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Research Article

Short-term exposure to clofibric acid induces haematological and biochemical changes in *Labeo rohita* (Hamilton, 1822) fingerlings

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Abstract

Clofibric acid (CA) is a commonly used pharmaceutical drug which is an active derivative substance of clofibrate and other fibrates designed to improve fat metabolism in the human body. The toxicological effects of CA are garnering increased attention owing to its extensive use as a lipid regulator and pharmaceuticals and discharge into aquatic environments. The detection of CA in ground, surface and drinking water up to 4 µgL^{-1}. In the present investigation, *Labeo rohita* fingerlings were exposed to 1, 10 and 100 µgL^{-1} concentrations of CA for 96 hours. The evaluated parameters, such as haemoglobin content and Total erythrocyte count (TEC) levels, showed mixed trends in all exposed concentrations. However, the Total leukocytes count (TLC) level was increased in all concentrations. Blood glucose levels were elevated, and serum total protein levels deviated in all treated groups. All parameters assessed in fish exposed to various CA concentrations were changed significantly (Pd"0.05). In summary, alterations in haematological and biochemical parameters can serve as biomarkers for evaluating the toxicity of CA in aquatic environments. However, further in-depth research is required on using particular biomarkers to track the utilization of human pharmaceuticals.

Keywords: Biochemical parameters, Clofibric acid (CA), Haematological parameters, *Labeo rohita*

Highlights

- Exposure to clofibric acid (CA) altered the haematological parameters of fish.
- Fish exposed to clofibric acid (CA) altered blood glucose and total protein levels.

INTRODUCTION

The global rise in urbanization and industrialization has led to a more severe water pollution problem (Adeniyi *et al*., 2019). The everyday usage of manufactured goods, such as plastics, medicines, and cleaning supplies, is expanding at an exponential rate (Thomaidis *et al*., 2012), and numerous pollutants from these products have been found in a range of settings (Chinnaiyan *et al*., 2018; Patel *et al*., 2019). Uncontrolled human activity has resulted in the quantification of numerous contaminants in significant proportions in both surface and ground waters, as well as inside organisms (Bu *et al*., 2013; Wilkinson *et al*., 2016), which has created a number of potential dangers for aquatic habitats (Gavrilescu *et al*., 2015; Wang and Wang *et al*., 2016; Shi *et al*., 2022). Because of their high persistence and slow rates of degradation, a significant portion of these pollutants, known as contaminants of emerging concern, or CECs, remain in the effluent after being treated by conventional wastewater treatment methods, which are still unable to effectively remove all contaminants from influents (Luo *et al*., 2014; Hwang *et al*., 2016; Rodriguez-Narvaez *et al*., 2017; Rathi *et al*., 2021).

Emerging contaminants have become a cause for concern (Hernández and Muñoz, 2020). These emerging contaminants include pharmaceutically active compounds (PhACs), endocrine disruption chemicals (EDCs), pesticides and personal care products (PPCPs) (Eletta *et al*., 2019). One of the most widely used pharmaceuticals is clofibric acid (CA), an active derivative of clofibrate and other fibrates that is intended to enhance the body's ability to metabolize fat. It is also a hypolipidemic compound and an active derivative substance of clofibrate, which has been reported in water bodies worldwide (Buser *et al*., 1998; Nunes *et al*., 2005). The continuous discharge and occurrence of pharmaceutical drugs in the aquatic ecosystem has become a major problem due to their

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long persistent period in the environment (Fedorova *et al*., 2014). Furthermore, they can persist in the environment in an active form and affect the life of various aquatic organisms. Furthermore, in the aquatic environment, CA may act as an endocrine disruptor (Pfluger and Dietrich, 2001). Clofibric acid is non-biodegradable, highly bio-accumulative and very persistent in the environment, with a half-life of 21 years and a water residence time of 1-2 years (Richardson and Bowron, 1985; Buser and Muller, 1998). The detection level of clofibric acid in treated wastewater of the USA was 0.8-2 μg L^{-1} (Hignite and Azarnoff, 1977). The detection levels in groundwater and surface water were 4 μg L^{-1} and 0.07-0.27 μgL^{-1}, respectively worldwide (Wuersch *et al*., 2005).

Several studies have reported that xenobiotic compounds in the aquatic ecosystem may induce haematological and biochemical alteration in aquatic species (Jaffer *et al*., 2017; Naz *et al*., 2021; Akram *et al*., 2022).

