Biomarker enzyme activities and ultrastructure of liver of the grass carp *Ctenopharyngodon idella* (Valenciennes, 1844) exposed to the organophosphate pesticide chlorpyrifos

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ABSTRACT

The present investigation analysed the toxic effects of the organophosphate pesticide chlorpyrifos (CPF) on activities of biomarker enzymes viz., alanine transaminase (ALT), aspartate transaminase (AST), alkaline phosphatase (ALP) and acid phosphatase (ACP) as well as ultrastructural alterations in hepatocytes of grass carp *Ctenopharyngodon idella* exposed to sublethal concentrations (1.44 and 2.41 $\mu\text{g l}^{-1}$) for 15, 30 and 60 days. The enzymes showed marked fluctuations post-pesticide treatment. The altered enzymes activities indicated disturbance in the structure and integrity of cell organelles. Ultrastructural alterations such as formation of crescent shaped nucleus along with condensed chromatin, margination of heterochromatin, severe breakdown and fragmentation of endoplasmic reticulum, deformed mitochondria with loss of cristae were observed in the pesticide exposed fishes. The findings revealed exposure period and concentration dependent response in hepatocytes. Ultrastructural changes could be correlated to corresponding alterations in the activities of marker enzymes. At both the toxicant concentrations, altered activities of AST and ALT as markers of glycolysis and protein metabolism indicated disturbances to the cellular metabolism. The observed changes in the liver enzyme levels could be used as sensitive biomarkers for pesticides contamination in grass carp.

Keywords: Chlorpyrifos, *Ctenopharyngodon idella*, Liver, Marker enzymes, Transmission electron microscopy

Introduction

Pesticides are recognised worldwide as a veritable means of controlling pests, at the same time such chemicals are highly toxic to non-target species in the environment (Rao, 2006a). Presently, there is an increasing concern world over on the indiscriminate use of such chemicals that result in environmental pollution and toxicity risk to non-target organism (Velisek *et al.*, 2007). Among the various organisms present in the aquatic ecosystem, fishes are the one that are relatively more sensitive to changes in their surrounding environment. The pesticide concentration in the aquatic organisms increased manifold due to biomagnification than the concentration present in the ecosystem (Martin and Knaeur, 1973). The pesticides liberated into aquatic ecosystem have a tremendous adverse effect on fish and thereby to man. Chlorpyrifos is a broad-spectrum organophosphate (OP) with wide application in the field of agriculture and has wide variation of toxicity among different species. It is the second largest selling OP agrochemical in India.

The liver is an important organ involved in metabolic processes and in detoxification of xenobiotics. Pesticides may accumulate in the liver and cause pathological changes (Braunbeck *et al.*, 1990). There are many studies on liver

ultrastructural alterations induced by pesticides in aquatic organisms (Khangarot, 1992). The type of liver injury is often dependent not only on the toxicant and its mechanism of action but also on the duration of exposure (Jacobson-Kram and Keller, 2006). Evaluation of enzyme activities in the tissues and organs of aquatic organisms in the diagnosis of the effects of pollutants is one of the emerging areas in toxicological monitoring and remediation programmes (Oluah *et al.*, 2005). Fluctuations in aspartate aminotransferase (AST) and alanine aminotransferase (ALT) activities reflect liver damage (Bernet *et al.*, 2001). Further, alkaline phosphatase (ALP) is composed of several isoenzymes that are present in all tissues of the body, especially in cell membranes. These enzymes catalyse the hydrolysis of monophosphate esters and have wide substrate specificity. Such responses allow the fish liver to be considered as a good indicator of fish health status (Bowser *et al.*, 1990; Brusle and Gonzalez, 1995). Investigations on biochemical and histological changes in fish liver has become an important tool to monitor the environmental exposure of fish to contaminants (Fernandes *et al.*, 2008; Carrola *et al.*, 2009). Keeping this in view, the present work was undertaken to evaluate the stress parameters by analysing biomarker enzyme levels and ultrastructural alterations in the liver of the grass carp *Ctenopharyngodon idella* (Valenciennes, 1844) exposed to chlorpyrifos.

