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Original Research Paper

Quinalphos Induced Oxidative Stress Biomarkers in Liver and Kidney of Common Carp, *Cyprinus Carpio*

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ABSTRACT

Oxidative stress responses were evaluated in liver and kidney of freshwater fish, *Cyprinus carpio* exposed to organophosphorus pesticide, quinalphos (25% emulsified concentration). Lipid peroxidation levels and antioxidant enzyme activities were measured spectrophotometrically in liver and kidney of fish, treated with two sub-lethal concentrations of quinalphos viz., 0.275 ppm and 0.55 ppm along with control as a reference for 4, 8, 16 and 32 days. The experimental concentration of quinalphos evoked different degrees of activity in both the tissues, the liver and the kidney. The significant increase in lipid peroxidation suggests quinalphos-mediated free radical production; the increase in the activity of glutathione-S-transferase with corresponding increase in the activity of glutathione reductase indicates the glutathione conjugation of the pesticide; activity of superoxide dismutase, catalase and glutathione peroxidase decreased drastically after 32nd day exposure to sub-lethal concentrations of pesticide. Overall, the results demonstrate that alteration in the antioxidant enzymes, glutathione system and induction of lipid peroxidation reflects the toxicity of quinalphos which may cause oxidative stress in the experimental fish. The study, therefore, provides a rational use of biomarkers of oxidative stress in biomonitoring programs.

INTRODUCTION

The use of biomarkers to analyse the effects of exposure to chemical contaminants in the aquatic environment is more extended in the actuality (De Luca-Abbott et al. 2005). Biomarkers of oxidative stress have been largely used for the assessment of the effects induced by several classes of chemical contaminants on the organisms. The assessment of alterations on key enzymatic activities of sentinel species following exposure to contaminated waters has been one of the major uses of biomarkers in environmental studies. A review by Livingstone (2001) reports the role of biomarkers of oxidative stress in ecotoxicology, showing the main perspectives for the future evolution and purpose of this special class of biomarkers. Oxidative stress biomarkers comply with basic requirements, such as: responsiveness; low cost; simple procedures; applicability under varied testing conditions; and sensitivity to a high number of environmental contaminants.

Oxidative stress is able to compromise many vital functions, and lipid peroxidation is a major mechanism involved in the oxidative cell injury (Bassi et al. 2001). The biomarkers of oxidative stress in all organisms include superoxide dismutase (SOD; E.C.1.15.1.1), catalase (CAT; E.C.1.11.1.16), glutathione peroxidase (GPx; E.C.1.11.1.9), glutathione-S-transferase (GST; E.C.2.5.1.18) and glutathione reductase (GR; E.C.1.6.4.2). An important feature of these enzymes is their inducibility under conditions of oxidative stress, which can serve as an important adaptation. These systems may also be inhibited, the process of which can lead to antioxidant mediated toxicities (Di Giulio et al. 1989).

Organophosphate based pesticides account for a major percentage of pesticides used in domestic, agricultural and industrial applications throughout the world. They are highly popular because they are effective, non persistent and relatively less expensive. Due to their rapid breakdown in water accompanied by low environmental persistence, organophosphate pesticides have largely replaced the use of organochlorines in recent years (Zhang & Li 2002). Quinalphos (O,O-diethyl O-quinoxalin-2-yl phosphoro-thiate) is a synthetic organo-phosphate, non-systemic, broad spectrum insecticide and acaricide, acting as a cholinesterase inhibitor with contact, stomach and respiratory action. The major use of quinalphos in farming is to protect corn, cotton and

fruit trees against insects. It is a hard insecticide, which has become a matter of concern because of its potentiality and hazardous effect. Since quinalphos is extensively applied in agriculture for pest eradication, it is pertinent to study its hazardous effect on the aquatic system as it is assumed that the residue might affect the aquatic vertebrates (Das & Mukherjee 2000).

Fish has been largely used to evaluate the quality of aquatic systems as bioindicators of environmental pollutants (Dautremepuits et al. 2004, Lopes et al. 2001). The freshwater fish, *Cyprinus carpio* was selected as experimental model because of its wide availability in local tanks and ponds. It also serves as a cheap protein rich source of food for the vast population of India. Hence, the present study is an attempt to evaluate oxidative stress biomarkers in liver and kidney of common carp, *Cyprinus carpio* exposed to sub-lethal concentrations of organophosphorus pesticide, quinalphos (25% EC: emulsifiable concentrate) for varying treatment durations.

