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Developmental age strengthens barriers to ethanol accumulation in zebrafish

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A B S T R A C T

Fetal Alcohol Spectrum Disorders (FASD) describes a wide range of phenotypic defects affecting facial and neurological development associated with ethanol teratogenicity. It affects approximately 1 in 100 children born in the United States each year. Genetic predisposition along with timing and dosage of ethanol exposure are critical in understanding the prevalence and variability of FASD. The zebrafish attributes of external fertilization, genetic tractability, and high fecundity make it a powerful tool for FASD studies. However, a lack of consensus of ethanol treatment paradigms has limited the interpretation of these various studies. Here we address this concern by examining ethanol tissue concentrations across timing and genetic background. We utilize headspace gas chromatography to determine ethanol concentration in the *AB*, *fli1:EGFP*, and *Tu* backgrounds. In addition, we treated these embryos with ethanol over two different developmental time windows, 6–24 h post fertilization (hpf) and 24–48 hpf. Our analysis demonstrates that embryos rapidly equilibrate to a sub-media level of ethanol. Embryos then maintain this level of ethanol for the duration of exposure. The ethanol tissue concentration level is independent of genetic background, but is timing-dependent. Embryos exposed from 6 to 24 hpf were 2.7–4.2-fold lower than media levels, while embryos were 5.7–6.2-fold lower at 48 hpf. This suggests that embryos strengthen one or more barriers to ethanol as they develop. In addition, both the embryo and, to a lesser extent, the chorion, surrounding the embryo are barriers to ethanol. Overall, this work will help tighten ethanol treatment regimens and strengthen zebrafish as a model of FASD.

Introduction

Fetal Alcohol Spectrum Disorders (FASD) describes a wide array of neurological defects and morphological malformations associated with ethanol teratogenicity (Elliott, Payne, Morris, Haan, & Bower, 2008). This array of ethanol-induced defects can be attributed to many different factors, including the timing and dosage of ethanol administration and genetic background (Riley, Infante, & Warren, 2011; Sulik, 2005). The complex interplay of these variables in humans makes understanding FASD challenging, necessitating the use of animal models.

Amniote model systems have been used by numerous labs to study various aspects of FASD. Consistent with what is found in early exposure in humans, particularly during gastrulation, binge levels of alcohol cause profound teratogenesis. Recently, even later exposures to ethanol have been shown to cause developmental anomalies (Lipinski et al., 2012). In addition, the availability of many mouse mutants has prompted an analysis of potential gene–ethanol interactions, particularly with the Sonic Hedgehog pathway, which has been implicated in ethanol teratogenesis (Ahlgren, Thakur, & Bronner-Fraser, 2002; Hong & Krauss, 2012; Loucks & Ahlgren, 2009; Sulik, 2005). In sum, this work has shown that these models are useful for understanding the influences of ethanol exposure on embryonic outcome.

The zebrafish is ideally suited for studying the effects of timing, dosage, and genetics on ethanol teratogenesis. Zebrafish are genetically tractable, highly fecundate, and fertilize externally, allowing ethanol to be consistently administered to precisely staged embryos. Additionally, the developmental programs regulating embryonic development are conserved between amniotes and fish (Sheehan-Rooney, Pálinkášová, Eberhart, & Dixon, 2010; Swartz, Sheehan-Rooney, Dixon, & Eberhart, 2011). Thus, findings in zebrafish can help guide the analyses of FASD in humans (McCarthy et al., 2013).

Zebrafish have been used to examine the teratogenic effects of ethanol (Ali, Champagne, Alia, & Richardson, 2011; Bilotta, Barnett,

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Hancock, & Saszik, 2004; Blader & Strähle, 1998; Dlugos & Rabin, 2003; Gerlai, Lahav, Gou, & Rosenthal, 2000; Gerlai, Lee, & Blaser, 2006; Li et al., 2007; Lockwood, Bjerke, Kobayashi, & Guo, 2004; McCarthy et al., 2013; Pan, Chatterjee, & Gerlai, 2012; Reimers, Flockton, & Tanguay, 2004; Stockard, 1910; Zhang, Ojiaku, & Cole, 2013; Zhang, Turton, Mackinnon, Sulik, & Cole, 2011). However, there is a lack of consensus regarding ethanol treatment regimens and how these regimens would relate to humans. In mammalian systems, blood alcohol levels directly correlate to tissue levels (Adalsteinsson, Sullivan, Mayer, & Pfefferbaum, 2006; Flory, O'Malley, Grant, Park, & Kroenke, 2010; Quertemont, Green, & Grant, 2003). However, there is some inconsistency in reported tissue levels in ethanol-exposed zebrafish embryos. To address the concern of tissue ethanol concentrations, we used headspace gas chromatography to determine ethanol concentrations across the variables of timing and genetic background. In this study, we demonstrate that the zebrafish embryo has some barrier to the accumulation of ethanol. This results in ethanol concentrations approximately 3–6-fold less than the medium, depending on embryonic age. In our study, we examined ethanol concentrations after an early exposure (6–24 hpf) and a later exposure (24–48 hpf). At 24 hpf, embryos contained 2.7–4.2-fold less ethanol than the medium, while the embryos contained 5.8–6.2-fold less at 48 hpf. Further, we found that genetic background did not alter ethanol tissue concentrations. Collectively, our results help elucidate the tissue accumulation of ethanol in exposed zebrafish and show that genetic background is not a common source of variation in tissue levels of ethanol.

