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Molecular and morphological changes in zebrafish following transient ethanol exposure during defined developmental stages

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ABSTRACT

Alcohol is a teratogen that has diverse effects on brain and craniofacial development, leading to a constellation of developmental disorders referred to as fetal alcohol spectrum disorder (FASD). The molecular basis of ethanol insult remains poorly understood, as does the relationship between molecular and behavioral changes as a consequence of prenatal ethanol exposure. Zebrafish embryos were exposed to a range of ethanol concentrations (0.5–5.0%) during defined developmental stages, and examined for morphological phenotypes characteristic of FASD. Embryos were also analyzed by in situ hybridization for changes in expression of defined cell markers for neural cell types that are sonic hedgehog-dependent. We show that transient binge-like ethanol exposures during defined developmental stages, such as early gastrulation and early neurulation, result in a range of phenotypes and changes in expression of Shh-dependent genes. The severity of fetal alcohol syndrome (FAS) morphological phenotypes, such as microphthalmia, depends on the embryonic stage and concentration of alcohol exposure, as does diminution of retinal *Pax6a* or forebrain and hindbrain *GAD1* gene expression. We also show that changes in eye and brain morphology correlate with changes in *Pax6a* and *GAD1* gene expression. Our results therefore show that transient binge-like ethanol exposures in zebrafish embryos produce the stereotypical morphological phenotypes of FAS, with the severity of phenotypes depending on the developmental stage and alcohol concentration of exposure.

1. Introduction

Alcohol is a teratogen that adversely affects brain and craniofacial development (Sulik et al., 1981; Sulik et al., 1986; Jones and Smith, 2003). In humans prenatal alcohol exposure results in congenital abnormalities affecting the development of numerous brain regions, which includes eyes (Stromland, 1985; Chan et al., 1991; Stromland and Pinazo-Duran, 1994), auditory structures (Church and Kaltenbach, 1997) and cerebral cortex (Clarren et al., 1978; Mattson and Riley, 1996). Understanding the mechanisms that lead to fetal alcohol spectrum disorders (FASD) is a critical emphasis of FASD research, and rodent studies have provided important insights into understanding the pathologies and likely molecular targets of ethanol exposure during fetal development. Rodent models have helped identify key molecular bases of FASD, but the processes by which these molecular disruptions lead to abnormal neurodevelopment and behavioral dysfunction are difficult to study in rodents given their inaccessibility during embryonic development. Zebrafish, with their clear chorion and accessibility of embryos, can provide the needed information about the processes of neuromolecular disruption by alcohol.

Since a wide range of developmental processes are perturbed as a consequence of ethanol exposure, it is critical that the genetic mechanisms that underlie the effects of ethanol are identified. A wealth of evidence indicates that ethanol may exert its effects on brain development via disruption of extracellular matrix (ECM) function, with genes encoding key ECM proteins, such as Fgf2 and Fgf8, being affected by ethanol exposure in prenatal mice (Hard et al., 2005; Rubert et al., 2006; Aoto et al., 2008). Ethanol also perturbs neuronal interactions with the ECM protein laminin, disrupting laminin-mediated axon growth and cell migration (Liesi, 1997). In limb patterning ethanol appears to disrupt this process via perturbation of Fgf8 and sonic hedgehog (Shh) signaling (Chrisman et al., 2004). Shh signaling appears to be a critical target of prenatal ethanol exposure, with disruption of this morphogen's function likely being responsible for the craniofacial abnormalities of FAS (Ahlgren et al., 2002; Arenzana et al., 2006; Li et al., 2007; Aoto et al., 2008; Loucks and Ahlgren, 2009; Zhang et al., 2013). Prenatal ethanol exposure during gastrulation and early neurulation also disrupts *Shh* gene expression in mouse and chick embryos, resulting in phenotypes characteristic of perturbed Shh signaling (Ahlgren et al., 2002; Loucks et al., 2007; Aoto et al., 2008). Many of these ECM molecules identified as targets of ethanol have their function modulated by interactions with heparan

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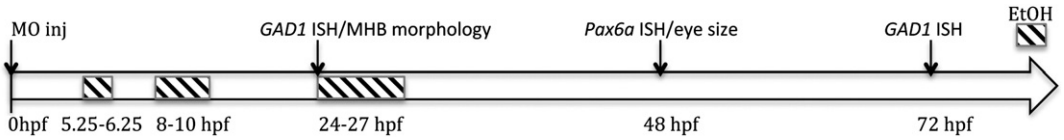

Fig. 1. Experimental design for ethanol exposure and MO treatment of zebrafish embryos. This schematic diagram summarizes the developmental times embryos were exposed to ethanol, and/or injected with MO, and then analyzed for morphological phenotypes (MHB or eye size) and gene expression changes.

sulfate proteoglycans (HSPGs), consistent with a reduction in heparan sulfate synthesis following ethanol exposure (Dow and Riopelle, 1990). A major focus of our laboratory has been the analysis of the function of \the HSPG agrin during zebrafish development, and in particular in response to ethanol exposure during zebrafish CNS development (Kim et al., 2007; Liu et al., 2008; Zhang et al., 2011, 2013). Our recent studies demonstrated that ethanol-mediated disruption of zebrafish ocular development and GABAergic neuronal differentiation results from perturbed agrin and Shh function (Zhang et al., 2011, 2013).

