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Ethanol-dependent toxicity in zebrafish is partially attenuated by antioxidants

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Abstract

Ethanol is a well-established developmental toxicant; however, the molecular and cellular mechanism(s) of toxicity remains unclear. It has been suggested that ethanol metabolism leads to oxidative stress resulting in an increase in cell death. Alcohol developmental toxicity has not been well studied in zebrafish; however, zebrafish represent an excellent vertebrate model for investigating and understanding normal and aberrant development. To evaluate ethanol metabolism dependent toxicity, chemical inhibitors of the ethanol metabolizing enzymes were utilized. Embryos co-exposed to ethanol and a combination of ethanol metabolism inhibitors led to a significant increase in the occurrence of pericardial edema. Further, in the presence of the inhibitor mixture there was an increase in developmental malformations at lower ethanol concentrations. Cell death has been implicated as a potential explanation for ethanol-dependent toxicity. Using cell death assays, ethanol significantly increased embryonic cell death. To determine if oxidative stress underlies cardiovascular dysfunction, embryos were co-exposed to ethanol and several antioxidants. The antioxidants, glutathione and lipoic acid, partially attenuated the incidence of pericardial edema. The effectiveness of the antioxidants to protect the embryos from ethanol-induced cell death was also evaluated. The antioxidants provided no protection against cell death. Thus, ethanol-mediated pericardial edema and cell death appear to be mechanistically distinct.

Keywords: Alcohol; Embryos; Ethanol; Ethyl alcohol; Development; Developmental toxicity; Fetal alcohol syndrome; Larvae; Oxidative stress; Teratogen; Teratogenicity; Toxicity; Zebrafish

1. Introduction

While the teratogenic properties of ethanol have been firmly established, the underlying mechanism(s) of toxicity remains unclear. One proposed mechanism suggests that the metabolism of ethanol to acetaldehyde produces oxidative stress and leads to physical dysmorphogenesis and central nervous dysfunction (reviewed in [37]).

Excessive cell death has been reported to underlie ethanol-induced nervous system pathogenesis during various stages of embryonic or fetal development [23]. Rat cerebellum Purkinje cells were particularly sensitive to ethanol during the fetal stage of development [6,20]. Further, during the embryonic stages, chick and mouse cranial neural crest cells were predominantly vulnerable to ethanol [7,8,12,13,18]. Mouse fetal cells, such as neural crest cells, underwent apoptotic cell death as a consequence of ethanol-induced oxidative stress [11,12,24].

Numerous studies have demonstrated that ethanol-induced oxidative injury in the embryo may contribute to the pathogenesis of ethanol-mediated developmental toxicity. A study conducted by Devi et al. documented ethanol-induced

Abbreviations: dpf, days post fertilization; FAS, fetal alcohol syndrome; hpf, hours post fertilization; S.E.M., standard error of the mean; S.D., standard deviation; TI, teratogenic index; ATA, 3-amino-1,2,4-triazole; 1-ABT, 1-aminobenzotriazole; 4-MP, 4-methyl pyrazole; MTC, maximum tolerable concentration.

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oxidative injury in fetal rat hepatocytes [17]. Additionally, cultured fetal rat brain cells exposed to ethanol led to an increase oxidative stress [16,17,21]. Further, in mice dosed with ethanol, there was a significant increase in oxidative stress and excessive cell death throughout the embryo but was concentrated in the brain region [24]. Studies have suggested that fetal tissues have a more compromised or less active antioxidant system than adult tissue; thus embryos would be more sensitive to oxidative injury [19,27] (reviewed in [22]).

Experimental manipulations with protective antioxidants support the hypothesis that ethanolic damage may result partially from increased oxidative stress. Antioxidant enzymes (catalase, superoxide dismutase, glutathione peroxidase, glutathione reductase and glutathione-*S*-transferase) and nonenzymatic modifiers (glutathione, α-tocopherol (vitamin E), and ascorbic acid (vitamin C)) protect against oxidative damage [44]. The use of nonenzymatic modifiers in culture attenuated ethanol-induced oxidative injury *in vitro* and *in vivo* studies (reviewed in [30]). For instance, ethanol-dependent oxidative stress led to a reduction in mouse neural crest cell viability and cell survival was enhanced with the addition of superoxide dismutase, catalase, and/or α-tocopherol [11]. This suggests that free radicals play a significant role in ethanol-mediated neural crest cell death.

