



## Histopathological alterations in adult zebrafish (*Danio rerio*) under acute fluoxetine exposure

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### Abstract

The presence of antidepressant residues, such as fluoxetine (FLX), in surface and underground waters worldwide has raised concerns regarding their potential impacts on non-target organisms, including fish. This study aimed to investigate the effects of short-term exposure to environmentally relevant concentrations of FLX on zebrafish (*Danio rerio*). A total of 120 zebrafish, with an average weight of  $0.43 \pm 0.05$  g and length of  $3.43 \pm 0.05$  cm, were divided into one control group and four treatment groups exposed to different FLX concentrations (0.1  $\mu\text{g/L}$ , 0.5  $\mu\text{g/L}$ , 10  $\mu\text{g/L}$ , and 100  $\mu\text{g/L}$ ) for 96 hours. Histopathological examination was conducted on the gill and kidney tissues to assess the effects of acute FLX exposure. The results revealed structural changes in the gills, including hyperplasia, aneurysm, clubbing, shrinkage, atrophy, and fusion of secondary lamellae. Similarly, the kidney tissues exhibited expansion of Bowman's space, increased thickness of Bowman's capsule, dilation of glomerular capillaries, tubular degeneration, and necrosis. The study underscores the toxic effects of FLX on zebrafish at environmentally relevant concentrations in the micrograms per liter range. Therefore, the potential adverse effects of residual FLX exposure should not be overlooked in fish populations.

**Keywords:** Zebra fish, Fluoxetine, Antidepressant, Tissue damages

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## Introduction

In recent decades, the global concern regarding the presence of pollutants in aquatic environments, including emerging pollutants like drugs of human or natural origin has escalated. These substances are typically found in low concentrations in water sources, and their diverse structures and mechanisms of action make their identification and removal challenging in wastewater treatment plants. Furthermore, the absence of well-defined maximum allowable concentrations for these compounds has resulted in limited regulatory measures to prevent their release into the environment (Quesada *et al.*, 2019). Pharmaceutical residues enter the environment through agricultural field runoff, landfill leachates, and inadequate destruction, removal, and eradication in sewage treatment plants. Given the bioactive nature of these drug residues and their metabolites, their presence in the environment adversely affects non-target organisms, particularly aquatic animals. One group of drugs frequently detected in aquatic ecosystems is selective serotonin reuptake inhibitor (SSRI) antidepressants (Correia *et al.*, 2023). Fluoxetine, an antidepressant belonging to the SSRI class, has been identified in numerous studies conducted on wastewater treatment plants (Batt *et al.*, 2008; Gros *et al.*, 2009; Sousa *et al.*, 2011).

Fluoxetine (FLX) was initially introduced in 1974. It received approval from the US Food and Drug Administration (FDA) in 1987 and was marketed as Prozac® in 1988 for the treatment of depression (Zucker *et al.*, 2017). The concerning issue regarding FLX drug residues is the identification of

these residues in fish tissues (Schultz *et al.*, 2010; Liu *et al.*, 2021). Zebrafish (*Danio rerio*), a freshwater fish belonging to the Cyprinidae family, has emerged as a valuable vertebrate model in recent years in various studies (Alavinezhad *et al.*, 2020). Despite anatomical and physiological differences between zebrafish and humans, many organs in zebrafish perform similar functions to their human counterparts (MacRae and Peterson, 2015). Additionally, zebrafish possesses advantageous features such as small size, ease of breeding, rapid growth, high egg production per spawning, and transparent larvae. These characteristics make zebrafish a valuable model organism and an alternative to mammals in studying various types of fish poisoning (Arayesh *et al.*, 2021; Kazempour and Kazempoor, 2022).

Several studies have investigated the effects of FLX exposure in fish, including Abreu *et al.* (2014) and (2015), Dorelle *et al.* (2017) in cichlid fish and de Farias *et al.* (2020) in zebrafish. Conducting studies on the ecotoxicological potential of environmental pollutants at low concentrations poses a significant challenge. The question often arises as to whether these compounds at environmental concentrations can disrupt the physiological functions of organisms in aquatic ecosystems. Therefore, it is crucial to investigate the effects of exposure to environmentally relevant concentrations of FLX. Furthermore, monitoring histopathological changes can provide valuable insights into the pathological effects of water pollution on fish (Kazempoor *et al.*, 2015). In this study, we examined the histopathological

effects of short-term exposure to FLX at concentrations relevant to the environment on the gills and overall health of zebrafish.

