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Effect of Dichlorvos (Nuvan) on Behaviour, Haematology and Histology of Freshwater Teleost *Labeo rohita*

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ABSTRACT

Nuvan (active ingredient dichlorvos) is a potent insecticide used wildly in agriculture and enters into the aquatic system as runoffs. The present study deals with the toxicity of nuvan towards major Indian carp, *Labeo rohita*. During the present study, when the fishes were exposed to nuvan, the behavioural changes were prominently observed, which included erratic swimming, gulping of air and excessive secretion of mucus. Our study shows the LC50 values for nuvan as 1.949 µg L-1 with a range of 1.718 to 2.211 µg L-1. The effect of exposure to sub-lethal concentrations of nuvan showed significant decrease in haemoglobin percent, Red Blood Corpuscle (RBC) count and increase in White Blood Corpuscle (WBC) count. All the histopathological observations during the study indicated that exposure to lethal concentrations of nuvan caused destructive effect in the gill, kidney and liver tissues of *L. rohita*.

INTRODUCTION

India is an agriculture based country and extensive uses of chemical fertilizers and pesticides/insecticides are in practice to take out more and more from the soil. The Government policies on use of such chemicals not only promote the farmers to use them but in fact encourage them to use them freely. The result is the much increased levels of such chemicals in the soil system.

Different kind of insecticides can cause serious impairment to physiological and health status of fishes. Pollutants such as insecticides may significantly damage certain physiological and hematological process when they enter into the organs of fishes (Banee *et al.,* 2011). Tilak (2007) studied the effect of phenol on certain blood compound of Indian major carps, *catla catla* (Ham.) *Labeo rohita* and *cirrhinus mrigala* (Ham). It is, therefore, become necessary to study the acute toxicity of the pollutants, where these pollutants remain under tested at very low concentration.

Rohu (*Labeo rohita*) is the most important among the three Indian major carp species used in carp polyculture systems. This graceful Indo-Gangetic riverine species is the natural inhabitant of the riverine system of Northern and Central India. The traditional culture of this carp goes back hundreds of years in the small ponds of the eastern Indian states.

Aquatic organisms, like fish, accumulate pollutants directly from contaminated water and indirectly through the food chain (Ashraf, 2005). Once the toxicant enters the body of the fish they may affect the organs leading to physiological and pathological disorders. An array of histopathological fluctuations is observed among fishes exposed to pollutants both in field and in laboratory conditions (Abdallah and Abdallah, 2008). Therefore the haematological and histopathological studies are potential tools to analyze the effect of toxicants on various target organs of fish in laboratory experiments and in field investigations (Kori- Siakpere *et al.,* 2005). Fishes are considered as one of the most significant indicators in freshwater systems for the evaluation of metal pollution (Rashed, 2001).

Nuvan 500 EC is an organophosphorus pesticide (active ingredient: dichlorvos), widely used in agriculture, animal husbandry, horticulture, food storage and even to control fleas on domestic pets. Since rohu is one of the major carp and economic backbone of the aquaculture industry in India, the present study is oriented towards studying the lethal effects of Nuvan, a

widely used organophosphate pesticide, on behavior, hematological changes and histological changes in vital organs of the rohu fish (*Labio rohita*) under laboratory conditions.

MATERIALS AND METHODS

The present study was conducted to analyze the effect of nuvan, on behaviour, mortality rate, and effects on fish haematology and histology of a major carp *Labeo rohita*.

Collection and preparation of experimental fishes

The test fishes (*L. rohita*) were collected from local aquaculture pond (Supataal) in the city of Jabalpur (India). Living and healthy *Labio rohita* of body size of 10 ± 1 cm and body weight of 30 ± 2 g were chosen for the study. The fishes were kept in glass aquaria containing 25 L of groundwater, with continuous aeration through aquaria pumps. Fishes were treated with 0.01% potassium permanganate solution to obviate dermal infections. The fishes were fed with commercially available fish food and acclimatized for 15 days before starting the experiment.

Experimental chemical

The technical grade insecticides Nuvan 76% (2, 2 dichlorovinyl dimethyl phosphate) was purchased from the local market, manufactured by Excel India Limited, New Delhi respectively.

