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RESEARCH ARTICLE

Histomorphometric and Hematological Profile of Grass Carp (*Ctenopharyngodon idella*) during Acute Endosulfan Toxicity

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ABSTRACT

This study was designed to investigate the acute toxicities of endosulfan on the hematopoietic, gastro-intestinal, respiratory systems of grass carp (*Ctenopharyngodon idella*). A total of 35 grass carp, 5-6 cm long were divided into five groups (E0, E1, E2, E3 and E4) having seven fish in each. The fish in group E0 were kept as control in plain water, while those in group E1, E2, E3 and E4 were exposed to 0.75, 1.00, 1.50 and 2.00 ppb of endosulfan in water, respectively for 96 hrs. Water and test chemical solution were renewed daily throughout the experiment. At the end of experimental period blood samples from fish in each group were collected. The morphometric parameters including total weight, length, standard length and organ weights showed non-significant difference. Samples from gills, intestine and liver were collected for the histopathological examination. A significant dose dependent decrease was noted in the leukocyte and erythrocyte counts, while the platelets were higher in the intoxicated fish. The values of Hb and PCV were also significantly lower in the high dosed group. There was fusion and disruption of gills along with severe congestion in the high dosed fish groups. The intestine showed atrophy of villi and increased goblet cells count. The hepatocytes showed degenerative changes including vacuolation, pyknosis etc. It was concluded that use of endosulfan in the water is extremely toxic for the fish health so a strong legislation must be implemented in the developing world as in the analogy of European Union.

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INTRODUCTION

The use of pesticide is an obligation of modern agriculture to obtain the yield in accordance with the need of ever increasing population, particularly in the developing world (Ali *et al*., 2014; Ghaffar *et al*., 2014). Pesticides, without doubt have been/are playing a significant role in the enhanced cereal production, however, they are also commonly found in aquatic habitats, like ponds, rivers and streams at varying concentrations because of direct overspray, drift, atmospheric transport, agricultural and residential runoff, individual misuse, and improper disposal (McConnell *et al*., 1998; LeNoir *et al*., 1999). The presence of these chemicals in water channels leads huge damage to non-target spp. like fish and other aquatic life (Rohr and Crumrine, 2005; Rohr *et al*., 2008; Tang *et al*., 2012). The residual transmission to the human food chain as has been reported by several researchers (Sparling *et al*., 2001; Wan *et al*., 2005; Relyea, 2009; Chishti and Arshad, 2013) in the tissue of aquatic animals pose at huge risk to human population.

Endosulfan is widely used to control insects, pests and mite in agriculture. The acute toxicity of endosulfan to the aquatic life has been widely reported (Sunderam *et al*., 1992; Berrill *et al*., 1998; Wan *et al*., 2005). The toxicities of endosulfan include neuromuscular excitation/endocrine disruption (Yadav, 2013), degenerative changes in respiratory system (particularly gills) and visual abnormalities (Bernabo *et al*., 2008; Procópio *et al*., 2014). It is also a well-known contact hepatotoxic (Nahla and El-Shenawy, 2010), repro-toxic (Ata *et al*., 2007) and induces abnormalities of the blood forming system (Da Cuna *et al*., 2011). In environment, several transformation products of endosulfan have been identified such as endosulfan sulfate, endosulfan diol, endosulfan lactone, endosulfan ether, endosulfan alcohol, and endosulfan α-hydroxy ether (Awasthi *et al*., 2000).

Endosulfan can be present at 700 ppb in pond water 10 m away from targeted application sites and 4 ppb in pond water 200 m away from such sites, when sprayed from a nozzle 3-4 m above the ground (Ernst *et al*., 1991). It has been reported at 0.5 ppb in ponds near apple orchards in Ontario, Canada (Harris *et al*., 1998), and 2.5 ppb in Australian aquatic environments (Muschal, 2001) while the U.S. Environmental Protection Agency assumes the expected environmental concentration for surface drinking water at 0.5 to 23.9 ppb (Rossi, 2002).

