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Multi-biomarker approach to evaluate the neurotoxic effects of environmentally relevant concentrations of phenytoin on adult zebrafish *Danio rerio*

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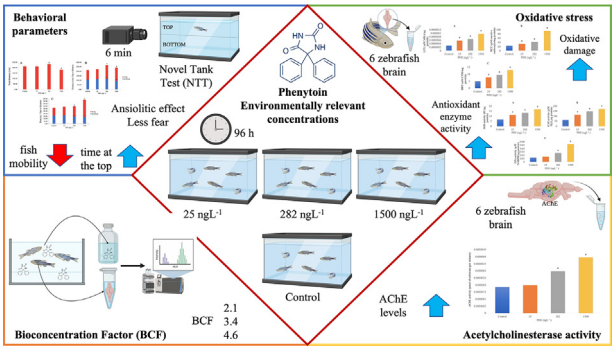
HIGHLIGHTS

- Environmentally relevant concentrations of phenytoin were neurotoxic to zebrafish.
- Oxidative stress plays an important role in the mechanism of phenytoin-induced neurotoxicity.
- Phenytoin bioconcentrates in zebrafish brain
- Phenytoin exposure showed an anxiolytic-like effect in zebrafish.
- An increase in acetylcholinesterase activity was observed.

GRAPHICAL ABSTRACT



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ABSTRACT

Several studies have reported the presence of phenytoin (PHE) in wastewater treatment plant effluents, hospital effluents, surface water, and even drinking water. However, published studies on the toxic effects of PHE at environmentally relevant concentrations in aquatic organisms are scarce. The present study aimed to determine the effect of three environmentally relevant concentrations of PHE (25, 282, and 1500 ng L^{-1}) on behavioral parameters using the novel tank test. Moreover, we also aimed to determine whether or not these concentrations of PHE may impair acetylcholinesterase (AChE) activity and oxidative status in the brain of *Danio rerio* adults. Behavioral responses suggested an anxiolytic effect in PHE-exposed organisms, mainly observed in organisms exposed to 1500 ng L^{-1}, with a significant decrease in fish mobility and a significant increase in activity at the top of the tank. Besides the behavioral impairment, PHE-exposed fish also showed a significant increase in the levels of lipid peroxidation, hydroperoxides, and protein carbonyl content compared to the control group. Moreover, a significant increase in brain AChE levels was observed in fish exposed to 282 and 1500 ng L^{-1}. The results obtained in the present study show that PHE triggers a harmful response in the brain of fish, which in turn generates fish have an anxiety-like behavior.

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1. Introduction

Phenytoin (PHE) is effective in the treatment of tonic-clonic and partial seizures. In low- and middle-income countries, PHE remains the most widely used antiepileptic drug (AEDs). Its primary mechanism of action is associated with limiting the spread of seizure activity by acting on neuronal voltage-gated sodium channels and thereby reducing seizure propagation. (Kola et al., 2018; Patocka et al., 2020). PHE has been continuously detected in wastewater, 111–2375 ng L^{-1} (Mijangos et al., 2018), surface water, 3.1–23 ng L^{-1} (Simazaki et al., 2015), groundwater, 10 ng L^{-1} (Huerta-Fontela et al., 2011), and even drinking water, 1.93 ng L^{-1} (Liu et al., 2019) at concentrations ranging from 1.3 to 2375 ng L^{-1}.

Despite its high consumption and detection in the environment, there are scarce studies on the effect of PHE on aquatic organisms. Up to date, for example, it has been reported that PHE caused adaptive alterations in CAT and GST (liver) activities in response to oxidative stress in *Lepomis gibbosus* in a concentration range of 6.25 to 100 μg L^{-1} (Brandão et al., 2013). Moreover, in another study, zebrafish larvae exposed to PHE showed a significant decrease in locomotion activity, with 50% less hydrodynamic power than the control group (Zhao et al., 2020). In agreement with the above study, PHE decreased locomotor activity and thigmotaxis in zebrafish larvae exposed to 500 μM, while larvae exposed to 20 μM showed an increase in locomotor activity (Liu et al., 2016). In the adult zebrafish brain, PHE was shown to have a complex interaction with the purinergic and cholinergic systems, which results in AMP hydrolysis at 500 and 100 μM by 65 and 64.8%, respectively (Siebel et al., 2010). Recently, Pieróg et al., 2021 have reported a significant anxiolytic-like effect after administration of 15 and 30 mg Kg^{-1} PHE in adult zebrafish.

Oxidative stress (OS) is widely implicated in the mechanism of toxicity of several drugs detected in water bodies, threatening the ecological balance of aquatic life (Almeida et al., 2015; Brandão et al., 2013; Cheng et al., 2020; Oliveira et al., 2017; Orozco-Hernández et al., 2021). In general, aquatic organisms have a system of enzymatic defenses to protect themselves from excessive production of reactive oxygen species. However, when the production of free radicals and reactive species exceeds the antioxidant defenses, a state of oxidative stress is generated (Wu et al., 2017; Yang et al., 2020). In parallel, changes in behavioral parameters have been described as endpoints for assessing the neurotoxic effects of drugs such as AEDs. Behavioral studies are essential as they are closely related to individual fitness and population persistence, allowing us to better understand the ecological impacts of pollutants in the aquatic environment (Chen et al., 2021a; Cunha et al., 2017). Furthermore, as PHE is a drug that acts directly on the central nervous system, it is important to assess acetylcholinesterase (AChE) activity. AChE plays an essential role in brain homeostasis by regulating ACh levels, directly influencing neuronal excitability and the release of other neurotransmitters (Bernardo et al., 2019). AChE has been recognized as a central biomarker of neurotoxicity (Métais et al., 2019). Induction and inhibition of its activity have been observed in laboratory and field studies in organisms exposed to pesticides, metals, polycyclic aromatic hydrocarbons, and pharmaceuticals (Chen et al., 2021b; Pullaguri et al., 2021; Ramesh et al., 2020; Severo et al., 2020; Xia et al., 2021).

The advantages of zebrafish (*Danio rerio*) as an animal model include their small size, relatively inexpensive, easy breeding and reproduction, rapid maturity, embryonic transparency, their genome was sequenced entirely (Collin and Martin, 2017). The morphology of *D. rerio* nerves is very similar to that of other vertebrates and is evolutionarily conserved. In addition, neurotransmitter systems are conserved between *D. rerio* and mammals. These features make it possible to use *D. rerio* as an animal model to understand the neurotoxic mechanisms underlying exposure to environmental pollutants.

