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Histopathological Alterations Induced by Diazinon in Rainbow trout (*Oncorhynchus mykiss*)

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ABSTRACT: Diazinon is a moderately persistent organ phosphorus pesticide largely used in agriculture. The purpose of this study was to evaluate the histopathological damages induced by diazinon in gills, kidney, spleen, and intestine tissues of rainbow trout, Oncorhynchus mykiss. The fishes were exposed to sub-lethal concentrations of diazinon (0.1 and 0.2 mg/L) for a period of 28 days. Tissues analyzed by a microscope were normal in the control group. The histological alterations in gills were characterized by epithelial hyperplasia, necrosis of epithelial filament and lamellar fusion, edema and curling of secondary lamellae. Glomerular lesions, shrinking of the glomerulus and enlargement of space inside Bowman's capsule, dwindling of the tubular lumen, degeneration and necrosis of renal tubules were observed in the kidney tissue of the exposed fish. The spleen tissue showed disorder in ellipsoids cellular and deposition hemosiderin in melanomacrophage centers. Exfoliate of mucosal epithelium, lymphocyte infiltration to lamina propria, reduction of the elastic properties and capillary bleeding were seen in intestine tissue of fish exposed to diazinon. In conclusion, these results indicate the existence of a direct relationship between the pollution of ecosystems by organ phosphorus pesticides such as diazinon and histopathological disorders in different tissues of exposed fishes.

Key word: Diazinon, Histopathology, Gill, kidney, Spleen, Intestine, Rainbow Trout

INTRODUCTION

Agriculture, as the largest consumer of freshwater and as a major cause of reduction of surface and groundwater resources through erosion and chemical runoff is directly correlated with the loss of water quality (Wauchope, 1978; Li *et al.,* 2012; Ashraf *et al.,* 2012; Mhadhbi and Boumaiza, 2012; Strohschoen *et al.,* 2013; Tajziehchi *et al.,* 2013; Garcia-Flores *et al.,* 2013; Etale and Drake, 2013; Ghaderi *et al.,* 2012; Miletic *et al.,* 2012). The processing industry associated to agriculture is also a significant source of organic pollution in most countries. Conventionally, in most countries, all types of agricultural practices and land use, including animal farming, are treated as non-point sources. The main characteristics of non-point sources are not simply determined or controlled directly and therefore, are difficult to regulate their impacts on ecosystem health (Garcia *et al.,* 2001; Koner *et al.,* 2012; Volmajer Valh *et al.,* 2012; Lopez-Pineiro *et al.,* 2012; Jiang *et al.,* 2011; Ma *et al.,* 2010; Kulluru *et al.,* 2010; Sengupta *et al.,* 2009). Non-point source pollutants are mainly transported overland and through the soil by runoff (Dubus *et al*., 2000). These pollutants ultimately find their way into groundwater, wetlands, rivers and lakes and, finally, to oceans in the form of sediment and chemical loads carried by rivers (U.S.A. EPA, 2003). Although monitoring the presence of pesticides in surface water and ground water are generally poor in much of the world and especially in developing countries, the effect of these pollutants on aquatic animals' health frequently was investigated (Chambers *et al*., 2002; Lam and Wu, 2003; Scott and Sloman, 2004; Box *et al*., 2007; Sun and Chen, 2008).

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Diazinon (O,O-diethyl O-(2-isopropyl-4-methyl-6-pyrimiinyl) phosphorothioate) is a moderately persistent organ phosphorus pesticide largely used in agriculture (Banaee *et al*., 2008; 2011). Several studies reported that some of surface waters and surrounding environments in Iran and other areas around the world were contaminated with organophosphate pesticides such as diazinon and its derivatives (Bagheri *et al*., 2000; Ghassempour *et al*., 2002; Lydy and Austin, 2004; Mast *et al*., 2007; Bouldin *et al*., 2007; Echols *et al*., 2008; Werimo *et al*., 2009; Arjmandi *et al*., 2010; Nasrabadi *et al.,* 2011; Ding *et al*., 2011; Hope, 2012). Previous studies evidenced that the highest levels of diazinon (about 1 mg/L water) were detected the day after spraying the pesticide and, there was no trace of diazinon two or three months after its application (Arjmandi *et al*., 2010; Shayeghi and Javadiane, 2001). However, diazinon has been reported to be a persistent pesticide in aquatic environment, because of its continual input into water (Wauchope, 1978; Bailey *et al*., 2000; U.S.A. EPA, 2005; Vryzas *et al*., 2009). Moreover, long-term exposures to sub-lethal diazinon concentrations have adverse effects on different biological and physiological aspects of aquatic organisms, especially fish (Dutta and Meijer, 2003; Khoshbavar-Rostami *et al*., 2006; Banaee, 2012).

