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Biochemical Patterns and Genotoxicity of the Endocrine Disruptor Metformin in the Freshwater Fish *Labeo rohita*

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Abstract: Metformin is one of the most extensively used drugs, making it one of the most likely endocrine disruptors in the environment, which may negatively affect fish and other freshwater animals. Still, there is a dearth of studies examining metformin's impact on freshwater creatures, like fish. This research aimed to identify the biochemical and genotoxicity effects of the endocrine disruptor metformin in the freshwater fish *Labeo rohita* at ecologically appropriate doses. Metformin's toxicity was evaluated by subjecting *L. rohita* to the drug over 28 days at two dosages (40 µg/L to 80 µg/L). The results indicated that 40 µg/L and 80 µg/L of metformin caused an increase in reactive oxygen species and the generation of free radicals in the body of *L. rohita*, which in turn caused impairment and alterations in total hemoglobin, red blood corpuscles, white blood corpuscles, oxidative stress, lipid peroxidation, protein carbonyl activity, respiratory burst activity, myeloperoxidase activity, and lysozyme activity. In addition, animals treated with the maximum metformin dose (80 µg/L) demonstrated substantial DNA damage in the genotoxicity experiment. Metformin's endocrine-disrupting actions may have unintended ramifications for the well-being of aquatic species in their natural habitats. Results of the study demonstrated a serious concern that metformin exposure might be harmful to aquatic life.

Keywords: *Labeo rohita*; metformin; hemoglobin; reactive oxygen species; lysozyme

Key Contribution: Metformin, an endocrine disruptor, has been found to augment cellular damage and perturb redox homeostasis. The outcome of this study would elicit notable concern regarding the possible influence of metformin exposure on aquatic fauna.

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1. Introduction

Diabetes is a persistent metabolic condition that is distinguished by elevated levels of glucose in the bloodstream. Diabetes encompasses various classifications, such as type 1 diabetes, type 2 diabetes, and gestational diabetes. Type 1 diabetes mellitus is a pathological state characterized by an autoimmune response that leads to the destruction of pancreatic beta cells responsible for insulin production. Type 2 diabetes mellitus represents the prevailing form of diabetes, characterized by insulin resistance or inadequate insulin secretion by the body. According to the World Health Organization, diabetes ranks as

the seventh leading cause of mortality in the United States and the ninth leading cause of mortality worldwide. In the last 20 years, the number of people with diabetes has more than doubled [1]. Metformin, an endocrine disruptor mainly used to treat type 2 diabetes, is among the most often prescribed antidiabetic pharmaceuticals that end up in the environment. Both metformin prescriptions and consumption skyrocketed globally [2,3]. This medication falls under the category of biguanides and functions by diminishing hepatic glucose production, enhancing insulin sensitivity, and reducing glucose absorption from the intestines. Metformin does not increase the insulin level in the body, but it helps lower blood sugar by improving the body's utilization of insulin. Metformin is commonly recommended as the primary pharmacological intervention for type 2 diabetes, in conjunction with lifestyle adjustments, such as a nutritious diet and consistent physical activity. It is typically taken orally, usually with meals, and comes in various forms, including tablets and extended-release tablets. Humans do not metabolize metformin before excreting it; hence, its elimination rate is >90% in sewage-treatment units and up to 3 μg/L in surface waters after intake [3]. Metformin can enter water bodies through various routes, primarily via human excretion. After being consumed, a significant portion of metformin is excreted by the body in its original form, without undergoing significant degradation. Consequently, metformin can end up in wastewater through sewage systems, as well as in surface water bodies, such as rivers and lakes. Once metformin enters the aquatic environment, it may cause a variety of side effects in fish and other aquatic creatures [4].

Metformin is abundant in the present wastewater treatment plant (WWTP) effluent and surface water, as has been reported in the past. WWTP effluent contains the drug in quantities ranging from 1 μg/L to 47 μg/L [5]. Metformin, at doses between 10 and 250 mg/kg daily, has been shown to affect CYP17 activity, fatty acid-loaded steroid acute regulatory protein (StAR), and the representation of steroidogenic enzymes, like cytochrome p450 (CYP)11A and 3-hydroxysteroid dehydrogenase (3-HSD), while doses of 1500 mg/day impact the levels of anti-Müllerian hormone (AMH) in PCOS patients [6]. Metformin's presence in aquatic environments raises concerns due to its potential effects on aquatic organisms and ecosystems. Research has shown that metformin can accumulate in fish tissues, affecting their physiology and behavior. It has been observed to interfere with fish metabolism, disrupt their hormonal systems, and impair their reproductive processes. Metformin exposure can alter the activities and physiology of fish. For example, it may affect swimming performance and feeding patterns, affecting hormone levels, changes in sex ratios, and reproductive functions [7].

