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Effects of Diclofenac on the Oxidative Stress Parameters of Freshwater Fish *Oreochromis niloticus*

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

The indiscriminate use and abuse of pharmaceuticals have led to pharmaceutical residues in the aquatic environment which has been receiving great attention since significant levels of contamination have been found. The present study investigated the acute and sub-lethal effects of a pharmaceutical drug diclofenac on oxidative stress parameters and the recovery ability in *O. niloticus*. The juveniles were exposed to different concentrations of diclofenac to determine the 96 h LC_{50}. The results indicated that diclofenac was toxic to *O. niloticus* with a 96 h LC_{50} of 0.489mg/L. The percentage mortality increased as the concentrations increased. Fish were exposed to a control (0.00 mg/L) and three sub-lethal concentrations of 0.48, 0.32, and 0.25 mg/L of diclofenac for 28 days and allowed to recover for 7 days. The result of the sub-lethal test indicated that the responses were dose and duration dependent. The oxidative stress results showed significant concentration- and time-dependent increases in the values of lipid peroxidation, glutathione peroxidase, glutathione reductase and reduced glutathione but reduction in catalase and superoxide dismutase in the liver of the exposed fish. Many of the oxidative parameters were found to be restored after the 7-day recovery period. These results showed that diclofenac exposure had a profound negative influence on the selected indices of *O. niloticus*.

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1. INTRODUCTION

All over the world, there has been growing concerns about environmental quality in recent years both locally and internationally. Pharmaceutical drugs have become the focus of environmental concerns as some of these drugs are not eliminated from environment by conventional wastewater treatment processes. In addition, these drugs also exit the organisms, either unchanged or as metabolites [1,2]. The extensive use of veterinary pharmaceuticals (especially in the treatment of multiple reinfections) and wastes resulting from direct disposal by manufacturing plants, hospitals, and homes contribute to the build-up of the drugs in the environment. The runoff of the pharmaceuticals and metabolites into surface waters stemming from the treatment of livestock and pets may result in the contamination of natural water systems and is becoming a potential risk to non-target organisms [3]. Diclofenac is a popular pharmaceutical drug often detected in aquatic environment. The use of pharmaceutical products is on the increase in our world today, and this is as a result of the rise in global population as well as the increasing need for geriatrics to depend on drugs [4].

According to Daughton [5] it is likely to increase further in developing countries such as Nigeria where pharmaceutical production companies are flourishing due to increasing dependence on pharmaceuticals drugs. In Nigeria, the presence of acetaminophen and diclofenac in groundwater and surface water body has been confirmed by Olaitan et al. [6]. Diclofenac belongs to the class of nonsteroidal anti-inflammatory drugs (NSAIDs) with analgesic and anti-inflammatory properties and is a widely prescribed drug [7]. Stepanova et al. [8] reported exposure to early stages of common carp (*Cyprinus carpio*) to 3 mg/L of diclofenac for 30 days observed mortality and oxidative stress.

Diclofenac has also been detected in Baltic Sea biota at levels above threshold values (e.g. in Perch) [9, 10] and previous studies have linked toxic effects in marine organisms to high concentrations of diclofenac.

Diclofenac was included on the EU first watch list (2013/39/EU) with the stated aim being to gather monitoring data for the purpose of facilitating the determination of appropriate measures to address the risk posed by those substances. Inclusion on such watch list is done when there is insufficient data to assess potential negative impacts on the environment, the assertion being based on results from the prioritization process of hazardous substances under the WFD, research results and similar reports.

It has been reported that Diclofenac can bio-accumulate if fish and other aquatic organisms [11-14]. It has been implicated in damages to the kidneys, [12, 15, 16] eggs and embryos [15] and altered gene expression [17].

It has been established that pharmaceutical drugs induce oxidative stress. Oxidative stress is the disturbances in the balance between the production of Reactive Oxygen Species (ROS) and oxidative defences. It usually results to tissue damage and disturbances in the normal redox state of cells. Oxidative stress can also cause base damage as well as unwanted gaps in the DNA [18]. This is as a result of the ROS generated in the cell. Examples of Reactive Oxygen Species include: hydrogen peroxide (H_2O_2), superoxide radical (O_2) and hydroxyl radical (OH). All these radicals make up the Reactive Oxygen Species [19] and cause diseases that are associated with oxidative stress in humans. These include -vitiligo (patchy loss of skin pigmentation), autism (neurological disorder), chronic fatique, Asperger's syndrome (having social defect), sickle cell disease [20]. Nwani et al. [21] noted that the level of damage caused to the cell is dependent on the level of the stress caused to cell. While severe oxidative stress causes death, mild stress causes slight changes which can normalize when the cell recovers.

