HHS Public Access

Author manuscript

Reprod Toxicol. Author manuscript; available in PMC 2021 November 12.

Published in final edited form as:

Reprod Toxicol. 2020 September ; 96: 249–257. doi:10.1016/j.reprotox.2020.07.013.

Folic acid reduces the ethanol-induced morphological and behavioral defects in embryonic and larval zebrafish (*Danio rerio*) as a model for fetal alcohol spectrum disorder (FASD)

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Abstract

The objective of this work was to determine whether folic acid (FA) reduces the embryonic ethanol (EtOH) exposure induced behavioral and morphological defects in our zebrafish fetal alcohol spectrum disorder (FASD) model. Teratogenic effects, mortality, the excitatory light-dark locomotion (ELD), sleep (SL), thigmotaxis (TH), touch sensitivity (TS), and optomotor response (OMR) tests were evaluated in larvae (6–7 days post-fertilization) using four treatment conditions: Untreated, FA, EtOH and EtOH+FA. FA reduced morphological defects on heart, eyes and swim bladder inflation seen in EtOH exposed fish. The larvae were more active in the dark than in light conditions, and EtOH reduced the swimming activity in the ELD test. EtOH affected the sleep pattern, inducing several arousal periods and increasing inactivity in zebrafish. FA reduces these toxic effects and produced more consistent inactivity during the night, reducing the arousal periods. FA also prevented the EtOH-induced defects in thigmotaxis and optomotor response of the larvae. We conclude that in this FASD model, EtOH exposure produced several teratogenic and behavioral defects, FA reduced, but did not totally prevent, these defects. Understanding of EtOH-induced behavioral defects could help to identify new therapeutic or prevention strategies for FASD.

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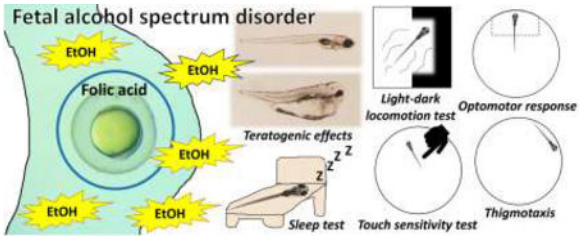
Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Graphical Abstract



Keywords

Zebrafish; Fetal alcohol spectrum disorder; Teratogenicity; Swimming activity; Sleep; Optomotor response; Thigmotaxis

1. Introduction

Fetal alcohol spectrum disorder (FASD) is caused by prenatal ethanol (EtOH) exposure, producing central nervous system damage, and its manifestations include physical, cognitive, and behavioral features. The global prevalence of alcohol use during pregnancy in the population was estimated to be 9.8% (WHO, 2018). FASD is possibly the most common cause of intellectual disability and preventable congenital disabilities (Patten et al., 2013). Congenital disabilities include craniofacial defects, organ deformities, cognitive impairment, and sensory and motor disabilities (Zhang et al., 2018). Embryonic EtOH exposure to vertebrate animal models can recapitulate defects seen in FASD patients (Muralidharan et al., 2015). Fish can be used as models to study human health problems or as indicators of environmental health (Kalueff et al., 2013). The zebrafish is an important animal model because due to non-adherent and transparent embryos, and embryonic development that is well-described (Spirita and Ahila, 2015; Basnet et al., 2019). Additionally, the genome of this species was fully characterized, and their physiology and neuroanatomy are homologous with those of humans (Kalueff et al., 2013) and other mammals.

Morphological defects were commonly described in animal models of FASD. Additionally, behavioral experiments associated with morphological defects can enhance the zebrafish of FASD model. Hence, we are studying fish behavior as a method to measure defects in a model of FASD using zebrafish (*Danio rerio*). According to Collier et al. (2020), the behavioral effects in zebrafish, produced by embryonic exposure to EtOH in the water, are very similar to those produced in other animal models like rodents. Also, zebrafish behavioral phenotypes provide important insights into normal and pathological brain function (Kalueff et al., 2013). Several mammalian and zebrafish behaviors are similar, suggesting evolutionary conservation of many behaviors across species (Basnet et al., 2019).

The zebrafish larvae show a pattern of swimming in response to light and dark conditions. This swimming behavior can be affected by neuroactive drugs (Basnet et al., 2019) like EtOH. Swimming patterns of zebrafish larvae showed an increase in the activity at low concentrations (1–2% v/v) and reduction at higher concentration (4% v/v) of EtOH in acute treatments (MacPhail et al., 2009; Irons et al., 2010; de Esch et al., 2012). Interestingly, the

EtOH exposure before the test affected swimming predominately during the light periods. Also, 1% EtOH exposure retarded the transition in activity from dark to light, and the habituation of activity in the dark, while 2% EtOH increased activity regardless of lighting condition (MacPhail et al., 2009). These studies mimic binge drinking in healthy animals at 6 dpf (days post-fertilization), not a FASD model. Collier et al. (2020) developed a FASD model to evaluate the effect of EtOH on 6 dpf offspring by feeding gelatin mixed with EtOH (0.1%) to adult female zebrafish, concluding that a significant increase in novelty-induced locomotor activity, but no effect on thigmotaxis. Similar results were seen in the larvae that consumed 0.1% EtOH-gelatin at 12 days post-fertilization. These findings show that EtOH exposure modified the swimming pattern behavior of zebrafish.

Zebrafish were also used in sleep research as a model of human sleep due to the similarities in genetics, pharmacology, and neuroanatomy (Lee et al., 2019). The ontogeny of sleep cycles in zebrafish and humans is remarkably similar. The sleep levels are highest early during development, and sleep and wake bouts gradually consolidate to form the adult sleep pattern (Sorribes et al., 2013). Alcohol produces several sleep disorders in humans (Crews et al., 2019), but the toxic effects of embryonic EtOH exposure in zebrafish sleep patterns remain unknown.

Alcohol consumption during pregnancy affects anxiety in human offspring (WHO, 2018; Abozaid et al., 2020). Thigmotaxis is accepted as an index of anxiety like-behavior in the zebrafish, where zebrafish normally avoid the center of an area and move to the edge (Baiamonte et al., 2016; Basnet et al., 2019). Finally, as previously described by our group (Muralidharan et al., 2015, 2018), embryonic EtOH exposure produced retinal defects in zebrafish. As a model for human eye disease, zebrafish offer several advantages to analyze these defects. At 5 days post-fertilization, zebrafish have measurable behavioral responses to visual stimuli. The optomotor response is employed to evaluate behavioral visual responses in zebrafish using an animation of a series of black and white lines that simulate motion, leading the zebrafish larvae to change their direction and align with the animation (Brastrom et al., 2019).

