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Catching a conserved mechanism of ethanol teratogenicity

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Abstract

Due to its profound impact on human development, ethanol teratogenicity is a field of intense study. The complexity of variables that influence the outcomes of embryonic or prenatal ethanol exposure compels the use of animal models in which these variables can be isolated. Numerous model systems have been used in these studies. The zebrafish is a powerful model system, which has seen a recent increase in usage for ethanol studies. Those using zebrafish for alcohol studies often face two questions: 1) How physiologically relevant are the doses of ethanol administered to zebrafish embryos and 2) Will the mechanisms of ethanol teratogenesis be conserved to other model systems and human? The current manuscript by Flentke et al helps to shed important light on these questions and clearly demonstrates that the zebrafish will be a valuable model system with which to understand ethanol teratogenicity.

Prenatal alcohol exposure has long been suspected of having deleterious effects on human development. The most severe of these effects was formally recognized in the 1970s with the diagnosis of Fetal Alcohol Syndrome (FAS) (Jones and Smith, 1973). Since then, a wide body of research has clearly demonstrated that *in utero* exposure to ethanol can cause a widely variable set of developmental defects in humans collectively termed Fetal Alcohol Spectrum Disorders (FASD) (Riley et al., 2011). Understanding the underlying cause of these varied developmental defects has been a source of intensive study for many decades.

Many different factors have been suspected to contribute to ethanol's broad effects on the developing embryo and fetus. These factors include timing and dosage of ethanol administration, as well as genetic background (Ali et al., 2011; Cartwright and Smith, 1995; Riley et al., 2011; Sulik, 2005). The interrelationship between these factors is complex and makes understanding ethanol teratogenesis in humans difficult. This ultimately compels the use of animal models to examine the effects of ethanol on embryonic development.

Chicken, mouse and rat have long been amniote model systems used to study a wide range of ethanol-induced developmental defects. While proving invaluable in gaining an understanding into the wide range of ethanol's effect on embryonic outcomes, each of these model systems has limitations. First used in 1910 (Stockard, 1910), fish have only recently gained momentum as a valuable model system with which to understand ethanol teratogenicity. The zebrafish in particular is ideally suited for these types of studies. Many hundreds of optically clear eggs are laid externally, allowing for precise developmental

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timing of ethanol administration and detailed cellular analyses. There are many zebrafish transgenic lines that have been used to characterize the development of organ tissues relevant to FASD (Higashijima et al., 2000; Shin et al., 2003; Wada et al., 2005). Analyses in transgenic lines such as these will allow for the real-time analyses of cell behaviors disrupted by ethanol, these types of analyses, in particular, are not readily performed in any other vertebrate model system. Additionally, there is a wide array of available zebrafish mutants. For instance, the zebrafish mutation project aims to mutate every predicted gene in the genome and at the time of writing has identified mutations in 45% of predicted genes (https://www.sanger.ac.uk/Projects/D_rerio/zmp/). Additionally, genome editing via TALENs or CRISPRs is becoming more and more commonplace (Auer and Del Bene, 2014; Bedell et al., 2012), increasing the availability of resources with which to model the genetic susceptibility to ethanol teratogenicity. Despite these strengths, there have remained questions to how well the zebrafish embryo models human FASD.

As with all animal models the critical question is how well do the animal results predict the human condition. The components of all major developmental signaling pathways are strongly conserved throughout vertebrates, including zebrafish and human (Postlethwait, 2006; Woods et al., 2005). Because of this conservation, studies in zebrafish have proven to be extremely informative for mammalian development, including neural and craniofacial development, which are often disrupted in ethanol teratogenesis (Eberhart et al., 2008; Ingham, 2009; Knight and Schilling, 2006; Neuhauss et al., 1996; Piotrowski et al., 1996; Sager et al., 2010; Schilling et al., 1996; Whitfield, 2002).

Conservation of developmental pathways in normal development would suggest that the mechanisms of ethanol teratogenesis may be conserved across vertebrate species. Consistent with this, the Sonic Hedgehog pathway is implicated in ethanol teratogenesis in mouse, chicken and zebrafish (Ahlgren et al., 2002; Kietzman et al., 2014; Zhang et al., 2013). Recently, mutation of *platelet-derived growth factor receptor a* (*pdgfra*) has been shown to predispose zebrafish embryos to craniofacial defects (McCarthy et al., 2013). Analysis of a human datasets showed that, similarly, SNPs in *PDGFRA* associate with ethanol-induced alterations to facial measures (McCarthy et al., 2013). This conservation likely extends to other FASD phenotypes as recent studies in zebrafish have demonstrated ethanol exposed zebrafish embryos will develop learning deficits and neurochemical alterations (Fernandes et al., 2014; Mahabir et al., 2014). These phenotypes are similar to children with FASD (Mattson and Roebuck, 2002; Riikonen et al., 2005). Collectively, these findings strongly suggest that the developmental pathways governing ethanol teratogenesis are conserved across vertebrate species.

