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Research report

Embryonic alcohol exposure impairs associative learning performance in adult zebrafish

Yohaan Fernandes [a], Steven Tran [a], Emil Abraham [b], Robert Gerlai [a,b,*]

[a] Department of Cell and System Biology, University of Toronto, Canada
[b] Department of Psychology, University of Toronto, Canada

HIGHLIGHTS

- Zebrafish embryos exposed to low alcohol concentrations were tested when adult.
- Embryonic alcohol exposed zebrafish exhibited no gross anatomical abnormalities.
- Associative learning performance of embryonic alcohol exposed fish was impaired.
- Motor function and food consumption of the alcohol exposed fish were unaffected.
- Even small amount of alcohol reaching the embryo leads to lasting cognitive deficit.

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ABSTRACT

The zebrafish has been proposed for modeling fetal alcohol spectrum disorders (FASD). Previous FASD research with zebrafish employed high concentrations of alcohol and/or long exposure periods. Here, we exposed zebrafish eggs to low doses of alcohol (0, 0.25, 0.50, 0.75 and 1.0% (vol/vol); external bath application of which 1/20th may reach the inside of the egg) at 16-h post-fertilization (hpf) and only for a short duration (2 h) in the hope to avoid gross morphological aberrations and to mimic the more frequent FASD exposure levels. Upon reaching adulthood the exposed and control zebrafish were tested for their associative learning performance in a plus-maze. Embryonic alcohol exposure led to no gross anatomical abnormalities and did not increase mortality. Unexposed (control) zebrafish showed excellent acquisition of association between a conditioned visual stimulus (CS) and food reward, demonstrated by their preference for the target zone of the maze that contained the CS during a probe trial in the absence of reward. However, alcohol-exposed fish showed no such preference and performed indistinguishable from random chance. Locomotor activity during training and the probe trial or the amount of food consumed during training did not differ between the embryonic alcohol exposed and unexposed (control) fish, suggesting that the impaired learning performance found was unlikely to be caused by altered motivation or motor function. Our results suggest that even very small amounts of alcohol reaching the embryo for only a short duration of time may have long lasting deleterious effects on cognitive function in vertebrates.

1. Introduction

Prenatal alcohol (ethyl alcohol, ethanol, EtOH) exposure produces a range of symptoms that are collectively labeled under the non-diagnostic umbrella term, fetal alcohol spectrum disorder (FASD) [1]. Fetal alcohol syndrome (FAS), a more specifically defined and severe form of FASD is characterized by craniofacial malformations, growth deficiencies as well as central nervous system (CNS) abnormalities including severe cognitive and/or behavioral impairment [2]. These CNS abnormalities include deficits in intelligence, executive functioning, language, visual-spatial ability, motor function, attention, activity, academic achievement, learning and memory [3]. The prevalence of FAS in the United States is 2.8 per 1000 live births [4]. Alcohol-related neurodevelopmental disorder, or ARND, is another clinical term that describes a symptom cluster associated with prenatal alcohol exposure. Individuals with ARND exhibit cognitive and behavioral problems but lack the physical malformations associated with FAS [5]. While FAS is the most severe outcome of prenatal alcohol expo-

* Corresponding author at: Department of Psychology, University of Toronto Mississauga, 3359 Mississauga Road North, Rm DV4023C, Mississauga, Ontario L5L 1C6, Canada. Tel.: +1 905 569 4255; fax: +1 905 569 4326.
E-mail address: robert_gerlai@yahoo.com (R. Gerlai).

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sure, ARND, which is thought to result from only moderate drinking by pregnant women, is more prevalent. Embryonic alcohol exposure induced problems are estimated to affect over 9 out of 1000 children [4,6] and the less severe cases that fall within the category of ARND represent more than two third of these cases. Thus elucidating the mechanisms that underlie the milder forms of fetal alcohol exposure induced problems (i.e. ARND) is of utmost importance. Animal models can significantly aid in this endeavor.

The zebrafish may be an ideal model organism with which the effects of embryonic alcohol exposure may be investigated [7]. One advantage of this species is the simplicity and precision of alcohol delivery. Since the chorion of the egg is somewhat permeable to alcohol [8], alcohol diffuses into the egg bathing the embryo. Thus the complexities associated with intra-uterine environment and maternal physiology inherent in mammalian animal models is absent in zebrafish. The timing of alcohol exposure is also precise. Eggs can be immersed and removed from the alcohol solution at will and thus the initiation and cessation of alcohol exposure can be precisely controlled [9]. A female zebrafish can produce 200 eggs in a single spawning [10] and the eggs are fertilized and develop externally. Thus large number of subjects may be exposed to alcohol treatment in a uniform manner, which is expected to reduce error variation and thus increase statistical power [9].

