Acute exposure to environmentally relevant concentrations of phenytoin induces oxidative stress and organ-specific histopathological damage in adult zebrafish (*Danio rerio*)

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Abstract

Phenytoin (PHEN), a widely prescribed antiepileptic drug, is increasingly detected in surface waters, yet its toxic effects on fish remain poorly characterized. This study evaluated the acute impact of environmentally relevant PHEN concentrations on adult zebrafish (Danio rerio). Fish (223 ± 40 mg; 3.4 ± 0.3 cm) were exposed for 96 h to 25 ng/L, 282 ng/L, and 1500 ng/L PHEN, alongside a control. PHEN exposure significantly increased lipid peroxidation (malondialdehyde, MDA), inhibited key antioxidant enzymes—catalase (CAT) and superoxide dismutase (SOD)—and elevated glutathione (GSH) levels in gut tissue ($p < 0.05$). Histopathological analyses revealed dose-dependent lesions, including hyperemia, branchitis, and lamellar degeneration in the gills; hyperemia and hepatic steatosis in the liver; and hyperemia, hemorrhage, and epithelial necrosis in the kidneys. Overall, short-term PHEN exposure impaired antioxidant defenses and induced oxidative and structural damage across multiple organs in a concentration-dependent manner, indicating potential ecological risks associated with pharmaceutical contamination in aquatic environments.

Keywords: Phenytoin; Antiepileptic drug; Oxidative stress; Histopathology; Zebrafish

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Introduction

The presence of pharmaceutical residues in aquatic environments has become a serious global concern due to their high environmental persistence (Brandão *et al*., 2013). Among these, antiepileptic drugs (AEDs) represent a class of pharmaceutical pollutants that are frequently detected in aquatic ecosystems. These compounds can enter the environment either directly or as active metabolites through human excretion, microbial transformation, and wastewater treatment processes—potentially posing serious risks to non-target organisms (Liu *et al*., 2023). Phenytoin (PHEN) is one of the most commonly used AEDs, primarily prescribed for the treatment of tonic-clonic seizures. It has been identified in groundwater (Phillips *et al*., 2015), surface water (Hoshina *et al*., 2009), wastewater (Bodur *et al*., 2024), and even drinking water (Liu *et al*., 2023).

Zebrafish (*Danio rerio*) is widely used as a model organism in environmental toxicology due to several advantageous features, such as low cost, small size, transparent embryos, ease of breeding, rapid development, a fully sequenced genome, and significant genetic similarity to humans. Moreover, their aquatic lifestyle facilitates exposure to waterborne chemicals and pharmaceuticals, making them ideal for toxicity assessments (Alavinejad *et al*., 2023).

Fish play a central role in aquatic food webs and are highly sensitive to even minor environmental disturbances (Sathya *et al*., 2012; Poopal *et al*., 2013). Environmental risk assessments of chemical and pharmaceutical contamination often rely on the use of biomarkers as early warning signals. These biomarkers can reflect not only individual organism health but also population-level and ecosystem-wide effects (Tkachenko and Kurhaluk, 2012). They provide insight into the biological mechanisms of toxicity, from initial cellular responses to broader physiological disruptions (Viarengo *et al*., 2007).

Despite the widespread use and environmental detection of PHEN, there is limited research on its toxicological impacts on aquatic organisms. Previous studies have mostly focused on its teratogenic and anticonvulsant effects in zebrafish embryos and larvae (Martinez *et al*., 2018), growth inhibition and antioxidant disruption in larvae (Cardoso-Vera *et al*., 2022b), behavioral changes in Japanese medaka (Sawada *et al*., 2024), neurotoxic effects in adult zebrafish (Cardoso-Vera *et al*., 2022a), and behavioral abnormalities, downregulation of *kisspeptin* expression, and reproductive impairment in Japanese medaka (Mitsunaga *et al*., 2024). However, to date, no study has comprehensively investigated the effects of PHEN on antioxidant defenses and histopathological responses in adult zebrafish.

