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Research Paper

Study on acute toxicity and haematological alterations induced by the exposure of diclofenac to common carp (Cyprinus carpio)

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Abstract-*Blood cell responses are important indicators of changes in the internal and /or external environment of animals.The present study was carried out to investigate the duration exposure time and toxicity on survival effects for freshwater fish C. carpio to different concentrations of diclofenac and alterations in haematological parameters.A 21 day experiment was carried out by using common carp (C. carpio) as the test organism.According to 96 h LC50=11.71 mg/L of diclofenac in C. carpio, Fish were exposed to nominal concentration of 0.25, 0.50, and 1 mg/L of diclofenac.Red blood cell (RBC) counts in fishes that had 7 and 14 days exposure to diclofenac were not significantly different, compared to control fishes, but there was a significant, P<0.01 reduction in RBC count in fishes that received 1 mg/L diclofenac for 21 days.Diclofenac treated fish did not elicit any significant changes (p<0.05) with regard to monocytes, basophils and eosinophils.To fully comprehend the mechanism of this medication, extra research is advised to look at the toxicokinetics and toxicodynamics of diclofenac in freshwater fishes.*

Keywords-*C. carpio, Diclofenac, RBC, WBC, Toxicity.*

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I. Introduction

Diclofenac, a non-steroidal anti-inflammatory drug (NSAID), is amongst the most widely prescribed medicines worldwide and, consequently, one of the most frequently detected compounds in waste and surface waters (McGettigan and Henry 2013). It is used in the treatment of pain, physical disability in rheumatic diseases as well as for its anti-inflammatory and antipyretic effects (Kołodziejska and Kołodziejczyk 2018). In general, diclofenac, as a nonselective cyclooxygenase (COX) inhibitor, acts on both types of COX, namely COX-2 synthesized during inflammatory processes (therapeutic effect) and COX-1 with physiological function in synthesis of prostaglandins, which are important for protection of gastrointestinal epithelial cells (Hawkey 2000), for blood pressure balance, correct thrombocyte aggregation and some other cardiovascular system characteristics (Al-Saeed 2011). Therefore, adverse effects are commonly associated with the above-mentioned systems (Al-Saeed 2011). Importantly, however, detoxification and elimination pathways of xenobiotics including diclofenac are located in the liver and kidney, where diclofenac can be concentrated and/or metabolized by the enzyme complex of cytochrome P450 (CYP450) to form toxic metabolites (Boelsterli 2002).

Previous studies have shown that when the water quality is affected by toxicants, any physiological change will be reflected in the values of one or more of the haematological parameters (Van Vuren, 1986). Undoubtedly, blood cell responses are important indicators of changes in the internal and/or external environment of animals. In fish, exposure to chemical pollutants can induce either increases or decreases in haematological levels. Presently, for environmental monitoring and research in toxicology, the different hematological parameters are investigated to evaluate the pathological and physiological changes and disease demonstration of fishery management and aquaculture (Bhattacharya, 2009). Moreover, the studies related to the detection of aberration in the fish (chemical composition and haematological profile) could provide symptoms of exposure to toxicants before any massive evidence become apparent and therefore are used as reliable indices of fish health. It is also worth to mention that changes in chemical composition and haematological profile are considered to provide symptoms about the type of toxicants and degree of pollution in the environment. Thus, the present study was carried out to investigate the duration exposure time and

toxicity on survival effects for freshwater fish *C. carpio* to different concentrations of diclofenac and alterations in haematological parameters.

II. Materials and Methods

Fish Preparation and Adaptation

A 21 day experiment was carried out within July to September 2018 by using common carp (*C. carpio*) as the test organism. Fish were obtained from Bhategaon Fish Form located in Kalamnuri taluka, Hingoli District, Maharashtra, India and transferred to the laboratory. Two weeks before the experiment, fish with an average body weight of 30±5 g; and average body length of 16.1±1.02 cm were stocked in aquaria (with volume of 140 liters of water) and aeration was provided with an air pump for 24 h. for adaptation. After the adaptation period, fish of similar mean weight were separated and survival test was performed with three replications: 20 fish were used in each replication, at a density of 3.5 gr/L. Aeration was provided at all times and a photo period of 12:12 (L:D) was used. Fish were fed at the rate of 1% body weight and 50% of water was exchanged daily.