Based on the information above, these methodologies are suitable for our FASD model. In summary, the zebrafish behavior can be used to analyze the effects of drugs that prevents EtOH-induced defects like folic acid (FA). FA is an essential micronutrient with antioxidant properties (Zhang et al., 2018), but embryonic EtOH exposure affects FA metabolism, including reduced maternal-to-fetal folate transfer and reduced expression of folate metabolizing enzymes (Muralidharan et al., 2013). FA supplement prevented EtOH induced morphological defects in our FASD model (Sarmah and Marrs, 2013; Muralidharan et al., 2015, 2018). Our experiments tested FA for its ability to protect against behavioral defects induced by EtOH exposure. In this framework, the objective of this work was to study whether FA reduction in the EtOH-induced morphology defects correlate with behavioral locomotor activity, sleep pattern, thigmotaxis, touch sensitivity and optomotor response in our zebrafish FASD model.

2. Material and methods

2.1. Zebrafish husbandry and egg production

The experiments were performed at Purdue School of Science at Indiana University – Purdue University Indianapolis. Adults zebrafish (*Danio rerio*; AB strain; 15-months-old) were raised and housed under standard laboratory conditions (Westerfield, 2000; OECD, 2013) on an Aquatic Habitats recirculating filtered water system (Pentair Aquatic Eco-Systems, Apopka, FL) following protocols approved by the Indiana University Policy on Animal Care and Use Committee. Adult male/female zebrafish with a 2:1 ratio (Westerfield, 2000) were isolated in spawning aquariums. The eggs were collected 30 min after fish crossing and then incubated at 28.5°C in an Ecotherm incubator. Fertilized eggs (normal developing blastula) were used to form experimental groups, while unfertilized eggs, with cleavage irregularities, injuries, or other malformations were discarded (Silva et al., 2019). Additionally, the criteria used in our experiments were based on OECD 236 (2013), where the overall fertilization rate of clutches was ≥ 70%. The survival of animals in the control group was ≥ 90% at the end of the 96 hpf in all tests. The hatching rate in the control group was ≥ 80% at the end of 96 hpf.

2.2. Reagents and preparation of stock solution

FA (lot # 121K01445, CAS number 59–30-3, purity of ≥ 98%, and HPLC grade) was purchased from Sigma (St. Louis MO, USA). EtOH anhydrous (lot # 160308, CAS number 64–17-5, purity of ≥ 99.99%) was purchased from Electron Microscopy Sciences (Hatfield, PA, USA). All other reagents utilized were of analytical grade. The stock solution of FA was made fresh before the experiments (Muralidharan et al., 2015) and prepared by dissolution in embryo medium, obtaining the final nominal concentration of 1,000 μM.

2.3. Ethanol treatment and raising ethanol-treated embryos

The methodology to fetal alcohol spectrum disorder (FASD) model was used as described by Sarmah et al. (2016). Briefly, healthy embryos were maintained in embryo medium until 2 hpf (hour post-fertilization). The development stages were identified according to Kimmel et al. (1995), and then, the animals were transferred to embryo medium (untreated, control); embryo medium containing 100 mM EtOH (EtOH group), a physiological blood concentration of a heavy drinker (Harrison et al., 2017); embryo medium containing 100 mM EtOH and 75 μM FA (Muralidharan et al., 2015) (EtOH+FA group); and, embryo medium containing 75 μM of FA (FA group) in Petri dishes wrapped with Parafilm®. This FA concentration was described to prevent some morphological defects in zebrafish in our FASD model (Muralidharan et al., 2013; Sarmah and Marrs, 2013; Muralidharan et al., 2015). EtOH treatment dishes were placed in chambers with 2% EtOH to minimize EtOH volatilization (Muralidharan et al., 2015). At 24 hpf (22-h of exposure), all solutions were replaced with a fresh embryo medium. Control and experimental groups were put into a light-dark cycle of 14:10 h and 28.5°C and raised until assayed. The scheme of experimental design is depicted Figure 1.

2.4. Morphological analysis

The embryos and larvae were rinsed in embryo medium and viewed under a stereomicroscope (Leica M212). Images of the larvae were collected using a camera to measure the effects of EtOH (n = 40 per group). Mortality was determined daily, and the determination of embryo lethality was assayed according to OECD 236 (OECD, 2013). The teratogenic effects and morphometry were evaluated at 3–7 dpf (days post-fertilization). Teratogenic effects analyzed were coagulation (COA), large inter-eye distance (ED), small eyes (EY), pericardial edema (PE), delayed swim bladder inflation (SB), spine deformation (SD), tail deformation (TD), yolk sac edema / unusual large yolk sac (YSE), delayed yolk sac resorption (YSR) and hatching rate (HR) and larvae paralysis (LP) (Lammer et al., 2009; Jonas et al., 2015; Lutte et al., 2015; Dubińska-Magiera et al., 2016; Yang et al., 2016; Silva et al., 2019).

Morphometry of larvae (n = 20 per group) in the dorsal view position were imaged at 7 dpf under a stereomicroscope. The parameters studied were (i.) Larval length (μm), distance from the larval mouth to the pigmented tip of the tail; (ii.) Ocular distance (μm), the distance between the inner edge of the two eyes (similar to the inner intercanthal distance in humans) (Altenhofen et al., 2017); (iii.) Eye diameter; (iv.) Head-width, maximum head measurement; and, (v.) Head-length (μm), larval mouth to the pectoral fin's measurement. The images were captured, and measurement was made using a Leica DFC450 C digital camera microscope and Leica Application Suite LAS 4.3.0 software (Leica Microsystem Switzerland, 2013).

2.5. Behavioral tests

Dead larvae or larvae that displayed severe morphological defects (teratogenic effects described in section 2.4) induced by EtOH exposure were not included in any behavioral test. The embryonic movement was tracked using the Zebrabox (Viewpoint Life Sciences, Lyon, France) tracking system equipped with an infrared camera. A total of 24 replicates (1 embryo per replicate placed individually in each plate well) were used per experimental group in the analysis with Zebrabox. Assessment of behavioral effects was performed by tracking the movement of each larva during periods of light and dark using two different protocols. The excitatory light-dark locomotion test (ELD Test) is based on the fact that light-dark transition increases locomotor activity of the fish (Basnet et al., 2019). This test started in the afternoon (> 2 PM) due to the steady activity of the zebrafish (de Esch et al., 2012) with 30 min of acclimatization in the dark and 6 cycles of 20-min alternating light (10 min) and dark (10 min) cycles. The second test is the sleep test (SL Test) based on the fact that the sleep and awake pattern of zebrafish is similar to that of humans (Sorribes et al., 2013) and possibly affected by EtOH embryonic exposure. This test had the acclimatization period of light for 14 h starting at 8 AM. The larvae were transferred to Zebrabox at 4 PM, followed by evaluation for 6 h of in the dark started at 10 PM. The hour that the light was turned off was the same as that used in the vivarium that the larvae were previously acclimatized. Movement analysis was performed at 6 dpf for ELD and SL tests. Finally, an integration period of 2 minutes was used for the ELD test and 10 minutes for the SL test. The distance moved was analyzed which was divided into small activity (between 4 and 8 mm/s) and high activity (> 8 mm/s) for the ELD test. The average inactivity (time spent

in inactivity < 4 mm/s) was used to determine as sleep period at night, the arousal periods (reduce inactivity) were compared qualitatively in the SL test.