Given that biochemical and histological biomarkers provide valuable insight into organ-specific toxicity caused by pharmaceutical residues (Capkin *et al*., 2009; Cardoso-

Vera *et al*., 2022b; Rezaeipour *et al*., 2024), the present study aimed to evaluate the acute effects of environmentally relevant concentrations of PHEN on adult zebrafish. Specifically, we examined changes in lipid peroxidation (measured via malondialdehyde [MDA] levels), antioxidant enzyme activities including catalase (CAT), superoxide dismutase (SOD), and the intracellular antioxidant glutathione (GSH), as well as histopathological alterations in the gills, liver, and kidneys.

Materials and methods

Experimental design and Toxicity exposure

Adult zebrafish (*Danio rerio*) with a mean body weight of 223±40 mg and a body length of 3.4±0.3 cm were obtained from a commercial aquaculture facility and acclimated under laboratory conditions for two weeks. During acclimation, the fish were housed in 64-liter glass aquaria containing 20 liters of dechlorinated tap water, equipped with a central aeration system. Environmental conditions were maintained at 25±1°C with a 14:10 hour light-dark cycle. To maintain water quality, 30% of the water was replaced daily to remove uneaten feed and waste. Fish were fed twice daily with a commercial diet (Biomar, France) at 5% of their body weight. During the experiment, the water quality parameters were monitored and maintained as follows: temperature 26±0.5°C, dissolved oxygen at 5.0 mg/L, pH between 7.2 and 7.4, and water hardness ranging from 50 to 60 mg/L.

Toxicity study

A stock solution of phenytoin (PHEN) was prepared by dissolving 10 mg of PHEN (Sigma-Aldrich, PHR1139-1G) in 1 liter of deionized water and stored at −20°C until use. The experimental exposure was based on environmentally relevant concentrations reported by Cardoso-Vera *et al*. (2022a) and lasted for 96 hours. Fish were randomly assigned to four groups, each with three replicates (ten fish per aquarium): a control group with no PHEN exposure, and three treatment groups exposed to 25 ng/L (T1), 282 ng/L (T2), and 1500 ng/L (T3) of PHEN, respectively. To maintain stable exposure conditions, 80% of the aquarium water was renewed daily, and appropriate amounts of stock solution were added to restore the PHEN concentration.

Sample collection

After 96 hours of exposure, fish were euthanized using the rapid cooling method by immersion in an ice-water bath (ice: water ratio of 5:1) at 2–4°C for at least 10 minutes, until opercular movements ceased, in accordance with Rezaeipour *et al*. (2024). The gastrointestinal tracts of nine fish from each treatment group were dissected, pooled, and transferred into microtubes for antioxidant analysis. Samples were kept on ice during transport and stored at −80°C until further analysis. For biochemical assessment, tissue samples were thawed on ice and homogenized using a tissue homogenizer with 600 µL of Tris-HCl buffer (100 mM, pH 7.4) or phosphate buffer (50 mM, pH 7.4).

Homogenates were centrifuged at 10,000× g for 15 minutes at 4°C, and the supernatants were collected for antioxidant parameter analysis. Additionally, three fish per replicate were selected for histological examination of the gills, liver, and kidneys. Following a ventral incision, organs were removed and fixed in 10% formalin according to the procedure described by Gyimah *et al*. (2020).

Antioxidant activity

The enzymatic activities of superoxide dismutase (SOD), catalase (CAT), glutathione (GSH), and the level of malondialdehyde (MDA) were measured in the centrifuged tissue samples using commercial assay kits (ZellBio, Germany; Cat. Nos. ZB-SOD96A, ZB-CAT96A, ZB-GSH96A, ZB-MDA96A), following the manufacturer's protocols. Total protein content in the supernatants was also determined based on the method by Eldar *et al*. (1999), using bovine serum albumin as the standard.

Histopathological analysis

After 24 hours of initial fixation, the formalin in the tissue samples was refreshed, and the specimens were stored until histological slides were prepared. The tissues were dehydrated through a graded series of ethanol, cleared in xylene, and embedded in paraffin wax. Paraffin blocks were sectioned using a microtome at a thickness of 4–5 µm. The sections were mounted on glass slides and stained with hematoxylin and eosin (H&E) for pathological evaluation. The prepared slides were then examined under a light microscope. Histopathological changes were assessed based on the severity of alterations observed in comparison to the control group, as described by Alavinejad *et al*. (2023).