The objectives of the experiment were to investigate the short exposure effects of clofibric acid on haematological and biochemical indices in *Labeo rohita* (Hamilton, 1822), which is a most cultivable and typically commercially important species in the Indian scenario (Karmakar *et al*., 2021).

MATERIALS AND METHODS

Experimental chemical: Clofibric acid [a- (p-Chlorophenoxy) isobutyric acid] (CAS No. 882-09-7) was purchased from Hind Biotech company. Dimethyl sulphoxide was used as a solvent in the present experiment.

Procurement and acclimatization of an experimental animal: With an average weight of 28.6±1.4 g (mean ± SD) and length of 12.8±1.2 cm (mean ± SD), the advanced healthy and uniformly proportioned *L. rohita* fingerlings were purchased from the Bengal Fish Hatchery in Sonarpur, Kolkata, and were securely transported via aluminium "Hundi" in the early morning to the Faculty of Fishery Sciences' wet laboratory (Department of Aquatic Environment Management). For acclimatisation, the fish were carefully moved to two FRP tanks with a 1000 L capacity and 950 L water volume. They had constant aeration and a photoperiod of 12:12 hours. Fish were given floating granules at a rate of 3% of body weight during the acclimatisation period. To prevent infection or sickness, the tanks underwent twice-daily cleanings and a thirty percent water exchange every other day.

Experimental set-up for short-term exposure studies: Based on the recent environmental concentrations of CA, three concentrations were selected for the present experiment, which were 1 (T_1), 10 (T_2) and 100 (T_3) μg L^{-1}, respectively. Each concentration had three replicates and one control. The short-term toxicity (96 hours) was carried out by EPA guidelines (EPA, 2002) in each glass aquarium (75 L capacity and 60 L volume) and simultaneously, the current study period was conducted strictly in accordance with the animal ethics norms of West Bengal University of Animal and Fishery Sciences, Kolkata, India. During the bioassay experiment, seven fish were added to each aquarium, and feeding was stopped. In order to keep the concentration of CA constant, fresh solution was added, and the test water was replenished after the 24-hour period. The following physicochemical parameters (APHA, 2005) were examined twice over the 96-hour study period.: The ranges of temperature, dissolved oxygen, pH, free carbon dioxide, and total alkalinity were 16-18°C, 5.7-6.0 mg L^{-1}, 7.3-7.6, 0.521-0.841 mg L^{-1}, and 159-178 mg L^{-1}, respectively during the short-term exposure of CA.

Collection of blood: Before drawing blood, each fish was anaesthetised with 50 μL^{-1} of clove oil (Apollo Pharmacy, India). Using a medical syringe (23G), blood was extracted from the caudal vein after being washed with 2.7% EDTA solution. To avoid haemolysis, the drawn blood was quickly transferred to EDTA test tubes and vigorously shaken. After being moved to Eppendorf tubes, some blood that had not been treated with anticoagulant was left to stand for two hours. The serum was then extracted by centrifugation, which was carried out for 15 minutes at 4°C and 4500 rpm (Ali *et al*., 2018).

Biochemical analysis: The commercial assay kits, namely Liquixx Glucose kit (BLT120235) and Liquixx total protein kit (BLT00054), respectively, were procured from Erba® Mannheim, Transasia Diagnostic BioMedicals Pvt. Ltd., Himachal Pradesh, India and performed as per the manufacturer's instruction provided. Blood glucose level and serum total protein were expressed in mg dL^{-1}.

Haematological analysis: A fresh blood sample was collected to determine haematological parameters. The haemoglobin (Hb) content was analysed following the cyanmethemoglobin method (Kampen Van and Zijlstra, 1961) and the following formula; Haemoglobin (g dL^{-1}) = [OD (T)/OD(S)] × (251/1000) × 60, Where T is the test, and S is the standard.

Using a hemocytometer, the total erythrocyte count (TEC) or red blood cell count (RBC) was measured according to Hendricks' (1952) instructions. White blood cell (WBC) or total leucocyte count (TLC) counts were performed using leucocyte dilution fluid (Qualigens) in a Neubauer's counting chamber of a hemocytometer, according to Shaw's (1930) method.

