on Alcohol Abuse and Alcoholism (Grant Number RO3AA016915). The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institute on Alcohol Abuse and Alcoholism or the National Institute of Health. This publication was made possible by NIH grant number RR016476 from the MFGN INBRE Program of the National Center for Research Resources.

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Figure 1. Representative photomicrograph of the neurocranium (A, D =dorsal side, B lateral side) and splanchnocranium (C= dorsal side) of a medaka hatchlings 10 dpf

The hatchlings were stained with alcian blue showing the different regions of the head skeleton considered for morphometric analysis.

Figure 2. Representative photomicrographs of the neurocranium (dorsal view) of medaka hatchlings showing the disruption in chondrification of neurocranial cartilages by ethanol during development

The embryos were exposed to ethanol (300 mM) 48 hpf and transferred to fresh hatching solution. The controls were maintained in hatching solution only. The hatchlings (10 dpf) were stained with alcian blue. It was observed that chondrification of trabecular cartilages (TC) and polar cartilages (PC) are disrupted by ethanol (B-D). A= control (no ethanol); B, C, and D= 300 mM ethanol (0-48 hpf). In A, a normal TC and PC are seen; in B, C, and D various levels of disrupted chondrification in TC and PC are seen (B= reduced EP with tiny TC and unequal PC; C= reduced EP with short and unequal TC and absence of *PC; D= reduced EP with no TC* and unequal PC).

Figure 3. Effect of ethanol on NAD⁺ and NADH status of medaka embryo during development
 Embryos were exposed to 200 and 400 mM ethanol 48 hpf and either used for coenzyme assay or maintained in hatching solution 6 dpf and used for the coenzyme assay. The corresponding controls were maintained in hatching solution only. To determine the basal value the fertilized eggs ~ 1 hpf were used. Each bar is the mean $\pm$ SD of 4-6 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 (#) indicates that the results are significantly different from the samples of zero day. A= NAD⁺, B= NADH, C= NAD⁺: NADH.

-705-5' -

caaaccgcggggaggttgtgttaggcaaattgggtgatggcgcgctgaaag-655
 ggaaagggtgacacctttcatgtcactagtaggaaataacctggtgctgtg-605
 atttttttttatgtttccatgcaaattttgacccgcaatgaataaaaacg-555
 tgtttgggtttctctttttattttatttgaaatcatccagatcataagat-505
 cttttatgaaggctctgctaaatgagactttcgctgagatgattttgcaaa-455
 gcaaagcgagcctgtttgcattgttagcaatgtacctcattgcagaaacc-405
 tcattgcaaaaatacaatacaaaagaaattacttctgagtgaaaatctgac-355
 tccccagttccagactaagtgcaggttcggcctgcacatttgtgtatgta-305
 aaactatgaataagtatgtaaaagcctcaactgttggagtcggggagaag-255
cgagtgtaccgggactgtgaggtgggtgggtggatgtgttcctcctccc-205
 tagcacgcacggctgggctcgttttatgtaaatgctcgaatttaattttc-155
 aaaaggacagccctttgctttgcccaatgaaaggggaacggcgacCGACAC-105
CTGGCTATAAACCCAGGAAGGAACCTAGAAGCCCGAATCAGAAATCTGCC-55
GAGGGAACCTAGAGACGCGCAGCACGCAAGCACAGCAGCGCAGCCACGGC-5
AGTCATGACTTCCAGTAAGATCGAGATCCCCGGAGAGGTGAAGAGCGACC+46
CGGCTGCCCTCATGGCATCCCTCCAGCTGATGCCCTCACCGGTACCCAAC+96
 CCTGAGCTCAAGTACACAAAGgtgcgttttttctccgcgctctgtgcgcactttaggc-3' +154

Figure 4. The nucleotide sequences of Aldh1A2 promoter of Japanese medaka (*Oryzias latipes*)

The nucleotide sequence (NT) information are originally obtained from Ensemble (www.ensembl.org/Oryzias_latipes/gene) and verified by PCR amplification, cloning and sequencing. The NT represented in upper case letters are the first exon (227 nt) where ATG is represented in bold letters. The underlined regions were used for designing primers for genomic DNA amplification. CpG dinucleotides are colored. There are 31 CpG dinucleotides present in the region of which 26 are considered for analysis. Numbers on the right represent NT number, starting with ATG as +1.