Statistical analyses

A combination of statistical methods was applied to analyze the experimental data. Paired samples t-tests were used to compare the means of the same treatment groups at different time points. One-way analysis of variance (ANOVA) was conducted to assess differences among the treatment groups. To determine specific group differences, Duncan's multiple range test was performed at a significance level of $p<0.05$. All statistical analyses were carried out using Microsoft Excel 2013 and SPSS version 19 software.

Results

Changes in antioxidant enzymes

The effects of PHEN exposure on oxidative stress-related biomarkers in zebrafish are presented in Table 1. By the end of the exposure period, malondialdehyde (MDA) levels had significantly increased in all treatment groups compared to the control (CNT) group ($p<0.05$), with the highest MDA concentration observed in the T1 group. Superoxide dismutase (SOD) activity showed a significant increase in the T1 and T2 groups compared to the control ($p<0.05$). However, no significant difference in SOD activity was found

between T1 and T2, or between the control and T3 groups (p>0.05). Catalase (CAT) activity significantly decreased in all PHEN-exposed groups compared to the control (p<0.05), with no significant difference observed between the T2 and T3 groups (p>0.05). Glutathione (GSH) levels significantly increased in all treatment groups compared to the control (p<0.05). However, there was no significant difference in GSH levels between the T2 and T3 groups (p>0.05).

Table 1: Comparison of mean values (Mean ± SE) for antioxidant parameters (CAT, SOD, MDA, GSH, and total protein) in zebrafish across control and PHEN-treated groups after 96 hours of exposure. Different lowercase letters within each column indicate statistically significant differences between groups (p<0.05).

Treatment	MDA (nmol/g protein)	SOD (ul/mg protein)	CAT (ul/mg protein)	GSH (nmol/mg protein)
Control	6.51±0.046 ^{d}	1.3±0.017 ^{b}	0.185±0.003 ^{a}	18.3±0.252 ^{c}
T1	9.16±0.031 ^{a}	1.36±0.015 ^{a}	0.159±0.002 ^{c}	23.5±0.173 ^{a}
T2	8.65±0.082 ^{b}	1.38±0.021 ^{a}	0.17±0.002 ^{b}	22.7±0.173 ^{b}
T3	8.35±0.021 ^{c}	1.25±0.015 ^{b}	0.157±0.002 ^{c}	23.1±0.153 ^{ab}

Gill, liver, and kidney histopathological analysis

Microscopic examination of the gill tissues revealed mild lymphoplasmacytic bronchitis in the control group, characterized by slight infiltration of mononuclear inflammatory cells, mainly lymphocytes and plasma cells. In the T1 and T2 groups, moderate lymphoplasmacytic bronchitis was observed, along with vascular congestion. In the T3 group, severe lymphoplasmacytic bronchitis was detected, accompanied by destruction of several secondary lamellae (Fig. 1).