Zebrafish are an excellent vertebrate model to study developmental toxicity because they share many molecular, biochemical, cellular and physiological characteristics with higher vertebrates [1,2,41,42,45]. The transparent embryos rapidly develop externally. Organogenesis is completed within the first 48 h of development. Since zebrafish embryos develop externally, changes in development may be continuously observed, which greatly facilitates developmental time course studies. Since zebrafish development has been well characterized, results from zebrafish are comparable to mammalian developmental studies. Finally, the practical advantages of this model allow for saturation mutagenesis screens [15,32] and knockdown approaches that can be used to identify genes involved in toxic responses [34].

Since zebrafish development has been well characterized, results from zebrafish can be compared to mammalian results. For instance, previous studies in zebrafish demonstrated that ethanol leads to craniofacial abnormalities, cardiac and structural malformations, and developmental delays similar to results observed in mammals [4,10,25,26,28,36]. The role that ethanol biotransformation may play in these ethanol-dependent endpoints in zebrafish remains unknown. This study was designed to investigate the mechanism by which ethanol perturbs vertebrate development. We report that inhibitors of ethanol metabolism increased ethanol toxicity. Furthermore, we demonstrated that antioxidants partially protected embryos from ethanol-induced pericardial edema. Ethanol exposure led to a significant increase in cell death throughout the embryo, but the addition of antioxidants did not reduce ethanol-dependent cell death. This suggests that ethanol-dependant oxidative stress may play a partial, but significant, role in embryotoxicity, and that zebrafish is a suitable model for investigating the underlying mechanisms of ethanol-mediated developmental toxicity.

2. Materials and methods

2.1. Materials

DL-Lipoic acid and 1-aminobenzotriazole were obtained from Acros Organics (Pittsburgh, PA). Glutathione reduced (L-glutathione or GSH), 3-aminobenzoate ethyl ester methanesulfonate salt (tricaine), methyl cellulose and 4-methyl pyrazole (4-MP) were purchased through Sigma Chemical Company (St. Louis, MO). TCI America (Portland, OR) supplied the antioxidant Trolox or water soluble Vitamin E (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid). All of the above chemicals were at least 98% pure. Vitamin C or L-(+)-ascorbic acid (USP-FCC, ≥99% purity) was purchased through JT Baker Analytical, a Division of Mallinckrodt Baker, Inc., Phillipsburg, NJ. The inhibitor 3-amino-1,2,4-triazole (≥95%) was purchased from Calbiochem, a brand of EMD Biosciences, Inc., San Diego, CA. Absolute ethyl alcohol USP, 200 proof, was purchased from AAPER Alcohol and Chemical Company, Shelbyville, KY. Glass exposure vials with Teflon®-lined lids were purchased from Fisher Scientific Company.

2.2. Fish care and husbandry

Adult AB strain zebrafish (*Danio rerio*) were raised and housed in accordance to the Institutional Animal Care and Use Committee protocols at Oregon State University. Zebrafish were reared in 2.0 l polycarbonate tanks on a recirculating system in which the water was maintained at 28±1 °C and a pH of 7.0±0.2. The fish were fed twice daily with either crushed TetraMin® Tropical Flake (Blacksburg, VA) or live artemia from INVE (Grantsville, UT). Newly fertilized eggs were collected and embryos were rinsed several times in water prior to their use. Normal dividing and spherical embryos at the 256 cell stage (2.5 hours post fertilization or hpf) through the oblong stage (3.7 hpf) were selected and utilized for all of the described studies. Embryos were staged as previously described [43], using the pectoral fin, yolk sac, anal pore, and swim bladder as indicators of developmental stage.

2.3. Embryonic exposure

All embryos were waterborne exposed using a static method with 12 embryos per vial per treatment. Embryos were exposed to ethanol alone, or with antioxidants, ethanol metabolism inhibitors in 20 ml glass vials sealed with Teflon®-lined lids (Fisher Scientific International, Pittsburgh, PA) to prevent losses by volatilization. The embryos were exposed to ethanol and the other chemicals from 3 through 24 hpf. This exposure period was selected because embryos were previously found to be most responsive to ethanol [36]. At the end of the exposure period, the embryos were washed several times with water and allowed to develop until 120 hpf.

To evaluate the interaction between ethanol metabolism and antioxidants, sublethal waterborne ethanol concentrations ≤200 mM were used [36]. Range finding experiments were initially conducted in order to determine the maximal tolerable