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Acute exposure to environmentally relevant concentrations of phenytoin damages early development and induces oxidative stress in zebrafish embryos

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ABSTRACT

Phenytoin (PHE) is an antiepileptic drug that has been widely used in clinical practice for about 80 years. It is mainly used in the treatment of tonic-clonic and partial seizures. The widespread consumption of this drug around the world has led to PHE being introduced into water bodies through municipal, hospital, and industrial effluent discharges. Since the toxic effects of this drug on aquatic species has been scarcely explored, the aim of this work was to investigate the influence of low (25–400 ngL^{-1}) and high (500–1500 ngL^{-1}) environmentally relevant concentrations of PHE on the development and oxidative status of zebrafish (*Danio rerio*) embryos. The toxicity of PHE was evaluated from 12 to 96 h after fertilization in *D. rerio* at concentrations between 25 and 1500 ngL^{-1}. In both the control group and the 0.05% DMSO system, no malformations were observed, all embryos developed normally after 96 h. The severity and frequency of malformations increased with increasing PHE concentration compared to embryos in the control group. Malformations observed included developmental delay, hypopigmentation, miscellaneous (more than one malformation in the same embryo), modified chorda structure, tail malformation, and yolk deformation. Concerning the biomarkers of oxidative stress, an increase in the degree of lipid peroxidation, protein carbonylation, and hydroperoxide content was observed ($p < 0.05$) concerning the control. In addition, a significant increase ($p < 0.05$) in antioxidant enzymes (SOD, CAT, and GPx) was observed at low exposure concentrations (25–400 ngL^{-1}), with a decrease in enzyme activity at high concentrations (500–1500 ngL^{-1}). Our IBR analysis demonstrated that oxidative damage biomarkers got more influence at 500ngL^{-1} of PHE. The results demonstrated that PHE may affect the embryonic development of zebrafish and that oxidative stress may be involved in the generation of this embryotoxic process.

1. Introduction

The global prevalence of epilepsy is reported at 700 per 100,000 people, affecting >70 million people worldwide (Espinosa-Jovel et al., 2018). The prophylactic use of antiepileptic drugs (AEDs) is considered the primary treatment for epilepsy (Dasgupta and Krasowski, 2020).

Unlike other drugs, AEDs are not classified by their mechanism of action. They can often be classified according to the year in which they were developed and introduced (generation). This is because drugs are not fully elucidated in their mechanism of action and may have several modes of action (Perucca, 2005). The "first generation" AEDs (1912–1978) include carbamazepine, ethosuximide, phenobarbital, primidone, valproate, and PHE (Cotterman-Hart, 2015). PHE is a drug that has been widely used in clinical practice for about 80 years to treat tonic-clonic and partial seizures (Abou-Khalil, 2016; Patocka et al., 2020). PHE binds to the inactivated state of the Na^{+} channel and thus prolongs the neuronal refractory period, having a highly selective inhibitory effect in the motor area of the cerebral cortex. In addition,

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Table 1
Occurrence of PHE in aquatic matrices.

Country	Occurrence media	Concentration (ngL^{-1})	References
U. S	Wastewater	287–402	(Vanderford and Snyder, 2006)
Korea	Surface water	1.1–8.9	(Kim et al., 2007)
Japan	River	4	(Hoshina et al., 2009)
Korea	Surface water	1.8–54	(Yoon et al., 2010)
Spain	Drinking water	10	(Huerta-Fontela et al., 2011)
U. S	Surface water	150	(Laws et al., 2011)
Saudi Arabia	WWTP	<20–440	(Alidina et al., 2014)
U. S	Well water	66	(Schaider et al., 2014)
U. S	Hospital effluent	60–100	(Oliveira et al., 2015)
Japan	Rivers and drinking water	3.1–23	(Simazaki et al., 2015)
U. S	Urban watersheds	145	(Bai et al., 2018)
Spain	WWTP	111–2375	(Mijangos et al., 2018)
Spain	Estuary	6–1401	(Mijangos et al., 2018)
Sri Lanka	Surface water	6.4–79	(Guruge et al., 2019)
China	Tap water	1.93	(Liu et al., 2019)

PHE is thought to exert its antiepileptic effect by stabilizing brain cell membrane function and increasing levels of inhibitory neurotransmitters (serotonin [5-HT] and γ-aminobutyric acid [GABA]) in the brain (Keppel Hesselink, 2017).

In addition, the safety and efficacy of PHE have been evaluated in >100 different disorders (Keppel Hesselink and Kopsky, 2017), from bipolar disorder (Patocka et al., 2020), wound healing (Keppel Hesselink, 2018; Kumar et al., 2020) bronchial spasms induced by bronchial asthma and chronic asthmatic bronchitis (Zhang et al., 2018), neuropathic pain (Hall et al., 2020), anti-arrhythmic (Zhang et al., 2018), to reduce migration and invasion in metastatic breast cancer (Kalaiarasi et al., 2018) and optic neuritis (Raftopoulos et al., 2016).

In conventional status epilepticus treatment protocols, PHE is considered the first-line drug when the patient does not respond to benzodiazepines in approximately 40–60% of cases. (Brigo et al., 2018; Li et al., 2020; Mishra et al., 2014; Nalisetty et al., 2020). Although new AEDs have been approved to treat status epilepticus in recent years, PHE remains the first-line drug in many countries, including Thailand (DeMott et al., 2020; Soontornpun et al., 2020). In the United States, according to the annual Medical Expenditure Panel Survey conducted by the Agency for Healthcare Research and Quality (AHRQ), PHE is in the top 300 drugs of 2018, ranking 269th. According to this survey, prescriptions in 2018 were approximately 1,643,497 (Kane, 2018). According to these data, PHE is widely consumed globally, further contributing to introducing this AED into water bodies through discharges of municipal, industrial, and hospital effluents.

Several studies have reported PHE in wastewater treatment plant (WWTP) effluents, hospital effluents, surface water, and even drinking water. These concentrations range from 1.1 to 2375 ngL^{-1} in different aquatic matrices, as shown in Table 1.

Disruption of cellular and/or molecular processes during normal physiological development can lead to toxicity as teratogenesis or mortality. Redox regulation, protein folding, and apoptosis play essential roles during normal development, and when these processes are disturbed by toxicants (endogenous or exogenous), they can lead to a wide range of malformations and even death (Kupsco and Schlenk, 2015). The oxidative stress has been associated with the pathogenesis of various abnormalities; within these, skeletal malformations and cardiovascular defects (Kovacic and Somanathan, 2014). ROS have an important role in embryonic development, since its can be to alter embryonic signal transduction pathways (Dennery, 2007; Wells et al., 2005).

Studies reported in the literature on the toxic effects of PHE on aquatic organisms are scarce. PHE toxicity was evaluated in *Lepomis gibbosus*, exposed to 6.25, 12.5, 25, 50, and 100 μgL^{-1}, focusing on oxidative stress parameters in the liver, digestive, and gill tissues. The results showed adaptive responses in catalase and glutathione S-transferase enzyme activities in the liver of the exposed organisms in response to the oxidative stress state (Brandão et al., 2013). Liu et al. (2016), exposed 5-day post-fertilization zebrafish larvae to 1,4,20,100 and 500 μM PHE. The results suggest a concentration-dependent effect on locomotor activity and thigmotaxis. Furthermore, at high concentrations, PHE caused a significant decrease in locomotor activity and thigmotaxis, and treatment with low doses increased locomotor activity. In a rehearsal conducted by Weigt et al. (2011), the teratogenicity of 10 compounds (including PHE) was evaluated in zebrafish embryos exposed for three days to concentrations of 31.25, 62.5, 125, and 250 μM. The LC_{50} and EC_{50} values were >250 μM and 386 μM, respectively, with a teratogenic index >1. Likewise, the teratogenic effect of PHE was evaluated in zebrafish exposed to 6.25, 12.5, 25, 50, and 100 μM, monitoring hatching, and morphology. Exposure of embryos to PHE induced a developmental delay and a teratogenic effect in a concentration-dependent manner reflected in hatching time and embryo morphology (Martinez et al., 2018).

