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Oxidative Stress Responses and Recovery Patterns in the Liver of *Oreochromis niloticus* Exposed to Chlorpyrifos-Ethyl

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Abstract Chlorpyrifos is the most common insecticide in freshwater ecosystems, and detected in agricultural and fishery product. In this study, *Oreochromis niloticus* were exposed to 5, 10 and 15 ppb sublethal concentrations of chlorpyrifos in order to determine the oxidative stress response in liver. Acetylcholinesterase (AChE) activity was significantly inhibited. Superoxide dismutase activity (SOD) increased after 15 days of chlorpyrifos treatments at all concentrations (146.95%, 53.04%, 208.70%, respectively). Malondialdehyde levels were higher than that of the control level after 15 days of 5 ppb (95.65%), 10 ppb (69.56%) and 15 ppb (252.17%) chlorpyrifos treatments. Malondialdehyde levels were also increased ranging from 59.09%, 113.63% to 195.46% after 30 days of 5, 10 and 15 ppb chlorpyrifos exposures. Glutathione S-transferase activity decreased except for 15 days low concentration exposure. Catalase (CAT) activity decreased while there is no significant alteration in glutathione peroxidase activity. After recovery period, the low concentration group of chlorpyrifos provided a protection in AChE activity during recovery, but fish were observed to be unable to overcome the inhibition of AChE activity at high concentration groups. CAT activity remained reduced, SOD activity increased whereas the other biochemical parameters recovered to control levels. Results of this study suggest that chlorpyrifos induces oxidative stress in the liver of *O. niloticus* and this effect is not related with anti-acetylcholinesterase activity of pesticide.

Keywords Insecticide · Oxidative stress · Fish · Liver

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Organophosphorus (OPs) insecticides are widely used in domestic and agricultural purposes for the control of pests. Chlorpyrifos (*O*,*O*-diethyl-*O*,-(3,5,6-trichlor-2-pyridyl) phosphorothioate) is a broad spectrum OP insecticide that has been used in the home and on the farm. Therefore, one can be exposed to these compounds as residues in agricultural products (Ncibi et al. 2008). Chlorpyrifos is also one of the major pesticides detected in fishery products (Sun et al. 2006). Sun and Chen (2008) analyzed marketable fish samples for chlorpyrifos residues and found detectable residues in farmed as well as wild fishes. These authors reported that chlorpyrifos in the farm fish were positively related to its existence in fish feed. The prevalence of chlorpyrifos in the aquatic environment, and their potential for adverse effects, makes it strong candidates for toxicological studies (Xing et al. 2010).

Chlorpyrifos affects nervous system by inhibition of Acetylcholinesterase (AChE) activity. On the other hand, the AChE active sites are widely distributed in liver cytoplasm and nuclei, and the inhibition of this enzyme activity could affect the cell function, metabolism and signal transduction (Achudume et al. 2010). However, the hepatotoxicity of chlorpyrifos is not extensively explored (Ncibi et al. 2008). The liver plays an important role in intermediate metabolism of an individual as well as in the metabolism of xenobiotics. Therefore, it is a good indicator of the health status of fish. The liver is important in oxidative stress studies since lipid peroxidation is a major cause of liver damage (Patlolla et al. 2009). Although increasing numbers of researchers have focused on the oxidative stress responses arising from xenobiotics, the results on fish species were very limited (Jin et al. 2010). Therefore, the present study was planned to determine responses of selected biomarkers of oxidative stress in *Oreochromis niloticus* exposed to chlorpyrifos to evaluate

the mechanism of liver toxicity of this insecticide. We investigated the effects of chlorpyrifos on oxidative stress biomarkers and AChE activity in the liver of *O. niloticus*. The activity of AChE was measured due to its wide usage for rapid detection in predicting early warning of organophosphorus pesticide toxicity (Oruc and Usta 2007). We also considered the reversibility of chlorpyrifos toxicity in pesticide free water.