Materials and methods

Procurement of fish and acclimatisation

Fingerlings of *Ctenopharyngodon idella* (10 ± 2 g, 10 ± 2 cm), were collected from Nanoke Fish Seed Farm, Nanoke, District Patiala, Punjab and were safely brought in aerated water packed polythene bags. They were acclimatised to the laboratory conditions for 15 days in glass aquarium and fed with pelletised supplementary feed once a day at least 1 h prior to replacement of water. The physico-chemical characteristics of water used in the experiment were determined in accordance with the standard methods (APHA, 2012) and the mean values recorded were: pH 7.2 ± 0.1 , temperature $25 \pm 2^\circ\text{C}$, dissolved oxygen 8.0 ± 0.3 mg l⁻¹, total alkalinity 40 ± 10 mg l⁻¹ and total hardness 90 ± 0.5 mg l⁻¹.

Chemicals

Chlorpyrifos (20% EC), commercial grade was purchased from Shivalik Insecticide Pvt. Ltd., India. Stock solution was prepared by dissolving the appropriate amount of chlorpyrifos in distilled water which was further used for preparing the working concentrations for acute toxicity tests. Other chemicals of analytical grade (CDH, New Delhi) were procured from local scientific suppliers, Chandigarh, India.

Experimental design

Static toxicity bioassay was performed following APHA (2012). The short term range finding tests were carried by exposing the fish to wide range of the pesticide concentrations. After performing exploratory experiments, the lowest ($6 \mu\text{g l}^{-1}$) and highest ($9 \mu\text{g l}^{-1}$) concentration of chlorpyrifos (CPF) were selected and three different concentrations of chlorpyrifos viz., 6, 7, 8, $9 \mu\text{g l}^{-1}$ were prepared for the determination of LC_{50} . Ten fish were exposed to each concentration of pesticide in dechlorinated tap water. Appropriate control was also maintained in chlorpyrifos free water. On the basis of percent mortality at each concentration, 96 h LC_{50} of CPF was calculated by Probit analysis (Finney, 1980).

For chronic toxicity studies, fish (in three different groups, 20 fish in each group) were exposed to $1/3^{\text{rd}}$ of LC_{50} ($2.41 \mu\text{g l}^{-1}$) (Group I) and $1/5^{\text{th}}$ of LC_{50} ($1.44 \mu\text{g l}^{-1}$) (Group II) of 96 h LC_{50} of CPF for 15, 30 and 60 days along with control (Group III). Experiments were conducted in duplicates. During the experiment, water was changed daily to avoid the accumulation of faecal matter and to maintain the toxicant concentration. Biochemical analysis of liver of fish exposed to the toxicant and of control was made on 15th, 30th and 60th day. For this, at the end of each exposure, 6 fish were sacrificed for further analyses.

Biochemical analysis

The treated and normal fish were euthanised following the guidelines of Institutional Animal Ethical Committee, Panjab University, Chandigarh (Ref no. IAEC/526). and liver tissues were removed for the preparation of homogenate. Tissue was washed in ice cold 0.9% saline until bleaching of haemoglobin and then chopped to prepare 10% homogenate in ice-cold 0.1 M Tris HCl buffer (pH 7.1) with the help of Porter-Elvehjem homogeniser at 4°C with motor driven teflon pestle. The homogenate was centrifuged in a refrigerated centrifuge at 12000 rpm for 15 min and supernatant was used for the estimation of transaminases and phosphatases. ALT and AST activities were estimated as per manufacturer's instructions (Reitman and Frankel, 1957) using colorimetric DNPH (2,4-Dinitrophenylhydrazine) method. Quantitative estimation of ACP and ALP was done as per Bergmeyer (1965). Test kits from Reckon and Erba, Mumbai were used for determination. All the measurements were made in duplicate. Protein was estimated by Lowry *et al.* (1951) using Bovine Serum Albumin as standard.