Keeping in view the magnitude of the problem, it was assumed that the microscopic toxicopathological effects might be present in the respiratory and gastrointestinal system of the exposed animals. So the present study was designed to investigate the histomorphometric and blood profile of the grass carp (*Ctenopharyngodon idella*) during acute endosulfan toxicity.

MATERIALS AND METHODS

All of the animal experiments were conducted according to the rules and regulations of the Animal Ethics Committee, at the University of Peshawar, Peshawar (Pakistan). A total of thirty five "Grass carp" (*Ctenopharyngodon idella*) 5-6 cm long were collected from Carp Hatchery and Training Centre Sherabad, Peshawar-Pakistan. The fish were transported to the laboratory at Department of Zoology, University of Peshawar in a container having optimum environmental conditions of oxygen, water temperature etc. Before starting the experiment, all the fish were physically examined and verified to be free from parasitic, fungal or bacterial infection. After the acclimatization period of one week, all the fish were divided into five groups (E0, E1, E2, E3 and E4) having seven fish in each. Five glass aquaria of 60 L capacity were filled with 30L of water. Seven fishes were selected randomly and stocked in each aquarium. The fish in group E0 were kept as control in plain water, while those in group E1, E2, E3 and E4 were exposed to 0.75, 1.00, 1.50 and 2.00 ppb of endosulfan in water, respectively for 96 hrs. These endosulfan concentrations were prepared from thiosulfan 35% EC (Jaffer Agro Services, Karachi, Pakistan) having 32.9% active ingredient. Water and test chemical solution were renewed daily throughout the experiment. During the experiment, water in each aquarium was aerated with the help of air pumps and temperature was maintained at 20-23°C by water heaters. The average values of physiochemical parameters of the laboratory water noted at different time points included, pH 7.6, TDS (total dissolved solids) 365 mg/l, dissolved oxygen 6.5 mg/l, total alkalinity 130 mg/l, calcium hardness 45 mg/l, chloride 19 mg/l, conductivity 820 ms/cm

Hematology: At the end of experimental period, blood samples from five fish in each group were collected by cardiac puncture and were immediately transferred to EDTA coated tube. The samples were subjected to different hematological parameters. RBC, WBCs and platelets count were determined by using hemocytometer. Hemoglobin (Hb) concentration was determined by cyanmethemoglobin method for which 20 µl of blood was added to 4 ml of cyanmethemoglobin reagent. After mixing the tube was allowed to stand at room temperature for three minutes then absorbance was taken against reagent blank in photoelectric colorimeter at 450 nm. The Hb concentration was calculated by using the formula:

HB = Abs. of the test sample/Abs. of the standard * Conc. of standard * dilution factor/100

Packed cells volume (PCV) was determined by using micro-hematocrit tube method.

Histomorphological examination: For histopathological examination five fish in each group were selected and their total length, weight and body depth were recorded. The fish were dissected and samples from gills, liver and intestine were collected. The tissue samples after weighing were transferred to neutral buffered formalin (NBF) and transported to Department of Animal Health (Section Pathology), the University of Agriculture Peshawar. After fixation, samples were washed in the running tape water for overnight and then dehydrated in ascending grades of alcohol, cleared in xylene and embedded in paraffin. Thin slices of 3-4 µm were obtained using rotary microtome and stained by H and E. The changes in the different organs were recorded and captured by camera fitted microscope.

RESULTS

Hematological parameters: A significant decrease in the red cells count was noted in the fish from all intoxicated groups as compared to the values noted in group E0 ($2.27\pm0.05 \times 10^{12}$/L). The least value ($0.51\pm0.02 \times 10^{12}$/L) was recorded in the fish exposed to highest concentration of endosulfan (2.00 ppb). A similar dose dependent decrease in the White Blood cells (WBCs) was also noted in the different groups. The exposure of fish to endosulfan led to a significant increase in the platelets count in all the groups except in E1, which was exposed to low concentration of the chemical. A significant lower concentration of Hb was noted in the fish from the intoxicated groups as compared to the values noted in their counterpart fish in group E0. A similar trend was also noted in the PCV values in the experimental groups (Table 1).