Exposure to Nuvan

The fishes were divided into groups, having 10 fishes in each group. The first group served as a control and received no insecticide. The other groups received different concentrations of nuvan. The fingerlings of *Labeo rohita* were exposed to the 6 concentrations of nuvan, i.e., 0.5, 1, 2, 5, 10.0 and 20 mg L^{-1} (Tilak and Kumari, 2009). Fish were fed daily with commercial diet at the rate of 3 % of their body weight in two fractions at an interval of 8 hours.

In both the cases, the fish behaviour was observed closely and recorded. The fish mortality was also recorded and the dead fishes were immediately removed. For haematological experiments, the fish blood was collected every 24 hours from the live fishes.

Determination of LC_{50} values

The 96 hr 50% Lethal Concentration (LC_{50}) was calculated using log of the concentration versus mortality rate at different time intervals and fitting a non-linear regression curve using Sigma Graphpad Prism® software, version 6.0.

Haematological tests

The fish collected every 24 hr was immediately processed for the determination of vital haematological parameters such as haemoglobin (Hb), RBC count, WBC count and haemtocrit (Hct) using standard haematological procedures. Statistically significant differences were calculated using one way ANOVA by Graphpad Prism version 6.0 software.

Histological examination

Histopathological changes in different organs are generally assessed to find the health condition of fish. For the study of effect of pesticides on the important organs of the *Labeo rohita*, the important fish organs, i.e., gill, liver and kidney were processed for the microscopic studies. Every time, a fish is dead during the experiment was dissected to remove the fish gills, liver and the kidney. Immediately after removal, the organs were washed with distilled water and kept separately in Bouin's fixative. After the fixation, the tissues were dehydrated, sectioned and stained with hematoxylin and eosin.

RESULTS

The fishes were exposed to six different concentrations of nuvan i.e., 0.5, 1, 2, 5, 10 and 20 mg L^{-1}. These concentrations were chosen based on the literature and the Lethal Dose LD_{50} values of nuvan in other experiments and included sublethal to lethal dose of nuvan. The changes in fish behavior, LD_{50}, changes in hematological parameters and changes in fish histology were observed during the experiments to ascertain the toxicity of nuvan to *Labeo rohita.*

Behavioral changes in L. rohita after exposure to nuvan

Behavioral changes in experimental fishes were observed with reference to the food consumption, swimming activity and mucous secretion through general body surface. The

changes were recorded with references to the control fishes that received no pesticide. In control group of experiments, where no pesticide was supplied, the given food was fully consumed by the *Labeo rohita* within 24 hr. The fishes started consuming the food immediately after supply. The fishes exposed to 0.5 mg L^{-1} concentration also consumed the food during a period of 24 hours. The fishes exposed to 1.0 and 2.0 mg L^{-1} nuvan concentrations did not consume the food completely and about 20 to 25% food per day was left unconsumed. Fishes exposed to the higher concentrations of the nuvan were not at all interested in food and most of the food was left after 24 hr.

As far as the swimming activity is concerned, the fishes in water with lower concentrations of toxicant nuvan (up to 1 mg L^{-1}) did not show much disturbed swimming behavior, except occasional restlessness and uncoordinated swimming in between. The fishes exposed to the higher concentrations of nuvan were sluggish and showed uncoordinated swimming movement.

Gradual reduction of mucous secretion was also observed after 4-5 days which was almost normal, and water tank turns to less milky white. With lower nuvan concentrations, the mucus secretion was more. After introducing the fish in to test solutions with higher nuvan concentration, copious secretion of the mucous was observed over the body.

In controlled set of experiments, the fishes showed normal respiratory activities. No restlessness was observed. Relatively increased respiratory activity in the beginning and reduced later as revealed by increased and decreased opercular movement was observed during the early hours of exposure of the fishes to the toxicants Nuvan.

Determination of 50% lethal concentration (LC_{50}) of nuvan

For the determination of nuvan concentration able to kill 50% of the fishes was calculated using the observations of fish deaths during 96 h period with different concentrations of nuvan. The LC_{50} values obtained by fitting a non-linear regression dose-response curve (Fig 1) was found to be 1.949 µg L^{-1} with a range of 1.718 to 2.211 µg L^{-1} (Table 1).