Transmission electron microscopy

For transmission electron microscopy, the liver tissue (1 mm) was washed in 0.1 M phosphate buffer (pH 7.2) fixed in 2.5% glutaraldehyde and 2% paraformaldehyde in 0.1 M phosphate buffer (pH 7.2, 4°C , 2 h) for 10-12 h and again washed in 0.1 M phosphate buffer (3 times), post-fixed in 1% osmium tetroxide in cacodylate buffer for 1 h at 4°C , rinsed with 0.1 M phosphate buffer to remove unutilised osmium tetroxide, dehydrated in increasing acetone concentration solutions and embedded in spur-low viscosity Epon. Sections were cut in an ultramicrotome (Reichert-Jung), stained with 1% toluidine blue and selected sections were loaded on copper grids for subsequent contrast enhancement with uranyl acetate and lead citrate (Reynolds, 1963). The samples were observed under transmission electron microscope (Leo-Morgagni 268d TEM, Fei company, Netherlands) at All India Institute of Medical Sciences (AIIMS) New Delhi, India.

Statistical analysis

Statistical analysis of data was carried out using one-way ANOVA with Tukey's test.

Results

Acute toxicity test

Acute static bioassay was conducted to determine LC_{50} of chlorpyrifos to *C. idella* and to select sublethal concentration for chronic toxicity studies. Fish were exposed to different concentrations ($6-9 \mu\text{g l}^{-1}$) of chlorpyrifos with 10 fish in each group for 96 h. Percentage

mortality after 96 h exposure to the pesticide at different concentrations were converted to probit scale (Finney, 1980). The 96 h LC_{50} was calculated using SPSS 18.0 and was found to be $7.24 \mu\text{g l}^{-1}$ (Table 1), with regression equation $Y = 0.925X + 4.1857$ and $r = 0.98$ (Fig. 1).

Enzyme analysis

In the present study, the activity of AST in liver decreased (0.85 and 2.6%) significantly ($p < 0.01$) after 15 days of exposure at lower and higher concentration of

26.87%) concentrations of CPF after 15, 30 and 60 days exposure, respectively (Fig. 4).

The activity of ACP showed significant reduction ($p < 0.05$) (37.8 and 43.59%) at 1.44 and $2.41 \mu\text{g l}^{-1}$ of CPF, respectively after 15 days exposure. Further, on 30th day of exposure, ACP activity showed significant ($p < 0.05$) decrease (42.8 and 47.2%) at lower and higher concentration of CPF respectively. On 60th day of exposure, further significant ($p < 0.05$) reduction in ACP activity (47.3 and 54.7%) at lower

Table 1. Mortality rate of *C. idella* after 96 h exposure at different chlorpyrifos concentrations and empirical probit values

Group	Conc. ($\mu\text{g l}^{-1}$)	Log conc.	No. of fish	Dead fish	% mortality	Probit kill
1	6	0.77	10	0	10	3.72
2	7	0.84	10	4	40	4.75
3	8	0.90	10	7	70	5.52
4	9	0.95	10	9	90	6.28

Fig. 1. Regression line between probit kill of *C. idella* and log concentration of chlorpyrifos

CPF, respectively. Similar significant ($p < 0.05$) decline in AST activity as compared to control (1.32 and 2.8%) at lower and higher concentration of toxicant was noticed after 30 days exposure respectively. Further, on exposure of the fish for 60 days, significant ($p < 0.05$) decrease by 1.03 folds (2.16%) in AST activity was observed at lower concentration of the toxicant, whereas at higher concentration, the change (3.7%) was found to be insignificant (Fig. 2).

The activity of ALT showed significant ($p < 0.05$) elevation (194 and 255%) at 1.44 and $2.41 \mu\text{g l}^{-1}$ of chlorpyrifos, respectively after 15 days exposure. On 30th day, similar marked ($p < 0.05$) elevation in ALT activity (188 and 209%) was found at lower and higher concentration of toxicant respectively. Further, after 60 days exposure, significant ($p < 0.05$) elevation in activity of ALT than control (180 and 201%) at lower and higher concentration of chlorpyrifos, respectively was observed (Fig. 3).