Materials and Methods

Oreochromis niloticus were obtained from fish culturing pools of Çukurova University and transferred to the laboratory. They were acclimated for at least 2 weeks at 20 ± 2°C, pH 7.4 ± 0.05, dissolved oxygen 8 ± 0.3 mg/L, total hardness 188 mg/L $CaCO_3$ with a 12:12 light:dark photoperiod. Fish were supplied daily with commercial fish food (Pinar, Turkey) at a rate of 3% body weight during acclimation. Animal maintenance and experimental procedures were in accordance with the Guideline of American Public Health Association (1985). The insecticide used in this study was a commercial formulation of Dursban (480 g/L Chlorpyrifos-ethyl, *O*,*O*-diethyl-*O*-(3,5,6-trichlor-2-pyridyl) phosphorothioate, 48% active ingredient). Stock solutions of the test substance were prepared by dissolving the insecticide in test water. These solutions were further diluted to obtain the experimental concentrations in aquaria.

Adult fish exposed to three different sublethal concentrations of chlorpyrifos (5, 10 and 15 ppb chlorpyrifos) for 15 and 30 days after acclimatization period. Subsequently, fish were transferred to pesticide free water for 15 days to determine the potential reversibility of biochemical parameters. Experiments were carried out in duplicate. No mortality occurred under any of these conditions. After each experimental periods, five fish were taken from each tank and decapitated, liver tissues of both control and treated fish were immediately dissected on an ice-cold plate. Tissues were homogenised to a 1/5 (w/v) ratio in 0.25 M pH 7.4 sucrose buffer and then centrifuged at 9500*g* for 30 min at 4°C. The supernatant was used for biochemical analyses.

AChE activity was determined at 412 nm wavelength at 30°C according to the method suggested by Ellman et al. (1961). SOD activity was measured at 505 nm at 37°C and was calculated using inhibition percentage of formazan formation (McCord and Fridovich 1969). The activity of GPx was assayed at 37°C and 340 nm by calculating the difference in absorbance values during the oxidation of NADPH (Beutler 1984). For quantitative determination of CAT activity 1 M Tris–HCl, 5 mM EDTA (pH 8.0), 10 mM H_2O_2 and H_2O were mixed and the rate of H_2O_2 consumption at 230 nm and 37°C was measured (Beutler 1984). GST activity was measured by following the change in absorbance at 340 nm of the substrate, CDNB, conjugated with reduced glutathione (Habig et al. 1974). The enzyme activities were measured as specific activities expressed in units of activity per mg of protein, one unit of which catalyses the formation of 1 μmol of product per minute under each assay conditions. Total protein was quantified according to the method developed by Lowry et al. (1951) using Folin's reagent, and egg albumin as standard. Lipid peroxidation was estimated by the formation of thiobarbituric acid reactive substances and quantified in terms of malondialdehyde (MDA), an end product of lipid peroxidation, according to the method described Ohkawa et al. (1979).

The data were analysed using SPSS statistical package program (SPSS 10.0 for Windows SPSS Inc., Chicago, IL, USA). Statistically significant differences between treatment and control groups were determined by analysis of variance (ANOVA). Significant differences ($p < 0.05$) were reanalyzed by LSD method to determine which one of the individual groups were significantly different from the control. All parameters were expressed as mean ± standard error.

Results and Discussion

All groups tested with chlorpyrifos in this study revealed significant inhibition of AChE activity in the liver of *O. niloticus* (Fig. 1, $p < 0.05$). Ansari and Kumar (1984)

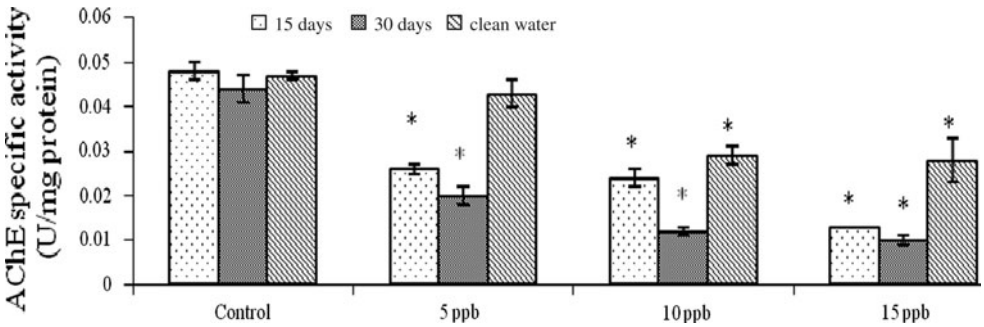

Fig. 1 AChE activity (U/mg protein) in the liver tissue of adult *O. niloticus* exposed to chlorpyrifos and its recovery response. Each value is the mean ± standard error (n = 5). *Asterisk* indicates significant differences between groups and control group ($p < 0.05$)

