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Embryonic Exposure to Ethanol Increases Anxiety-Like Behavior in Fry Zebrafish

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Abstract

Aims: Fetal alcohol spectrum disorder (FASD) is an umbrella term to describe the effects of ethanol (Eth) exposure during embryonic development, including several conditions from malformation to cognitive deficits. Zebrafish (*Danio rerio*) are a translational model popularly applied in brain disorders and drug screening studies due to its genetic and physiology homology to humans added to its transparent eggs and fast development. In this study, we investigated how early ethanol exposure affects zebrafish behavior during the initial growth phase.
Methods: Fish eggs were exposed to 0.0 (control), 0.25 and 0.5% ethanol at 24 h post-fertilization. Later, fry zebrafish (10 days old) were tested in a novel tank task and an inhibitory avoidance protocol to inquire about morphology and behavioral alterations.
Results: Analysis of variance showed that ethanol doses of 0.25 and 0.5% do not cause morphological malformations and did not impair associative learning but increased anxiety-like behavior responses and lower exploratory behavior when compared to the control.
Conclusion: Our results demonstrate that one can detect behavioral abnormalities in the zebrafish induced by embryonic ethanol as early as 10 days post-fertilization and that alcohol increases anxious behavior during young development in zebrafish.

INTRODUCTION

Alcohol is a legalized drug of high social acceptance worldwide. This psychotropic substance is, by far, the most widely used drug in the world, which outweighs alcohol consumption and surpasses all illicit drugs together (Lima, 2003; Grinfeld, 2009; Guerri and Pascual, 2010). Although the higher incidence of addictive behavior are the major contributors to global indices of morbidity and premature death (Lim *et al.*, 2012; Gowing *et al.*, 2015), the trade of alcoholic beverages brings high profitability for the economy, favoring the often encouraged consumption (de Melo Freire *et al.*, 2005; World Health Organization, 2019).

One of the biggest concerns about alcoholic beverages intake is its consumption by pregnant women. The National Survey on Drug Use and Health shows that about 20% of women reported the use of alcohol during pregnancy and postpartum (Tebeka *et al.*, 2020), an increase of more than 10% in data for the past 10 years (Abuse, 2007). Ethanol exposure during embryonic developmental phase can cause a range of abnormalities, which includes long-lasting physiological and behavioral alterations with significative effects for the developing fetus and through child lifespan (Sadrian *et al.*, 2013; Gil-Mohapel *et al.*, 2019). The group of conditions that covers the deficits caused by maternal alcohol ingestion is known as fetal alcohol spectrum disorders (FASD). Still, the variability of symptoms resulting from FASD comes from different factors as the amount of alcohol intake, frequency of the alcohol ingestion and phase of the gestational period when exposure occurred (Guerri *et al.*, 2009).

Prenatal alcohol exposure is a public health concern. The placenta does not have the physiological capacity to metabolize ethanol as

the liver does, and the drug chemical structure favors a fast diffusion across biological membranes being delivered directly to the amniotic fluid and the fetus (Heller and Burd, 2014). Within 1 h of drug exposure, amniotic fluid and fetal blood reach equivalent ethanol levels to the maternal blood (Idänpään-Heikkilä *et al*., 1972; Burd *et al*., 2007). The fetal liver does not have an effective ethanol metabolizing system yet; it has been shown that the primary mechanism for ethanol metabolism is catalase and not alcohol dehydrogenase (Tran *et al*., 2007). The slow process leads to the accumulation of toxic substances for child development. The final consequences may be intrauterine growth retardation, delayed development, congenital malformations, besides other aspects that place FASD among the current most common cause of mental and behavioral deficits (Warren *et al*., 2011; Sadrian *et al*., 2013).

For the developing neural system, ethanol exposure affects several molecular mechanisms, such as alterations in the regulation of gene expression (Rifas *et al*., 1997; Guerri *et al*., 2009; Kalisch-Smith *et al*., 2016), interference in neural stem cell migration and differentiation (Miller, 2006; Mooney *et al*., 2006; Muralidharan *et al*., 2016; Kashem *et al*., 2018) and alterations in critical functions of glia (Guerri and Renau-Piqueras, 1997; Guerri *et al*., 2009). Studies also found alcohol inducing microcephaly, synaptogenesis alterations and neuronal cell loss (Guerri and Renau-Piqueras, 1997; Tenkova *et al*., 2003; da Silva *et al*., 2018; Rah *et al*., 2019; West *et al*., 2019). These effects are ultimately related to the child behavior after birth, who present several levels of cognitive deficits and behavioral issues, like attention deficit, reflex motor control impairment, hyperactivity, visuospatial abilities and deficits in fine and gross motor control (Carvan *et al*., 2004; Wozniak *et al*., 2004, 2017; Kalberg *et al*., 2006; Mattson *et al*., 2006; Popović *et al*., 2006; Shan *et al*., 2015; Carter *et al*., 2016). Although many studies have focused on effects of premature alcohol exposure, most of them focus on high doses of intoxication because it is usually detected earlier. The less prominent cases, in which the mothers were not aware of the pregnancy or do not admit a small amount of alcohol use, are more challenging to approach and many fewer extreme cases of FASD are not even identified. In this sense, studies with moderate ethanol effects are still needed to bridge the gap on those superficial responses that may go unnoticed throughout the child's growth or even treated as another type of disability (Marquardt and Brigman, 2016; Inkelis and Thomas, 2018; Agnihotri *et al*., 2019). For instance, children with attention deficits and severe anxiety are usually not related to FASD and treated as other mental disorders because of the poor behavioral diagnosis for ethanol-exposed children (American Psychiatric Association, 2000; Glass *et al*., 2013).