Zebrafish has emerged as an excellent vertebrate model for lethality and teratogenicity testing due to the following advantages: the maintenance of a zebrafish colony is simple, they occupy little space, are inexpensive and produce a high number of embryos (high fecundity), development is rapid and can be visualized from embryogenesis to the complete organism (Kimmel et al., 1995), toxicity during development can be assessed in a short time, the embryos are optically transparent, which allows teratogenicity tests to be carried out thoroughly, as their developmental pathways are evolutionary conserved, which makes zebrafish very sensitive to teratogenic compounds (Raghunath and Perumal, 2018).

According to the background mentioned above and since PHE has been shown to generate oxidative stress and teratogenesis at concentrations above those found in aquatic environments in different hydrobionts. In addition, embryogenic effects have been limited to the evaluation of limited endpoints. In light of filling the current knowledge gaps on the embryotoxicity effects that PHE at environmentally relevant concentrations can induce in aquatic species, we conducted a study in *D. rerio* embryos. We hypothesized that environmentally relevant concentrations of PHE are capable of disrupting zebrafish embryonic development through an oxidative stress mechanism.

2. Material and methods

2.1. Ethical approval

This research protocol was reviewed and approved by the Ethics and Research Committee of the Autonomous University of the State of Mexico (UAEM) to ensure that experiments were conducted following institutional standards for animal care (approval ID: RP.UAEM.ERC.142.2021). The provisions of the Mexican official standard on the breeding, care, and use of laboratory animals (NOM-062-ZOO- 1999) were also considered.

2.2. Chemicals

All reagents were of analytical grade and were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise indicated. PHE (>99% purity, CAS No 57-41-0) was purchased from Sigma-Aldrich (St. Louis, MO). The stock solution was prepared with 10 mg PHE dissolved in DMSO (99.9% purity, Sigma-Aldrich) in 1 L deionized water. The other PHE concentrations tested were prepared from this stock solution, the final concentration of DMSO in the exposure solutions was 0.05% (v/v).

2.3. Animal husbandry and collection of eggs

Adult zebrafish (*D. rerio*, Wild-type, AB strain) were maintained at the Autonomous University of State of Mexico (Toluca, Mexico) under laboratory conditions. Zebrafish were maintained in 120 L aquaria, 1 L of water per fish, at a temperature of 28 ± 1 °C, with 14/10 h light/dark cycles. The fish were fed with Spirulina flakes (Ocean Nutrition, US) twice a day and *Artemia* sp. *nauplii* larvae once a day. The aquarium water was constantly aerated and maintained at a pH of 7.5–8. Embryos were obtained from natural mating. For oocyte collection, fish were transferred to a spawning chamber the evening before the start day of the experiments, in a ratio of 2 females: 1 male. Eggs were allowed to mate and spawn, and oocytes were collected in clear glass dishes 1 h after spawning.

2.4. Toxicity exposure protocol

In the present study, OECD test guideline 236 (OECD, 2013), an acute fish embryo toxicity (FET) test with zebrafish (*D. rerio*), was followed. The concentrations tested of PHE (1500, 1400, 100, 500, 440, 287, 150, 78, 54 and 25 ngL^{-1}) were selected for their environmental relevance (Table 1). In addition to the control group, a system with 0.05% DMSO was set up. Viable oocytes were selected with the aid of a stereomicroscope and incubated in 24-well plates. Oocytes were incubated individually in 2 mL of medium, forming batches of 24 oocytes for each concentration tested, each concentration was tested in triplicate. The microplates were placed in an incubator at 28 ± 1 °C for four days, with 14:10 h light/dark cycles. To detect alterations to embryonic development and/or embryo death due to PHE, stereomicroscopic observations were made at 12, 24, 48, 48, 72, and 96 hpf.

After 96 hpf of exposure, alive, dead, and malformed embryos were counted. With the data obtained, a maximum likelihood linear regression analysis was performed to calculate the mean lethal concentration (LC_{50}) and the effective malformation concentration (EC_{50}) with their 95% confidence intervals ($p < 0.05$). With these data, the teratogenic index (TI) was calculated using the LC_{50}/EC_{50} ratio.

2.5. Embryonic development evaluation

Based on the visible morphology of the embryos, the evaluation of structural abnormalities and developmental delay of the embryos exposed to PHE was carried out by comparison with a reference embryo, as follows Kimmel et al. (1995). The factors assessed were: tail development, eye development, somite formation, movement, blood circulation, head-body pigmentation, tail pigmentation, pectoral fin emergence, mouth protuberance, and hatching; all of which are assigned a value according to Hermsen et al. (2011), and one unit was not scored for each abnormality or delay in any of the characteristics to be assessed.

2.6. Determination of oxidative stress in D. rerio embryos

Ten environmentally relevant concentrations of BPA (1500, 1400, 100, 500, 440, 287, 150, 78, 54, and 25 ngL^{-1}) were tested, each with 1600 *D. rerio* embryos (4hpf) divided into eight glass Petri dishes (30 mL of solution), to ensure enough surviving larvae for each trial. The control groups (control solution and 0.05% DMSO) contained 900 embryos (4hpf) each. Three replicates were performed for each group. During the exposure period, all treatments were maintained at 28 ± 1 °C with photoperiods of 14 h light and 10 h dark. Half of the solution of each treatment was renewed daily to maintain the appropriate PHE concentration. After 72 and 96 h of PHE exposure, about 600 embryos were homogenized in 1 mL of ice-cold phosphate buffer solution (PBS, pH 7.4). The homogenate was separated into two tubes and proceeded as follows: tube 1, 300 μL of homogenate and 300 μL of a 20% trichloroacetic acid (TCA) solution, centrifuged at 11495*g* at 4 °C for 15 min, the precipitate was used to determine the protein carbonyl content (PCC), and the supernatant was used to determine the level of lipoperoxidation (LPX) and the hydroperoxide content (HPC); tube 2, 700 μl of the homogenate was centrifuged for 15 min at 12500*g* at 4 °C, the supernatant was used to determine the activity of the enzymes superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) and total protein content. The level of each biochemical parameter was normalized to its protein content.

2.6.1. Evaluation of cellular oxidation

The level of lipid peroxidation was assessed following the procedures reported by Buege and Aust (1978). 50 μL of supernatant from tube 1, 450 μL of Tris-HCl solution (150 mM), and 1 mL of trichloroacetic acid-thiobarbituric acid (TCA-TCB) solution were mixed in glass tubes. Heat shock was produced by immersing the tubes in boiling water. The tubes were incubated for 30 min at 37 °C. They were then centrifuged at 3500*g* for 10 min. The absorbance was measured at 535 nm.

