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Original Article

Observation on the Histopathological Alterations in the Liver of Cypermethrin Affected Fish, *Cirrhinus mrigala* (Hamilton)

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ABSTRACT

The freshwater fish, *Cirrhinus mrigala,* was exposed to lethal and sub-lethal concentrations of cypermethrin (5.13 µg/l and 1.026 µg/l) and the histopathological changes were observed in liver tissues. Compared to the structure of control fish, exposed tissues were initially exhibited disarray of liver lobes, mild degree of degeneration of cytoplasm, occasional blood clots, congregation of nuclei, cloudy swelling of hepatocytes, granulisation of cytoplasm, hypertrophic, pyknotic nuclei, atrophic, hepatocytic nuclei, focal necrosis, vacuolation, shrinkage of hepatocytes, granular degeneration, rupture of blood vessels, necrosis dissolution of laminar structure and cytoplasmic disintegration in hepatocytes. In the sub-lethal concentration of cypermethrin, tissues initially exhibited few changes like slight disarray of liver lobes, mild degree of degeneration of cytoplasm, occasional blood clots, congregation of nuclei and cloudy swelling of hepatocytes, granulisation of cytoplasm, hypertrophic and pyknotic nuclei on 1 and 7 days. But on 14 and 21 day, certain degree of reorganisation in the structure of liver cords was observed.

Keywords: Cypermethrin, Toxicity, Pesticide, Liver, *Cirrhinus mrigala*

INTRODUCTION

Pesticides are found as common contaminants in soil, air and water, and on non-target vegetation in our urban landscapes. Environmental pollution is one of the most serious problems that face mankind in this century, which has now become a global issue among scientists and researchers working in this area[1]. Unfortunately, several toxic pollutants, few are even unknown or un-identified to the biota, are being regularly introduced in large quantities into the environment, especially into the aquatic environment. Pollution of water is an important dimension of environmental degradation. Pesticides are known to be the major toxicants polluting the aquatic environment[2]. A diversity of pesticides and their residues are present in a wide variety of aquatic habitats[3]. The pesticides manifest their toxic effect not only on aquatic organisms but also ultimately affect human beings through the aquatic food sources. The pesticides affect the eco system, reproduction and behaviour by causing pathological and physiological changes[3]. A toxicant induces its effect first at cellular or even at molecular level, but ultimately causes physiological, histopathological and bio-chemical alterations[4]. As the fishes are economically important non-target organisms, they are quite sensitive to a wide variety of toxicants and are used as pollution indicator in the water-quality management. Acute toxicity tests are generally used to determine the concentration of a toxicant that produces a specific adverse effect on a specified percentage of test organisms in a short span of time. Because of death is normally and easily detected and obviously one of the most important adverse effects, the most common acute toxicity test is acute lethality test. Synthetic pyrethroids constitute a potent group of insecticides. Although many analogs of natural pyrethroids have been synthesised, few have succeeded commercially and only recently developed ones exhibit sufficient photostability to show promise for wide-scale use in

agriculture or forestry. It is known that arthropods and fishes are highly sensitive to synthetic pyrethroids[5]. Cypermethrin is a synthetic pyrethroid insecticide widely used throughout the world to control different crop pests, flies and mosquitoes because of the high photostability, degradability, non-persistent nature and low mammalian toxicity[6]. It has been found that their entry into the aquatic environment causes great havoc to the fishes[7].

The histopathological changes are irreversible, while altered functional systems are considered as a reversible effect. The severity of histological damages in any particular aquatic organism is directly proportional to the concentration of a pollutant in the medium[4]. Moreover, the histopathological picture of the organs can corroborate with the biochemical changes accounting for the functional disruptions in the activity of the organs due to cellular damage[8]. The liver being the main organ of various key metabolic pathways, the effects of a chemical usually appears primarily in the livers. This, in turn, provides toxicologists a significant site for investigation[9]. Toxicants impair the metabolic and physiological activities of the organisms but such studies alone do not satisfy the complete understanding of pathological condition of tissues under toxic stress. Hence, it is useful to have an insight into histological analysis regarding the extent of damage of the tissue, liver when cypermethrin enters the body of *Cirrhinus mrigala*.

MATERIALS AND METHODS

Procurement and Maintenance of Fish

Freshwater fish, *Cirrhinus mrigala* (length 10±1 cm; weight 15±1 g) fingerlings, was obtained from Karnataka State Fisheries Department Fish Farms, Dharwad, India. The species were reared and acclimated to laboratory conditions (26±1 °C) in a large cement tank. During acclimation they were fed with rice bran and oil cakes in the ratio of 2:1 on every alternate day. Water of the tank was changed daily to avoid fungal and bacterial contaminations, if any.