Morphometric studies: A non-significant difference was noted in the total weight, total length, standard length and organs weight of the fish in all the intoxicated groups compared to the values noted in the control group. The gills, liver and intestine weight was lower in the experimental fish however these differed non-significantly from those noted in the control group (Table 2).

Histopathological changes

Gills: The gills in group E0 (control group) showed normal structure gill filament, primary and secondary gill lamellae (Fig. 1). The vascular histology was normal with no congestion or accumulation of cells in the gill structure. No deposition/mineralization was observed. In group E1 (0.75 ppb endosulfan), there was recorded lifting of the secondary gills lamellae. The vascular changes were observed as congestion and mild degree of cellular accumulation. There was mild to moderate degree of disorganization in the gills structure. With increasing the

Table 1: Hematological profile (mean±SD) of fish exposed to different concentrations of endosulfan

Groups (Endosulfan ppb)	RBC (10^{12}/L)	WBC (10^{9}/L)	Platelets (10^{9}/L)	Hb (g/dL)	PCV (%)
E0 (0.00)	2.27±0.05^{a}	132.54±6.32^{a}	141.80±8.23^{c}	9.88±0.29^{a}	29.68±2.75^{a}
E1 (0.75)	2.05±0.07^{b}	105.63 ±4.16^{b}	247.20±39.72^{b}	8.54±0.48^{b}	25.00±1.30^{b}
E2 (1.00)	1.66±0.33^{b}	93.38±3.68^{c}	537.20±37.37^{a}	7.04±0.43^{c}	18.34±1.18^{c}
E3(1.50)	0.68±0.02^{c}	44.76±2.09^{d}	597.80±26.41^{a}	5.17±0.52^{d}	6.10±1.54^{d}
E4 (2.00)	0.51±0.02^{d}	21.63±1.43^{e}	642.40±33.12^{a}	2.68±0.64^{e}	2.50±0.42^{e}

The values in each column denoted by different superscript letter are significantly different from each other (P≤0.05) .

Table 2: Morphometric and gross anatomical parameters (mean±SD) of the fish exposed to different concentrations of endosulfan

Groups (endosulfan ppb)	Total weight (g)	Total length (cm)	Standard length (cm)	Body depth (cm)	Gills weight (g)	Liver weight (g)	Intestine weight (g)
E0 (0.00)	14.75±4.63	14.82±5.46	12.15±4.53	2.73±1.38	1.22±0.93	1.36±1.27	1.15±0.90
E1 (0.75)	14.22±7.72	11.07±2.36	9.10±1.88	2.03±0.68	0.61±0.40	0.36±0.18	0.56±0.34
E2 (1.00)	14.44±7.14	11.08±2.20	9.00±1.66	1.92±0.35	0.57±0.32	0.34±0.17	0.50±0.26
E3 (1.50)	13.42±6.00	11.30±1.48	9.25±1.24	1.93±0.31	0.56±0.24	0.24±0.15	0.50±0.18
E4 (2.00)	19.31±5.87	12.32±2.95	9.85±2.44	2.35±0.83	0.70±0.50	0.32±0.20	0.65±0.48

dose of endosulfan i.e. in group E2 (1 ppb endosulfan) these changes became more apparent in the gills histology. In group E3 and E4, where endosulfan was added at 1.5 and 2 ppb concentration in the water there were severe congestion, shortening of both primary and secondary gills lamellae and lifting of lamellae.

Intestine: In group E0 (0 ppb endosulfan) the intestine showed no deviation from the normal histological structure, the height of villi, the vascular structures and epithelial cells were all normal. In group E1 (0.75 ppb endosulfan), the changes of mild degree were observed in the intestinal histology. These changes included atrophy and disruption of villi at some places. In the high dose groups (E2, E3 and E4) which were exposed to 1, 1.5 and 2 ppb endosulfan respectively, the histological changes comprised of a dose dependent increasing trend. The major changes included fusion of villi, villus atrophy, accumulation of inflammatory cells, and congestion. The number of goblet cells per microscopic field also showed dose dependent increasing trend in the intestine (Fig. 2).

