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Evaluation of Acute Toxicity and Histopathological Examination of the Freshwater Fish *Labeo rohita*

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The current research looks at the acute toxicity of fungicides, like carbendazim and mancozeb on the freshwater fish *Labeo rohita*. For 96 hr, the fish were exposed to various concentrations of carbendazim and mancozeb. The 96 hr LC_{50} value of carbendazim and mancozeb was found to be 0.076 g/L. Several histopathological abnormalities in fish organs were found, which will be beneficial in determining the harmful impact of carbendazim and mancozeb. Microscopically, histopathological alterations in the gills, liver and kidneys revealed growing and decreasing damage in tissues treated with carbendazim and mancozeb in comparison to that of control groups that had normal architecture. The study gives an awareness of the usage of fungicides, such as carbendazim and mancozeb in the future as the usage may cause histopathological issues.

KEYWORDS

Carbendazim, Mancozeb, Fungicide, Histopathological alterations

1. INTRODUCTION

Rohu (*Labeo rohita*) is the most important among the three Indian major carp species used in carp polyculture systems. *Labeo rohita* is a prime cultured and important staple freshwater fish generally found in rivers, ponds and reservoirs; it is popular in Thailand, Bangladesh, Northern India and Pakistan [1]. Satyavani investigated the effect of certain expired pesticides on *Labeo rohita* in acute exposure test [2]. Rohu is an eurythermal species and does not thrive in temperature below 14^{0}C. It is a fast growing species and can grow upto 35-45 cm in length and 700-800 gm in weight within one year under normal culture condition. When fed properly, the species attain maturity within 2 years. The diameter of eyes is 4-6 cm in length of the head, 1.5-2 diameters from end of the snout and 3 are apart. The inter-orbital space is flat. Dorsal profile is more convex than that of the abdomen, it is a little concave over the orbit. The greatest width of the head equals its length excluding the snout. The snout is obtuse and depressed, scarcely swollen but projects beyond the jaws. Lips are thick and fringed with a distinct inner fold above and below. The gill rakers are stiff and are half as long as the eye. There is a short and thin maxillary pair of barbel (Figure 1).

The use of pesticides has been recognized as part of agricultural practices throughout the world; pesticides have no doubt been a boon to human civilization in sustaining agriculture revolution but at the same time bared its ravaging face on humanity pushing it to a point of almost no return. Modern agriculture practices, despite their remarkable contribution to the enhancement of crop production, have at the same time, also widely polluted the aquatic environment. The pesticides when reaches aquatic environment may have impacts on non-target organisms, such as fish. World Health Organization reported that roughly 3 million cases of pesticide poisoning occur annually, resulting in 220,000 deaths worldwide. Many of these chemicals are mutagenic and lead to the development of cancers. The extensive use of pesticides, insecticides, herbicides and fungicides is being promoted by Government of India to enhance crop production to meet the demand of the growing population [3]. There are 234 kinds of pesticides used in India of which 24 are used widely. The environmental conditions, like temperature, pH and dissolved oxygen play major role in increasing pesticide toxicity in the presence of residual molecules. The accumulation of pesticides produces some physiological, biochemical and morphological responses in freshwater fauna by influencing several activities of metabolites and enzymes [4]. Bioaccumulation of pesticides is a threat to the long-

Figure 1. *Labeo rohita*

term survival of fish by disrupting ecological relationships between organisms, leading to loss of biodiversity. Long-term exposure to pesticides induces physiological disturbance, behavioural changes, histopathological damage, haematological alterations, biochemical changes, immune suppression, hormone disruption, diminished intelligence, reproductive abnormalities and cancer. Extensive use of pesticides in agro-ecosystem has resulted in global contamination of the environment. Only 0.1% of the applied pesticides reach the target pests and the remaining 99.9% find their way to different components of the environment [5].