Physico-chemical Characterisation of Water

The physico-chemical characteristics of the water used for fish bioassay were determined according to the procedure described in Standard[10]. Water quality parameters were given Table 1.

Table 1: Showing physico-chemical parameter

S.No.	Parameter	Testing water
1.	Temperature	28±1° C
2.	pH value at 28° C	8±0.2
3.	Dissolved (DO) oxygen	6.7 to 7.2
4.	Biological Oxygen Demand (BOD)	11
5.	Chemical Oxygen Demand (COD)	2.8
6.	Carbon dioxide	9.0
7.	Total Hardness	115
8.	Chloride (as Cl)	46.3
9.	Sodium	1.22
10.	Potassium	30.5
11.	Calcium	17.04
12.	Magnesium	1
13.	Oxygen per cent<R> saturation	57 (as $CaCO_3$)
14.	Specific gravity	1.00374
15.	Ammonium (NH_4)	0.8
16.	Nitrite (NO_2)	1.4
17.	Nitrate (NO_3)	14
18.	Fluoride (as Fl)	1.5

* Except Temperature and pH, value express in mg/L

Toxicant Selected and Preparation of Stock Solution

Technical grade of cypermethrin {(R.S.) µ Cyno – 3 phenoxybenzyl 2, 2 dimethyl (IR, IS) *cis*, *trans* 3 (2, 2 dichlorovinyl) cyclopropane carboxylat} (95%) was obtained from Rallis India Ltd, Bangalore. The pesticide stock solution was prepared by dissolving 10 mg of cypermethrin in 10 ml of analytical grade acetone. For experimental purposes, the pesticide was drawn from the prepared stock solution. The maximum amount of acetone contained in the highest concentration tested was less than 0.1 ml and the same quantity of acetone was added to the controls. Acetone was found to be non-toxic to fish[11].

Toxicity Evaluation

In order to understand the influence of time over toxicity, the effect of lethal concentration of cypermethrin on *Cirrhinus mrigala* was studied at different periods of exposure. Before experimentation, healthy fishes were collected from the large cement tank with the help of nylon net and hand net. They were acclimatised to laboratory conditions in glass troughs for 15 days. Each trough contained 15 litre of water with uniform sized fish (length 10 cm; weight 15 g). During acclimation period, the fishes were fed with commercially available standard palletised feed (Lipton India Ltd.). Aeration was provided.

After 15 days, if fishes were in normal behavioural activity and good health conditions, those species were selected for experiment purpose.

Fixation of Exposure Periods

For histropathological studies, fishes were divided into three groups of 10 each. Static bioassay method of[12] was used to expose fishes to pesticides. Fishes of group of one and two were exposed to lethal concentration 5.13 mg/l of cypermethrin (1, 2, 3 and 4 days) and sub-lethal concentration of one-fifth of LC50 value 1.026 mg/l (1, 7, 14 and 21 days) were chosen to observe the short-term and long-term effects. They were exposed separately in glass aquaria of 15 litre capacity. Third group was simultaneously maintained without pesticide served as control. No aeration was provided during the experimental periods. Water was renewed after every 24 h to maintain the pesticide concentration.

Histopathological Studies

The histopathological studies were observed in liver, *Cirrhinus mrigala*, exposed to lethal (1, 2, 3 and 4 day) and sub-lethal (1, 7, 14 and 21 day) concentrations of cypermethrin. To study the histopathology of tissues, the method described by[13] was followed. After the end of each day exposure period fishes from all groups were taken out from respective aquarium one by one and dissected and liver tissue of treated and control fish were isolated. It was fixed in boun's fluid for 24 h at room temperature. Then it was taken out from boun's fluid and repeatedly washed with 70% alcohol till all the traces of boun's fluid were removed. Dehydration process was carried out by washing the tissue with alcohol (90% and 100%), alcohol-benzene in different ratios (3:1, 1:1 and 1:3) followed by pure benzene and benzene-paraffin wax (1:1). After the process, the organs were embedded in paraffin (58–60^{o}C). Sections were taken (5 mm thickness) and stained with[14] and counter stained with eosin. All sections were mounted with Di-N-Butyle Phthalate in Xylene (DPX) mounting and histopathological changes were observed under light microscope.