Although PHE has been reported to cause behavioral and biochemical alterations in the nervous system of some aquatic organisms, these effects have been reported at concentrations higher than those found in different aquatic matrices. Moreover, the neurotoxic effects of phenytoin have been limited to the assessment of isolated endpoints, leaving a significant gap in the relationship between neurobehavioral responses and biochemical-molecular mechanisms. Therefore, herein, we aimed to find an integral or complementary PHE toxicity mechanism in adult zebrafish. The present study hypothesizes that exposure of adult zebrafish to environmentally relevant concentrations of PHE (25, 282, and 1500 ng L^{-1}) can induce biochemical alterations in the zebrafish brain, such as oxidative stress and changes in AChE activity, altering zebrafish behavior.

2. Material and methods

2.1. Chemicals

Phenytoin (purity >99%, CAS number 57–41-0) and DMSO (purity 99.9%) were purchased from Sigma-Aldrich (Mexico). A stock solution of 10 mg L^{-1} PHE dissolved in DMSO (0.5 mL) was prepared in 1 L of deionized water. The concentrations used in this study were obtained from this stock solution. The final concentration of DMSO in the exposure solutions was 0.05% (*v*/v). Unless otherwise indicated, all other reagents were of analytical grade and were purchased from Sigma-Aldrich (St. Louis, MO).

2.2. Test organisms and exposure systems

Six months old *D. rerio* adults (AB strain) were maintained in 50 L glass aquaria supplied with UV-sterilized and dechlorinated water (1 fish/L). Water from aquaria was renewed every other day. The organisms were maintained on a 14:10 h light/dark cycle and fed with a commercial spirulina-based food (Ocean Nutrition, US) twice daily. Moreover, the temperature of the water was always kept constant (27 ± 1 °C). Conductivity 300–1500 μS, pH 7.5; chlorine 25–50 mg L^{-1}, nitrate <50 mg L^{-1}, nitrite <0.1 mg L^{-1}, ammonia <0.02 mg L^{-1}, dissolved oxygen >6.0 mg L^{-1}, and salinity 0.5–1 g L^{-1} were measured in all aquaria to ensure quality of water.

The use of vehicle controls with 0.1% DMSO has been reported to be safe and is widely used in the screening of zebrafish for chemicals (Maes et al., 2012; Vliet et al., 2017). Furthermore, in a recent study conducted in our research group, zebrafish embryos were exposed to a control group with 0.05% DMSO, the concentration used in our study, in which no toxic effects from the solvent were observed (Cardoso-Vera et al., 2022).

For the exposure of fish, twenty-four *D. rerio* adults were allocated into four aquaria of 6 L of capacity. Each aquaria contained six fish and represented each of the PHE concentrations tested in this study (0, 25, 282, and 1500 ng L^{-1}), for each experimental group, exposure was performed in triplicate. Exposure lasted 96 h, and the medium was renewed every other day. Temperature, as well as light/dark cycles, were the same as in the fish maintenance.

2.3. Determination of PHE in water and zebrafish brain

The methodology described by Ahrer et al., 2001 was followed for the determination of PHE in water. PHE concentrations in the exposure medium were measured at the beginning of the test (0 h) and after 96 h of exposure. A water sample (20 mL for each group) was acidified with concentrated hydrochloric acid. 1 mL at a time of the pre-treated sample was extracted by mechanical shaking with 1:1 n-hexane/ethyl acetate for one minute, repeating this procedure twice. Subsequently, the organic phase was evaporated under a stream of nitrogen gas and the residue was subjected to a derivatization procedure for subsequent HPLC analysis. Water samples were analyzed per triplicate.

The PHE concentration in the zebrafish brain was determined according to the method described by Pieróg et al., 2021 with slight modifications. Zebrafish brain was homogenized in a 2:1 water-methanol solution for 20 s using rotor-stator homogenizer (Ultra-turrax T25, IKA, Germany) at 10,000 rpm. The homogenate was centrifuged for 10 min at 10000 rpm (Centurion Scientific K241R, UK). The supernatant (10 μL) was injected directly onto the column for HPLC analysis. The analyses were performed using an Agilent 1260 HPLC system coupled to an API 5500 Qtrap MS with a Turbo V ion spray source. Separation was performed using a Xbridge

C18 column (50 mm × 3.0 mm, particle size 3.5 μm). The mobile phase consisted of acetonitrile and water; acetonitrile was mixed with 50 nM potassium dihydrogen phosphate buffer (pH = 3) in a ratio (*v*/v) of 40:60. Chromatographic analyses were performed at 21 °C and a wavelength of 214 nm. The samples were eluted at a flow rate of 100 μL min^{-1}, and the injection volume was 10 μL. Instrument control, data acquisition, and data processing were performed with Analyst 1.6 software. No interferences were observed in the chromatograms, indicating a high selectivity of the developed method. PHE concentrations were expressed in ng Kg^{-1} tissue. Using the PHE concentrations in the brain samples and in water, the bioconcentration of PHE in the zebrafish brain was assessed using the bioconcentration factor (BCF), which is defined by the following formula: $BCF = C_b/C_w$, where C_b is the concentration of PHE in brain and C_w is the concentration in water. Brain samples were analyzed per triplicate. We performed a five-point calibration curve by spiking ultrapure water with PHE at a concentration ranging from 0 ng L^{-1} to 2000 ng L^{-1}. To confirm the accuracy of the proposed method, we spiked ultrapure water with PHE at three different levels 80%, 100%, and 120%.

2.4. Determination of oxidative stress

After NTT, all fish were immediately euthanized following the hypothermic shock method (2–4 °C) described by Elizalde-Velázquez et al. (2022). Once fish became immobilized due to the hypothermic shock, the brains of all fish were extracted and enclosed in Eppendorf tubes previously re-filled with PBS pH 7.4. For each concentration of PHE (0, 25, 282, and 1500 ng L^{-1}), we got two Eppendorf tubes, each with three brains of zebrafish, which were used to evaluate oxidative stress biomarkers and AChE activity.

