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Research Article

STUDIES DELINEATING THE EFFECT OF CHLORPYRIFOS ON *HETEROPNEUSTES FOSSILIS*: HISTOPATHOLOGICAL AND HEMATOLOGICAL ASPECTS

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ABSTRACT

Pesticides are a fatal threat to the non-target organisms in the aquatic system. The present study was aimed to explore the effect of chlorpyrifos (CPF), an organophosphate (OP) on a fresh water teleost, *Heteropneustes fossilis*. LC_{50} for 96h was found to be 1.921 mg/l. The fishes were sacrificed after 96h of exposure and tissues (gill, liver, and kidney) were collected and were further processed for histopathological studies. Collected blood was processed for haematological studies. Significant decrease ($p < 0.05$) in various haematological parameters i.e. total number of RBCs, % haemoglobin, hematocrit, MCV, MCH and MCHC was noted while a significant increase in WBCs particularly, neutrophils was recorded ($p < 0.01$) upon exposure. Histopathological studies of the treatment group showed significant destruction of histo-architechture of all of the tissues in comparison to control. This preliminary study reflects the impact of CPF on organ and blood physiology of *Heteropneustes fossilis*.

INTRODUCTION

The highly consumed aquatic organism throughout the world is the fishes (Mohammad, 2001). To protect and increase the yield of crops nowadays there is increase in the use of different pesticides which contaminate all kind of water resources (Singh *et al*., 2017). As a result, the aquatic organisms get exposed to and severely affected metabolically by these pesticides. The fishes are richest and cheapest source of protein for common people in India and they get exposed to high concentration of these pesticides due to biomagnification through food chain. When the pesticides are used in the field to control the pests, fishes (the non-target organisms) also get affected involuntarily (Gupta and Dalela, 1986). Thus, studies on the impact of these pesticides, particularly organophosphates on the organ and blood physiology in fishes are most relevant in the present context. Reports on such studies are scanty and therefore the present study was aimed to note the effect of chlorpyrifos on hematological parameters and histopathology of fresh water teleost, *Heteropneustes fossilis*.

MATERIALS AND METHODS

Animals and maintenance

Healthy fishes (average length 18 ± 2 cm, average weight 41 ± 2 gm) were obtained from local fish market of Allahabad, Uttar-Pradesh, India (25.43° N, 81.84° E). Fishes were treated with potassium permanganate solution (0.5% w/v) for 1 min to remove sub-cutaneous adherents and were acclimatized under laboratory conditions for two weeks in glass aquarium containing de-chlorinated aerated tap water at room temperature (25 ± 2ºC) with commercially available food palette (Tokyu, India) *ad libitum*.

Procurement of chemicals

The pesticide (Chlorpyrifos; commercially known as Bilbo, Bharat Insecticide Limited, New Delhi, India) and other chemicals were procured from local supplier of Allahabad, Uttar-Pradesh, India.

Assessment of water quality

Before exposure, the quality of water was evaluated as per APHA guideline (APHA, 1985). The parameters were noted as adequate for fishes as published elsewhere (Singh *et al*., 2017)

Experimental design

The LC_{50} for chlorpyriphos exposure was determined as 1.921mg/l (Tiwari *et al*., 2017). Fishes were divided into three different groups (control, 5% of LC_{50} and 10% of LC_{50}, n=10/group) for 96 h of exposure.

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Sample collection

Upon sacrifice, blood was collected in heparinised vial and was processed for haematological parameters and the desired tissues (gill, liver, and kidney) were further processed for histopathological studies.

Preparations for histopathology

The histo-pathological preparations were done following the protocol published elsewhere (Singh *et al*., 2017). In brief, the tissues (gill, liver and kidney) were fixed in 10% Neutral Formaline, washed under running tap water, dehydrated using graded alcohols and passed through xylene (xylene + absolute alcohol) and xylene. Further, they were transferred to xylene + wax (50:50) and three grades of waxes and block was prepared and 6µm thin sections were cut using a microtome (Leica Microtome, Leica, Leitz, Germany). Sections were spread on 0.1% gelatin coated slides and the slides were processed for Hematoxylene-Eosin counter staining. The slides were dipped in xylene, passed through down-grade alcohols and was stained in Ehlrich's hematoxylin. The slides were counter-stained by eosin, dehydrated by different grades of alcohol, cleared in xylene and was mounted in DPX. Slides were snapped by Nikon, 80i, Japan with a camera setup attached to the microscope and computer.

