Effect of subleathal concentrtion of imidacloprid on the histology of heart, liver and kidney in *Labeo rohitr*

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Abstract: Randomly selected 500 fingerlings of *Labeo rohita* were collected from fish seed hatchery Muzaffar Garh. Six groups of 40 fish each were exposed to subleathal concentration of Imidacloprid (120 mg L^{-1}) under short-term (2, 4 and 8 days) and long term (16, 32 and 64 days) conditions to assess the effect of this most extensively used insecticide on the heart, liver and kidney of economically most important fresh water fish, *L. rohita*, in Pakistan. Separate control groups were used for each Imidacloprid treatment. No drastic histopathological changes were observed in heart structure following short and long term treatment with Imidacloprid. Exposure of liver to Imidacloprid resulted in severe degenerative changes, which were directly related to exposure time. Wrinkling of hepatocyte cell membrane, hepatocyte necrosis and degeneration, dislocation of nucleus, dilation of blood sinusoid and pycnosis of hepatic nuclei were the visible changes in liver histology in various experimental treatments. Comparison of treated and untreated kidney of *L. rohita* revealed wide Bowman's space, renal tubular lumen enlargement, necrosis and inflammation of cells. Our results are the pioneer report indicating that imidacloprid can act as strong toxic agent for *L. rohita*.

Keywords: Imidacloprid, *Labeo rohita*, histopathology, liver, kidney, heart.

INTRODUCTION

It is estimated that chemical insecticides preserve twenty percent of annual crop yield (Blacquiere *et al*., 2012), making them crucial for sustaining global food supplies. However, the widespread use of pesticides in agriculture resulted in a series of toxicological and environmental problems, have received extensive concerns (Li and Randak, 2009; Qadir *et al*., 2014). Pesticides are the pollutants increasingly present in the surrounding environment, which are often persistent and can be bioaccumulated through the biological chains such as soil-plant-food or water-aquatic organism-food (Preston, 2002; Qadir *et al*., 2015).

Imidacloprid is one of the major representatives of the new generation of neonicotinoid insecticides. It is a nicotine derived compound (neonicotinoid) with a large potential distribution due to its agonistic action on insect acetylcholine receptors and its selective toxicity to insects over vertebrates (Tomizawa and Casida, 2003) Nicotinoid insecticides are synthetically derived from nicotine, an alkaloid compound, which is found in the leaves of many plants including tobacco. Imidacloprid belongs to chloronicotinyl nitroguanidine chemical family (Bhardwaj *et al.,* 2010). The International Union of Pure and Applied Chemistry (IUPAC) named it as 1-(6-chloro-3-pyridylmethyl)-N-nitroimidazolidin-2-ylideneamine (Watanabe *et al.,* 2007),

Heart, liver and kidney are vital organs of body responsible to maintain the homeostasis. Liver is the center of metabolism and detoxification while kidney is involved in removal of the waste chemicals from body and selective reabsorbtion and heart is responsible to maintain the blood pressure, which is involved in the transportation of materials (Iqbal *et al*., 2005). Histopathology is considered as an ultimate tool to find out the effect of pollutants like Imidacloprid on fish tissue, because these insecticides interfere with the normal physiology of the animal (Atamanalp *et al*., 2008; Pathan, *et al*., 2009; Bhardwaj *et al.,* 2010; Qadir *et al.,* 2014)

Present study was conducted to evaluate the effect of subleathal concentration of most commonly used insecticide in Pakistan, Imidacloprid, on the histology of vital organs of commercially most important fish, *L. rohita*, under short and long term experimental conditions. The histological changes observed in the vital organs indicate that Imidacloprid is a toxicant for *Labeo rohita* as it severely affected the histology of liver and kidney and it has not only affected the normal fish histology but can also indirectly effect the fish consumers in food web.

MATERIALS AND METHODS

Randomly selected 500 fingerlings of freshwater Cyprinid fish Rahu *(Labeo rohita)* of both sex (body length 4.2-16.1cm and body weight 0.83-57.79g) were purchased from the fish seed hatchery Muzaffar Garh and transported to the fisheries laboratory at Bio Park, Bahauddin Zakariya University Multan. Fish were acclimated to laboratory conditions for two weeks in fiberglass containers with recirculation-aerated system (RAS). All experiments were carried out in semi-static

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conditions with water renewal after every 24h. Temperature (25.6±2.5^{o}C), pH (7.2±0.31) and oxygen concentration (7.8±0.45) mgL^{-1} in water were maintained throughout the experimental duration following Umer *et al.* (2011).

