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The protective role of ascorbic acid (vitamin C) against chlorpyrifos-induced oxidative stress in *Oreochromis niloticus*

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Abstract Ability of ascorbic acid (vitamin C) to attenuate oxidative damage was evaluated in liver and brain tissues of *Oreochromis niloticus* (*O. niloticus*) experimentally exposed to sublethal concentrations of chlorpyrifos (CPF). *O. niloticus* was exposed to sublethal concentrations of CPF at 12 μg/L (CPF1) and 24 μg/L (CPF2) for 96 h. The fish of vitamin C (Vit C) and CPF2 + Vit C groups were fed with Vit C supplemented diet (200 mg Vit C/100 g feed). A significant increase in thiobarbituric acid-reactive substances (TBARS) level ($P < 0.05$) was observed in brain of CPF-exposed fish although liver TBARS level was not changed compared to control group. This result showed that lipid peroxidation (LPO) was elevated in brain of fish exposed to CPF. Glutathione peroxidase (GSH-Px) activity in liver and brain tissues was significantly elevated ($P < 0.05$) by exposure to CPF1 and CPF2. Catalase (CAT) activity was significantly increased ($P < 0.05$) in liver but decreased in brain of treated fish by CPF2 concentration. Superoxide dismutase (SOD) activity was decreased in liver, but increased in brain by exposure to CPF1 and CPF2 concentrations. Levels of TBARS were increased in brain of CPF-treated animals, but tended to decrease by the effect of Vit C. Vit C treatment for CPF-intoxicated animals normalized the otherwise raised activities of GSH-Px, CAT, and SOD within normal limits. The results clearly indicate that exposure to CPF caused a dose-dependent increase in oxidative stress brain and to a lesser extend in liver of fish and the ability of Vit C to attenuate CPF-induced oxidative damage.

Keywords Chlorpyrifos · *Oreochromis niloticus* · Oxidative stress · Antioxidant · Vitamin C

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Introduction

The widespread use of pesticides has resulted in the pollution of many aquatic habitats worldwide. Pesticides enter to the aquatic systems by different ways such as direct application, urban and industrial discharges, including agricultural soil, aerosol, particulate deposition, and rainfall (Galloway and Handy 2003). Organophosphate pesticides (OPs) are the most widely used synthetic chemicals for controlling variety of pests. The main target of OPs action is the central and peripheral nervous system in animals (Kwonq 2002). Besides, many authors claim that these compounds in both acute and chronic intoxication disturb the redox processes, changing the activities of

antioxidative enzymes and causing enhancement of lipid peroxidation (LPO) in many organs in organisms (Kavitha and Rao 2008; Thomaz et al. 2009).

Chlorpyrifos (O,O-diethyl O-3,5,6-trichloro-2-pyridyl phosphorothioate) is an organophosphate pesticide used heavily throughout the world for agriculture and domestic purposes. Chlorpyrifos (CPF) passes via air drift or surface runoff into natural waters, where it is accumulated in different organisms living in water, especially in fish, thus making it vulnerable to several discernible effects (Varo et al. 2002). CPF treatment in previous studies resulted in increased oxidative stress and altered activities of superoxide scavenging enzymes in different tissues of fish (Kavitha and Rao 2008).

Reactive oxygen species (ROS), such as superoxide anion radicals, hydrogen peroxide, and hydroxyl radicals, are continuously formed in oxygen-consuming organisms. Exposure to xenobiotics or toxic chemical pollutants may produce an imbalance between these endogenous and exogenous ROS and can subsequently induce a decrease in antioxidant defenses or cause oxidative damage outright in organisms (Valavanidis et al. 2006). Defence systems that tend to inhibit ROS formation include the antioxidant enzymes such as superoxide dismutase (SOD; EC 1.15.1.1), catalase (CAT; EC 1.11.1.6), and glutathione peroxidase (GSH-Px; EC 1.11.1.9) (Van der Oost et al. 2003). Like other organisms, fish combat elevated levels of ROS with protective ROS-scavenging enzymes (Craig et al. 2007). LPO has also been used as a bioindicator of oxidative damage in aquatic organisms exposed in polluted environmental conditions. The most used assay for LPO is the thiobarbituric acid-reactive substances (TBARS) test (Valavanidis et al. 2006).