Chlorpyrifos is widely used organophosphate pesticide and it is the second largest selling pesticide in India. It is used for more than a decade to control pests on cotton, paddy field, pasture and vegetable crops [6]. Carbendazim is the internationally approved standard fungicide with common name methyl 2-benzimidazole carbamate. It is a systematically active benzimidazole fungicide that inhibits the synthesis of beta tubulin. The chief non-target species that are very much affected by these pesticides are fishes which are economically important and contribute much to our nation's protein value [7]. Carbendazim inhibits the microtubule polymerization in fungal and mammalian cells by acting with the β-tubulin causing a disruption of microtubule assembly, which leads to impaired segregation of chromosomes during cell division. Humans are exposed to carbendazim either directly (oral ingestion, inhalation and dermal contact) or indirectly (ingestion of contaminated water, food items and occupational exposure). Mancozeb, a fungicide belonging to ethylene bis-dithiocarbamate group, is used to protect and to give an attracting aspect to vegetables. Despite its low acute toxicity, mancozeb has been shown to cause detrimental effects both on humans' and experimental animals' organ systems. Mancozeb [1,2-ethane diyl-bis-[carba-modithioate] manganese is a fungicide, subclass of carbamate pesticides called dithiocarbamates. They have a similar action to carbamate insecticides they affect the nervous system through their main metabolite, carbon disulphide and ethylene thiourea.

Mancozeb is marketed by trade names dithane, manzeb, nemispot and manzane. It is used to protect many fruits, nuts and field crops from a wide spectrum of fungal diseases. Mancozeb exposure was strongly associated with increased incidence of thyroid disease in pesticide applicators that can affect nervous system. Symptoms of exposure range from fatigue, headache, blurred vision to nausea. At high doses, slurred speech, confusion or slowed heartbeat are common. In one study a worker developed rash as well as inflammation of eyelid after handling seedlings which had been treated with mancozeb. In another study prolonged low-level exposure to mancozeb affected several aspects of immune functioning and association existed between mancozeb and neural tube defects. Changes in body colour, behavioural changes, such as irregular swimming movements, loss of equilibrium, restlessness and excess secretion of mucous suggest that *L. rohita* has undergone chemical stress when exposed to pesticide and this could be taken as indicator of aquatic pollution [8].

Histopathological investigations have long been recognized to be reliable biomarkers of stress in fish. Histopathological studies allow examining specific target organs, including gills, kidney, liver, that are responsible for vital functions, such as respiration, excretion and accumulation and biotransformation of xenobiotics in fish. Since gills and gastrointestinal tracts in fishes are considered the main passage for entrance of pollutants to internal body organs, like liver and kidney through the blood. Gills are the target of waterborne pollutants due to their constant contact with external environment. Gills are the first organs which come in contact with environmental pollutants. Paradoxically, they are highly

Figure 2. *L. rohita grown in laboratory*

Figure 3. *Acute toxicity test at different concentrations (T1: 0.12 mg, T2: 0.15 mg and T3: 0.18 mg)*

vulnerable to toxic chemicals because firstly, their large surface area facilitates greater toxicant interaction and absorption and secondly, their detoxification system is not as robust as that of liver [4]. Carbendazim and mancozeb are fungicides of major concern due to suspected damage to fish organs. The objective of this study was to investigate the acute toxicity of fungicides, such as carbendazim and mancozeb on *Labeo rohita* and to conduct histopathology examinations on control and fungicide-treated fish.

2. MATERIAL AND METHOD

2.1 Sample collection

Freshwater healthy fishes, *L. rohita*, were chosen for the experiment and were gathered from the KRP dam in Krishnagiri and were reared in biotechnology laboratory at Arignar Anna College for further research (Figure 2).

2.2 Acute toxicity test

Toxicity tests were conducted in accordance with standard methods. Stock solution of carbendazim and mancozeb with a concentration of 1 mL/L (equivalent to 1 ppt) was prepared in distilled water and different dilutions were prepared by adding required amount of distilled water (Figure 3). After the addition of toxicant into the test tank with 10 L of water having twenty fishes, mortality was recorded after 24, 48, 72 and 96 hr. Five replicates were maintained simultaneously. The concentration at 50% survival/mortality occurred in carbendazim and mancozeb and these treated fishes were taken as median lethal concentration (LC_{50}) for 96 hr. Experiment was conducted in water temperature of 27 ± 1^0C, DO of 4.2 mL and pH 7.7. The behavioural changes of *L. rohita* were observed in lethal concentrations of carbendazim and mancozeb during bioassay test.