In the case of the tube used for oxidative stress, brains were homogenized in an mL of PBS, and the content was then divided into two Eppendorf tubes. Tube 1 enclosed 300 μL of homogenate and the same amount of trichloroacetic acid at 20%. Meanwhile, tube 2 contained only 700 μL of the former. Both tubes were then centrifuged at 11,495 and 12,500 rpm for 15 min, respectively, and the supernatant was used to evaluate the whole oxidative stress biomarkers battery. The level of each biochemical parameter was normalized with respect to its protein content, which was determined by the Bradford method (Bradford, 1976). To measure the level of lipid peroxidation, we followed the procedure described by Buege and Aust, 1978. In consequence, the absorbance was measured at 535 nm, and the result was expressed as nM malondialdehyde (MDA) using the MEC of 1.56×10^5 M^{-1} cm^{-1}. The hydroperoxide content was obtained following the methodology of Jiang et al., 1992. Accordingly, the absorbance was measured at 560 nm, and the result was expressed as nM cumene hydroperoxide (CHP) mg^{-1} protein wet weight. The protein carbonyl level was measured at 366 nm using the protocol described by Levine et al., 1994 with slight modifications (Burcham, 2007; Parvez and Raisuddin, 2005). So, the absorbance was measured at 366 nm, and the results were expressed as nM reactive carbonyls mg protein wet $tissue^{-1}$. The enzymatic activity of superoxide dismutase (SOD) in the whole brain was determined by the methodology described by Misra and Fridovich, 1972. Consequently, the absorbance was measured at 480 nm (30 s and 5 min), and the results were expressed as U SOD mg protein wet $tissue^{-1}$. To determine the enzymatic activity of catalase (CAT), we followed the procedure described by Radi et al., 1991. As a result, the absorbance was measured at 0 and 60 s at 240 nm, and the result was expressed as μM H_2O_2 mg protein wet $tissue^{-1}$. Finally, the glutathione peroxidase (GPx) activity was determined by Flohé and Günzler, 1984. Hence, the absorbance was measured at 340 nm at 0 and 60 s and the results were expressed as UI mg^{-1} protein wet weight. A PBS blank with the same treatment as the samples were used in all assays. Samples from all concentrations were analyzed three times.

2.5. Measurement of D. rerio brain AChE levels

Brains assigned for evaluation of AChE activity were also homogenized in an mL of PBS. Acetylthiocholine (ASCh) was used as a substrate to determine AChE activity. According to the method described by Ellman et al., 1961, the conjugation product between 5,5-dithiobis-2-nitrobenzoic acid (DTNB) and thiocholine (degradation product of ASCh) was measured at 414 nm for 6 min at intervals of 30 s. For enzyme determination, ASCh (0.8 mM) was used in 1.5 mL test solutions with 100 nM phosphate buffer (pH 7.5) and DTNB (1.0 mM). Protein concentration was previously determined (Bradford, 1976) and the linearity of the absorbance with respect to time. Samples from each PHE concentration were evaluated in triplicate. AChE activity was expressed as μM of hydrolyzed substrate per minute per milligram of protein. Samples from all concentrations were analyzed three times.

2.6. Evaluation of behavioral parameters (Novel Tank Test)

Novel Tank Test (NTT), used to assess anxiety and locomotor activity, was carried out as described by Cachat et al., 2013; Faria et al., 2018 with minor modifications. Briefly, experiments were conducted in an isolated and noise-proof behavioral room at 28 ± 1 °C. One hour before the start of the tests, the fish (50:50 ratio of females to males) were brought into the behavioral room so they could acclimate to the new environment. The NTT was carried out in experimental tanks of 20 cm length X 20 cm width X 25 cm height, which contained 7 L of water at a temperature of 28 °C. The standard test was performed by video-recording (MPEG-4 format) the PHE-exposed and control fish for 6 min. After recording, each video was analyzed with ToxTrac version 2.95 Hotfix software. The experimental tank was divided into two equal horizontal virtual zones. For each test, the total distance traveled (cm), the distance the fish traveled at the top and bottom of the tank (cm), the latency (s), the number transitions, the time spent at the top and bottom (s), and the freezing time at the top and bottom were determined. All behavioral experiments were conducted between 8:00 and 13:00 h and on different days.

2.7. Statical analysis

The statistical package Sigma plot 12.5 (Sigma Stat) was used for statistical analysis of the data. Normality and homoscedasticity were checked by Shapiro-Wilk and Bartlett tests, respectively. Once normality and homoscedasticity were verified, a one-way ANOVA was performed, followed by a post hoc test (Student Newman Keuls). Results are presented as mean ± SEM. In addition, a Pearson correlation between the behavioral endpoints and the other biomarkers was performed using R software. All statistical analyses were performed at a significance level of 0.05.

3. Results

3.1. Determination of PHE in water and zebrafish brain

Determination of PHE concentration in zebrafish brain homogenate confirmed that the drug was absorbed after administration and crossed the blood-brain barrier (Table 1). Following above mentioned, PHE concentrations in water samples decreased at 96 hpf compared to nominal concentrations. Thus, PHE was bioconcentrated in the brain. BCF for each of the concentrations were 0.21, 0.34, and 0.46, respectively.

3.2. Oxidative stress biomarkers in the zebrafish brain

Lipoperoxidation (LPX) was significantly increased ($F(3,8) = 168.215$; $p < 0.001$) in all treatment groups compared to the control group (Fig. 1A). Since the LPX increase in the brain of fish was in a concentration-dependent manner, the highest peak was observed at the 1500 ng L^{-1} concentration (3.3 times higher than in the control group). Similar to LPX, oxidative protein damage (PCC) in the brain showed a significant increase ($F(3,8) = 56.883$; $p < 0.001$) in a concentration-dependent manner compared to the control group. Thus, protein carbonylation levels in the groups exposed to 25, 282, and 1500 ng L^{-1} increased significantly 1.4, 1.8, and 4 times, respectively, compared to the control group. Concerning the hydroperoxide

Table 1
PHE concentrations in water at the beginning of exposure and after 96 h of exposure, and PHE concentrations and bioconcentration factor (BCF) in zebrafish brains exposed for 96 h.