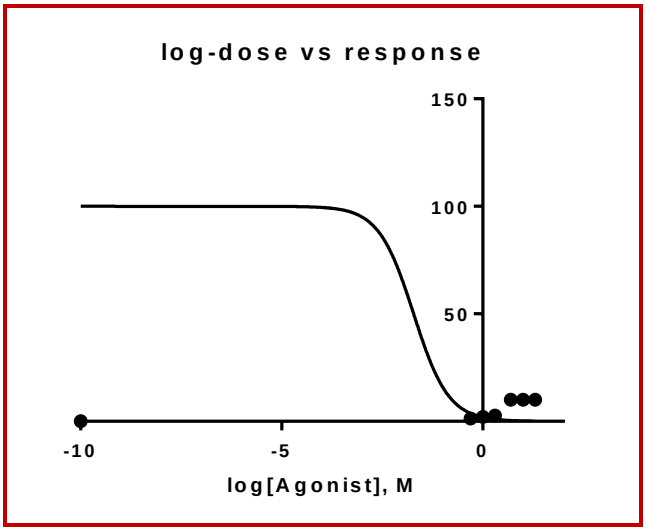

Fig 1: Regression curve plotted for Log dose versus mortality for identifying the 50% lethal dose of nuvan against *Labeo rohita*

Table 1: Detailed statistical data for the calculation of LC_{50} value of nuvan against *Labeo rohita* during the present study.

log(inhibitor) vs. normalized response -- Variable slope	
Best-fit values	
$LogLC_{50}$	0.2898
HillSlope	1.361
LC_{50}	1.949
Std. Error	
$LogLC_{50}$	0.02616
HillSlope	0.1032
95% Confidence Intervals	
$LogLC_{50}$	0.2351 to 0.3446
HillSlope	1.145 to 1.577
Range LC_{50}	1.718 to 2.211
Goodness of Fit	
Degrees of Freedom	19
R square	0.9788
Absolute Sum of Squares	598.8

Changes in fish hematology due to exposure to nuvan

The control fishes showed hemoglobin content as 12.7 ± 0.25 % after 24 hr and after 96 hours of experiment, the hemoglobin was almost stable and found to be 13.0 ± 0.15%. With 0.5 µg L^{-1} nuvan concentration the hemoglobin content reduced to 10.0 ± 0.42% after 96 hours, while with 1.0 and 2.0 µg L^{-1} concentrations of nuvan, the Hb% reduced to 9.0 ± 0.06 and 8.7 ± 0.21 % respectively. With nuvan concentrations higher than 2.0 µg L^{-1}, the fish deaths were higher between 48 hr and 72 hr, the fishes after 96 hr were not available for the blood sampling. However, the Hb% was lowest as 5.6 ± 0.16 after 72 hours with 20 µg L^{-1} **nuvan concentration.**

Table 2: Changes in hemoglobin concentrations (%) in fishes exposed to varying concentrations of nuvan in aquaria water for a period of 96 hr. Data are presented as mean ± Standard deviation (n=3). Different letters in superscript show significant difference from control (one way ANOVA, p<0.05).

Sr. No.	Time (h)	Control	Concentration of nuvan (µg L^{-1})					
			0.5	1.0	2.0	5.0	10.0	20.0
1	24	12.7 ± 0.21[a]	11.8 ± 0.25 [a]	11.1 ± 0.13 [a]	11.4 ± 0.06 [a]	9.9 ± 0.15 [a]	9.7 ± 0.11 [a]	9.7 ± 0.18 [a]
2	48	12.6 ± 0.10 [a]	11.2 ± 0.45 [a]	10.5 ± 0.15 [b]	10.1 ± 0.10[b]	9.6 ± 0.25 [b]	9.1 ± 0.14 [b]	8.8 ± 0.12 [b]
3	72	12.9 ± 0.06 [a]	10.3 ± 0.55[b]	9.1 ± 0.10 [b]	NT*	6.0 ± 1.17 [b]	6.1 ± 0.98 [b]	5.6 ± 0.16 [b]
4	96	13.0 ± 0.15 [a]	10.0 ± 0.42 [b]	9.0 ± 0.06 [b]	8.7 ± 0.21 [b]	NT	NT	NT

*NT=No fish available for taking blood samples due to the mortality of the fishes.