The thigmotaxis test (TH Test) was used to evaluate the tendency of the larvae to move near to the wall of plates (2019) for evaluating the anxiety like-behavior in zebrafish (Brastrom et al., 2019) because EtOH affects this behavior (Abozaid et al., 2020). Before the optomotor response test, after 10 min of acclimatization in 48-well plates (48 larvae per experimental group) in a light environment, the TH test was carried out and the response was recorded when larvae remain near to the wall. Finally, the touch sensitivity test (TS Test) was used to evaluate the larval response to mechanical stimuli. The larvae were touched on the tail or head and the response was recorded if the larva elicited an escape (Ashlin et al., 2018). We consider an escape behavior when larvae exhibit burst swim or swim behaviors (Kalueff et al., 2013). The scan sampling method (Silva et al., 2019) was used in the TH and TS tests to collect behavioral data of larvae (6 dpf).

The possible visual impairment induced by EtOH was analyzed by the optomotor response test (OMR Test) according to Brastrom (2019). Briefly, larvae with 6 dpf (72 per group) were transferred to 48-well plates acclimatized in a light environment for at least 2 h, and then submitted a 30 sec of OMR animation (one direction with black and white lines moving from right to left) using an application loaded in a 10-inch tablet. Videos were made with a cellphone camera and the start and end positions of the larvae were scored in four different areas representing low-alignment (70% of the well), scores 1 and 2 according to Brastrom et al. (2019), and high-alignment (30% of the well), scores 3 and 4 according to the same authors.

2.6. Statistical analysis

Statistical planning for the experiments was performed by two-level 2^2 experimental design considering as factors presence or absence of FA (0 and 75 μM), and presence or absence of EtOH (0 and 100 mM). The data are presented by mean ± SD. The results of morphometry and behavioral data of the entire testing periods were analyzed by one-way or two-way ANOVA. When the difference was significant, the means were compared by Tukey's test with $p < 0.05$. Results of the OMR test were analyzed by McNemar test of symmetry with $p < 0.05$. Statistical analyses were performed using the Origin Pro Academic 2015 (Origin Lab. Northampton, MA USA) and BioStat 6.9.1.0 (AnalystSoft Walnut, CA USA).

3. Results

3.1. Morphological analysis

The results revealed less than 10% mortality in the embryos at 2 dpf in all studied groups, and the reason for mortality appeared to be blood coagulation according to OECD 236 (2013). After 5 dpf, the mortality again was less than 10% in all groups, and the probable reason for mortality was defects induced by EtOH exposure. Importantly, teratogenic effects were observed in zebrafish groups exposed to EtOH and EtOH+FA, visualized at 72 hpf. We examined the affected larvae in our FASD model, and at least three teratogenic effects described in the methodology section (see section 2.4) were observed per animal. The

animals developed teratogenic effects in the EtOH (25.3%) and EtOH+FA (14.7%) groups after 7 dpf. The animals had a similar set of morphological defects, but FA reduced the severity of EtOH effects in the affected animals. Interestingly, it was possible to divide the larval population into the exposed groups without morphological defects according to our criteria (see section 2.4) and animals with several teratogenic effects. The teratogenic effects typically found in the larvae are shown in Figure 2. As expected, when fish were exposed to EtOH, there was a significant decrease ($p < 0.05$) in the eye diameter and high variation in the ocular distance compared to all other groups (Figure 3). FA reduced these morphological defects. Note: the animals with morphological defects were excluded from the behavioral tests because of severely impaired or lack of swimming activity.

3.2. Behavioral tests

The results of the excitatory light-dark locomotion test (ELD Test) are shown in Figure 4. As previously shown (Basnet et al., 2019), zebrafish larvae were more active in the dark than in light conditions for all experimental groups (Figure 4A and 4C). Regarding the small activity (between 4 and 8 mm/s), embryonic EtOH exposure affected only the total distance moved in the dark condition with a reduction of almost 70% in the swimming activity of larvae, indicating hypoactivity (Figure 4B). Interestingly, the FA reduced the activity by almost 35% compared to the control. The exposure of EtOH+FA increased 25% of the activity compared to the EtOH group (Figure 4B, $p = 4.73E\text{-}7$). EtOH only affected the total distance moved in the high activity (> 8 mm/s) in the dark condition with a reduction of 70%, like that seen in small activity. Again, the FA reduced the activity by almost 48% compared to the control group, and the exposure to the EtOH+FA increased 20% of the activity compared to the EtOH group (Figure 4D, $p = 0.011$). The time spent and the number of movements (count) in small and high activity were also analyzed, and similar results as distance moved were obtained (data not shown). In summary, EtOH produces hypoactivity, FA reduces the distance moved by larvae, and EtOH+FA maintained the activity to the same level as FA alone.

The results of the SL test revealed that comparations between experimental groups and zeitgeber time were different (Figure 5A). Control larvae exhibited more inactivity in the early evening, and this inactivity decreases during the night, producing arousal periods (Figure 5A). The animals exposed to FA had a low variation of inactivity during the night, maintaining a pattern with few arousal periods. However, EtOH and EtOH+FA groups had a small increase in the activity during the night, with the EtOH group being more inactive. EtOH and EtOH+FA groups had several arousal periods (Figure 5A). These results explain the plots found in Figure 5B where the average inactivity of all experimental groups was significantly different than control. The variation found in the control group was explained by the increase in the activity during the night. The small variation in the FA groups was due to constant inactive behavior, and EtOH and EtOH+FA groups had moderate variation due to several arousal periods. FA may maintain the activity, not the arousal periods.

Using TH test, embryonic EtOH exposure affected thigmotaxis, the ability of larvae to move near to the wall of the plate, reducing the affinity for the wall (Figure 6A). FA reduced this defect in the EtOH exposed larvae (EtOH+FA group), and FA alone (FA group) did not

affect this parameter. Mechanical stimuli were used to evaluate if EtOH exposure affects the response to touch in the TS test. According to our results (Figure 6A), this parameter was not affected in the larvae tested. It should be noted that only larvae without morphological defects were used for this test, and fish with teratogenic effects (larvae paralysis) were excluded from our behavioral tests. Finally, the results (Figure 6B) of the OMR test revealed that EtOH exposure produced visual system impairment in zebrafish. Larvae exposed to EtOH during embryogenesis did not respond normally to the OMR animation. On the other hand, the larvae of the EtOH+FA group were protected responding normally to the OMR animation, like the control and FA groups.