The hydroperoxides content was evaluated with the technique of Jiang et al. (1992), 100 μL of the supernatant from tube 1 and 900 μL of the reaction mixture (10 mL of solution A ($FeSO_4 + H_2SO_4$) and 20 mL of solution B (dehydroxytoluene butylate + xylenol orange) were mixed in a tube, left to stand for 60 min at room temperature and protected from light. The absorbance was measured at 560 nm.

The level of protein carbonyl was quantified using the method of Levine et al. (1994) modified by Burcham (2007); Parvez and Raisuddin (2005). Briefly, precipitate from tube 1, and 150 mL of dinitrophenylhydrazine (DNPH)/HCl 10 mM were mixed and left to stand for one hour in the dark at room temperature. After that time 500 ML of ATC (20%) was added, the tubes were incubated at 4 °C for 15 min before centrifugation at 11000*g* for 5 min. The supernatant was discarded, and the button was washed with a 1:1 ethanol: ethyl acetate solution to obtain a white button dissolved in 1 mL of 6 M guanidine and incubated for 30 min at 37 °C. The absorbance was measured at 366 nm. A PBS blank with the same treatment as the samples were used in all assays.

2.6.2. Antioxidant activity assays

The activity of SOD in embryos was assessed following the protocols of Misra and Fridovich (1972). For the assay, 40 μL of the supernatant from tube two was added directly into the cell, 260 μL of carbonate buffer and 200 μL of adrenaline (30 nM) were added, absorbance was measured at 480 nm (30 s and 5 min).

CAT activity was measured with the method of Radi et al. (1991). 30 μL of supernatant from tube two was placed directly into the cell, 420 μL of isolation buffer (0.3 M sucrose, 1 mM EDTA, 5 mM HEPES and 5 mM KH_2PO_4) and 300 μL of hydrogen peroxide (20 mM) were added, absorbance was measured at 240 nm (0 and 60 s).

The activity of GPx in whole embryos was ascertained using the technique of Flohé and Günzler (1984). 100 μL of the supernatant from tube two was placed directly into the cell, 290 μL of reaction buffer, 100 μL of hydrogen peroxide (20 mM), and 12 μL of glutathione reductase were added, absorbance was measured at 340 nm (0 and 60 s). A PBS blank with the same treatment as the samples were used in all assays.

2.6.3. Determination of total protein content

Total protein content was determined spectrophotometrically by the method described by Bradford (1976). 13 μL of supernatant were taken from tube two and mixed with 75 μL of distilled water and 1.25 mL of Bradford's reagent. This mixture was homogenized in a Vortex for one minute, protected from light and incubated at room temperature for 5 min. The absorbance was measured at 595 nm, using a PBS blank with the same treatment as the samples. The absorbances obtained were extrapolated to a standard curve (bovine serum albumin) to determine the total protein concentration.

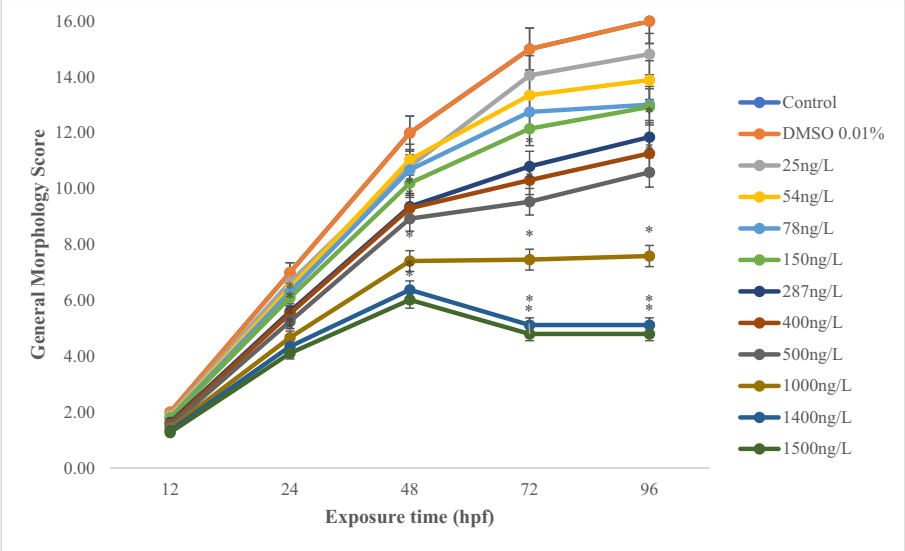


Fig. 1. Exposure time-response curve of the general morphology score according to different concentrations of PHE on *D. rerio* embryos. Values are the mean of three replicates ± SEM. *Significantly different from control group ($p < 0.05$).

2.7. Phenytoin determination

Water withdrawn daily for renewal of the exposure medium was stored in amber glass bottles. At that time, the pH was adjusted to 2 with concentrated hydrochloric acid for preservation and stored at 4 °C until extraction. No >14 days were allowed to elapse from collection to extraction. Samples were collected from 0, 12, 24, 48, 72, to 96 h of exposure. PHE determination was performed according to the methodology described by Ahrer et al. (2001). An HP 110 HPLC system equipped with an HP 1050 autosampler (Agilent, Palo Alto, CA) was used for liquid chromatographic separations. A flow rate of 150 mL/min and a ternary gradient of 20 mM ammonium acetate solution adjusted to pH 5.5 with 1 M acetic acid (A), methanol (B), and water (C) at different ratios (10:15:75, 10:50:40, and 10:90:0) was used from 0 to 20 min. An HP 5989B quadrupole system (Agilent) equipped with a radiofrequency hexapole (Analytica of Bradford, CT) using an HP 59987A pneumatically assisted electrospray ionization interface (Agilent) was used. The drying gas-flow rate was 7 L/min. Nitrogen 5.0 was used for nebulization at a pressure of 550 kPa.

2.8. Integrated Biomarker Response (IBR)

Biomarkers measured in *D. rerio* embryos were used to calculate the Integrated Bioindicator Response Index (IBR). In the present study, the biomarkers used were SOD, CAT, GPx, LPX, PCC, and HCP. According to Beliaeff and Burgeot (2002), the IBR index was obtained and modified by Sanchez et al. (2013), which was represented in star graphs. The procedure was as follows: a comparison was made between the individual data of each biomarker (X_i) against reference data (X_0), and a logarithmic transformation reduced the variance.

$$Y_i = log\ (X_i/X_0)$$

For Yi, the mean (μ) and standard deviation (σ) were calculated, and standardization was performed.

$$Z_i = (Y_i - \mu)/\sigma$$

A biomarker deviation index (A) was defined as the mean of the standardized biomarker response (Zi) and the mean of the reference biomarker data (Z_0).

$$A = Z_i - Z_0$$

Finally, to obtain the IBR index, the absolute value of the A-parameters calculated for each biomarker was summed.