RESULTS

Histology of controlled liver of fish comprises a continuous mass of large hexagonal hepatic cells (Hepatic parenchyma). Hepatic cells are of round dish or polygonal shape containing clear spherical nucleus. They are located among sinusoids forming cord-like structures known as hepatic cell cords. In fish these structures are generally obscure. Bile canaliculus is centrally located in each cord. There is no clear division of hepatic cells into lobules. These cells contained granular cytoplasm and with distinct nuclei either exocentric or slightly centrally placed. Hepatic cells have many vital functions other than the secretion of bile. They play an important role in protein, lipid and carbohydrate metabolism. They serve as storage site for some nutrients. Detoxification is another important function. A large number of blood sinusoids and lipid glycogen granules are found in the hepatic mass (Plate 1: Figures 1, 2 and 3).

Histological changes in the liver of exposed fish shows, on day 1 of exposure to the lethal concentration of cypermethrin, enlarged nuclei and vacoulation in hepatic cells. Liver cords were seen disarrayed (Plate 1: Figures 4 and 5). On day 2 of exposure, the paranchymatous nature of the liver was greatly disrupted with congested blood vessels. The hepatocyte cell membranes were ruptured and granular degeneration was evident in most of the hepatocytes. Nuclei became slightly hypertrophic (Plate 2: Figures 1 and 2). On further exposure, day 3, severe degrees of atrophic changes were noticed in the liver cords. Haemorrhagic condition was prominent with heavy vacuolisation in the liver tissue. At some regions, exfoliation and congregation of hepatocytic nuclei and focal necrosis were seen (Plate 2: Figures 3 and 4). This was followed by the severe degree of vacuolation, shrinkage of hepatocytes, atrophy, granular degeneration, rupture of blood vessels, necrosis dissolution of laminar structure and cytoplasmic disintegration in hepatocytes on day 4 of exposure (Plate 2: Figures 5 and 6).

Compared to the structure of the liver of control fish, fish exposed to sub-lethal concentration of cypermethrin initially exhibited few changes like slight disarray of liver lobes, mild degree of degeneration of cytoplasm, occasional blood clots and congregation of nuclei on day 1 (Plate 3: Figures 2 and 3) and cloudy swelling of hepatocytes, granulisation of cytoplasm, hypertrophic and pyknotic nuclei on day 7 (Plate 3: Figures 4 and 5). However, on further exposure on day 14, certain degree