found similar results after malathion exposure. Specific AChE activity decreased in *P. reticulata* after diazinon exposure (Sharbidre et al. 2011). Methyl parathion exposures also caused a reduction in AChE activity decreased in *O. niloticus* (Tridico et al. 2010). Chlorpyrifos exerts its toxicity by irreversibly binding to a serine hydroxyl group on the AChE resulting in cholinergic over-stimulation (Osterloh et al. 1983). The degree of AChE inhibition in liver of *Tilapia mossambica* in relation to the interacting effects of aging and sublethal concentrations of dichlorvos showed a positive correlation with insecticide concentration and the time of exposure (Rath and Mishra 1981). In the current study, AChE activity in fish exposed to chlorpyrifos did not fully recover except for the low concentration group of chlorpyrifos when the fish were transferred into pesticide-free water. The patterns of inhibition and recovery of AChE activity of exposed fish species can be useful biomarker for better explanation of environmental effects of pesticide. Recovery is a significant increase in the AChE activity that occurs following stopping of exposure to anticholinesterase agents (Kumar et al. 2010). Organophosphorus pesticides are irreversible AChE inhibitors, because the phosphorylation rate of the bound enzyme proceeds at an insignificant rate. Therefore, the inhibitory effects of OPs exposure may be long lasting, with recovery depending on new enzyme synthesis (Habig and Di Giulio 1991). It has been shown that a greater reduction in AChE activity requires longer recovery periods. Chlorpyrifos may bioaccumulate in fish and recovery period should be more prolonged than 15 days to provide de novo synthesis of AChE (Carr et al. 1997). Decreased AChE activity by RPR-II in the liver of *O. mossambicus* was not recovered after 7 days recovery period (Venkateswara Rao 2006). A decreasing AChE activity (79%) was observed in *Gambusia affinis* after monochrotophos exposure, and subsequent recovery indicated that the fish have the ability to overcome the stress of toxicant (Kavitha and Venkateswara Rao 2007). Inhibited AChE activity by chlorfenvinphos recovered after 8 days depuration period about 81% in *C. carpio* (Dembele et al. 2000). Recovery of AChE activity following reduction in exposure to OPs has been found to be a process that takes time depending on factors such as insecticide class, species, the range of AChE inhibition and the bioactivation potential of the anti-cholinesterase insecticides (Morgan et al. 1990; Abdullah et al. 1994). Anticholinesterase activity of chlorpyrifos can be associated with free radical formation. Tsakaris et al.(2000) suggested that the activity of AChE inhibited by free radical formation. Venkataraman et al. (2008) showed that PCB altered cholinergic function by inducing oxidative stress which can be protected by melatonin, considered as a broad spectrum antioxidant that is more powerful than glutathione in neutralizing free radicals and that can protect cell membranes from oxidative tissue damage.

The use of molecular oxygen during aerobic metabolism produces reactive oxygen species which react with biomolecules and damage to cells (Halliwell and Gutteridge 1984). An imbalance between pro-oxidant and antioxidant defense mechanisms result in oxidative stress. The resulting damage may cause cancer, neurodegenerative, and immune system diseases. Pesticides may induce oxidative stress, leading to lipid peroxidation (Kehrer 1993). The key enzymes for the detoxification of reactive oxygen species in all organisms are superoxide dismutase (SOD; EC 1.15.1.1), catalase (CAT; EC 1.11.1.6) and glutathione peroxidase (GPx; EC 1.11.1.9). An important feature of these enzymes is their inducibility under conditions of oxidative stress and such inductions can serve as an important adaptation to these conditions (Di Giulio et al. 1989). Superoxide dismutase activity showed increasing activity after 15 days of chlorpyrifos treatments at all concentrations being 146.95%, 53.04%, 208.70%, respectively, over control values (Fig. 2, $p < 0.05$). Chlorpyrifos did not change SOD activity in the liver of *O. niloticus* after 30 days exposure ($p > 0.05$). After recovery period, enzyme activity was found to be higher than the control level ($p < 0.05$), and SOD activity increased up to at 88.52% 5 ppb, 71.31% at 10 ppb and 175.41% at 15 ppb pesticide treatments. Induction of SOD could occur during