Endogenous enzymatic and non-enzymatic antioxidants are essential for the conversion of ROS to harmless metabolites as well as to protect and restore normal cellular metabolism and functions (Bebe and Panemangalore 2003). Vitamin C (Vit C) or ascorbic acid is one of the non-enzymatic antioxidants that can convert ROS to limit their effect, thus preventing tissue damage (Fetoui et al. 2008). Vitamin C has the potential to protect both cytosolic and membrane components of cells from oxidant damage. Several authors have reported that Vit C supplementation in diets for aquatic organisms has prevented the negative effects of stress, minimized toxicity by water contaminants, and increased immune response (Guha et al. 1993; Korkmaz et al. 2009).

Liver plays a central role in the detoxification process and faces the threat of maximum exposure to xenobiotics and their metabolic by-products. It is the major organ of accumulation, biotransformation, and excretion of contaminants. Because of the low levels of antioxidant enzymes and readily oxidizable substances such as polyunsaturated fatty acids and high rate of oxidative metabolic activity, the central nervous system (CNS) is particularly susceptible to the damaging effect of ROS (Mehta et al. 2009). The brain is among the organs most vulnerable because of its high oxygen consumption and because its cell membrane lipids are high in oxidizable polyunsaturated fatty acids (Gupta 2004). Thus, both of tissues are have very susceptible to oxidative stress.

Fish have been widely used as models to evaluate the health of aquatic ecosystems in toxicologic pathology (Peixoto et al. 2006). In the present study, *Oreochromis niloticus* was chosen as an experiment model, because of its wide availability and suitability for toxicity testing (Thomaz et al. 2009). We have studied the effects of sublethal concentrations of chlorpyrifos (12 and 24 μg/L) for 96 h on oxidative stress biomarkers in liver of brain tissues of *O. niloticus* and protective role of vitamin C (ascorbic acid).

Materials and methods

Chemicals

A technical formulation of the organophosphate insecticide chlorpyrifos (O,O-diethylO-3,5,6-trichloro-2-pyridyl phosphorothioate) was used pure of 99.9%. All of chemicals and reagents were purchased from Sigma-Aldrich Chemical Corporation (USA).

Test animals and treatment

Juvenile *O. niloticus* (mean weight: 13.0 ± 0.9 g, mean length: 11.1 ± 1.0 cm) were obtained from Mersin University Fisheries Faculty Aquaculture Department and transferred to laboratory to where the temperature was kept at 24 ± 2°C (12:12 L:D). Throughout the experiments, dechlorinated tap water with pH value of 7.85, an alkalinity of 326 mg/L

$CaCO_3$, and oxygen concentration of 6.70 mg/L was used. The fish were allowed to acclimatize to these conditions for 2 weeks. The fish were fed at a rate of 2% body weight/day with a commercial pellet diet (Çamlı-Yem, İzmir-Turkey) during the acclimation period. The commercial diet contained 44% protein, 18% lipid, 3% cellulose, 12% ash, and 160 mg/kg Vit C.

Experimental diets were prepared in the laboratory from commercial pellet diet. Vit C was obtained as powder from Sigma (L-ascorbic acid 2-phosphata trisodium salt). The Vit C-supplemented diet was prepared by adding 200 mg/100 g to the commercial pellet and then sealing the vitamin to the pellet by spraying 2.5 mL fish oil/100 g feed (Ortuno et al. 2003; Korkmaz et al. 2009). The control diet was prepared by spraying 2.5 mL fish oil/100 g on the commercial pellet diet.

Experiments were conducted in glass aquaria containing 100 L test solution. Fish were exposed to 12 and 24 μg/L sublethal concentrations of chlorpyrifos (Sigma 99.9%) for 96 h. These sublethal concentrations were chosen according to 96-h LC_{50} value previously determined for juvenile *O. niloticus* (98.67 μg/L) (Oruç 2010). Stock solution was prepared by technical chlorpyrifos and diluting it in acetone to give the dosing concentrations. The water was refreshed every 2 days to compensate for the pesticide lost in the exposure medium.

Forty fish were divided into five groups ($n = 8$ in each group). The control group was exposed to acetone at the highest concentration of stock solution used in the chlorpyrifos-exposed groups (the absence of CPF) and was fed with control diet. The vitamin C group (Vit C) was exposed to tap water (the absence of CPF and acetone) and was fed with ascorbic acid-supplemented diet. The chlorpyrifos 1 group (CPF1) was exposed to chlorpyrifos concentration of 12 μg/L and was fed with control diet. The chlorpyrifos 2 group (CPF2) was exposed to 24 μg/L concentration of CPF and was fed with control diet. The chlorpyrifos 2 + Vitamin C group (CPF2 + Vit C) was exposed to 24 μg/L concentration of CPF and was fed with ascorbic acid-supplemented diet.