According to previous investigations, endocrine disruptors in WWTP effluents negatively impact fish and other aquatic organisms; it has been found to exhibit toxicity toward aquatic organisms, such as algae, fish, and invertebrates. It can interfere with the metabolic processes of these organisms, potentially leading to adverse effects on aquatic ecosystems [7]. Specifically, metformin exposure constrains complex I gluconeogenesis of the electron transport chain and activates metabolic enzymes and transcript features, thus affecting the cellular energy balance [3]. When administered to aquatic animals, metformin causes mutagenesis and intersex by connecting insulin signaling with steroidogenesis. It has been recognized as a potential contributor to the development of antibiotic resistance. Studies have suggested that continuous exposure to low concentrations of metformin in water may promote the proliferation of antibiotic-resistant bacteria, reducing the effectiveness of antibiotics in both aquatic and terrestrial environments. Niemuth and Klaper [8] experimentally proved that 28-day chronic exposure of metformin in fathead minnow (*Pimephales promelas*, FHM) male fish exposed to 40 μg/L resulted in liver mobilization as the mRNA for the egg protein vitellogenin (VTG) was programmed. This phospholipid glycoprotein is usually produced in the livers of females who are capable of producing eggs, where metformin acts as an endocrine disruptor (EDC). In addition, the male testis of *Pimephales promelas* exposed to metformin exhibits the considerable induction of intersex after being treated with 40 μg/L metformin for one year. In addition, the common carp liver is a target organ attacked by the toxic environmental pollutants chlorpyrifos and

4-tert-butylphenol [9,10]. Excess environmental pollutants can cause oxidative stress [10,11] and immunosuppression [9]. Oxidative stress and immunity are involved in the molecular mechanism in *Luciobarbus capito* (*L. capito*) under a high-salinity environment [12].

The mechanistic action of metformin is that it stimulates the estrogen receptor (ER), which results in ER's movement from the cytosol to the nucleus, which tempts the transcription of endocrine-related genes, and this indicates that metformin is an effective endocrine-disrupting compound functioning via an ER-independent mechanism [4]. Metformin is one of the most common medications found in WWTP discharge, and although its hazardous effects on aquatic creatures have been researched mostly in terms of their metabolic effects, its immunological, biochemical, and genotoxic aspects have not been explored at all. The environmental concentrations of metformin are typically much lower than therapeutic levels. Nevertheless, due to the widespread use of metformin and its continuous release into the environment, its cumulative effects on aquatic ecosystems warrant attention [3].

In ecotoxicology evaluation studies, fish are often utilized as bioindicators to identify environmental contamination. *L. rohita*, the Indian major carp, is frequently farmed by Indian aquaculture farmers. Nevertheless, the precise toxicological mechanism of metformin, an antidiabetic drug, and its impact on the biochemistry and enzymology of *Labeo rohita*, a commonly farmed fish species in India's major ponds, remains relatively unexplored despite its regular exposure to various harmful contaminants. This investigation examined the effects of oxidative stress on various hematopoietic components, including total hemoglobin (Hb), red blood cells (RBC), and white blood cells (WBC), as well as non-specific immune components, such as respiratory burst activity (RBA), myeloperoxidase activity (MPO), and lysozyme activity (LYZ), and the genotoxic potential in *L. rohita*, a species of Indian major carp.

2. Materials and Methods

2.1. Chemicals

The standard solution was produced using metformin (1,1-dimethylbiguanidine hydrochloride), which was purchased from Sigma-Aldrich (Germany). The conventional method involved the combination of 50 mg of metformin with 10 mL of ethanol and 490 mL of Milli-Q water. The experimental concentrations of metformin at 40 g/L and 80 g/L were achieved by introducing 2 mL and 4 mL, respectively, of a standard solution into 5 L of double-distilled water. The ethanol standard for the control group was composed of 10 mL of ethanol and 490 mL of double-distilled water. The ethanol concentration of every control tank was generated through the combination of 4 mL of a standard solution with 5 L of double-distilled water [9].