$$IBR = \Sigma|A|$$

2.9. Statistical analysis

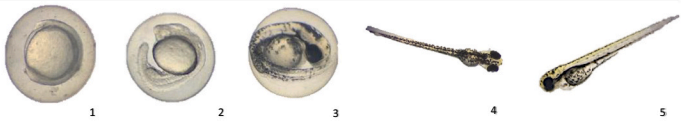
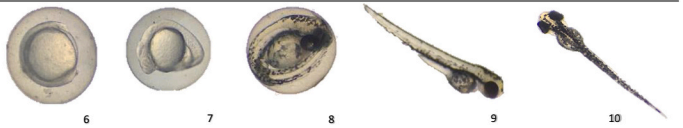
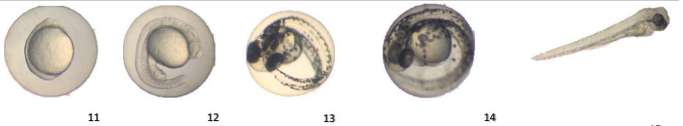
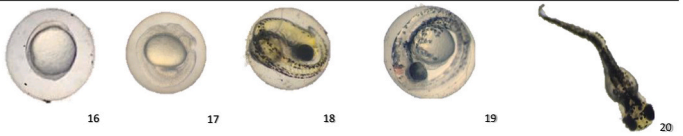
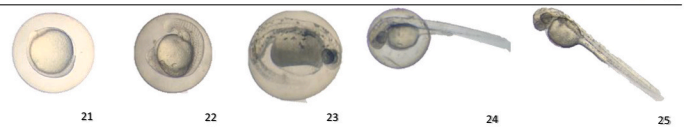
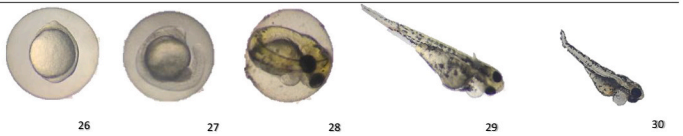
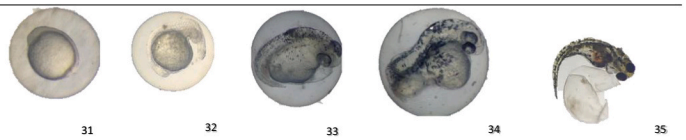
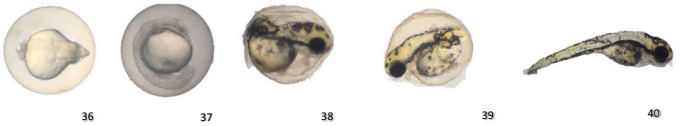
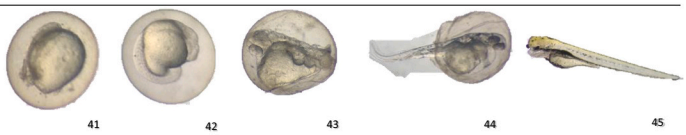
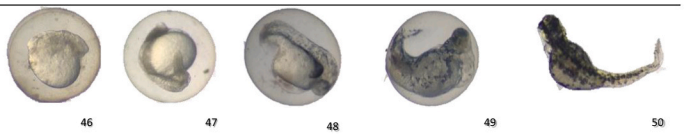
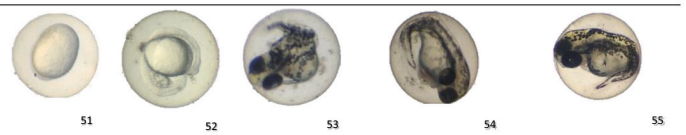
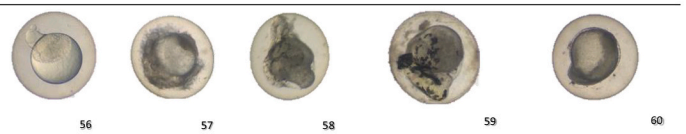
The LC_{50} and EC_{50} were calculated using the PROBIT analysis (EPA analysis Program v 1.5). Fisher's exact test analyzed data on alterations to embryonic development and teratogenic effects; significance was accepted when $p < 0.05$, using SPSS v9 software (SPSS, Chicago, IL). Oxidative stress data were examined by a two-way analysis of variance (ANOVA). Differences between means were examined using a Tukey-Kramer multiple comparisons test with a 95% confidence limit.

3. Results

3.1. Developmental toxicity and embryonic evaluation

In the present study, PHE toxicity was evaluated from 12 to 96 h post-fertilization in *D. rerio* embryos at environmentally relevant concentrations. In both the control group (0 ngL^{-1} of PHE) and the 0.05% DMSO system, no malformations were observed, all embryos normally developed at the end of 96 h. The frequency and severity of malformations increased with increasing PHE concentration compared to embryos in the control groups. The mortality rate of *D. rerio* embryos increased significantly when exposed to 1400 and 1500 ngL^{-1} of PHE until 96 h (Fig. 1). In addition, PHE induced morphological abnormalities during embryonic development, including developmental delay (38%), eye deformation (5.3%), hypopigmentation (27.3%), hemorrhaging in the head (5.1%), Hemorrhaging in the yolk (16.4%), miscellaneous (32.2%), modified chorda structure (31.5%), malformation of the head (18.5%), malformation of the tail (34%), pericardial edema (18%), yolk deformation (22%) and yolk edema (16%) [Table 2]. Malformation rates were observed with a concentration-dependent increase, with 1500 ngL^{-1} of PHE having the maximum value at 96 h. Table 2 summarizes the teratogenicity due to exposure to environmentally relevant concentrations of PHE. Embryos coagulation was highest in the group exposed to 1500 ngL^{-1} (90.3%), followed by the group exposed to 1400 ngL^{-1} (87.5%). Developmental delay, which was mainly observed in modified chorda structure and short tail, was more frequent in the group exposed to 1500 ngL^{-1} and less in the group exposed to 25 ngL^{-1} PHE.

Table 2
Major malformations present in *D. rerio* embryos exposed to environmentally relevant concentrations of PHE.

Concentration	12hpf	24hpf	48hpf	72hpf	96hpf
0 (Control)	 1 2 3 4 5				
DMSO 0.05%	 6 7 8 9 10				
25 ngL^{-1}	 11 12 13 14 15				
54 ngL^{-1}	 16 17 18 19 20				
78 ngL^{-1}	 21 22 23 24 25				
150 ngL^{-1}	 26 27 28 29 30				
287 ngL^{-1}	 31 32 33 34 35				
400 ngL^{-1}	 36 37 38 39 40				
500 ngL^{-1}	 41 42 43 44 45				
1000 ngL^{-1}	 46 47 48 49 50				
1400 ngL^{-1}	 51 52 53 54 55				
1500 ngL^{-1}	 56 57 58 59 60				