At the end of exposure period, eight fish were removed from each tank and killed by transaction of the spinal cord. The liver and brain tissues of both control and treated fish were dissected. Tissues were homogenized to 1/5 (w/v) ratio in physiological saline solution (0.8% NaCl) with homogenizer and then centrifuged at 13,500 rpm for 10 min in a Sigma 2–16 K centrifuge at +4°C and supernatant was used for biochemical analyses.

The ethic committee of Mersin University evaluated this study. The number of the ethic committee agreement for animal experimentation is 2011/09.

Measurement of TBARS levels

The levels of TBARS homogenized tissue, as an index of LPO, were determined by thiobarbituric acid reaction using the method of Yagi (1998). Malondialdehyde and other aldehydes when boiled with thiobarbituric acid at acid pH give a pink-colored product that can be assayed spectrophotometrically. Briefly, a 50 μL of tissue homogenate was mixed with 750 μL of TBARS reagent. The mixture was incubated for 30 min in a boiling water bath. After cooling, the mixture was centrifuged at 3,500 rpm for 15 min. Absorption was measured at 532 nm, and the values are expressed as nanomoles of TBARS/mg protein.

Enzyme assays

The total GSH-Px activity was assayed by Jocely method (1970), using H_2O_2 and NADPH as substrates. The conversion of nicotinamide adenine dinucleotide phosphate (NADPH) to nicotinamide adenine dinucleotide phosphate (NADP) was followed by recording the changes in absorption intensity at 340 nm, and one unit of GPx activity was defined as the amount of protein that oxidizes 1molar NADPH per min and is expressed as unit of tissue protein content.

The CAT activities of tissues were determined according to the method of Aebi (1974). The enzymatic decomposition of H_2O_2 was followed directly by the decrease in absorbance at 240 nm. The difference in absorbance per unit time was used as a measure of CAT activity.

SOD activity was measured by the inhibition of nitroblue tetrazolium (NBT) reduction due to O_2 generated by the xanthine/xanthine oxidase system (Sun et al. 1988). One unit of SOD activity was defined as the amount of protein causing 50% inhibition of the NBT reduction rate. The reduction in NBT by superoxide anion to blue formazan was

measured at 560 nm. The enzyme activities are given in U/mg protein.

Tissue protein contents were measured only to determine the specific activity of antioxidant enzymes according to the method developed by Lowry et al. (1951) using bovine serum albumin as standard. Absorbances of samples were measured at 750 nm wavelength by spectrophotometer.

Statistical analysis

Data were expressed as mean ± standard error (SE) and analyzed using with SPSS 10.0 for Windows computer program. ANOVA and Duncan's multiple range tests were used to analyze differences between groups. The differences were defined as statistically significant when $P < 0.05$.

Results

In this experiment, no mortality was observed. The levels of TBARS in tissues are given in Fig. 1. There is no significant change in tissues TBARS content in Vit C group compared to control. The liver TBARS levels of exposed fish were not significantly changed in CPF1 and CPF2 concentrations as compared with control (Fig. 1). Also, CPF2 + Vit C group was not significantly affected compared with the other groups. But, TBARS levels of brain tissue were significantly increased by 41% in CPF1 group and 130% in CPF2 group ($P < 0.05$) compared to after 96-h exposure. The administration of Vit C in CPF2 + Vit C group decreased TBARS contents in brain tissue compared to CPF2 group without reaching control values.

The GSH-Px activities of fish are shown in Fig. 2. Vit C alone has not produced any significant changes in both tissue activities of GSH-Px compared to control. Exposure to concentrations of pesticide significantly increased ($P < 0.05$) by 49% in CPF1 group and 89% in CPF2 group in liver, 86% in CPF1 group, and 179% in CPF2 group in brain at the end of the 96 h. Treatment with Vit C tends to regulate the activity of enzyme as compared with CPF2-intoxicated fish, normalize in liver (−33%, $P < 0.05$), and reduce significantly in brain (−35%, $P < 0.05$) without reaching control values (Fig. 2).