One of the challenges of analyzing neurodevelopmental abnormalities in vertebrates as animal models for human diseases is that rodents—the most common scientific model—like other mammals, develop inside the uterus. Besides the fact that it is not possible to follow immediate drug effects, ethanol concentrations used and exposure time are difficult to determine as the mother's metabolic functions must be considered. Therefore, vertebrates with external fertilization have gained field (Nakatsuji, 1983; Peng *et al*., 2004; Matsui *et al*., 2006; Marrs *et al*., 2010; McClure *et al*., 2011; Fainsod and Kot-Leibovich, 2017) and the zebrafish appeared as an ideal model for FASD studies (Mahabir *et al*., 2014; Shan *et al*., 2015; Baggio *et al*., 2018). Comparisons between human, rodents and zebrafish show similar craniofacial anomalies after high concentration ethanol exposure on developmental phase, that is small eyes, smaller head and malformed body structures (Warren *et al*., 2011; Murawski *et al*., 2015), as seen in fetal alcohol syndrome, the most serious cases of FASD. In this sense, less severe FASD may take advantage of the zebrafish model, and behavioral indicators of low alcohol exposure could be screened for future use in diagnosis.

Zebrafish has been very well applied for studies in learning tasks (Karnik and Gerlai, 2012; Roberts *et al*., 2013; Manuel *et al*., 2014; May *et al*., 2016; Roy and Bhat, 2016), including in our own research group (Chacon and Luchiari, 2014; Oliveira *et al*., 2015; Amorim *et al*., 2017). Here, we sought to investigate the effects of ethanol exposure on zebrafish behavior, first performing a behavioral screening through a Novel Tank Task, followed by an Inhibitory Avoidance Test. Quick reactions to noxious stimuli in zebrafish are one of the instinctive abilities crucial to survival, mainly because these animals do not have parental care, which demands precocious aversive learning to guarantee the physical integrity and to avoid predators. Thus, we hypothesized that embryonic exposure to moderate ethanol concentration does not affect morphology but hinder learning and lead to behavioral deficits that can be observed during the initial growth phase in zebrafish.

MATERIALS AND METHODS

Animals housing and ethanol exposure

Adult male and female zebrafish (*Danio rerio*, wild-type, 6 months old, 0.58 ± 0.11 g) were bred following a previously established protocol (Westerfield, 2007) to obtain zebrafish embryos for ethanol exposure. Two females and one male were set up in breeding tanks (15 × 20 × 10 cm tank with a removable tray with holes that allow eggs to fall through) and left overnight for spawning on the first hour of light in the next morning. Eggs were collected, counted and maintained in Petri dishes with system water (water from the system where adult fish were held) at 28°C till 24 h post-fertilization (hpf). This time point has been previously chosen for embryo exposure to ethanol (Bilotta *et al*., 2004; Mahabir *et al*., 2014; Bailey *et al*., 2015; Baggio *et al*., 2018) because it matches the period when the brain development take place and synapses start to be functional (Kimmel *et al*., 1990). This stage equals the end of the first gestational trimester in humans (Fernandes *et al*., 2015), a critical phase that may negatively affect neuronal morphology. At the given time, eggs were submerged in ethanol solution at three different concentrations for 2 h: Eth 0% (control), Eth 0.25% and Eth 0.5%. Ethanol was previously prepared by diluting absolute ethyl ethanol 99.8% PA (Dynamics, Contemporary Chemistry Ltd.) in system water. Fernandes and Gerlai (2009) previously measured ethanol concentration within the egg after 2 h of exposure, founding that levels reach the embryo with about 1/25 to 1/30 of the alcohol concentration employed. The concentrations were chosen because they do not cause gross morphological deformations (Fernandes and Gerlai, 2009; Buske and Gerlai, 2011), since this is not the objective of this work, while higher concentrations were found to cause teratogenic effects in zebrafish (Arenzana *et al*., 2006). After the exposure period, embryos were washed twice in clean system water for 20 min. Then, eggs were placed to grown in batches from 20 to 30, in 1 L static water until fish completed 10 days (stage named as fry, it occurs between the larva and juvenile phases) and were tested. Adults and embryos were kept in the same room under controlled temperature, pH, oxygenation (28°C, pH ~ 6.7, O2 ~ 6 mg/L) and photoperiod set at 12 h light/12 h dark. The feeding regime started at 5dpf with mixed *Artemia salina* nauplii and dry food (Alcon Alevinos, 44% protein and 5% fat). All animals used in the following tests were bred, raised and housed in the same environment.