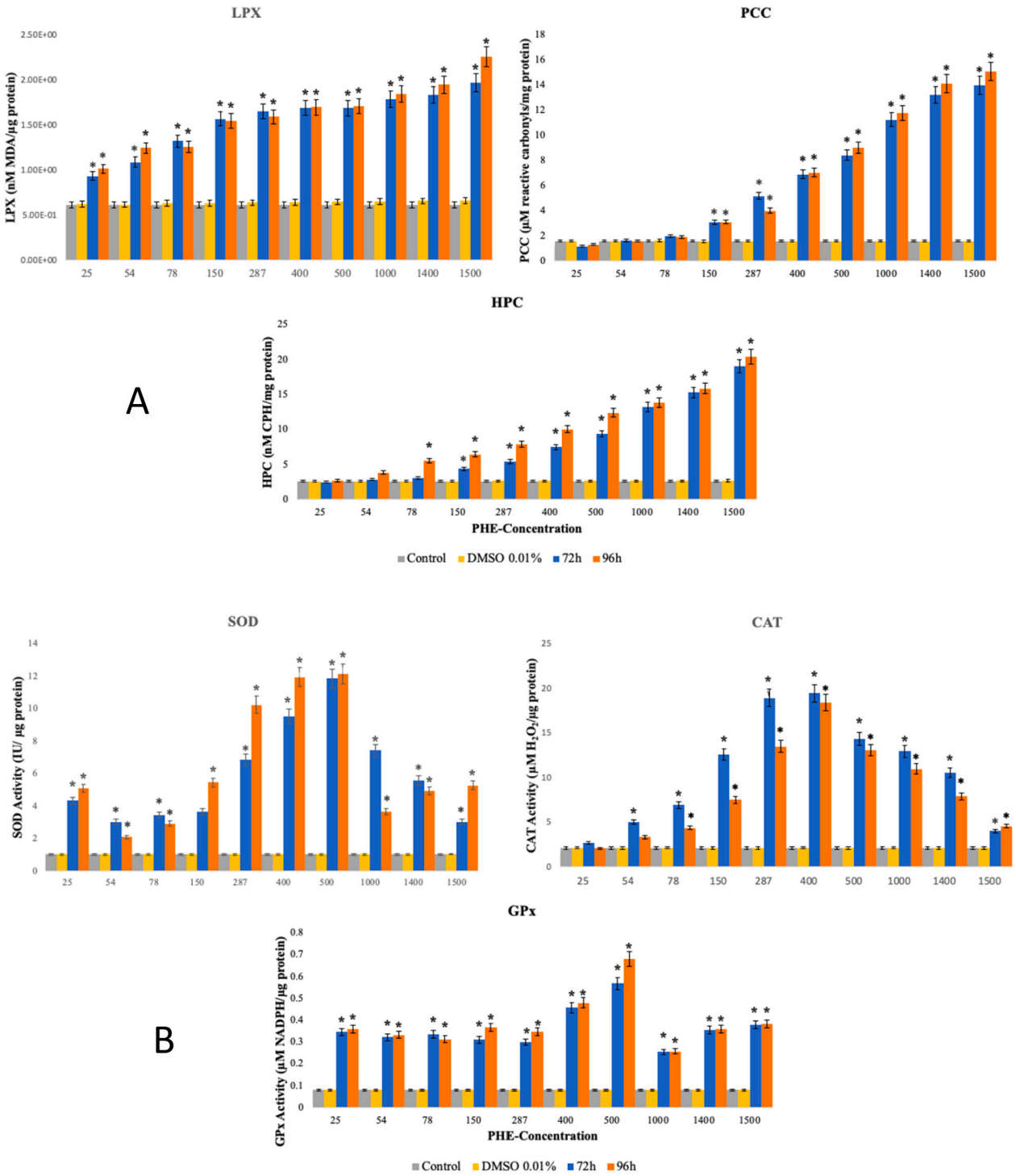


Fig. 2. Effects of PHE exposure on biomarkers of cellular oxidation (A) and antioxidant activities of SOD, CAT and GPx (B) in the larvae of zebrafish after 72 and 96 hpf. Values are mean ± SD of three replicate. * Significantly different from control group ($p < 0.05$).

Table 2 represent images of *D. rerio* embryos at 12 to 96 hpf. The control group (1–5) and the system with 0.05% DMSO (6–10) showed normal development until 96 hpf. Microscopic images of representative malformations in embryos exposed to 10 different environmentally relevant concentrations of PHE, including developmental delay (24, 33, 34, 38, 39, 43, 51–60), eye deformation (28, 44, 45), hypopigmentation (15, 23, 24, 25, 45), hemorrhaging in the head (19), hemorrhaging in the yolk (23, 34, 48, 54), miscellaneous (34, 35, 48, 49, 50, 53, 59), modified chorda structure (20, 34, 35, 40, 49, 50), malformation of the head (43, 44, 45, 49, 50), malformation of the tail (20, 30, 44, 49, 50), pericardial edema (28, 29, 30, 44, 49, 54), yolk deformation (23, 33, 42, 43, 48) and yolk edema (23, 43, 48), in zebrafish larvae after embryonic exposure to PHE.

3.2. Determination of oxidative stress in D. rerio embryos

Fig. 2 shows the results of the different biomarkers of oxidative stress. A significant concentration-dependent increase in cellular oxidation biomarkers ($p < 0.05$) was observed with respect to the control and DMSO groups. Antioxidant enzyme activity showed a

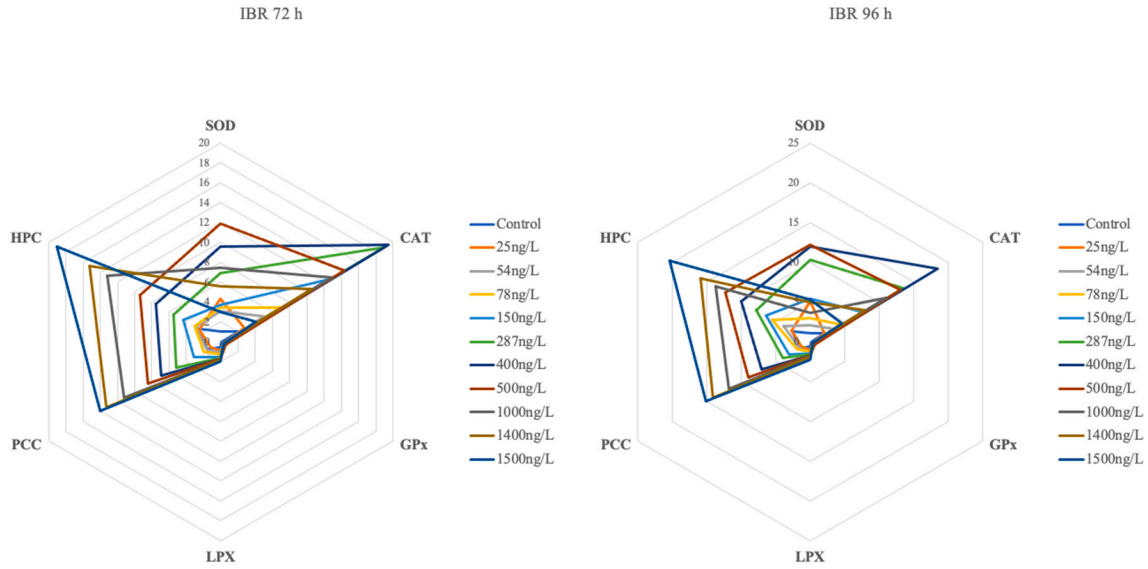


Fig. 3. Integrated Biomarker Responses Index for *Danio rerio* larvae exposed to environmentally relevant concentrations of PHE. The graphical comparison was based on six biomarkers of oxidative stress at 72 and 96 hpf. The IBR for the concentration tested at 72 h were 1.6, 0.9, 2.17, 2.2, 1.5, 1.9, 4.1, 3.2, 4.5, 4.9 and for 96 h 4.1, 3.3, 7.6, 3.8, 1.3, 10.9, 18.1, 6.3, 2.7 and 3.7 respectively.

significant increase from 25 ngL^{-1} to 400 ngL^{-1}. Subsequently, it decreased with respect to the control and DMSO groups ($p < 0.05$).