2.2. Experimental Animals

Experimental animals utilized in this study were maintained in accordance with the experimental protocols previously reported by Sibiya et al. [13]. Juvenile *L. rohita* (n = 150; 7.3 ± 0.4 g; 6.7 ± 0.4 cm; mean ± SD) (30 males and 30 females) samples were procured from an aquaculture service located in Trichy, Tamil Nadu, India. The fish specimens were meticulously transported to the designated testing facility, where they were subsequently accommodated for a period of 15 days within 1000 L capacity tanks that were fully supplied with dechlorinated freshwater. The physicochemical parameters that are considered essential were measured and monitored using a multiparameter water quality meter (HORIBA U-52, Kyoto city, Japan). These parameters included temperature (28.45 ± 1.1 °C), pH (7.45 ± 0.3), salinity (0.24 ± 0.05 ppt), total ammonia (0.085 ± 0.01 mg N-NH_4 L^{-1}), dissolved oxygen (6.15 ± 0.4 mg L^{-1}), conductivity (345.65 ± 16.2 µs/cm), total hardness (137.55 ± 9.6 mg $CaCO_3$/L), and alkali (45.75 ± 6.2 mg $CaCO_3$/L). The experimental animals were provided with regular pellet food from Tairoun Feed Company in Taipei, Taiwan, and were subjected to a 12:12 h light/dark photoperiod, receiving two daily feedings. Prior to commencing the experimental period, the fish underwent

a 24 h fasting period to mitigate potential aquatic contamination resulting from feces. The experimental strategies and fish management methods employed in this research were evaluated and sanctioned by the Board of Animal Maintenance and Use, which is a part of the Faculty of Science at Alagappa University.

2.3. Experimental Setup and Sample Collection

Metformin, an antidiabetic drug, was tested in a 28-day chronic toxicity trial using juvenile *L. rohita*. A total of 30 fish of uniform size and weight were preserved as replicas for each group. All physiochemical parameters were monitored to keep fish in groups 1 (control), 2 (low dosage metformin 40 µg/L), and 3 (high dose metformin 80 µg/L) at a consistent level [14]. Animals had their water replaced and were given a dose of metformin that was safe for the environment every day before noon [9]. The water was routinely changed throughout the experimentation to prevent the animals from exposure to water pollution and keep them stress free. One night of fasting was forced upon every fish after the research period in order to gather samples. Using a sterile needle, blood samples were collected from the posterior vein of each fish; ten fish were sampled from each group. Liver samples were also collected and stored in PBS buffer since it is the primary organ for eliminating toxins from the body. After collecting samples, the serum was separated by centrifuging them at 5000 rpm for around 20 min. The samples were then stored at −80 °C for additional research [13]. In order to get more accurate results, the hematological parameters were determined using freshly drawn blood cells.

2.4. Hematological Analysis

Hematological analysis was performed on freshly drawn blood samples from individual fishes' posterior veins using a single-use, sterilized disposable needle. Hematological indices, including Hb, RBC, and WBC counts, were estimated from the obtained blood samples. Drabkin's [15] cyanmethemoglobin technique was used to calculate total Hb. Sivanandan's [16] technique was used to quantify red blood cells and white blood cells. Conventional methods were also used to evaluate and quantify the erythrocytes' hematocrit (Hct), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), and mean corpuscular hemoglobin concentration (MCHC), which was measured in accordance with [17].

$$\text{MCV (fl)} = \text{Hct (\%)} \times 10/\text{RBC count in millions/mm}^3$$

$$\text{MCH (pg)} = \text{Hb (g/dL)} \times 10/\text{RBC count in millions/mm}^3$$

$$\text{MCHC (g/dL)} = \text{Hb (g/dL)}/\text{Hct (\%)}$$

2.5. Sample Preparation for Biochemical Assays

The liver was of particular interest in this investigation; therefore, when the experiments had been completed, the livers of three animals from each group were taken and dissected thoroughly with the use of sterile dissection kits. The liver was removed, placed in a PBS buffer, homogenized using a mortar and pestle, and centrifugated at 5000 rpm for 20 min immediately after dissection. The supernatant was kept at −80 °C to analyze all biochemical parameters. Three test fish also had blood drawn from their posterior veins using a sterile needle. After centrifuging at 5000 rpm for 20 min, the blood supernatant was stored at −80 °C.

2.6. Indicators of Cellular Damage

Cellular damage activity and lipid peroxidation (LPO) were assessed based on the by-product malondialdehyde (MDA) and measured in µmol of MDA formed per mg protein in accordance with the procedure of Buege and Aust [18]. Tissue samples were mixed with TBARS and kept for incubation, which leads to the formation of malondialdehyde (MDA). Reznick and Packer [19] used the 2,4-dinitrophenylhydrazine method to assess

the protein carbonyl activity (PCA), which was represented in µmol carbonyl per mg of protein. Similarly, dinitrophenyl hydrazones formed from 2,4-dinitrophenylhydrazine were detected at 360 nm.

