



Organophosphorus Insecticide, Methyl Parathion Toxicity in *Labeo rohita*

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Abstract

The effects of three sublethal concentrations of Methyl Parathion (MP) toxicity were evaluated in freshwater fish *Labeo rohita*. Lipid peroxidation (LPO) and antioxidant enzymes *viz.*, superoxide dismutase (SOD, EC 1.15.1.1), catalase (CAT, EC 1.11.1.6), glutathione peroxidase (GPx, EC 1.11.1.9), glutathione S-transferase (GST, EC 2.5.1.18) and total reduced glutathione (GSH EC 1.6.4.2) were estimated in liver and the neurotoxic effects were investigated by the determination of acetylcholinesterase (AChE, EC 3.1.1.7) activities in brain of *Labeo rohita* of size 75 ± 6 g at sub lethal exposure. The exposure to MP at 1.0 mg L^{-1} increased LPO by two-fold and in 45 days almost five-fold increase was observed when compared to control. SOD activity increased by three-fold (0.5 mg L^{-1}) and six-fold (1 mg L^{-1}) at sub lethal exposure of MP. The AChE activity decreased with increase in the concentration of MP. The results show that changes in antioxidant enzymes system and inhibition of AChE activity can be used as biomarkers of exposure to MP in aquatic ecosystems.

Keywords: Methyl parathion, *Labeo rohita*, oxidative stress, antioxidant enzymes, acetylcholinesterase

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Introduction

Though the use of chemical pesticides is well recognized as an economic approach for control of pests, such chemicals are highly toxic to other species sharing the environment. There is growing

concern worldwide over the indiscriminate use of such chemicals that results in environmental pollution and toxicity risk to non-target organisms (Rao, 2006). Many environmental pollutants induce oxidative stress in fish. Pesticides may induce oxidative stress through generation of reactive oxygen species (Banerjee et al., 1999). Reactive oxygen species are highly reactive molecules, which indiscriminately interact with essential macromolecules such as DNA, protein and lipids, leading to the disturbance of physiological processes (Cnubben et al., 2001). Lipid peroxidation has been suggested as one of the molecular mechanisms involved in pesticide-induced toxicity (Khrer, 1993).

Oxidative stress resulting from the production of reactive oxygen species has gained considerable interest in the field of ecotoxicology (Lemaire et al., 1994). Brain of fish contains low levels of antioxidants and higher levels of oxidizable catecholamines and peroxidizable unsaturated lipids (Lasner et al., 1995), which are vulnerable to peroxidative damage compared with other tissues. The highly reactive hydroxyl radical ($\text{OH}\cdot$), which is one of the reactive oxygen species generated in the process leading to oxidative stress, is considered to be responsible for the formation of carbonyl groups in proteins (Oliver, 1987). Protein oxidation can lead to loss of critical sulfhydryl groups in addition to modification of amino acids leading to the formation of carbonyl and other oxidized moieties (Stern, 1985). High oxygen tension in many areas of the circulation favours reactive oxygen species formation and membrane proteins are cross-linked. Oxidative modification leads to proteolytic degradation, which may affect the structure, function and integrity of proteins (Bainy et al., 1996).

Methyl parathion (MP) is an organophosphorus pesticide intended only for out door use and is classified as category I (*viz.*, most toxic) by US

Environmental Protection Agency. MP is rapidly metabolized to biologically active methylparaxon, which can chemically bind to acetylcholine esterase leading to a variety of clinical manifestations. Most of the toxicological studies in fish are confined to the effects of pesticides on fingerlings and not many studies are available in juvenile/ adult fish. The aim of the study is to evaluate lipid peroxidation, and antioxidant enzyme activities in liver and acetylcholinesterase activity in brain tissues of fish (*Labeo rohita*) exposed to sublethal doses of MP.

Materials and Methods

Rohu (*Labeo rohita*) weighing about 75 ± 6 g and having mean body length 23 ± 5 cm were collected from a fish farm at Thiruvankulam near Ernakulam, Kerala, India. The fish were brought to the laboratory and acclimatized for more than 15 days in plastic tanks before starting the experiment. pH of water was maintained at 6.74 ± 0.4 and temperature at $32 \pm 2^\circ\text{C}$. The fish tanks were well aerated and the physical and chemical parameters were kept nearly constant.

MP-50% (O, O dimethyl-O-4-nitrophenyl Phosphorothioate - Bayer, Germany), a synthetic organophosphorous insecticide was obtained from the market in Cochin. All other experimental chemicals were purchased from Sigma (USA), Merck (Germany) and SRL (India).

The LC_{50} of MP in *Labeo rohita* has been studied and the LC_{50} for 96 h exposure was found to be 10.2 mg L^{-1} . Three concentrations viz., 1/10, 1/20, 1/40 of 96 h LC_{50} (0.25, 0.5 and 1.0 mg L^{-1}) were selected for sub lethal exposures for 15, 30 and 45 days. One group was maintained as control in a tank containing MP free water. Fishes were fed with commercial fish feed and 20 percent water was replaced on daily basis during the experimental period. The pesticide loss during this procedure was compensated by adding it to water. After fifteen days of exposure, fishes were killed by decapitation and liver was analysed for antioxidant enzymes and lipid peroxidation and the brain was analysed for acetylcholinesterase activity. Liver tissues were dissected, washed in physiological saline (0.9% NaCl), and kept at -20°C until analysis. The tissue was homogenized for 5 min in ice-cold 0.1M Tris-HCl buffer solution pH 7.2 (1:5 w/v) using Polytron homogenizer (Polytron Model PT3000, Kinematica-Switzerland) and centrifuged (Remi-India) at

8000 rpm for 30 min. Supernatants were used for determination of enzymes.