MATERIALS AND METHODS

Quinalphos: Quinalphos, which is a trade name of Ekalux EC 25 [O,O-diethyl-O-(2-quinoxalinyl)- phosphorothiate] supplied by Chethana Agro Chemicals, Mangalore, India was used throughout the study. For each experiment the required concentrations were prepared from fresh stock solutions.

Chronic toxicity study: Common carp fingerlings measuring 7.0±0.5 cm in total length and weighing 10.0±1.0 g were selected for the present study. The fish were divided into 3 groups, with the first group held in tap water serving as a control and the others as the experimental groups. The experimental groups were subjected to the assessment of chronic toxicity by exposing them to two selected sublethal quinalphos (25% EC) concentrations of 0.275 ppm ($1/10^{th}$ of LC_{50}/ 96 hr) and 0.55 ppm ($1/5^{th}$ of LC_{50}/ 96 hr). The 96 hr LC_{50} of quinalphos for fingerlings *C. carpio* was previously determined as 2.75 ppm.

Ten healthy animals were stocked in 50 litre of freshwater in a glass aquarium. Triplicates were run for each concentration. Renewal assay for static test was applied (APHA 1981). The water with insecticide was replaced every 24 hr to maintain constant quinalphos (25% EC) nominal concentration. The control fish were maintained in the same manner. There were no fish mortalities during the experiment. Control and experimental fish were fed once daily with biogold floating feed, at a rate of approximately 3% of their body weight. The temperature was recorded and water quality was determined for DO_2, pH at the beginning of the experimental period. The specimens were sampled after 4 days, 8 days, 16 days and 32 days of exposure for antioxidant enzyme assays and lipid peroxidation studies.

Sample preparations: The fishes were sacrificed by decapitation and the test organs, liver and kidney were immediately dissected out, the post-mitochondrial fraction from the pooled liver and kidney samples were washed in ice-cold 1.15% KCl solution blotted and weighed. The tissues were homogenized with a homogenizing solution (50 mM phosphate buffer pH 7.4 containing 1 mM EDTA, 1 mM dithioth-reitol (DTT), 0.15 M KCl and 0.01% (w/v) PMSF). Homogenization was performed at 4 °C using motor driven Remi Homogenizer (RQ-127A) and centrifuged at 10,000 rpm for 20 min at 4°C using refrigerated centrifuge (Eppendorf 5810 R). The supernatant was decanted and stored at -20 °C until biochemical analysis.

Assay of lipid peroxidation: Lipid peroxidation level was assayed by measuring malondialdehyde (MDA), a decomposition product of polyunsaturated fatty acids. Lipid peroxidation concentration was determined by the thiobarbituric acid (TBA) reaction (Ohkawa et al. 1979). The absorbance was read at 532 nm after removal of any flocculated material by centrifugation. The amount of thiobarbituric acid reactive substances (TBARS) present was then calculated using an extinction coefficient of $1.56\times10^5\ M^{-1}\ cm^{-1}$ (Wills 1969) and expressed as nmol TBARS formed/mg protein.

Assay of antioxidant enzymes: Superoxide dismutase activity was measured according to Paoletti et al. (1990). The method is based on inhibition of oxidation of NADH by SOD. One unit of enzyme activity was defined as 50% inhibition of oxidation of NADH in the reaction. The reaction rate was recorded at 340 nm and expressed as Units/mg protein. Catalase activity was determined based on the decomposition rate of H_2O_2 by the enzyme (Aebi 1974). Absorbance was measured at 240 nm and enzyme activity was expressed as Units/mg protein. One unit of catalase activity was defined as decomposition of 1.0 nmol of H_2O_2 to oxygen and water per minute at pH 7.4 and 25 °C. Glutathione peroxidase activity was determined following the oxidation of NADPH with cumene-hydroperoxide as substrate (Paglia & Valentine 1967). Absorbance was recorded at 340 nm and enzyme activity was expressed as nmol NADPH oxidized/min/mg protein, using a molar extinction coefficient of 6.22 $mM^{-1}\ cm^{-1}$. Glutathione-S-transferase activity was measured using 1-chloro-2, 4-dinitrobenzene as a substrate (Habig et al. 1974). The reaction rate was recorded at 340 nm and enzyme activity was expressed as nmol CDNB conjugate formed/min/mg protein using a molar extinction coefficient of 9.6 $mM^{-1}\ cm^{-1}$. Glutathione reductase activity was measured following the rate of NADPH oxidation at 340 nm in the presence of GSSG (Massey & Williams 1965).