The CAT activities in tissues of fish are given in Fig. 3. Vit C alone did not produce any significant changes in tissue CAT activities as compared with control. CAT activity in liver of fish was significantly increased (35%, $P < 0.05$) by exposing the highest concentration of CPF (24 μg/L) at the end of the 96 h, while at lower concentration of CPF, there were no significant changes compared to control (Fig. 3). CAT activity of brain was significantly decreased (−52%, $P < 0.05$) with effect of 24 μg/L concentration as compared with control. Administration of Vit C decreased (−14%, $P < 0.05$) in liver without

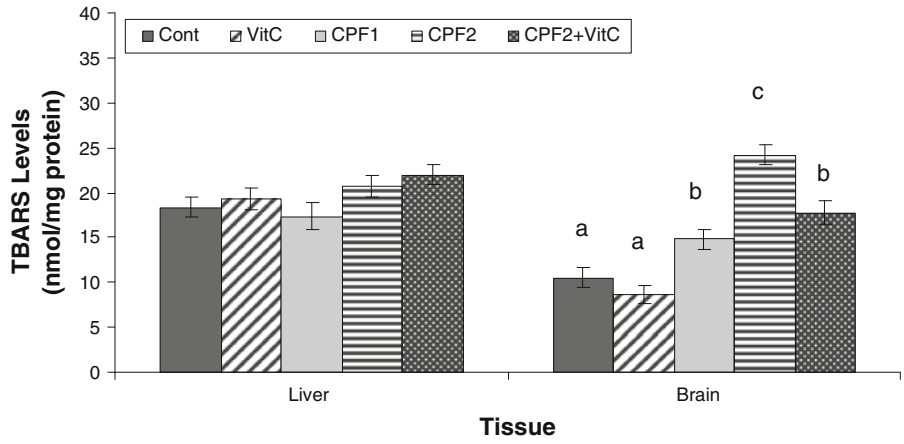

Fig. 1 TBARS levels in liver and brain tissues of *O. niloticus* exposed to sublethal concentrations of chlorpyrifos: 12 (CPF1) and 24 μg/L (CPF2), with or without a dietary supplementation of vitamin C. Each value is the mean ± SE ($n = 8$). Multiple comparisons were made separately for each tissue and means with *different superscript* in tissues are significantly different ($P < 0.05$)

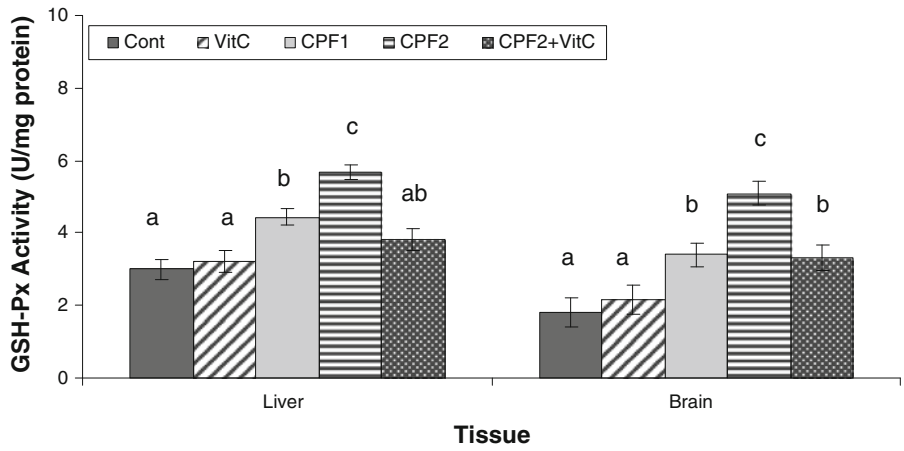

Fig. 2 GSH-Px activity in liver and brain tissues of *O. niloticus* exposed to sublethal concentrations of chlorpyrifos: 12 (CPF1) and 24 μg/L (CPF2), with or without a dietary supplementation of vitamin C. Each value is the mean ± SE ($n = 8$). Multiple comparisons were made separately for each tissue and means with *different superscript* in tissues are significantly different ($P < 0.05$)