## Materials and methods

### *Fish species and test conditions*

A total of 150 adult zebrafish (weight:  $335 \pm 45$  mg; length:  $3.32 \pm 0.29$  cm) were sourced from a zebrafish breeding and rearing facility located in Sari, Iran. Before commencing the experiment, the fish underwent a 14 day acclimation period in spacious glass tanks ( $40 \times 40 \times 40$  cm) filled with 16 liters of tap water that had been treated to remove chlorine. During the acclimation period, the fish were fed twice daily with commercial feed from Beumar, France. The water temperature was maintained at  $26 \pm 0.5^\circ\text{C}$ , and the dissolved oxygen level was maintained at 5.0 mg/liter (mg/L). The pH of the water was within the range of 7.2 - 7.4 and the water hardness was kept at 50-60 mg/L. Continuous aeration was provided in the tanks, which were not equipped with any filtration system. The lighting cycle was set at 16 hours of light and 8 hours of darkness. Throughout the entire exposure experiment, feeding of the zebrafish was carried out on a daily basis.

### *Experimental design*

The fish were randomly distributed into 15 experimental tanks, with each tank containing 12 fish. An acute toxicity test was conducted based on the methodology described by Duarte *et al.* (2019) and followed OECD guidelines (test no. 203). The test duration was 96 hours, and four concentrations of fluoxetine hydrochloride (Sigma-Aldrich, PHR1394-1G) were used, along with a control

treatment. The concentrations tested were 0, 0.1, 0.5, 10, and 100  $\mu\text{g/liter}$ . Each concentration was replicated three times. The selected concentration range for this study falls within the reported environmental concentrations of antidepressants and their metabolites and (Duarte *et al.*, 2019). Stock solutions of FLX hydrochloride (Sigma-Aldrich, PHR1394-1G) were prepared using distilled water and stored at  $-20^\circ\text{C}$ , following the methodology described by Duarte *et al.* (2019). To maintain the desired FLX concentration throughout the experiment, 80% of the water in the tanks was renewed daily, and the appropriate amount of FLX was added back to the tanks from the stock solution. After the 96-hour exposure period, samples were collected for histopathological examination to assess any potential pathological effects.

### *Histopathological changes in tissues*

To prepare tissue slides of the gills and kidneys, three zebrafish from each treatment group were euthanized using the rapid chilling method. The fish were immersed in an ice water bath (5 parts ice to 1 part water) at a temperature of  $2-4^\circ\text{C}$  until the opercular movement ceased, following the protocol described by Matthews and Varga (2012). Subsequently, the fish were dissected along the ventral line, and the gill and kidney tissues were carefully collected. The collected tissues were then placed in 10% formalin solution for fixation. After 24 hours, the formalin solution was replaced, and the samples were stored until further processing for histological slides. For the preparation of histological slides, the fixed samples were transferred

to 70% ethanol for preservation. The samples were then subjected to dehydration using a series of increasing alcohol concentrations. Following dehydration, the samples were cleared in xylene and embedded in paraffin wax. Using a microtome, tissue sections with a thickness of 4-5  $\mu\text{m}$  were prepared. The tissue sections were mounted on slides for pathological analysis and stained with hematoxylin-eosin. Finally, the slides were examined under a microscope (Olympus BX51; Olympus, Tokyo, Japan). Histopathological changes were assessed by comparing the severity of the observed changes in the treated groups to the control sections, as described by Alavinejad *et al.* (2023).

## Results

### *Histopathological changes in gill tissues induced by fluoxetine*

Table 1 and Figure 1 present the histopathological changes observed in the gill tissues. In the control group, the primary and secondary lamellae of the gill tissue appeared normal. However, in the fish treated with fluoxetine (FLX), structural alterations were evident, and the severity of these changes intensified with higher concentrations of fluoxetine. The gills of the fluoxetine-treated fish exhibited various degrees of hyperplasia, aneurysm formation, clubbing, shrinkage, atrophy, and fusion of the secondary lamellae.

