Permethrin-induced oxidative damage in liver of rainbow trout (*Oncorhynchus mykiss*) and its attenuation by vitamin C

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Summary

The objective of this study was to investigate the propensity of permethrin (PTN) to induce oxidative stress and changes in enzyme activities in liver of rainbow trout and its possible attenuation by vitamin C. Forty-eight fish were randomly assigned to 1 of 6 treatment groups and their livers were used for liver perfusion method: control (0 μgL^{-1} permethrin and 0 mgL^{-1} vitamin C), PTN-0.16 (0.16 μgL^{-1} permethrin), PTN-0.32 (0.32 μgL^{-1} permethrin), PTN-0.64 (0.64 μgL^{-1} permethrin), Vit. C (17.2 mgL^{-1} vitamin C), and PTN-0.64 + Vit. C (0.64 μgL^{-1} permethrin and 17.2 mgL^{-1} vitamin C). Results obtained showed that permethrin significantly ($P<0.05$) increased ALT, AST and LDH activities in the liver perfusion medium and malondialdehyde (MDA) level in liver tissue. The values of reduced glutathione (GSH) and total antioxidant capacity (FRAP) in the liver tissue were significantly decreased due to permethrin administration. Pearson's correlation analysis revealed a positive correlation between MDA concentration and ALT, AST and LDH activities in the permethrin groups, suggesting that the enhanced lipid peroxidation may be linked to hepatic damage caused by permethrin. On the other hand, treatment with vitamin C in the PTN-0.64 + Vit. C group increased the values of GSH and FRAP, and decreased the level of MDA and the activities of hepatic enzymes, when compared to the PTN-0.64 group. The present study revealed that vitamin C could ameliorate permethrin-induced oxidative damage by decreasing lipid peroxidation and altering antioxidant defense system in liver of rainbow trout.

Key words: Liver, Oxidative stress, Permethrin, Rainbow trout, Vitamin C

Introduction

Pyrethroid insecticides can enter the aquatic environment through different processes such as atmospheric deposition, river runoff and municipal treatment discharges (Alonso *et al*., 2012). Unlike most animals, in which pyrethroids have a short life and are readily metabolized, fish are reported to be deficient in enzymes that hydrolyze these insecticides (Haya, 1989). Pyrethroids have been shown to be lethal to fish at concentrations 10-1000 times lower than the corresponding values for animals and birds (Ural and Saglam, 2005). Permethrin is a synthetic pyrethroid pesticide with high insecticide potency and low mammalian toxicity. Permethrin is one of the most frequently found pyrethroids in urban and agricultural watersheds (Delgado-Moreno *et al*., 2011). Environmental concentrations of permethrin dissolved in surface water have been found in excess of 17 µg/L (Delgado-Moreno *et al*., 2011); a concentration more than the reported 96 h LC_{50} for rainbow trout (6.43 μgL^{-1}, Kumaraguru and Beamish, 1981).

The pesticide toxicity in fish may be related to an increased production of reactive oxygen species (ROS) leading to oxidative damage (Oruc *et al*., 2010). Under normal physiological conditions, ROS are rapidly eliminated in fish and other vertebrates by antioxidant system consisting of antioxidant enzymes and non-enzymatic substances like reduced glutathione (GSH) and vitamin C (Jin *et al*., 2011; Heidarpour *et al*., 2012). Oxidative stress and changes in antioxidants and lipid peroxidation levels have been reported in different fish species, following exposure to cypermethrin and deltamethrin (Uner *et al*., 2001; Sayeed *et al*., 2003; Sentürk *et al*., 2009; Jin *et al*., 2011).