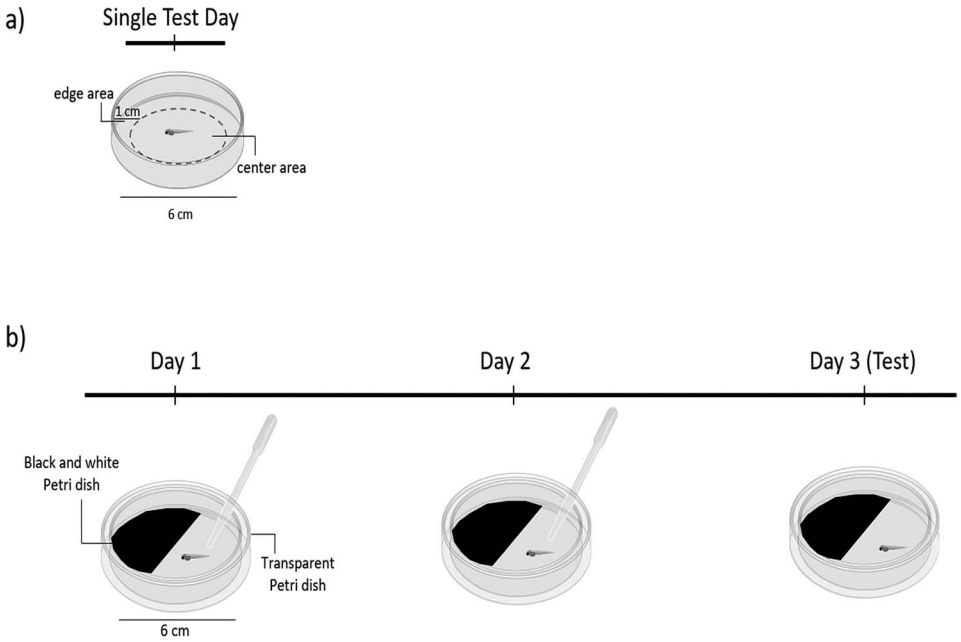

Fig. 1. Schematic outline of the experimental procedure for behavioral tests. (**a**) Novel Tank Test—animals ($n = 54$) were tested in a 6 cm arena, for 10 min. Time on edge area, center area and latency to cross areas were analyzed. (**b**) Inhibitory avoidance—Fish fry ($n = 45$) were tested in a 6 cm shuttle dish and the aversive stimulus consisted in water flow in the fishtail. The protocol consisted of 2 days of training followed by the test day (Day 3). Time on black or white side and latency to cross areas were analyzed. For both tasks, the distance to the center, movement speed, immobility time and total distance traveled were also consider.

The Animal Ethics Committee of the Federal University of Rio Grande do Norte has approved this study (CEUA 007/2016).

Behavioral tests

Novel tank The novel tank test is a fish adapted version of the Open Field Test, commonly used protocol to obtain behavioral data related to animal locomotion and anxiety. This kind of task help to identify pharmacological effects of drugs applied to various species as rodents (Karl *et al.*, 2003; Deacon, 2006; Gould *et al.*, 2009), primates (Ferguson and Bowman, 1990; Dal-Pan *et al.*, 2011) and recently fish (Champagne *et al.*, 2010; Rosemberg *et al.*, 2011; Stewart *et al.*, 2012; Collier *et al.*, 2017; Baker *et al.*, 2018). When previously applied for zebrafish, the novel tank protocol disclosed similarities between rodents and zebrafish regarding strategies adopted in the exploration of the novel environment. For example, zebrafish and rodents exhibit thigmotaxis (Lamprea *et al.*, 2008; Blaser *et al.*, 2010) and habituation responses (change in behavior as the exploration of new environment takes place) (Bolivar *et al.*, 2000; Wong *et al.*, 2010). At 10dpf, zebrafish present functioning sensory and motor systems; thus, a novel tank test can be applied to evaluate previous exposure to the mentioned ethanol doses.