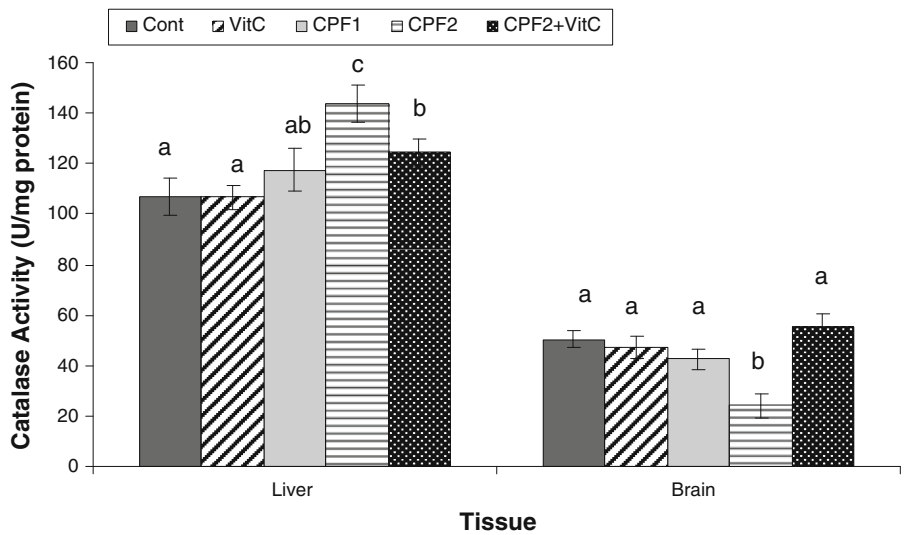

Fig. 3 CAT activity in liver and brain tissues of *O. niloticus* exposed to sublethal concentrations of chlorpyrifos: 12 (CPF1) and 24 μg/L (CPF2), with or without a dietary supplementation of vitamin C. Each value is the mean ± SE ($n = 8$). Multiple comparisons were made separately for each tissue and means with *different superscript* in tissues are significantly different ($P < 0.05$)

reaching control values and improved CAT activity in brain of CPF2 + Vit C group.

The SOD activities in tissues of fish are shown in Fig. 4. Vit C alone did not make any significant changes in tissues SOD activities compared to control. 12 and 24 μg/L concentrations of CPF caused a significant inhibition by 29 and 52% ($P < 0.05$), respectively, in liver of fish. Administration of Vit C improved this enzyme activity in CPF2 + Vit C group compared to CPF2 group. After 96-h exposure, the increased values observed were 112 and 62% ($P < 0.05$) over control values at increased concentrations of CPF, respectively, in brain of fish. Also, administration of Vit C reduced this enzyme activity in CPF2 + Vit C group compared to CPF2 group without reaching control values.

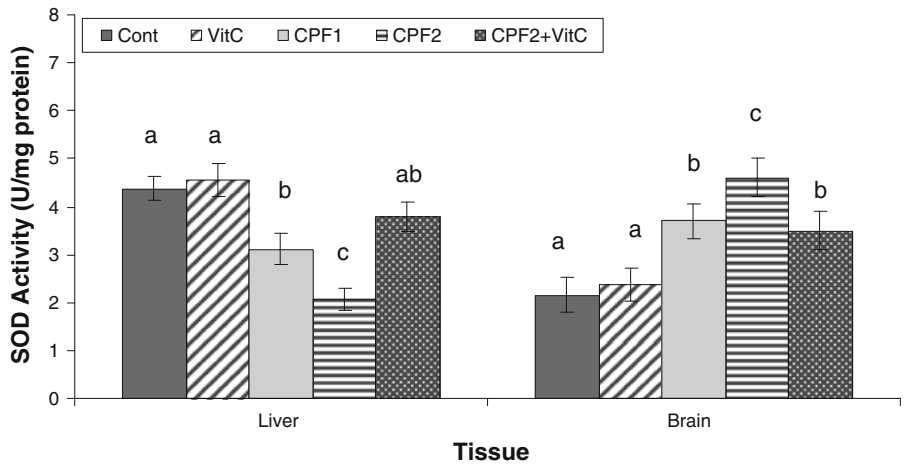

Fig. 4 SOD activity in liver and brain tissues of *O. niloticus* exposed to sublethal concentrations of chlorpyrifos: 12 (CPF1) and 24 μg/L (CPF2), with or without a dietary supplementation of vitamin C. Each value is the mean ± SE ($n = 8$). Multiple comparisons were made separately for each tissue and means with *different superscript* in tissues are significantly different ($P < 0.05$)