Table 1: Water quality parameters: Temperature (°C), DO_2 (mg/L) and pH

Quinalphos exposure	Water quality parameters		
	Temperature	DO_2	pH
Control	27.9 ± 0.35	7.44 ± 0.06	7.21 ± 0.04
0.275 ppm	28.3 ± 0.31	7.29 ± 0.04	7.07 ± 0.08
0.550 ppm	27.5 ± 0.59	7.36 ± 0.06	6.96 ± 0.05

The values are presented as mean ± S.D. (n = 6).

Enzyme activity was expressed as nmol NADPH oxidized/min/mg proteins using a molar extinction coefficient of 6.22 $mM^{-1}\,cm^{-1}$. Protein concentrations were estimated by using bovine serum albumin as standard (Lowry et al. 1951). Samples were treated with folin-phenol reagent and the absorbance was measured at 750 nm. All biochemical parameters were assayed using a Thermo Fisher Scientific (Evolution 300) UV-Visible spectrophotometer.

Statistical analysis: The biochemical data were subjected to statistical analysis employing ANOVA to compare variables among controls and treatments, and Duncan's multiple range test at $P<0.05$ (Duncan 1995, Snedecor & Cochran 1968) to determine which individual groups were significantly different from control. All the data are expressed as mean ± standard deviation (SD) of the mean of six observations.

RESULTS AND DISCUSSION

The water quality parameters are given in Table 1. No alterations were observed in temperature, DO_2 and pH with quinalphos contamination. The liver was chosen as the major site of accumulation, biotransformation and excretion of xenobiotic compounds, whereas kidney was selected as the major route for elimination and rapid clearance of xenobiotic chemicals (Triebskorn et al. 1997). Lipid peroxidation is one of the major mechanisms involved in the cellular membrane damage by pesticides. The lipid peroxidation values, as evidenced by TBARS levels, were found to increase significantly in the liver and kidney of the fish under both the experimental concentrations of the pesticide (Table 2), which might also be due to the direct interaction of the pesticide with cellular plasma membrane, as organophosphorus compounds were found to possess such a potential (Durmaz et al. 2006). Elevation of lipid peroxidation in both the test tissues suggests, quinalphos-mediated free radical production can be the cause of oxidative stress in these tissues.

The SOD-CAT system provides the first defence against oxygen toxicity. SOD catalyses the dismutation of the superoxide anion radical to water and hydrogen peroxide, which detoxified by the CAT activity. Usually a simultaneous induction response in the activities of SOD and CAT is observed when exposed to pollutants (Dimitrova et al. 1994). The primary enzymatic antioxidant machinery of the liver and kidney of the fish comprising superoxide dismutase and catalase showed contrasting activities exhibiting a declension dependent on duration and concentration. The decreased activity of these primary antioxidative enzymes after 32nd day exposure (Tables 3 and 4), signifies the inability of the enzyme in countering the toxic effects of the pesticide. It has also been reported that the long-term treatment with organophosphorus leads to a gradual exhaustion of SOD, GPx and GST or brings about an increase of antioxidative defence systems (Gultekin et al. 2000).

Low activities of GPx after 32nd day exposure (Tables 3 and 4) demonstrates the inefficiency of tissues in neutralizing the impact of peroxides. It is conceivable that substrate competition between GPx and CAT might be the cause of the reduction in GPx (Cheung et al. 2004). As the activity of glutathione peroxidase showed declension at both the concentrations, superoxide dismutase and catalase failed to cope up with the increasing oxidative stress (as evidenced by enhanced levels of lipid peroxidation) and the accumulating superoxide radicals might have resulted in tissue injury. Detoxification enzymes, especially GST help in eliminating reactive compounds by forming their conjugates with glutathione and subsequently eliminating them as mercapturic acid, thereby protecting cells against ROS induced damage (Rodriguez-Ariza et al. 1993). The increase in the activity of glutathione-S-transferase with a corresponding increase in the activity of glutathione reductase, at high as well as a low concentration of the pesticide, vividly indicates the glutathione conjugation of the pesticide or its metabolites due to the activity of glutathione-S-transferase facilitating the conjugation excretion.