The protocol consisted of an arena (6 cm petri dishes, containing system water) divided into two areas virtually determined: edge and center (see Fig. 1a for details). Animals were individually placed in the center of the arena, and behavior was recorded for 10 min. During this period, the researcher was outside the testing room to avoid any disturbances. The arena was washed, and the system water replaced at the end of each trial. A total of 18 fry zebrafish was tested for each ethanol treatment ($n = 54$). All sessions were recorded from above using a webcam, and behavior was analyzed in tracking software.

Inhibitory avoidance The inhibitory avoidance task is also a known protocol, based on conserved mechanisms, widely used as a tool to study fundamental mechanisms of animals learning and memory formation, as well as screen for drugs that may affect these mechanisms (Roozendaal and McGaugh, 1996; Quevedo *et al.*, 1999; Machado *et al.*, 2009). For zebrafish, this has been a standard test to study aversive responses (Blank *et al.*, 2009; Gorissen *et al.*, 2015; Amorim *et al.*, 2017). However, there are no references to this protocol applied to 10dpf zebrafish, even though it has been previously shown that zebrafish larvae have excellent performance in short- and long-term memory, associative and also social learning (Roberts *et al.*, 2013).

Here, the half-white and half-black tanks used to inhibitory avoidance in adult fish were adapted to fit in 6 cm diameter Petri dishes. The arena had no physical dividers but presented white and black bottom as visual cues (Fig. 1b). First, individual fry fish were transferred to a transparent Petri dish for 5-min habituation to minimize any disturbance related to handling and novel tank stress. After that, the transparent dish was placed on a black and white background, and the fish was always positioned to the black side (non-preferred side at this stage) (Kalueff *et al.*, 2013; Bai *et al.*, 2016). As an aversive stimulus, fish received a water flow (0.2 mL of system water, applied with a Pasteur pipette) close to its tail every time it entered the white side of the dish. Previous studies have shown zebrafish responding with an escape behavior to water flow, the same way as it reacts to a predator strike (McHenry *et al.*, 2009; Stewart *et al.*, 2013).

The avoidance learning protocol lasted 3 days, with a 5-min trial per day. On Day 1, fry were individually placed at the black side of the dish, and as soon as it crosses to the white side, it received a water flow close to its tail, making fry respond in a startle reaction. Animals that did not cross to the white side during the first 5 min were discarded. On the next day (Day 2), the procedure was repeated: fish fry was placed on the black side of the dish and had 1 min to cross to the other side, where it was stimulated with the water flow. If it did not happen, the transparent Petri dish was slowly rotated until

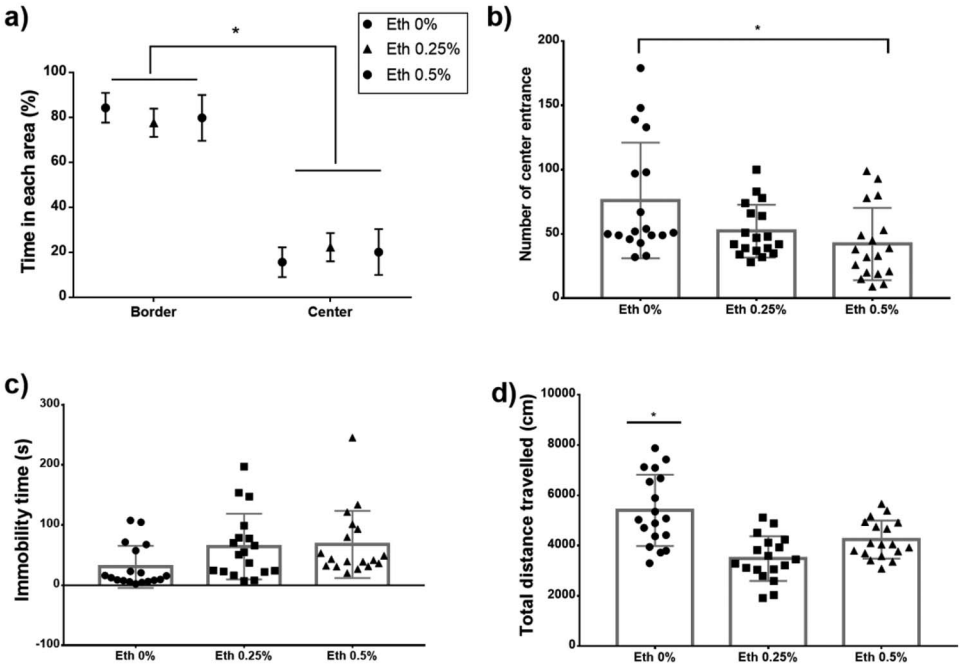

Fig. 2. Behavioral parameters for fry zebrafish at novel tank test. (**a**) Time spent in each area of the arena, (**b**) number of entrances at each area, (**c**) immobility time, (**d**) total distance traveled. Animals were submitted to 0 (control), 0.25 or 0.5% of ethanol at 24hpf and tested at 10dpf. Data are expressed as mean ± SEM; in all cases $P < 0.05$.

the fish was on the white side, and then it received the water flow as if it had crossed sides. On the day after, the test trial took place. During the test, fry fish were placed on the black side of the dish and behavior was recorded from above for 5 min. No water flow was applied, and fish was expected to avoid the white side due to association with punishment. A total of 15 fry zebrafish was tested for each ethanol treatment ($n = 45$). The behavior was analyzed in tracking software.