2.7. Non-Specific Immune Parameters

The lysozyme activity was determined using the method of Ellis et al. [20] using *Micrococcus lysodeikticus* (Sigma, ATCC 4698). Blood serum of 100 mL was mixed with 2 mL of Micrococcus lysodeikticus that had been diluted to a 0.02% (*w*/*v*) concentration in PBS buffer (0.05 M, pH 6.2). The absorbance at 530 nm was used to distinguish differences between fish treated with metformin before and after incubation. Following the method of Secombes et al. [21], respiratory burst activity was assessed, in which the serum samples were incubated for 60 min, while 50 mL of NBT (0.2%) was added to stop the incubation. This was followed by continuously washing the serum 3 times with PBS solution (pH 7.2). Further, the cells were fixed in methanol (30%, *w*/*v*) for about 3 min and dried at room temperature with 60 mL of potassium hydroxide (2 N) and 70 mL of dimethyl sulfoxide (DMSO). Finally, the optical density was measured at 540 nm. The MPO action was assessed employing the methodology proposed by Kumari and Sahoo [22]. Blood serum that was diluted was mixed with Hank's Balanced Salt Solution (HBSS) in 96-well plates. The mixture was then incubated at 30 °C for 30 min with the peroxidase substrate tetramethylbenzidine hydrochloride (TMB) and 5 mM H_2O_2 (hydrogen peroxide). Subsequently, a volume of 35 mL of sulfuric acid (H_2SO_4, 4 M) was introduced to terminate the reaction. Subsequently, the measurement of absorbance was conducted at a wavelength of 450 nm, and the results were expressed in units of U mL^{-1}.

2.8. Genotoxicity (Comet Assay)

The comet test, used to detect DNA damage in liver tissues, was performed. The comet test was performed in accordance with the procedure of Singh et al. [23], by means of some changes. At first, following some preliminary testing, liver tissues were extracted and lysed in lysis buffer. Furthermore, a microscopic comet slide with agarose gel (1.5% concentration) was prepared. Afterward, the comet slides were incubated in a fridge at 4 °C overnight. Then, the samples were run through electrophoresis in a denaturation buffer (for 25 min at 25 V), followed by a 2 min neutralization. Once the comet slides were neutralized, they were stained with ethidium bromide (EtBr) to be seen under a fluorescence microscope.

2.9. Statistical Analysis

One-way scrutiny of variance and Tukey's HSD were performed to compare the effects of metformin, over time and at varying doses, on oxidative stress and immunological markers in fish. The Shapiro–Wilk and Levene statistics were used to determine the regularity and variance homogeneity of each experiment, which were all carried out in triplicate. The obtained data were classified based on whether or not they showed a significant difference between the treatment and control groups (mean standard deviation). Microsoft Excel was used to create all graph comparisons for the study's statistical analysis.

3. Results and Discussion

3.1. Hematological Analysis

Non-target organism toxicity from pharmaceutical drugs and environmental contaminants was quantified using a battery of biomarkers. Several hematological measures and indices (Hb, Hct, RBC, WBC, MCV, MCH, and MCHC) were considered as a standard against which the toxic pressure, physiological state, and efficient status of animals living in the contaminated environment and the sublethal toxicity of chemicals could be evaluated [24]. According to the findings of the present investigation, both low (40 µg/L) and high (80 µg/L) doses of metformin reduced RBC and MCHC activities (Figure 1). Metformin's toxicity, as measured by reduced RBC counts, is best shown by the drug's ability to destroy hematopoietic centers, which are responsible for RBC generation. After

being exposing Indian major carp, *Cirrhinus mrigala*, to the non-steroidal anti-inflammatory medicine ibuprofen for 35 days, a similar drop in RBCs and the MCHC was observed [24]. The red blood cell count in *Cyprinus carpio* was shown to decrease 14 and 21 days after carbamazepine administration, according to research by Rezaei et al. [25]. In this investigation, both a low dosage (40 µg/L) and a high dose (80 µg/L) of metformin resulted in increases in Hb and Hct activity. Exposure to high levels of pollution may cause RBC growth, impaired respiratory performance, and stress, all of which lead to replacing oxidized, denatured Hb through the supply of fresh oxygenated blood to tissues [24]. White blood cells (WBCs) perform a significant role in the control of immunological activity in many species. There was a significant increase in the white blood cell count when metformin was administered to fish, suggesting a robust immune system response and defense mechanism. Fish may have a higher white blood cell (WBC) count as a result of the toxin's immune-stimulating characteristics and the protective precaution of decaying lymph cells from lymphomyeloid tissue [26]. In this study, researchers found that metformin improved anemia by increasing MCV and MCH while decreasing MCHC (Figure 1).

