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Zebrafish retinal defects induced by ethanol exposure are rescued by retinoic acid and folic acid supplement

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ABSTRACT

Fetal Alcohol Spectrum Disorder (FASD) is caused by prenatal alcohol exposure, producing craniofacial, sensory, motor, and cognitive defects. FASD is highly prevalent in low socioeconomic populations, which are frequently accompanied by malnutrition. FASD-associated ocular pathologies include microphthalmia, optic nerve hypoplasia, and cataracts. The present study characterizes specific retinal tissue defects, identifies ethanol-sensitive stages during retinal development, and dissects the effect of nutrient supplements, such as retinoic acid (RA) and folic acid (FA) on ethanol-induced retinal defects. Exposure to pathophysiological concentrations of ethanol (during midblastula transition through somitogenesis; 2–24 h post fertilization [hpf]) altered critical transcription factor expression involved in retinal cell differentiation, and produced severe retinal ganglion cell, photoreceptor, and Müller glial differentiation defects. Ethanol exposure did not alter retinal cell differentiation induction, but increased retinal cell death and proliferation. RA and FA nutrient co-supplementation rescued retinal photoreceptor and ganglion cell differentiation defects. Ethanol exposure during retinal morphogenesis stages (16–24 hpf) produced retinal defects like those seen with ethanol exposure between 2 and 24 hpf. Significantly, during an ethanol-sensitive time window (16–24 hpf), RA co-supplementation moderately rescued these defects, whereas FA co-supplementation showed significant rescue of optic nerve and photoreceptor differentiation defects. Interestingly, RA, but not FA, supplementation after ethanol exposure could reverse ethanol-induced optic nerve and photoreceptor differentiation defects. Our results indicate that various ethanol-sensitive events underlie FASD-associated retinal defects. Nutrient supplements like retinoids and folate were effective in alleviating ethanol-induced retinal defects.

Introduction

Fetal alcohol spectrum disorder (FASD) caused by prenatal ethanol exposure is the most frequent preventable birth defect syndrome (May et al., 2009). FASD prevalence ranges from 0.3 to 5% within most populations, reaching as high as 8.9% in some low socioeconomic populations (May et al., 2009). Ethanol-induced defects include craniofacial, cardiac, central nervous system, learning, motor, sensory, and ocular defects. Ocular defects are frequently seen in children diagnosed with FASD, which include microphthalmia (reduced eye size), coloboma (incomplete optic fissure closure), optic nerve hypoplasia (ONH), cataracts, scotopic vision loss, low visual acuity, and abnormal electroretinograms (Hug, Fitzgerald, & Cibis, 2000; Strömland & Pinazo-Durán, 2002).

Ethanol exposure in vertebrate animal models recapitulates retinal defects seen in FASD patients. Experiments using mice showed that *in utero* ethanol exposure induced specific defects in rod photoreceptor sensitivity and dark adaptation (Katz & Fox, 1991). Studies on zebrafish embryos showed reduced electroretinogram responses, ONH, retinal lamination defects, inhibition of photoreceptor outer segment growth, and reduced visual acuity due to ethanol exposure during gastrulation through neurulation stages (2–24 hpf) (Arenzana et al., 2006; Bilotta, Saszik, Givin, Hardesty, & Sutherland, 2002; Matsui, Egana, Sponholtz, Adolph, & Dowling, 2006). Ethanol exposure during zebrafish retinal neurogenesis (24–48 hpf) also induced persistent microphthalmia (Kashyap, Frederickson, & Stenkamp, 2007). Shorter periods of ethanol exposure (12–24 hpf) were sufficient to induce microphthalmia, similar to that produced by longer treatments (Bilotta et al., 2002). However, cellular details of ethanol effects on retinal cell specification, differentiation, and potential protective measures remain unclear.