Behavioral analysis and statistics Behavioral records were stored as AVI files, and behavior was tracked using Zebtrack software (Pinheiro-da-Silva *et al.*, 2017), developed in MATLAB (R2014a, Math Works, Natick, MA). For Novel Tank test, the parameters considered for analyses were time spent in *edge and center* of the arena, latency to reach the edge area, immobility time, total distance traveled, movement speed [= total time in movement − immobility time/total distance traveled], number of entrance center and distance to the center of the arena. For the inhibitory avoidance test, the parameters considered for analyses were time spent in black and white sides of the arena, latency to cross areas, immobility time, total distance traveled, movement speed, and the number of entrances in each side of the arena.

Data were analyzed for normality using the D'Agostino—Pearson normality test. For Novel Tank, time in each side of the arena was analyzed using two-way analysis of variance (ANOVA) considering as factors ethanol exposure (0, 0.25 and 0.5%) and side of the arena (edge and center), followed by Tukey's multiple comparison test when needed. For the inhibitory avoidance, data on the time in black and in white areas were analyzed using two-way ANOVA considering as factors: ethanol exposure (0, 0.25, and 0.5%) and testing day (first and last). For all other behavioral parameters, one-way ANOVA was applied, followed by Tukey's multiple comparison test when needed. All analyses were performed using GraphPad Prism 7.0, error rate (alpha) set to 0.05 in all cases.

RESULTS

Novel tank

Figure 2 presents the behavioral results from the Novel Tank Task for fry fish exposed to ethanol at the embryo phase. Two-way ANOVA showed no effects of ethanol concentration [$F(2, 34) = 0.22$; $P = 0.80$], but significant effect of the side of the arena [$F(1, 17) = 166.8$; $P < 0.0001$] (Fig. 2a). The interaction terms ethanol concentration vs. side of the arena were non-significant [$F(2, 34) = 0.91$; $P = 0.40$]. Tukey's *post hoc* test showed that all groups remained more time closer to the edge than in the center area during the 10-min task (Fig. 2a).

We also analyzed the distance to the arena's center and how many times the fish crossed from edge to center area, that is, the number of center entrances. The ethanol-treated groups presented no statistical significance for the distance to the center of the arena [one-way ANOVA: $F(2, 51) = 0.99$, $P = 0.37$]. However, one-way ANOVA showed statistical significance for the entrances to the center of the arena [one-way ANOVA: $F(2, 51) = 5.05$; $P = 0.01$]. Tukey's test indicated that Eth 0% group showed more entrances to the center than Eth 0.5% ($P < 0.05$) (Fig. 2b). The time fish remained immobile during the task was also evaluated by one-way ANOVA, although presenting a marginal value and increased data dispersion for the treated groups, the test showed no significance between treatments [$F(2, 51) = 3.11$; $P = 0.053$] (Fig. 2c). Besides, we analyzed the fish mean speed while moving, named here as movement speed. This parameter showed a similar pattern between the three groups, and there was no statistical significance between them [$F(2, 51) = 1.55$; $P = 0.22$]. Regarding exploration ability, one-way ANOVA showed statistical significance on total distance traveled between groups

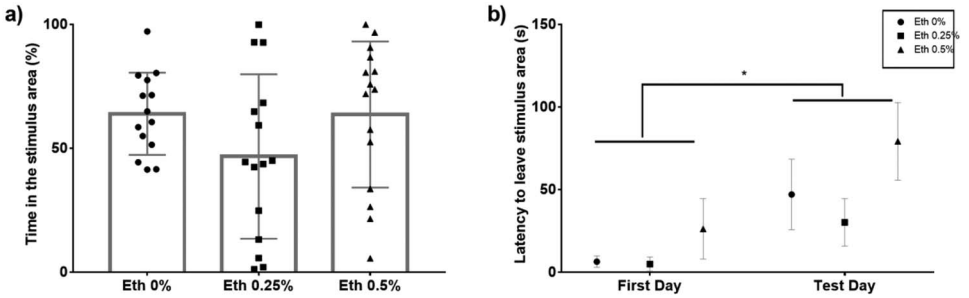

Fig. 3. Locomotor behavior of zebrafish at Inhibitory Avoidance test. During the test, animals did not receive any punishment, but learning was evaluated by (**a**) time spent in the stimulus side (white area) and (**b**) latency to cross areas at first x last day. Values are means ± SEM. (*) $P \leq 0.05$.