Liver: The liver of fish exposed to 0 ppb endosulfan (Group E0), showed no histological alteration. The hepatocytes were normal with foamy cytoplasm, the hepatic triad was normally placed and no vascular changes were observed. In group E1 (exposed to lowest dose of endosulfan i.e. 0.75 pbb), a mild degree of vacuolation was observed, however the vascular histology was normal with no congestion or cellular infiltration. In group E2 (1 ppb endosulfan) a moderate degree of changes were observed in the liver histology, including vacuolation suggestive of fatty changes, cellular swelling, and infiltration of inflammatory cells at some places. These changes were more prominent in the sections of liver from group E3 and E4 exposed to higher doses of endosulfan (1.5 and 2 pbb), respectively.

DISCUSSION

The exposure of endosulfan to grass carp (*Ctenopharyngodon idella*) resulted in a significant decrease in the red and white cells count, Hb level and PCV. However, the numbers of platelets were higher in the fish from the intoxicated groups. In the published literature no information is available regarding endosulfan induced hematological profile for *Ctenopharyngodon idella*, however, Sarma *et al*. (2012) reported a significant decrease in Hb level in freshwater fish within 12-24 hrs of acute endosulfan exposure (8.1 µg/L), thereafter the values increased and at the end of 72 h returned to normal levels. A similar trend in was observed in red cells count. The WBC count showed an inverse relation i.e., increasing trend with increase in the duration of acute endosulfan exposure. In another experiment, working with *Channa punctatus*, same group of researcher recorded a significant decrease in Hb level, RBC and WBC counts during endosulfan toxicity for longer duration of 90 days. The reversal towards normal during chronic exposure might be an adaptive response of the fish to the toxicant. The decrease in the hematological parameters noted in the present can be associated with inhibition of erythropoiesis, hemosynthesis, osmoregulatory dysfunction, increase in the rate of hemolysis or increased lipid peroxidation induced oxidative damage to hemoglobin (Jenkins *et al*., 2003) or decrease intestinal iron absorption (Banik *et al*., 1996).

A non-significant difference was noted in the total weight, total length, standard length and organs weight of the fish exposed to endosulfan among all the groups compared to the values noted in the control group. However, the gills, liver and intestine weights were lower in the experimental fish but showed a non-significant difference compared to the values noted in the control group. These results might be due to short time acute exposure of the fish to endosulfan which might not get time to affects these parameters. Kalsoom *et al*. (2005) reported an improvement (significant) in the length and breadth of fish (*Cyprinion watsoni*) at 0.75 ppb, while the same parameters were decreased at 1 ppb endosulfan exposure for 30 alternate days. Salvo *et al*. (2008) recorded a significant difference (as compared to untreated group) in liver weight while non-significant differences in the body weight and length of the fish (*Cyprinus carpio*) exposed to sub-lethal concentration (0.001 mg/L) of endosulfan for 15 days.

The histological changes observed in the gills of grass carp included lifting of epithelial at secondary gills lamellae, congestion, accumulation inflammatory cells, and disorganization in the gills structure. In the intestine, villus atrophy, fusion of villi, mononuclear cells accumulation and the most exciting change the increase in goblet cells count. Liver showed vacuolation (suggestive of fatty change), cellular swelling, and infiltration of inflammatory cells at some places. These histological alterations noted in liver and intestine of the fish are typical marker of generalized stress (Glover *et al., al*., 2007) to the fish.

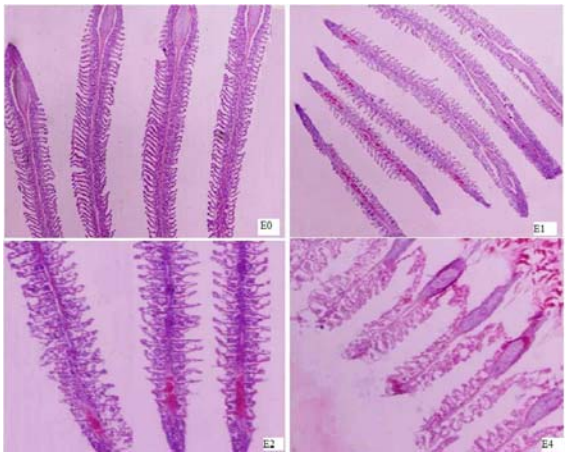

Fig. 1: Histomicrograph of gills of the fish exposed to different concentration of endosulfan. E0 - Gills having normal length of primary and secondary gills lamellae along with no observable vascular changes in control group (100X); E1 - lifting of secondary lamellae, congestion and disorganization are prominent (40X); E2 - congestion is evident (400X); E4 shortening of primary and secondary lamellae, severe congestion and lifting of the lamellae can be seen (100X). Fish of E1, E2 and E4 were exposed for 96 hours to 0.75, 1 and 2 ppb endosulfan, respectively. H&E stain.