4. Discussion

Many conditions induce human congenital defects, but EtOH consumption during pregnancy is the most common known cause (Patten et al., 2013). Embryonic EtOH exposure induced morphological defects can be reproduced in the zebrafish animal model (Sarmah et al., 2013), and now behavioral defects were also analyzed. In our work, EtOH exposure produced teratogenic and behavioral defects in zebrafish. In the first 48 hpf, it is not possible to associate the mortality with EtOH exposure, because control and FA groups exhibited similar levels of mortality. According to OECD 236 (OECD, 2013), the mortality rate of less than 10% was expected in the control group by 96 hpf. However, after hatching several teratogenic effects (Figure 1) were observed only in the EtOH and EtOH+FA groups. FA co-treatment with EtOH reduced the proportion of affected animals but did not eliminate them. Embryonic ethanol exposure produces a wide range of phenotypic effects, including morphological defects in zebrafish (Ali et al., 2011). In our study and those of others, some animals displayed morphological and behavioral defects, while other animals show less effect. In humans, this phenomenon of FASD variability with similar ethanol exposure was observed (Ali et al., 2011). Variability may be due to environment or genetic difference, but this variation is still poorly understood.

Observed teratogenic effects like large inter-eye distance, small eyes, high ocular distance and small eye diameter (Figure 3) are characteristic of eye defects. Previous work (Muralidharan et al., 2015, 2018) showed that when zebrafish were exposed to EtOH, the altered retinal cell differentiation can produce severe ocular defects as microphthalmia and abnormal photoreceptor differentiation, and FA alleviates these defects. Antioxidant functions of FA may partially protect the retina from EtOH-induced defects (Muralidharan et al., 2015) during the exposure time (2–24 hpf). However, molecular details about these protective effects remain unknown.

Pericardial and yolk sac edemas are easy to observe and are the most common teratogenic effects observed in our study. The edemas and blood coagulation indicated circulatory defects in the zebrafish FASD model. Previous studies (Sarmah and Marrs, 2013, 2017) evaluated the heart defects induced by EtOH, concluding that FA protected against a spectrum of cardiac defects induced by EtOH exposure. We observed a reduction in the number of affected animals (14.7%) with edemas in the EtOH+FA group, corroborating the previous observations (Sarmah and Marrs, 2013, 2017). These cardiac defects affect normal circulation, possibly producing edema. EtOH exposure also affected swim bladder inflation,

indicating swimming defects. Ali et al. (2011) observed at 5 dpf a developmental delay in swim bladder inflation when embryos were exposed to 1.64 M EtOH for 1 h. Similar results were obtained in our work using 100 mM EtOH exposure from 2–24 hpf.

Larvae paralysis was also observed in the EtOH exposed groups, and EtOH exposure induced central nervous system defects (Muralidharan et al., 2013). EtOH effects on swimming activity help to explain our findings. Finally, EtOH exposure caused other morphological defects like spine and tail deformation. As reported by Abosaid et al. (2020), severe morphological defects were found in the larvae zebrafish FASD model, which is compatible with dose and time of exposure in our experiment.

Teratogenic effects were fatal in some embryos. FA protected animals from EtOH-induced defects. FA functions as a cofactor, and it has antioxidant properties, affecting a wide range of developmental processes (Muralidharan et al., 2013). The mechanism of action for FA in our model remains unknown. Several larvae in the EtOH exposed groups do not exhibit detectable teratogenic effects. The behavioral tests were performed in groups where severely affected larvae were excluded, and these tests reveal effects of EtOH exposure in larvae, despite apparently normal development.

Our ELD results may be due to visual system or locomotor behavior defects in larvae without morphological defects. In this case, larvae either did not notice or had difficulty swimming when the light turned off. Collier et al. (2020) developed an FASD model by feeding gelatin mixed with EtOH to adult zebrafish, and the larval offspring showed hyperactivity locomotion behavior in the light, perhaps due to an increase in hypothalamic hypocretin neurons. In another study (Abozaid et al., 2020), embryos were exposed to EtOH for 2 h at different developmental stages (8 – 40 hpf), and again, larvae showed hyperactivity in the light condition. Our results with the embryonic exposure to EtOH produced different behavioral defects than those seen by these authors. The different results indicate that different periods of development, exposure time, and EtOH concentration produced variations in behavioral defects. FA reduced the swimming activity, but EtOH+FA prevented behavioral defects seen in the ELD test to a level similar to FA alone. FA acts in multiple important metabolic pathways, including the development of the central nervous system (Muralidharan et al., 2013). Embryonic exposure to FA possibly modifying the development and maturation of the fish, reducing the swimming activity behavior like that see in more mature fish. Silva et al. (2019) evaluated the behavior of adults zebrafish, and they exhibited low swimming and rest behaviors like our FA larvae group.

We evaluated the sleep pattern of zebrafish. The control group exhibited the same sleep pattern as seen by other authors (Ashlin et al., 2018; Lee et al., 2019). However, embryonic EtOH exposure increased the arousal periods and increased inactivity. The increased inactivity was found in the ELD test (Figure 3) and SL test (Figure 4), increasing the inactivity in response to EtOH exposure. EtOH consumption induces sleep disorders in humans (Crews et al., 2019). Our finding indicates that this effect was also produced in our zebrafish FASD model. Harrison et al. (2017) described that alcohol consumption affects the function of the thalamus, which regulates the arousal states in humans. Possibly, the thalamus was affected in our FASD model. When zebrafish were exposed to EtOH+FA,

the inactivity was reduced. However, frequent arousal periods remain, indicating that FA reduces some EtOH behavior effects but does not protect against all defects. FA exposure also modified the sleep pattern of zebrafish larvae, showing a low variation of inactivity during the night with the low arousal periods. Sorribes et al. (2013) evaluated sleep in zebrafish at different ages and compared them to human sleep behaviors. These authors concluded that the number of arousal periods reduced in the fish as they mature like humans. This statement remains in agreement with our suggestion that FA modified the maturation of the fish compared to the control group.

Thigmotaxis was affected by EtOH embryonic exposure. Our larvae were housed in an unfamiliar environment to evaluate this response. The reduced thigmotaxis may also be associated with the reduction of the swimming activity in larvae. Thigmotaxis is associated with fear or anxiety-like behaviors (Abozaid et al., 2020). Baiamonte et al. (2016) obtained similar results, showing a reduction in the thigmotaxis in 9 dpf larvae after EtOH exposure for 2–4 dpf using 20 – 50 mM EtOH. These authors concluded that an EtOH exposed larvae showed less anxiety-like behavior. In the TS test, all groups reacted when mechanical stimuli were applied, showing that all larvae were able to move and react. Together, our data showed that behavior defects were produced in the EtOH exposed larvae, even with the morphologically defective fish being excluded from the assays.

EtOH exposure produced larvae with severe ocular defects, which were excluded from the behavioral tests, but the larvae without obvious morphological defects also may have visual system impairment because they did not respond normally in the OMR test. According to Brastrom (2019), the optomotor response can be used to detect visual system impairment in the larvae. Our results confirmed the utility of this test in our FASD model. Photoreceptor differentiation is reduced in the zebrafish FASD model (Muralidharan et al., 2015), possibly interfering in the perception of black and white lines in the OMR animation. EtOH exposure affects morphogenesis and retinal development, which may produce visual system impairment (Muralidharan et al., 2015). According to the same authors, FA treatment protected retinal morphogenesis during EtOH exposure. This effect may be explained due to the physiological functions of FA as a cofactor for methyl group transfers in 1-carbon metabolism or antioxidant properties that affect developmental processes, including eye development (Muralidharan et al., 2015), which is consistent with our results. Finally, EtOH consumption interferes with folate absorption, and the FA deficiency may cause developmental defects (Muralidharan et al., 2013). The exogenous FA used in our experiments probably reduced EtOH toxic effects, as evidenced by the morphological and behavioral data in our experiments.