Nominal exposure concentrations (ng L^{-1})	Measured concentrations in water (ng L^{-1})		Concentration in zebrafish brain (ng Kg^{-1})	BCF
	0 h	96 h	96 h	
Control	ND	ND	ND	–
25	25.3 ± 0.122	21.5 ± 0.118	4.6 ± 0.301	0.21
282	280.3 ± 2.05	245.3 ± 2.34	83.4 ± 5.56	0.34
1500	1498 ± 9.5	1380 ± 9.0	634.8 ± 3.21	0.46

ND not detected. "–" no data. LOQ: limit of quantification (1 ng L^{-1}). The data are expressed as mean ± SEM.

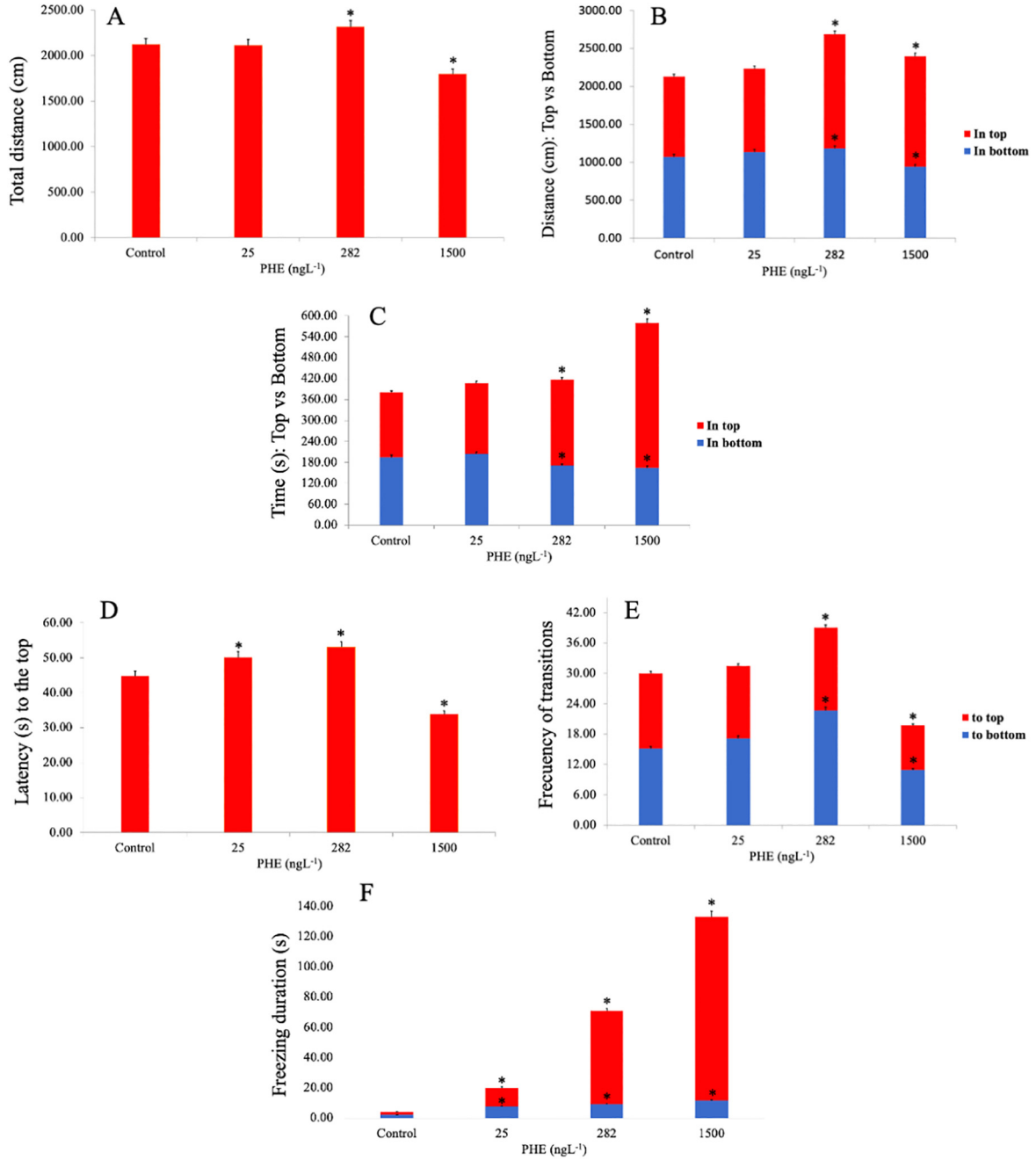

Fig. 1. Oxidative damage biomarkers. Graphs represents the changes in nM MDA/mg protein as a measure of lipid peroxidation (A), μM reactive carbonyls/mg protein as a measure of protein carbonylation (B), and nM CPH/mg protein as a measured of hydroperoxide content (C) in the whole brain of zebrafish following PHE exposure. Values are expressed as mean ± SEM, $N = 18$ per group. Asterisks (*) represent significant differences to the control group ($p < 0.05$).