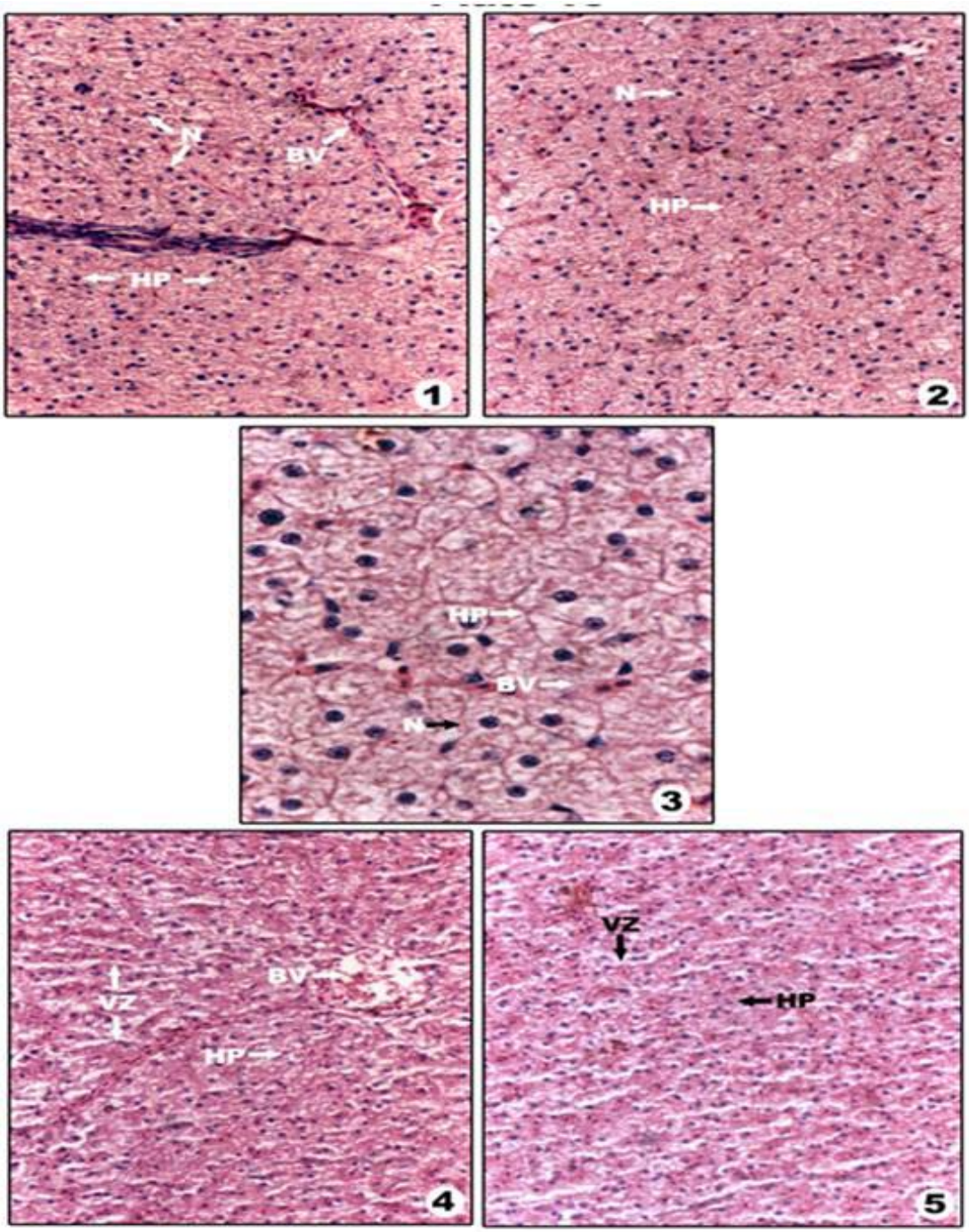

Plate 1: (1 & 2) Section of liver of control fish, *Cirrhinus mrigala* showing normal structure. HP=hepatocytes, N=nucleus, BV= blood vessel. Haematoxylin and eosin: x400; **(3)** Section of gill of control fish *Cirrhinus mrigala* a part of liver enlarged showing polygonal hepatocytes (HP) and nucleus (N) and blood vessels (BV). Haematoxylin and eosin: x1,000; **(4 & 5)** Section of liver of fish, *Cirrhinus mrigala,* exposed to cypermethrin (5.13 mg/l) for 24 h showing vacuolization (VZ), cytoplasmic degeneration, shape of hepatocytes was changed (HP) and damage of blood vessel (BV). Haematoxylin and eosin: x400.