In fish, diazinon is absorbed through gills, skin or alimentary ducts (Schlenk, 2005; Banaee *et al*., 2011; Banaee, 2012). Diazinon is metabolized to diazoxon due to oxidative desulfuration by cytochrome P450 enzymes and then, diazoxon is hydrolyzed by A and B esterase to water soluble metabolites and excreted into bile and urine (Keizer *et al*., 1995; Schlenk, 2005). Reactive oxygen species (ROS) produced during the diazinon detoxification process in liver tissue may react with vital macromolecules such as lipid, protein, carbohydrate and nucleic acid and result in oxidative damage to aquatic organisms (Üner *et al*., 2006). ROS derived damage to natural and structure cellular components are generally considered as a serious mechanism involved in the histological disorders (Sepici-Dinçel *et al*., 2009). On the other hand, organophosphate pesticides through methylation and phosphorylation of cellular proteins (Murray *et al*., 2003) may lead to a reduction in the reconstruction of necrotic tissues. The deterrent effect of organophosphate pesticides on acid phosphatase and alkaline phosphatase activity in different tissues of fish can also adversely affect on nucleic acid synthesis (Das and Mukherjee, 2000). Authors found out that different kinds of pesticides can cause serious impairment to physiological and health status of fishes (Behrens and Segner, 2001; Todd and Leeuwen, 2002; Tripathi *et al*., 2003; Begum, 2004, 2005; Monteiro *et al*., 2006; Banaee *et al*., 2008; Wang *et al*., 2009; Benli and Özkul, 2010; Banaee *et al*., 2011; Banaee, 2012). Since fishes are important sources of proteins and lipids for humans and domestic animals, so health of fishes is very important for human beings. Recently, many studies have been conducted to determine the mechanisms of pesticides' damage in fishes, with the ultimate goal of monitoring, controlling and possibly intervening in xenobiotics exposure and theirs effects on the aquatic ecosystem. Histopathological investigations on different tissues of exposed fish are useful tools for toxicological studies and monitoring water pollutions.

The present study was performed to evaluate the sub-lethal effects of diazinon on histopathological alterations in target tissues such as gill, kidney, spleen and intestine of rainbow trout (*Oncorhynchus mykiss*) as a laboratory animal model. The rainbow trout was selected for the bioassay experiments since it is one of the most economically important freshwater fish that is extensively cultured in Iran and other countries.

MATERIALS & METHODS

Rainbow trout (Oncorhynchus mykiss) was used as experimental animal according to National Ethical Framework for Animal Research in Iran (Mobasher et al., 2008). Immature rainbow trouts, *Oncorhynchus mykiss* (body weight: 80.5 ± 15 g; body length: 14.0 ± 0.4 cm), were purchased from a fish farm (Rainbow trout Farm, kordan village, karaj, Iran, 35°, 57´N/ 50°, 51´E). Fish were transferred to the aquaculture laboratory of Fishery and Environmental Department of Tehran University and acclimated to the laboratory conditions for 2 weeks before the experiments. Fishes were randomly parcelled in 12 closed 1000-L recirculating tanks supplied with oxygenated water maintaining constant dissolved oxygen at 6.5 ± 0.5 mg/L, temperature at 15.5 ± 1 ºC, pH at 7.4 ± 0.2, water hardness 150 ± 5 mg/L $CaCO_3$ and photoperiod at 16L:8D. During acclimation and all experimental tests, fishes were fed with commercial trout pellets at the manufacturers recommended rate (2% of their body weight twice a day). Fishes were starved for 1 day before the experiments started, and they were deprived of food for 24 h before sacrifice.