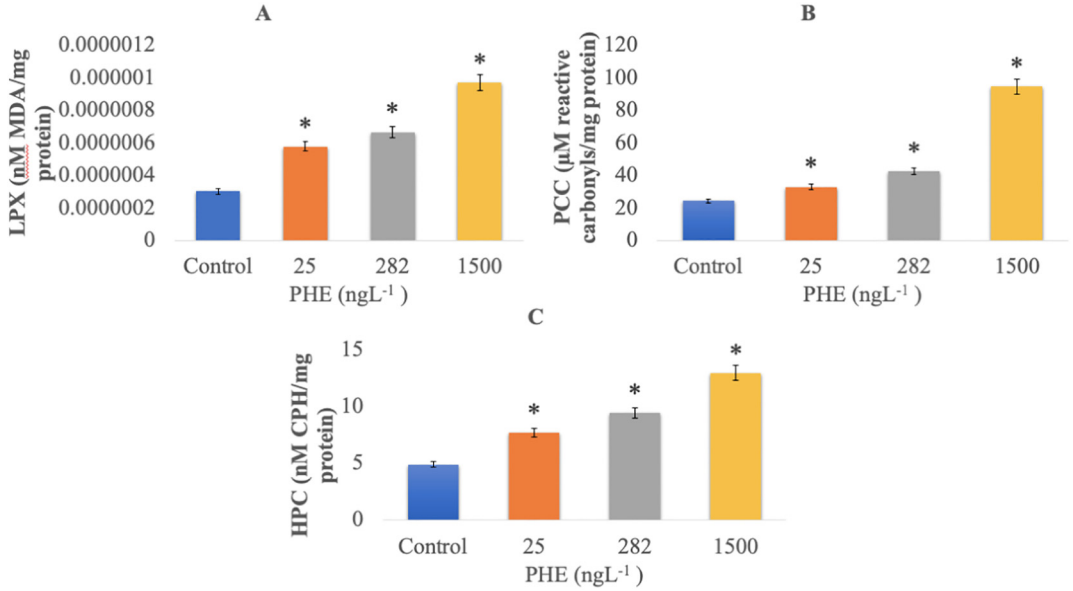

Fig. 2. Antioxidant enzyme activity. Graphs representing changes in the concentration of SOD (IU/mg protein, A), CAT (µM H_2O_2/mg protein, B) and GPx (µM NADPH/mg protein, C) activity in the whole brain of zebrafish following PHE exposure. Values are expressed as mean ± SEM, $N = 18$ per group. Asterisks (*) represent significant differences to the control group ($p < 0.05$).

content (HPC) in the brains of fish exposed to PHE, there was a significant 1.6-, 2.2- and 2.8-fold increase ($F(3,8) = 63.009$; $p < 0.001$) at the concentrations of 25, 282, and 1500 ng L^{-1}, respectively.

The effects of exposure to PHE for 96 h on the activities of antioxidant enzymes showed a similar tendency, with a significant increase in their activity observed in a concentration-dependent manner in exposed fish compared to controls (Fig. 2A-C). SOD activity ($F(3,8) = 65.335$; $p < 0.001$) in the 25, 282, and 1500 ng L^{-1} treatments increased 1.7, 2, and 2.6-fold, respectively, after 96 h treatment than the activity observed in the brain of control fish. Similarly, CAT levels ($F(3,8) = 60.895$; $p < 0.001$) increased 1.9-, 2.5- and 3.1-fold after 96 h of exposure compared to the control group. In contrast, GPx enzyme activity ($F(3,8) = 260.950$; $p < 0.001$) only showed significant differences in the 282 and 1500 ng L^{-1} treatments after 96 h of treatment, increasing 2.2 and 4.1-fold, respectively.

3.3. AChE activity

Exposure to 25 ng L^{-1} PHE did not significantly change AChE activity in the zebrafish brain compared to the control group (Fig. 3). Nonetheless, AChE activity ($F(3,8) = 30.407$; $p < 0.001$) significantly increased in the 282 and 1500 ng L^{-1} PHE exposure groups by 1.5- and 2.6-fold, respectively, after 96 h of exposure.

3.4. Effects of PHE exposure on behavioral parameters

No signs of systemic toxicity (macroscopic morphology or lethality) were observed at the three PHE concentrations tested. At the concentration of 25 ng L^{-1}, no significant differences were observed in the total distance traveled, distance traveled in top and bottom, time spent in top and bottom,

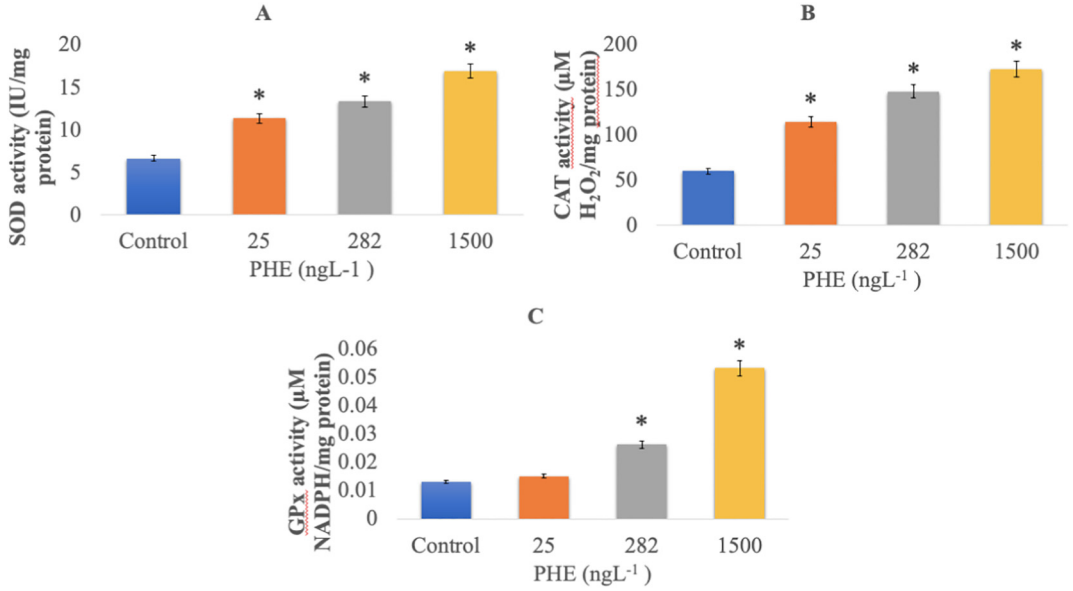

Fig. 3. Acetylcholinesterase (AChE) activity in the brains of zebrafish exposed during 96 h to PHE. Values are expressed as mean ± SEM, $N = 18$ per group. Asterisks (*) represent significant differences to the control group ($p < 0.05$).

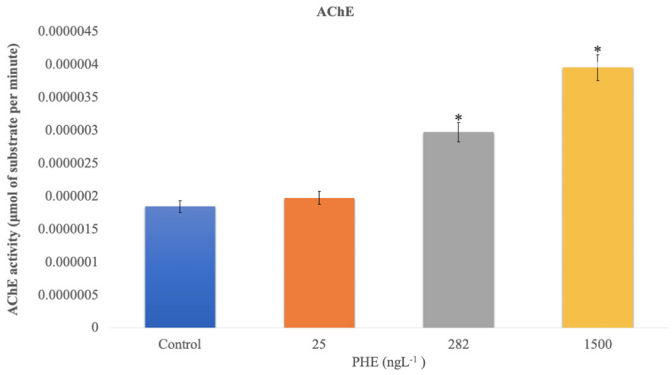

Fig. 4. Effects of PHE on behavioral parameters evaluated in zebrafish. A- total distance traveled, B- distance traveled in the top and bottom, C- number of transitions (top > bottom and bottom > top), D- time spent in the top and bottom, E- latency to top zone entry, and F- time of freezing. Values are expressed as mean ± SEM. Asterisks (*) represent significant differences to the control group ($p < 0.05$).