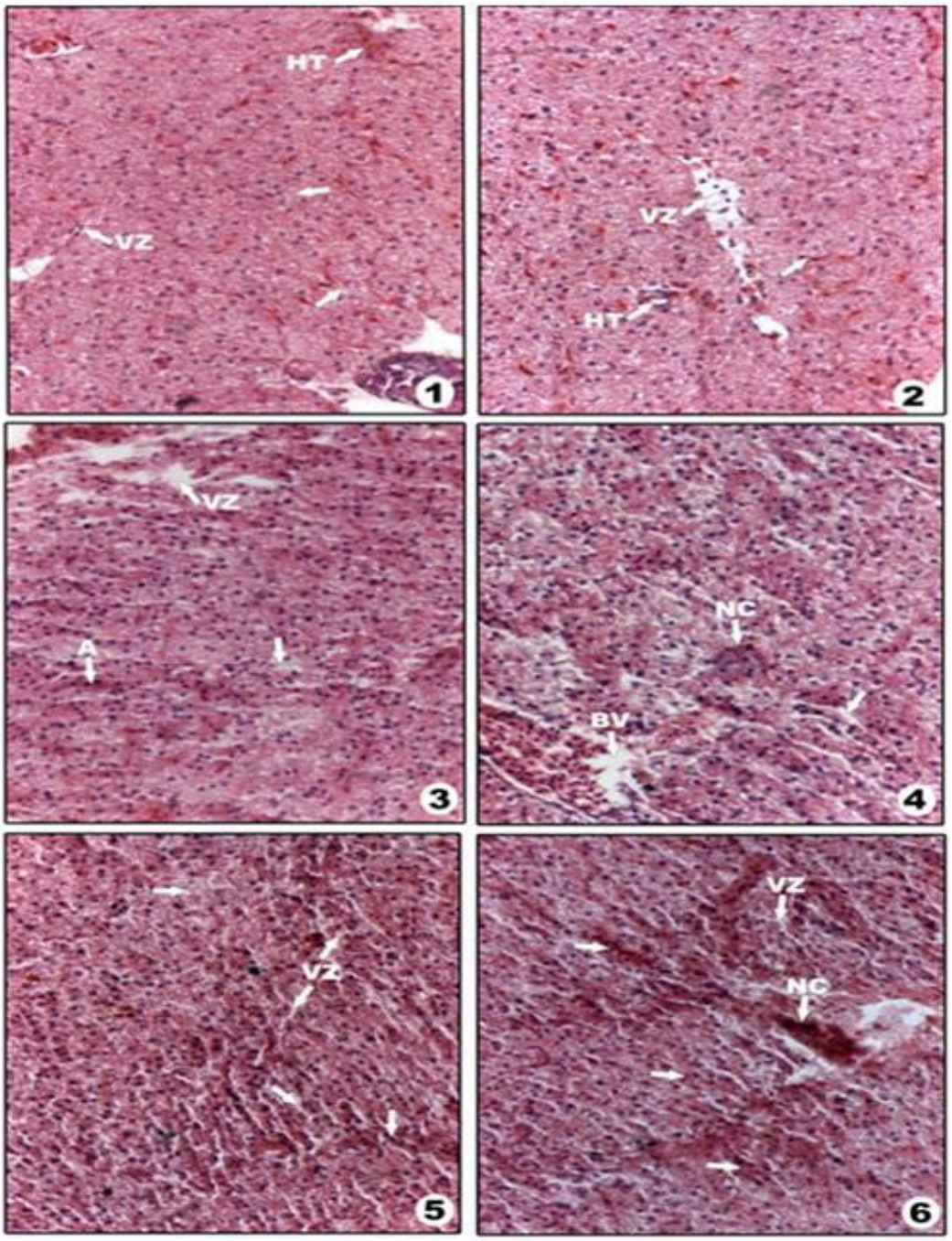

Plate 2: (1&2): Section of liver of fish, *Cirrhinus mrigala,* exposed to cypermethrin (5.13 mg/l) for 48 h showing vacuolization of hepatocytes, lymphocytic degeneration and hypertrophy. Haematoxylin and eosin: x400; **(3 & 4)** Section of liver of fish, *Cirrhinus mrigala,* exposed to cypermethrin (5.13 mg/l) for 72 h showing diffused necrosis, cytoplasmic degeneration, damage of blood vessel and vacuolization of hepatic cells. Haematoxylin and eosin: x400; **(5 & 6)** Section of liver of fish, *Cirrhinus mrigala,* exposed to cypermethrin (5.13 mg/l) for 96 h showing sever necrosis, cytoplasmic degeneration, lymphocytic infiltration and vacuolization of hepatic cells. Haematoxylin and eosin: x400.