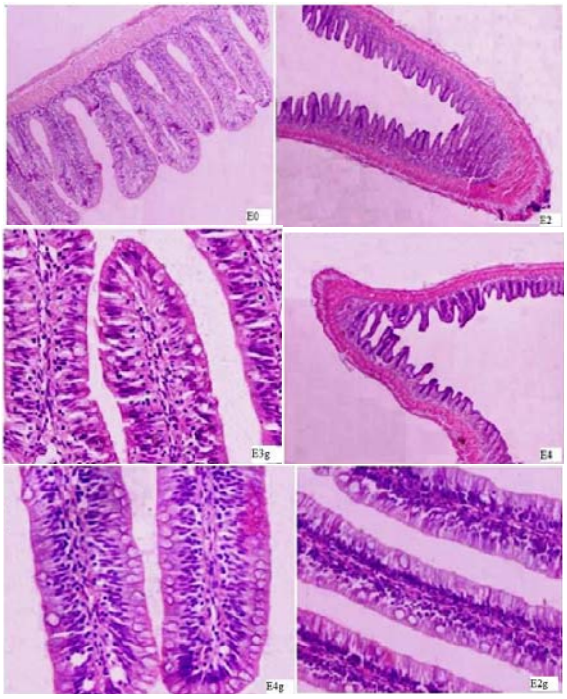

Fig. 2: Histo-micrographs of intestine of fish exposed to different concentration of endosulfan. E0: (control group) showing normal height of villi, the epithelial cells contains normal proportion of goblet cells (40X). In E2: atrophied villi and accumulation of cells (100X), higher number of goblet cells along with cellular infiltration (400X) while in E4: stunting and fusion of villi (40X) can be seen. Compare abundant goblet cells in E4g while few goblet cells in E2g group (400X). Fish of E2, E3 and E4 were exposed for 96 hours to 1, 1.5 and 2 ppb endosulfan, respectively. H&E stain.

Glycogen depletion, lipidosis, villi depletion and fusion have been reported by several researchers in the different spps of fish excluding grass carp (*Ctenopharyngodon idella*) in response to endosulfan toxicity (Altenok and Capkin, 2007; Berntssen *et al*., 2010; Sarma *et al*., 2012). The dramatic increase in the number of intestinal goblet cells may be linked to activation of host defense against the environmental pollutant. As the goblet cells are highly polarized secretory cells that play an important protective role in the intestine by synthesizing and secreting several mediators, including the mucin MUC2 and trefoil factor 3 (TFF3). Under basal conditions or under the influence of host or bacterial stimuli, goblet cells release MUC2-containing granules into the lumen, where they hydrate and form the structural basis for the mucus gel layer overlying the intestinal epithelium (Van Klinken *et al*., 1999). This mucus layer plays important physiological roles in the gut by i) lubrication to the intestinal surface, ii) limits passage of luminal molecules into the mucosa, iii) functions as a dynamic defensive barrier against enteric pathogens (Specian and Oliver, 1991). TFF3 on the other hand is considered to be potent inducer of cell migration and an inhibitor of apoptosis (Taupin *et al*., 2000), synergize with colonic mucins to enhance the protective barrier properties of the mucus layer against toxins (Kindon *et al*., 1995). These protective barriers might have been in activation to decrease the absorption of the toxicant from the intestinal lumen.

Conclusion: It could be concluded that endosulfan was toxic for the grass carp at as low as 0.75 ppb and could induce pathological alterations in hemopoietin, gastro-intestinal, respiratory system.

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