Discussion

In the present study, TBARS level was not changed in liver although a significant increase was seen in brain of fish exposed to CPF concentrations for 96 h. LPO is one of the main processes induced by oxidative stress and the first step of cellular damage caused by OP insecticides (Kavitha and Rao 2008). The induction of ROS could increase the oxidation of polyunsaturated fatty acids and lead to peroxidation (Zhang et al. 2008). Monteiro et al. (2006) found that hepatic LPO content did not vary significantly after methyl parathion treatment in *Brycon cephalus*. These authors supposed that this organ resist to the oxidative stress by antioxidant mechanisms preventing LPO increases. Hai et al. (1997) reported that level of LPO was elevated in liver and brain of catfish (*Ictalurus nebulosus*) exposed to dichlorvos. Also, increased level of LPO products in rat brain after exposure with a toxic OP compound such as CPF was reported earlier by Mehta et al. (2005) and Verma et al. (2007, 2009). The increase in LPO products shows that the pesticide induces ROS that are not totally scavenged by the antioxidant enzymes (Ballesteros et al. 2009). In this study, increase of TBARS level in brain is a result of LPO induction in this tissue. Liver is known as the most important target organ of metabolism and detoxification for many toxicants, whereas the brain may be particularly susceptible to oxidative damage because it contains a large amount of polyunsaturated fatty acids (Song et al. 2006). In the previous study, it was found that brain was a more sensitive target organ to oxidative damage than liver (Song et al. 2006; Zhang et al. 2008).

In this study, we found that GSH-Px activities were increased in liver and brain tissues of CPF-treated fish. Similar results have also been reported in other fish species exposed to pesticides. Hai et al. (1997) showed that GSH-Px activation is enhanced (approximately 200%) in brain of *Cyprinus carpio* exposed to dichlorvos. Likewise, GSH-Px activities were elevated (57%) in the livers of *C. auratus* exposed to 2,4-dichlorophenol (Zhang et al. 2004) and to malathion (Huculeci et al. 2008). The biological function of GSH-Px is to reduce H_2O_2 and lipid hydroperoxides (Verma et al. 2007). These data in our study suggest that CPF treatment may result in increased formation of oxygen-free radicals that could stimulate GSH-Px activity to cope with this increased oxidative stress and protecting membranes from damage due to LPO products (Van der Oost et al. 2003).

In the present study, we observed an increase in CAT activity following 96 h of toxicity by CPF concentrations but a decrease SOD activity of liver tissue. These results are parallel to the results of many authors. Zhang et al. (2004) observed that CAT and GSH-Px activities in the liver of *C. auratus* were

increased although SOD activity was inhibited gradually with 2,4-dichlorophenol concentration increasing. Thomaz et al. (2009) reported that CAT activity was increased and SOD activity was decreased in liver of *O. niloticus* exposed to the insecticide trichlorfon for 96 h. Lushchak et al. (2009) found that the activity of CAT in liver and kidney of *C. auratus* was elevated by exposure to glyphosate. Exposure to methyl parathion resulted in a significant induction of CAT activity in *Brycon cephalus* liver (Monteiro et al. 2006). CAT and SOD enzymes have related functions. SOD catalyzes the dismutation of the superoxide anion radical to H_2O and H_2O_2, which is detoxified by both CAT and GSH-Px activities. Due to the inhibitory effects on ROS formation, the SOD–CAT system provides the first defense line against oxygen toxicity and usually used as an indirect biomarker indicating ROS production (Pandey et al. 2003; Van der Oost et al. 2003). An increase in CAT enzyme activity is probably a response toward increased ROS generation in pesticide toxicity (Monteiro et al. 2006). Usually, an induction of hepatic SOD activities was observed when exposed to organic pollutant (Palace et al. 1996); however, the excess production of superoxide radicals or after their transformation to H_2O_2 causes an oxidation of the cysteine in the enzyme and deactivates SOD (Dimitrova et al. 1994). The decreased antioxidant enzymes result in increased oxidative stress, an indication of impaired antioxidant defense mechanism due to excessive generation of free radicals generated by CPF (Kavitha and Rao 2008). The toxicity of CPF in present study may be caused by the unbalance between free radicals and antioxidants, which might have resulted in inhibition of SOD activity.