Fishes were stocked in a rectangular aquaria containing seasoned tap water. These aquaria are well aerated. During acclimatization period, water was renewed daily and fishes were fed once in a day with artificial food pellets available from the local market. The fishes were acclimatized for 15 days and the well acclimated fishes were divided into 7 groups, each containing 10 fishes. These groups of fishes were placed separately in the fish tanks containing 10 liter of well aerated water. Feeding was stopped 24 hours prior to the exposure of fishes to the tested compounds for acute toxicity tests.

Warangal district, Telangana state, India. The voucher specimens were all kept in the same place. Plant components were collected, cleaned thoroughly under running tap water, dried in the shade, and then triturated into fine powders using an electric grinder. This powder was kept at room temperature in airtight brown bottles.

Test Compound and Preparation of Stock Solution

For the present study, diclofenac was chosen as toxicants based partially on the probability of their having biochemical effects. In all, 20 film-coated diclofenac tablets (manufactured by RANBAXY Laboratories Ltd, India) were purchased from a pharmaceutical store in Nanded and was used to prepare the stock solution and prepared at different concentrations 5, 10, 15, 20, 25 and 30 mg/L due to their low water solubility to determine the LC50.

Assessment of Median Lethal Concentration (LC50)

Test systems consisting in 120×80×40cm glass tanks filled with water reconstituted from the following salts: NaHCO3 (174 mg/L), MgSO4 (120 mg/L), KCl (8 mg/L) and CaSO4.2H2O (120 mg/L) were maintained at room temperature with constant aeration and a natural light/dark photoperiod. Static systems were used, and no food was provided to specimens during the exposure period.

To establish the target concentration to be used in evaluating reproductive health, biochemical analysis of tissue, respiratory parameters, the median lethal concentration (LC50) of Diclofenac was determined (Banaee, et al., 2011). To this end, six experimental systems containing different concentrations of NSAIDs (5, 10, 15, 20, 25 and 30 mg/L) in reconstituted water and a seventh NSAIDs-free control system were set up, and ten carp randomly selected from the stock (using the random number method) were placed in each system. 10 fish per each group were used in the LC50 determination.

Duration of the exposure period was 96 h, at the end of which the number of dead specimens in each system was counted. The assay was performed in triplicate. The 96-h LC50 of NSAIDs and its 95 % confidence limits ($P<0.05$) were estimated by Probit analysis.

Haematological study

Experimental Groups and Dosage

After being starved for 24 h, fish (n=42) were gathered and then randomly distributed into 7 glass tanks. Each tank contained six fish and 25L of test solution, with three tanks used in each treatment group. The control fishes were included in group-I, fishes exposed to diclofenac 0.25, 0.50 and 1 mg/L for 21 days are included in group-II, group-III and group-IV respectively.

From the above four groups, except group I, the remaining three groups of fishes were exposed to their respective sub-lethal concentration (0.25, 0.50 and 1 mg/L) of diclofenac for 21 days respectively. Group I was maintained as control. All the groups received the same type of food and other conditions were maintained similarly. At the end of each exposure period (7th day, 14th day and 21st day), fish specimens were anesthetized with olive oil to enable the collection of blood samples.

Blood collection

The method for blood collection was done following the procedure of Bello et al. (2014). Fish specimens were not sacrificed but rather they were immobilised and blood was collected from surviving fish, after which they were put in a separate fish tank to recover. Sterilized 3G needles and heparinized syringes of 5 ml were used to collect blood from the fish. A distance of 3–4 cm from the genital opening of each fish was punctured and wiped with dry tissue paper to avoid contamination with mucus. The needle was inserted at a right angle to the vertebral column of the fish. Blood was taken under gentle aspiration until about 3 ml had been obtained. The needle was gently withdrawn and the blood was transferred into an anticoagulant plastic tube. Collection of blood was carried out at 7th, 14th, and 21st day of exposure. Blood samples were collected from different surviving individuals exposed to the same concentration and transferred immediately (10–20 min) to the laboratory for haematological analysis.