Proposed mechanisms underlying ethanol-induced ocular defects include increased cell death, developmental delay, and

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reduced cell differentiation (Kashyap et al., 2007). Developmental defects may be due to ethanol-induced retinoic acid (RA) signaling disruption, reactive oxygen species (ROS) generation, and epigenetic defects (Brocardo, Gil-Mohapel, & Christie, 2011; Kot-Leibovich & Fainsod, 2009; Marrs et al., 2010; Singh, Shiue, Schomberg, & Zhou, 2009; Zhou et al., 2011). In addition, low socioeconomic populations show increased FASD incidence, which correlates with nutritional deficiencies. Reduced absorption and increased excretion of essential vitamins in adults caused by ethanol consumption aggravates malnutrition (Lieber, 2003). Several studies showed nutritional compounds, including retinoids, folate, choline, and vitamin E partially rescued ethanol-induced developmental defects (Heaton, Paiva, & Siler-Marsiglio, 2011; Kot-Leibovich & Fainsod, 2009; Marrs et al., 2010; Mitchell, Paiva, & Heaton, 1999; Sarmah & Marrs, 2013; Thomas, Idrus, Monk, & Dominguez, 2010; Wang et al., 2009; Yelin et al., 2005). RA and FA were very effective in rescuing various developmental defects (Ballard, Sun, & Ko, 2012; Marrs et al., 2010; Sarmah & Marrs, 2013; Yelin et al., 2005).

Retinal structure and developmental mechanisms are highly conserved among vertebrates. Rapid and well-characterized developmental events in zebrafish offer opportunities to examine specific ethanol-induced retinal defects and design rescue experiments to study cellular details. Vertebrate retinal morphogenesis occurs through a series of tightly regulated processes involving retinal cell specification, lamination, and differentiation into various cell types which are tightly orchestrated by signaling pathways, including BMPs, Shh, FGFs, and RA (Lupo et al., 2005; Ohkubo, Chiang, & Rubenstein, 2002).

RA is a derivative of vitamin A (retinol), and RA signaling plays a crucial role during embryonic development. During retinal morphogenesis, RA performs distinct functions. RA is a morphogen for retinal dorsoventral patterning and RA induces terminal differentiation of unspecified photoreceptor progenitors and precursors into rod and cone photoreceptors in the outer nuclear layer (ONL) of the retina (Hyatt, Schmidt, Fadool, & Dowling, 1996; Prabhudesai, Cameron, & Stenkamp, 2005; Rhinn & Dollé, 2012). Several alcohol/aldehyde dehydrogenases (ADHs/ALDHs) tightly regulate RA biosynthesis, and RA-degrading enzymes control its catabolism during development. Retinaldehyde dehydrogenase enzymes are expressed in the dorsal (Raldh2) and ventral (Raldh3) regions of the zebrafish retina during retinal morphogenesis. Early *in vitro* studies showed competitive inhibition of ADHs by ethanol (Mezey & Holt, 1971) produces ethanol-induced RA signaling deficits during development, causing embryonic malformations (Duester, 1991), but it is unclear what other developmental signaling mechanisms are also disrupted by embryonic ethanol exposure.

FA is an essential vitamin that participates in nucleic acid synthesis and repair (Kamen, 1997). FA also plays a crucial role as a cofactor in 1-carbon metabolism as tetrahydrofolate, which is needed in DNA and histone methyl transfers. More recent studies identified ROS scavenging properties of FA (Ibrahim, Tousson, El-Masry, Arafa, & Akela, 2012; Joshi, Adhikari, Patro, Chattopadhyay, & Mukherjee, 2001). FA deficiency, consequently, produces a wide range of birth defects including severe ocular defects, such as microphthalmia, delayed lamination, and optic cup abnormalities (Maestro-de-las-Casas et al., 2013). Embryonic ethanol exposure affects FA metabolism, including reduced maternal-to-fetal folate transfer and reduced expression of folate metabolizing enzymes (Hutson, Stade, Lehotay, Collier, & Kapur, 2012). Ethanol-induced FA deficiency could alter histone and DNA methylation patterns as seen in ethanol-treated cell culture models (Mason & Choi, 2005; Singh et al., 2009; Zhou et al., 2011). Importantly, studies showed that FA supplementation rescued overall ethanol-induced morphological defects, particularly cardiac defects (Ballard et al., 2012; Sarmah & Marrs, 2013; Serrano, Han, Brinez, & Linask, 2010). Prenatal FA supplementation significantly reduces the risk of neural tube defects, congenital heart defects, and cleft lip/palate, and thus, FA is a recommended dietary supplement for pregnant mothers (Taruscio, Carbone, Granata, Baldi, & Mantovani, 2011).