Experimental design

Experimental design had short and long-term phases. During short term experiments, 3 treatment groups (each having 40 fish) were exposed to sub lethal concentration of 120mg L-^{1} Imidacloprid for 2, 4 and 8 days respectively while in long term experiments, fish were exposed to above mentioned dose for 16, 32 and 64 days. Separate control groups were used for each treatment. All fish were fed with ordinary fish diet used in fish farms (24% Protein). All the experimental procedures and fish handling protocols were approved by ethical committee of Zoology Department at Bahauddin Zakariya University, Multan.

Histological studies

At the end of short and long term experimental phase's heart, liver and kidney were surgically removed from each treated and untreated fish for histopathological study. Tissues were sliced and fixed in fixative solution (containing ethanol, formaldehyde and glacial acetic acid in 60:30:10 ratio) for 5h at room temperature. Tissues were dehydrated in series of ethanol grades, cleared in clove oil and transferred in a mixture of molten paraffin and benzole (1:1) for 20min at 60^{o}C followed by paraplast phase twice for 12h at 60^{o}C to complete the process of block formation. The sections were cut out of paraffin block at 6µm by using Reichert microtome. Sections were affixed to pre cleaned albumenized glass slides, deparaffinized at 60^{o}C and rehydrated in descending grades of ethanol (100, 90, 70, 50 and 30% each for 2-5 min), washed in tap water and stained in hematoxylin, dipped in tap water for 10 minutes in order to stain cell nucleus with hematoxylin. Slides were dehydrated in ascending grades of ethanol (30, 50, 70 and 90% each for 2-5) and counter stained with eosin (cytoplasm staining) and cleared in xylene for 10 minutes. Photographs of selected areas in slides were taken by Nikon Optiphoto (Olympus, New York) research microscope equipped with digital camera (Sony, Japan) and were compared between treated and untreated fish groups.

RESULTS

Short term imidacloprid exposure

Effect on heart

No drastic histopathological changes observed in heart following short-term treatment with Imidacloprid. The heart of untreated *L. rohita* was composed of mass of cardiomayocytes that were regularly arranged. Interstitial connective tissue was evenly distributed among cardiomayocytes (fig. 1A) In 2, 4 and 8 days imidacloprid treated *L. rohita* cardiomayocytes showed healthy cardiomayocytes with scattered fibrocytes (fig. 1 B-D).

Effect on liver

The liver of *L. rohita* is made up of homogenous mass of hepatic cells containing large, round, centrally located nuclei in cytoplasm. Normally hepatocytes are polyhedral cell having different size and shapes. Channel like sinusoids separates the hepatic tissue in to lobules and cords. Hepatocyte encloses bile canaliculi that open in to sinusoid (fig. 2A).

Exposure of liver to sub lethal concentration of Imidacloprid (120mg L^{-1}) for 2, 4 and 8 days showed sever degenerative changes directly related to exposure time. Liver morphology was indefinite in all cases and compact liver was not observed in any treatment. Major histopathalogical changes were observed in liver following short-term treatment of Imidacloprid included wrinkling of hepatocyte cell membrane, hepatocytes necrosis and degeneration, dislocation of nucleus, dilation of blood sinusoid and pycnosis of hepatic nuclei.

In 2 days Imidacloprid exposed experiment, normal shape of hepatocytes was lost, cell margins were wrinkled and lobules were disorganized. Many irregular lesions were visible due to degeneration of cells. Specific cord pattern was lost and hepatocyte arrangement was also disturbed. Cloudy swelling in the hepatocytes was visible. Karyorheixis and karyolysis were seen in many of the liver cells (fig. 2B).

Exposure of fish to Imidacloprid for four days resulted in hepatocytic necrosis and degeneration with swelling of nucleus (fig. 2C). Following 8 days treatment of Imidacloprid, *L. rohita's* liver showed karyorhexis (destructive fragmentation of the nucleus of a dying cell) of hepatocyte nucleus. Intensive degeneration of hepatocyte and dislocation of nucleus were also visible. Moreover cell borders fused together and definite cord like pattern of hepatocytes in liver was missing (fig. 2D).