The role of vitamin C in neutralizing free radicals has been attributed to its property of being water soluble, allowing it to work both inside and outside cells to combat free radical damage (Badgujar *et al*., 2015). Most fish species cannot synthesize vitamin C, and have to depend on external sources to meet their needs. The vitamin C requirement for normal growth and survival is quite low; however, a higher level is required to improve the stress resistance of fish (Garcia *et al*., 2007). The supplementation of ascorbic acid at higher doses could counter the adverse effects of the pesticides in *Clarias gariepinus* (Datta and Kaviraj, 2003) and *Catla catla* (Vani *et al*., 2011). Few studies have evaluated the protective role of vitamin C against metal-induced oxidative stress in fish species (Vijayavel *et al*., 2006; Harabawy and Mosleh, 2014). Isolated perfused fish livers have been used to study a variety of hepatic

functions. In contrast to alternative *in vitro* preparations (e.g., liver slices, isolated hepatocytes, or subcellular fractions), isolated perfused livers retain their three-dimensional structure-function. Advantages of this technique over whole-animal sampling methods include control over perfusate composition and flow rate, and the ability to isolate hepatic functions from systemic influences (Nichols *et al*., 2009). A review of the literature reveals that there is a paucity of information on permethrin-induced oxidative stress and its effect on liver tissue in fish. Permethrin was chosen for study as it is the most commonly used pyrethroid in agriculture and has been found in the aquatic environment at concentrations potentially detrimental to fishes (Delgado-Moreno *et al*., 2011).

The aim of this study, therefore, was to assess the impact of exposure to permethrin on liver tissue in rainbow trout by liver perfusion method. Whether oxidative stress is a mechanism involving hepatic disrupting effects of permethrin was also studied. An attempt has also been made to assess protective effects of vitamin C on permethrin-induced oxidative stress.

Materials and Methods

Chemicals

All reagents used in the present study were of the highest quality available and were obtained from Sigma Aldrich (Germany) or Merck (Germany).

Animals

The fish, rainbow trout (*Oncorhynchus mykiss*), weighing approximately 500 g were purchased from commercial sources and acclimatized in the laboratory at 11 ± 2°C with 12 h:12 h light and dark photoperiods before being used for experiments. Under this condition, the animals were acclimatized and starved for 48 h prior to the experiment.

Experimental design

Forty-eight fish were weighed and randomly divided into six groups (n=8 fish/dose group). The doses and exposure schedule is given below in the Table 1. The dose of vitamin C was selected based on the dose that was seen to be most effective in lowering toxicity induced by pyrethroids (Datta and Kaviraj, 2003). For this purpose, the food intake was registered 3 days before food deprivation. Based on recommendations for the most effective dose of vitamin C (1000 $mgkg^{-1}$ diet) and registered food intake (12.4 ± 0.75 g of food kg^{-1} body weight), the final concentration of vitamin C in perfusate solution (360 ml) in a fish weighing 500 g was calculated as 17.2 mgL^{-1}. The doses of permethrin were based on its LC_{50} of 6.43 μgL^{-1} in rainbow trout (Kumaraguru and Beamish, 1981). Thus, three test doses of permethrin were 0.16 μgL^{-1} (1/40th LC_{50}), 0.32 μgL^{-1} (1/20th LC_{50}) and 0.64 μgL^{-1} (1/10th LC_{50}). The protective effect of vitamin C was evaluated against high dose of permethrin (0.64 μgL^{-1}).

Liver perfusion

The perfusion technique was an open recirculating system which perfuses at constant pressure. Two funnels with tubings were connected to the liver via a three-way stopcock. The preparation of perfusate solutions, surgical procedure and perfusion protocol were performed based on Brett *et al*. (1998). Total perfusion period was 120 min.

Table 1: Doses and exposure schedule for *in vitro* hepatotoxicity of permethrin in liver perfusion method

Treatment	Dose/Concentration	Period of exposure
Control	0 μgL^{-1} + 0 μgL^{-1}	120 min
PTN-0.16	0.16 μgL^{-1}	120 min
PTN-0.32	0.32 μgL^{-1}	120 min
PTN-0.64	0.64 μgL^{-1}	120 min
PTN-0.64 + Vit. C	0.64 μgL^{-1} + 17.2 mgL^{-1}	120 min
Vit. C	17.2 mgL^{-1}	120 min

PTN: Permethrin

Sample collection

In all experiments, the liver was allowed to equilibrate in the perfusion system for 30 min before treatment was initiated. The perfusate was collected at the end of perfusion period (120 min) from a polyethylene tube (PE-240). The liver was also collected at the end of the exposure period and used for oxidative stress evaluation.