Materials and methods

Zebrafish (Danio rerio*) care and use*

All embryos were raised and cared for using established IACUC protocols (Westerfield, 1993) approved by the University of Texas at Austin. Three strains were utilized in this study, AB, Tubigan (Tu, in the text), and *Tg(fli1:EGFP)*^{y1} transgenic embryos (Lawson & Weinstein, 2002; *fli1* in the text). Embryos were treated with 1% ethanol diluted in embryo media from either 6–24 hpf or 24–48 hpf. Embryos were then gathered for determination of tissue ethanol concentrations.

Embryo volume and weight calculations

In order to calculate tissue concentrations of ethanol in zebrafish embryos, the average volume of an embryo is needed. Because embryo volume is small, we used displacement of a relatively large volume of water to reduce error. 0.5 mL microcentrifuge tubes (Fischer Scientific) were filled with 250 μL (250 mg) of water using a P200L Pipetman pipette (Gilson, Inc.) and a fill line was marked representing 250 μL. The water was removed and 10 embryos were placed into the tube at a time, which was then refilled to the 250 μL marked line. This process eliminates the concern about fluid clinging to the embryos as any carryover was included in the final measurement. All of the water was then carefully removed from the samples; any sample where an embryo was pipetted was not used. While it is impractical to remove all residual water in all of our treatments, we estimated the residual liquid by using a Kimwipe® to remove liquid stuck to the embryo and microcentrifuge tube sides in 10 samples of 24 hpf embryos (10 embryos per sample). Based on our weight measures we were able to determine that there was approximately 0.067 μL of residual water, per embryo. This is a small fraction of the calculated volume for the embryos (see Results section) and, importantly, would be similar across all treatments. The weight of the final water sample was subtracted from the initial 250 mg of water weight. Volume was directly determined from the weight of the removed water.

To determine dry weight of the embryos, the 10 embryos previously measured for volume were placed on pre-weighed glass coverslips (22 mm × 22 mm) and baked at 70 °C for 2 h. The samples were allowed to cool and were weighed (Mettler-Toledo XS64; accurate to 0.01 mg). Baking the embryos at 70 °C for longer than 2 h did not further decrease the weight of the embryos, demonstrating that baking for 2 h completely dried the embryo samples. The P200L Pipetman used for these analyses was calibrated with a systemic error (representing accuracy) of 0.09 μL and a random error (representing precision) of 0.03 μL (http://www.pipetman.com/RequestLiterature.aspx – Verification Procedures for Accuracy & Precision). The pipette and the Mettler–Toledo scale are calibrated multiple times a year.

Measurement of ethanol concentration using headspace gas chromatography

To determine ethanol tissue concentration relative to media exposure, headspace gas chromatography (GC) was used. Embryos, either with their chorions intact or removed prior to ethanol treatment, were exposed to media ethanol at 1% from 6 to 24 or 24–48 hpf. Samples were taken at 24 hpf for the early exposure period and 48 hpf for the later exposure period. Samples consisted of 10 pooled embryos that were rinsed once for 1 s in fresh media lacking ethanol to eliminate ethanol adhering to the exterior of the samples, and then treated with 50 μL of Pronase (2 mg/mL – Roche Applied Science) for 10 min to aid in removal of the chorion in the chorionated samples. Dechorionated embryos were also treated with pronase for consistency. 450 μL of 5 M NaCl was then added to each sample and the samples were then vortexed for 10 min to homogenate the embryos. Silica beads were added to the 48 hpf old embryos to aid in homogenization. For each sample, an aliquot of 2 μL was transferred to a 2 mL GC vial and sealed with a PTFE silicon septum and plastic cap. For each time point, 6 treated and untreated embryo samples were also collected and homogenized. In addition, 6 media reference samples were collected and diluted 10 fold in 5 M NaCl and aliquotted to a 2 mL GC vial and sealed. A Varian CP 3800 gas chromatograph with flame ionization detection and a Varian CP 8400 headspace autosampler, heated to 58 °C, were used to analyze the concentrations of ethanol in the homogenized samples. The stationary phase was an HP Innowax capillary column (30 m × 0.53 mm × 1.0 μm film thickness) and helium was the mobile phase. Resulting ethanol peak heights were recorded using Varian Star Chromatography Workstation software. The GC signal-to-noise ratio was 7 and calibration was achieved using external standards from 0.3125 to 40 mM ethanol in 5 M NaCl as described in Doyon et al. (2003). The accuracy and precision of the calibration was verified against a 1% ethanol standard in triplicate and was within 0.013% of the targeted 1% ethanol concentration with a coefficient of variation of 0.018. Quantification of ethanol concentrations of all samples was performed by comparing peak heights obtained from the Varian Star Chromatographic analysis with the external standards.

Statistical analysis

Ethanol concentrations in embryo samples were compared to one another, untreated controls, and media using a 1 way ANOVA with a Tukey's *post hoc* test and a student's *t* test in Graphpad Prism v5.02 (Graphpad Software Inc., La Jolla, CA).

Results

Zebrafish were first used to examine the teratogenic effects of ethanol as early as 1910 (Stockard, 1910), and more recent work has