Effect on kidney

Histopathological analysis showed coarse morphological changes in kidney tissue of *L. rohita* upon exposure to 120mgL^{-1} of Imidacloprid. Mostly the degenerative changes were observed. Prolonged exposure period resulted in more severe degenerative changes accordingly. In control group, kidney section showed normal cuboidal cell in renal tubule with prominent nucleus and eosinophillic cytoplasm. Interestitium of the tubules is infiltered with hematopoitic tissues, which contain round to polygonal cells having nuclei (fig. 3A). Histological changes were evident in histopathological sections like wide Bowman's space, renal tubular lumen enlargement, necrosis and proliferation of inflammatory cell.

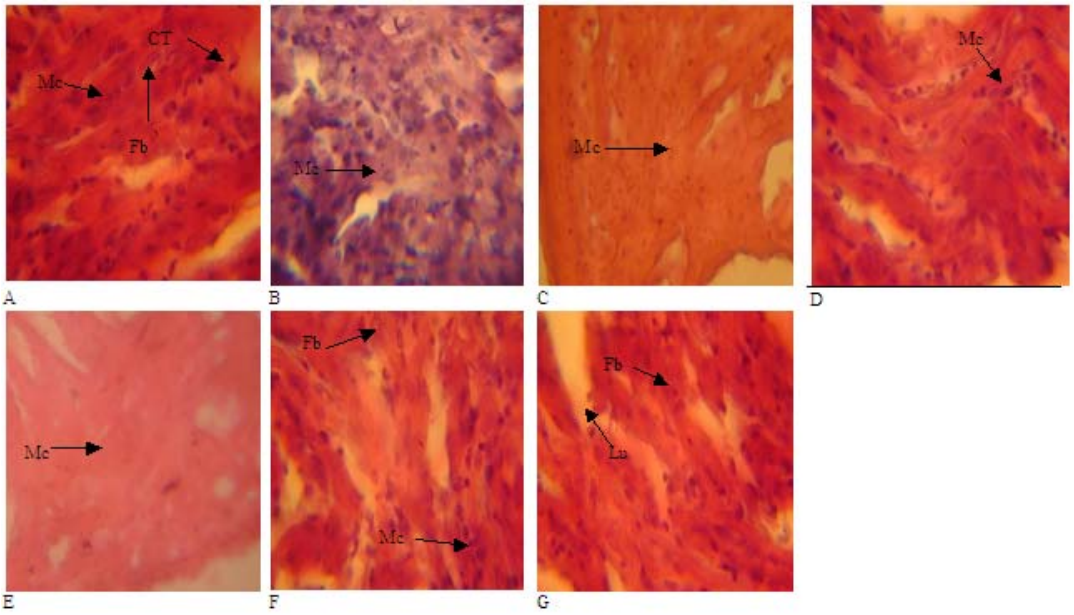

Fig. 1: Effect of Imidacloprid exposure on *Labeo rohita*'s heart under short-term experimental conditions. A is untreated heart, B, C and D are the groups exposed to 120mgL^{-1} imidacloprid. (Lu: luman, Fb: fibrocyte, Mc: mayocytes, CT: Interstial Connective tissue). A. Control heart with normal mayocytes (Mc), Interstial Connective tissue (CT) and fibrocyte (Fb) are present. B, C and D are heart section treated with imidacloprid for 2, 4 and 8 days respectively, normal mayocardium with healthy nucleus of mayocardiac cells show no adverse effect of pesticide on heart. E-G. Effect of Imidacloprid exposure on *Labeo rohita*'s heart under long-term experimental conditions. E, F and G are heart section exposed to imidacloprid for 16, 32 and 64 days respectively. Normal histology of heart was observed devoid of any abrupt change due to chemical exposure.