5. Conclusion

The present study evaluated whether FA reduction in the EtOH-induced morphological defects also reduced behavioral defects. We conclude that in our FASD model, EtOH exposure produced teratogenic and behavioral defects, and FA partially protected these defects. FA alone produced different behavioral responses, indicating that FA may independently modify fish development. This zebrafish behavior on FASD model also provides an opportunity to test whether other compounds reduce the EtOH toxic effects.

Understanding EtOH-induced behavior defects could help us identify new therapeutic or prevention strategies for FASD.

Acknowledgments

The authors would like to thank members of the Marrs laboratory for helpful discussion and students of *Laboratório de Ecofisiologia e Comportamento Animal* – LECA (Brazil) for inspiration, *Coordenação de Aperfeiçoamento de Pessoal de Nível Superior* – CAPES (PRInt #88887.364263/2019-00) for the fellowship to Pabyton G. Cadena. This work was supported by NIH/NIAAA 1 R21 AA026711.

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Research Highlights

- Embryonic EtOH exposure induced morphological defects in the zebrafish FASD model.
- FA prevented defects on morphology, swimming, thigmotaxis, and optomotor response in larvae.
- EtOH exposure affected the sleep pattern, inducing several arousal periods in zebrafish.
- EtOH produced developmental defects, and FA reduced partially these defects.

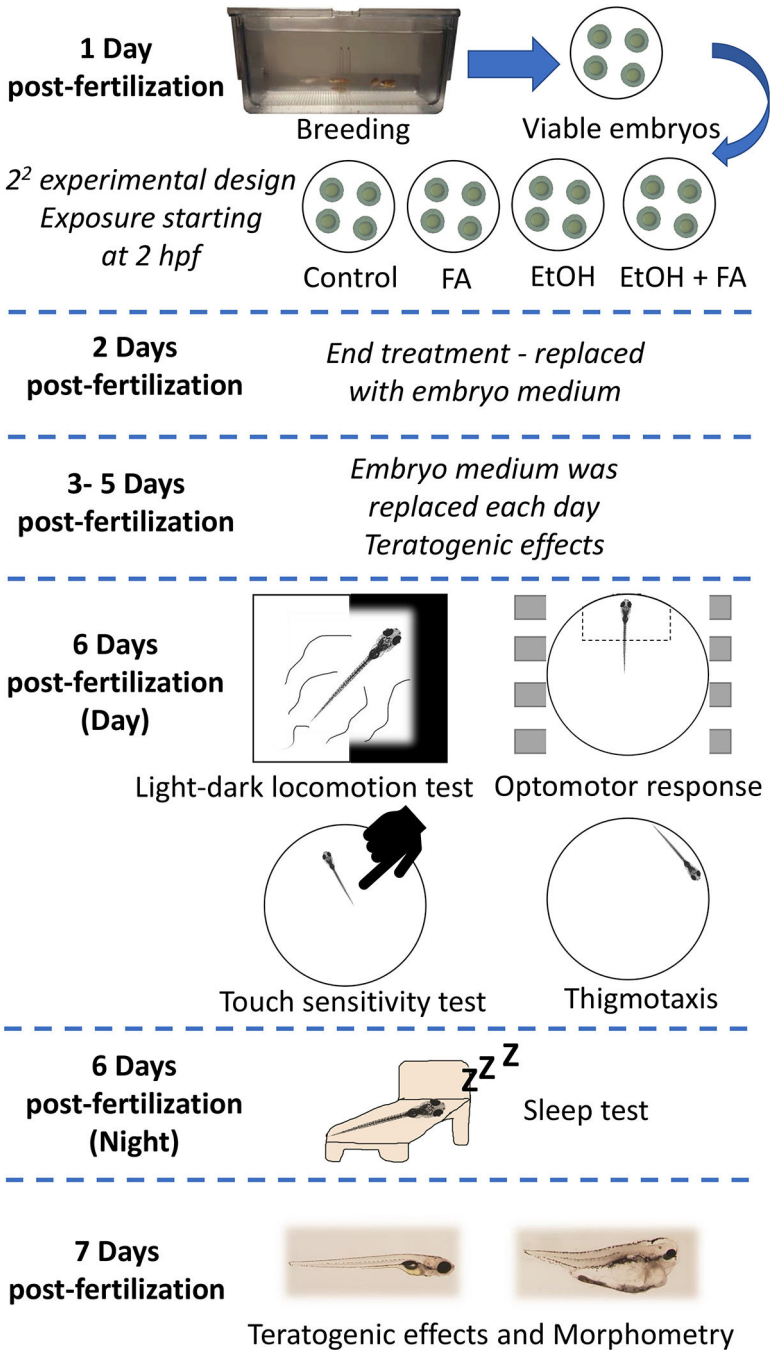

Figure 1.

A scheme of experimental design that tests EtOH and folic acid exposure on zebrafish (*Danio rerio*) model for fetal alcohol spectrum disorder (FASD). Legend: FA – Folic acid; EtOH – Ethanol.