Haematological Analysis

The total red blood cell count was done by instrument-Neubauer Haemocytometer. The total white blood cell count is also made by using Neubauer haemocytometer slide and Turk's fluid is used as a WBC diluting fluid. The white cells are counted in the four large corner squares. For the determination of leucocytes, 0.02 ml of blood was pipetted into a small test tube containing 0.38 ml of WBC diluting fluid (Turk's) make a 1:20 dilution of the blood sample.

Statistical Analysis

In the acute toxicity assay (96h LC50 of NSAIDs), Probit analysis was performed and significance assessed by the degree of 95 % LC50 overlap. The $\chi 2$ linear adjustment test was not significant at $P<0.05$. Results of sublethal toxicity assays were statistically evaluated by one-way analysis of variance (ANOVA), The analysis of each blood parameter was repeated three times and result were subjected to statistical analysis with students't' test for significance. All statistical analyses were performed in Microsoft Excel, and statistical significance was defined as $p<0.05$.

III. Results and Discussions

Acute toxicity

Diclofenac was used as toxicant to determine the acute toxicity. Stock solution of 30 mg/L was prepared and then test fishes were exposed to different concentrations (5, 10, 15, 20, 25 and 30 mg/L) of diclofenac. There was low (10%) mortality observed at 5 mg/L concentration of diclofenac after 24 h exposure period.

Table-1. Per cent and probit mortality of Cyprinus carpio in different concentrations of Diclofenac for 96 hours of exposure period (LC50 = 11.71)

S.N	Concentration of DCF (mg/L)	Log concentration	Number of Fish			Mortality	
			Exposed	Alive	Dead	Percent	Probit
1	5	0.698	10	7	3	30	4.48
2	10	1	10	5	5	50	5
3	15	1.176	10	4	6	60	5.25
4	20	1.301	10	3	7	70	5.52
5	25	1.397	10	2	8	80	5.84
6	30	1.477	10	1	9	90	6.28

There was 30%, 50%, 60%, 70%, 80% and 90% mortality observed at 5 mg/L, 10 mg/L, 15 mg/L, 20 mg/L, 25 mg/L and 30 mg/L respectively at the end of 96 h. Above data of acute toxicity test has been given in Table-1. The regression equation with 95% fiducial limits with observed and calculated values are given in Figure-1. According to 96 h LC50=11.71 mg/L of diclofenac in *C. carpio*, Fish were exposed to nominal concentration of 0.25, 0.50, and 1 mg/L of diclofenac.

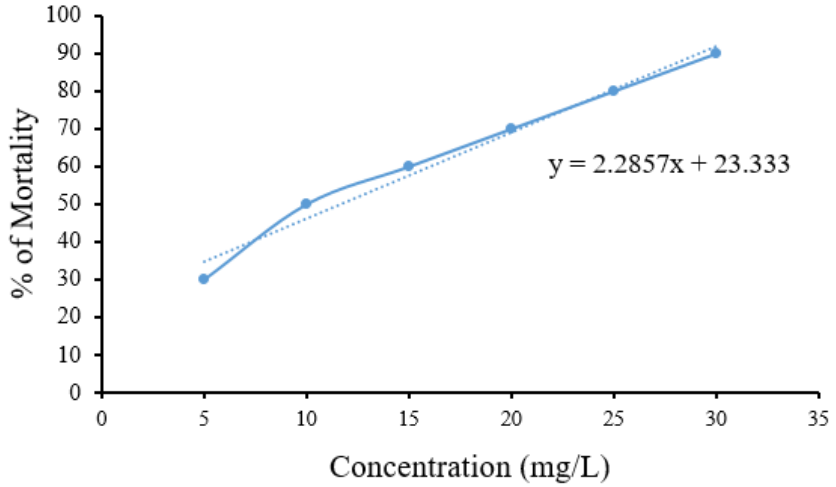

Figure-1. Linear graph of Percentage of mortality at different concentration of diclofenac for 96 hours of exposure period

Haematological Alterations

Blood is a pathophysiological reflector of whole body and therefore blood parameters are an asset in diagnosing the structural and functional status of body organs exposed to toxicants. The blood is said to be a mirror in which all the vital processes, taking place in the organism are reflected. Hence, the study of blood picture of an organism is an important feature that provides a key for diagnosis of various disease and stress due to pollutants. This has promoted, the present investigation to study on the hematology of common carp Cyprinus carpio fish, exposed to diclofenac and ibuprofen.