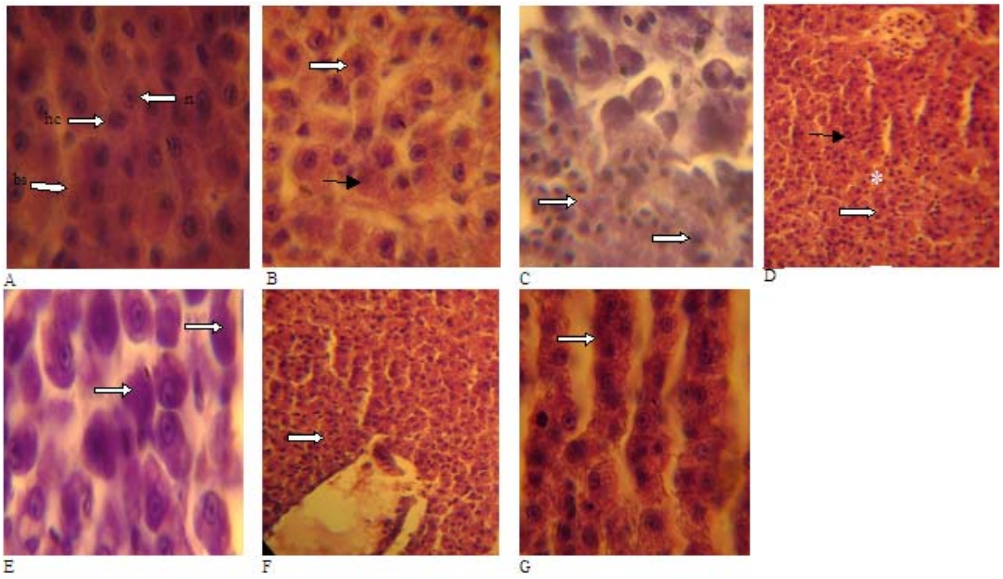

Fig. 2: Effect of Imidacloprid exposure on *Labeo rohita*'s liver under short-term experimental conditions. A is untreated liver slide showing healthy hepatocyte. B, C and D are the liver section exposed to Imidacloprid. (hc: Hepatocyte, bc: Bile canaliculi, bs: Blood sinusoid. n : Nucleus). B. 2 days exposure: Cloudy swelling of hepatocyte (white bold arrow), karyorhexis and karyolysis (narrow black arrow). C. 4 days exposure: Degeneration of hepatocyte with swelling of nucleus. D. 8 days exposure: Karyorhexis of hepatocytes, degeneration of cell nucleus (white bold arrow). Fusion of cell borders (narrow black arrow), Loss of cord like pattern of hepatocyte (*). E-G. Effect of Imidacloprid exposure on *Labeo rohita*'s liver under long-term experimental conditions. E. 16 days imidacloprid exposure: Eccentric position of hepatocyte neucleus. F. 32 days exposure: Karyohexis in hepatocyte. G. 64 days exposure: Vacuolar degeneration of Hepatocyte with widening of sinusoidal spaces.