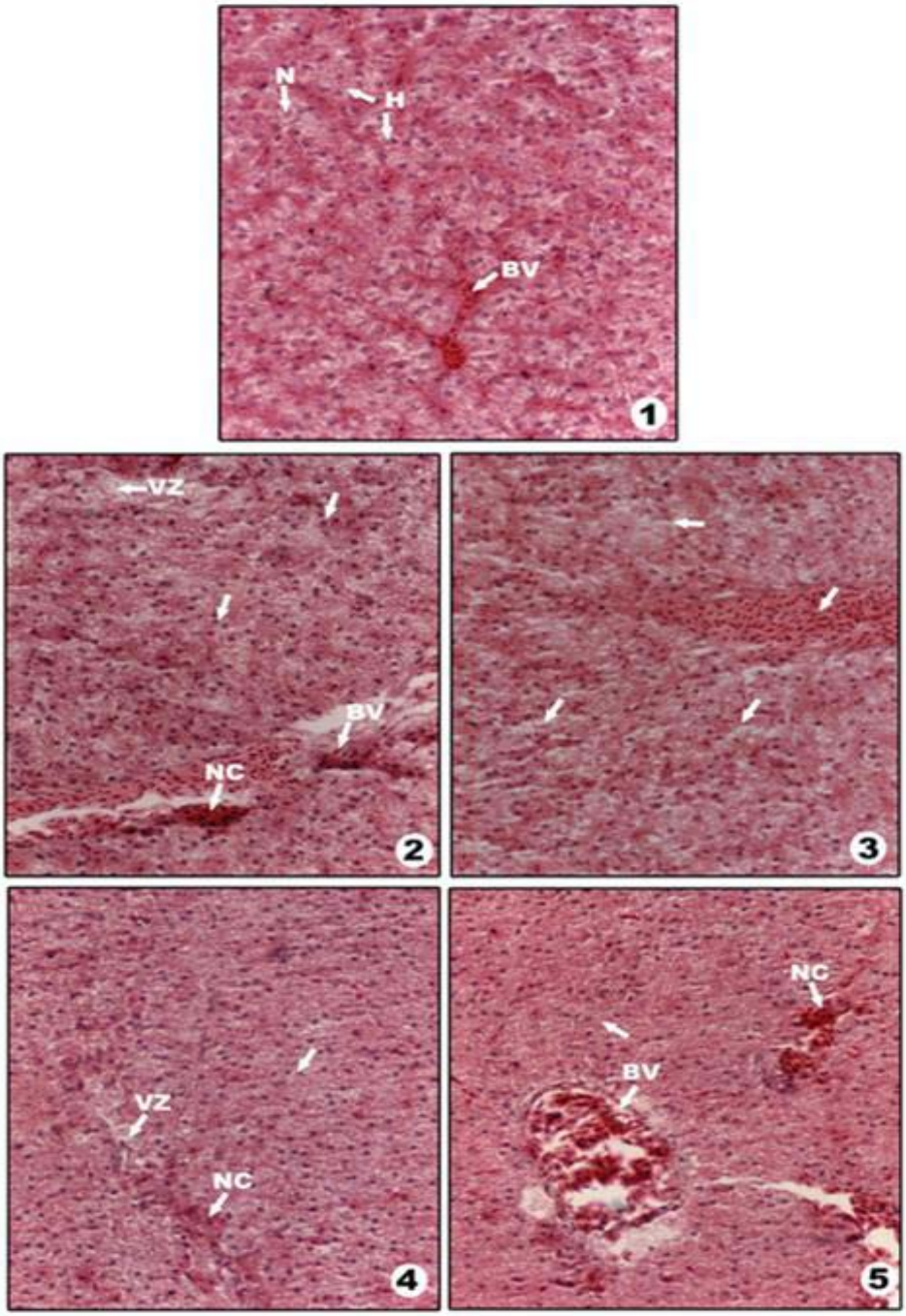

Plate 3: (1): Section of liver of control fish, *Cirrhinus mrigala* showing normal structure H=hepatocytes, N=ncleus, BV=blood vessel. Haematoxylin and eosin: x400; **(2 & 3)** Section of liver of fish, *Cirrhinus mrigala,* exposed to cypermethrin (1.02 mg/l) for 1 day showing slight necrosis (NC), damage of blood vessel (BV) and vacuolization (VZ). Haematoxylin and eosin: x400; **(4 & 5)** Section of liver of fish, *Cirrhinus mrigala,* exposed to cypermethrin (1.02 mg/l) for 7 day showing diffused necrosis (NC), cytoplasmic degeneration, sever damage of blood vessel (BV) and vacuolization of hepatic cells (VZ). Haematoxylin and eosin: 400.