Glutathione-S-transferase-mediated conjugation may be an important mechanism for detoxifying peroxidised lipid breakdown products which have a number of adverse biological effects when present in high amounts. GST responded in a different manner, that it registered a continuous increase in activity at both the concentrations in liver and kidney (Tables 3 and 4). The changes in the activity of GST show the role of this enzyme in protection against the lipid peroxidation (Leaver & George 1998). GST catalyses the conjugation of GSH with electrophilic metabolites, which are involved in the detoxification of both reactive intermediates and oxygen radicals. Increased activities of GST are known to serve as protective responses to eliminate xenobiotics (Smith & Litwack 1980). Elevated GST activity may reflect the possibility of better protection against pesticide toxicity and it is used as a biomarker for environmental biomonitoring (Oruc et al. 2004). When GST

Table 2: Effect of quinalphos on activitiy of lipid peroxidation, LPx, nmol of TBARS formed/mg protein in liver and kidney of *Cyprinus carpio.*

Tissue	Test groups	Exposure time			
		4 days	8 days	16 days	32 days
Liver	Control	1.850 ± 0.06	1.864 ± 0.04	1.798 ± 0.05	1.868 ± 0.03
	0.275 ppm	2.875 ± 0.04	3.990 ± 0.08*	4.619 ± 0.08*	7.261 ± 0.05*
	0.550 ppm	4.383 ± 0.46*	5.821 ± 0.06*	6.526 ± 0.05*	6.943 ± 0.06*
Kidney	Control	1.166 ± 0.04	1.083 ± 0.06	1.300 ± 0.04	1.239 ± 0.04
	0.275 ppm	2.375 ± 0.04	2.768 ± 0.06*	2.422 ± 0.07	3.036 ± 0.52*
	0.550 ppm	3.183 ± 0.06*	2.629 ± 0.06*	3.299 ± 0.06*	3.949 ± 0.05*

Data are means ± S.D., n = 6, *Significant differences compared with control value for a test group along time (P < 0.05).

Table 3: Effect of quinalphos on activities of antioxidant enzymes (superoxide dismutase, SOD, U/mg protein; catalase, CAT, U/mg protein; glutathione peroxidase, GPx, nmol of NADPH oxidized/min/mg protein; glutathione-S-transferase, GST, nmol of CDNB conjugate formed/min/mg protein; glutathione reductase, GR, nmol of NADPH oxidized/min/mg protein) in liver of *Cyprinus carpio.*

Indices	Test groups	Exposure time			
		4 days	8 days	16 days	32 days
SOD	Control	1.018 ± 0.02	1.043 ± 0.07	1.085 ± 0.06	1.043 ± 0.03
	0.275 ppm	1.150 ± 0.02	2.192 ± 0.05	2.235 ± 0.04	2.293 ± 0.06*
	0.550 ppm	2.160 ± 0.06	3.438 ± 0.05*	3.240 ± 0.04*	2.607 ± 0.07*
CAT	Control	0.485 ± 0.03	0.578 ± 0.03	0.525 ± 0.04	0.548 ± 0.04
	0.275 ppm	1.078 ± 0.05*	0.979 ± 0.08	0.963 ± 0.05	0.675 ± 0.05
	0.550 ppm	0.920 ± 0.03	1.143 ± 0.04*	0.914 ± 0.06	0.446 ± 0.02*
GPx	Control	12.59 ± 0.32	12.41 ± 0.31	12.82 ± 0.39	13.52 ± 0.34
	0.275 ppm	21.45 ± 0.40	26.97 ± 0.56*	28.65 ± 0.66*	25.57 ± 0.82*
	0.550 ppm	22.74 ± 0.77*	24.22 ± 0.49*	27.69 ± 0.67*	23.93 ± 0.58*
GST	Control	23.51 ± 0.43	24.20 ± 0.30	24.99 ± 0.32	25.32 ± 0.38
	0.275 ppm	31.88 ± 0.58	43.21 ± 0.30*	35.22 ± 0.81	39.02 ± 0.36*
	0.550 ppm	34.32 ± 0.87	40.51 ± 0.34*	38.84 ± 0.53*	44.15 ± 0.26*
GR	Control	4.246 ± 0.04	4.134 ± 0.07	4.283 ± 0.05	4.361 ± 0.05
	0.275 ppm	13.81 ± 0.49*	17.36 ± 0.34*	16.56 ± 0.53*	20.23 ± 0.32*
	0.550 ppm	11.92 ± 0.58*	15.17 ± 0.57*	19.87 ± 0.58*	20.41 ± 0.88*

Data are means ± S.D., n = 6, *Significant differences compared with control value for a test group along time (P < 0.05).