For experimental exposure to sub-lethal concentrations of diazinon, 90 healthy fishes were distributed to 1000 L equipped fiberglass tanks with aerator in three groups of 30 individuals each. Fishes were exposed to diazinon at a nominal concentration of 0.0 mg/L (control group), 0.1 mg/L and 0.2 mg/L , respectively, which were equivalent to approximately 10% and 20% of 96 h LC_{50} value (1.17 mg/L) for 28 days toxicity testing (Banaee *et al*., 2011). Ten fishes per each exposure concentration were captured and anesthetized with clove powder extract (1:5000) after 28 days of diazinon exposure. The water was changed daily to reduce the build-up of metabolic wastes and to keep concentrations of diazinon near the nominal level. At the end of the experimental period, on the 28th day, nine fish per treatment were captured and anaesthetized with a clove powder extract. After observing bleeding from a dorsal aorta of fish, they were dissected and tissues were rapidly collected and washed by buffered normal saline. Tissues were fixed into bouin's solution (prepared with saturated picric acid, formaldehyde and acetic acid), for 48 h and then dehydrated through graded alcohol series (70 to 100%), cleared in xylene and embedded in paraffin. 5 to 6 μm thick paraffin sections were cut and stained with haematoxylene-eosin and investigated under a light microscope (XSZ-801BN model, China) equipped with 12.1 mega pixels' camera (Casio, EX-Z450, Japan).

RESULTS & DISCUSSION

A fundamental contributor to the Green Revolution has been the development and application of pesticides for the control of a wide variety of insectivorous and herbaceous pests which would otherwise diminish the quantity and quality of food production (Ecobichon, 2001). Unfortunately, in spite of its advantages, chemistry has great disadvantages as well. Pesticides are threatening the long-term survival of major ecosystems by disruption of ecological relationships between organisms and loss of biodiversity. Pesticides can also have significant consequences on aquatic organisms' health (Richards and Kendall, 2002; Cengiz, 2006; Banaee *et al*., 2008; Banaee *et al*., 2011; Banaee and Ahmadi, 2011). The purpose of this study was to evaluate the histopathological damages induced by sub-lethal concentrations of diazinon in rainbow trout. In the present study, no mortality was recorded during the experimental period for all treatment groups studied. Behavioral changes are the most sensitive indicators of potential toxic effects. The behavioral and the swimming patterns of the fish in control group were normal, but in the experimental groups, abnormal swimming behavior increased with increasing concentration and exposure time. In some cases, fish exposed to diazinon exhibited vertical and downward swimming patterns, swimming near the water surface, lethargic and erratic swimming, loss of schooling behaviour, hyperactivity, seizures and convulsion (fish suffering from severe muscular contraction and tremor), loss of buoyancy, increased cough rate, increased gill mucus secretions, and flaring of the gill arches. Behavioral alterations may be caused by impaired activity of the acetylcholine esterase enzyme as it was described in a previous study (Banaee *et al*., 2011).

Morphological lesions observed in gill, kidney, spleen and intestine of rainbow trout, *Oncorhynchus mykiss*, revealed important alterations throughout the experiment. Tissue damages and injuries after 28-day diazinon exposure are illustrated in Fig.1&4. Individuals in the control group did not display any histological changes in any of the examined tissues.

Fish gills have many important functions including exchange of gases, transport of many mono and divalent ions, excretion of waste nitrogen, and uptake and excretion of various xenobiotics (Zayed and Mohamed, 2004; Evans *et al*., 2005). Histopathology of gill is the appropriate bio-indicator to pollution monitoring.

Fish exposed to 0.1 mg/L diazinon showed hyperplasia of gill lamellae and increased gill lamellae thickness resulting in fusion and necrosis. Dilatation of blood capillaries, hyperplasia of the epithelial lining of the secondary lamellae, necrosis and shortening of the secondary lamella, abnormal raising or swelling of the epithelium, as well as fusion of the secondary lamellae and excessive mucus secretion, were observed in fish exposed to 0.2 mg/L diazinon. Damage to gill tissue may interfere with gas exchange performance of gill and cause respiratory disorders, ion-regulation and osmoregulation dysfunction and inefficacy of the excretion of waste nitrogen metabolite in exposed fish (Nero *et al*., 2006; Cengiz and Unlu, 2006; Velmurugan *et al*., 2007). Gill histopathological damage was also observed after exposure of mosquitofish (*Gambusia affinis*) to deltamethrin (Cengiz and Unlu, 2006), yellow perch and (*Perca flavescens*), goldfish (*Carassius auratus*) to oil sands (Nero *et al*., 2006), yellow perch (*Perca flavescens*) to naphthenic acid (Nero *et al*., 2006), carp (*Cyprinus carpio*) to deltamethrin (Cengiz, 2006), and rainbow trout (*Oncorhynchus mykiss*) to maneb and carbaryl (Boran *et al*., 2010) and gourami (Trichogaster trichopterus) to paraquat (Banaee *et al.*, 2013).