Hepatic enzymes

Lactate dehydrogenase (LDH), alanine transaminase (ALT), and aspartate transaminase (AST) activities in the perfusion medium were measured using commercial kits (Pars Azmoon, Iran).

Oxidative stress estimation

At the end of the exposure period, liver was collected and used for oxidative stress evaluation. Liver homogenates were prepared in chilled phosphate buffer saline (PBS), pH = 7.4 (10% w/v) and 0.02 M ethylenediamine tetraacetic acid (EDTA) (used for GSH estimation only) under ice cold conditions. The homogenates were centrifuged at 4000 g for 10 min to yield a supernatant that was used for the assay of oxidative stress related parameters.

The concentration of malondialdehyde (MDA), as a marker of lipid peroxidation in liver was determined as thiobarbituric acid reactive substances according to Placer *et al*. (1966). The concentration of GSH was determined in the homogenate using dithionitrobenzoic acid (DTNB) method. The total antioxidant capacity of the homogenized tissues was measured using ferric reducing antioxidant power (FRAP) assay (Benzie and Strain, 1996).

Statistical analysis

Statistical analysis was conducted using SPSS for windows (release 16, SPSS Inc., Chicago, IL) with a p-value of <0.05 as statistically significant. Data were expressed as mean ± standard deviation (SD). One way

ANOVA was used to compare means among the different groups. Following analysis of variance, significant between-group differences were detected by the Tukey's post-hoc test. For determination of the correlation between oxidative damage marker (MDA) and hepatic enzymes in permethrin groups (PTN-0.16, PTN-0.32 and PTN-0.64), Pearson's method analysis was performed on the paired data obtained by permethrin received groups.

Results

Effect on hepatic enzymes

Significant and dose dependent increase ($P<0.05$) in the AST and ALT activities was observed in liver perfusates exposed to different doses of permethrin as compared to control. The activity of LDH was increased in the liver perfusates exposed to medium and high doses of permethrin when compared with control. Treatment with vitamin C in the PTN-0.64 μgL^{-1} group caused significant reduction ($P<0.05$) in the activities of ALT, AST and LDH. The activity of hepatic enzymes in the perfusate of liver that received PTN-0.64 + vitamin C was comparable to that of control (Table 2).

Effect on lipid peroxidation

MDA level was significantly ($P<0.05$) higher in the permethrin-treated groups (PTN-0.16, PTN-0.32 and PTN-0.64), when compared with control. MDA concentration increased in a dose dependent manner, the increase being significantly higher at ($P<0.05$) PTN-0.64 group when compared to PTN-0.16 and PTN-0.32 groups. Concurrent administration of vitamin C in PTN-0.64 dose group caused significant decrease ($P<0.01$) in the MDA content compared to PTN-0.64 alone treated dose group. MDA content in the liver tissue of PTN-0.64 + vitamin C group was comparable to that of control (Table 3).

Effect on GSH

Permethrin treatment (at all three doses) significantly decreased ($P<0.05$) GSH level in the liver. GSH level in the PTN-0.64 group was significantly lower ($P<0.05$) than that in the PTN-0.16 and PTN-0.32 groups. Conversely, GSH level in the liver tissue was significantly ($P<0.05$) restored following administration of vitamin C against PTN-0.64 group. Its concentration in the PTN-0.64 + vitamin C group was comparable to that of control (Table 3).

Effect on total antioxidant capacity

FRAP level of liver was significantly decreased ($P<0.05$) at medium and high doses of permethrin as compared to control. FRAP was decreased in dose dependent manner, the decrease being highly significant ($P<0.05$) at PTN-0.32 and PTN-0.64 doses when compared to PTN-0.16 group. Concurrent administration of vitamin C in the PTN-0.64 + vitamin C group resulted in significant increase ($P<0.05$) in the FRAP level. FRAP level in PTN-0.64 + vitamin C group was comparable to that of control group (Table 3).