2.2 Histopathological studies

Surviving fishes were randomly selected after exposure period for histopathological examination. Organs, like gill, liver and kidney were isolated from normal and experimental fish. Physiological saline solution (0.85% NaCl) was used to rinse and clean the tissues. They were fixed in aqueous Bouin's solution for 48 hr processed through graded series of alcohols, cleaned in xylene and embedded in paraffin wax. Gills alone were processed by double embedding technique. Sections of 6 μ thickness were cut, stained with ehrlich hematoxylin/eosin (dissolved in 70% alcohol) and mounted on Canada balsam. Sections were observed under digital microscope.

3. RESULT AND DISCUSSION

3.1 Behavioural manifestation

The behavioural changes in the pesticide (carbendazim) treated fishes, such as low food intake, enormous mucus, fast jerky movement were observed which may be due to stress given to fish by pesticides. Vutukuru, has also observed similar behavioural changes in *Labeo rohita* exposed to higher concentrations of potassium dichromate [9]. The secretion of mucus layer over the gill lamellae has been observed during heavier stress [10]. The change in body colour and behaviour, such as irregular swimming movements, loss of equilibrium, restlessness and excess secretion of mucous suggest that *L. rohita* has undergone chemical stress when exposed to pesticides and this could be taken as pollution indicator of aquatic ecosystem.

3.2 Acute toxicity

The acute toxicity result was recorded as 10-90% mortality during the experiment; no mortality was found in control fishes at 24, 48, 72 and 96 hr. The LC_{10} values of pesticides at different concentrations of 0.12 mg,

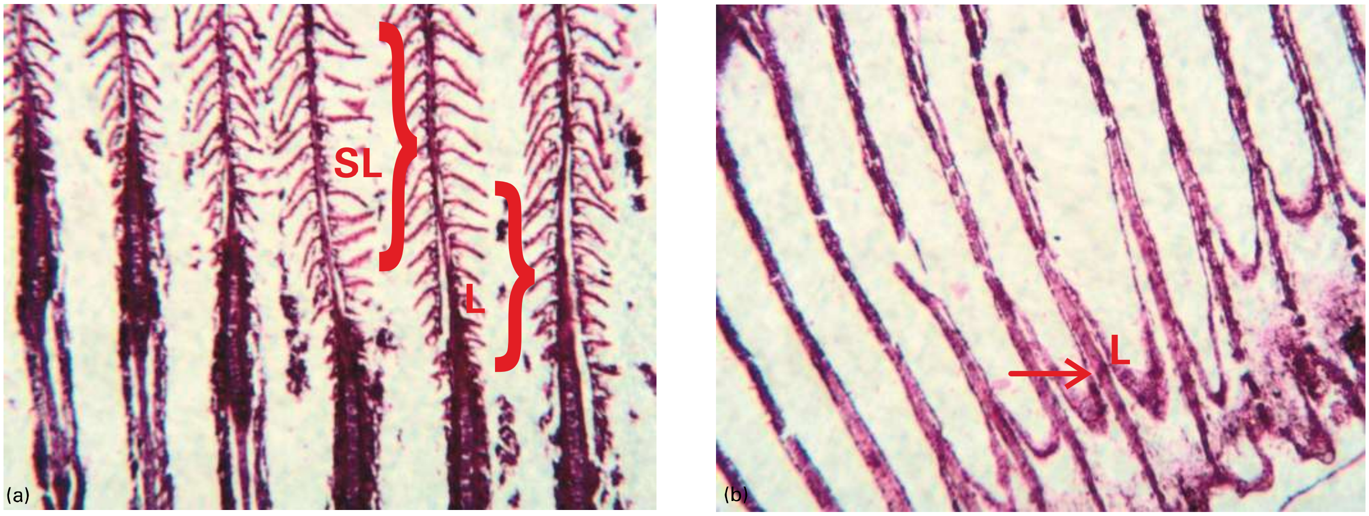

Figure 4. *Histopathological study on gills of (a) control fish and (b) carbendazim and manacozeb treated fish (SL- secondary lamellae, L- lamellae)*

0.15 mg, 0.18 mg and 3 L for 24, 48, 72 and 96 hr. Similarly, LC_{50} values were observed for 0.23 mg and 0.24 mg for 24 , 48, 72 and 96 hr. 100% mortality was also observed.