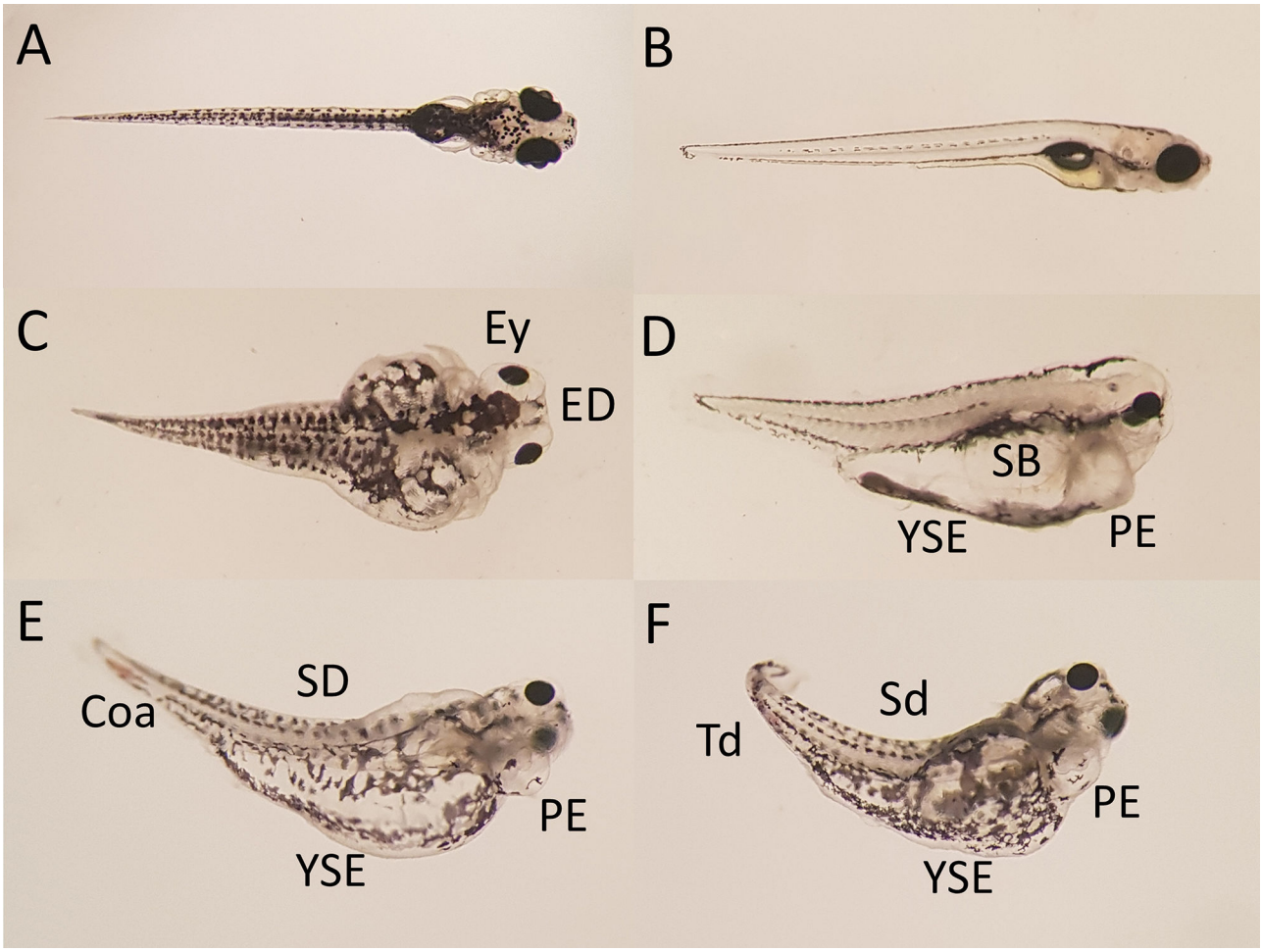

Figure 2.

Observed teratogenic effects of ethanol (EtOH) exposure in embryos from 2–24 hpf at 7 dpf. A – Control in the dorsal view; B – Control in the lateral view; C-F Fish exposed to EtOH. Arrows and abbreviations indicate main teratogenic effects as COA – Coagulation; ED – Large inter-eye distance; EY – Small Eyes; PE – Pericardial Edema; SB – Delayed Swim Bladder Inflation; SD – Spine Deformation; TD – Tail Deformation; YSE – Yolk Sac Edema / Unusual Large Yolk Sac; YSR – Delayed Yolk Sac Resorption.

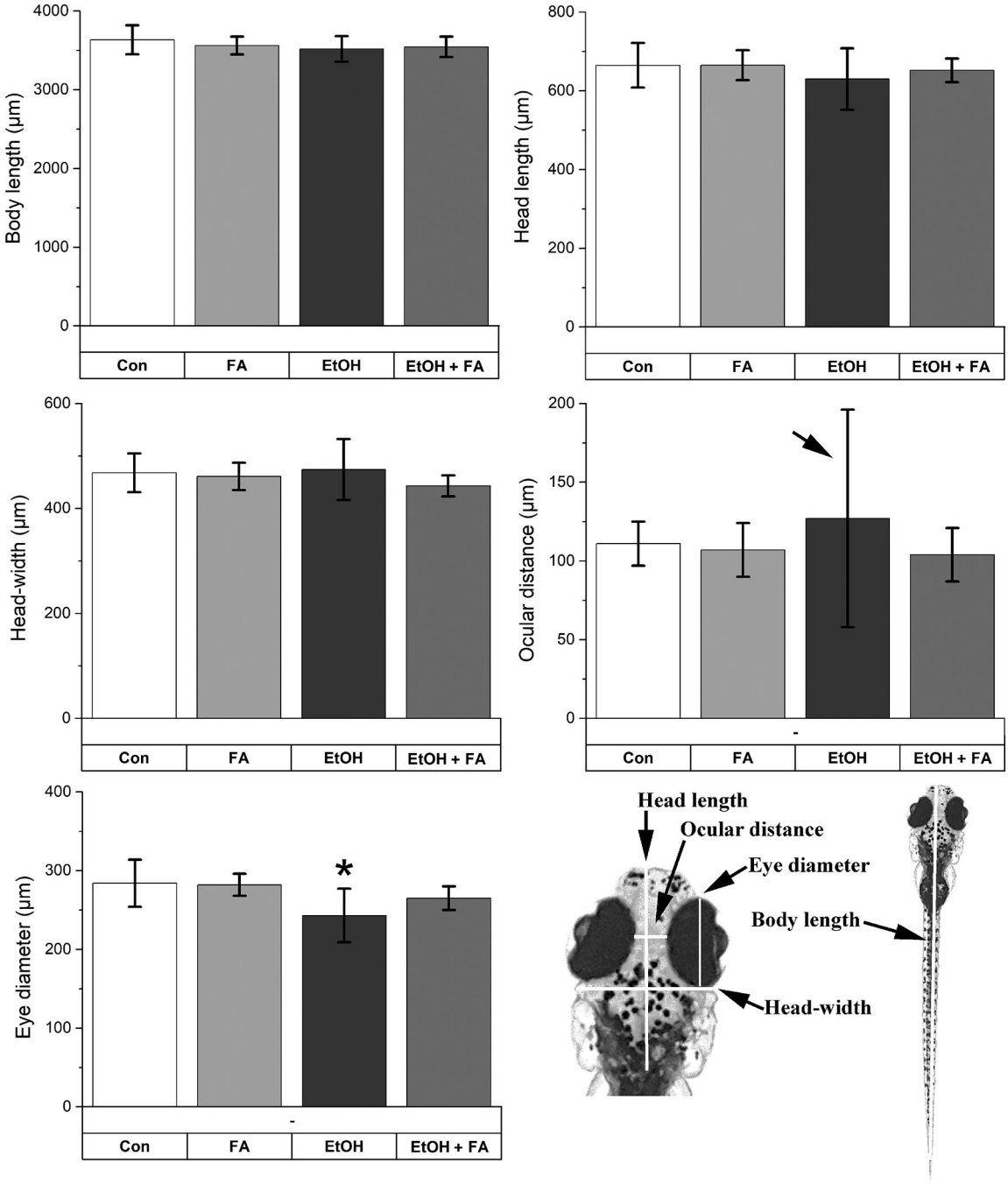

Figure 3.