An antioxidant defence system (ADS) is needed to protect bio-molecules from the harmful effects of ROS [22]. Fish are endowed with defensive mechanisms to neutralize the impact of Reactive Oxygen Species (ROS) resulting from metabolism of various chemicals. These include various antioxidant defense enzymes such as superoxide dismutase (SOD) Catalase (CAT), glutathione peroxidase (GPx), glutathione S-transaferase (GST) and glutathione reductase (GR). Low molecular weight antioxidants such glutathione reductase (GSH), ascorbate (Vitamin C), Vitamin A and E are also reported to contribute in the reduction of oxy radicals. ROS which is not neutralized by this antioxidant

defense system damages bio-molecules. One of the most important targets of ROS is membrane lipids which undergo peroxidation (LPO). Thus LPO estimation has also been successfully employed to signify oxidative stress induced in aquatic animals by such chemicals [23]. The present study was designed to investigate the acute and sub-lethal effects of a pharmaceutical product diclofenac on the oxidative stress parameters of Tilapia fish (*O. niloticus)*. The study will also investigate the recovery ability of the fish after exposure to the drug.

2. MATERIALS AND METHODS

2.1 Experimental Fish and Maintenance

Three hundred healthy juveniles of fresh water Tilapia, *O. niloticus* (Family: Cichlidae; Order: Perciformes; Genus: Oreochromis) with the mean weight of 29 ± 4.10g were collected from a private fish farm and acclimatized for 14 days in concrete pond using non-chlorinated tap water at Zoology and Environmental Biology Department laboratory of University of Nigeria, Nsukka. They were fed 3% of their body weight in divided rations, twice daily (7.00am and 7.00pm) with Coppens commercial feed, containing 35% crude protein. The fish were subjected to a bath treatment with tetracycline to avoid possible dermal infection as a result of injuries sustained from the stress of transportation. The faeces, dead fishes and other waste materials were removed to avoid contamination of the set-up. The water temperature (28.7 ± 0.15 °C), conductivity (234.10 ± 0.35 µM cm^{-1}), dissolved oxygen (7.9 ± 0.26 mg /L), pH (7.5 ± 0.04) and hardness (135.50 ± 0.60 mg/L) were determined following the methods of APHA [24]. The feeding of the fish was stopped a day before the acute toxicity test to prevent contamination by faeces [25]. The experiment was carried out in an indoor experimental system under normal photoperiod of day/night (12:12) cycle prevalent at Nsukka, Nigeria. The data on mortality of the fish were used to calculate the 96h LC_{50}. The safe levels of diclofenac at the 96 h duration were calculated by multiplying the 96 h LC_{50} by the various application factors [26-31].

2.2 Determination of Sub-Lethal Concentrations

A total of 10 fish specimens each were exposed to five different concentrations (0.35, 0.45, 0.55, 0.65 and 0.75mg/L) of diclofenac in 40 l glass aquaria (60 × 30 × 30 cm size) for 96 h to determine the 96h LC_{50}. A control group without diclofenac was also set up. All the experiments were set in triplicate and the mortalities were recorded. The data obtained from the mortality (Table 1) were used to calculate the 96 h LC_{50} [32]. The 96-h value of diclofenac was 0.489 mg/L. Based on the 96 h $LC_{50,}$ three different sublethal concentrations (0.25, 0.32, and 0.48 mg/L) were selected for the sublethal exposure. A total of 120 acclimated fish were used in the sublethal experiment. The sample was divided into four groups (Groups 1, 2, 3 and 4) in separate 40-L glass aquaria. The fish in groups 1, 2, and 3 were exposed to 0.25, 0.32, and 0.48 mg/L of diclofenac, respectively. The fish in group 4 were designated as the control and only exposed to tap water. A total of 30 fish were randomly distributed to each of the four groups without regard to the sex. Each group was further divided into three with 10 fish per replicate. The set up lasted for 21 days and another 7-days recovery during which the fish were fed small quantity of feed (approximately 1% of the body weight) to avoid mortality arising from starvation. There were no mortalities during the 28–day exposure period. Three fish from each of the experimental and control groups were removed for sampling at the end of every week. The fish were anesthetised with a solution of tricainemethanesulfonate (MS 222) at a concentration of 0.1 g/L to minimise stress. The liver was dissected out, carefully washed in an ice-cold 1.15% KCl solution, blotted and weighed. The samples were homogenized in pre-chilled phosphate buffer (0.1M, pH 7.2). Some parts of the homogenate were used for the estimation of thiobarbituric acid reactive substances (TBARS), while the other part was further centrifuged at 12,500 × g for 10 min at 4°C for estimation of other oxidative stress biomarkers.