Effect of diclofenac on RBC and WBC

There were no significant changes in hematocrit levels between diclofenac exposed and control fishes (Table-2). Red blood cell (RBC) counts in fishes that had 7 and 14 days exposure to diclofenac were not significantly different, compared to control fishes, but there was a significant, $P < 0.01$ reduction in RBC count in fishes that received 1 mg/L diclofenac for 21 days (Table-2). The RBC count in group IV fishes (1mg/L diclofenac treated) at 21 day of exposure, was decreased from 6.99+0.53 to 4.10+0.81 (x106 mm-3) and it is statistically significant ($p<0.01$).

Table-2. Effect of diclofenac in fish *Cyprinus carpio* with reference to RBC and WBC ($x10^6$ mm-3) (Values are mean ± SE).

Tissue	Day of Exposure	Experimental Groups			
		Group I (Control)	Group II (0.25 mg/L)	Group III (0.5 mg/L)	Group IV (1 mg/L)
RBC ($x10^6$ mm^{-3})	7	6.99±0.53	6.12±0.41	5.24±0.18*	4.0+0.44*
	14	6.99±0.53	6.69±1.18	6.80±0.63	5.20+0.39*
	21	6.99±0.53	6.13±0.71	5.78±0.24*	4.10+0.81*
WBC ($x10^6$ mm^{-3})	7	4.35±0.26	4.25±0.60	3.93±0.54	2.01+0.22
	14	4.35±0.26	4.20±0.33	4.43±0.48	5.20+0.19*
	21	4.35±0.26	5.55±0.52*	6.23±0.40**	7.90+0.70**

Data expressed as mean ± SEM. * Significant at $P < 0.01$; ** Significant at $P < 0.001$.

Effect of Diclofenac on Differential Count of WBC

Changes in the white cell differentials of *C. carpio* are presented in Table-3. Diclofenac elicited a dose and duration-dependent decrease in neutrophil count. The maximum neutrophil count was at 21 day in group-IV fishes is 27.65 and lowest at 21 day in the same group was 24.67. The levels of neutrophils were in group IV fishes at 21st day of exposure was highly statistically significant ($p<0.01$). There is no statistical significance in the neutrophil count at 7 and 14 day exposure in group II and group III fishes when compared to group I (control) fishes.

Diclofenac significantly increased lymphocyte count in a dose and duration dependent pattern. The maximum count was at 21 day for 69.04 in group IV fishes and lowest at 14 day for the 61.44 in group III fishes (Table-3). Data presented in Table-3 further showed that administration of Diclofenac (1 mg/L) showed an

increase in lymphocyte count at 21 day of exposure from 55.92+4.32 to 69.04+2.95 relative percentage and it is highly statistical significant at $p<0.01$.

Table-3. Effect of diclofenac in fish *Cyprinus carpio* with reference to WBC differential count (Values are mean ± SE and in relative %)

Tissue	Day of Exposure	Experimental Groups			
		Group I (Control)	Group II (0.25 mg/L)	Group III (0.5 mg/L)	Group IV (1 mg/L)
Lymphocyte count	7	55.92±4.32	57.44±3.00	56.94±2.50	59.04±2.88
	14	55.92±4.32	59.77±2.60	61.44±3.70	64.34±2.30*
	21	55.92±4.32	63.44±3.80*	66.14±4.89	69.04±2.95**
Neutrophil count	7	34,44±2.75	33.60±1.60	31.84±2.50	27.65±2.76
	14	34,44±2.75	31.64±2.10	30.92±2.60	26.50±2.50
	21	34,44±2.75	28.46±3.55	27.04±3.00	24.67±2.86**
Monocyte count	7	1.5±0.03	1.4±0.01	1.4±0.01	1.5±0.02
	14	1.5±0.03	1.5±0.08	1.6±0.05	1.7±0.07
	21	1.5±0.03	1.5±0.06	1.6±0.09	1.7±0.03
Basophil count	7	1.3±0.06	1.3±0.07	1.4±0.02	1.3±0.09
	14	1.3±0.06	1.4±0.03	1.4±0.09	1.3±0.07
	21	1.3±0.06	1.2±0.04	1.3±0.06	1.2±0.07
Eosinophil count	7	1.4±0.09	1.4±0.06	1.4±0.08	1.5±0.07
	14	1.4±0.09	1.6±0.02	1.6±0.09	1.4±0.06
	21	1.4±0.09	1.5±0.04	1.4±0.07	1.3±0.09