Table 1
Group classification of medaka embryos used during ethanol treatment and methylation analysis

Groups	Stages of development (Iwamatsu, 2004) during initial ethanol exposure	Ethanol (mM)	Duration	DNA preparation	Alteration in Aldh1A2 promoter methylation
A	2-8 (2-4 hpf)	100 and 400	48 h (day 0-2)	6 dpf	No
B	17-20 (24-26 hpf)	100 and 400	48 h (day 1-3)	6 dpf	No
C	23-25 (48-50 hpf)	100 and 400	48 h (day 2-4)	6 dpf	No
D	28-30 (72-75 hpf)	100 and 400	48 h (day 3-5)	6 dpf	No
E	30-32 (96-100 hpf)	100 and 400	48 h (day 4-6)	6 dpf	No
Adult fish	2-8 (2-4 hpf)	300	48 h (day 0-2)	Whole fish	No
Adult fish	2-8 (2-4 hpf)	300	48 h (day 0-2)	Brain	No
Adult fish	2-8 (2-4 hpf)	300	48 h (day 0-2)	Eye	No
Adult fish	2-8 (2-4 hpf)	300	48 h (day 0-2)	Heart	No
Adult fish	2-8 (2-4 hpf)	300	48 h (day 0-2)	Liver	No

Table 2

Effect of ethanol in neurocranial cartilage length (µm) of medaka embryo

Groups	EtOH (mM)	NRC	EP	TC	EB	AOC	ABC	BP	PC
Control A									
	100	1077±12.3	241±6.7	227±5.5	314±5.1	301±5.8	497±4.9	491±7.3	80±1.9
	200	1052±8.8	237±7.8	210±7.6	328±5.7	272±10.4	485±1.6	457±7.8	76±6.5
B									
	200	1008±13.4	234±5.2	160±12.8 [*]	292±6.5	277±14.0	479±4.6	470±6.5	58±5.5
	300	907±14.9 [*]	182±6.1 [*]	63±5.1 [*]	277±5.6 [*]	264±8.4	448±5.3 [*]	436±4.2 [*]	54±5.4
C									
	100	975±111.6 [*]	234±7.1	192±6.5	295±4.6	273±9.7	461±9.0 [*]	444±15.0	77±5.0
	200	908±20.0 [*]	207±9.6	156±13.4 [*]	276±4.2 [*]	242±12.1 [*]	426±7.8 [*]	430±9.5 [*]	55±6.9
D									
	300	911±12.2 [*]	215±9.8	114±22.5 [*]	232±9.9 [*]	232±9.0 [*]	436±6.8 [*]	410±7.5 [*]	42±9.7 [*]
	400	994±19.3 [*]	198±11.2 [*]	214±6.8	286±3.6	259±12.4	466±9.5	463±15.0	70±3.7
E									
	200	947±22.8 [*]	195±9.8 [*]	187±8.9	279±6.0 [*]	253±11.6 [*]	426±8.5 [*]	447±9.7	61±7.2
	300	951±25.8 [*]	205±9.8	170±19.3	260±7.0 [*]	211±14.2 [*]	431±9.4 [*]	398±20.0 [*]	40±8.0 [*]
	400	841±27.2 [*]	178±15.3 [*]	95±24.2 [*]	251±11.8 [*]	213±19.6 [*]	417±9.4 [*]	409±13.1 [*]	47±8.0
	100	966±14.8 [*]	195±13.7 [*]	204±7.7	297±4.1	248±6.8 [*]	460±5.7 [*]	455±6.6	64±8.2
	200	961±23.8 [*]	204±7.9	197±13.1	288±7.4	252±11.7	455±6.9 [*]	459±9.0	82±2.6
	300	897±13.1 [*]	170±5.8 [*]	199±20.0 [*]	274±6.6 [*]	216±6.1 [*]	421±4.4 [*]	417±8.6 [*]	54±8.8
	400	843±18.5 [*]	161±6.9 [*]	189±25.1 [*]	252±7.7 [*]	227±11.0 [*]	387±8.0 [*]	405±11.2 [*]	29±9.9 [*]
	100	1016±20.2	204±8.2	215±6.8	292±4.6	277±10.6	467±6.9	483±7.5	77±5.3
	200	968±15.1 [*]	181±6.6 [*]	222±9.0	287±6.2	249±5.8 [*]	448±7.8 [*]	429±7.8 [*]	81±2.4
	300	902±16.9 [*]	166±13.9 [*]	192±11.7	299±12.7	239±11.7	428±6.8	452±8.3	80±4.3
	400	856±26.8 [*]	143±9.2 [*]	187±11.9	255±13.8 [*]	233±14.7 [*]	414±8.0	410±19.0	69±6.1

Medaka embryos were exposed to ethanol developmentally (Table 1) and then allowed to hatch. The hatchlings were stained with alcian blue and the images were captured at constant magnification by using an Olympus B-max 40 microscope. The images were used for morphometric analysis by using image analysis software. The results (µm) are expressed as mean ±SEM of 8 or more hatchlings.

NRC= Neurocranium, EP=Ethmoid plate, TB=Trabecular cartilage, EB=Epiphyseal bar, AOC=Anterior orbital cartilage, ABC= Anterior basicranial commissure, BP= basilar plate, PC= polar cartilage

* Asterisks indicate that the value is significantly different from the controls.