The total RBCs were counted using hemocytometer and the results are presented in table 3. The control fishes showed mean RBC count as 5.8 ± 1.12 x 10^6 cells per µl of blood. With 0.5 µg L^{-1} nuvan concentration the RBC count reduced to 5.1 ± 1.11 x 10^6 cells per µl after 96 hours, while with 1.0 and 2.0 µg L^{-1} concentrations of nuvan, the RBC count reduced to

4.3 ± 0.27 x 10^6 cells and 4.1 ± 0.42 x 10^6 cells per µl respectively. However, the RBC count was lowest as 4.2 ± 0.71 x 10^6 cells per µl after 72 hr with 20 µg L^{-1} nuvan.

Table 3: Changes in total RBC counts (x10^6 cells µL^{-1}) in fishes exposed to varying concentrations of nuvan in aquaria water for a period of 96 hr. Data are presented as mean ± standard deviation (n=3). Different letters in superscript show significant difference from control (one way ANOVA, p<0.05).

Sr. No.	Time (h)	Control	Concentration of nuvan (µg L^{-1})					
			0.5	1.0	2.0	5.0	10.0	20.0
1	24	5.8 ±1.12 [a]	5.8 ± 0.93 [a]	5.9 ± 1.12 [a]	5.8 ± 1.11 [a]	5.9 ± 1.32 [a]	5.7 ± 1.02 [a]	5.6 ± 0.95 [b]
2	48	5.8 ± 1.21 [a]	5.7 ± 0.87 [a]	5.6 ± 0.87 [a]	5.1 ± 1.29	5.1 ± 0.79	5.0 ± 0.45	4.8 ± 0.56
3	72	5.7 ± 1.51 [a]	5.2 ± 1.12 [b]	5.1 ± 0.34 [b]	NT	4.6 ± 0.64 [b]	4.6 ± 0.56 [b]	4.2 ± 0.71 [b]
4	96	5.7 ± 1.32 [a]	5.1 ± 1.11 [b]	4.3 ± 0.27 [b]	4.1 ± 0.42 [b]	NT	NT	NT

*NT=No fish available for taking blood samples due to the mortality of the fishes.

The control fishes showed mean WBC count as 1.45 ± 0.06 x 10^3 cells per µl of blood, which increased to 1.8 ± 0.028 x 10^3 cells per µl. With 0.5 µg L^{-1} nuvan concentration the WBC count increased from 1.36 ± 0.07 (in 24 hr) to 1.83 ± 0.02 x 10^3 cells per µl after 96 h. With 1.0 and 2.0 µg L^{-1} concentrations of nuvan, the WBC count increased to 2.88 ± 0.06 x 10^3 cells and 3.88 ± 0.1 x 10^3 cells per µl respectively. The WBC count was highest as 7.1 ± 0.79 x 10^3 cells per µl after 72 hr with 20 µg L^{-1} nuvan (Table 4).

Table 4: Changes in total WBC counts (x10^3 cells µL^{-1}) in fishes exposed to varying concentrations of nuvan in aquaria water for a period of 96 hr. Data are presented as mean ± standard deviation (n=3). Different letters in superscript show significant difference from control (one way ANOVA, p<0.05).

Sr. No.	Time (h)	Control	Concentration of nuvan (µg L^{-1})					
			0.5	1.0	2.0	5.0	10.0	20.0
1	24	1.45 ± 0.06 ^{a}	1.36 ± 0.076 ^{a}	2.03 ± 0.07 ^{a}	4.58 ± 0.07 ^{b}	4.58 ± 0.07 ^{b}	5.2 ± 1.02 ^{b}	5.3 ± 0.95 ^{b}
2	48	1.53 ± 0.07 ^{a}	1.56 ± 0.057 ^{a}	2.25 ± 0.08 ^{b}	4.75 ± 0.05 ^{b}	4.75 ± 0.07 ^{b}	5.8 ± 0.45 ^{b}	5.8 ± 0.86 ^{b}
3	72	1.75 ± 0.06 ^{a}	1.68 ± 0.076	2.75 ± 0.05 ^{b}	NT	5.33 ± 0.28 ^{b}	6.7 ± 0.65 ^{b}	7.1 ± 0.79 ^{b}
4	96	1.8 ± 0.028 ^{a}	1.83 ± 0.028 ^{b}	2.88 ± 0.06 ^{b}	3.88 ± 0.10 ^{b}	NT	NT	NT

*NT=No fish available for taking blood samples due to the mortality of the fishes.