Data expressed as mean ± SEM.
Statistically significant comparison of control group and other treated groups ($p < 0.05$), ***highly significant ($p < 0.01$)

Diclofenac treated fish did not elicit any significant changes ($p > 0.05$) with regard to monocytes, basophils and eosinophils (Table-3).

Various investigators determined acute toxicity and LC50 values of pesticides using different test fishes for different exposure periods. Reports on comparative toxicity of diclofenac and ibuprofen are less. Deoray and Wagh (1987) have reported 0.1560, 0.5012 and 0.400 ppm LC50 values for thiodon, nuvan and dithane M-45 respectively after 96 h exposure to fresh water fish *Bariliusbendelisis*.

Focusing on freshwater invertebrates, some studies investigated the accumulation of NSAIDs in invertebrates from different ecosystems worldwide. Measureable concentrations of DCF (12.4 ng/g dry weight) and IBU (183 ng/g dry weight) were found in Hydropsyche spp. individuals from the River Segre (Huerta et al., 2015).

Neutrophils are vital in the defense against invading microorganisms that harm the body and they may be increased in the blood by tissue damage induced by toxicant stress. In this study, ibuprofen elicited significant dose and duration-dependent neutropenia and lymphocytosis. The duration dependent decreases and increases in the percentage subpopulation of lymphocytes and neutrophils may be associated with drug-induced stress and defense against the stressor. Lymphocytosis is therefore a response by the fish to cope with stress induced by the drug. Neutrophils are phagocytotic and consequently a decrease means that the phagocytotic capacity of the fish blood has been compromised. Suppression of the neutrophil count has also been shown in studies with fish exposed to fenthion (Nwani et al. 2016). According to Lohner et al. (2001) reduced neutrophil numbers are possibly indicative of reduced or disrupted phagocytotic capacity and reduced disease resistance. Nwani et al. (2014) reported a significant increase in WBCs in Clariasgariepinus exposed to pharmaceutical chloramphenicol and Reddy (2013) found the same for the freshwater fish *Catlacatla* exposed to cadmium.

In this study, monocytes, basophils and eosinophils were scarcely observed and there were no significant difference between the control and treated groups. This indicated an extremely low concentration of these cell types. Similar findings have been reported in fish exposed to various concentrations of toxicants (Ogueji and Ibrahim 2012). According to Nwani et al. (2006), it is hard to preserve basophils and this is the main reason why basophils are difficult to identify in fish blood. Modra et al. (1998) reported a decreased concentration of eosinophils and the absence of basophils in several fish species, including *Cyprinus carpio*, *Tincatinca*, *Silurisglanis* and *Oncorhinchus mykiss*.

IV. Conclusion

Acute toxicity of DCF to *C. carpio* was determined for 96h. The LC50 of DCF was determined to evaluate the effect on hematological alterations against *C. carpio*. According to 96 h LC50 of DCF (11.71 mg/L) in *C. carpio*, it is concluded that, the fishes are less toxic to this concentration and for further studies the fish were exposed to nominal concentration of 0.25, 0.50, and 1 mg/L of DCF. Based on the current results, it could be concluded that administration of diclofenac, may induce immunological perturbations, and their toxicity may increase depending on the dose. Further studies are desired to evaluate their toxicity in the biological system. However, additional studies are also recommended to investigate the toxicokinetics and toxicodynamics of the diclofenac in freshwater fishes to obtain a comprehensive understanding of the mechanism of this drug.

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