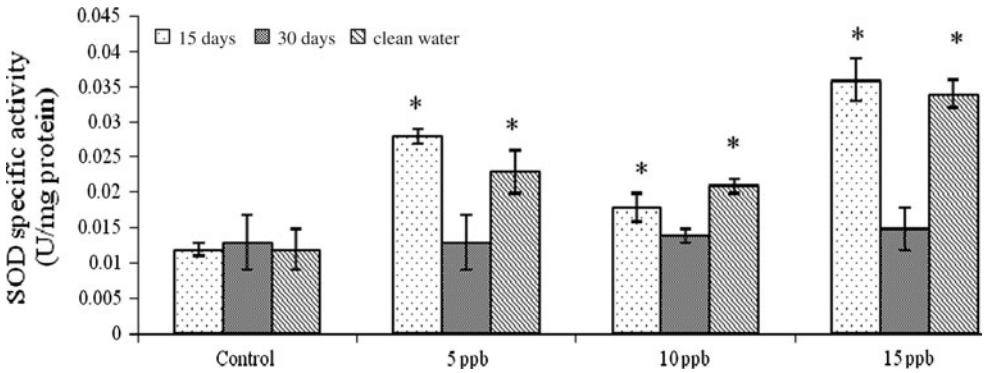

Fig. 2 SOD activity (U/mg protein) in the liver tissue of adult *O. niloticus* exposed to chlorpyrifos and its recovery response. Each value is the mean ± standard error (n = 5). *Asterisk* indicates significant differences between groups and control group ($p < 0.05$)

high production of superoxide anion radical. Therefore, an increase in the SOD activity indicates an increase of superoxide anion radical production and a compensatory upregulation of antioxidant defense mechanisms. SOD and CAT convert superoxide anions into H_2O_2 and then into H_2O and O_2. An induction in these enzyme activities contributes to the elimination of reactive oxygen species following exposure to xenobiotics. Results concerning CAT activity are presented in Fig. 3 ($p < 0.05$). CAT activity was found to decrease in all experimental concentrations and durations. At the end of recovery, CAT activity was observed to be still low. Catalase activity was found significantly decreased in the liver of *Channa punctatus* exposed to deltamethrin (Sayeed et al. 2003). Aminotriazole decreased CAT activity in *Carassius auratus* (Vasylkiv et al. 2011). Deltamethrin exposure also caused significant decreases in CAT activity in liver tissues of *Channa punctatus* (Sayeed et al. 2003). The treatment of menadione led to a decreasing CAT activity in *Ameliurus nebulosus* (Hasspieler et al. 1994). Monochrotophos exposed fish, *G. affinis*, exhibited significant induction in antioxidant enzyme activities and gradually restored to the control levels by day 16th (Kavitha and Venkateswara Rao 2007). Superoxide radicals are known to inhibit catalase activity (Kono and Fridowich 1982; Brainy et al. 1996). The different responses of the CAT activity may indicate different mechanisms for the regulation of gene expression for this enzyme (Zhang et al. 2004).

The Liver GST activity did not show any alteration after low concentration exposure of chlorpyrifos on day 15 of exposure (Fig. 4, $p > 0.05$). However, the activity decreased following 10 ppb and 15 ppb chlorpyrifos exposures at the same duration ($p < 0.05$). After 30 days of exposure to chlorpyrifos, the fish showed a significant decrease in the liver GST activity. The GST activity in fish exposed to chlorpyrifos recoverd at the end of 15 days of recovery period. Our study is in agreement with Mansour and Mossa (2009) observed the significantly decreased level in GST activity after chlorpyrifos exposure. *Carassius carassius* exposed to Roundup decreased GST activity in the liver (Lushchak et al. 2009). GST activity maintain high protection against lipid peroxidation and there is a increasing correlation between susceptibility to toxic substances and GST activity. Glutathione S-transferases are detoxifying enzymes that catalyze the conjugation of a variety of electrophilic substrates to the thiol group of glutathione, producing less toxic form. The significant decrease of GST activity may show insufficient detoxication of insecticide (Mansour and Mossa 2010). The lower GST activity was stated to have been resulted from a decrease in the synthesis of GST proteins at the molecular level, and the loss of GST activity was associated with