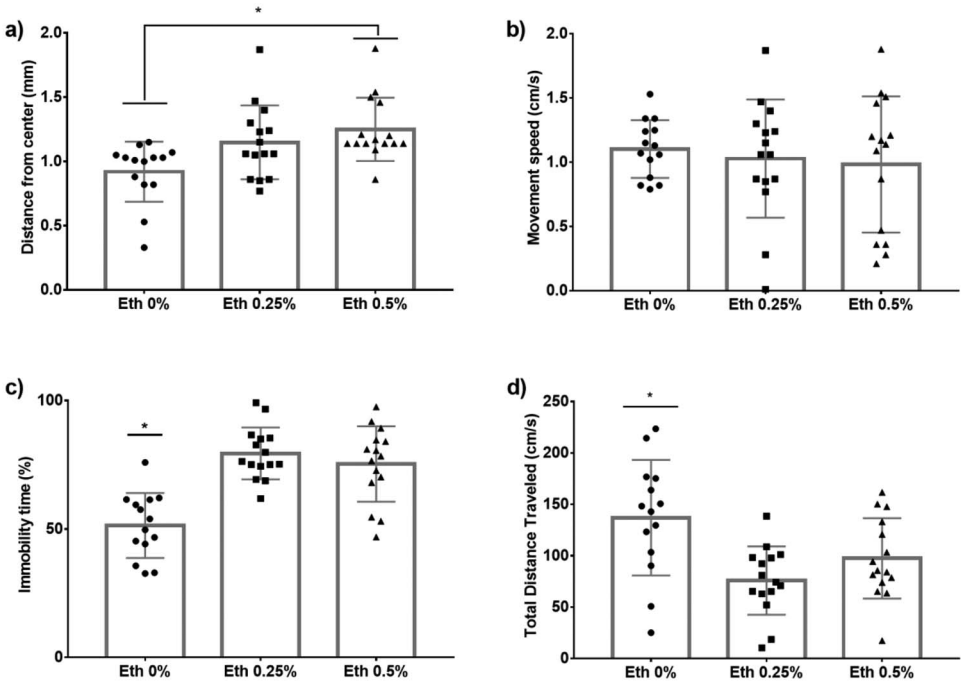

Fig. 4. Behavioral parameters for fry zebrafish at Inhibitory Avoidance test. (**a**) Distance from arena center, (**b**) movement speed, (**c**) immobility time, (**d**) total distance traveled. Animals were submitted to 0 (control), 0.25 or 0.5% of ethanol at 24hpf and tested at 10dpf. Data are expressed as mean ± SEM; in all cases $P < 0.05$.

[$F(2, 51) = 14.96$; $P < 0.0001$], and Tukey's test indicated that the control group presented higher total distance traveled than Eth 0.25% and Eth 0.5% groups ($P < 0.05$) (Fig. 2d).

Inhibitory avoidance

Figure 3 depicts the behavioral parameters analyzed for the inhibitory avoidance test. One-way ANOVA showed no statistical significance in time spent in each area by fish fry [$F(2, 41) = 1.89$; $P = 0.16$] (Fig. 3a). The latency to leave the black side (stimulus area) for the three groups is shown in Fig. 3b. One-way ANOVA showed no statistical significance between treatments in any of the days [$F(2, 41) = 3.06$; $P = 0.057$]. However, there was a statistical significance in latency to leave the black area between the first day and test day [RM ANOVA: $F(1, 41) = 7.67$; $P = 0.008$].

Other behavioral parameters were tested to evaluate the animal's performance and exploration after the aversive stimulus and are shown in Figure 4. One-way ANOVA indicated a statistical significance for the distance to the arena center between groups [$F(2, 41) = 0.37$; $P = 0.004$], and the Tukey's test showed that Eth 0% group differed from Eth 0.5% ($P < 0.05$) (Fig. 4a). There was no statistical significance regarding the movement speed (one-way ANOVA: $F(2, 41) = 0.28$; $P = 0.75$] (Fig. 4b). For immobility time, one-way ANOVA showed a statistical significance between groups [$F(2, 41) = 20.69$; $P < 0.0001$], and the Tukey's test indicated that Eth 0% showed reduced immobility compared to the other groups (Fig. 4c). For total distance traveled, one-way ANOVA showed a statistical significance between groups [$F(2, 41) = 7.29$; $P = 0.002$], and the Tukey's test showed that Eth 0% group traveled higher distance than the other groups (Fig. 4d).