The head kidney of teleost fish is composed of a variety of cells, including parenchymal cells, lymphoid and hematopoietic tissues (Mela *et al*., 2007). The functional unit of the kidney is nephron. Morphologically, the nephron of bony fish includes glomerulus, tubules and collecting ducts. Hypertrophy of epithelial cells, morphological alterations in renal tubules and degenerations of nephritic cells, reduced lumen in the Kidney tissue of fish exposed to 0.1 mg/L diazinon. In the fish exposed to 0.1 mg/L diazinon, disorientation in the glomerular structure, dilation in the inter space of urinary tubular, cloudy swelling were observed. Histopathological damage in the kidney tissues of fish exposed to 0.2 mg/L diazinon were

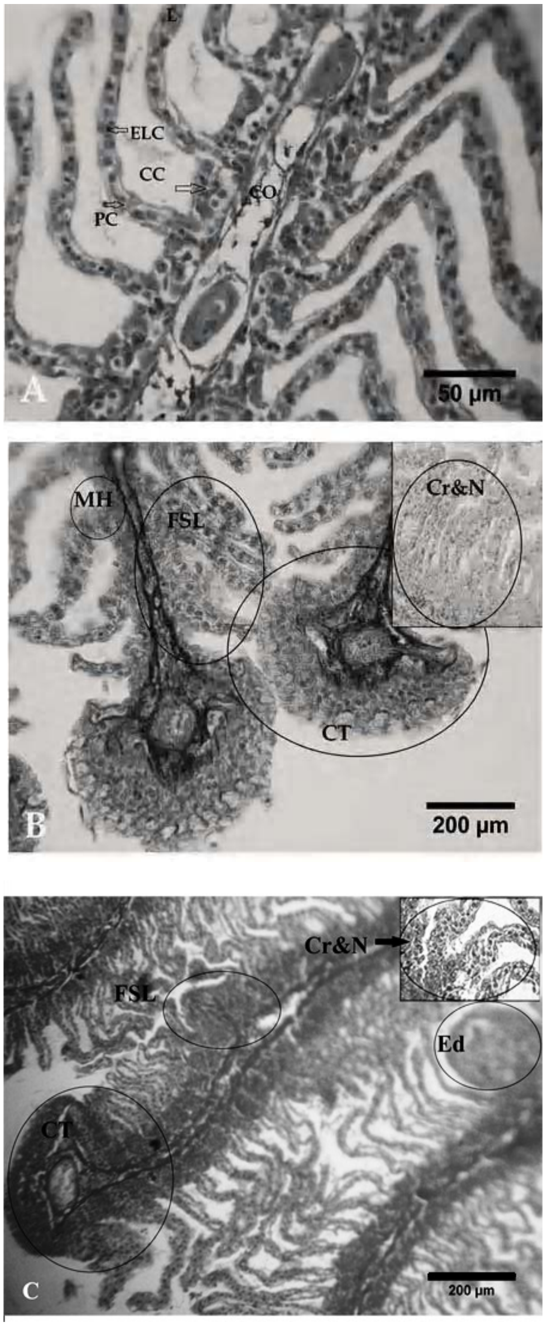

Fig.1. Gills of rainbow trout from the control group (A): Lamella (L), erythrocyte in lamellar capillary (ELC), Chloride cell (CC), Pillar cell (PC), Chondrocytes (CO); Gills of rainbow trout exposed to 0.01 mg/L diazinon (B): crusting and necrosis of secondary lamellae's epithelium (C&N), mucosa cell hyperplasia (MH), clubbing tips of gill filaments (CT); Gills of rainbow trout exposed to 0.02 mg/L diazinon (C): Changes in cartilage tissue of the gill filament, fusion of secondary lamellae (FSL), edema and epithelial hyperplasia (Ed), and the loss of the secondary filaments