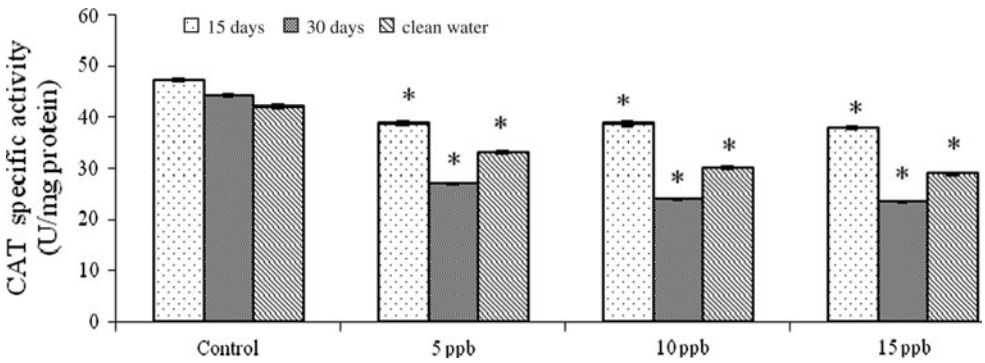

Fig. 3 CAT activity (U/mg protein) in the liver tissue of adult *O. niloticus* exposed to chlorpyrifos and its recovery response. Each value is the mean ± standard error (n = 5). *Asterisk* indicates significant differences between groups and control group ($p < 0.05$)

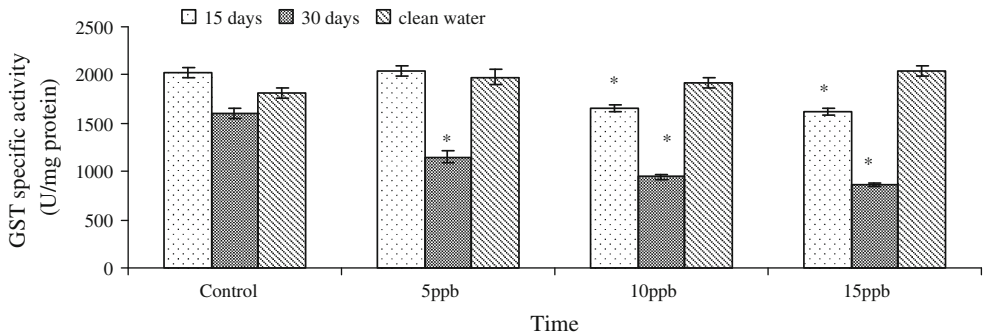

Fig. 4 GST activity (U/mg protein) activity in the liver tissue of adult *O. niloticus* exposed to chlorpyrifos and its recovery response. Each value is the mean ± standard error (n = 5). *Asterisk* indicates significant differences between groups and control group ($p < 0.05$)

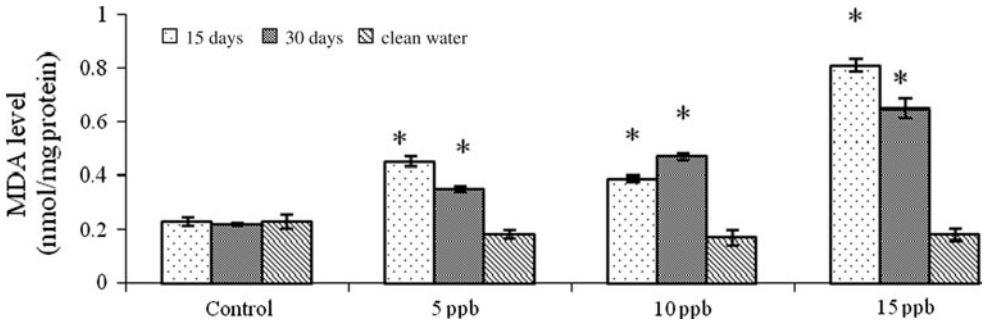

Fig. 5 MDA content (nmol/mg protein) in the liver tissue of adult *O. niloticus* exposed to chlorpyrifos and its recovery response. Each value is the mean ± standard error (n = 5). *Asterisk* indicates significant differences between groups and control group ($p < 0.05$)