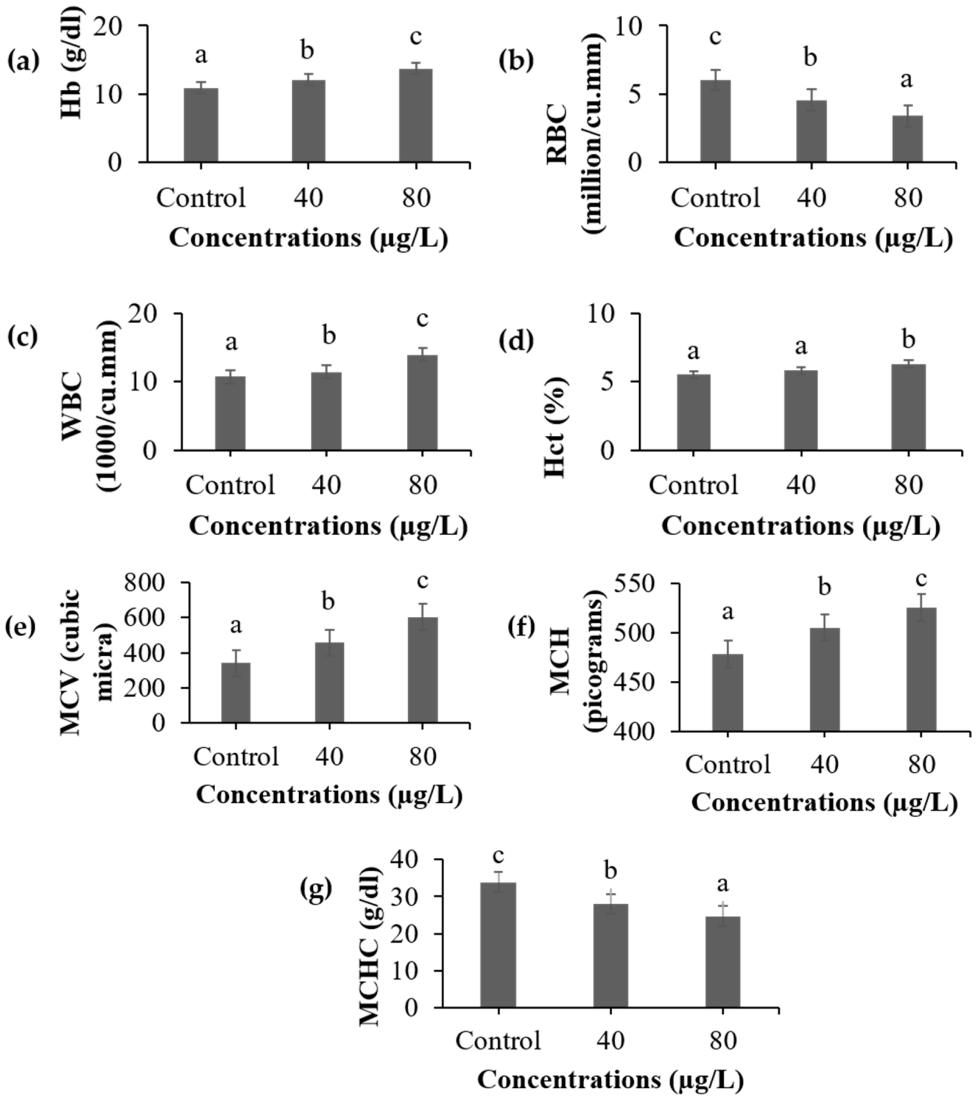

Figure 1. Changes in hematological parameters. (**a**) Changes in the Hb content. (**b**) Changes in the RBC count. (**c**) Changes in the WBC count. (**d**) Changes in the Hct values. (**e**) Changes in the MCV value. (**f**) Changes in the MCH values. (**g**) Changes in MCHC values in the freshwater fish *L. rohita* treated with varying concentrations of metformin. Values expressed in mean ± SD, ANOVA followed by Tukey's post hoc, which showed a significant level $p < 0.05$ (Lowercase letters).