Morphometry of EtOH-induced defects and protection in EtOH+FA evaluated in zebrafish larvae at 7 dpf. Data are expressed as the mean ± SD of 20 animals analyzed individually for each group compared to the control group using one-way ANOVA followed by Tukey's test. * = $p < 0.05$ and Arrow represent a high ocular distance. Legend: Con – Control; FA – Folic acid at 75 µM; EtOH – Ethanol at 100 mM; EtOH+FA – Ethanol at 100 mM plus Folic acid at 75 µM. One-way ANOVA results: Body length ($F(3, 78) = 1.94$, $p > 0.05$), Head-width ($F(3, 78) = 2.02$ $p > 0.05$), Eye diameter ($F(3, 80) = 11.22$, $p < 0.05$ (Tukey post hoc test (FA

p = 0.99, EtOH p = 1.43E-5, EtOH+FA p = 0.07)), Head length ($F(3, 78) = 1.78$, $p > 0.05$), Ocular distance ($F(3, 78) = 1.48$, $p > 0.05$).

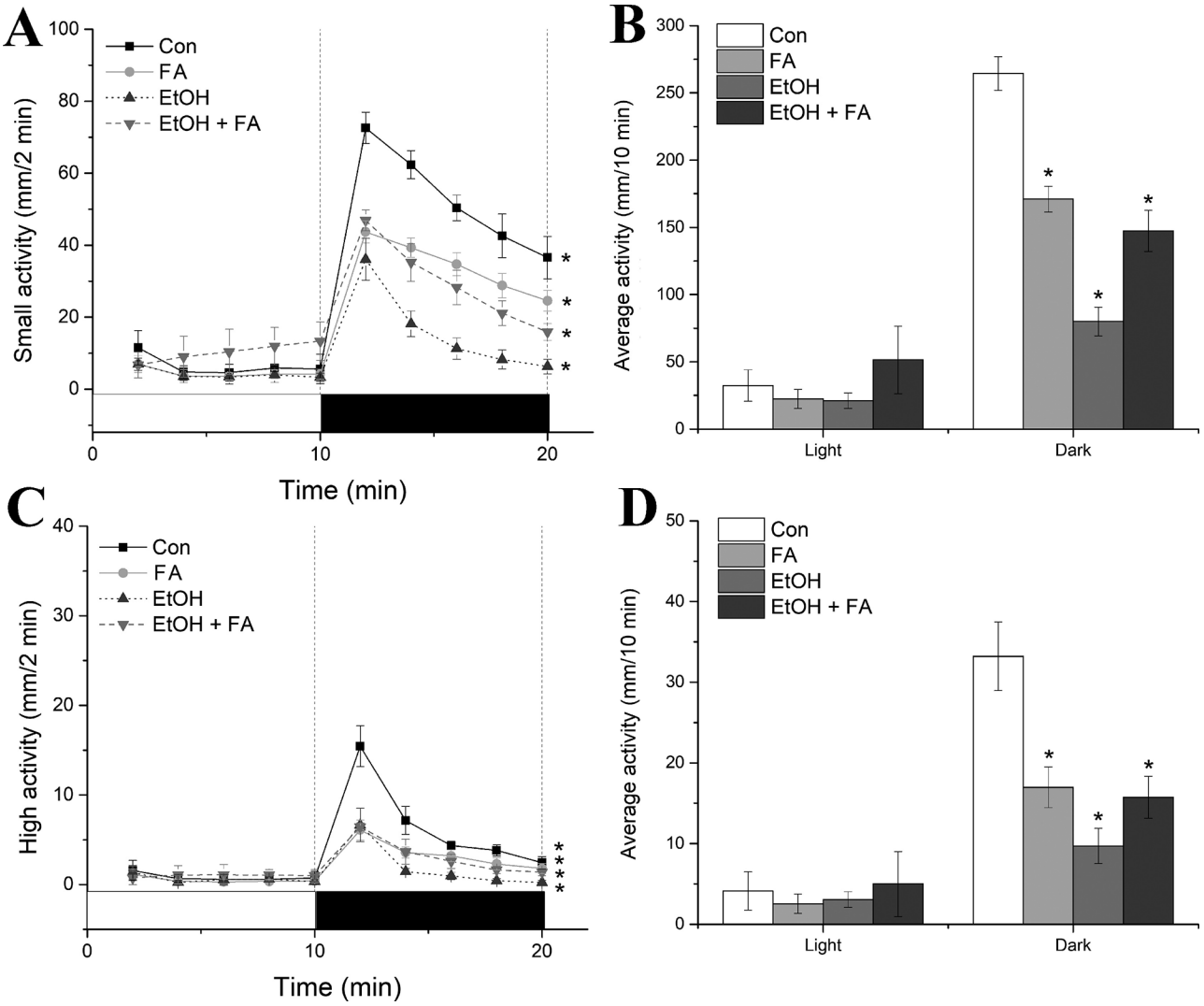

Figure 4.

Results of the excitatory light-dark locomotion test (ELD Test) on 6 dpf zebrafish larvae. Data are expressed as distance moved, showing small activity (between 4 and 8 mm/s) and high activity (> 8 mm/s). A and C show the swimming pattern of the larvae in the light and dark conditions with the period (light and dark), and time (0 – 20 min) analyzed by one-way ANOVA followed by Tukey's test for each group. B and D bars compared the average of total movement for each cycle (10 min) for the fish compared to the control group by one-way ANOVA followed by Tukey's test. * = $p < 0.05$. The experimental groups were Con – Control; FA – Folic acid at 75 µM; EtOH – Ethanol at 100 mM; EtOH+FA – Ethanol at 100 mM plus Folic acid at 75 µM. Small activity (A): One-way ANOVA results: Period (Con ($F(1, 59) = 303.95$, $p < 0.05$), FA ($F(1, 59) = 433.08$, $p < 0.05$), EtOH ($F(1, 59) = 30,48$ $p < 0.05$), EtOH+FA ($F(1, 59) = 66.92$, $p < 0.05$); Time (Con ($F(9, 59) = 249.68$, $p < 0.05$), FA ($F(9, 59) = 272.31$, $p < 0.05$), EtOH ($F(9, 59) = 78,17$ $p < 0.05$), EtOH+FA ($F(9, 59) = 47.92$, $p < 0.05$). Average small activity (B): One-way ANOVA results: Light ($F(3, 23) = 5.52$, $p < 0.05$ (Tukey post hoc test (FA $p = 0.65$, EtOH $p = 0.56$, EtOH+FA $p = 0.14$)); Dark ($F(3, 23) = 234.02$, $p < 0.05$ (Tukey post hoc test (FA $p = 1.63E\text{-}5$, EtOH $p = 1.63E\text{-}5$,

EtOH+FA p = 1.63E-5)). High activity (C): Period (Con ($F(1, 59) = 41.38$, $p < 0.05$), FA ($F(1, 59) = 83.56$, $p < 0.05$), EtOH ($F(1, 59) = 7,56$ $p < 0.05$), EtOH+FA ($F(1, 59) = 29.59$, $p < 0.05$); Time (Con ($F(9, 59) = 116.63$, $p < 0.05$), FA ($F(9, 59) = 63.49$, $p < 0.05$), EtOH ($F(9, 59) = 44,49$ $p < 0.05$), EtOH+FA ($F(9, 59) = 27.63$, $p < 0.05$). Average high activity (D): One-way ANOVA results: Light ($F(3, 23) = 1.16$, $p > 0.05$); Dark ($F(3, 23) = 67.35$, $p <$ 0.05 (Tukey post hoc test (FA p = 1.63E-5, EtOH p = 1.63E-5, EtOH+FA p = 1.63E-5)).