Table 4: Effect of quinalphos on activities of antioxidant enzymes (superoxide dismutase, SOD, U/mg protein; catalase, CAT, U/mg protein; glutathione peroxidase, GPx, nmol of NADPH oxidized/min/mg protein; glutathione-S-transferase, GST, nmol of CDNB conjugate formed/min/mg protein; glutathione reductase, GR, nmol of NADPH oxidized/min/mg protein) in kidney of *Cyprinus carpio.*

Indices	Test groups	Exposure time			
		4 days	8 days	16 days	32 days
SOD	Control	0.378 ± 0.04	0.474 ± 0.05	0.531 ± 0.05	0.479 ± 0.06
	0.275 ppm	1.271 ± 0.05*	1.437 ± 0.05*	1.345 ± 0.04*	1.006 ± 0.06
	0.550 ppm	1.454 ± 0.04*	1.413 ± 0.05*	1.374 ± 0.05*	1.084 ± 0.05
CAT	Control	0.268 ± 0.04	0.262 ± 0.03	0.309 ± 0.02	0.275 ± 0.03
	0.275 ppm	0.380 ± 0.03	0.510 ± 0.03	0.468 ± 0.04	0.223 ± 0.03*
	0.550 ppm	0.410 ± 0.04	0.398 ± 0.03	0.557 ± 0.02	0.331 ± 0.02
GPx	Control	7.172 ± 0.05	7.526 ± 0.08	7.389 ± 0.06	7.487 ± 0.06
	0.275 ppm	6.236 ± 0.05	8.108 ± 0.08	12.08 ± 0.29	11.46 ± 0.37
	0.550 ppm	6.874 ± 0.05	9.625 ± 0.07	10.16 ± 0.10	7.007 ± 0.07*
GST	Control	21.03 ± 0.20	21.51 ± 0.28	20.77 ± 0.35	20.56 ± 0.54
	0.275 ppm	34.94 ± 0.76	36.38 ± 0.37	44.21 ± 0.55*	42.58 ± 0.72*
	0.550 ppm	31.33 ± 0.51	33.16 ± 0.29	46.50 ± 0.49*	44.02 ± 0.64*
GR	Control	2.616 ± 0.08	2.540 ± 0.05	2.652 ± 0.06	2.577 ± 0.07
	0.275 ppm	8.531 ± 0.09*	8.841 ± 0.10*	10.92 ± 0.10*	9.805 ± 0.50*
	0.550 ppm	9.960 ± 0.11*	14.09 ± 0.17*	13.70 ± 0.34*	15.77 ± 0.71*

Data are means ± S.D., n = 6, *Significant differences compared with control value for a test group along time (P < 0.05).

activity is inhibited, accumulation of lipid peroxidation products occurs. GSTs play a primary important role in celluer detoxification of toxic aldehydes.

In living organisms, oxidation of GSH to GSSG occurs due to many biochemical reactions. GR converts GSSG to its reduced form GSH, which is important both as a substrate for peroxide scavenging enzymes (i.e., GPx and GST) and as direct scavenger of oxy-radicals. The changes in the activities of glutathione reductase were more or less similar to the response of glutathione-S-transferase. The level of glutathione reductase enhanced significantly in both the test tissues after exposure to the two experimental concentrations of quinalphos when compared to that of control group (Tables 3 and 4), suggests its active role in recycling pathways. Fishes from polluted sites have high GR activity due to higher peroxidative components in the polluted aquatic sites (Stephensen et al. 2002).

CONCLUSIONS

Quinalphos, a commonly used organophosphorus pesticide by the agricultural sector at sub-lethal concentrations can alter the antioxidant mechanisms of fish exposed to it for a long period of time. Depletion of two primary antioxidative enzymes together with increased lipid peroxidation, suggesting quinalphos-mediated free radical production causes oxidative stress. Since the typical reaction during oxidative stress is peroxidative damage to unsaturated fatty acids, the oxidative stress response could conveniently be used as biomarker in biomonitoring of aquatic pollution.

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