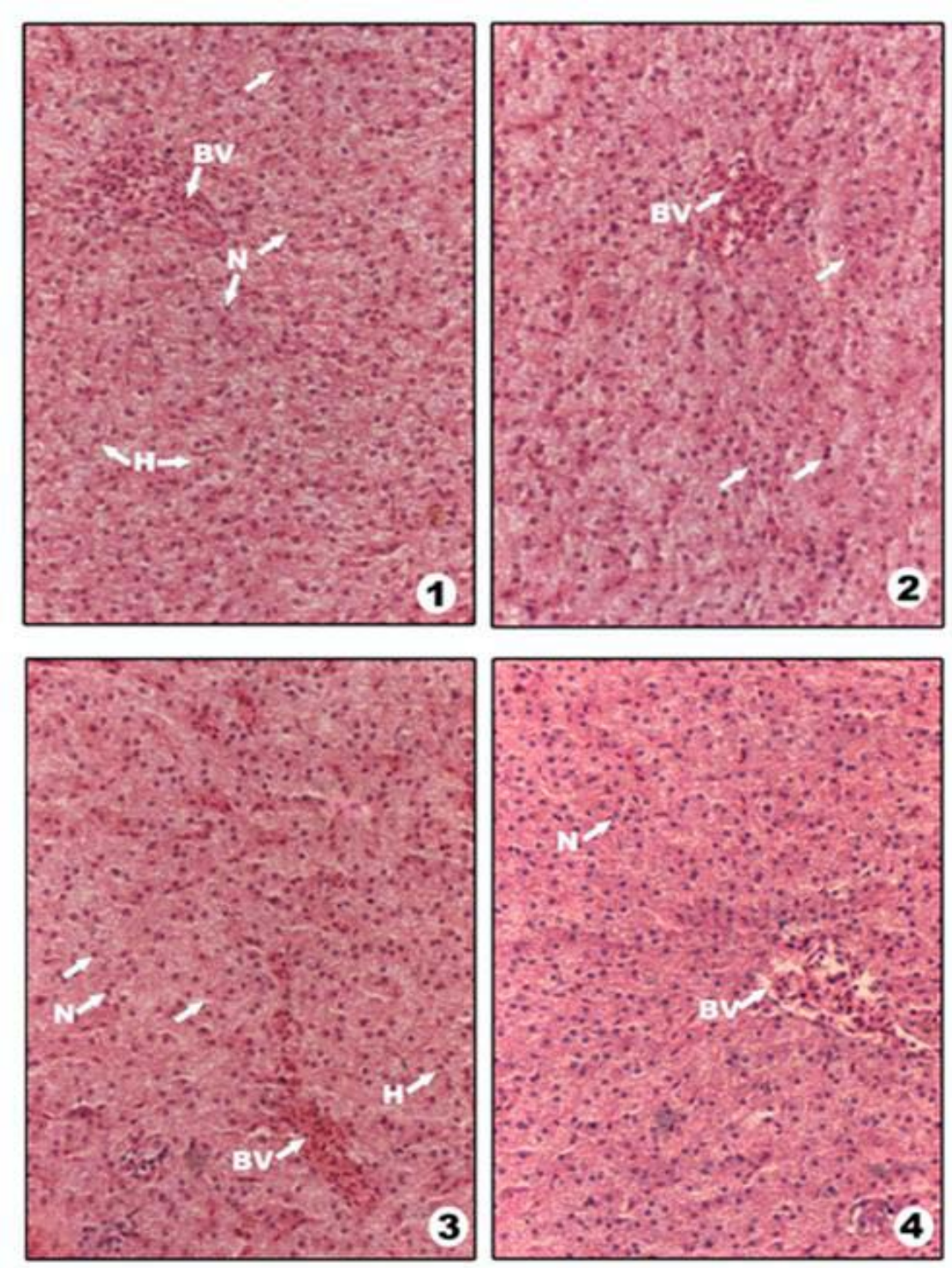

Plate 4: (1 & 2) Section of liver of fish, *Cirrhinus mrigala,* exposed to cypermethrin (1.02 mg/l) for 14 day showing less damage of hepatocytes, hepatic card and blood vessels. Haematoxylin and eosin: x400; **(3 & 4)** Section of liver of fish, *Cirrhinus mrigala,* exposed to cypermethrin (1.1 mg/l) for 21 day showing a recovery liver structure. Haematoxylin and eosin: x400.

of reorganisation in the structure of liver cords was observed. The nuclei appeared normal, with a very little degree of cytoplasmic vacuolisation (Plate 4: Figures 1 and 2). At 21 days of exposure, no significant changes were seen different from controls, except a slight degree of hyperchromatic condition of the nuclei (Plate 4: Figures 3 and 4).

DISCUSSION

Liver is involved in the metabolism of most toxicants, which can usually be detoxified, but many of them can be bioactivities and in turn becomes more toxic. The toxicology of liver is complicated by the variety of liver injuries caused. The liver has a high concentration of xenobiotic-metabolising enzymes, some of which activate the toxicants to induce lesions locally[15]. Toxicants induced changes in the liver of fishes can be regarded as an index for the identification of pollution stress on fishes[16]. In the present investigation, the appearance of degenerative changes in the liver of fish exposed to the lethal concentration of cypermethrin supports the metabolic disorders observed in it. The disarrayed liver cords, vacuolation in hepatic cells, dilated sinusoids, coagulation of blood cells, serves degree of nuclear atrophy followed by the shrinkage of hepatocytes and dissociation of laminar structure suggest that the depletion in its glycogen reserves (Plate 1). The pathological changes in liver due to insecticides have been reported by a number of workers[17,18,4]. These pathological changes may be associated with the accumulation of the pesticide. Mandal and Kulshrestha[19] reported histopathology of liver exposed to sumithion. The changes in the liver was characterised by necrosis, heptate cells lost their original shape, cell boundaries begin to rupture and disintegrate, which lead to the formation of multinucleated giant cells. Similar responses were also observed in the fish subjected to malathion treatment[20]. The necrosis of hepatocytes vacuolisation and swelling of liver cords were noticed by some workers in different fishes treated with various toxicants. The liver of blue gills treated with methoxychlor showed cell vacuolisation and swelling of liver cords. Other investigators in different fishes treated with various toxicants also noticed these changes. Vinod Ghanathay[21] reported vaculation of connective tissue and grouping of hepatocytes culminating in focal necrosis, rupture of cell membrane, etc., in *Channa punctatus* on exposure to benzenehexachloride (BHC). Battacharya[22] *reported swollen liver cells with irregular surface in Clarius batrachus exposed to various c*oncentration of endrin. The cells were either binucleated or the nucleus was enlarged. Degenerative changes were shown by rupture and vaculation of hepatic cells, sometimes with the appearance of inter cellular spaces indicating sever necrotic condition. The damage to liver was more in fishes at higher concentrations of cypermethrin but the damage at lower concentration was not significant. Similar changes were also observed in the fish *Orechromis niloticus* exposed to glyphosate[23].