3.3 Histopathology studies

3.3.1 Gills: The gills are important organs for respiration, osmoregulation, acid base balance and nitrogenous waste excretion. It is possible that the damage to the gills should be direct result of salts, heavy metals, pesticides, sewage and fertilizers which are exposed in water [11]. Gills are among most valuable structures of the teleost fish because of the external location and intimate contact with water. In the present histopathological study, gills of control group fish were observed with normal developed respiratory epithelium; they are often observed in cases where phase transformation front moves quickly, leaving behind two solid products as in rapid cooling of eutectoid systems (Figure 4a). In treated fishes, gills were in atrophy of secondary lamellae with fusion. Atrophy of epithelial cells with lymphatic infiltrate in interstitial cells and hypoplasia of cartilages were observed (Figure 4b). Similar results were obtained by Haldi on *Tilapia zillaii* exposed to aluminium; the gills showed typical structural organization of lamellae in untreated fishes but when exposed to aluminium resulted in several forms of histopathological changes, such as cellular hypertrophy or hyperplasia in epithelial layer of primary filaments and fusion of secondary lamellae [12]. The lifting of lamellar epithelium is another histological change observed, probably stimulated by incidents of severe oedema [13]. Similar observations were also seen by other workers [14-16].

On other hand, fusion of secondary lamella decreased inter-lamellar distance, resulting in reduction in diffusion conductance of the gills to respiratory gases [17]. Karlsson mentioned that increase in cellular layers of lamellar epithelium may be due to an increase in the number of mitotic divisions of the lamellar epithelium [18]. Kantham suggested that hyperplasia in gills may increase epithelial thickness so as to retard into the blood stream [19]. Lamellar telangiectasia resulted from rupture of pillar cells and capillaries under effect of heavy metals pollution and leads to an accumulation of erythrocytes in the distal portion of the secondary lamellae [20]. These histopathological changes of the gills likely resulted in hypoxia and respiratory failure problems with ionic and acid-base balance [21]. Also, the pathological changes in the chloride cells may indicate osmoregulatory dysfunction, which is main function of chloride cells [22].

3.3.2 Liver: The liver is made-up of hepatocytes that are not oriented into distinct lobules but arranged in branched laminate two cell thickness spate by sinusoids hepatocytes nucleus densely stained and exposure of *Labeo rohita* to carbendazim induced obvious histopathological changes in the liver. An intense migration of cells, such as red blood cells sinusoid to hepatic parenchyma is observed in the tissues of the fish which are exposed to carbendazim and mancozeb for longer duration [23]. In this present histopathological study, controlled fish showed normal hepatocytes alongwith sinusoids and bile duct and scattered kupler cells (Figure 5a). Treated fishes showed hydropic degeneration of hepatocytes with kupfer cells prominence sinusoid in congestion with RBCs and absence of bile ducts was