In the present study, we observed low CAT activity following 96 h of toxicity by CPF concentration in brain tissue although SOD activities increase. Similarly, to our study, Bagnyukova et al. (2005) reported a reduction of CAT activity by a systemic herbicide, 3-amino 1,2,4-triazole in the brain of *Carassius auratus* and the activity of GSH-Px increased. The authors suggested that this increase might represent a compensatory response to lowered CAT activity, and these changes could provide compensatory mechanisms for detoxifying H_2O_2 or elevated amounts of hydroperoxides. The increased activities of GSH-Px suggest that free radical scavenging processes in the cell are generally cooperative as CAT and GSH-Px combine to metabolize H_2O_2 produced by different substrates. Our study indicated a significant elevation in the activity of GSH-Px, but decreased activity of CAT in brain CPF-exposed fish. Increase of GSH-Px activity in brain could appear as a result of LPO induction in this tissue, which is more susceptible to ROS than other organs with higher polyunsaturated fatty acid contents (Ballesteros et al. 2009). In present study, CAT inhibition in brain of fish exposed to CPF might lead to increased concentrations of H_2O_2 and result in increased LPO intensity due to elevated production of hydroxyl radicals (Ballesteros et al. 2009). Induction of SOD in brain tissue of fish could be an adaptive response to the toxicant stress and to neutralize the impact of ROS generated (Hussain 2008).

The activity of the antioxidant enzymes could be increased or inhibited by xenobiotic exposure depending on the intensity and the duration of the stress applied, as well as the susceptibility of the exposed species. It is not a general rule that an increase in xenobiotic concentrations induces antioxidant activity (Oruç and Usta 2007). Antioxidant enzymes also show tissue-specific differences in activities that reflect the functions of the tissues and the oxidative stress load that they experience (Lushchak et al. 2009). In the present work, the studied enzymes responded in a different level in liver and brain tissues. The response of antioxidant system to oxidative stress in various tissues shows differences from one species to another due to the differences in free radical generation and different antioxidant potential of these tissues.

The results of the present study indicate that activities of GSH-Px, CAT, and SOD in liver and brain of fish were altered by CPF and were normalized by Vit C supplementation. Also, the present results showed that vitamin C treatment for CPF-intoxicated fish improved level of TBARS in brain tissue. Korkmaz et al. (2009) and Datta and Kaviraj (2003) evaluated efficiency of supplementation of ascorbic acid to remove stress of pesticides (cypermethrin and deltamethrin) in tissues of *O. niloticus* and *Clarias gariepinus,* respectively. El-Gendy et al. (2010) showed that Vit C improved imidacloprid-induced oxidative damage by decreasing LPO and altering antioxidant defence system in liver of mice. Fetoui et al. (2008) reported that dietary supplementation of Vit C protected against increased LPO in brain of rats.

The cell has several ways to alleviate the effects of oxidative stress either by repairing the damage or by directly reducing the pro-oxidative state via enzymatic and non-enzymatic antioxidants (Korkmaz et al. 2009). Vit C acts as an electron donor (antioxidant) in non-enzymatic reactions and therefore a reducing agent. Thus, Vit C is called an antioxidant because it prevents other compounds from being oxidized. It can reduce the initiating ROS so that initial or continued lipid peroxidation is inhibited (Padayatty et al. 2003). Consequently, Vit C could ameliorate the tissues damage induced by CPF exposure in this study.

Conclusion

The results indicated the potential effects of CPF to induce oxidative damage in liver and brain tissues of *O. niloticus* and the ability of Vit C to attenuate CPF-induced oxidative damage. Compared to liver, brain was more sensitive to the oxidative damage. The increased LPO and alterations in the antioxidant defense system can be used as biomarkers in the pesticide-contaminated aquatic streams. The increased or decreased levels of antioxidant enzyme (GSH-Px, CAT, and SOD) activities and increased level of TBARS in the exposed fish can also be effectively used for better assessment of chlorpyrifos toxicity in biomonitoring of aquatic environment. However, more experiments are needed to validate these parameters as biomarkers of oxidative stress in large-scale environmental monitoring programs. In conclusion, Vit C could be able to improve CPF-induced oxidative stress by decreasing lipid peroxidation and altering antioxidant defense system in tissues. Thus, dietary supplementation of Vit C may be useful in aquaculture that is occupationally exposed to insecticides. Suggesting that vitamin C may be beneficial in preventing CPF-induced oxidative stress.

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