and frequency of transitions compared to the control group (Fig. 4A-F). However, at the 282 ng L^{-1} and 1500 ng L^{-1} concentrations, we observed significant changes compared to the control group. For instance, fish exposed to 282 ng L^{-1} of PHE showed a significant increase in total distance traveled ($F(3,20) = 47.896; p < 0.001$), distance traveled in top and bottom ($F(3,20) = 34.800; p < 0.001$), time spent in top ($F(3,20) = 1203.071; p < 0.001$), latency to enter the top ($F(3,20) = 19.620; p < 0.001$), number of transitions to the bottom ($F(3,20) = 46.722; p < 0.001$), and freezing duration in the top ($F(3,20) = 1245.709; p < 0.001$) compared to the control group. Unlike the 282 ng L^{-1} concentration, fish exposed to the highest concentration of PHE showed a significant decrease in the total distance traveled ($F(3,20) = 34.678; p < 0.001$), latency to enter the top ($F(3,20) = 14.356; p < 0.001$), number of transitions to the top and the bottom ($F(3,20) = 18.487; p < 0.001$), time spent in the bottom ($F(3,20) = 27.371; p < 0.001$), and distance traveled in the bottom ($F(3,20) = 28.089; p < 0.001$) compared to the control group.

3.5. Pearson correlation between oxidative stress biomarkers and AChE levels with behavioral changes

The correlation between oxidative stress biomarkers, AChE levels, and BCF concerning behavioral changes in zebrafish exposed to PHE for 96 h is depicted in Fig. 5. The colors show the strength of the correlation of variables has with each other; as the intensity of the color increases, the correlation between the variables becomes stronger. The blue indicates a positive correlation between the variables, while the red indicates a negative correlation. A positive correlation can be observed between all oxidative stress biomarkers and our results of AChE activity. Moreover, AChE activity and oxidative stress biomarkers showed a strong positive correlation with the distance fish traveled at the top, the time they spent at the top, the time they remained frozen, and the amount of PHE that their brain bio concentrated. However, it is noteworthy to indicate that the correlation between oxidative stress biomarkers and AChE with all other behavioral endpoints was negative.

4. Discussion

The present study aimed to assess the effects of PHE exposure in adult zebrafish specimens, using a multi-biomarker study where behavioral parameters, AChE activity, and six biomarkers of oxidative stress were evaluated in the zebrafish brain exposed PHE for 96 h.

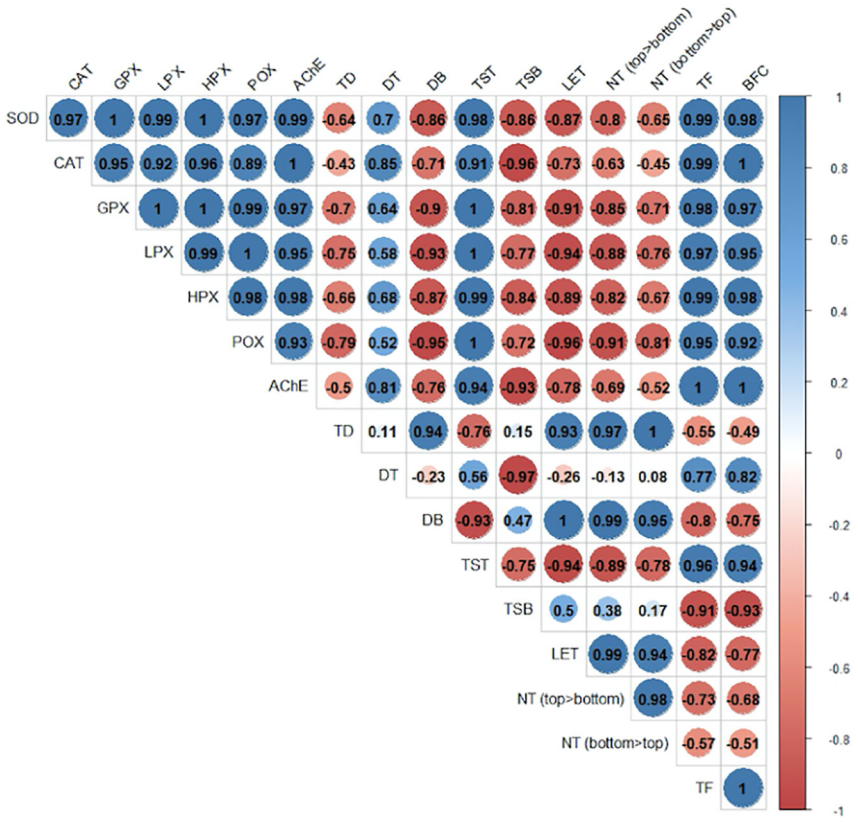

Fig. 5. Correlation between oxidative stress biomarkers, AChE levels and BCF with behavioral changes. TD (total distance), DT (distance in top), DB (distance in bottom), TST (time spent in top), TSB (time spent in bottom), LET (latency to enter the top), NT (number of transitions) AND TF (time frozen).

4.1. Biochemical endpoints

The brain is particularly vulnerable to damage caused by oxidative stress, mainly due to three factors: high oxygen consumption, low concentration of antioxidant defenses, and its high content of polyunsaturated fats susceptible to oxidation (Cobley et al., 2018). Biological molecules are prone to constant oxidation, which causes structural change and consequently a loss of function. These relatively stable modifications are used as markers of oxidative stress (Cenini et al., 2019). PHE is neurotoxic to rodents, showing reduced brain weight (Hatta et al., 1999), impairment of the cerebellar system and hippocampus, and behavioral deficits in learning tasks and hyperactivity (Hatta et al., 1999; Inman et al., 1999; Vorhees and Minck, 1989). Our data showed that exposure to PHE for 96 h has effects in lipid peroxidation (MDA), protein carbonylation, and hydroperoxide content, as the content of these biomarkers increased in a concentration-dependent manner in all three PHE exposure groups relative to the control group. It has been reported that reactive oxygen species are produced during PHE biotransformation, resulting in increased levels of lipid lipoperoxidation (Liu et al., 1998), protein carbonylation, and even damage to nucleic acids (Kasapinovic et al., 2004; Mahle and Dasgupta, 1997). Similarly, PHE acted as an inducer of oxidative stress in chicken eggs, showing increased lipid peroxidation in the liver and brain of embryos exposed to the drug (Hilscherova et al., 2003). In addition, an increase in MDA formation, translated into lipid peroxidation, was observed in rodents chronically exposed to PHE (Reeta et al., 2009). In contrast to our findings, Brandão et al., 2013 did not observe any oxidative damage in the liver, digestive, and gill tissue of fish (*Lepomis gibbosus*) exposed to PHE. On the contrary, they observed a significant decrease in TBARS levels at a concentration of 25 μg L^{-1} in the gills.