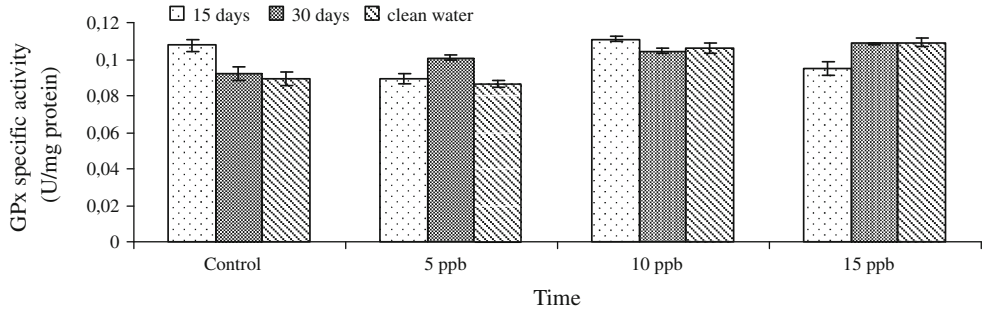

Fig. 6 GPx activity (U/mg protein) in the liver tissue of adult *O. niloticus* exposed to chlorpyrifos and its recovery response. Each value is the mean ± standard error (n = 5). Exposure to sublethal concentrations of chlorpyrifos did not change GPx activity ($p > 0.05$)

down regulation of GST isozyme expression at the m-RNA level (Gallagher and Sheehy 2000).

Malondialdehyde levels were higher than that of control level after 15th days of 5 ppb (95.65%), 10 ppb (69.56%) and 15 ppb (252.17%) chlorpyrifos treatments ($p < 0.05$, Fig. 5). Malondialdehyde levels were also increased ranging from 59.09%, 113.63% to 195.46% after 30 days of 5, 10 and 15 ppb chlorpyrifos exposures. MDA level returned to control level in the liver after recovery period in pesticide free water. Increased MDA level was also found in *Onchorhynchus mykiss* after diazinon exposure (Isik and Celik 2008). Miron et al. (2008) observed a significant increase in lipid peroxidation in *Leporinus obtusidens* after clomazone exposure. This results suggest that a large amount of lipid peroxidation has occured in the exposed fish (Jin et al. 2010). Malondialdehyde, a major oxidation product of peroxidized polyunsaturated fatty acids, has been considered as an important indicator of lipid peroxidation. Giordano et al. (2007) suggested that OPs may lead to promote higher reactive oxygen species (ROS) levels and higher lipid peroxidation. They also stated that cytotoxicity and ROS production of OPs may not be a result of AChE inhibition. Lipid peroxidation is a complex process resulting from free radical reactions in biological membranes, which are rich in polyunsaturated fatty acids. Elevation of lipid peroxidation after pesticide exposures suggests participation of free-radical induced oxidative cell injury in mediating the toxicity of pesticides (Lackner 1998). Lipid peroxidation decreases membrane fluidity, increases the leakiness of the membrane, and lead to complete loss of membrane integrity (Halliwell and Gutteridge 1984).

Glutathione peroxidase activity did not show any alteration due to chlorpyrifos exposure compared with control group (Fig. 6, $p > 0.05$).

The results of this study suggest that chlorpyrifos induces oxidative stress in the liver of *O. niloticus* and this effect is not related with anti-acetylcholinesterase activity of pesticide. The high concentrations of chlorpyrifos increase lipid peroxidation in the liver of *O. niloticus*. However, an adaptive response takes place after exposure to low concentration of chlorpyrifos. With regards to SOD and CAT levels, mainly during the recovery period, significant induction on oxidative stress biomarkers were observed in chlorpyrifos exposed *O. niloticus*. Therefore, SOD and CAT activities can be used as biomarkers in environmentally biomonitoring studies.

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