DISCUSSION

In this study, we tested if embryonic ethanol exposure effects on behavior can be observed at developmental stages as early as 10 days post-fertilization (fry stage) in zebrafish. We tested moderate ethanol

concentrations, which do not cause morphological malformations (0.25 and 0.5% ethanol) and used the novel tank and the inhibitory avoidance test to evaluate anxiety-like behavior and aversive associative learning. We observed that moderate ethanol exposure during embryogenesis leads to increased anxiety and decreased locomotion in fry, while it does not affect learning to avoid an aversive stimulus.

The low to moderate embryonic ethanol exposure effects in humans are challenging to be diagnosed because it is usually associated with other causes. When malformation is combined with behavioral issues, the whole picture is brighter, and treatments can be started sooner. However, dealing exclusively with behavior will demand a more detailed and more profound approach. Several studies on FASD focus on the most critical cases of FAS, which are well described in the literature (Eason *et al*., 2017; Popova *et al*., 2017; Wilhoit *et al*., 2017; Montgomery *et al*., 2018; Rohac *et al*., 2019). Other studies evaluate FASD in young adults or adults, missing the important interval of development in which different treatments and therapies could be applied to alleviate and smooth the effect of the drug on the behavioral repertory (Brintnell *et al*., 2011; Denny *et al*., 2017).

In the present study, we tested zebrafish behavior at 10dpf in search for behavioral indicators of embryonic ethanol exposure. The increased anxiety-like behavior and decreased locomotion observed in fry previously exposed to 0.25 and 0.5% ethanol depict the signs of behavioral change that can be observed early (Fig. 2). In zebrafish, thigmotaxis is a behavioral measure for the tendency that an individual has to remain close to the walls, as opposed to actively exploring the environment during a test (Champagne *et al*., 2010; Richendrfer *et al*., 2012; Best *et al*., 2017). We observed that all groups (Eth 0%, 0.25% and 0.5%) spent more time close to the edges of the arena in the novel tank test, a result that is expected as a reaction to novelty (Wong *et al*., 2010; Stewart *et al*., 2012). However, both groups treated with ethanol showed reduced locomotion and reduced exploration of the center of the arena (Fig. 2b and d), behaviors that indicate high levels of anxiety along with the test. The exploratory behaviors help in anti-predation and foraging abilities, being considered an important feature, especially during developmental phases in zebrafish (Adriaenssens and Johnsson, 2013; Baker *et al*., 2018). The diminished exploratory activity induced by ethanol treatment replicates previous results described by Baggio *et al*. (2018) in adult FASD zebrafish. Other authors have also reported embryonic ethanol exposure led to higher anxiety levels both in larvae zebrafish (Ramlan *et al*., 2017; Abozaid *et al*., 2020) and juvenile rats (Brolese *et al*., 2014; Diaz *et al*., 2016; Ramlan *et al*., 2017; Rouzer *et al*., 2017; Balaszczuk *et al*., 2019). In the present study, we corroborate with cited embryonic ethanol effects showing that behavioral responses can be observed during early stages (10dpf), what contributes to the syndrome thorough understanding and suggests that the attentive look at the first phases of growth may help to diagnose and initiate early treatment for FASD cases.

Other behavioral parameters observed herein, as reduced total distance traveled and increased immobility time (Figs 2 and 4), are also indicative of anxiety, while embryonic ethanol exposure did not impair locomotion capacity (measured by the movement speed). Acute and chronic ethanol exposure has previously been found to affect freezing and exploratory responses in adults (Rosemberg *et al*., 2012; Pannia *et al*., 2014; Amorim *et al*., 2017) and larvae zebrafish (Baiamonte *et al*., 2015). However, the anxiolytic effects of embryonic ethanol exposure can jeopardize the healthy development of the nervous system (Eckardt *et al*., 1998; Ramlan *et al*., 2017; Gil-Mohapel *et al*., 2019) leading to the disturbing anxious reaction that we showed in this study and was also observed in adult FASD zebrafish (Seguin *et al*., 2016; Baggio *et al*., 2018). While anxiety is an important feature to prevent one from being extra exposed to risk and prepare itself for several stressful situations, increased levels of anxiety may affect individual performance (Jesuthasan, 2012). In recent years, the increasing cases of mental disorders, particularly the ones associated with anxiety, have a serious negative impact on individuals and society (Wittchen and Jacobi, 2005). To date, we are not aware of any study that has investigated ethanol anxiolytic effects during development using novel tank test in fry zebrafish. Still, Lockwood *et al*. (2004) obtained similar results for acute ethanol exposure inducing thigmotaxis behavior when they tested 7dpf larvae. Currently, thigmotaxis is a well-known anxiety indicator (Kalueff *et al*., 2013) used in many studies. Based on our results on zebrafish from the early developmental stage (10dpf) compiled with data investigation of adult zebrafish behavior (Rico *et al*., 2007; Rosemberg *et al*., 2012), it is very likely that embryonic ethanol exposure causes increased anxiety that is expressed throughout the ontogenesis. However, further studies, as measures of stress levels, are needed to confirm whether exposure to embryonic ethanol has doubtless affected anxiety-like behavior.