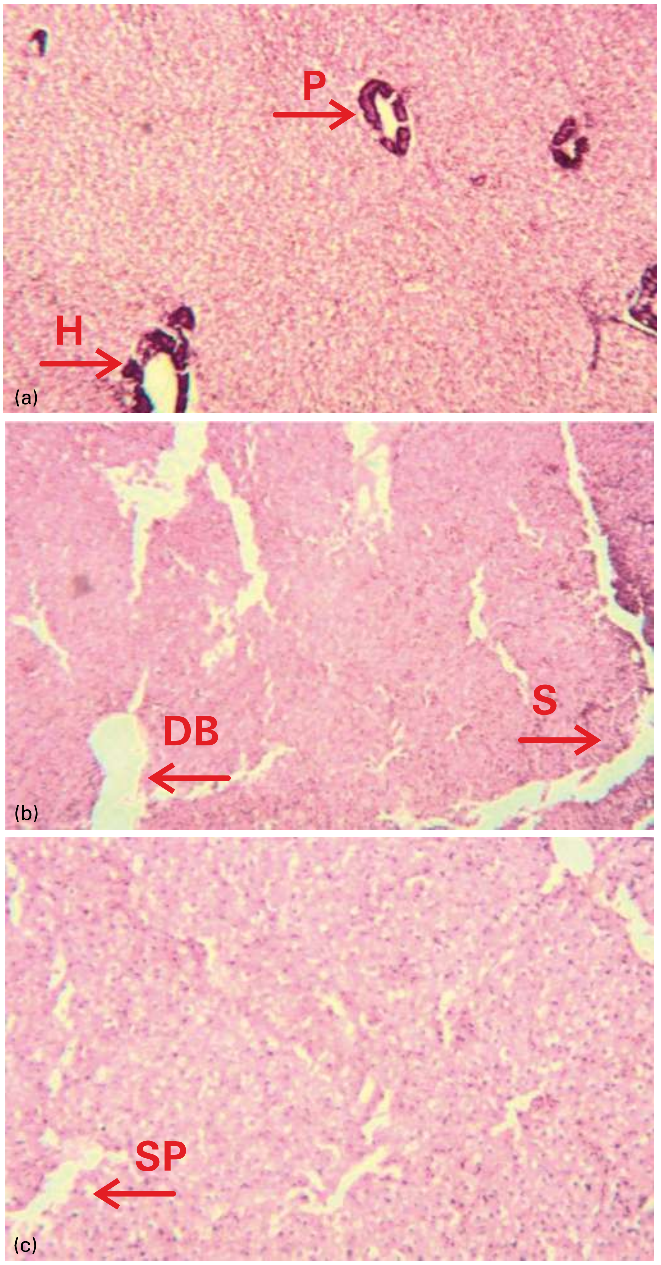

Figure 5. *Histopathology of (a) control fish and (b,c) treated liver sample (P- phagocyte accumulation, S- sinusoids, H- hepatocytes, DB- damaged blood vessels, Nu- enlarged nucleus of hepatocytes)*

observed (Figure 5b,c). The results correlated with study conducted by Sridhar of histopathological studies on *Cyprinus carpio* liver tissue exposed to carbendazim [24]. In liver section of normal *Cyprinus carpio,* hepatocytes form a cord like pattern. These cords are arranged around tributaries of the hepatic vein; the liver cells are larger in size, polygonal in shape with homogeneous eosinophilic cytoplasm and centrally located nuclei. Similar changes were also observed in the liver after exposure to various contaminants.

Melano-macrophage aggregation at the site of blood congestion as these develop in association with chronic inflammatory lesions. Hypertrophy is generally characterized by increase in cellular size. Exposure to compounds that induce proliferation of the endoplasmic reticulum membranes can be regarded as an example of hypertrophy [15]. The vacuolization of hepatocytes might indicate an imbalance between rate of synthesis of substances in the parenchymal cells and rate of their release into systemic circulation [25]. Cellular degeneration and necrosis may be due to accumulative effect of metals in hepatic tissue. Fish commonly accumulate greater concentrations of elements in liver than flesh and gills [26]. Tilak reported acute toxicity of phenol in liver of *Catla catla*, sinusoids became enlarged and contained numerous blood cells, vacuolated and atrophic areas were visible [27]. In kidney, extravasations and necrosis of the nephron were reported. In severe cases the kidney lost its affinity towards hematoxylin-eosin staining to a considerable degree with some parts of the structure obliterated.

3.3.3 Muscle: Histopathological changes have been widely used in evaluating the health of fish exposed to contamination in the laboratory and filed studies on the exposure to carbendazim for 1 day showed mild degenerative changes in the muscle of *Labeo rohita* [28]. In this histopathological study, control fishes consist of compactly packed muscle fibers with definite inter-muscular spaces, there was no splitting in muscle fibers and inter-muscular spacers appeared to be filled with viscous fluid. Round to spindle fiber shaped nucleus was found distributed all over the bundle length (Figure 6a). In treated test, fishes showed fragmentation of muscles with thin fiberal internalization of nuclei, increased fibroblast lymphoblast in between fibers (Figure 6b).