The current work by Flentke et al., demonstrate that this conservation in developmental pathways is likely to carry over to a precise mechanism of ethanol teratogenesis. In an avian model of FAS, the same authors demonstrated that blocking calcium transients protects neural crest cells from ethanol-induced apoptosis (Flentke et al., 2011). In this system, there was also a coincident depletion of nuclear beta catenin in neural crest progenitors within the dorsal neural folds. Beta catenin electroporation reduced the levels of ethanol-induced apoptosis. Additionally, beta catenin levels and Wnt signaling activity were restored in chicken embryos treated with ethanol and a calcium chelator. Collectively, these results

suggest that Calcium signaling, upstream of canonical Wnt signaling, mediates a portion of the neural crest response to ethanol.

In their most recent work, the same authors show that ethanol induces Ca-mediated cell death in zebrafish (Flentke et al., 2014). As observed in chicken, inhibiting Calcium-mediated signaling reduced the levels of ethanol-induced apoptosis. It remains to be seen if this calcium-mediated cell death impinges upon canonical Wnt signaling in zebrafish.

Some questions regarding the physiological relevance of work in zebrafish are based around the sometimes astonishingly high doses of ethanol used in some studies. Indeed, the studies using zebrafish embryos to examine ethanol teratogenesis have used many different ethanol treatment regimens. Timing and dosage of ethanol exposure widely varies, from 20 minutes to 2 weeks and .25% to 10% (42.75 to 1710 mM). Ethanol tissue concentrations have been reported in a subset of these studies and have used a variety of assay methods (Table 1). In these studies, a wide range of embryonic tissue concentrations have been reported, from .42% to 70% as a ratio of embryo to media concentration, using a variety of reporting methods. These different reporting measures make it difficult to directly compare tissue concentration levels between these studies. Therefore, the lack of a consensus on the tissue levels of ethanol in exposed zebrafish has hampered researchers desiring to use this species.

The present work solidifies a growing consensus that the zebrafish embryo contains approximately 24–37% of media ethanol concentrations (Flentke et al., 2014; Lovely et al., in review; Reimers et al., 2004; Zhang et al., 2013). While these studies use differing assays for reporting ethanol concentration, there is similarity in experimental design allowing for comparison. Collectively, these results demonstrate that ethanol treatment in the range of 1% to 1.5% is physiologically relevant and helps explain why such a steep gradient from the extracellular solution to the embryonic tissue is formed.

It is clear that the zebrafish embryo contains substantially less ethanol than a free diffusion model would predict. In contrast, work in rodent models has shown that tissue levels directly correlate to blood alcohol levels (Adalsteinsson et al., 2006; Flory et al., 2010; Quertemont et al., 2003) consistent with a free diffusion model. The kinetic analysis performed by Flentke et al. hints at a possible mechanism for this observed difference. These authors show a linear dose curve of ethanol across a wide range of ethanol and lasting for several hours. Due to the energy requirements, these kinetics are inconsistent with active transport or degradation and would seem to favor a passive transport model similar to that used in yeast. Given that fish and other aquatic species are more likely to encounter ethanol in the environment, it is likely that the Aquaglyceroporins present in zebrafish function similarly to those in yeast to remove ethanol.

This work, in combination with other recent work, demonstrates that studies in zebrafish are physiologically relevant and likely to translate to human. The unique characteristics of zebrafish that make it a powerful model for developmental biology also make it a powerful model organism for studies of ethanol teratogenesis. As the zebrafish model system becomes accepted in the FASD research community, we will all reap the benefits of a set of analyses that are not readily performed in other systems. Indeed, it is the combination of work across

model organisms, each with their own strengths, which will provide the deepest and most profound insights into FASD in human.

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Table 1

Study	Ethanol Dosage ^a	Number and Length of Washes ^b	Assay ^c	Embryonic Ethanol Concentration ^d
Diugos and Rabin, 2003	.25 – 1%	NA	Enzymatic	1–3 µg/mg [#]
Lockwood, <i>et al.</i> 2004	1.5 – 3%	1X for 30 sec	Enzymatic	25–71 mM
Reimers <i>et al.</i> 2004	1.98%	2X	Enzymatic	31–35%
Bradfield, <i>et al.</i> 2006	50 or 500 mM	3X	HGC/ ¹⁴ C	70%
Li, <i>et al.</i> 2007	.25 – 2%	NA	Enzymatic	.71–7.4 mM
Ali, <i>et al.</i> 2011	10%	1X for 30 sec	MRS	0.86%
Zhang, <i>et al.</i> 2013	.5 – 2%	1X – 3X	Enzymatic	32%
Flentke, <i>et al.</i> 2014	50 – 300 mM	1X	Analox	35%
Lovely, <i>et al.</i> In review	1%	1X	HGC	24–37%

^aReported as % v/v or mM concentration.

^bNumber and length of washes (where listed) to remove excess ethanol from the embryo surface before embryo processing.

^cAssays used to assess embryonic ethanol concentration. MRS – Magnetic Resonance Spec.; HGC – Headspace Gas Chromatography; ¹⁴C – ¹⁴Carbon Radiometry; Enzymatic – Ethanol assay kit measuring NADH production using Alcohol dehydrogenase; Analox – analyte analyzer.

^dReported as % v/v or mM concentration except – [#]Reported as µg ethanol/mg brain wet weight.