ALP activity showed significant reduction ($p < 0.05$) at lower (1.59, 4.73 and 17.08%) and higher (2.52, 13.37 and

Fig. 2. Variation in the activity of AST in liver of *C. idella* on exposure to chlorpyrifos

Fig. 3. Variation in the activity of ALT in liver of *C. idella* on exposure to chlorpyrifos

Fig. 4. Variation in the activity of ALP in liver of *C. idella* on exposure to chlorpyrifos. Data presented as Mean±S.D., n = 6; *p<0.05; Significant difference; control vs. 1.44 and 2.41 µg l⁻¹ CPF treated group; #p<0.01; Significant difference; control vs. 1.44 and 2.41 µg l⁻¹ CPF treated group

Fig. 5. Variation in the activity of ACP in liver of *C. idella* on exposure to chlorpyrifos. Data presented as Mean±S.D., n = 6; *p<0.05; Significant difference; control vs. 1.44 and 2.41 µg l⁻¹ CPF treated group; #p<0.01; Significant difference; control vs. 1.44 and 2.41 µg l⁻¹ CPF treated group

and higher concentrations of chlorpyrifos, respectively was observed (Fig. 5).

Liver ultrastructure

The ultrastructure of control fish liver showed ovoid nucleus having scattered heterochromatin and distinct nucleolus. Rough endoplasmic reticulum (RER) with extensive stacks of cisternae was interspersed with some mitochondria, formed continuous sheath around the nucleus (Fig 6 a, b). The RER were found to be regularly bordered by spherical peroxisomes and were regularly separated from glycogen fields. Mitochondria often observed were the most prominent organelle of hepatocytes and were characterised by double membranes with closely packed prominent cristae (Fig 6 a, b).

Marked disruption in the hepatic ultrastructure was observed in *C. idella* on exposure to chlorpyrifos for 15 days.

In some cells, nuclei were found to be deformed with condensed chromatin and a cluster of clumped mitochondria was seen surrounding nucleus at both the concentrations of the toxicant. The cytoplasm showed elongated mitochondria with irregular shape and size and their vacuolisation with distorted cristae, dilated and fragmented smooth endoplasmic reticulum (SER), scattered glycogen granules and lipid droplets (Fig 7 a-d).

On exposure of the fish to CPF for 30 days at 2.41 and 1.44 µg l⁻¹ sublethal concentrations, more pronounced alterations in the cellular components were observed (Fig 8 a-d). The hepatocyte nucleus lost its shape and became elongated. The double layered nuclear membrane got dilated and separated. Cytoplasm was found to be occupied with large number of glycogen granules. Numerous vacuoles, swollen mitochondria with loose network of few dilated cristae, dilated cisternae of endoplasmic reticulum were also seen. Numerous dilated RER were found to be coiled forming circular structures. Granular ribosomes got detached and scattered in the cytoplasm.

Fig. 6. Transmission electron micrographs of hepatocytes of *Ctenopharyngodon idella* of control group (a, b) showing ovoid nucleus (N), scattered heterochromatin, distinct nucleolus (Nu), rough endoplasmic reticulum (RER) with extensive stacks of cisternae, smooth endoplasmic reticulum (SER), mitochondria (M), spherical peroxisomes (Pr) and glycogen fields (Gly)

Fig 7. Transmission electron micrographs of hepatocytes of *Ctenopharyngodon idella* exposed to $1.44 \mu\text{g l}^{-1}$ (a, b) and $2.41 \mu\text{g l}^{-1}$ (c, d) of chlorpyrifos for 15 days showing deformed nuclei (DN) with condensed chromatin (CC), clumped mitochondria (CM), elongated mitochondria (EM), dilated and fragmented SER, scattered glycogen granules (a,c,d) and lipid droplets (LD)