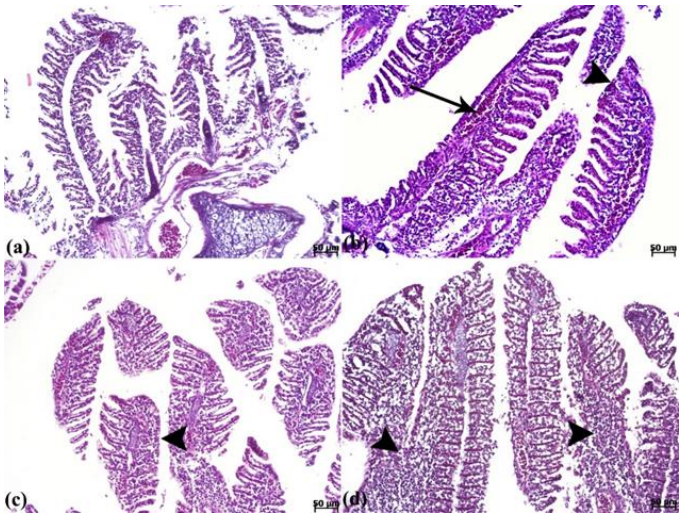

Figure 1: Histopathological evaluation of gill tissue in different experimental groups of adult zebrafish. (a) Normal gill architecture in the control group. (b–c) Vascular congestion (arrow) and moderate lymphoplasmacytic bronchitis (arrowhead) observed in T1 (b) and T2 (c) groups. (d) Severe lymphoplasmacytic bronchitis along with degeneration of multiple secondary lamellae (arrowheads) in the T3 group. Staining: Hematoxylin and Eosin (H&E).

In the liver, no pathological lesions were observed in the control group, and the tissue appeared normal. In both T1 and T2, except for vascular congestion, no other histological alterations were noted. However, in the T3 group, moderate hepatic steatosis was evident, characterized by cytoplasmic vacuolization within hepatocytes (Fig. 2).

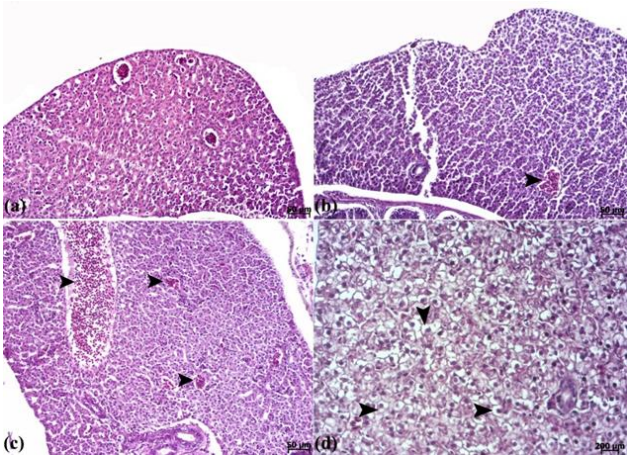

Figure 2: Histopathological examination of liver tissue in different treatment groups of adult zebrafish. (a) Normal liver tissue in the control group. (b–c) Vascular congestion (arrowheads) observed in T1 (b) and T2 (c) groups. (d) Hepatocellular steatosis indicated by cytoplasmic vacuoles (arrowheads) in the T3 group. Staining: Hematoxylin and Eosin (H&E).

Histopathological analysis of kidney sections showed normal tissue architecture in the control group. In T1, mild multifocal hemorrhages were noted in the renal parenchyma along with vascular congestion. In T2, in addition to these changes, epithelial necrosis was observed in a few renal tubules. In the T3 group, moderate necrosis of tubular epithelial cells was seen in a greater number of renal tubules (Fig. 3).

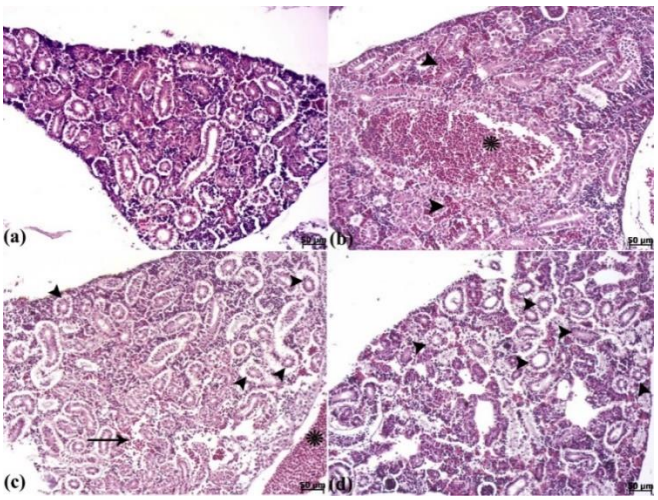

Figure 3: Histopathological examination of kidney tissue samples from different treatment groups of adult zebrafish. (a) Normal kidney tissue in the control group. (b) Vascular congestion (*) and mild multifocal hemorrhages (arrowheads) in the renal parenchyma of the T1 group. (c) Vascular congestion (*), mild multifocal hemorrhages (arrow), and epithelial necrosis of renal tubules (arrowheads) in the T2 group. (d) Epithelial necrosis of renal tubules (arrowheads) in the T3 group. Staining: Hematoxylin and Eosin (H&E).