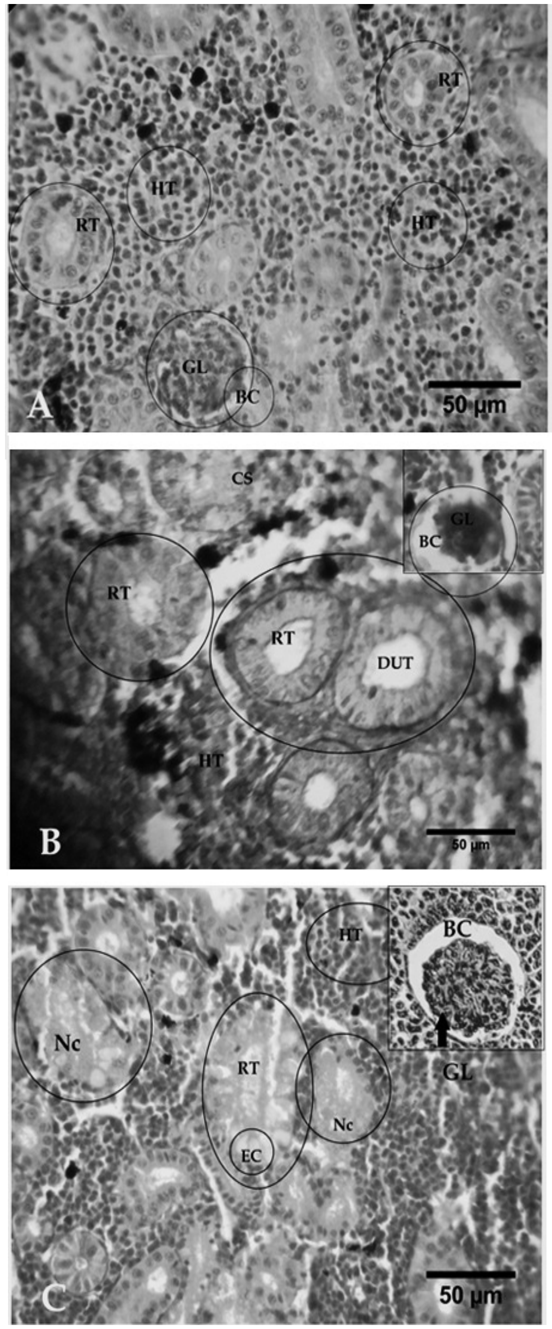

Fig. 2. Kidney of rainbow trout from the control group (A): Hematopoietic tissue (HT), renal tube (RT), Glomerulus (GL), Bowman capsule (BC); kidney of rainbow trout exposed to 0.01 mg/L diazinon (B): dilation in inter space of urinary tubular (DUT), cloudy swelling (CS), glomerular destruction; kidney of rainbow trout exposed to 0.02 mg/L diazinon (C): degeneration of tubular epithelial cells (EC), proliferation of epithelial cells in Bowman capsule, cloudy swelling (CS), severe necrosis in nephritic tissue (Nc)