Statistical analysis: All the values were evaluated through one-way analysis of variance (ANOVA) using SPSS software version 22.0 (SPSS Inc., Chicago, USA). Subsequently, a Duncan multiple range test (DMRT) was applied to identify significant differences ($P \leq 0.05$) among the concentrations for each parameter.

RESULTS

Haematological indices: Alterations of haematological indices are represented with specific data in Table 1 and Fig. 1, 2 and 3. In the case of Hb content, the overall difference among the treatments remained highly significant ($P<0.05$), but no significant difference was evidenced between C and T_2 and between T_1 and T_2. However, the highest mean Hb content was obtained in T_3 and the lowest in T_1. In the present experiment, the RBC count in *L. rohita* differed significantly ($P<0.05$) among the treatment groups having the highest mean value in C, followed by T_3, T_2, and T_1. However, no noteworthy difference was found between C and T_3 and between T_1 and T_2. WBC count in *Labeo rohita* differed significantly ($P<0.05$) among the treatment regimes in the present study, having the highest mean value in T_3 followed by T_2, T_1 and C. However, between T_2 and T_3, no significant difference remained.

Table 1. Mean value (mean ± SD) of haematological indices of *L. rohita* subjected to various clofibric acid concentrations during 96 hours of exposure

Concentration ($\mu g\ L^{-1}$)	Haematological indices		
	Hb ($g\ dL^{-1}$)	TEC ($10^6/mm^3$)	TLC (cu. per mm^3)
C	15.18±0.95^b	5.80±0.28^b	6266.33±42.39^a
T_1	12.32±0.86^a	4.29±0.32^a	6718.53±34.55^b
T_2	13.4±0.84^{ab}	4.38±0.36^a	6916.03±42.08^c
T_3	18.52±1.16^c	5.30±0.29^c	6958.88±63.71^c

Column wise alphabetical superscript expresses the significant difference

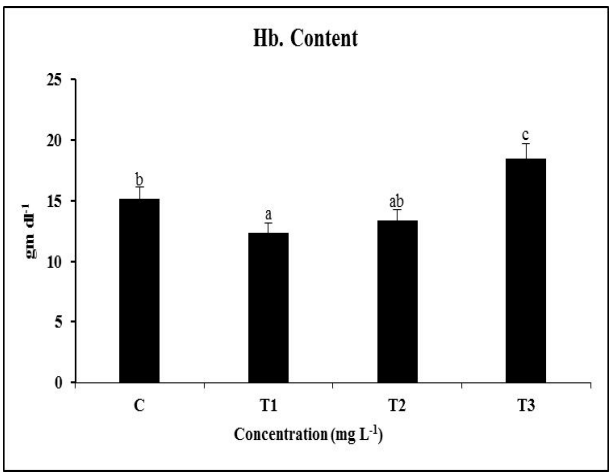

Fig. 1. Haemoglobin content (gm dL^{-1}) of *L. rohita* fingerlings during the short-term exposure to CA throughout the experimental period

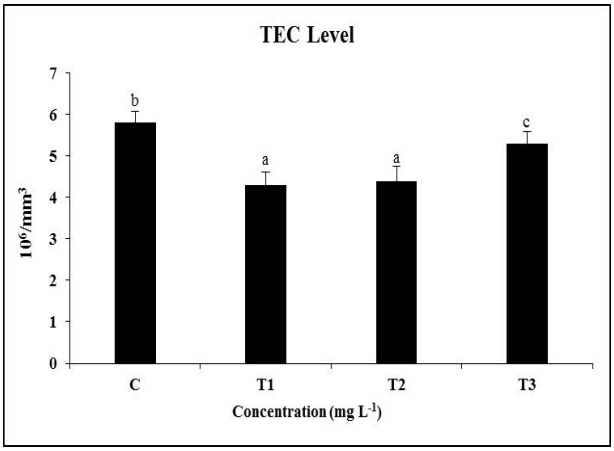

Fig. 2. Total erythrocyte count (106/mm3) of *L. rohita* fingerlings during the short-term exposure to CA throughout the experimental period