Hematological preparations

Total RBC and WBC (by Neubaur's Hemocytometer), gram percentage (g%) hemoglobin (by Sahli's hemoglobinometer), DLC by Giemsa stain and hematocrit were estimated by using standardized protocols. Mean Corpuscular Volume (MCV), Mean Corpuscular Hemoglobin (MCH) and Mean Corpuscular Hemoglobin Concentration (MCHC) were calculated using proper formula.

Statistical analyses

The data of total RBC and WBC counts, g% hemoglobin, DLC, hematocrit, MCV, MCH and MCHC are represented as mean ± SEM and were analyzed by One Way Analysis of Variance (One Way ANOVA). For the variations between control *vs* experimental groups and among the experimental groups (5% and 10% groups), Duncan's Multiple Range Post-hoc Test was applied. The results were considered to be significant at $P \leq 0.05$ and $P \leq 0.01$ levels. All of the statistical analyses were done in accordance to Brunning and Knitz, 1977.

RESULTS

Histo-pathological observations

Gill

The general histo-architecture of gills was noted in control groups with intact primary and secondary gill lamellae along with gill filament attached to gill raker (Fig. 1A). In 5% group, the primary and secondary gill lamellae lost their basic structure (due to degeneration in gill filament) as compared to control (Fig. 1B). But, in 10% group, the entire structure of both primary and secondary gill lamellae along with gill filament was not only degenerated but also they are dispersed from their attachment to gill raker (Fig. 1C).

Liver

The normal histo-architecture of liver was noted in control groups where intact hepatic capsules (with assembled hepatocytes) and hepatic artery were observed (Fig. 2A). But, in 5% group, the integrity of hepatic capsules was partially lost (as identified by increased intercellular spaces). Hepatocytes and kupffer cells were present in the dilated hepatic artery (Fig.2B). But, in 10% group, the integrity of hepatic capsules was severely affected along with high disposition of hepatocytes and kupffer cells in more dilated hepatic arterial area (Fig. 2C).

Kidney

The basic histo-architecture of kidney with intact glomerulus was noted in control fishes (Fig. 3A). But, the histo-anatomy of kidney is lost in 5% treatment group as marked by swelling of glomerulus and Bowman's capsule (Fig. 3B). In case of 10% treatment group, the degeneration of kidney is more marked by swelling of Bowman's capsule and increased inter cellular spaces.

Hematological parameters

Total RBC Count

A significantly low level of total RBC count in 5% and 10% chorpyrifos treatment groups ($p < 0.01$) in comparison to control was observed. But, among the experimental groups the variation was more significant being significantly low in 10% groups than 5% groups ($p < 0.01$, Table-1).

% Hb

We noted significantly low level of percentage of Hb in 5% and 10% chlorpyrifos treatment groups ($p < 0.05$) in comparison to control. Among the experimental groups the variation was significantly low in 10% groups than 5% ($p < 0.05$, Table-1).

% Hematocrit

A significantly low level of % hematocrit in 5% and 10% chlorpyrifos treated group ($p < 0.01$) in comparison to control was observed. Among the experimental groups (between 5% and 10%) the variation was significantly low in 10% than 5% ($p < 0.05$, Table-1).

MCH level

A significantly decreased level of MCH in 5% and 10% chlorpyrifos treated group ($p < 0.01$) in comparison to control was noted. But, among the experimental groups (5% *vs* 10%) the variation was significant ($p < 0.01$, Table-1).

MCHC level

We noted significantly low level of MCHC in 5% and 10% chlorpyrifos treated group ($p < 0.05$) in comparison to control. But, among the experimental groups (5% *vs* 10%) the variation was significant ($p < 0.05$, Table-1).

MCV level

A significantly high level of MCV in 5% and 10% chlorpyrifos treated group ($p < 0.01$) in comparison to control was found.