Discussion

This study examined the acute effects of environmentally relevant concentrations of phenanthrene (PHEN) exposure, with results reflecting disruptions in immune responses and tissue damage in zebrafish exposed to this compound. Evaluating oxidative stress biomarkers alongside histopathological changes serves as a reliable approach for assessing the impact of pharmaceutical residues on aquatic organisms (Naderi *et al*., 2022; Alavinejad *et al*., 2022; Cardoso-Vera *et al*., 2022a,b).

One of the earliest responses observed in aquatic organisms subjected to environmental pollutants is oxidative stress (Cardoso-Vera *et al*., 2022b). Oxidative stress results from increased production of reactive oxygen species (ROS), which can damage cellular membranes (Muthulakshmi *et al*., 2018). The current findings demonstrated significant disturbances in both the levels and activity of oxidative stress-related factors in zebrafish exposed to PHEN. PHEN induces ROS generation, leading to enhanced lipid peroxidation (Liu *et al*., 1998). The breakdown of lipids due to elevated ROS produces molecules such as malondialdehyde (MDA), a well-known biomarker for oxidative stress and cellular damage (Dogan *et al*., 2011). MDA content significantly increased in all PHEN-treated groups compared to controls; however, this increase was not dose-dependent, with the highest MDA level observed at the lowest PHEN concentration. These findings are consistent with those of Cardoso-Vera *et al*. (2022b), who reported elevated MDA levels in zebrafish larvae exposed to PHEN, and Chen *et al*. (2014), who observed similar effects in freshwater mussels exposed to carbamazepine, another antiepileptic drug. Additionally, Liu and Wells (1994) documented increased lipid peroxidation in mice treated with PHEN and Reeta *et al*. (2009) reported heightened MDA formation in rodents following chronic PHEN exposure. Thus, the elevated lipid peroxidation observed can be attributed to the induction of oxidative stress by PHEN.

Antioxidant enzyme activities, including superoxide dismutase (SOD), catalase (CAT), and levels of the non-enzymatic antioxidant glutathione (GSH), were also investigated. These enzymes and molecules constitute essential components of the cellular defense system and serve as important biomarkers in toxicological studies (Santos *et al*., 2018; Cardoso-Vera *et al*., 2022b). Previous research indicates that cytotoxic effects of PHEN are mediated through modulation of SOD, CAT, and intracellular antioxidants such as GSH (Gallagher and Sheehy, 2001; Abramov and Wells, 2011). Generally, the results showed a decrease in SOD activity alongside increases in CAT activity and GSH levels. Catalase plays a critical role in decomposing hydrogen peroxide generated by SOD activity (Brandão *et al*., 2013). The suppression of SOD activity suggests impairment in the antioxidant defense mechanism of PHEN-exposed fish. Similarly, Cardoso-Vera *et al*. (2022b) reported

reductions in SOD and CAT activities in fish exposed to PHEN concentrations between 500 and 1500 ng/L, aligning with these observations, while at lower concentrations (54–400 ng/L), both SOD and CAT activities increased (Cardoso-Vera *et al*., 2022b). In this study, the lowest enzyme activities were recorded at the highest PHEN concentration.

Furthermore, Mahle and Dasgupta (1997) reported decreased antioxidant function in patients treated with PHEN, a finding of interest given the genetic similarities between zebrafish and humans. Several oxidants formed during oxidative stress inhibit catalase activity, exacerbating oxidative damage (Gebicka and Krych-Madej, 2019). Similarly, Ni *et al*. (2019) and Hema *et al*. (2023) documented decreased CAT activity in zebrafish exposed to maduramycin and the anticancer drug cyclophosphamide, respectively, which supports these results.

