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The Histopathological Effects of 2,4- Dichlorophenoxyacetic Acid on Intestine Tissue of Zebrafish (Danio rerio)

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ABSTRACT
The histopathological effects of 2,4- dichlorophenoxyacetic acid (2,4- D) intestine tissues of the *Danio rerio* were determined by light microscopy. The fish were experimentally exposed to sub-lethal concentrations (0,1 ppm, 0,5 ppm and 1 ppm) of 2,4-D for 5 days. Tissues were normal in the control group. In the intestine tissues of fish exposed to 2,4 dichlorophenoxyacetic acid, oedema, necrosis and atrophy of epithelial cells were observed. The present study proves its toxic potential in terms of the damages induced by 2,4- D in organ level.

Introduction

2,4-Dichlorophenoxyacetic acid (2,4-D) which is a white-yellow crystalline powder, commonly used as a broadleaf herbicide in commercially-available products, generally in liquid formulations. The application of herbicides in land and water management has posed potential health hazards to wildlife and humans.

Although phenoxyherbicides are relatively slightly toxic, their ubiquitous distribution and prolonged exposure may impose a substantial health risk on subjects living in rural areas. Among herbicides widely used, with potential toxicity against humans are phenoxy compounds such as 2,4- dichlorophenoxy acetic acid (2,4-D); (2,4,5-trichlorophenoxy) acetic acid (2,4,5-T); (4-chloro-2-methylphenoxy) acetic acid (MCPA) and their respective esters. Among these 2,4- D is the most widely used herbicide in the world (Wauchope, 1992; Karasu Benli et. al., 2007). 2,4-D (2,4-dichlorophenoxyacetic acid) is a common herbicide used around the home and garden, on golf courses, ball fields, parks, in agriculture and forestry.

Agricultural uses include pasture land, wheat, corn, soybeans, barley, rice, oats and sugar cane. 2,4-D functions by maintaining high levels of the plant hormone auxin, resulting in overstimulation of plant growth and ultimately death. Aquatic toxicity of 2,4-D on non-target organisms is either incomplete or lacking. Although some formulations of 2,4-D were reported highly toxic to fish; others were less so (Karasu Benli et. al., 2007).Acute toxicity of phenoxyacetic acid derivatives measured as the LD50 dose varies between 100 and 1200 mg/kg body mass for various species of experimental animals (Hayes and Laws 1991, Wafa et al. 2011). 2,4-D is easily adsorbed into the human organism from the alimentary tract and skin and is subsequently excreted in the urine in nearly unchanged form (Brand, 2002). The teratogenic, neurotoxic, immunosuppressive, cytotoxic and hepatoxic effects of 2,4-D have been well documented (Blakely et al., 1989; Charles et al., 2001; Madrigal- Bujadar et al., 2001; Osaki et al., 2001; Tuschl and Schwab, 2003). There are studies in literature concerning the accumulation of 2,4-D, its derivatives, and other agricultural chemicals in tissues (Koziolleket al., 1996). Oruc and Uner (2000) studied the combined effects of 2,4-D and azinphosmethyl on antioxidant enzymes for clarifying mode of action of these chemicals.

The zebrafish *(Danio rerio)*, which lives in India and Pakistan naturally, is a member of the Cyprinidae family. It is widely used as laboratory model, especially in developmental biology. The zebrafish model has become more and more popular because it is easy to produce. In the laboratory, zebrafish can be stimulated to breed throughout the year and the development from the fertilised egg to the reproducing stage takes only about 3-4 months. Their short generation (three months) makes them an ideal candidate for genetic studies and their susceptibility to mutagens, carcinogens, teratogens and toxins makes them ideal as environmental models.The principal advantages of the zebrafish model discussed above make this species also a suitable model for toxicological purposes. Zebrafish have been used as a general vertebrate toxicity model (Hill et al., 2005), but also as an ecotoxicological test species to determine effects of chemicals on fish survival, growth and reproduction.

In this study, we investigate the histopathological effects of different sublethal doses of 2,4-D on intestine tissues of zebrafish. Intestine histology, the shapes of the cells and detailed description of histological structure of the tissue was observed with light microscopy.