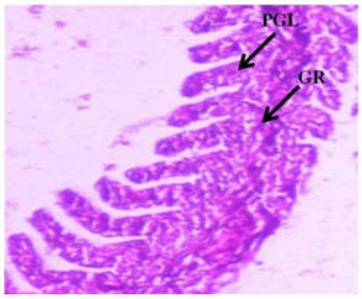

Fig. 1A

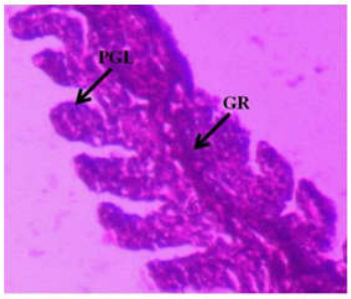

Fig. 1B

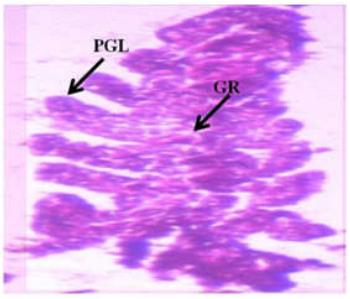

Fig. 1C

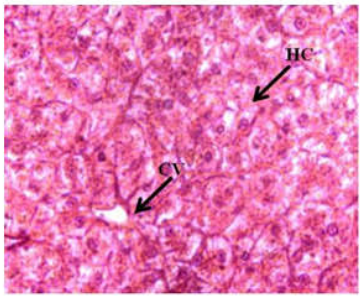

Fig. 2A

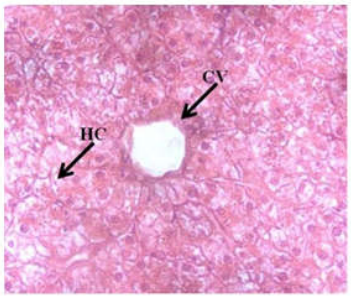

Fig. 2B

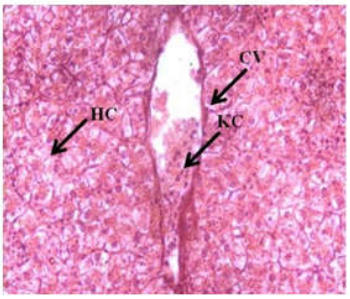

Fig. 2C

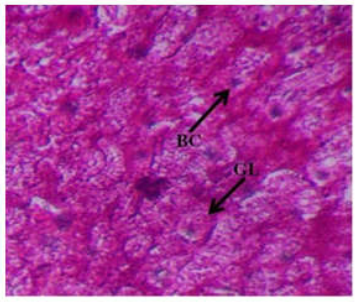

Fig. 3A

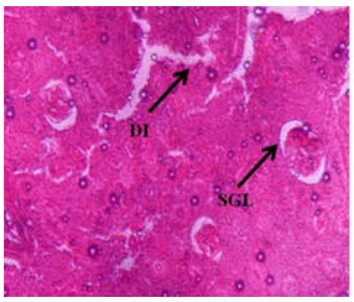

Fig. 3B

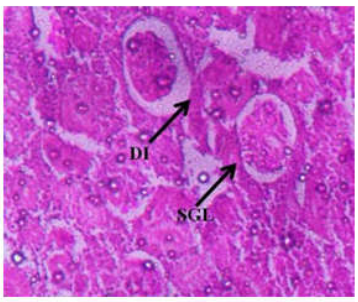

Fig.3C

Fig. 1A. showing histo-architecture of control gill. GR Gill Raker, PGL: Primary Gill Lamellae.
Fig. 1B: showing histo-architecture of 5% Chlorpyrifos treated gill. GR Gill Raker, PGL: Primary Gill Lamellae.
Fig. 1C: showing histo-architecture of 10% Chlorpyrifos treated gill. GR Gill Raker, PGL: Primary Gill Lamellae.
Fig. 2A: showing histo-architecture of Control liver. HC: Hepatic cord (Glisson's Capsule), CV: Central Vein.
Fig. 2B: showing histo-architecture of 5% Chlorpyrifos treated liver. HC: Hepatic cord (Glisson's Capsule), CV: Central Vein.
Fig. 2C: showing histo-architecture of 10% Chlorpyrifos treated liver. HC: Hepatic cord (Glisson's Capsule), CV: Central Vein, KC: Kupffer Cells.
Fig. 3A: showing histo-architecture of Control kidney. BC: Bowman's Capsule, GL: Glomerulus.
Fig. 3B: showing histo-architecture of 5% Chlorpyrifos treated kidney. DI: Degenerated interstitium, SGL: Shrunken Glomerulus.
Fig. 3C: showing histo-architecture of 10% Chlorpyrifos treated kidney. DI: Degenerated interstitium, SGL: Shrunken Glomerulus.

Among the experimental groups the variation was also significant being lesser in 10% in comparison to 5% group ($p < 0.01$, Table-1).

Total WBC count

We noted significantly high levels of total WBC count in 5% and 10% ($p < 0.01$) chlorpyrifos treated group when compared to control. The total WBC count was more significantly high in 10% chlorpyrifos treated group than 5% ($p < 0.01$, Table-1).