The decrease in SOD and CAT activities may be attributed to the inhibitory effects of excessive ROS and elevated superoxide anion concentrations (Moreno *et al*., 2005; Ni *et al*., 2019). Contrastingly, Brandão *et al*. (2013) observed a significant increase in CAT activity in the livers of fish exposed to PHEN. Differences in findings may be explained by variations in experimental design, exposure duration, PHEN concentration, fish age, sex, species, and the specific organs studied. The liver, in particular, is not only a primary organ showing antioxidant response alterations but also the main site of biotransformation and toxicity of PHEN (Brandão *et al*., 2013).

In this study, glutathione (GSH) levels increased in zebrafish exposed to PHEN. Considering the role of glutathione S-transferase (GST) in conjugating GSH with xenobiotics and reactive oxygen species to protect tissues (Zager and Johnson, 2022), it appears that GST was either not effectively induced or was suppressed following PHEN exposure in this experiment. Similar decreases in GST activity have been reported in zebrafish exposed to maduramycin and the anticancer drug cyclophosphamide (Ni *et al*., 2019; Hema *et al*., 2023). In contrast, other studies have commonly reported intracellular GSH depletion as a typical outcome of PHEN exposure (Raya *et al*., 1995; Brandão *et al*., 2013). It has also been suggested that metabolites formed during the biotransformation of PHEN lead to oxidation of reduced GSH, which may cause a decrease in GSH levels at higher PHEN concentrations (Harris *et al*., 1995; Cardoso-Vera *et al*., 2022b). This phenomenon could explain the lower GSH levels observed in fish exposed to higher PHEN concentrations in this study.

The gill is the first organ to come into contact with foreign compounds dissolved in water and thus plays a critical role in evaluating the effects of pollutant exposure, since the presence of any toxic environmental compound causes damage to this organ (Rodrigues *et al*., 2015; Wolf *et al*., 2015). Exposure to PHEN in this study caused vascular

congestion, lymphoplasmacytic branchitis, and secondary lamellar destruction in zebrafish gill tissue. Gill tissue damage following exposure to pharmaceutical residues has been reported previously (Ramesh *et al*., 2018; Rezaeipour *et al*., 2024). However, it is important to note that structural changes induced by pollutants in the gills are not specific and vary depending on pollutant concentration and exposure duration (Rašković *et al*., 2010; Rezaeipour *et al*., 2024).

PHEN exposure also induced vascular congestion and lipid accumulation in the liver tissue of zebrafish. Unlike severe and irreversible liver damage such as nuclear degeneration, cellular edema, and necrosis reported in other studies (Jarrar and Taib, 2012), such extreme lesions were not observed here. Nevertheless, similar liver alterations have been documented in fish exposed to pharmaceutical residues previously (Ramesh *et al*., 2018; Hema *et al*., 2023). The liver is a major organ affected by aquatic pollutants and undergoes metabolic and physiological changes in response to contaminants (Ahmed *et al*., 2013). Hepatocytes, however, exhibit considerable resistance to high levels of chemical exposure (Fernandes *et al*., 2008).

The kidney is another key organ for histopathological assessment of pollutant effects in fish, as it receives a high volume of blood from the caudal vein (Mishra and Mohanty, 2008). In this study, PHEN exposure caused vascular congestion, hemorrhage, and epithelial necrosis in kidney tissue. Even low concentrations of pharmaceutical residues have been reported to cause kidney tissue damage in fish (Galus *et al*., 2013; Rezaeipour *et al*., 2024). The mechanisms underlying these changes are thought to involve oxidative stress induced by pharmaceutical residues in fish, which, as observed here, can trigger pathological alterations in kidney tissue (Cao *et al*., 2015).

Conclusion

The present study demonstrates that exposure to phenytoin induces disruption of antioxidant enzyme function and causes histopathological lesions in zebrafish. Considering that the concentrations used in this study correspond to those reported in the environment, the findings carry significant ecological relevance. Given the limited number of studies investigating the effects of this pharmaceutical on fish, these results provide valuable and novel insights into the impacts of phenytoin exposure in aquatic organisms. This highlights the urgent need to implement effective strategies to control and reduce water pollution caused by pharmaceutical residues such as phenytoin.

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