Table 3

Effect of ethanol on splanchnocranial cartilage length (µm) of medaka

Groups	EtOH (mM)	MC	QU	BH	CH	BB1-3	BB-4	CBR1	CBR2	CBR3	CBR4	CBR5
Control A												
	100	147±3.0	380±10.0	215±9.3	322±4.5	393±6.8	49±2.2	256±6.3	255±5.3	267±5.5	287±5.9	163±3.1
	200	151±3.3	392±6.7	221±6.0	315±4.4	383±6.0	49±2.4	255±6.2	253±6.3	260±5.7	277±9.2	170±7.4
	300	144±3.4	371±10.6	212±6.3	328±6.5	381±7.4	51±2.2	265±6.6	261±6.1	270±4.7	278±8.1	165±6.0
B												
	100	131±3.5	356±6.5	191±4.7	308±6.5	363±8.1	44±1.7	236±6.7	235±5.4	245±6.2	276±7.2	142±3.0
	200	127±3.4	338±7.5	199±5.1	292±8.0	337±11.5	44±2.3	217±8.7	216±7.9	238±9.3	246±7.4	145±4.9
	300	123±3.5	331±12.6	192±7.4	280±9.1	308±6.3	44±1.3	209±7.4	218±6.6	226±9.1	245±10.3	141±4.6
C												
	100	133±5.1	354±6.8	218±9.2	311±8.9	351±10.4	45±1.8	242±8.2	248±4.0	248±6.7	264±8.0	145±2.8
	200	136±4.3	356±8.0	207±7.2	297±11.0	333±11.8	39±1.5	246±7.2	253±11.3	236±7.7	256±9.3	151±5.4
	300	124±3.5	341±9.2	194±9.3	290±8.3	327±10.8	36±1.2	219±8.7	225±7.8	235±6.0	251±7.7	133±4.1
D												
	400	116±3.8	304±8.9	179±4.9	273±6.1	301±6.9	33±0.6	221±9.2	218±11.9	218±8.2	235±10.6	130±5.7
	100	137±4.2	359±9.2	206±12.9	291±6.9	353±6.7	43±1.6	220±7.4	221±6.8	233±7.5	274±5.5	152±4.4
	200	131±6.2	342±6.7	214±11.8	284±10.3	352±8.9	39±1.3	240±7.2	231±6.8	241±8.8	273±11.9	151±3.3
E												
	300	116±3.2	319±5.8	179±8.5	273±5.0	312±4.6	36±1.3	214±5.4	218±5.8	221±5.3	239±4.8	135±2.2
	400	112±3.4	297±4.3	167±6.5	252±7.2	288±5.4	34±1.4	197±5.8	201±6.5	214±6.2	239±5.1	120±3.3
	100	139±3.9	361±8.7	231±15.4	315±4.2	358±3.9	43±1.8	234±13.2	244±3.7	258±3.2	269±3.9	145±4.7
	200	136±2.7	354±8.7	227±8.7	291±5.7	328±5.4	37±0.3	232±7.8	226±6.4	241±2.8	258±3.0	141±3.8
	300	124±3.1	304±11.5	165±5.5	263±6.1	322±8.9	39±0.9	199±4.2	202±3.7	215±8.1	223±11.0	121±4.0
	400	118±4.0	289±11.6	170±8.0	268±3.7	308±4.3	34±0.4	204±6.1	201±7.1	211±9.2	227±9.8	110±6.6

Medaka embryos were exposed to ethanol developmentally (Table 1) and then allowed to hatch. The results are expressed as mean ±SD of 8 or more hatchlings.

MC= Meckel's cartilage, QU= quadrate; BH= basihyal, CH= Ceratohyal, BB= basibranchial, CBR=ceratobranchial.

* Asterisks indicate that the value is significantly different from the controls.

Table 4
List of primers used for amplification of genomic DNA and bisulfite treated genomic DNA

	Forward primer (5'-3')	Reverse Primer (5'-3')	Product size (bp)
Genomic DNA	CAAACCGCGGGGAGGTTGTG	GCCTAAAGTGCGCACAGAG	859 [#]
Bisulfite-treated genomic DNA	GAAAGGGAAAGGTGATATTTT	CTAAAAATCATAACTACCAT	668 [*]
	TTATG	AAC	
	GAATTAGAAATTTGTTGAGGG	ACCTAAAATACACACAAAAC	225 ^{\$}
	GAGTGAAAATTTGATTTTGTAG	ATAACCAAATACCGATCACC	273 ^{&}
	TTTGTAG	GTTC	

[#] Amplified 859 bp of genomic DNA from -705 to +154 which contains 31 CpG dinucleotides 3 of which are included within the primers.

^{*} Amplified 668 bp from -659 to +9 which contains 18 CpG dinucleotides 1 of which is included within the reverse primer

^{\$} Amplified 225 bp from -70 to +154 which contains 15 CpG dinucleotides 2 of which are included within the primers.

[&] Amplified 273 bp from -369 to -99 which contains 7 CpG dinucleotide 1 of which is included in the primer. This region is the overlapping region which contains part of the product 668 and part of 225.