Differential Leukocyte Count

In fishes, two major classes of WBCs were identified which are granulocytes and agranulocytes. Among the granulocytes, % neutrophil level was found to be significantly high in 5% ($p < 0.05$) and 10% ($p < 0.01$) chlorpyrifos treated groups than control. The level of increase was more significantly high in 10% group than 5% ($p < 0.01$).

Basophils showed significantly high level in 5% and 10% groups in comparison to control ($p < 0.01$, Table-1). Level of eosinophil was significantly high in 5% treated group ($p < 0.01$) being more significantly high in 10% group in comparison to 5% group ($p < 0.05$). Among agranulocytes, % monocyte level was noted to be significantly high in 5% and 10% chlorpyrifos treatment groups ($p < 0.05$ and $p < 0.01$ respectively, Table-1). The variation in % lymphocyte level was significantly high only in 10% treated group ($p < 0.01$, Table-1).

DISCUSSIONS

Studies on histopathological and hematological changes are indicators of environmental impacts of pesticides (Kumari and Kumar, 1997; Parry, 1998) as the pesticide exposure significantly damages a number of physiologically important organs which in turn brings about different behavioral changes like loss of equilibrium, irregular movements, increase in opercular movements, imbalance and finally leading to death.

Table 1 Impact of Chlorpyrifos on hematological parameters of *Heteropnuestes fossilis*. Data represented as Mean ± SEM, n=10/group). (+ indicates % increase; - indicates % decrease). (*p < 0.05 control vs experimental groups; ** p < 0.01 control vs experimental groups; [a] p < 0.05, 5% of LC_{50} vs 10% of LC_{50}; [b] p < 0.01, 5% of LC_{50} vs 10% of LC_{50}

Sl. No	Conc. of Triazophos (mg/l)	Total RBC Count ($x10^6/mm^3$)	Hematocrit (%)	MCH (pg)	MCHC (g/dl)	%Hb	MCV	Total WBC Count ($x10^3/mm^3$)	Differential Leukocyte Count (%)				
									Neutrophil	Basophil	Eosinophil	Lymphocyte	Monocyte
1	Control (0)	4.22 ± 0.02	73.75 ± 1.75	21.5 ± 1.09	0.11±0.002	4.23±0.13	178.55±1.80	10.85±0.78	31.78±1.34	1.88±0.30	12.48±1.10	25.32±1.26	27.78±1.62
2	5% of LC_{50}	3.24 ± 0.03** (-23.22)	54.5 ±1.94** (-26.10)	14.85 ± 1.13** (-30.93)	0.09±0.006* (-18.18)	3.24±0.12* (-23.4)	159.35±3.11** (-10.75)	19.88±0.94** (+83.23)	37.63±1.27* (+18.41)	4.1±0.42** (+118.08)	19.1±1.21** (+53.04)	21.38±1.13 (-15.56)	20.73±1.24* (-25.38)
3	10% of LC_{50}	3.03 ± 0.02**b (-28.20)	47.5 ± 1.55**a (-35.59)	10.85 ± 0.75**b (-49.53)	0.04±0.002*a (-63.64)	2.85±0.09*a (-32.6)	142.85±1.60**b (-19.99)	27.2±0.39**b (+150.69)	46.88±1.82**b (+47.51)	5.25±0.37** (+179.26)	14.45±1.26a (+15.79)	18.38±1.38** (-27.41)	18.23±1.32** (-34.38)

In the present study, histological alterations were found in different tissues (gill, kidney and liver) of chlorpyrifos exposed fish (*Heteropneustes fossilis*). It is evident from the above findings that, even the sub-lethal doses of chlorpyrifos (i.e. 5% and 10% LC_{50}) are highly toxic to fish. The histological changes in the organs of experimental fish increased with increasing dose exposure. Kumari and Kumar (1997) reported histopathological changes in kidney, liver and gills, due to impact of industrial effluents in the fish, *Channa punctatus* and *Hetropneustes fossils*.