Correlation between oxidative stress markers and hepatic enzymes

Pearson's correlation (r) analysis of the paired data obtained by permethrin received groups revealed the existence of a significant positive correlation between MDA concentration and the activities of AST ($r=0.571$, $P=0.004$), ALT ($r=0.567$, $P=0.004$) and LDH ($r=0.545$, $P=0.006$).

Discussion

The results of the present study indicate that permethrin in the liver of rainbow trout causes specific effects on the antioxidant defense mechanisms (reduction of FRAP and GSH). These results are in agreement with Sayeed *et al*. (2003) and Sentürk *et al*. (2009) who have reported reduction in antioxidants in pyrethroids-exposed fish. These antioxidants are likely consumed as free radical scavengers during the oxidative process in the permethrin-exposed fish. GSH is an important intracellular antioxidant that protects cells against endogenous and exogenous oxidants (Parke and Piotrowski, 1996). GSH with its -SH group functions as a catalyst for disulfide exchange reactions, and

Table 2: Hepatic enzyme activities in liver perfusate of rainbow trout exposed to permethrin and vitamin C

Hepatic enzymes	Control	PTN-0.16	PTN-0.32	PTN-0.64	Vit. C	PTN-0.64 + Vit. C
AST (IU/L)	125.6 ± 24.1^{a}	191.2 ± 16.0^{b}	234.7 ± 22.2^{c}	302.0 ± 38.8^{d}	132.6 ± 15.3^{a}	142.1 ± 27.9^{a}
ALT (IU/L)	14.5 ± 4.5^{a}	30.1 ± 4.1^{b}	49.3 ± 11.2^{c}	86.1 ± 15.9^{d}	13.0 ± 4.0^{a}	12.5 ± 3.5^{a}
LDH (IU/L)	386.9 ± 43.2^{a}	438.1 ± 64.1^{a}	511.7 ± 42.1^{b}	594.6 ± 28.6^{c}	423.7 ± 24.3^{a}	407.7 ± 25.8^{a}

Values (mean±SD, n=8) bearing at least one common superscript letter in the same row do not differ significantly between groups at $P<0.05$

Table 3: Oxidative stress markers in liver tissue of rainbow trout exposed to permethrin and vitamin C

Oxidative stress markers	Control	PTN-0.16	PTN-0.32	PTN-0.64	Vit. C	PTN-0.64 + Vit. C
MDA (nmol/mg)	9.4 ± 1.2^{a}	18.8 ± 2.4^{bc}	25.5 ± 11.5^{cd}	32.1 ± 5.5^{d}	10.4 ± 2.4^{ab}	10.5 ± 3.9^{ab}
GSH (nmol/mg)	44.4 ± 8.8^{a}	26.6 ± 6.3^{b}	18.5 ± 3.8^{bc}	10.9 ± 4.7^{c}	42.6 ± 13.3^{a}	43.7 ± 16.2^{a}
FRAP (nmol/mg)	286.9 ± 48.4^{a}	227.1 ± 33.8^{a}	147.0 ± 20.2^{b}	92.1 ± 20.0^{b}	291.4 ± 64.2^{a}	282.3 ± 68.7^{a}

Values (mean±SD, n=8) bearing at least one common superscript letter in the same row do not differ significantly between groups at $P<0.05$

contributes in H_2O_2 detoxification. GSH plays a major role in antagonizing the oxidative action of the herbicides or insecticides (Parke and Piotrowski, 1996). Depletion of GSH by oxidants may alter the redox status of the cell and present a stressful and toxic situation. Falcioni *et al*. (2010) showed the primary role of GSH in maintaining a redox cytosol status following oxidative stress induced by permethrin in rats. Furthermore, a previous work showed that a reduction of GSH content in rat liver and kidney led to higher oxidative damage (Hashimoto *et al*., 2008).