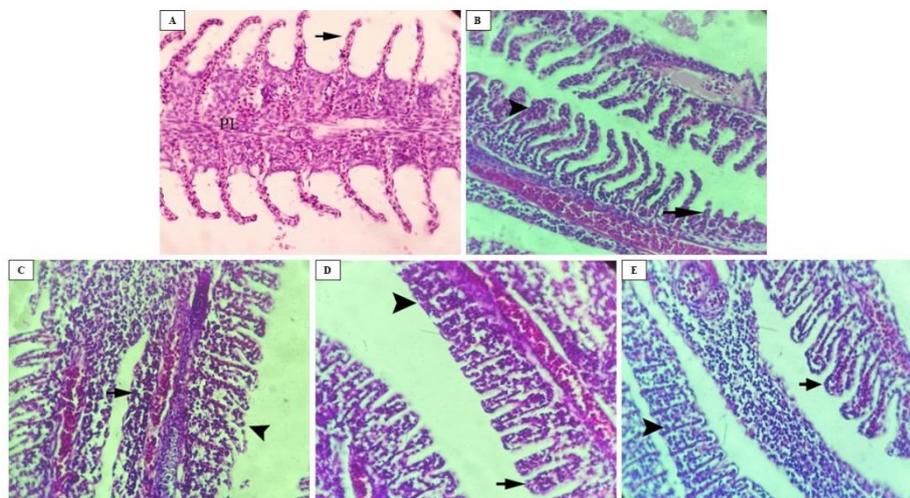
**Table 1: Histological Alterations in Gill Tissues Following Exposure (Scoring System: 0 - No Injury; 1 - Injury in <25% of Cells; 2 - Injury in >25% and <50% of Cells; 3 - Injury in >50% and <75% of Cells; 4 - Injury in >75% of Cells).**

Histopathological alteration	Treatment				
	C	T1	T2	T3	T4
Secondary lamellae shrinking	0	1	2	2	3
Lamellar fusion	0	0	3	4	4
Lamellar aneurism	0	1	1	1	1
Lamellar clubbing	0	1	2	2	3
hyperplasia	0	0	3	3	3

### *Effects of fluoxetine on histopathological changes in kidney tissues*

Table 2 and Figure 2 present the histopathological changes observed in the kidney tissues. In the control group, the urinary tubes and glomeruli in the kidney tissue appeared normal. However, in the fish treated with fluoxetine (FLX), structural alterations

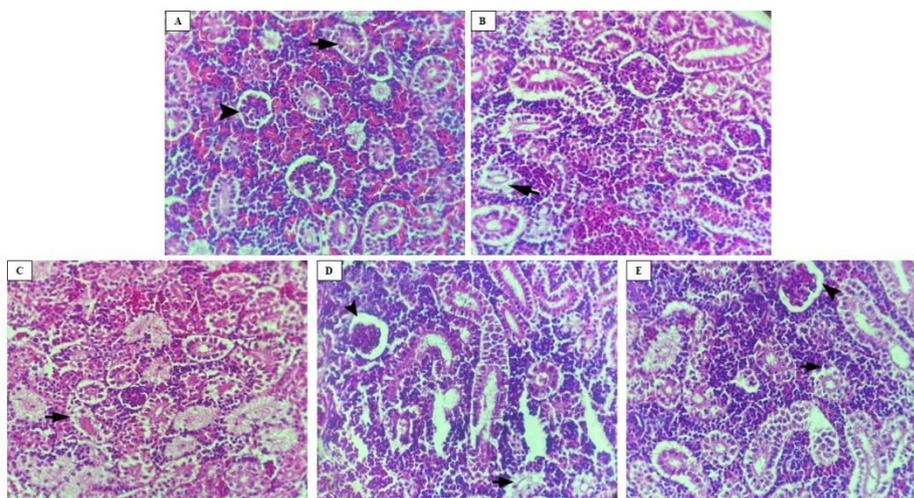
were evident in the kidneys. These included varying degrees of expansion of Bowman's space, increased thickness of Bowman's capsule, dilation of glomerular capillaries, as well as degeneration and necrosis of the tubules. These changes were observed in the kidney tissue of all fish treated with fluoxetine.



**Figure 1: Histopathological Alterations in Gill Tissues Following Exposure.** A) FLX 0 µg/L (Control): Normal primary lamellae (PL) and normal secondary lamellae (arrow); B) FLX 0.1 µg/L (Treatment 1): Clubbing of secondary lamellae (arrowhead) and shrinkage and atrophy of secondary lamellae (arrow); C) FLX 5 µg/L (Treatment 2): Severe clubbing and fusion of secondary lamellae (arrowhead) and severe shrinkage and atrophy of secondary lamellae (arrow); D) FLX 10 µg/L (Treatment 3): Severe clubbing of secondary lamellae (arrow) and severe fusion of secondary lamellae (arrowhead); E) FLX 100 µg/L (Treatment 4): Severe clubbing of secondary lamellae (arrow) and severe fusion of secondary lamellae (arrowhead).