2.3 Assay of Oxidative Stress and Antioxidant Enzymes

The LPO was determined according to the method described by Wallin et al. [33]. The CAT activity was assayed by the method of Sinha [34]. The SOD activity was determined by measuring the inhibition of antioxidation of epinephrine at pH 10.02 at 30 °C [35]. Glutathione reductase (GR) activity was assayed by measuring NADPH oxidation at 340 nM [36], the activity being expressed as U/mg protein. The activity of glutathione peroxidase (GPx) was measured by the method of Lawrence and Buck

[37], with the specific activity being determined using the extinction coefficient of 6.22 mM/cm. The activity of glutathione (GSH) was assayed as described by King and Wootton [38].

2.4 Statistical Analysis

The median lethal concentration was calculated following the probit analysis method of Finney [32]. One-way analysis of variance using SPSS (version 16.0) was used to analyse the data followed by Duncan multiple range post-hoc test at 95% significant level to separate the means of treatment. Analysis and sample percentages were also used where applicable.

3. RESULTS

3.1 Fish Mortalities and Safe Levels of Diclofenac

Our results indicate concentration and duration dependent increase in mortality of *C. gariepinus* exposed to diclofenac (Table 1). There was however no mortality in the control group after 96 h exposure. The mortality in the fish group exposed to 0.35 mg/l diclofenac was 20% after 96 h. At a higher diclofenac concentration of 0.75 mg/L, the mortality rate after 96 h increased to 100%. The safe level of diclofenac as obtained by multiplying the LC_{50} by various application factors, ranged between 4.89×10^{-2} to 4.89×10^{-6} mg/L (Table 2).

3.2 Effects of Lipid Peroxidation and Antioxidant Enzyme

The effect of different sub-lethal concentrations of diclofenac on lipid peroxidation in the form of TBARS formation and the responses of other antioxidants enzymes (CAT, SOD, GPx, GSH-R and GSH) in the liver of tissue of *O. niloticus* are presented in Table 3. Diclofenac was associated with oxidative stress in *O. niloticus* in a manner dependent on the drug concentration in the aquatic medium and the duration of exposure. The activity of LPO increased significantly on exposure to the drug ($p < 0.05$) and the effects of the drugs appeared more pronounced at higher concentration on prolonged exposure. There was slight recovery after the 7-day withdrawal. The activities of the oxidative stress biomarkers SOD and CAT where significantly reduced by diclofenac as the concentration increases throughout the exposure period. Reduction in SOD and CAT activities were more on day 21 compared to previous noted days and effects diclofenac on the activities of SOD and CAT were similar in magnitude. The GR and GPx activities increased significantly in fish exposed to the drug, the effect of the drug concentrations was significant for the duration of exposure. The was significant recovery after the 7-day withdrawal.

There was significant increase in GSH activity in fish exposed to diclofenac as the concentration increases throughout the exposure time. There was recovery after the withdrawal phase in the exposed fish.

Table 1. Cumulative mortality of *O. niloticus* exposed to various concentrations of Diclofenac

	Cumulative mortality (h)					
Concentration (mg/L)	**24**	**48**	**72**	**96**	**% Mortality**	**% Survival**
Control	00	00	00	00	00	100
0.35	02	04	05	06	20	70
0.45	03	06	08	10	34	60
0.55	04	08	12	16	54	46
0.65	08	15	21	26	87	13
0.75	10	20	25	30	100	00

Table 2. Safe levels of diclofenac for *O. niloticus* after 96 h exposure

Drug	**96 h LC_{50} (mg/L)**	**Method**	**AF**	**Safe level (mg/L)**
Diclofenac	0.489	Hart et al. (1948)*	-	8.13×10^{-3}
		Sprague (1971)	0.1	4.89×10^{-2}
		CWQC (1972)	0.01	4.89×10^{-3}
		NAS/NAE (1973)	0.1 – 0.00001	4.89×10^{-2} – 4.89×10^{-6}
		CCREM (1991)	0.05	2.445×10^{-2}
		IJC (1977)	5 % LC_{50}	2.445×10^{-2}

**C = 48h LC_{50} x 0.03/S^2, where C = presumable harmless concentration and S = 24 h LC_{50}/48h LC_{50}*

Table 3. Changes in oxidative stress biomarkers of *O. niloticus* on 21-day exposure to Diclofenac