The Hct% in control showed mean as 25.8 ± 2.12 % at 24 hr which remained unchanged up to 96 h and read as 26.2 ± 1.26%. With 0.5 µg L^{-1} nuvan concentration the Hct % reduced from 26.2 ± 2.13 to 25.2 ± 1.19 % after 96 h, while with 1.0 and 2.0 µg L^{-1} concentrations of nuvan, the Hct % reduced to 24.3 ± 2.34 and 23.1 ± 1.34 % respectively. The Hct % was lowest as 14.7 ± 1.06 % after 72 hours with 20 µg L^{-1} nuvan concentration.

Table 5: Changes in hematocrit value (%) in fishes exposed to varying concentrations of nuvan in aquaria water for a period of 96 hours. Data are presented as mean ± standard deviation (n=3). Different letters in superscript show significant difference from control (one way ANOVA, p<0.05).

Sr. No.	Time (h)	Control	Concentration of nuvan (µg L^{-1})					
			0.5	1.0	2.0	5.0	10.0	20.0
1	24	25.8 ± 2.12 [a]	26.2 ± 2.13 [a]	26.0 ± 1.08 [a]	25.7 ± 1.34 [a]	24.5 ± 1.43 [a]	23.1 ± 1.01 [a]	20.2 ± 1.06 [b]
2	48	25.8 ± 1.23 [a]	25.8 ± 1.33 [a]	25.8 ± 1.02 [a]	24.8 ± 2.11 [a]	23.2 ± 0.97 [a]	18.9 ± 1.12 [b]	17.2 ± 1.21 [b]
3	72	26.1 ± 1.11 [a]	25.9 ± 1.02 [a]	25.6 ± 2.11 [a]	NT	21.2 ± 1.23 [b]	17.2 ± 2.13 [b]	14.7 ± 1.06 [b]
4	96	26.2 ± 1.26 [a]	25.2 ± 1.19 [a]	24.3 ± 2.34 [a]	23.1 ± 1.34 [b]	NT	NT	NT

*NT=No fish available for taking blood samples due to the mortality of the fishes.

Effect of nuvan on fish histology

Changes in Gill Histology

The gills were observed for histological changes after the hematoxylin and eosin staining of fixed gill tissue. The gills from control fishes, which received no pesticide, showed the general structure of gills. The histopathological changes in the gills after 24 h exposure to the lethal dose of nuvan, the showed no marked changes except some signs of hemorrhage and increased blood channels in the gills. After 48 h of exposure, destruction of the primary gill lamellae was evident by hemorrhage and hyperplasia. The secondary gill lamellae started showing detachment from the basement membrane and the piller cells were more distorted. After 72 h of exposure, the epithelial cells of secondary gill lamellae were degenerated and only leaving by distorted pillar cell. Secondary gill lamellae were found detached by primary gill lamellae and basement membrane through vasodilation. After 96 h, completely damaged gill structure was seen. The primary and secondary gill lamellae were disintegrated and detached from the basement membrane. Higher amount of hemorrhage was seen in most part of the slide (Fig 2).

Changes in Liver Histology

By visualizing the transverse section of H&E stained liver exposed to the lethal dose of nuvan for 24 hr showed less hemorrhage, as compared to the fishes exposed for 72 to 96 h. No significant changes in liver tissues, except for mild congestion of the blood vessels and some hemorrhage was seen. The congestions of the blood vessels increased in fishes exposed for 48 h with more signs of hemorrhages in liver tissues. Shrinkage of the hepatic cells could well be observed with pycnotic nuclei. The liver from fishes exposed to 72 h showed clear signs of liver damage evident by marked swelling of hepatocytes with areas of necrosis. The normal architecture of liver tissue was marketedly disrupted for the fishes exposed to 96 h to nuvan. Sinusoids in most cases were distended and central veins appeared severely damaged due to degeneration of endothelial lining cells (Fig 2).