The induction in the levels of MDA in the present study is in agreement with the study performed by others, who indicated that MDA levels were significantly increased in all tissues, namely liver, kidney, and gill of fish species following exposure to cypermethrin and deltamethrin (Uner *et al*., 2001; Sayeed *et al*., 2003). Lipid peroxidation of the liver also increased in permethrin-treated fish (*Sciaenops ocellatus* and *Fundulus heteroclitus*) compared to control animals after 24 h (Parent *et al*., 2011). High lipid peroxidation may be due to oxidation of molecular oxygen to produce superoxide radicals. This reaction is also the source of H_2O_2, which causes the production of MDA by initiating the peroxidation of unsaturated fatty acids in the membrane (Uner *et al*., 2001). The significant depletion of GSH and FRAP and marked elevation in MDA content in the liver tissues exposed to permethrin clearly suggest oxidative stress. Oxidative stress on the liver of rainbow trout increased with increasing concentration of permethrin, because the highest value of MDA and the lowest values of GSH and MDA were observed in the liver tissues exposed to high dose of permethrin (0.64 μgL^{-1}).

In the present study, an increase of hepatic enzymes was observed in the liver perfusate of rainbow trout after exposure to permethrin. In agreement with the present study, increased hepatic enzymes activities following cypermethrin and deltamethrin exposure have been reported in rainbow trout (Velisek *et al*., 2006; Velisek *et al*., 2007). The increase in hepatic enzymes activity of fish exposed to pyrethroids was interpreted to reflect hepatocellular damage in response to the toxicant (Devi and Gupta, 2014). Hence, cellular damage caused by permethrin was accompanied by increasing cell membrane permeability and enzyme leakage. It is obvious that we noted a high level of MDA coupled with increase in the activities of hepatic enzymes in the liver perfusate. The positive correlation between MDA concentration and ALT, AST and LDH activities in permethrin-exposed groups suggests that the enhanced lipid peroxidation may be linked to hepatic damage. These data are also supported by previous studies where oxidative processes were involved in oxidative damage of cells following permethrin treatment (Falcioni *et al*., 2010).

Reduction in the level of vitamin C has been reported following treatment of pyrethroids. The depletion of vitamin C is probably a defensive reaction of fish to combat stress produced by the pesticide. Thus, the requirement for vitamin C is increased during stress (Datta and Kaviraj, 2003). The obtained data indicated that the treatment with vitamin C reduced the amounts of MDA and maintained the levels of antioxidants, which were reduced in the presence of permethrin, and this is in agreement with Badgujar *et al*. (2015), who found the protecting effects of vitamin C against pesticide-induced oxidative stress in mice. Our data is also in agreement with Mozhdeganloo *et al*. (2015), Harabawy and Mosleh (2014) and Vijayavel *et al*. (2006), who have shown protective effect of vitamin C against metal-induced oxidative stress in fish species. The primary role of vitamin C is to neutralize free radicals; it can work both inside and outside the cells. Free radical will seek out an electron to regain their stability, vitamin C is an excellent source of electrons; therefore, it can donate electrons to free radicals such as hydroxyl and superoxide radicals and quench their reactivity (Badgujar *et al*., 2015). Apart from up-regulating endogenous antioxidant defenses, vitamin C protects the DNA of cells from damage caused by free radicals (Badgujar *et al*., 2015). Treatment with vitamin C in permethrin-exposed group also restored liver injury markers, i.e., perfusate hepatic enzymes. Therefore, it seems that enhanced antioxidant defense mechanism and diminished lipid peroxidation, resulting from vitamin C treatment, could be able to protect liver from oxidative damage caused by permethrin.

The present study concludes exposure of rainbow trout liver to permethrin caused alteration in oxidative stress parameters. These alterations were reversed to a great extent with administration of vitamin C. The protective effect of vitamin C, observed in the present study, could be important for protecting the different tissues against the oxidative injury induced by permethrin.

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