**Table 2: Histopathological Alterations in Kidney Tissues Following Exposure (Scoring System: 0 - No Injury; 1 - Injury in <25% of Cells; 2 - Injury in >25% and <50% of Cells; 3 - Injury in >50% and <75% of Cells; 4 - Injury in >75% of Cells).**

Histopathological alteration	Treatment				
	C	T1	T2	T3	T4
Dilation of bowman's space	0	0	0	1	1
Increased bowman's capsule thickness	0	0	0	1	1
Dilation of glomerular capillary	0	1	1	2	2
Tubular degeneration	0	1	3	3	3
Tubular necrosis	0	0	0	1	2



**Figure 2: Histopathological Alterations in Kidney Tissues Following Exposure to Fluoxetine.** A) FLX 0 µg/L: Normal urinary tubes (arrow) and normal glomerulus (arrowhead); B) FLX 0.1 µg/L: Mild degeneration of urinary tubes (arrow); C) FLX 5 µg/L: Significant degeneration of urinary tubes (arrow); D) FLX 10 µg/L: Significant degeneration of urinary tubes (arrow) and expansion of the glomerular space (arrowhead); E) FLX 100 µg/L: Significant necrosis of the epithelium of urinary tubes (arrow) and expansion of the glomerular space (arrowhead).

## Discussion

Histopathological examination of fish organs exposed to pharmaceutical residues, such as FLX, is a valuable approach for evaluating biological changes and serves as an indicator of animal health (Galus *et al.*, 2013; de Farias *et al.*, 2020). Organ changes are easier to identify than functional changes and are considered early warning signs of damage. Therefore, in this study, we focused on assessing histopathological alterations in the gills and kidneys of zebrafish following acute exposure to low concentrations of FLX. Compared to other pharmaceutical residues, there has been limited research on the effects of antidepressant residues, including FLX, on fish. However, some studies have investigated the behavioral effects of FLX residue exposure in fish (Martin *et al.*, 2017; Duarte *et al.*, 2019; de Farias *et al.*, 2020). Additionally, investigations have examined the impact of FLX exposure on various organs such as the liver and intestine tissues of adult zebrafish (de Farias *et al.*, 2020), the liver of larval zebrafish (Nowakowska *et al.*, 2020), and the spleen and kidney head in cichlid fish (Rey Vázquez *et al.*, 2020). Furthermore, several studies have demonstrated the absorption and accumulation of FLX in aquatic organisms' tissues (Schultz *et al.*, 2010; Liu *et al.*, 2021). However, our review of existing studies did not uncover any specific research addressing the effects of FLX exposure on the gills and posterior kidney tissues of fish.

The gills of fish are particularly susceptible to toxic compounds as they

are the first organs exposed to foreign substances in the surrounding environment (Wolf *et al.*, 2015). Consequently, they serve as a vital organ for studying the direct impact of pollutants in aquatic ecosystems (Rodrigues *et al.*, 2015). Previous research has indicated that the effects of selective serotonin reuptake inhibitors (SSRIs), such as FLX, are primarily significant in cases of chronic exposure and as a result of long-term anxiety effects (Vera-Chang *et al.*, 2019). However, in our study, exposure to FLX had detrimental effects on the gill tissue of zebrafish, leading to various pathological changes, including blood congestion, leucocyte infiltration, apical fusion of lamellae, complete fusion of several lamellae, capillary dilation, epithelial lifting of lamellae, hyperplasia of epithelial cells, hypertrophy and proliferation of mucous cells, and necrotic areas.

While fish gills are known to respond rapidly to environmental changes, it is important to note that the structural changes observed in this tissue as a result of pollutant exposure are not specific and can vary depending on the concentration and duration of exposure (Raskovic *et al.*, 2010). Nonetheless, considering the previous findings of FLX accumulation in fish gill tissue reported by Liu *et al.* (2021), the occurrence of histological lesions in this organ due to FLX exposure is not unexpected. The observed changes in our study are consistent with the investigations of Nunes *et al.* (2015), Rodrigues *et al.* (2019), and Rodrigues

*et al.* (2019) on the effects of other pharmaceutical residues in fish. The histological changes observed in the gills can be classified into two main types, each indicating different aspects of the impact of toxic substances. The first type includes direct effects such as blood congestion and capillary dilation, which demonstrate the immediate influence of the toxic compound. The second type comprises adaptive changes, such as hyperplasia of epithelial cells and fusion of lamellae, which serve as protective mechanisms by reducing the contact surface and increasing the diffusion distance for toxic compounds within the gills (Rodrigues *et al.*, 2017; Rodrigues *et al.*, 2019). In our study, we observed both types of changes.