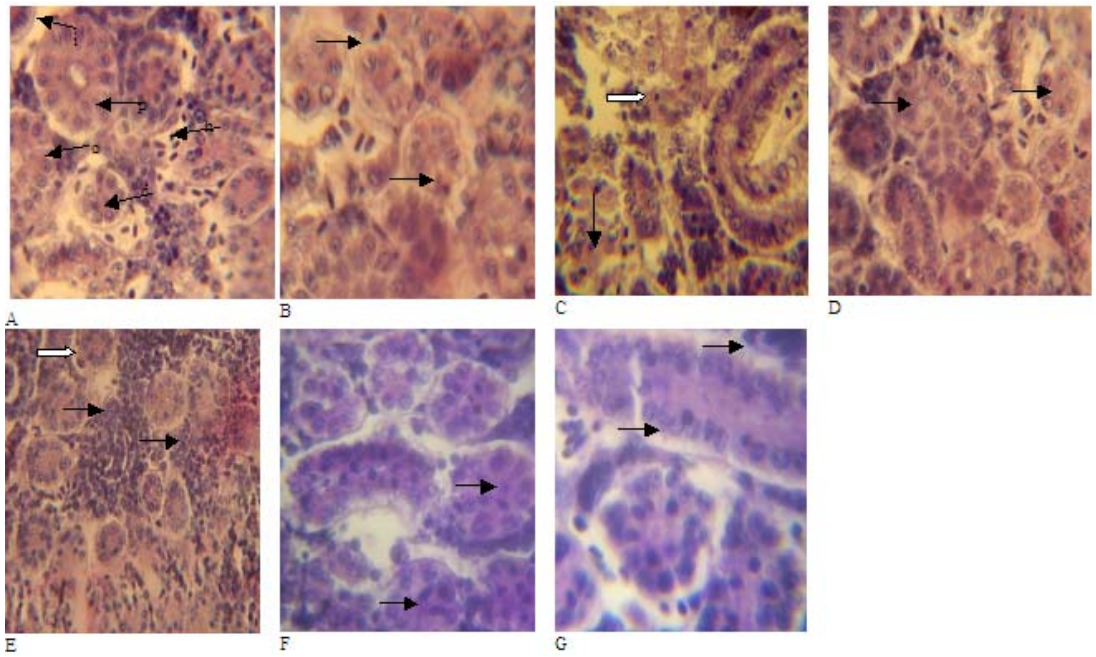

Fig. 3: Effect of Imidacloprid exposure on *Labeo rohita*'s kidney under short-term experimental conditions. A is untreated kidney slide; B, C, and D are the kidney section exposed to Imidacloprid. (d: distal tubule, p : proximal tubule, c: collecting duct, h: hematopoetic, I: Interstitial Space in kidney Parenchyma). In treated groups B. 2days exposure: Vacuolation of renal tubules. C. 4 days: Pyknosis of Nucleus in Renal Tubular cells (narrow black arrow). Focal Necrosis of renal tubular epithelial cells (bold white arrow). D. 8 days exposure: Swelling of renal tubular epithelial cell resulting in occlusion of tubular lumen. E-G. Effect of Imidacloprid exposure on *Labeo rohita*'s kidney under long-term experimental conditions. E. 16 days exposure: infiltration of leucocyte (bold white arrow) and increased hematopoietic tissues (narrow black arrow). F. 32 days exposure: Narrowing space in renal tubules, Condensation of epithelial cells of tubules. G. 64 days exposure: fusion of individual cell margins, fuzzy cell borders. Karyorrhexsis and karyolysis is evident at various stages in Renal Tubular Cells

A number of vacuolation in renal tubular cell were found in 2 days treatment group. Pycnosis in nucleus of renal tubular cells and coagulation necrosis of renal tubular epithelial cells were observed in 4 days exposed group. Vaccular degeneration and cloudy swelling were present in renal epithelial cells (fig. 3B-C). In 8 days exposed group swelling of renal tubular epithilial cell resulting in occlusion of tubular lumen was found (fig. 3D).

Long term imidacloprid exposure

Effect on heart

Following long-term treatment of Imidacloprid, no specific histopathological evidence was found in heart of *L. rohita*. In 16 and 32 days Imidacloprid exposed treatments exposed normal shapes of mayocytes persisted and no inflammation lesions were observed. Mayocyte fibre area was arranged. There were no signs of necrosis (fig. 1E-F). In 64 days pesticide treated heart of *L. rohita*, cardiac mayocyte having healthy nucleus and eosinophilic cytoplasm was visible. Intrestitial space was devoid of any type of inflammation (fig. 1G).

Effect on liver

Long-term treatment of *L. rohita* with Imidacloprid for 16, 32 and 64 days also provided evidences of liver damage directly related with chemical exposure time. 16 days exposure of Imidacloprid resulted in dislocation of hepatocyte nucleus, which found at eccentric position in hepatocytes (fig. 2E). Karyorhexis was visible in hepatocytes nucleus in *Labeo rohita* liver exposed for 32 days to pesticide. While vacuolar degeneration of hepatocytes with enlarged sinusoidal spaces were observed in 64 days exposure to Imidacloprid (fig. 2F-G).

Effect on kidney

Kidney of *Labeo rohita*, exposed to Imidacloprid for 16 days, showed infilteration of leucocyte that spread over large area (fig. 3E). Upon 32 days of pesticide exposure renal tubules of the kidney shrinked. Cell margins were fusioned in 64 days Imidacloprid treatment making cell borders fuzzy and Karyorrhexsis and karyolysis is also evident at various stages in Renal Tubular Cells (fig. 3F-G).