Kidney filters water toxicants as well as other contaminants in animal body. Elsan treatment in *Channa punctatus* also revealed significant decrease in the dimension of Bowman's capsule and glomerulus, and the tubules lost their regular shape due to precipitation of cytoplasm and karyolysis (Banerjee and Bhattacharya, 1995). Hypertrophy of renal cells, changes in the nuclear structure, formation of vacuoles, necrosis and degeneration of renal components were noticed on the renal cells of *Cyprinus carpio* exposed to malathion and sevin (Dhanapakiam and Sampoorni, 1998; Cengiz *et al*., 2001) demonstrated lesions in the kidney tissues of fish exposed to deltamethrin, tubular degeneration observed in catfish, *Ictalurus punctatus* upon exposure to methyl mercury (Kendall, 1975). Sublethal concentration of phenolic compounds exhibited degeneration and dissolution of epithelial cells of renal tubules, hypertrophy and necrosis in *Notopterus notopterus* (Gupta and Dalela, 1986).

Fish liver can be regarded as the body's detoxification center and hence a target organ for various xenobiotic substances. Necrosis, which is a passive and unregulated mode of cell death, shows that the capacity to maintain homeostasis was affected. Thus, occurrence of necrosis may be one of the important reasons for decreased lysosomal membrane stability leading to the leakage of lysosomal marker enzyme acid phosphatase to the soluble fraction. Pycnotic nuclei observed indicate that the cells became hypofunctional. Pycnosis results in irreversible condensation of chromatin in the nucleus of a cell. Acute toxic injury usually includes cloudy swelling or hydropic degenerations and pycnosis, karyorrhexis and karyolysis of nuclei (Hinton *et al*., 1988). Cloudy swelling, bile stagnation, focal necrosis, atrophy and vacuolization have been reported in the *Corydora spaleatus* exposed to methyl parathion by Cengiz *et al*., 2001 who also reported hypertrophy of hepatocytes, increase of kupffer cells, circulatory disturbance, narrowing of sinusoids, pycnotic nuclei, fatty degeneration and focal necrosis in the liver of *Gambusia affinis* exposed to deltamethrin. The cellular degeneration in the liver may be also due to oxygen deficiency as a result of gill degeneration and/or to the vascular and intravascular dialations haemolysis with subsequent stasis of blood (Mohamed, 2001). The teleost gills are critical organs which perform respiratory, osmoregulatory and excretory functions. Due to their (Polat *et al*., 2002). The main feature observed in gills exposed to sublethal concentration of pesticide was partial degeneration of epithelium of secondary gill lamellae. In some places adjacent secondary gill lamellae appeared to adhere each other. Fusion of secondary gill lamellae resulting in reduction of respiratory surface and vacuolization was also observed. Our results are inconsistent with the earlier reports (Butchiram *et al*., 2009), studied effect of sublethal concentration of malathion chloride on the histopathology of the gills of *Channa gachua* and observed hyperplasia, hypertrophy vacillation in primary gill lamellae, pycnotic nuclei and increase in volume of pillar cells. Our findings are in agreement with Singh *et al*., 2017 in *Channa punctatus* who reported that the effects of triazophos (both 5% and 10% concentrations of LC_{50}) have induced marked pathological changes in fish gills. The changes included lost their original shape and cutting of secondary gill filaments, pillar cell nucleus showed necrosis and developed vacuoles in the secondary gill epithelium. The degeneration in gill is due to intimate contact of gills with toxicant may lead to desertion of normal respiratory area i.e. damage of gill tissue which in turn may reduce the diffusion capacity of the gill.

The diffusion capacity of gill is directly associated with the oxygen carrying capacity of hemoglobin. The total RBC count, % Hb content, % Hematocrit value, MCV, MCH and MCHC were found to be significantly low in a dose dependent manner upon pesticide exposure in fish. Similar reports of RBC count and other hematological parameters are available in other fish models like *Cyprinus carpio* and *Puntius ticto* (Satyanarayan *et al*., 2004). Thus, lower levels of these parameters indicate that utilization of oxygen is differential and might have channelized to modulate different physiological processes including immunity.

In the cell-mediated immune response parameters, the TLC and DLC were found to be significantly high in exposed fishes especially the percent neutrophil level. This may be due to the effect of pesticides on the primary line of defense such as mucus and skin of fishes got injured and to maintain the homeostasis the Cell-mediated immune parameters were high. These findings are in agreement with other reports (Yonar, 2010; Kathya *et al*., 2010).

CONCLUSION

Depending upon our preliminary results, we may suggest that the detrimental impact of chlorpyrifos on various organs on non-target organisms is in coherence with the changes in histopathological studies. The hematological observations also validate our observations on histo-architecture of different

tissues. However, biochemical and molecular studies will further pin point the exact mechanism of action of chlorpyrifos (CPF an OP) in *Heteropneustes fossilis*.

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Conflict of Interest

None of the authors has any conflict of interest in submitting this manuscript (including financial).

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