Materıal Methods

Chemicals

2,4-Dichlorophenoxyacetic acid (2,4-D) is a common systemic pesticide / herbicide used in the control of broadleaf weeds. It is the most widely used herbicide in the world. 2,4-D is a synthetic auxin (plant hormone), and as such it is often used in laboratories. 2,4-D is primarily used as a herbicide. It is sold in various formulations under a wide variety of brand names. 2,4-D can be found in lawn herbicide mixtures such as "Weed B Gon MAX", "PAR III", "Trillion", "Tri-Kil". In aquatic environments microorganisms readily degrade 2,4-D and breakdown by sunlight is not a major reason for loss. Rates of

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breakdown increase with increased nutrients, sediment load and dissolved organic carbon. Under oxygenated conditions the half-life can be short, in the order of one week to several weeks.The zebrafishes are divided into four groups (n=30) concerning their different 2,4-Diclorophenoxy acetic acid doses (Group I: 0,1 ppm 2,4-diclorophenoxy acetic acid, Group II: 0,5 ppm 2,4-diclorophenoxy acetic acid, Group III: 1 ppm 2,4-diclorophenoxy acetic acid, Group IV: control group).

Model Organism

The zebrafish (*Danio rerio*) is a small fish about 6 cm in length, characterized by a series of five pigmented stripes running the entire length of each side of its body. The zebrafish's hardiness makes them excellent stress test subjects, as they can survive fairly severe environmental changes without succumbing, surviving long enough to show developmental defects. Finally, zebrafish are easy and inexpensive to raise, requiring only filtered water and a minimal investment in fish food, making them an ideal animal model for research labs with limited funding. All of these characteristics have contributed to making zebrafish the model of choice in this study. Ideal breeding conditions were maintained to ensure a maximum yield. The zebrafish received fourteen hours of daylight and ten hours of darkness every night. The temperature and humidity were kept at 28.5°C and 61% respectively.

Experimental analyses

A static toxicity bioassay was performed according to standard method (APHA 1992) to determine the 96-h LC50. The zebrafish were divided into 4 groups: a control group and three experimental groups (n=30). 2,4-D stored at +4 °C was diluted to give the stock material, and dosing solutions were prepared by dilution to give concentrations of 0.1, 0,5 and 1 ppm. The dosing volume never exceeded 0.2 ml, and control group received acetone alone. Following the preliminary experiment, all determinations were repeated twice. Mortality was assessed at 24, 48, 72 and 96 h after the start of the tests.

Dead individuals were removed immediately. Behavioral changes were followed closely. Control group was kept in tap water, no solvent was included since 2,4-D is soluble in water; all other conditions were same as experimental groups. After giving different doses of 2,4-diclorophenoxy acetic acid, fishes were dissected in the fifth day of the study.

Zebrafish were fed daily with *Artemia* sp. and Tetra- Min© Hauptfutter (Tetra Werke, Germany) under standardized conditions (20-L glass aquaria, 28±1 °C, light/dark cycle = 14 h/10 h).After 5 days, all fish were taken into post-mortem dissection.The fishes were killed instantly by placing in a jar with a fewdrops of formalin then they were fixed in 10% neutrally buffered formalin.The ovary of adults were fixed in Bouin's fixative for 18 h and stored in 70% methanol. Dehydration was carried out in an ascending series of ethanol and the tissue was cleared in xylene. The tissues were then embedded in paraffin wax and cut into 5µm sections on a microtome. The sections were mounted on glass slides and stained with haematoxylin (H&E).

Results

Control Group

Histopathological changes were not observed in the intestine tissue of the control fish. Figure 1 shows the normal histological structures of the intestine. The intestine of *D.rerio* has a mucosa, submucosa, muscularis, and serous membrane.

The mucosa epithelium (Fig.1) has thin and elongated absorptive cells (enterocytes),intestinal lümen, goblet cells, and lymphocytes. The enterocytes are a single layer of columnar cells with apical brush border and basal elongated nucleus withone nucleolus. There are also often lymphocytes at the basal and apical regions of the epithelium.