Fig 8. Transmission electron micrographs of hepatocytes of *Ctenopharyngodon idella* exposed to $1.44 \mu\text{g l}^{-1}$ (a, b) and $2.41 \mu\text{g l}^{-1}$ (c, d) chlorpyrifos for 30 days showing dilated nuclear membrane (NE) (b), condensed chromatin (CC), glycogen granules (Gly), vacuolisation (V) (a, c), swollen mitochondria with loose network of dilated cristae (b, d) and dilated cisternae of RER (c, d)

On exposure of the fish to chlorpyrifos at lower concentration for 60 days, many degenerative changes were observed. There was formation of crescent shaped nucleus along with condensed chromatin, severe breakdown and fragmentation of endoplasmic reticulum and clumped mitochondria was also seen. There was total destruction of the cell with severe vacuolisation and scattered glycogen granules (Fig 9 a, b). At higher concentration ($2.41 \mu\text{g l}^{-1}$) of chlorpyrifos, severe mitochondrial malformation along with condensation of its matrix, disorganisation of RER and loss of ER in some cells and accumulation of lipid droplets were noticed (Fig 9 c, d).

Discussion

Based on 96 h LC_{50} , it was observed that *C. idella* is more sensitive to chlorpyrifos than *Oncorhynchus mykiss*

($24 \mu\text{g l}^{-1}$) (Mayer and Ethersieck, 1986); Nile tilapia (1.57 mg l^{-1}) (Gul, 2005); *Oreochromis mossambicus* ($25.7 \mu\text{g l}^{-1}$) (Rao *et al.*, 2003) and *Cyprinus carpio* ($580 \mu\text{g l}^{-1}$) (Xing *et al.*, 2011). Jindal and Kaur (2015) rated chlorpyrifos as highly toxic to fish. The difference in LC_{50} value could be attributed to physico-chemical characteristics of water (Yorulmazlar and Gul, 2003) and variation in susceptibility and tolerance related to differences in rates of accumulation, biotransformation and excretion of toxicant (Pandey *et al.*, 2009).

Enzyme analysis and ultrastructural changes in liver have proven to be suitable and sensitive indicators of toxicant-induced injury and have been used as biomarkers of chemicals in environmental risk assessments (Boeger *et al.*, 2003). ALT and AST are more sensitive measures of hepatotoxicity and can be assessed within a shorter period

Fig. 9. Transmission electron micrographs of hepatocytes of *Ctenopharyngodon idella* exposed to $1.44 \mu\text{g l}^{-1}$ (a, b) and $2.41 \mu\text{g l}^{-1}$ (c, d) chlorpyrifos for 60 days showing crescent shaped and ruptured (RN) nucleus (a), condensed chromatin (b), severe breakdown and fragmentation of endoplasmic reticulum (a-d), clumped mitochondria (b, c), severe vacuolisation (c), scattered glycogen granules (a, b), severe mitochondrial malformation (MM), disorganised and fragmented (Fr) RER and SER (b, d) and karyolysis (Kr).

of time (Balint *et al.*, 1997). Initial marked elevation in ALT activity as well as initial reduction in ALP and ACP activity in liver as observed during the present study indicate damage and alteration in function of the organ.

During the present investigation, fluctuation in AST and ALT activity in liver of *C. idella* was observed. This is in agreement with the findings in *Heteropneustes fossilis* exposed to malathion (Goel *et al.*, 1992); *C. idella* exposed to fenvalerate (Shakoori *et al.*, 1996); *Catla catla* exposed to fenvalerate (Susan *et al.*, 1999) and deltamethrin (Vani *et al.*, 2011). The observed fluctuations in AST and ALT activity in the above studies were attributed to pesticide toxicity, disturbance in TCA cycle and leakage of enzymes from the organ. They also stated that oxaloacetate and glutamate are not available to Krebs cycle through this route transamination. Correlation was observed between aminotransferases activity and mitochondrial integrity, as any modification in mitochondrial structure is bound to alter associated enzyme system, evidenced by transmission electron microscopy of the liver (Bonitenko, 1974). Fluctuation in the activity of transaminases might be due to the organ dysfunction under the effect of oxidative stress by enhanced generation of reactive oxygen species (Nayak *et al.*, 2004). Amin and Hashem (2012) while working on the effect of deltamethrin on *Clarias gariepinus* inferred that ALT and AST are the most commonly used biochemical markers of cellular necrosis. Present findings are in concurrence with the findings of above workers.