DISCUSSION

The natural freshwater contamination with pesticide residues has becoming very serious problem worldwide due to their toxic impacts on the aquatic fauna (Indirabai *et al*., 2010; Magar and Dube, 2013; Muhammad *et al*.,

2012; Naz and Javed, 2012). These pollutants are transported from industry, agriculture run off, municipal and manmade activities and causing alarmingly high levels of xenobiotic chemicals in the aquatic ecosystems (Brack *et al*., 2002; Diez *et al.*, 2002; Naeem *et al*., 2011; Qadir *et al*., 2014; Qadir *et al.*, 2015). Some chemicals are biologically degradable while others are non-degradable and sustain in the aquatic environments for a long period of time and their toxicological impact extended to non-target organism like fish and human. Therefore, there is a growing concern all over the world with respect to indiscriminate use of pesticides that become dangerous to aquatic organisms (Weber *et al*., 2010).

Somehow, the physiological and the metabolic activities of the organisms are damaged by the toxicants. Physiological studies alone are not much enough to satisfy the pathological conditions of tissues under chemical stress. Hence, it is important to have an insight into histological analysis as they act as biological markers to assess the toxicity (Jayantha *et al*., 1985; Tilak *et al*., 2001; Srivastava *et al*., 2008). Frequency and intensity of tissue lesions of a fish depend on the concentrations of pesticides and the length of the period exposed to toxins (Fanta *et al*., 2003).

Scanty research work has been reported regarding the changes in cardiac muscle histology upon sublethal exposure to pesticide. Mager and Dube (2013) had reported structural variations like congestion and atrophy in cardiac muscle of *Channa puncctatus* upon 96 hours sub lethal exposure to malathion. Das and Mukherjee (2000) had documented moderate thickness in pericardium and leucocytes infiltration in *labeo rohita* upon hexachlorocyclohexane exposure. In the present study, we did not observe any significant alteration in histological sections from heart of *labeo rohita* upon imidacloprid exposure under both short and long term conditions indicating that cardiac tissue is impermeable to this pesticide.

Liver is a vital organ that is most affected by the contaminants in the water due to its role in detoxification and biotransformation processes. Present study reveals that liver of *L. rohita* upon Imidacloprid exposure show degenerative changes like cloudy swelling of hepatocyte, karyorhexis, karyolysis, degeneration of hepatocyte with swelling of nucleus, degeneration of cell nucleus, fusion of cell borders, loss of cord like pattern of hepatocyte, eccentric position of hepatocyte nucleus and vacuolar degeneration of hepatocyte with widening of sinusoidal spaces in both short term and long term treatment group. Sarkar *et al*. (2005) has reported vacuolation, hyperplasia, hepatocytes disruption, focal necrosis and disorganization of hepatic canaliculi in *L. rohita* upon cypermethrin exposure. Anomalies like hepatocytes swelling and infiltration of blood vessels were seen in liver of *L. rohita* upon exposure to hexachlorocyclohexane (Das and Mukherjee, 2000) and due to fenvalerate (Susan *et al*., 2012).

The kidney performs an important function related to electrolyte and water balance and maintains a stable internal environment (homeostasis) by excreting nitrogenous waste product like ammonia, urea and creatinine (Gernhofer *et al*., 2001). In both treatment groups of present study, kidney of *labeo rohita* showed vacuolation of renal tubules, pyknosis of nucleus in renal tubular cells, renal tubular epithelial cell focal necrosis and swelling resulting in occlusion of tubular lumen, infiltration of leucocyte and increased hematopoietic tissues, narrowing space in renal tubules, condensation of epithelial cells of tubules, fusion of individual cell margins, fuzzy cell borders, Karyorrhexsis and karyolysis was evident indicating toxicity of Imidacloprid. Similar degenerative changes like necrosis of tubular epithelium and distention of tubules of kidney tissues in kidney of *L. rohita* has been reported upon exposure to hexachlorocyclohexane and fenvalerate (Das and Mukherjee, 2000; Susan *et al*., 2012).

In conclusion, the histological changes observed in the vital organs indicate that Imidacloprid is a toxicant for *Labeo rohita* as it severely affects the histology of liver and kidney and would have not only affected the normal fish physiology and metabolism but also has indirect effects on fish consumers in food web.

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