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Disruption of circulation by ethanol promotes fetal alcohol spectrum disorder (FASD) in medaka (*Oryzias latipes*) embryogenesis

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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 24 h. 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.

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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 irreverse array of developmental anomalies associated with embryonic alcohol exposure (Riley and McGee, 2005). Alcohol consumption during pregnancy is the prime cause of the development 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 and Wittbrodt, 2004). These models have several advantages over mammalian models with regard to fertilization, development and transperancy of the egg chorion. The effects of ethanol on zebrafish embryos have been studied by a number of investigators (Bilotta 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,b; 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 skelatal 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

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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

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 1 mL hatching solution (17 mM NaCl, 0.4 mM KCl, 0.36 mM $CaCl_2$, 0.6 mM $MgSO_4$, $NaHCO_3$) 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 1 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 (1 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 48 h (groups A, B, C, D and E) or 24 h (F,G and H) following one-time change of ethanol at 24 h (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)

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)	TTCAACAGCCCT GCCATGTA	GCAGCTCATAGCT CTTCTCCAGGGAG	Internal Standard	359	S74868
β-actin[c](2)	CCTGACCCTG AAGTATCCCA	GAGCTATGAG CTGCCTGACG	Internal Standard	542	S74868
Eif1a[b]	AGCGACAAGAT 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.