3.2.1. Evaluation of cellular oxidation

The results for biomarkers of cellular oxidation are shown in Fig. 2A PHE produced a significant increase ($p < 0.05$) over the control group in the biomarker of lipid peroxidation (MDA) in a concentration-dependent manner, with increases at 72 h from 152 to 320% and at 96 h from 165 to 367%. In the present study, it was observed that there was no statistically significant difference ($p < 0.05$) in PCC levels at exposure to 25, 54 and 78ngL^{-1} PHE), while at exposure concentrations of 150, 287, 400, 500, 1000, 1400 and 1500ngL^{-1} there was a significant increase ($p < 0.05$) in a concentration-dependent manner at 72 h of 196%, 328%, 437%, 534%, 712%, 838% and 886% and at 96 h of exposure of 198%, 253%, 446%, 573%, 747%, 894% and 956% respectively. For the results of the hydroperoxide content, a statistically significant difference ($p < 0.05$) with respect to the control group only from the concentration of 150ngL^{-1} (170%), 287ngL^{-1} (211%), 400ngL^{-1} (291%), 500ngL^{-1} (362%), 1000ngL^{-1} (512%), 1400ngL^{-1} (592%) and 1500ngL^{-1} (738%) for the 72 h of exposure; and for 96 h of exposure from 78ngL^{-1} (214%), 150ngL^{-1} (250%), 287ngL^{-1} (306%), 400ngL^{-1} (389%), 500ngL^{-1} (479%), 1000ngL^{-1} (535%), 1400ngL^{-1} (615%) and 1500ngL^{-1} (790%), the increase in HPC was observed in a concentration-dependent manner.

3.2.2. Antioxidant activity assays

The results for SOD activity are shown in Fig. 2B. The enzyme activity was significantly ($p < 0.05$) higher at all concentrations tested compared to the control group. Showing an increase for 25ngL^{-1}, 54ngL^{-1}, 78ngL^{-1}, 150ngL^{-1}, 287ngL^{-1}, 400ngL^{-1}, 500ngL^{-1}, 1000ngL^{-1}, 1400ngL^{-1} and 1500ngL^{-1} at 72 h of 425, 297, 340, 360, 676, 936, 1165, 730, 547 and 297% and for 96 h of 500, 205, 287, 535, 1000, 1174, 1194, 360, 483 and 517% with respect to the enzyme activity in the control group. With respect to CAT enzyme activity, a significant increase was observed ($p < 0.05$) compared to the larvae of the control group from the concentration of 54ngL^{-1} at 72 h of exposure (239%), 78ngL^{-1} (329%), 150ngL^{-1} (601%), 287ngL^{-1} (902%), 400ngL^{-1} (927%), 500ngL^{-1} (684%), 1000ngL^{-1} (523%), 1400ngL^{-1} (376%) and 1500ngL^{-1} (218%), for 96 h of exposure a significant increase was observed ($p < 0.05$) with respect to the control group from 78ngL^{-1} and up to 1500ngL^{-1} of 208%, 357%; 642%; 876%, 624%, 523%, 376% and 218%, respectively. PHE induced a significant increase ($p < 0.05$) with respect to the control group of GPx enzyme activity at all exposure concentrations (25 to 1500ngL^{-1}) at 72 h 431, 401, 48, 387, 373, 571, 709, 318, 443 and 474% and for 96 h of 446, 415, 391, 460, 432, 598, 848, 322, 447 and 478% respectively.

3.3. Phenytoin determination

Table 4 shows the values of PHE concentrations in the water of the exposure systems. A decreasing trend in PHE concentration concerning exposure time is observed. However, the concentration in all systems remained above 80% of the initial concentration.

3.4. Integrated Biomarker Response (IBR)

In the present study, a battery of biomarkers related to oxidative stress was chosen to assess whether oxidative stress occurs in zebrafish larvae exposed to PHE. Therefore, for ease of interpretation, the responses were integrated by calculating the IBR index for each biomarker evaluated (Fig. 3). In ascending order, the observed IBR values for 72 h were 54 < 278 < 25 < 400 < 78 < 78 < 150 < 1000 < 500 < 1400 < 1500ngL^{-1} and for the 96 h of exposure 278 < 1400 < 54 < 1500 < 150 < 25 < 25 < 1000 < 78 < 400 < 500ngL^{-1}. The IBR values showed that the stress caused by the 500ngL^{-1} concentration at 96 h of exposure was the highest, with a value of 18.1.

4. Discussion

Several studies have used very high concentrations of xenobiotics, which do not reflect the natural environment scenario, so we decided to work with environmentally relevant concentrations of PHE, which reflect the use and presence of this drug in the environment. It has been shown that changes in hatching, malformations, and mortality during embryonic development can be used as good indicators of toxicity to assess stress caused by environmental toxicants during development (Pohl et al., 2019; Qiang et al., 2016; Xia et al., 2017; Zhou et al., 2019). In the present study, PHE was observed to decrease the hatching rate, increase mortality rates and malformations with an apparent concentration- and time-dependent response.

Table 3
Survival, mortality, and malformations data in *D. rerio* embryos exposed to PHE.

DFH concentration ngL^{-1}	Number of embryos exposed	Survival (%)	Mortality (%)	Malformations (%)
0 (control)	72	100	0	0
DMSO 0.05%	72	98.61	1.38	0
25	72	87.5	12.5	12.5
54	72	73.61	26.38	20.83
78	72	69.44	30.55	38.88
150	72	63.88	36.11	47.22
287	72	56.94	43.05	50
400	72	54.16	45.83	55.55
500	72	48.61	51.38	59.72
1000	72	26.38	73.61	69.44
1400	72	12.5	87.5	76.38
1500	72	9.72	90.27	83.33
			LC_{50} = 279.25 CI = [189.5–408.5] **TI = 1.1**	CE_{50} = 252.60 CI = [200.9–315.6]

TI = Teratogenic index.

The results obtained show that the LC_{50} and EC_{50} of malformations at 96 h of exposure were obtained at 279.25 and 252.60 ngL^{-1}, respectively (Table 3). Concentrations evaluated in other studies gave an LC_{50} of >250 μM and an EC_{50} of 386 μM giving a TI >1(Weigt et al., 2011). The teratogenic index (TI = 1.1) obtained in our study shows that PHE is teratogenic for *D. rerio* embryos exposed to environmentally relevant concentrations. Furthermore, this was confirmed by identifying the different malformations.

The toxicity of PHE during the embryonic development of *D. rerio* has been investigated in previous studies. Martinez et al. (2018), report a delay in development, reflected in the reduction in hatching in a dose-dependent manner in PHE-exposed embryos compared to the control group, reaching 35–40% of hatched embryos at 48 h of exposure. However, none of the concentrations studied affected the general morphology of the larvae. Weigt et al. (2011), reported for the first time the ability of *D. rerio* embryos to activate pro-teratogenic substances (such as PHE) without the need for the addition of an exogenous metabolic activation system. Zebrafish embryos showed phase I enzyme activity at very early stages of development. There was an increase in the percentage of embryos with teratogenic effects exposed to PHE, with 43.3% reported as the maximum level of teratogenic effects. In the PHE treatment groups, a statistically significant difference was obtained at all concentrations tested. With 51.4% tail malformation was the most frequently occurring malformation, concluding that hatching impairment resulted from this malformation and not the cause.

Toxicity of PHE has been proposed due to a free radical intermediate, which is generated during bioactivation by prostaglandin H synthase (Parman et al., 1998), and may also form protein adducts by CYP450 (Munns et al., 1997). In addition, PHE can increase genetic recombination (Winn et al., 2003) as it oxidizes DNA and forms 8-hydroxyguanine (Liu and Wells, 1995). Liu and Wells (1994), demonstrated that PHE could lead to lipid peroxidation and protein oxidation in embryonic cells. Therefore, lipid, protein, and DNA damage are believed to cause developmental toxicity caused by PHE.