The neurochemical changes caused by excessive anxiety are not fully understood. However, common brain areas are involved in learning and memory, as well as in behavioral and physiological responses to fear and anxiety. In this sense, anxiety has been found context dependent by many authors (Luca and Gerlai, 2012; Parker *et al*., 2014; Baiamonte *et al*., 2015; Seguin *et al*., 2016; Baggio *et al*., 2018). Thus, we tested whether the anxiolytic response caused by embryonic ethanol exposure could interfere with the learning of a fear-related task. A growing number of studies have shown that embryonic ethanol exposure impairs learning (Hamilton *et al*., 2003; Lee *et al*., 2009; Norman *et al*., 2009; Fan *et al*., 2016). However, the harmful effects seem to depend on ethanol exposure concentration, time length and embryo developmental stage (May *et al*., 2013; Veazey *et al*., 2015; Squeglia and Gray, 2016; Facciol *et al*., 2017; Fernandes *et al*., 2019).

In the inhibitory avoidance test used in this study, fry zebrafish spent more time on the non-preferred side of the arena irrespective of ethanol treatment, indicating associative learning of the arena background to the negative stimulus (Fig. 3). Fish from all treatments also showed increased latency to cross the areas during the test. These results suggest that avoidance learning was not affected by early ethanol exposure. Increased inhibitory avoidance behavior is indicative of associative skills, corroborating with results for past publications using black/white and light/dark preference (Blaser and Rosemberg, 2012; Dahlén *et al*., 2019) and electroshock stimulus (Manuel *et al*., 2014; Amorim *et al*., 2017). However, opposite to other studies, the ethanol concentration applied here was not enough to change animal learning ability (Obernier *et al*., 2002; Brady *et al*., 2012; Gomez *et al*., 2013; Pittman and Lott, 2014). All those studies have in common the use of a higher dose of ethanol or binge drink protocol model, frequently exposing the animals to the drug.

Although animals showed to associate the aversive stimulus to a context (black or white side), we must consider the nature of the stimuli applied in our study. For 10dpf zebrafish, the water flow is a strong and aversive signal that elicits a startle reaction, a reflex that serves to the protection and facilitates escape response. The water flow has been mentioned as an effective aversive stimulus in other studies, once the lateral line system could originate an escape response since very early in life (McHenry *et al*., 2009; Liao, 2010; Stewart *et al*., 2013). Thus, we speculate that such a severe stimulus

triggers instinctive and necessaire reaction to preserve the integrity of the animal and that its associative capacity is maintained even after ethanol exposure. However, zebrafish subjected to the inhibitory avoidance test was not tested again for anxiety and we cannot discard the effects of consecutively stressing the animal with the water flow to its behavior repertory. It is known that contexts in which one experience punishment can be associated with the negative experience and unleash behavioral changes related to anxiety in eventual context exposure (Roeckner *et al*., 2017). Future studies should approach the forthcoming effects of adverse experiences during the early developmental phase in FASD individuals. It would serve to understand better how embryonic ethanol exposure and adverse experiences mold an individual's life later.

In the present study, we used simple tasks (novel tank and inhibitory avoidance test) to approach moderate ethanol exposure during embryonic development. Although we did not observe harmful effects on learning, it is also important to point out that the FASD range is substantially varied, especially in cases of embryo exposure to moderate ethanol concentration. Although the effects on organisms are not severe, it changes different behavioral parameters indirectly related to cognition (Marrs *et al*., 2010; Brady *et al*., 2012; Muralidharan *et al*., 2015; Roozen *et al*., 2016). Conduct disorders are known to be related to embryonic ethanol exposure, for example, autistic spectrum, depression and social difficulties (Caldwell *et al*., 2008; Sadrian *et al*., 2013; Parker *et al*., 2014; Tsang *et al*., 2016; Cananzi and Mayhan, 2017; Baggio *et al*., 2018; Roozen *et al*., 2018). In this study, the increased anxiety-like behavior observed in animals exposed to 0.25 and 0.5% ethanol indicates such effects. More than that, due to the difficulty in diagnosing mild cases of FASD, the early monitoring and identification of altered behavior such as increased anxiety are beneficial. Our study shows that FASD signals can be observed in age as early as 10dpf in zebrafish and reinforces the need to investigate mild cases of ethanol exposure during fetal development, encompassing different doses of ethanol and also behavioral and cognitive testing at longer time intervals to reach as many time points as possible in order to build a bigger picture of the ethanol consequences. In this regard, new and specific therapies need to be devised for specific interventions, as is the case of anxiety disorders related to FASD. Thus, the sooner it is identified and diagnosed, the sooner it could be treated.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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