A potential limitation of many previous ethanol experiments in zebrafish is that the studies utilized chronic exposures of zebrafish embryos to ethanol, sometimes exceeding one day. These typical ethanol exposure times likely suffer from not representing the behavior of a pregnant woman drinking alcohol during pregnancy. For example, the widely used 6–24 h post-fertilization (hpf) exposure time likely would be equivalent to a pregnant woman drinking throughout a significant portion of the first trimester of pregnancy. Using this chronic exposure protocol, the majority of zebrafish embryos exposed to high-dose ethanol do not survive past the larval stage (Zhang et al., 2011, 2013). Thus, our goal in the present studies was to use transient ethanol exposures in zebrafish that more accurately mimic binge-like alcohol abuse by a pregnant woman and binge-like ethanol exposure during rodent fetal development. The current studies were designed to test the hypothesis that transient ethanol exposure during defined periods of zebrafish embryogenesis would result in morphological and gene expression phenotypes characteristic of FAS and FASD, and similar to our previous observations following chronic alcohol exposure during zebrafish development.

2. Materials and methods

2.1. Animals

Zebrafish were obtained from Zebrafish International Resource Center. The AB strain was used in these studies and fish were housed in automatic fish housing systems (Aquaneering, San Diego, CA) at 28.5 °C. All procedures using zebrafish were approved by the NCCU IACUC.

2.2. Ethanol treatment of zebrafish embryos

Zebrafish embryos in fish water containing a 1:500 dilution of 0.1% methylene blue (to prevent fungal infection) were exposed to 0.5%, 1%, 3% or 5% ethanol from 5.25 to 6.25, 8 to 10 or 24 to 27 hpf. We focused on three embryonic stages for analysis: 5.25–6.25 hpf, the first hour of zebrafish gastrulation; 8–10 hpf, the transition from gastrulation to neurulation in zebrafish; and 24–27 hpf, a key CNS developmental stage characterized by the formation of the 5-vesicle brain. Fig. 1 summarizes the main experiments summarized in Results, showing time of ethanol exposures, MO treatments and analyses conducted. Ethanol was diluted with fish water to its final concentration, and at the selected developmental stage embryos were placed in fresh fish water containing ethanol. Embryos were incubated in 100 mm plates with fish water containing ethanol, with 8–30 embryos per plate. Each experiment was repeated at least 3 times, and the combined number of embryos used to calculate ratios of embryos showing effects in response to ethanol exposure. At the end of the exposure period fish water containing ethanol was removed, embryos were washed once with fresh fish water, and then transferred to fresh fish water for the remainder of the experimental time-course. Ethanol concentrations were analyzed as previously described (Zhang et al., 2013) in embryos with intact chorion that were exposed to 1%, 3% or 5% ethanol.

Eye size was measured at 2 dpf as previously described (Zhang et al., 2011), and involved measuring the longest axis along the eye, and calculated against a standard 10 μm ruler under the same magnification. For 2 dpf eye, we designated a diameter less than 240 μm as small eye phenotype, since all untreated eyes were at least 250 μm diameter. Malformation of the MHB was assessed visually based on absence of the defined border between the midbrain

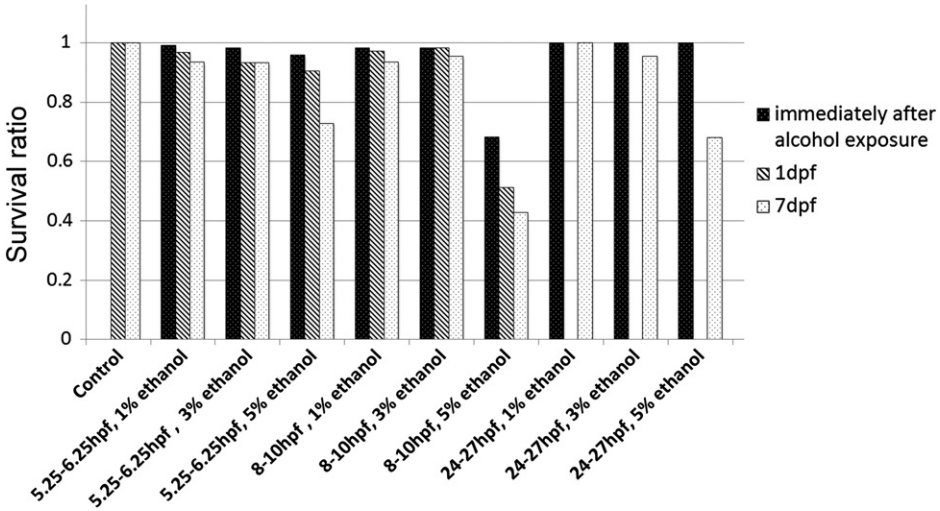

Fig. 2. Quantitation of embryo survival following ethanol exposure. Data are shown as percent survival and number of embryos surviving from the total number of embryos treated.