Dose, duration, and timing of ethanol exposure greatly affect the severity of FASD, which is also influenced by intrinsic (genetic background) and extrinsic (nutrition and environment) factors. Here, experiments are presented that characterize effects of ethanol on cell differentiation pathways that produce retinal defects. Experiments were used to identify ethanol-sensitive developmental stages and examine effects of nutrient supplementation with RA and FA.

Materials and methods

Zebrafish husbandry and ethanol, RA, FA, inhibitor treatments

Zebrafish (*Danio rerio*; Hamilton; TL strain) were raised and housed under standard laboratory conditions (Westerfield, 2000) in accordance with Indiana University Policy on Animal Care and Use. The embryos were treated with 1-phenyl-2-thiourea (0.003%) from 6 hpf (shield) onward in order to prevent melanogenesis. Embryos were exposed to ethanol by incubation in the embryo medium containing ethanol (100 mM and 150 mM) for different periods between 2 and 24 hpf in Petri dishes wrapped with Parafilm® and maintained at 28.5 °C. Ethanol treatment dishes were placed in chambers with 2% ethanol to minimize ethanol volatilization. After treatment, embryos were rinsed with pre-warmed embryo medium and incubated in embryo medium until the desired stage was achieved.

RA (0.1 mM) and DEAB (1 mM) stock solutions (Sigma, St. Louis, MO, USA) were dissolved in DMSO. Citral (1 mM; T.C.I., Portland, OR, USA) stock solution was freshly prepared prior to treatment. The medium with citral was replaced with embryo medium containing freshly diluted citral (1 and 10 μM) every hour for 8 h (16–24 hpf) due to lability of citral in water. FA (1 mM, Sigma) stock solution was made fresh prior to treatment and diluted in embryo medium. RA and FA treatments were performed as previously described (Marrs et al., 2010; Sarmah & Marrs, 2013). Other concentrations of RA and FA have been tested previously (Marrs et al., 2010; Sarmah & Marrs, 2013), and 1 nM RA and 75 μM FA were chosen as optimal concentrations for treatments that minimized their toxic effects and displayed maximum rescue phenotypes.

Immunofluorescence

Whole-mount immunostaining was performed as previously described (Clendenon, Sarmah, Shah, Liu, & Marrs, 2012) using primary antibodies against HuC/D (Sigma, 1:1000), zpr-1 (ZIRC, Eugene, OR, USA; 1:1000), zpr-3 (ZIRC, 1:500), zrf-1 (ZIRC1:1000), zn-5 (ZIRC, 1:500), acetylated tubulin (Sigma, 1:500), and phosphohistone-3 (Millipore, Bellerica, MA, USA; 1:500). Alexa Fluor 488-conjugated anti-mouse and anti-rabbit secondary antibody (Molecular Probes, Grand Island, NY, USA) was used at 1:200 dilution. Alexa Fluor 488-conjugated phalloidin (Molecular Probes) was used at a 1:100 dilution. Nuclear staining was performed using TO-PRO-3 iodide at a 1:1000 dilution incubated for 1 h.

Apoptosis imaging

Acridine orange staining was performed to visualize apoptotic nuclei by incubating the dechorionated embryos with 5 μg/mL acridine orange for 3 min, followed by several washes in embryo