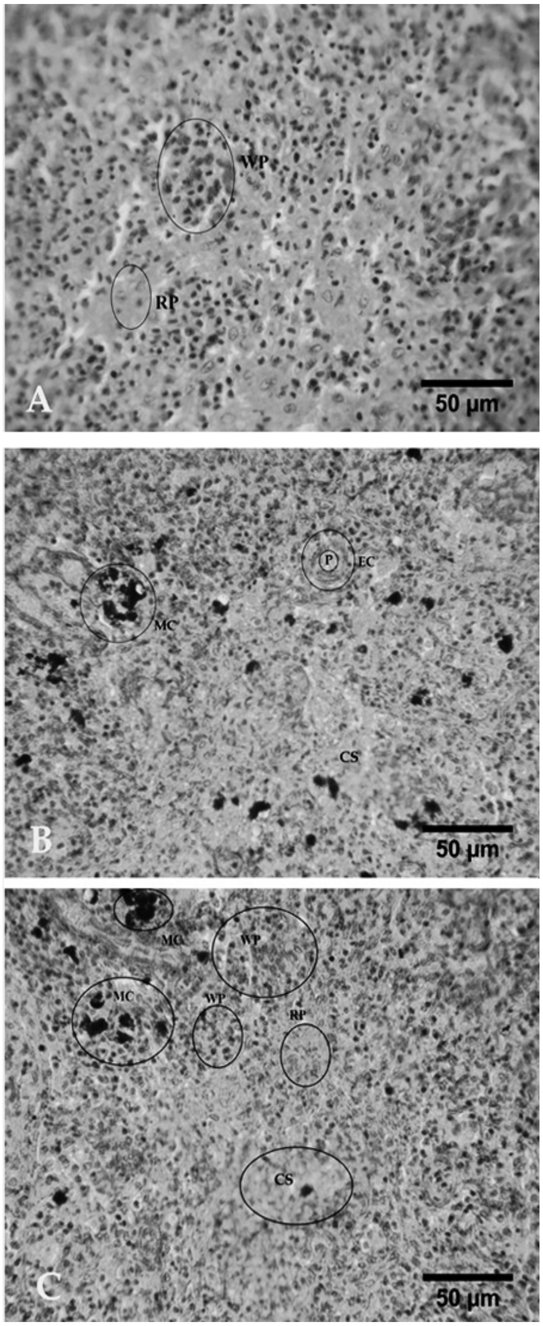

Fig. 3. Spleen of rainbow trout from the control group (A): With pulp (WP), Red Pulp (RP), Spleen of rainbow trout exposed to 0.01 mg/L diazinon (B): Melanomacrophage centers (MC), Ellipsoid cells (EC), Penicillus (P), Expansion of splenic red pulp and melano-macrophage centers, disorientation in ellipsoid cells; Spleen of rainbow trout exposed to 0.02 mg/L diazinon (C): Disorder in ellipsoid cells, the increase of number and size of melano-macrophage centers, and cloudy swelling in spleen tissue (CS)

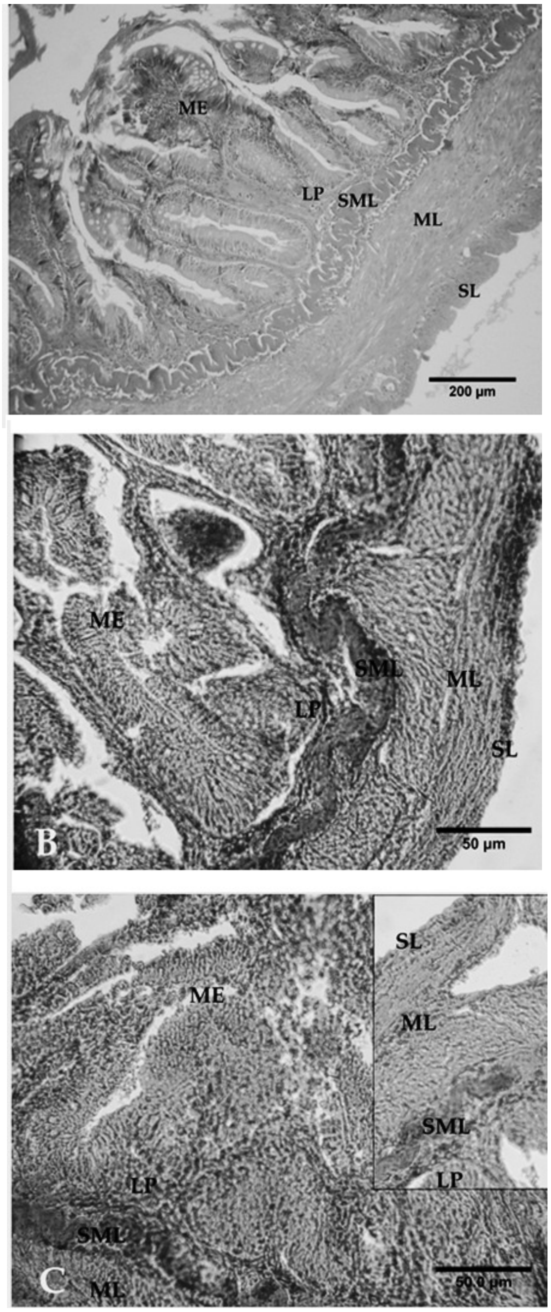

Fig. 4. Intestine of rainbow trout from the control group (A): Mucosal epithelium (ME), Lamia's propria (LP), Sub-mucosa layer (SML), Muscularis layer (ML), Serosa layer (SL). Intestine of rainbow trout exposed to 0.01 mg/L diazinon (B): Desquamating of the mucosal epithelium; Intestine of rainbow trout exposed to 0.02 mg/L diazinon (C): Mucosal epithelial hyperplasia, severe necrosis in the mucous layer, loss of the intestine microvilli, disorganization in arrangement of circular and longitudinal muscles in muscularis layer, loss of muscle elasticity and flexibility of the intestine