Changes in Renal Histology

The histology of kidney tissues in transverse sections showed mild degenerative changes in tubular epithelium after 24 h of exposure to the lethal dose of nuvan. After 48 h of exposure, the necrosis was clearly visible. The lumen of the tubules were found dilated. Cell membranes were found ruptured and clumping of the cytoplasm could be seen at higher magnification. The Bowman's space was also dialted. After 72 h of exposure to nuvan, the kidney of *Labeo rohita* showed shrunken nucleus, damaged glomerulus, dilated luman and clumping of cytoplasm in the Bowman's capsule which capturing the cell of membrane, and distorted brush border in the proximal segment. After 96 h of exposure, the kidney of *Labeo rohita* showed complete damage to the renal histology. The Bowman's capsule was completely lost and was hardly visible in the slides. Cytoplasm was clumped due to the rupturing of the cell membrane.

DISCUSSION

The organophosphates, i.e. nuvan (dichlorovos) are modern synthetic insecticide and are potent neurotoxic molecules (Lundbaye *et al.*, 1997). In India and in Nepal and other developing countries, nuvan is more in use and more deaths of human and livestock has been reported because of these two pesticides (Paudyal, 2008).

Behavioural changes are physiological responses shown by the animal, which are often used as the sensitive measure of stress syndrome in the organism experiencing it. During the present study, when the fishes were exposed to nuvan, the behavioural changes were prominently observed, which included erratic swimming, gulping of air and excessive secretion of mucus. Rao *et al*. (2005) observed same behavioural changes in the mosquito fish, *Gambusia affinis* exposed to the higher concentrations (400 and 500 µg/L) of chlorpyrifos. Rao *et al.* (2003) showed behavioral changes in euryhaline fish, *Oreochromis mossambicus* due to the toxic effects of profenofos. In another study by Marigoudar *et al.* (2009), impact of cypermethrin on behavioural responses in *Labeo rohita* showed Irregular, erratic and darting movements followed this with imbalanced swimming activity.

Our study shows the LC_{50} values for nuvan as 1.949 µg L^{-1} with a range of 1.718 to 2.211 µg L^{-1}. Bhat *et al.* (2012) observed the 96 hr LC_{50} of nuvan (dichlorvos) against *Labeo rohita* as 16.71 ppm, which was much higher in comparison to present study. The LC_{50} values of nuvan (dichlorvos) has been reported by various workers as in *Cyprinus carpio* as 0.34 ppm for 96 hr (Verma *et al.*, 1981), in *Cirrhinus mrigala* it was 9.1 ppm for 96 hr (*Velmurugan et al*., 2009) and in *Ctenopharyngodon idella* it was 13.1ppm for 24 hr (Tilak and Kumari 2009).