3.2. Indicators of Cellular Damage

Metformin has been shown to induce oxidative stress in fish, causing an imbalance between the production of reactive oxygen species (ROS) and the ability of the fish to detoxify them. This oxidative stress can lead to cellular damage and affect various physiological functions. Toxicity from aquatic environments may alter metabolic traits significantly by acting on organisms at the cellular or molecular level. We may better understand the mechanism and mode of action of metformin if we can understand how medications affect the biochemical characteristics of fish. Fish that have been exposed to toxicants may serve as a useful biochemical biomarker of exposure and impact, which might aid in water quality evaluations and the identification of substances that need a more in-depth risk assessment [27]. For this study, we used the freshwater fish *L. rohita* treated with varied doses of metformin, which had elevated levels of LPO and PCA (Figure 2). Considering that mutually LPO and PCA are useful indices of oxidative impairment, they illustrate how oxidative stress compromises cell physiology and functions in aquatic species. The study's findings demonstrate that LPO and PCA emerge from the inability of antioxidant enzyme-activated defense mechanisms to remove the surplus of reactive oxygen species (ROS) created under stressful conditions. Thus, obtained outcomes are corroborated by the work of Li et al. [27], who reported an uptick in LPO and PCA activity in *O. mykiss* after 21 days of carbamazepine treatment, which implies that these markers are more sensitive indicators of oxidative stress. In addition, both LPO and PCA activities in *O. mykiss* were increased after treatment with carbamazepine. After 42 days of study in *O. mykiss* [28], it is shown that, the ROS produced under stressful circumstances causes non-specific immune parameters to be stimulated and activated, as quantified by various assays.

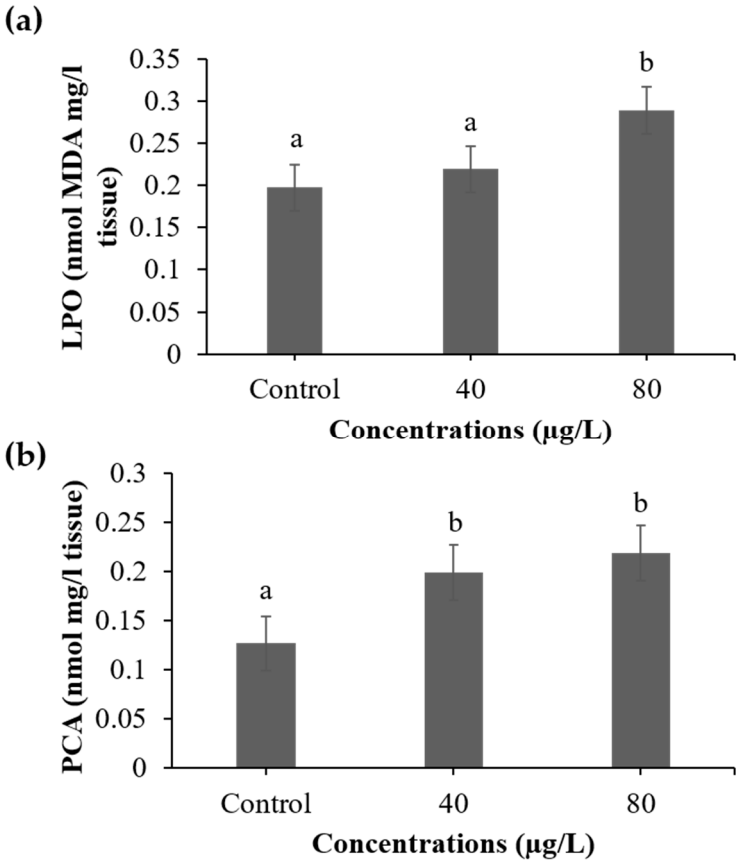

Figure 2. Cellular damage indicators lipid peroxidation (**a**) and protein carbonyl activity (**b**) in the liver of the freshwater fish *L. rohita* treated with varying concentrations of metformin. Values expressed in mean ± SD, ANOVA followed by Tukey's post hoc, which showed a significant level $p < 0.05$ (Lowercase letters).