		Duration (day)				
Parameter	**Conc.(mg/L)**	**1**	**7**	**14**	**21**	**7-day withdrawal**
LPO (U/L)	Control	1.56 ± 0.09^{a1B}	1.91 ± 0.29^{b1B}	2.68 ± 0.45^{b1A}	1.80 ± 0.38^{b1B}	2.41 ± 0.52^{b1A}
	0.48	2.08 ± 0.41^{a1B}	4.68 ± 0.12^{a2B}	5.45 ± 0.44^{a3A}	6.85 ± 0.46^{a4A}	4.04 ± 0.74^{a2A}
	0.32	1.66 ± 0.43^{a1B}	4.13 ± 0.20^{a2B}	5.41 ± 0.27^{a3A}	6.77 ± 0.42^{a4A}	4.33 ± 0.11^{a2A}
	0.25	1.43 ± 0.17^{a1B}	4.31 ± 0.18^{a2B}	5.70 ± 0.20^{a3A}	6.34 ± 0.22^{a4A}	4.30 ± 0.31^{a2A}
SOD(U/L)	Control	8.25 ± 0.32^{a1A}	8.62 ± 0.57^{a1A}	9.76 ± 0.53^{a1A}	8.76 ± 0.38^{a1A}	8.33 ± 0.32^{a1A}
	0.48	6.91 ± 1.18^{b2A}	4.47 ± 0.23^{c1A}	5.74 ± 0.34^{b1A}	4.18 ± 0.16^{b1A}	8.39 ± 0.26^{a3A}
	0.32	8.32 ± 0.58^{a3A}	5.90 ± 0.35^{b2A}	5.47 ± 0.30^{b2A}	4.54 ± 0.17^{b1A}	7.88 ± 0.51^{b3A}
	0.25	8.08 ± 0.68^{a3A}	5.82 ± 0.35^{b2A}	5.74 ± 0.30^{b2A}	4.68 ± 0.28^{b1A}	7.69 ± 0.40^{b2A}
CAT(U/L)	Control	0.65 ± 0.01^{a2A}	0.77 ± 0.07^{a3A}	0.60 ± 0.04^{a1A}	0.60 ± 0.02^{a1A}	0.62 ± 0.03^{a1A}
	0.48	0.59 ± 0.07^{a4A}	0.39 ± 0.06^{b2A}	0.24 ± 0.02^{b12A}	0.16 ± 0.06^{c1A}	0.44 ± 0.03^{b3A}
	0.32	0.63 ± 0.04^{a2A}	0.37 ± 0.08^{b1A}	0.31 ± 0.08^{b1A}	0.30 ± 0.15^{b1A}	0.58 ± 0.03^{ab2A}
	0.25	0.67 ± 0.04^{a2A}	0.36 ± 0.05^{b1A}	0.27 ± 0.04^{b1A}	0.22 ± 0.01^{b1A}	0.54 ± 0.02^{ab2}
GR (U/L)	Control	11.10 ± 0.57^{a1A}	11.50 ± 0.67^{b1A}	10.82 ± 0.56^{b1A}	10.89 ± 0.52^{b1A}	11.30 ± 0.34^{a1A}
	0.48	11.60 ± 0.64^{a1B}	14.83 ± 0.34^{a2B}	15.89 ± 0.90^{a2A}	16.25 ± 0.57^{a2A}	12.58 ± 0.44^{a1A}
	0.32	12.15 ± 0.46^{a1A}	15.41 ± 0.41^{a2A}	15.96 ± 0.28^{a2A}	15.71 ± 0.61^{a2A}	12.73 ± 0.79^{a1A}
	0.25	12.02 ± 0.46^{a1B}	14.38 ± 0.73^{a2A}	16.11 ± 0.63^{a23A}	15.37 ± 0.60^{a2A}	12.18 ± 0.23^{a1A}
GPx (U/L)	Control	4.87 ± 0.08^{a1A}	6.14 ± 0.20^{b1A}	4.80 ± 0.58^{b1A}	5.98 ± 0.42^{b1A}	4.92 ± 0.24^{a1A}
	0.48	5.30 ± 0.33^{a1A}	9.03 ± 0.33^{a2A}	8.44 ± 0.48^{a2A}	9.88 ± 0.40^{a2A}	6.37 ± 0.32^{a1A}
	0.32	5.50 ± 0.23^{a1A}	8.37 ± 0.07^{a2A}	8.40 ± 0.20^{a2A}	9.07 ± 0.45^{a2A}	5.17 ± 0.17^{a1A}
	0.25	4.60 ± 0.43^{a1A}	8.59 ± 0.35^{a2A}	8.70 ± 0.21^{a2A}	9.16 ± 0.19^{a2A}	5.69 ± 0.52^{a1A}
GSH (U/L)	Control	2.68 ± 0.28^{b1A}	3.57 ± 0.08^{a1B}	3.56 ± 0.40^{b1A}	2.94 ± 0.27^{b1A}	2.77 ± 0.31^{a1A}
	0.48	3.37 ± 0.92^{a1B}	3.86 ± 0.17^{a1B}	4.63 ± 0.50^{a1B}	3.86 ± 0.19^{a1B}	3.64 ± 0.37^{a1A}
	0.32	3.65 ± 0.33^{a1B}	3.77 ± 0.53^{a1B}	4.69 ± 0.22^{a1B}	3.67 ± 0.25^{ab1B}	3.74 ± 0.15^{a1A}
	0.25	3.91 ± 0.23^{a1B}	3.88 ± 0.58^{a1B}	4.31 ± 0.17^{b1B}	3.76 ± 0.17^{ab1B}	3.62 ± 0.17^{a1A}