Among the pathological alterations observed in our study, lamellar hyperplasia was a commonly observed change, leading to either complete or partial fusion of lamellae. Raskovic *et al.* (2010) described the complete fusion of secondary lamellae as a peripheral gill disease. It is important to note that while the hyperplasia of gill epithelial cells, by increasing the diffusion distance between blood capillaries and the surrounding environment, helps reduce the entry of toxic compounds into the body, it may also affect the respiratory, excretory, and osmotic regulation functions of this organ (Wolf *et al.*, 2015; Yancheva *et al.*, 2016). The structural damage to fish gills, which affects their functioning, can result in metabolic and physiological complications (Stoyanova *et al.*, 2015).

As the gills play a crucial role in fish for respiratory and osmotic regulation functions, the observed damage to gill tissues due to FLX exposure in our study may have detrimental effects on oxygen consumption and the ability of fish to regulate osmotic balance.

In freshwater bony fishes, the kidneys play a crucial role in regulating pH, water balance, and solute excretion (Galus *et al.*, 2013). Due to their significant blood supply from the postbranchial region, kidney lesions in fishes have been considered as reliable indicators for investigating environmental pollution (Mishra and Mohanty, 2008). In our study, the kidneys of zebrafish in the control group exhibited a normal structure. However, exposure to FLX had adverse effects on this tissue, resulting in various pathological changes, including expansion of Bowman's space, increased thickness of Bowman's capsule, expansion of glomerular capillaries, tubular degeneration, and necrosis in the kidney tissue of fish. Although the damage in the gills was more apparent than in the kidney tissue in our study, this could be attributed to the direct contact of gills with pollutants in the water. Nevertheless, considering that the accumulation of FLX in fish kidney tissue, similar to the gills, has been previously reported by Liu *et al.* (2021), the occurrence of histological lesions in the kidneys due to FLX exposure is not unexpected. Other studies have also reported kidney tissue damage in fish exposed to pharmaceutical residues, which supports our observations in all

FLX-exposed fish tissues (Tribskorn *et al.*, 2007; Galus *et al.*, 2013; Choi *et al.*, 2018; Ogunwole *et al.*, 2021). Galus *et al.* (2013) observed significant effects, including kidney tubule damage, in zebrafish exposed chronically to low doses of pharmaceutical residues, indicating that even low concentrations of pharmaceutical residues can cause harm. However, Galus *et al.* (2013) suggested that studying the effects of drug exposure on kidney function would require larger fish species than zebrafish.

The histological lesions observed in the kidney in our study can be attributed to the occurrence of oxidative stress in fish exposed to pharmaceutical residues, as reported by Li *et al.* (2010). Excessive production of reactive oxygen species (ROS), as indicated by Cao *et al.* (2015), can induce pathological changes in kidney tissue. These pathological changes can affect the plasma concentration of xenobiotics and disrupt the homeostatic control of water, pH, and ions (Galus *et al.*, 2013). Regarding the differences between our observations and other studies investigating the effects of various pollutants on tissue pathology changes in fish, it is important to note that results are influenced by factors such as the exposure methods, drug concentration or chemical composition used, duration of exposure, age, species, gender of the fish, as well as the specific endpoints evaluated (Correia *et al.*, 2023). These variations can contribute to differences in the observed outcomes across studies.

## Conclusion

The present study aimed to assess the toxic effects of FLX on the gills and kidneys of zebrafish using histopathological examinations. Despite the entry of drugs and their active metabolites into water through sewage effluents, there have been limited investigations into the effects of short-term exposure to pharmaceutical residues on gill tissues and whole fish. Hence, it is necessary to investigate the impact of short-term exposure to these compounds on aquatic organisms, particularly fish. The results clearly indicate that even exposure to fluoxetine at concentrations within the micrograms per liter range for a short duration can induce damage to the gill and kidney tissues of fish. This study emphasizes the potential underestimation of the adverse effects of FLX residue exposure on wild fish populations. Therefore, it is essential to implement control measures and decrease FLX drug residue levels in the aquatic environment.

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