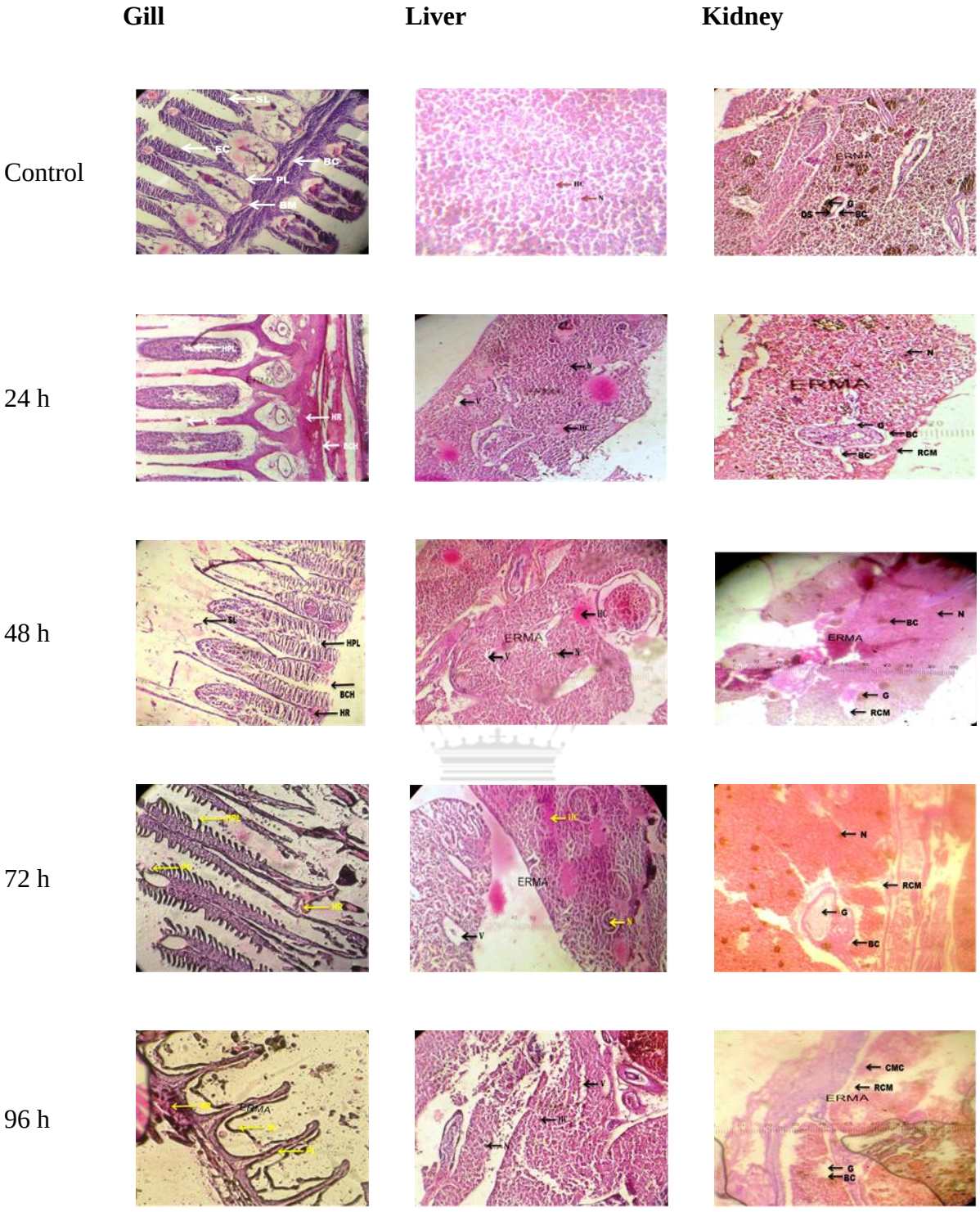

Fig 2: Transverse section of gill, liver and kidney of *Labeo rohita* from control as well as for fishes exposed for the different exposure times (X400). Primary gill lamellae (PL); secondary gill lamellae (SL); Basement membrane (BM); Epithelial cell (EPL); Mucous cell (MC); Pillar cell (PC); Blood capillaries (BC); Blood channel (BCH), Hepatic cell (HC); Blood Sinusoids (BS); Nucleus (N), Glomerulus (G), Bowman's capsule (BC) Distal segment (DS); Proximal I segment (PI); Proximal II Segmental (P II).

Das and Mukherjee (2003) showed the effect of exposure to sub-lethal concentrations of cypermethrin blood of *Labeo rohita*, and shown the decrease in Hb% and RBC count in sub-lethal concentrations after 45 days. Since our study used the concentrations in sublethal to lethal doses, the effect was more prominent after 96 hr. Similar reduction in RBC was reported for freshwater common carp (*Cyprinus carpio* L.) treated with diazinone (Svoboda et al., 2001) and African catfish (*C. gariepinus*) treated with diazinone (Adedeji *et al*., 2009).

Velmurugan *et al.* (2009) showed the histopathological effects on gill and liver tissues in *Cirrhinus mrigala* chronically exposed to dichlorvos (nuvan). The most common changes were hyperplasia, desquamation, and necrosis of epithelial, epithelial lifting, oedema, lamellar fusion, collapsed secondary lamellae and curling of secondary lamellae. During our study, we also found the hyperplasis of the epithelial cells and collapse of primary and secondary gill lamellae with lethal dose of nuvan. During the study, kidney tissues were also shown to affected by nuvan. The Bowman's capsule was completely lost. Cytoplasm was clumped due to the rupturing of the cell membrane. Similar findings with distended kidney tubules and marked necrotic changes in tubular cells of posterior kidney were observed when *L. rohita* was exposed to sublethal concentrations of hexachlorocyclohexane (Das and Mukherjee, 2000).

All the histopathological observations during the study indicated that exposure to lethal concentrations of nuvan caused destructive effect in the gill, kidney and liver tissues of *L. rohita*. As a conclusion, the findings of the present histological investigations demonstrated a direct correlation between pesticide exposure and histopathological disorders observed in several tissues.

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