Oxidative stress has been reported to be the primary response in aquatic organisms to exposure to environmental pollutants. Oxidative stress occurs when ROS production exceeds antioxidant enzymes. Excessive ROS production can alter cell membrane integrity (Muthulakshmi et al., 2018). The induction of oxidative stress in our study due to PHE exposure is evidenced by cellular oxidation and antioxidant enzyme activity biomarkers.

Free radicals abstract hydrogen atoms from a methyl group (CH_2), leaving an unpaired electron on the carbon atom (·CH) in the polyunsaturated fatty acids of the cell membrane. Consequently, the radical carbon forms a conjugated diene and can react with an oxygen molecule to form a lipid peroxyl radical (LOO·). These radicals can attract more hydrogen atoms, propagating LPX and causing damage to the cells (Taziki et al., 2013). When lipid degradation occurs, substances such as malondialdehyde (MDA) are formed. MDA (LPX) accumulation is considered a late biomarker of oxidative stress and cell damage (Dogan et al., 2011). In our study, MDA content increased significantly ($p < 0.05$) compared to the control group at all PHE concentrations in a concentration-dependent manner. Other studies have shown that PHE increases LPX levels. Liu and Wells (1994), evaluated the oxidative damage caused by PHE in embryonic and maternal tissue. They were injecting pregnant mice at the organogenesis stage. Lipid peroxidation was significantly increased in maternal hepatic microsomes, cytosol, mitochondria, nuclei, and plasma, in embryonic microsomes, cytosol, and mitochondria following phenytoin treatment. Therefore, the increased lipid peroxidation levels in our study can be attributed to the induction of oxidative stress.

PHE, when hydroxylated by cytochrome P450 (CYP) enzymes, forms its phenolic metabolite, 5-(*p*-hydroxyphenyl), 5-phenylhydantoin (HPPH), which can be oxidized to form the catechol PHE (Munns et al., 1997). Several studies have shown that PHE catechol forms protein adducts. The present study showed a significant increase ($p < 0.05$) in the level of protein carbonyls from 150 to 1500ngL^{-1} PHE. Similarly, (Winn and Wells, 1997), used a mouse embryo culture model to determine whether the molecular mechanism for generating teratogenesis by PHE is through bioactivation by prostaglandin H synthase (PHS) and the resulting oxidative damage to proteins and DNA. By reacting the carbonyl groups of proteins with 2,4-dinitrophenylhydrazine, protein oxidation was detected. PHE significantly enhanced protein oxidation. This study concluded that embryonic bioactivation (PHS) and oxidation of proteins and DNA by ROS constitutes a molecular mechanism by

Table 4
Determination of PHE concentration in the water of the drug-exposed embryo system.

Nominal concentrations of PHE (ngL^{-1})	Measured PHE concentrations at different exposure times (ngL^{-1})					
	0 hpf	12 hpf	24 hpf	48 hpf	72 hpf	96 hpf
Control	<LoQ	<LoQ	<LoQ	<LoQ	<LoQ	<LoQ
0.05% DMSO	<LoQ	<LoQ	<LoQ	<LoQ	<LoQ	<LoQ
25	24.98 ± 0.03	24.89 ± 0.03	24.76 ± 0.02	24.66 ± 0.02	22.32 ± 0.01	20.97 ± 0.03
54	53.84 ± 0.06	53.09 ± 0.06	53.07 ± 0.06	52.75 ± 0.05	49.08 ± 0.05	43.92 ± 0.05
78	77.90 ± 0.08	77.56 ± 0.08	77.01 ± 0.09	76.87 ± 0.1	70.80 ± 0.1	63.56 ± 0.1
150	148.9 ± 0.11	148.54 ± 0.11	147.96 ± 0.13	147.77 ± 0.13	136.88 ± 0.13	124.59 ± 0.13
287	286.94 ± 0.18	286.78 ± 0.18	286.03 ± 0.19	285.01 ± 0.19	265.95 ± 0.19	230.32 ± 0.19
400	398.01 ± 0.21	396.17 ± 0.21	390.32 ± 0.21	387.65 ± 0.25	352.12 ± 0.25	327.32 ± 0.25
500	496.12 ± 0.27	490.34 ± 0.27	487.45 ± 0.27	483.76 ± 0.31	445.85 ± 0.31	412.46 ± 0.31
1000	995.03 ± 0.30	974.12 ± 0.30	961.56 ± 0.30	937.43 ± 0.42	912.23 ± 0.42	879.34 ± 0.42
1400	1396.34 ± 0.45	1367.23 ± 0.45	1311.65 ± 0.45	1294.67 ± 0.56	1278.34 ± 0.56	1243.89 ± 0.56
1500	1496.32 ± 0.93	1456.43 ± 0.93	1425.12 ± 0.93	1402.56 ± 1.1	1398.34 ± 1.1	1357.74 ± 1.1

Values represent the mean ± standard deviation of each concentration. LoQ: limit of quantification (5 ngL^{-1}).

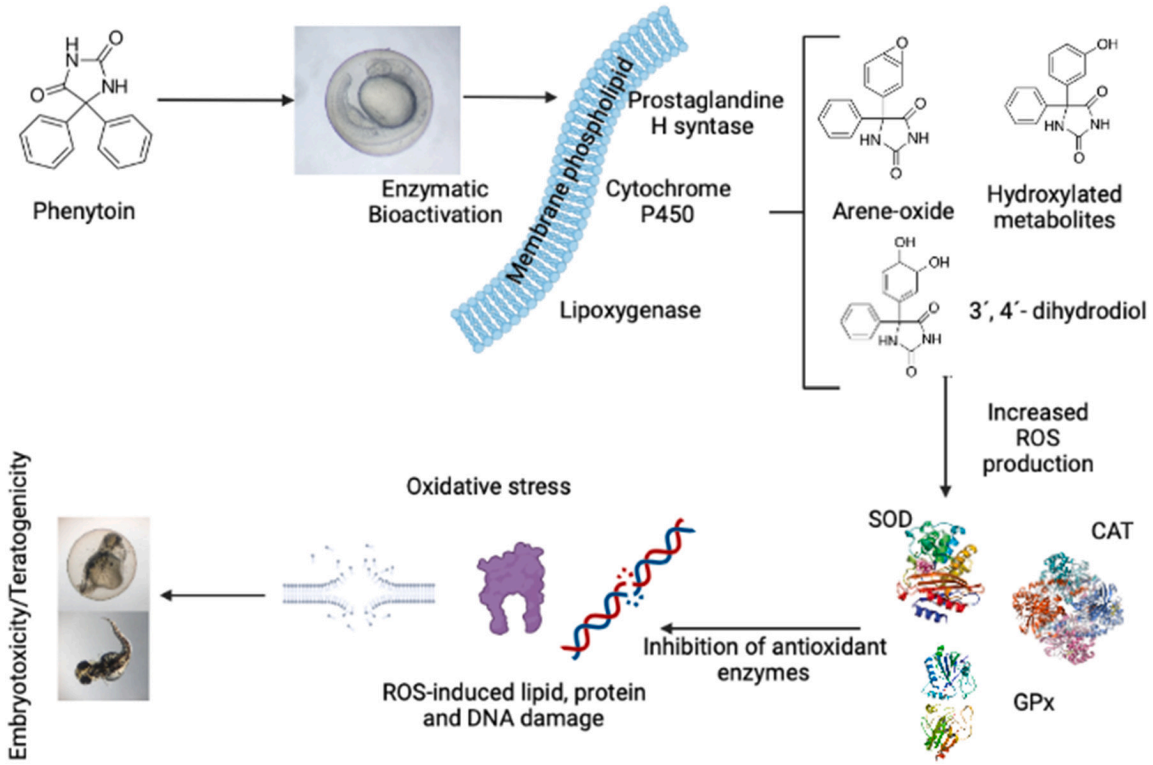


Fig. 4. Potential mechanism involved in the generation of PHE-induced oxidative stress and embryotoxicity in zebrafish embryos.

which PHE causes.