This result correlated with similar study of Lakshmaiah [29]. They worked on exposure of CSTP for period of 1 day, mild changes were observed in the structure of the muscle of fish *Cyprinus carpio*; they exhibited longitudinal splitting with cellular degeneration. Muscle fibers exhibited longitudinal splitting with pyknotic nuclei on exposure for a period of 30 days; further degeneration in the structure of the muscle was observed. A study on muscle showed slight structural reorganization of muscle structure of fish observed at day 30 of exposure; CSTP gives support to some extent that helps fish to resist sub-lethal stress and repair damage caused to muscle by enhancing the protein synthetic potential associated activities of the cell. The histopathological responses of fish in present study reveal the degree of damage caused by this pesticide to the muscle tissues

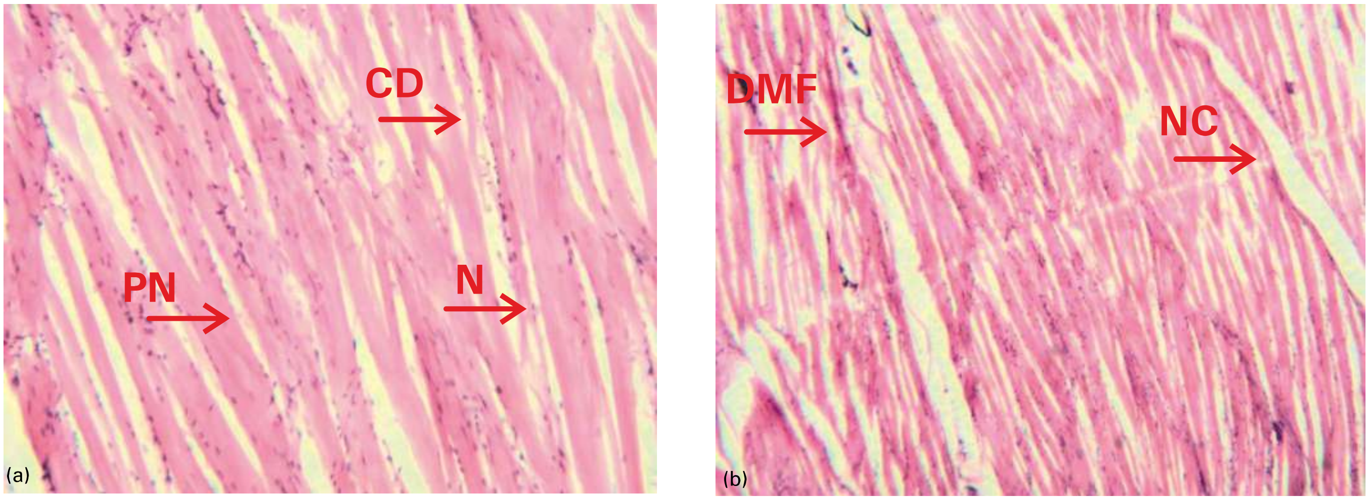

Figure 6. *Histopathology of (a) control muscle sample and (b) treated muscle sample (PN- pycnotic nucleus, N- nucleus, CD- cellular degeneration, DMF- degeneration of muscle fibers, NC- necrotic changes)*

of the fish. The extent of damage caused by phorate to muscle of fish and degenerative changes that occurred in muscle were progressive over period of exposure to CSTP, suggesting that the histopathological responses depend on concentration of pesticides and also on the length of fish exposure period to pesticides [29].

4. CONCLUSION

The present study showed that histopathological biomarkers of toxicity in fish organs are useful indicator of environmental pollution. The organ damage in the experimental fish was due to direct toxicity of carbendazim and mancozeb on the gills, liver and kidney. Hence it is concluded that the minimum use of fungicides (carbendazim and mancozeb) for agriculture by farmers would decrease the effect of damage caused to living organisms in future. This study also provides awareness regarding the use of these fungicides to farmers.

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