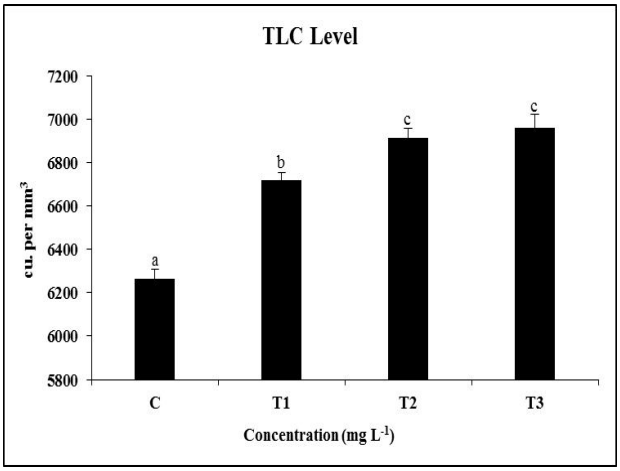

Fig. 3. Total leucocyte count (cu. per mm3) of *L. rohita* fingerlings during the short-term exposure to CA throughout the experimental period

Biochemical parameter: Changes in biochemical indices are represented with specific data in Table 2 and Fig. 4 and 5. The highest mean value of plasma glucose (mg dL^{-1}) in T_1 followed by C, T_2 and T_3, whereas a significant alteration ($P<0.05$) was observed among the treatment regimes in the current investigation. However, no significant difference was found between T_1 and T_3. The serum total protein level in the exposed fish group in all treatments was decreased compared to the control but was statistically significant ($P<0.05$). The highest complete serum protein was found in the T_1 group, and the lowest level was in the T_3 treatment group.

Table 2. Mean value (mean ± SD) of biochemical indices of *L. rohita* subjected to various clofibric acid concentrations during 96 hours of exposure

Concentration (mg L^{-1})	Biochemical indices	
	Blood glucose (mg dL^{-1})	Serum total protein (mg dL^{-1})
C	152.42±5.51[a]	3.34±0.13[d]
T_1	313.89±7.51[c]	2.25±0.1[b]
T_2	296.79±6.3[b]	2.71±0.15[c]
T_3	309.81±7.57[c]	1.84±0.16[a]

Column wise alphabetical superscript expresses the significant difference

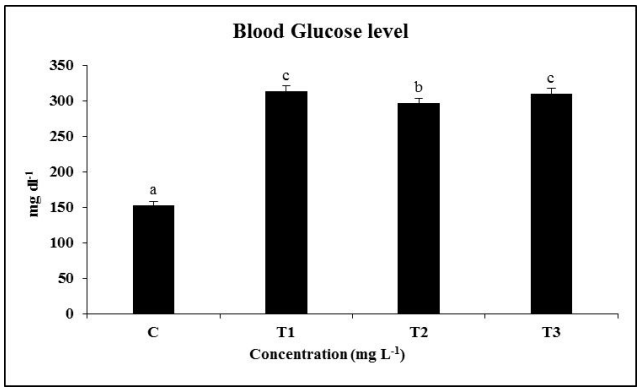

Fig. 4. Blood glucose level (mg dL^{-1}) of *L. rohita* fingerlings during the short-term exposure to CA throughout the experimental period

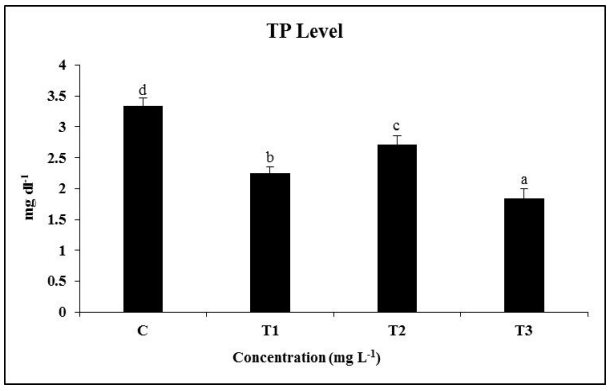

Fig. 5. Total protein level (mg dL^{-1}) of *L. rohita* fingerlings during the short-term exposure to CA throughout the experimental period

DISCUSSION

In the present era, the occurrence of CA is a significant issue in the majority of nations due to its toxicological effects. The current study clarified that fish can have a variety of physiological problems due to unregulated use of CA and its subsequent build-up in aquatic environment. The toxicity of CA may lead to altered haematological and biochemical parameters.