Oxidative stress has been considered the first response of aquatic organisms to the action of environmental stressors (Livingstone, 2001). Parallel activation of the cell's antioxidant defense system reduces the generation of ROS. In addition, it has been reported that zebrafish brain cells can stimulate antioxidant defenses to counteract the attack of environmental pollutants (Richetti et al., 2011; Sarkar et al., 2014). Antioxidant enzymes, such as SOD, CAT, and GPx, play an essential role in cellular protection against oxidative damage. In the present study, the increase in the levels of reactive oxygen species, which is reflected in damage to biomolecules, induced a significant increase in the activity of SOD, CAT enzymes at the three concentrations tested and a significant increase in GPx activity at the concentrations of 282 and 1500 ng L^{-1}. Similar results have been reported by Brandão et al., 2013, who reported that PHE exposure caused a significant increase in CAT and glutathione S-transferase (GST) activities in sunfish (*Lepomis gibbosus*) liver homogenates. In addition, (Abramov and Wells, 2011) exposed transgenic mice expressing human CAT to PHE. Results showed 2-fold elevations in embryonic and fetal brain catalase activities, demonstrating that catalase protects embryos from both physiological and PHE-generated stress. These data and those obtained in the present study are consistent with the proposal that PHE-induced neurotoxicity may be due to the generation of reactive intermediates resulting from enzymatic bioactivation of the drug. Our results show that the level of enzyme induction in the zebrafish brains exposed to PHE was insufficient to ensure detoxification of the reactive oxygen species and reactive intermediates generated, resulting in a state of oxidative stress.

Several studies have shown that acetylcholine is related to anxiety and fear behaviors, as brain areas involved in anxious traits exhibit high cholinergic drive (Ferlemi et al., 2014; Imam et al., 2018; Pidoplichko et al., 2013; Vasilopoulou et al., 2016). In the present study, we observed that concentrations of 282 and 1500 ng L^{-1} PHE caused an up-regulation in AChE activity, which we can relate to a PHE-induced toxicity condition. These results agree with those obtained by Shahid et al., 2004, who observed a significant increase in AChE activity in the brain of mice after administration of 12 mg kg^{-1} PHE compared to control. The increase in AChE activity is associated with increased degradation of acetylcholine and decreased stimulation of neurotransmitter receptors, causing adverse effects on motor and cognitive functions (Aldenkamp and Vermeulen, 1995; Braakman et al., 2017; Gupta et al., 2013). Contrary to our findings, Sudha et al., 1995, reported a reduction in AChE activity in the hippocampus and striatum of rats treated with therapeutic doses of PHE (75 mg Kg^{-1}), attributing impaired learning, memory, and cognitive functions to the neurochemical changes caused by PHE. Moreover, Mishra and Goe, 2015 observed that 30 mg kg^{-1}PHE treatment harmed cognitive performance in mice, attributing this effect to the persistent increase in AChE activity, which leads to a reduction in acetylcholine and thus reduces synaptic plasticity and memory. Finally, in the present study, we can attribute the induction in AChE activity to the behavioral and neurotoxic effects induced by PHE. This increase could be influenced by excessive free radical production and oxidative stress state (Mohan and Krishna, 2018; Pestana et al., 2010), causing rapid degradation of the neurotransmitter acetylcholine and consequently a decrease in receptor stimulation. In addition, previous studies have shown that lipid membrane modifications, evidenced in our study by increased lipoperoxidation in zebrafish brains exposed to PHE, may be responsible for inducing AChE activity (Senger et al., 2011).

4.2. Behavioral endpoints

Behavioral parameter analysis has been considered a valuable tool in assessing the effects of chemicals. Performing behavioral analysis in zebrafish does not require expensive specialized equipment or complex toolkits or reagents, and inducing behavioral responses in zebrafish is easier than in rodents (Gerlai, 2020). In addition, the assessment of zebrafish behavior was chosen because the regulation of zebrafish behavior is related to the interaction between the central and peripheral nervous systems and the neuroendocrine system (Matsuda et al., 2011; Zhang et al., 2013). A widely used method for assessing stressful conditions in zebrafish is the novel tank test (NTT) (Levin et al., 2007). Adult zebrafish are reported to spend about 50% of a 5-min session at the bottom of the tank, “bottom-dwelling”, and nicotine is reported to decrease this preference. Drugs (anxiolytics) such as buspirone, diazepam, and fluoxetine have also been reported to decrease this preference (Maximino et al., 2012). In this study, the locomotor activity of adult zebrafish was not affected. Studies by Berghmans et al., 2007 and Martinez et al., 2018 in zebrafish larvae exposed to PHE also demonstrated this drug did not affect locomotor activity. Furthermore, Tonelli et al., 2013 demonstrated that PHE in the range of 1–50 mg kg^{-1} does not affect spontaneous locomotor activity. Zebrafish exposed to 282 ng L^{-1} showed an increase in total distance traveled. On the other hand, fish exposed to 1500 ng L^{-1} showed a decrease in total distance traveled, latency and top transitions, and an increase in time spent at the top. These results suggest that PHE has anxiolytic properties without producing motor impairment in zebrafish. Our results agree with those of Pieróg et al., 2021, who performed a color preference test, observing an anxiolytic effect of PHE in adult zebrafish. The authors observed an increase in the number of zebrafish entries in the yellow aversive zone versus the red preference zone. Moreover, Dubey et al., 2015 demonstrated a similar anxiolytic effect in zebrafish exposed to 450 μM of PHE.