3.3. Non-Specific Immune Parameters

Biomarkers may be found in non-specific immune systems due to their susceptibility to environmental contamination. This research found that the metformin-treated *L. rohita* had lower LYZ, MPO, and RBA activity levels than the untreated control group (Figure 3). This means that greater doses will elicit stronger immune responses. LYZ activity in fish is a widely sought-after biomarker for identifying the negative consequences of pharmaceutical drugs because it can detect a wide range of non-specific immune responses [29]. In phagocytic cells of the fishes, the principal defense mechanism stimulates reactive oxygen species (ROS) to destroy invasive pathogens, which may be measured based on respiratory burst activity. After 28 days of treatment with either 40 µg/L or 80 µg/L metformin, the RBA activity was suggestively decreased compared to that in the control group. Low levels of the microbicidal peroxidase enzyme suggest very dangerous circumstances for fish [30]. Myeloperoxidase activity in *L. rohita* was significantly decreased after treatment with waterborne metformin at a low dosage of 40 µg/L and a high dose of 80 µg/L. The pharmaceutical metformin has molecular impacts on biochemical and physiological activities in aquatic organisms, but these effects have been poorly understood until recently. Our results highlight the need for further research into the unique mechanism(s) entangled in metformin's fatal impact on aquatic environments, demonstrating the necessity of such a study. These underlying mechanisms are linked to metformin's fatal activity.

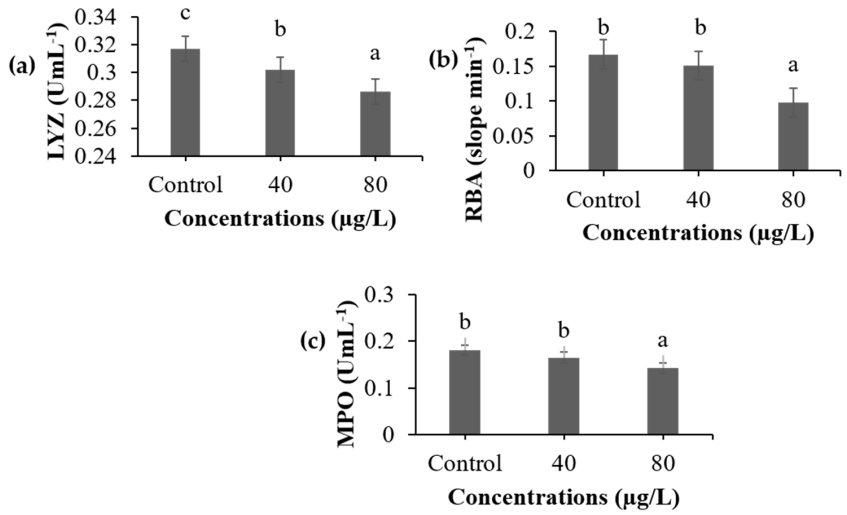

Figure 3. Non-specific immune parameters. (**a**) Respiratory burst activity, (**b**) lysozyme activity, and (**c**) myeloperoxidase activity of the freshwater fish *L. rohita* treated with varying metformin concentrations. Values expressed in mean ± SD, ANOVA followed by Tukey's post hoc, which showed a significant level $p < 0.05$ (Lowercase letters).

3.4. Genotoxicity

Today, there are more genotoxic pollutants than ever in the aquatic environment, spurring researchers to create rapid monitoring tools. Due to their accessibility, speed, and sensitivity, comet test methodologies are frequently used in ecotoxicology and biomonitoring [31]. Direct DNA damage may occur when environmental contaminants, such as pharmaceutical medications, combine with aquatic creatures; indirect DNA damage can occur when these toxins generate reactive oxygen species (ROS), which have the potential to disrupt the consistency of DNA pieces. One of the most popular methods for identifying DNA strand breaks in aquatic species is the comet test, which enables the assessment of the genotoxic potential of pollutants. It can recognize the distinction between alkali-labile regions and single and double DNA strand breaks in different types of cells [32].

Studies investigating the effects of metformin on DNA damage in fish are limited, and most of the available research has focused on the potential genotoxicity (DNA-damaging properties) of metformin in aquatic organisms. The present research demonstrated that

L. rohita was much more susceptible to DNA injury in its liver cells when treated with increasing concentrations of metformin (Figure 4). On the other hand, DNA impairment was less severe in fish subjected to sub-lethal doses of metformin. Our research only found DNA damage in liver tissue since the liver is the primary organ responsible for detoxification. According to the study's results, a lack of antioxidant defense against oxidative stress and the possible consequences of higher metformin dosages might lead to a higher degree of genotoxicity when animals are subjected to very high concentrations of pollutants and stress. Metformin has been shown to possess antioxidant properties, and it can modulate oxidative stress in various organisms. Oxidative stress can induce DNA damage. Some studies have suggested that metformin's antioxidant effects may help protect against DNA damage caused by oxidative stress in fish. Our findings are supported by the fact that planarians exposed to 10 mmol/L of metformin for 1, 3, and 5 days exhibited 10.2%, 25.4%, and 36.8% increases in DNA damage, whereas planarians exposed to 50 mmol/L of metformin experienced increases of 40.6%, 62.8%, and 65.4% in DNA damage [31]. The research on the effects of metformin on DNA damage in fish is still limited, and more studies are needed to fully understand the potential risks. Efforts are being made to mitigate the environmental impact of pharmaceuticals, like metformin. As per previous literature, more focus was given to the development of numerous advanced treatment technologies for wastewater treatment contaminated with metformin and other pollutants.