Values with different small letter alphabet superscript between different drug concentrations along a column were significantly different; while values with different numeric superscript across a row were significantly different; and values with different capital letter superscript along a column were significantly different between same concentrations ($p < 0.05$). of Diclofenac

4. DISCUSSION

The effect of pharmaceutical drugs on non-target organisms has been on the increase due to the ever growing population. Fent et al. [39] reported that although pharmaceutical drugs are usually in low concentration, and are also considered to be non-toxic compounds, they can exert toxic effects on non-target species. The increase in LPO suggests that there is increase in production of reactive oxygen species (ROS). The interaction of ROS with biological molecules may cause increase in LPO, DNA damage and protein oxidation resulting in the disturbance of the physiological processes [20]. The elevation in LPO may be due to the stress associated with exposure to the drug as earlier reported in rats administered albendazole [21]. Some related pharmaceuticals, notably benznidazole and mebendazole [40] have been reported to stimulate the production of ROS and to cause oxidative damage and lipid peroxidation in animals.

Antioxidant enzymes play significant roles in preventing cellular damage in animals [21]. The inhibition of SOD and CAT activity in the liver tissues contributed to higher LPO values in the exposed fish, indicating that in aquatic environment diclofenac could induce oxidative stress in fish. Inhibition of SOD and CAT that lead to oxidative stress was also reported in *Clarias gariepinus* exposed to primextra herbicide [21]. Ahmed (2015) also reported that simultaneous treatments with vitamin E and/or lycopene resulted in a significant decrease in the tissue SOD activities. The decrease in SOD activity can be attributed to the inhibition of superoxide radical formation or the potential free radical scavenging activity of vitamin E and/or lycopene (Ahmed, 2015). According to Puerto et al. [41], decrease in SOD and CAT were attributed to direct damage of its protein structure by the drug and increasing amounts of hydrogen peroxide produced. The low level of SOD and CAT when compared to the control indicates the high risks of cell injuries.

The GPx depletion in the stress–treated fish may be connected with increased exposure of the plasma membrane to peroxide attack, as reflected in changes in LPO levels. The depletion of GPx further enhances the susceptibility of the lymphoid tissues to oxygen metabolites and acid-mediated cell damage. These effects may subject livers to higher risk of damage from oxidative stress and more limited antioxidant responses. The continuous oxidative damage caused to the cells could paralyse them and eventually degrade completely the self defence mechanisms of the cells [42]. Our result is in agreement with Ajima et al. [43] who reported that structural and functional alterations in the liver result in changes in the levels of these enzymes in circulation.

5. CONCLUSION

This present study shows that diclofenac is toxic and may cause significant alterations in the oxidative stress of *O. niloticus*. Thus, it can be said that diclofenac at various doses and duration of study can cause adverse effects on liver resulting in oxidative stress. High concentration of diclofenac above its safe level is highly toxic to tilapia fish and could be toxic to non- target organisms. Thus, caution should be exercised in the clinical use of the drug for therapeutic purpose, which should be limited to the lowest dose and treatment duration required to achieve the best therapeutic effect to avoid being toxic to non- target organisms. There is a need for further studies to determine the accurate effects of this drug on several other biological organisms, and also to determine whether the effects are similar when fish are subjected to longer exposures to lower concentrations; a combined toxicity study will satisfy this need.

ETHICAL APPROVAL

The experiment was conducted according to the approved guidelines of the Animal Ethics Committee of the Enugu State University of Science and Technology (ESUT).

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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