Behavioral changes caused by embryonic alcohol exposures have not been the primary focus of investigation using zebrafish. Instead, most studies analyzed gross anatomical aberrations and/or gene expression changes induced by high doses of, or extended exposure to alcohol. For example, Arenzana et al. treated zebrafish embryos for approximately 20 h with 0.5% or 2.4% (vol/vol) alcohol and found cyclopia (the fusion of 2 eyes) along with cytoarchitectural abnormalities [11]. Loucks & Ahlgren treated zebrafish embryos with alcohol concentrations ranging from 0.2% up to 2.5% (v/v) from 4.3 h post-fertilization (hpf) up to 24 hpf and reported embryonic alcohol exposure to lead to reduced expression of genes, e.g. that of *six3b* and *gli1*, notochord defects and ocular distance abnormalities [12]. After exposing zebrafish embryos to alcohol concentrations ranging from 0.5% to 2.0%, from 6 to 10 hpf, 10–18 hpf, 6–24 hpf, 24–48 hpf, or 48–72 hpf, Zhang et al. reported reduced gene expression in the forebrain and hindbrain of exposed fish [13]. While the analysis of changes induced by larger doses of and longer exposures to alcohol may illuminate mechanisms associated with the most severe forms of human FAS, animal models that recapitulate the changes seen in less severe forms of the disease (e.g. ARND) may be even more important as such cases are more prevalent in the clinic. Cognitive impairment is a characteristic feature of these milder forms of fetal alcohol exposure cases. The abnormalities, which are often life-long, include deficits in intelligence, executive functioning, language, visual-spatial ability, motor function, attention, activity, academic achievement, and learning and memory [3].

In the current study, we investigate, for the first time, the effects of a 2 h long exposure to lower bath concentrations of alcohol (0, 0.25, 0.50, 0.75 or 1%) at 16 h post fertilization. Notably, the concentration of alcohol inside the egg has been found to be approximately 1/20th–1/30th of that of the external bath when using the above dose range [14,15]. Previously, we have found that similar doses of alcohol employed at 24 hpf resulted in no gross anatomical changes or increased mortality and only led to subtle but significant behavioral abnormalities [14]. In the current study, we explore the effect of alcohol when delivered at 16 hpf.

The choice of this developmental stage (the 16th hpf age of the embryo) when alcohol is administered was somewhat arbitrary. Although zebrafish embryonic development (including the development of the brain) is well mapped, the question of how certain developmental stages of zebrafish correspond to human embryonic development is complex. The 16 hpf stage appeared reasonable for us as the time point of manipulation because at this stage the brain has started to form but has not finished developing. For example, the prominent subdivisions of the zebrafish brain, the telencephalon, diencephalon, midbrain and hindbrain have already developed by 16 hpf, and the neural crest migration in the head and trunk is underway [16].

We decided to focus our analysis of the adult zebrafish previously exposed to alcohol during their embryonic development on learning and memory performance to explore possible long lasting consequences of the exposure. We chose our focus to be learning and memory because a prevalent abnormality seen in ARND children has been cognitive deficits associated with impaired attention, learning and memory. For example, Mattson et al. found children with prenatal alcohol exposure to be able to recall fewer words using a word list memory task [17] and later also described both verbal and non-verbal memory impairment [18].

Zebrafish have been increasingly employed to study learning and memory [19]. Zebrafish are highly social and when offered the opportunity under experimental conditions have been found to be motivated to seek out and stay close to their conspecifics [20–23]. This feature of zebrafish has been utilized in learning studies in which the sight of conspecifics has been found to be a salient unconditioned stimulus, a strong motivator [24]. Unfortunately, however, the sight of conspecifics may not be appropriate as a motivator in the current study because previously we found embryonic alcohol exposure to induce significant impairments in the way the treated zebrafish respond to their conspecifics [14,25]. The alcohol treated fish showed reduced preference for staying in close proximity to conspecific images presented on a computer screen [14] or to their shoal mates presented using freely moving live shoals [25]. Although the behavioral mechanism of this impairment is not known, it is likely to represent abnormal social behavior.

To avoid the possible complications arising from this (altered motivation as a confound in our learning study), we decided to train our zebrafish in an associative learning task in which the conditioned stimulus (a visual cue) would be paired with a rewarding stimulus other than the sight of conspecifics. We chose food to be the reinforcement. Zebrafish have been found to be able to learn the association between a visual cue and food reward in the plus maze before but food reward was found to diminish in reward value as the training progressed, presumably because the poikilothermic zebrafish satiated fast [26]. In the present work we employ a similar plus-maze but utilize a modified food reward and modified delivery method that increases delivery efficiency and rewarding value, and also makes the visual cue more salient (see below).

We investigate the effect of embryonic alcohol exposure on the behavior of adult zebrafish in this food rewarded associative learning task using the plus maze and report significant learning performance deficits without alterations in amount of food consumed or in locomotor activity.

2. Methods

2.1. Animals and housing

Sexually mature adult zebrafish of the AB strain were bred at the University of Toronto Missisauga Vivarium (Mississauga, Ontario, Canada) to obtain fertilized eggs. The progenitors of this population were obtained from the ZFIN Center (Eugene, Oregon, USA). AB is one of the most frequently studied zebrafish strains which is often used in forward genetic (mutagenesis) studies [27]. Approximately 240 fertilized eggs were collected 2 h post-fertilization (hpf) and washed with system water; deionized and sterile water supplemented with 60 mg/l Instant Ocean Sea Salt (Big Al's Pet Store, Mississauga, ON, Canada). At 16 hpf, collected eggs were randomly divided into 5 equal groups, with approximately 40–50 eggs per group.

The low alcohol concentration and short exposure time were chosen to model the low levels and small frequency of drinking more commonly seen during pregnancy that are associated with ARND. Eggs were placed in a container with 100 ml of solution of the corresponding alcohol concentration (0, 0.25, 0.50, 0.75, or 1.00 vol/vol%) for 2 h. The eggs were subsequently washed with system water and