characterized by degeneration in the epithelial cells of renal tubule, necrosis in the hematopoietic tissue, degeneration of glomerulus, dilation of glomerular capillaries, appearance of vacuoles in cytoplasm epithelial cells of renal tubules with hypertrophied cells and narrowing of the tubular lumen. Increase of ROS production in the diazinon metabolism process may play an important role in lipid peroxidation of components of cellular membrane resulting in reduced nephron number, glomerular lesions, and reduced glomerular filtration rate. Similar studies showed the toxic effect of pesticides on the histology of kidney of Atlantic salmon (*Salmo salar*), rainbow trout, *O.mykiss*, exposed to endosulfan, as pesticide and captan, as the fungicide, respectively (Glover *et al*., 2007; Boran *et al*., 2012). These results conform to the deltamethrin and heavy metals effects on kidney tissue of carp that have been reported by Cengiz, (2006) and Vinodhini and Narayanan (2009).

The spleen has a fibrous capsule, and small trabeculae extend into the parenchyma, which can be divided into a red and white pulp. In rainbow trout, the aggregations of melano-macrophages are less well-defined and lack a capsule, but the association with blood vessels and lymphocytes is maintained (Press and Evensen, 1999). Expansion of red pulp with vascular congestion and a significant deposition of hemosiderin granules in a melano-macrophage center are important histopathological damages observed in spleen of fish exposed to both concentrations of diazinon. One of the typical macroscopic changes associated with diazinon toxicity is enlargement and roughness of the spleen. These results are similar to the paraquat, 3,4-dichloroaniline and captanin effects that have been described in gourami (Trichogaster trichopterus), common goby (*Pomatoschistus microps*) and rainbow trout, respectively (Monteiro *et al*., 2006; Boran *et al*., 2012Banaee *et al.,* 2013).

The intestine of rainbow trout has a mucosa, submucosa, muscularis and serous membrane. The mucosa epithelium has thin and elongated absorptive cells or enterocytes, goblet cells and lymphocytes. Enterocytes are a single layer of columnar cells with apical brush border and basal elongated nucleus with one nucleolus. There are also often lymphocytes at the basal and apical regions of the epithelium. Atrophy and necrosis of mucosal cell, exfoliate of mucosal epithelium, lymphocyte infiltration to lamina propia, reduction in the elastic properties and capillary bleeding in intestine tissue are important histopathological alterations observed in fish exposed to both concentrations of diazinon. Necrosis, degeneration, and accumulation of lymphocyte in lamina propia were observed in the intestine of mosquitofish, *Gambusia affinis*, exposed to Thiodan and deltamethrin (Cengiz *et al*., 2001; Cengiz and Unlu, 2006) and *Cirrhinus mrigala* treated with lambda-cyhalothrin (Velmurugan *et al*., 2007). This result is similar to the observations by Glover *et al*. (2007) in Atlantic salmon (*Salmo salar*) to dietary endosulfan exposure.

CONCLUSION

In conclusion, the histopathological analysis performed in gills, spleen, kidney and intestine of rainbow trout exposed to sub-lethal concentration of diazinon reported significant damage in all tissues indicating that it was a useful methodology for monitoring the long-term effects of diazinon on cultured fish. Exposure to sub-lethal concentrations of diazinon resulted in significant histopathological alterations and behavioral changes. Tissues injuries and damages in organs can result inthe reduced survival, growth and fitness, the low reproductive success or increase of susceptibility to pathological agents. On the other hand, these changes may be potentially disruptive for the survivability of rainbow trout in aquaculture farms. This fact should be taken into consideration when this pesticide is used for pest control in agriculture fields surrounding freshwater sources of fish cultivation.

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