Acid and alkaline phosphatases are lysosomal hydrolytic enzymes present in all the tissues. They are concerned with the process of transphosphorylation, that hydrolyses the phosphoesters in acidic medium and are also associated with the transportation of metabolites, metabolism of phospholipids, phosphoproteins, nucleotides and carbohydrate and synthesis of proteins (Srivastava and Pandey, 1982). During the present study, the chlorpyrifos exposed fish showed decreased levels of phosphatase (ACP and ALP) activity in liver. This could be attributed to altered structural integrity of cell organelles as evidenced by TEM studies and decreased rate of transphosphorylation or coupling of oxidative phosphorylation (Saha and Kaviraj, 2009; Magar and Shaikh, 2013). Rao (2006a, b) reported ACP and ALP activity inhibition in liver and related this to increased lysosomal mobilisation and cell necrosis due to pesticide toxicity. Begum (2004) explained that inhibition of phosphatase activity is related to breakdown of glycogen to meet the energy demand under toxicant stress. Gabriel and George (2005) also stated that under various adaptive conditions, transamination is the main pathway for synthesis and deamination of amino acids, activating carbohydrates and protein metabolism during energy demands of the fish.

Alterations in liver are suitable indicators of exposure to environmental stressors. The loss of regular cytoplasmic compartmentation as noticed during the present study is a typical ultrastructural response of fish hepatocytes which depicts disturbance of hepatocellular homeostasis (Gernhofer *et al.*, 2001). Further, changes observed in the hepatocyte nuclei and condensation of heterochromatin could be the result of accumulation of chlorpyrifos in the liver. Toxicant induced ultrastructural alterations like condensation of chromatin and mitochondrial vacuolisation reported in the liver of snakehead *Channa punctatus* (Khangarot, 1992) were similar to those found in the present study.

Degeneration of mitochondria observed in the hepatocytes of *C. idella* may be attributed to the impairment of oxidative potential of hepatocytes in CPF exposed fish. Marked ultrastructural alterations in mitochondria including their swelling with loss of functional cristae have also been reported in freshwater fish exposed to methyl parathion (Tripathi and Shukla, 1990). Marked swelling of mitochondria could be attributed to the enhanced energy requirements of the cells to overcome stress induced by the toxic effect of chlorpyrifos. Similarly, swelling of mitochondria and ER cisternae in the liver of treated *Dicentrarchus labrax* have also been observed by Giari *et al.* (2008). In the present study, long period of exposure of chlorpyrifos resulted in scattered glycogen content and caused disintegration of cytoplasmic organelles. This indicated that chlorpyrifos can disturb the metabolic pathways of hepatocytes (Cheville, 1994). Lee *et al.* (2014) reported black granules in lysosomes, indistinct nuclear membrane and non-spherical nucleus in *Cyprinus carpio* induced by zinc. Chlorpyrifos also induced similar alterations in hepatocytes of *C. idella*.

To conclude, biochemical and electron microscopic studies revealed that exposure period and concentration of chlorpyrifos affected response in hepatocytes of the exposed fish to chlorpyrifos. Ultrastructural changes could be correlated to corresponding alterations observed in activity of marker enzymes. At both the toxicant concentrations, activity of alanine aminotransferase as marker of cytosolic glycolysis and protein metabolism got altered indicating disturbances in the cellular metabolism. Thus, the observed alterations in the marker enzyme levels can be used as sensitive biomarkers for chlorpyrifos contamination. It could also be inferred that chlorpyrifos is a potent metabolic obstructer to *C. idella* even at sublethal concentrations.

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