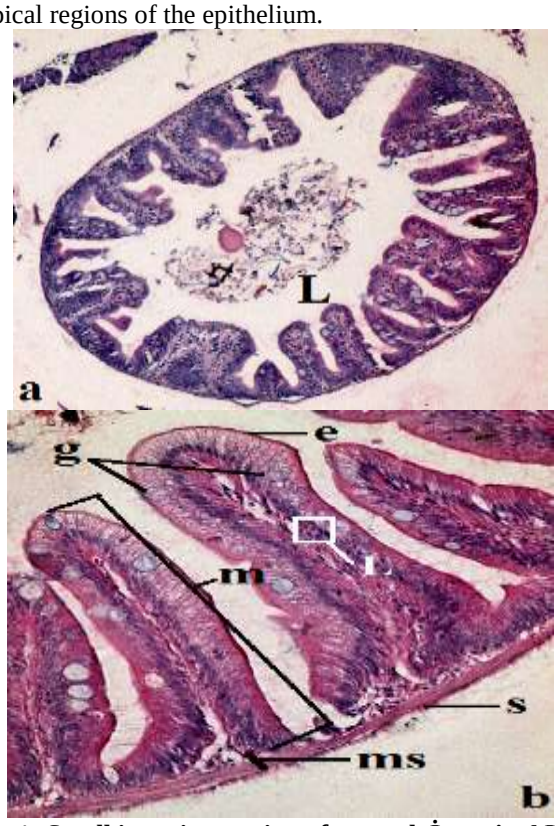

Figure 1: Small intestine section of control. İntestinal Lümen (L), Goblet cells (g), submucosa (s), mucosa (m), muscularis (ms), enterocytes (e), lymphocytes (L). HE a-x4, b-x40

Treatment group

In the intestine tissues of fish exposed to 2,4-D concentrations of 0,1; 0,5 and 1 ppm, necrosis of epithelial cells and oedema were observed in figures. The pathological findings in the intestine of *Branchydanio rerio* included atrophy severe degenerative and necrotic changes in the intestinal mucosa and submucosa with necrotized cells aggregated in the intestinal lumen. Changes in the structure of villi caused by atrophy and degenerations in epithelial cells in the intestine of zebrafish treated with 0,1 ppm 2,4-D. Hypersecretion of goblet cell and perinuclear vacuolization were also noted in 0,1 ppm 2,4-D dose group (Fig.2).

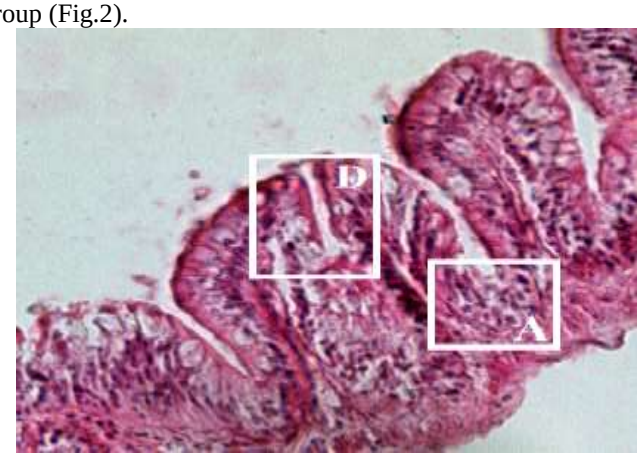

Figure 2. Changes in the structure of villi caused by atrophy (A) and degenerations (D) in epithelial cells in the intestine of zebrafish treated with 0,1 ppm 2,4-D. HE x40.g

There was a slight increase in the number of goblet cells, volume expansion of nucleus in small intestine of zebrafishs in 0,5 ppm 2,4-D group (Fig.3) . In 1 ppm 2,4-D group, there was excessive mucus accumulation in the apical cytoplasm,

perinuclear vacuolization, degeneration and desquamation of epithelial cells, atrophic changes in duedonum mucosa and degeneration of the villi's structure in comparison with controls (Fig.4). The histopathologic changes determined in 1 ppm 2,4-D dose group were similar to those in 0,5 ppm 2,4-D dose group but the degree of degeneration was found to be heavier (Fig.4) .