The effect of PHE on the behavioral response of zebrafish larvae has been evaluated at different doses and illumination conditions (Liu et al., 2016). The results show that under light or dark conditions, PHE exerted a stimulatory effect at low doses but an inhibitory effect at high doses on zebrafish larvae. It can be compared with the results obtained in our study. For example, at the concentration of 282 ng L^{-1}, a stimulatory effect was observed, while zebrafish exposed to 1500 ng L^{-1} showed a decrease in the activity. Although the common use of PHE is as an antiepileptic drug, it has been reported to have diverse neuroactivities and has been tested as a treatment in diseases such as bipolar disorder (Patocka et al., 2020), neuropathic pain (Hall et al., 2020), optic neuritis (Raftopoulos et al., 2016). This suggests that the results found in the present work may be related to different pathways at the molecular level. Alterations in the gamma-aminobutyric acid (GABA) ratio to glutamate has been reported to influence neuronal excitability (Guerriero et al., 2015). The main inhibitory neurotransmitter in the brain is GABA, and it seems to be involved in

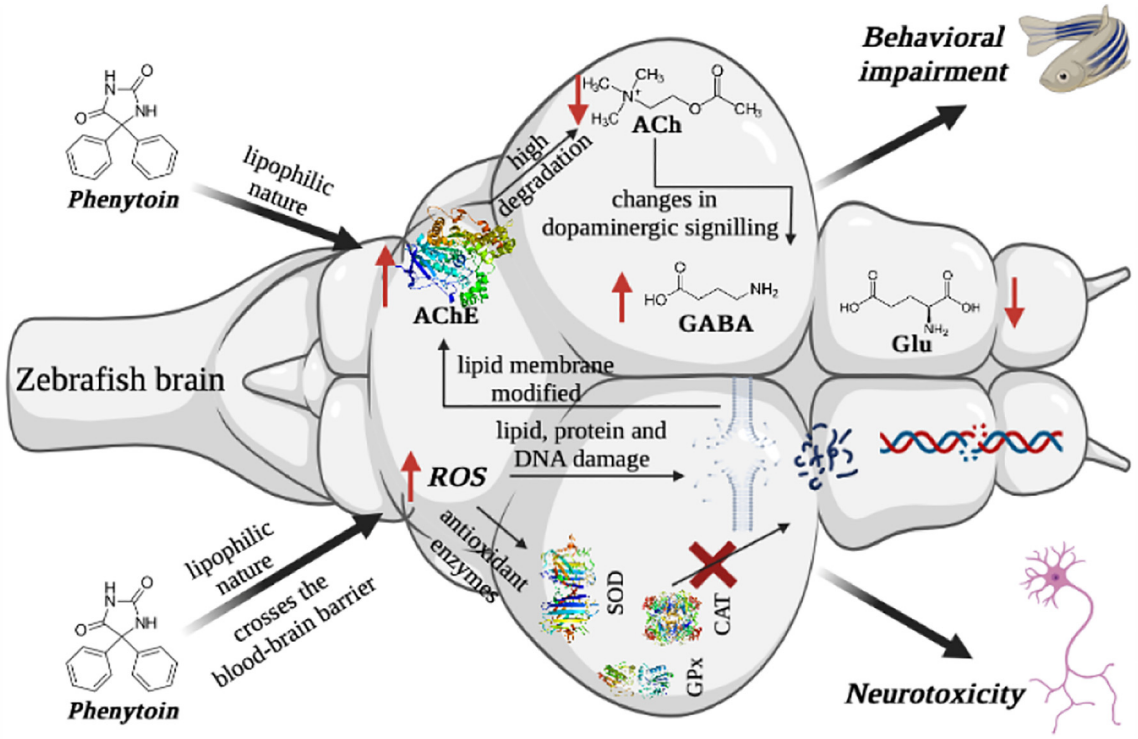

Fig. 6. Potential mechanism by which PHE induces neurotoxicity in zebrafish. The brain is particularly vulnerable to oxidative damage because of its high oxygen consumption, low concentration of antioxidant enzymes and high content of polyunsaturated fats. PHE crosses the blood-brain barrier and undergoes biotransformation processes, generating reactive intermediates, free radicals and reactive oxygen species (ROS). Increased levels of oxidative molecules induce the activity of antioxidant enzymes; suggesting adaptive changes to the oxidative stress state. However, antioxidant enzyme induction is insufficient to ensure detoxification of ROS and the reactive intermediates generated. This leads to increased levels of lipoperoxidation, protein carbonylation and hydroperoxide content. When biomolecules are oxidized, this causes a structural change and, consequently, a loss of function. On the other hand, PHE may be acting as an agonist/antagonist at the dopaminergic receptor, which plays a crucial role in the regulation of locomotor activity. In addition, PHE has effects on the relationship between gamma-aminobutyric acid (GABA) and glutamate (Glu), which gives it its anxiolytic and anti-seizure effects. Finally, AChE is closely related to sensory and locomotor functions. Increased AChE activity is associated with high acetylcholine degradation and a decrease in neurotransmitter receptors, causing adverse effects on motor and cognitive functions. Modifications in lipid membranes may be responsible for the induction of AChE activity (Liu et al., 2016; Mohan and Krishna, 2018; Cobley et al., 2018).

the pathogenesis of anxiety, as drugs such as benzodiazepines (GABA receptor stimulants) have anxiolytic and anticonvulsant effects, while blocking the GABA receptor causes episodes of severe anxiety (Liu et al., 2016). This suggests that PHE may be altering the relationship between GABA and glutamate in the zebrafish in our study (Fig. 6).

5. Conclusion

Exposure of *Danio rerio* to PHE altered brain activities of antioxidant enzymes, AChE, and behavioral parameters assessed. Exposure of fish to environmentally relevant concentrations of the drug increased biomarkers of oxidative damage in a concentration-dependent manner. In addition, oxidative stress may be responsible for increasing AChE levels, potentially leading to cognitive and memory impairment, which was not assessed in the study. Future chronic behavioral studies are suggested to verify this possibility. Therefore, this study contributes to elucidating the effects of PHE at environmentally relevant concentrations in the zebrafish brain and the repercussions on the behavior of this organism, therefore of great ecological relevance.

CRediT authorship contribution statement

JDCV, JMOH, GHG and KERP performed all the exposure experiments.
LMGO and JDCV were involved in the conception.
LMGO, JDCV, HIF, SGM and GAEV were involved in the design and interpretation of the data and the writing of the manuscript with input from MGM.

Declaration of competing interest

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.

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