In addition, during the enzymatic biotransformation of PHE, reactive intermediates can be formed that can lead to an increased amount of ROS, which can cause DNA oxidation. This oxidation can induce breaks in the DNA double-strand; during the repair of these breaks, homologous recombination can be generated, and deleterious genetic changes can be induced. Winn et al. (2003), used a cell line to determine whether PHE increases homologous recombination to test whether these changes are involved in PHE-induced developmental toxicity. This showed that PHE increases both DNA oxidation and homologous recombination in a concentration- and time-dependent manner. They were suggesting that PHE-initiated DNA damage may be a mechanism mediating PHE-induced developmental toxicity in zebrafish embryos in this study.

A group of antioxidant enzymes is part of the cellular defense system (Santos et al., 2018). SODs, which require a metal cofactor for their activity, are metalloenzymes that catalyze the reaction that dismutates the superoxide radical into hydrogen peroxide and molecular oxygen. CAT is the enzyme responsible for degrading or reducing hydrogen peroxide into water and molecular oxygen (using iron or manganese as a cofactor) and is present in almost all living tissues. GPx, an intracellular enzyme, breaks down hydrogen peroxide into water and lipid peroxides into their corresponding alcohols. Its activity depends on selenium as a cofactor. It plays a crucial role in protecting the cell from oxidative stress by inhibiting the process of lipid peroxidation. These enzymes have been proposed as biomarkers of toxicity, as the interaction between them is of utmost importance in the defense mechanism against ROS (Ighodaro and Akinloye, 2018).

SOD activity in *D. rerio* is constant throughout morphogenesis and up to 7 dpf (Gauron et al., 2016). In our study, an increase was observed from the concentration of 25 to 50 ngL^{-1}; from 1000 to 1500 ngL^{-1} a depletion in SOD activity was observed. This effect may reflect the oxidative stress experienced by the groups exposed to higher PHE concentrations. This is consistent with a study by Mahle and Dasgupta, 1997, which reports a lower antioxidant status in sera from patients treated with PHE compared to sera obtained from healthy volunteers.

CAT enzyme activity has been reported to increase after 48 hpf when morphogenesis is almost complete (Gauron et al., 2016). The results of our study show that PHE had a stimulatory effect on CAT activity, from 54 to 400ngL^{-1}. Furthermore, from 500 to 1500 ngL^{-1} activity was observed to decrease gradually. Previous studies have shown that high concentrations of superoxide anion can inhibit CAT and SOD activity (Moreno et al., 2005).

GPx has been proposed as biomarkers of toxicity, as the interaction between them is of utmost importance in the defense mechanism against ROS (Ighodaro and Akinloye, 2018). Data from our study show a constant GPx activity up to a concentration of 287ngL^{-1}, with a maximum in activity at 500 ngL^{-1} and a decrease in activity at higher concentrations. GPx can reduce hydroperoxides to hydroxylated compounds using GSH as a substrate, thus detoxifying the excess ROS by oxidation of GSH to GSSG (Muthulakshmi et al., 2018). However, during PHE biotransformation, a catechol-PHE is formed; it has been reported that catechol may undergo further redox reactions, which has been shown to result in net GSH oxidation (Harris et al., 1995). This may explain the decrease in the activity of this enzyme at higher concentrations.

In the present study, the IBR index was chosen to determine whether exposure to environmentally relevant concentrations of PHE leads to an oxidative stress process in exposed fish. The IBR index was calculated using the oxidative stress biomarkers to integrate the response and facilitate its interpretation. IBR values are shown in Fig. 3. A significant increase in IBR values was observed at all tested PHE concentrations; according to these results, *D. rerio* embryos exposed to 1500ngL^{-1} suffered higher stress levels at 96 h of exposure than control organisms. Furthermore, the results demonstrate the ability of *D. rerio* embryos to respond to environmentally relevant concentrations of PHE. With the standardization (S) of the values, it is possible to define which biomarker had the highest weight in the IBR index (Iturburu et al., 2018). Of the selected biomarkers, the activity of the antioxidant enzymes SOD, CAT, and GPx is more sensitive to 400 and 500ngL^{-1}, while the biomarkers of cellular oxidation were more sensitive to higher exposure concentrations (1400 and 1500ngL^{-1}).

With our findings and those identified in other works in Fig. 4 shows the possible mechanism of action by which PHE can generate oxidative stress and embryotoxicity in zebrafish embryos the possible mechanism of action by which PHE can generate oxidative stress and embryotoxicity in zebrafish embryos. After absorption, PHE can be enzymatically bioactivated by CYP450, Prostaglandin H synthase and lipoxygenase. As shown in the Fig. 4, the main metabolites are hydroxylated derivatives, 3′, 4′-dihydrodiol and arene oxide. Arene oxide is a highly reactive and extremely unstable metabolite, to which the carcinogenic effects of PHE are attributed (Patocka et al., 2020). In addition, hydroxylated metabolites can be biotransformed to a catechol that can subsequently be oxidized to form quinone and semiquinone species. Furthermore, during the synthesis of eicosanoids and prostaglandins, PHE can be oxidized by hydroperoxidases, giving rise to intermediate free radicals that upon reaction with oxygen produce reactive oxygen species. When not detoxified by antioxidant enzymes, free radicals can generate a state of oxidative stress. The results of the present study support the hypothesis that during enzymatic biotransformation of PHE, free radicals and reactive intermediates capable of generating reactive oxygen species are generated, which can oxidize lipids, proteins and DNA, leading to embryotoxicity and teratogenesis (Liu and Wells, 1995; Patocka et al., 2020; Winn et al., 2003; Cardoso-Vera et al., 2021).

5. Conclusion

This study demonstrated developmental toxicity in *D. rerio* embryos exposed to environmentally relevant concentrations of PHE, as evidenced by developmental delay, delayed hatching, morphological deformities. We also demonstrate that PHE can increase cellular oxidation biomarkers in a concentration-dependent manner. Biomarkers of antioxidation increased from 25 and up to 400 ngL^{-1} PHE, subsequently at higher concentrations of the anticonvulsant decreased. Our IBR analysis demonstrated that oxidative damage biomarkers got more influence at 500ngL^{-1} of PHE. Teratogenic effects and developmental alterations increased in frequency and severity in a time- and concentration-dependent manner. The data obtained in the study help provide information about the mechanism of PHE-induced toxicity and oxidative stress during *D. rerio* embryonic development. We suggest that these conclusions can be verified in other aquatic species, using environmentally relevant concentrations, to obtain complete information on the developmental toxicity of PHE.

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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