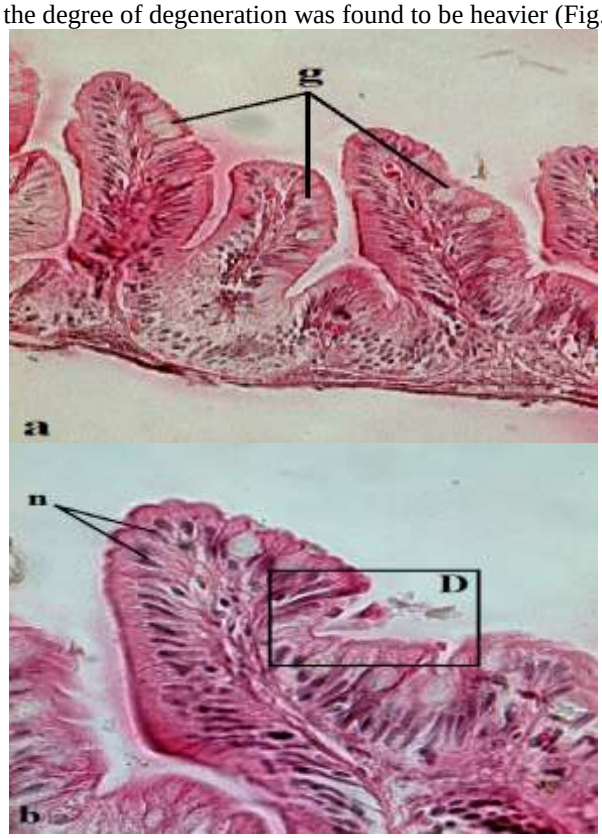

Figure 3. Intestine tissue of zebrafish treated with 0,5 ppm . There was a slight increase in the number of goblet cells (g), volume expansion of nucleus (n) and degenerations (D) in small intestine. HE a-x20, b-x40

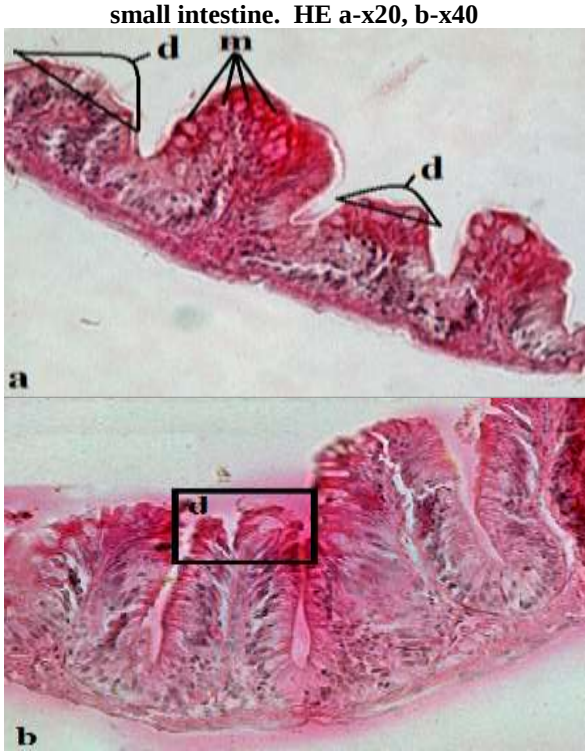

Figure 4. Changes in the structure of villi degeneration (d) and heavy degenerations(d) in epithelial cells, mucus accumulation (m) treated with 1 ppm 2,4-D. HE a-x10, b-x100.

Dıscussıon

The histopathological changes observed in this study are not characteristic of 2,4-D herbicides, but represent nonspecific changes induced by exposure to herbicides. Cytoplasmic vacuolation due to exposure to herbicides has been reported by a number of workers in different tissues of fish.

King (1962) has given a detailed account of various histopathological symptoms of guppies and brown trout fry exposed to sublethal concentrations of DDT, mainly in the liver, intestine, and kidneys, and with special reference to cell vacuolation. Sastry and Malik's (1979) study was undertaken to examine the effects of a 20-day exposure to a sublethal concentration of Dimecron on the digestive system of a teleost fish, *Channa punctatus.* Anees (1978) observed tissues of the intestine with changes and disturbances in distribution of seric proteins in *Channa punctatus* after exposure to the organophosphates methyl patrathion, diazinon, and dimetoate. *C. paleatus*, when exposed to a sublethal dose of OP by food, exhibited a large number of lipoid vesicles at the apical region of enterocytes. In the intestine and pyloric caeca, the villi were highly degenerated and the tips of villi ruptured. The cytoplasm of the columnar epithelial cells was degenerated and the mucosa presented a syncytial appearance. The intestine is another site for biotransformation of insecticides (Larini, 1979), and therefore, local intoxication of the enterocytes can damage their structure. This was noticed by the partial lateral separation of enterocytes in *C. paleatus*.