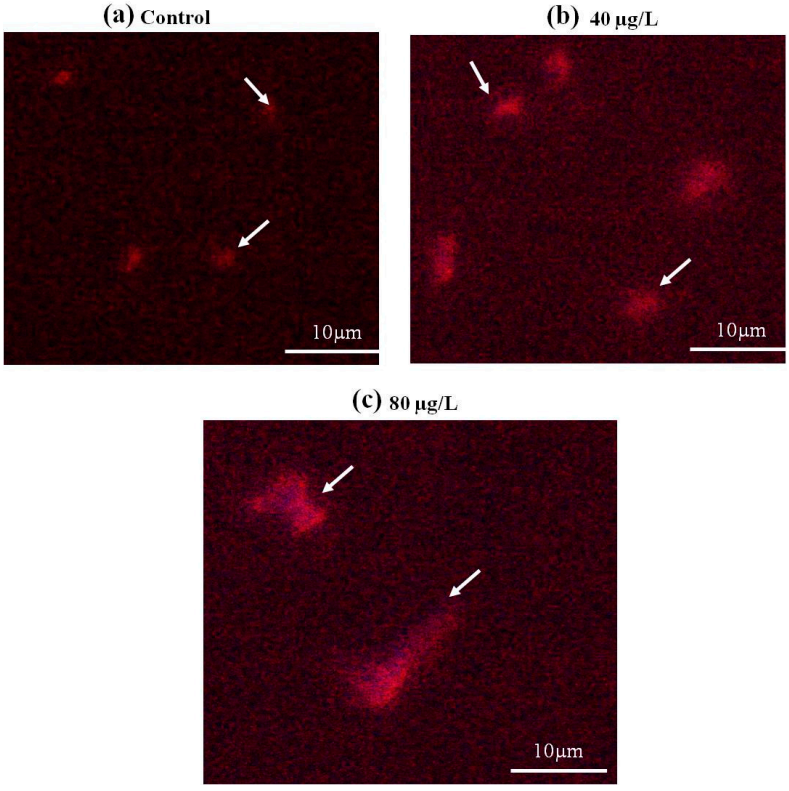

Figure 4. Fluorescent images of the comet assay using *L. rohita*, showing the increasing degree of DNA damage (original magnification: 20×), and the arrow indicating the DNA damage. (**a**) Control; (**b**) DNA damage in the liver tissue of *O. mossambicus* exposed to 40 μg/L metformin and (**c**) 80 μg/L metformin; data are presented as the mean ± SE with a significant difference between the control and treated groups within the same exposure period ($p < 0.05$).

Additionally, promoting the proper disposal of medications and effective measures can help minimize their presence in aquatic environments. Overall, while research on the specific impacts of metformin in aquatic fishes is still ongoing, evidence suggests that metformin exposure can have various effects on fish habits, physiology, endocrine functions, and oxidative stress.

4. Conclusions

This research demonstrated the chronic effects of long-term exposure to metformin, at doses relevant to the environment, on the freshwater fish *L. rohita.* Total hemoglobin, RBCs, and WBCs were all negatively affected by a higher concentration of metformin, as were indicators of oxidative stress, for instance, lipid peroxidation and protein carbonyl activity, as well as non-specific immune indicators, like respiratory burst activity, myeloperoxidase activity, lysozyme activity, and genotoxicity. The ecological significance of this work is that it illustrates new threats posed by rising levels of reactive oxygen species (ROS), which have a devastating impact on the freshwater fish *L. rohita*. The long-term use of these medications at higher concentrations can potentially affect the whole ecosystem by altering all physiochemical parameters. Future studies will focus on long-term views on assessing the risks of climate change, understanding its effects on the ecosystem, and safeguarding the biota. Results of the study suggest that efforts should be made to reduce the discharge of pharmaceuticals into the environment and to improve wastewater treatment processes for minimizing the potential risks posed by metformin and other pharmaceutical compounds to aquatic ecosystems.

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