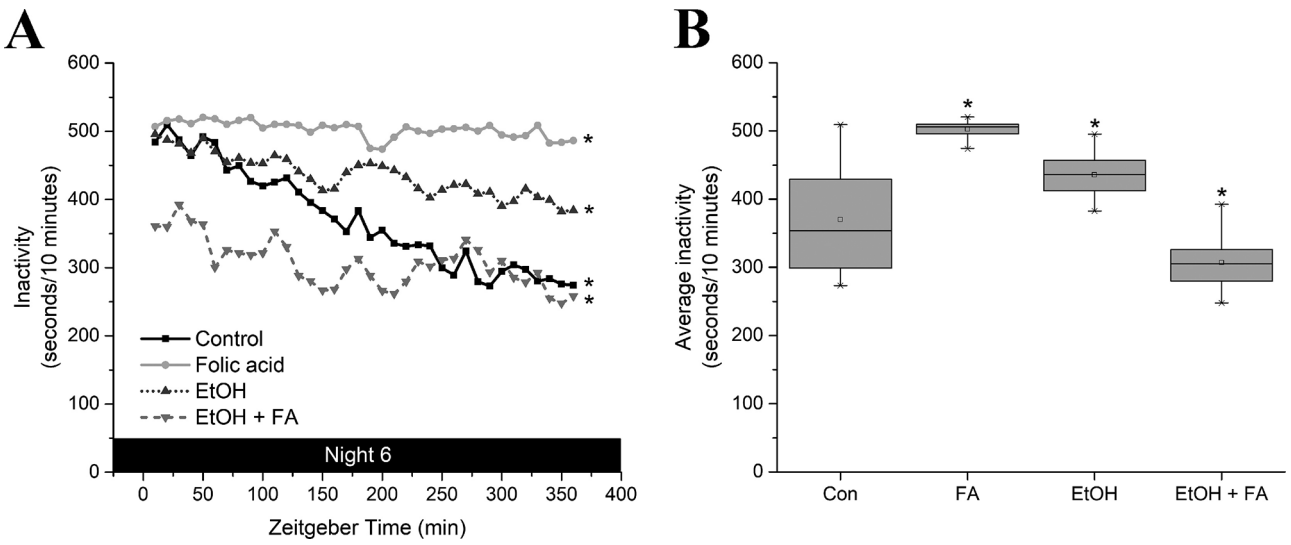

Figure 5.

An inactivity plot (A) for 6 h of night cycle reveals the different patterns of sleep between the treated groups in 6 dpf zebrafish with groups and zeitgeber time (0 – 6 h) compared by two-way ANOVA (group ($F(3, 143) = 244.99$, $p < 0.05$) and time ($F(35, 143) = 4.64$, $p < 0.05$)). Boxplot (B) of average inactivity representing the sleep pattern of each experimental group compared to the control group by one-way ANOVA ($F(3, 143) = 128.27$, $p < 0.05$) followed by Tukey's test (against control group (FA p = 5.35E-7, EtOH p = 1.99E-8, EtOH+FA p = 7.70E-8)). The arousal periods were compared qualitatively. * = $p < 0.05$. The experimental groups were Con – Control; FA – Folic acid at 75 μM; EtOH – Ethanol at 100 mM; EtOH+FA – Ethanol at 100 mM plus Folic acid at 75 μM.

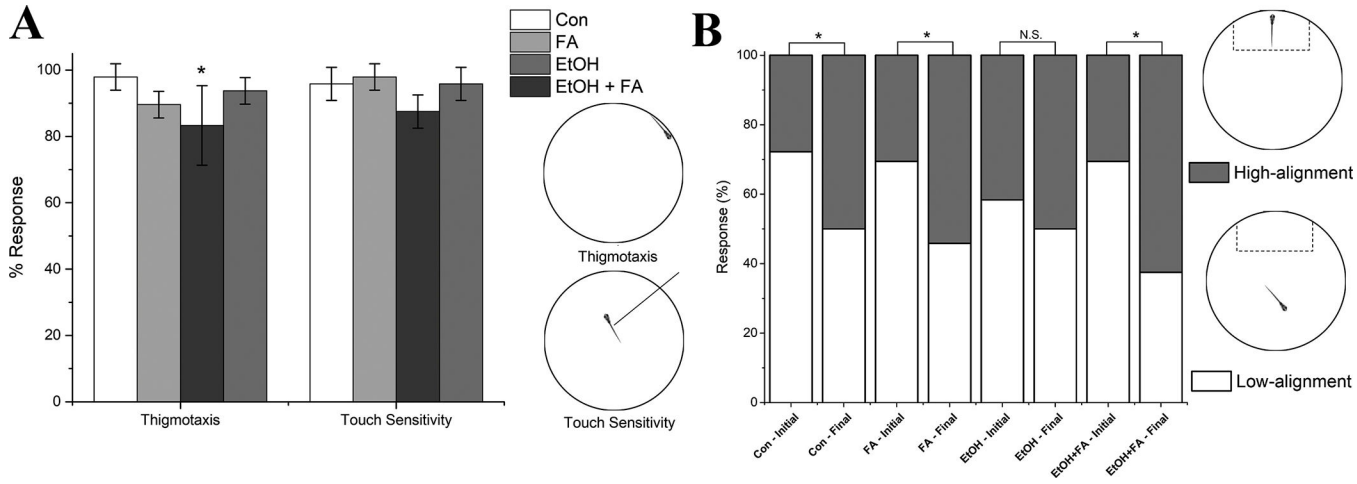

Figure 6.

The thigmotaxis test showed that EtOH reduced the thigmotaxis response, which was protected in EtOH+FA group, but did not affect the touch sensitivity response. (A). The response for each group was compared to the control group by one-way ANOVA followed by Tukey's test (thigmotaxis ($F(3, 16) = 4.02$, $p < 0.05$ (Tukey post hoc test (FA $p = 0.25$, EtOH $p = 0.02$, EtOH+FA $p = 0.74$); touch sensitivity response ($F(3, 15) = 3.93$, $p < 0.05$ (Tukey post hoc test (FA $p = 0.92$, EtOH $p = 0.11$, EtOH+FA $p = 1.00$)). The optomotor response analysis (B) of the zebrafish plotted as a percentage of the total and compared by the McNemar test of symmetry on the same group between the initial and final positions. * = $p < 0.05$. N.S. = not significant (Con $p = 0.002$, FA $p = 0.001$, EtOH $p = 0.258$, EtOH+FA $p = 6.03E\text{-}6$). Legend: Con – Control; FA – Folic acid at 75 μM; EtOH – Ethanol at 100 mM; EtOH+FA – Ethanol at 100 mM plus Folic acid at 75 μM.