Mandal and Kulshrestha (1980) described the lesion formation in villi of *Clarias batrachus* after exposure to sumithion. Khangarot (1982) reported that the gills of living animals treated with zinc were covered by a film of coagulated mucus. Desquamation of epithelial cells of intestine in treated animals was a consistent finding with the reports of Areechon (1988) in catfish given malathion. The intestine is a very important absorption place for the toxic compounds (Timbrell, 1991). Richmonds and Dutta (1989) reported that after malathion exposure, an excessive amount of mucus was found over the gills of live bluegill fish.

Histological analysis of intestine tissue of *Channa striatus* and *Heteropneustes fossilis* inhabiting the polluted water showed degenerative changes in the serosa, mucosa and sub-mucosal layers, focal necrosis, proliferation and desquamation of the superficial parts of villi (Kumari and Kumar, 1997) . According to Ozelmas and Akay (1995) malathion showed different degrees of degenerative changes in small intestines of animals in their experiment. Excessive increase in the secretion of goblet cells was predominant in all treated animals. This is similar to what occurs with other species when they feed after a longer period of food deprivation (Freiberger, 1996).

Histological studies are recommended for the evaluation of fish health. Naphthalene treated fish exhibited histological changes in the intestinal tissue, such as disjoinment of intestinal layer might be due to the toxic effect of naphthalene and its metabolites. According to Bhatnagar et al., (2007) observed irritation and destruction of the mucosa membrane of the intestine, hampering absorption were due to fluoride toxicity. The pathological alterations in the intestine of the studied fish were in agreement with those observed by many investigators about the effects of different toxicants on fish intestine due to pesticides and heavy metal (Mishra et al., 1988; Fatma et al., 2009). The effects of the malachite green dye on the intestine of the fish also included necrosis, desquamation and degeneration of epithelial cell lining, cytolysis and increase in goblet cell population, rupture of tip of intestinal villi, breakage of mucosal

folds, necrosis and disorganisation of muscularis and serosa (Srivastava et al., 1998b).

Histological analysis of intestine tissue of *Channa striatus* and *Heteropneustes fossilis* inhabiting the polluted water showed degenerative changes in the serosa, mucosa and sub-mucosal layers, focal necrosis, proliferation and desquamation of the superficial parts of villi (Kumari and Kumar, 1997). Braunbeck and Appelbaum (1999) have also found that in the intestine, exposure to endosulfan is associated with changes in the epithelial lining, which indicates disturbance of intestinal absorption. Cengiz et al.(2001) reported oedema, degeneration, accumulation of lymphocytes in the lamina propria, pycnotic state of nuclei, and necrosis in the intestine of *Gambusia affinis* exposed to endosulfan. In another study, infiltration of mononuclear leucocytes and eosinophils towards lamina propria, necrosis were noticed in the intestine of fish after exposure to deltamethrin (Cengiz and Unlu, 2006).

Histopathological observation indicated that exposure to sub-lethal concentrations of lambda-cyhalothrin caused destructive effect in the gill, liver, intestine and kidney tissues of *C. mrigala*. Gill, kidney, liver and intestine histopathological alterations, such as those observed in this study and findings from previous studies, may result in severe physiological problems, ultimately leading to the death of fish (Velmurugan, 2007).

According to Velmurugan et al. (2007) atrophy of epithelial cells, necrosis of epithelial cells, desquamation of mucosal epithelium and infiltration of lymphocytes into the lamina propria were detected in intestine tissues of fish after exposure to fenvalerate.

All the histopathological observations indicated that exposure to sub-lethal concentrations of 2,4-D caused destructive effects in the intestine tissue of *Danio rerio*. Intestine histopathological alterations, such as those observed in this study and findings from previous studies, may result in severe physiological problems, ultimately leading to the death of fish.

In conclusion, the findings of the present histological investigations demonstrate a direct correlation between pesticide exposure and histopathological disorders observed in several tissues. Therefore, the substantiation of pathological alterations in organs sequentially in contact with toxicants seems useful as a biomarker of pollutant exposure and effect.

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