The extent of liver damage observed in the present investigation indicates that chronic exposure always causes impairment to the architecture of the tissue. Since liver is involved in detoxification of pesticides[24]. It is susceptible to a greater degree of disruption in its structural organisation due to toxic stress.

Lipophilic aromatic hydrocarbon converted by reduction, oxidation, hydrolysis or conjugation to more water-soluble metabolites, facilitating their elimination from the body[18]. Primary and secondary metabolites were formed through G series of enzymatic reactions, consequences of metabolites production are conversion of toxic, hydrophobic molecules to more soluble compounds that may be readily excreted and reduction of toxic effects. Metabolism of some compounds can result in the formation of metabolites that are more toxic than toxic parent compounds and that may possess carcinogenic effect because of their binding to cellular macromolecules[8].

Oxidative metabolism of polyaromatic hydrocarbon is accomplished through highly electrophonic arene oxides, some or which may bind covalently to macromolecules such as DNA, RNA or proteins, and result in mutagenic or toxic effects[25]. Polyaromatic hydrocarbons (PAH) are active inducers of enzymatic activity in hepatic tissue of fish. Activity of the hepatic enzyme is altered for the metabolism of the PAH, but when the secondary metabolites are getting accumulated rather than excretion would attack on protein and macro-molecules and would cause mutation and cancer.

Results in this work showed severe necrosis, pyknosis and disintegration of hepatocyte cells were very much evident this test level. These responses can cause severe physics-metabolic dysfunction leading to death. Furthermore, recovery is not possible after longer period of exposure as evidenced by[26]. Fish mortalities observed in such situation may be related to complete hepatocyte degeneration, resulting in tumour formation and syncytium formation, and also indicative of its carcinogenic toxicity.

Mandal and Kulshrestha[19] reported changes in the liver of the fish exposed to 1 p.p.m. sumithion between 45 and 90 days of exposure. The pathology in the liver was characterised by necrosis, which progressively became more severe from 45th day to 90th day. The hepatic cells lost their original shape. On the 90th day, they observed that the cells were almost devoid of any cytoplasmic contents. From the 75th day onwards, the cell boundaries at places begin to rupture and disintegrate, which led to the formation of multinucleate gaint cells. Eller[27] after chronic treatment of cut throat trout *Salmo clarki*, with endrin, reported cord disarray in liver and swollen binucleated hepatocytes. In some cells, he observed that the nuclei were enlarged and in others acidophilic pigmentation with eccentric nuclei were seen. Several reports on pesticide toxicity reveal that such changes in the liver of *Channa punctatus* exposed to endrin are also noticed[28].

Jayantha Rao[16] stated that the concentration of pesticide is more important in bringing the histological changes in liver of fish; hence, these changes could be used as a tool for assessing the toxic effects of the pesticides in aquatic environment. The differences in the degree of liver damages noticed in the concentrations of the pesticide in the present study may be due to its mode of action, accumulation, persistence and concentration.

The fish also have mild to moderate damages caused to the liver at day 1 and 7 on exposure to the sub-lethal concentration, as evident by the slight disarray of liver lobes, swellings of hepatocytes, hypertrophy and pyknotic nuclei of liver of the vital organs of the fish, *Cirrhinus mrigala*. But the fish slowly developed good resistance to the influx of lower doses of cypermethrin as in organs on days 14 and 21. It appears that these animals vigour in order to detoxify or eliminate the accumulated cypermethrin. The recovery from the suppression of oxidative metabolism and the domination of protein synthesis might have facilitated them to activate the structural reorganisation. The liver of the fish does not show the diversity of pathology seen in higher animals probably as a result of the lack of kupffer cells in the liver sinusoids. However, it is susceptible to a number of toxic and metabolic differences. Acute and extensive necrosis of liver cells may occur in toxic condition[23,29,30].

It is concluded that more or less similar pathological changes are induced in the liver of different fishes by different biocides but the extent of damage varies depending upon the dose of biocide, duration damage varies depending upon the dose of biocide, duration of exposure, toxicity of biocide and susceptibility of fish.

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