Blood parameters can provide information on the stress level and potential extent of damage by assessing physiological and biochemical abnormalities in fish (Osman *et al*., 2010). The lysis of erythrocytes and the suppression of erythrocyte formation may be the cause of the reduced amount of haemoglobin content (Karmakar *et al*., 2021). Similarly, Mekkawy *et al*. (2011) reported that the blood cell's apoptosis caused the haemoglobin content to drop, which is similar to the findings of the present study. According to Saravanan *et al*. (2011), Hb content is deviated, which may be due to the inhibition of erythropoiesis or by the destruction of red cells. Furthermore, the replacement of oxidized denatured Hb and increased oxygen availability to tissues may have contributed to the raised level of Hb content in the CA exposure at higher conditions (Saravanan *et al*., 2011), which was corroborated with the outcome of the present study.

Yaghoobi *et al*. (2017) provided evidence to support the theory that suppressed antioxidant enzymes cause a rise in free radicals, which in turn causes a decrease in RBC. Similar factors during CA exposure may have contributed to the lowering of RBC in the current investigation. Karmakar *et al*. (2021) also justified same reason for the reduction of the RBC counts. On the other hand, the RBC level also increased, which might be due to the replacement of oxidised denatured Hb supplying huge oxygen in tissues (Saravanan *et al*., 2011). Carvalho *et al*. (2006) documented that immature RBC counts increased due to an increase in MCV value, which was the same reason that occurred in the present study.

WBCs play a significant role in the control of immune function in many organisms, and variations in WBC numbers in response to contaminants represent the decline in the fish's general immunity (Kavitha *et al*., 2010). In the current study, increases in WBC count during CA exposure are similar to the studies of Kavitha *et al*. (2010) and Yaghoobi *et al*. (2017). According to Karmakar *et al*. (2021), potential causes of increasing WBC were residual damages like tissue necrosis and inflammation, which supports the findings of the present study.

Sayed and Soliman (2018) documented that the blood glucose level elevated due to the secretion of stress response cortisol. Saravanan *et al*. (2011) reported that the blood glucose level increased due to the synthesis of glucagon and adrenocorticotropic hormone in *Cyprinus carpio* exposed to clofibric acid, which was in accordance with the findings of the present study. Saravanan and Vijayakumar (2012) found that during exposure to clofibric acid and diclofenac, the blood glucose level of *Cirrhinus mrigala* was also increased due to the same reason which supports the outcome of the present experiment. The present findings were also supported by the findings of Li *et al*. (2010), who reported that a higher level of plasma glucose in rainbow trout *O. mykiss* exposed to CBZ might be increased due to an increase in the circulation of corticosteroid hormone, which corroborated with the outcome of the present investigation.

Karmakar *et al*. (2021) pointed out that the hyperglycaemic reaction of glycogenolysis and gluconeogenesis process may result in a drop in serum protein levels. According to Sayed and Soliman (2018), the hyperglycaemic response of the gluconeogenesis and glycogenolysis processes causes the deviation in serum protein levels. The findings of the present study may account for such physiological responses of CA-exposed fishes. Karmakar *et al*. (2021) illustrated that the reduction in serum total protein might have occurred due to malnutrition and starvation. The significant decrease in plasma protein level may be caused by impaired protein synthesis (Kavitha *et al*., 2010; Saravanan *et al*., 2011) which was justified for the findings of the present investigation. Jenkins *et al*. (2003) pointed out that a decrease in protein levels may be attributed to the stress-mediated mobilization of toxic compounds by the fish to fulfil an increased demand for energy. Martin *et al*. (2010) documented that the decline in protein level may be related to tissue repairing and the detoxification mechanism during stress conditions, which supports the observation of the present experiment.

The current study demonstrates that a 96-hour exposure to clofibric acid has multiple acute toxicological effects on *Labeo rohita* fingerlings. This exposure leads to various abnormalities in haematological and biochemical indices due to the presence of these emerging contaminants. The findings of this study could assist policymakers in understanding the eco-toxicological impacts of CA, thereby aiding in the regulation of its usage and disposal.

Conflict of 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.

Author's contribution: SB: Investigation, validation, software and original draft preparation; SK: Methodology, visualization, validation, review and editing of the manuscript; GSP, AJ, SM: Visualization, validation, editing of the manuscript; SB: Review and edit the manuscript; SKR: Conceptualization, supervision, reviewing and editing of manuscript.

Data availability statement: Data will be available on request.

Ethical statement: The current study was conducted strictly in accordance with the animal ethics norms of West Bengal University of Animal and Fishery Sciences, Kolkata, India.

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