Lipid peroxides (LPO) (Ohkawa et al., 1979), superoxide dismutase (SOD) (Misra & Fridovich, 1972), catalase (Takahara et al., 1960), glutathione peroxidase (GPX) (Pagila et al., 1967), total reduced glutathione (GSH) (Ellman, 1959) and glutathione S-transferase (GST) (Habig et al., 1974) in liver were estimated by standard methods. Acetylcholinesterase (AChE) activity was assayed by the method of Ellman et al. (1961).

One-way analysis of variance (ANOVA) was run using SPSS 10.0 statistical system for windows. ANOVA was employed followed by Duncan's test to calculate the significant difference between control and experimental fishes (Daniel, 1987).

Results and Discussion

Lipid peroxide (LPO) level in liver of *L. rohita* after exposure to MP at sub lethal levels increased compared to the control (Table 1). There was a significant ($p < 0.05$) increase in LPO level with an increase in the concentration of MP. At 0.25 mg L^{-1} MP concentration, there was only marginal increase (8%) but at 1.0 mg L^{-1} concentration, lipid peroxidation doubled. The 15th day exposure showed 17% increase at a concentration of 0.5 mg L^{-1} compared to control. Exposure to MP at 1.0 mg L^{-1} increased LPO over two-fold and at the end of 45 days the increase was almost five-fold compared to control. LPO is considered a valuable indicator of oxidative damage of cellular components. The results of sub lethal experiments suggest that exposure to MP enhanced ROS synthesis in the liver of *L. rohita* and the antioxidant defenses were not totally able to effectively scavenge them, thus leading to lipid peroxidation. LPO has been reported as a major contributor to the loss of cellular function under oxidative stress (Storey, 1996). Considering that the typical reaction during ROS-induced damage involves the peroxidation of unsaturated fatty acids, the results clearly show that exposure to MP at sub lethal level leads to oxidative stress, with increase of LPO values in liver, compared to the control group. The increased hydroperoxide production in the present study suggested that ROS-induced oxidative damage could be one of the main toxic effects of MP. It has been reported that LPO may be induced by a variety of environmental pollutants (Oakes & Van der Kraak, 2003; Oakes et al., 2004).

SOD activity in liver of *L. rohita* after exposure to MP at sub lethal levels was compared to control (Table 2). SOD activity significantly ($p < 0.05$) increased with increase in the concentration of MP. SOD activity was increased in *L. rohita* during exposure to three different concentrations of MP for 15, 30 and 45 days. On 15 days exposure to 0.25 mg L⁻¹ MP concentration, there was a two-fold increase in SOD activity, which increased by three- fold and six-fold respectively for 0.5 mg L⁻¹ and 1 mg L⁻¹ of MP. However, exposure to higher concentrations for longer duration showed decreasing activity, which was more pronounced with further increase in MP concentration. The generation of free radicals due to exposure to MP, induced the LPO reaction, which might have exceeded the ability of the superoxide dismutase to dismutate the superoxide radicals, resulting in membrane damage and inhibition of the free radical scavenging enzymes. Similar decrease in SOD activity levels was found in erythrocytes of

Cyprinus carpio exposed to MS 222 (Peixoto et al., 2006). Free radicals ($O_2^{\cdot-}$) are dismutated by SOD to H_2O_2 . Induction of SOD could occur during high production of superoxide anion radical. Therefore, an increase in the SOD activity indicates an increase in $O_2^{\cdot-}$ production. Increased SOD levels were reported in similar studies in *Leuciscus cephalus* (Lenartova et al., 1997).

Catalase (CAT) converts hydrogen peroxide, to hydrogen and water. CAT activity increased ($p < 0.05$) by 70% upon exposure to MP (0.25 mg L⁻¹) for 15 days compared to control. Further increase in the MP concentration however decreased the activity. For a particular MP concentration, CAT activity gradually decreased with increasing duration of exposure (Table 2). The reduced activities of CAT and SOD in the presence of MP may result in the accumulation of $O_2^{\cdot-}$, H_2O_2 or their products of decomposition and may cause oxygen intolerance

Table 1. The level of LPO and GSH at sub-lethal exposure of methyl parathion on *Labeo rohita*

Conc. (mg L ⁻¹)	15 days		Duration 30 days		45 days	
	LPO	GSH	LPO	GSH	LPO	GSH
Control	0.53 ± 0.04 ^a	1.19 ± 0.2 ^a	0.52 ± 0.03 ^a	1.97 ± 0.3 ^a	0.54 ± 0.01 ^a	1.35 ± 0.2 ^a
0.25	0.57 ± 0.02 ^a	2.11 ± 0.3 ^b	0.64 ± 0.01 ^b	3.01 ± 0.3 ^b	0.60 ± 0.03 ^b	2.25 ± 0.4 ^b
0.50	0.62 ± 0.01 ^a	2.58 ± 0.4 ^b	0.94 ± 0.02 ^c	3.78 ± 0.5 ^c	1.08 ± 0.01 ^c	2.36 ± 0.2 ^b
1.00	1.00 ± 0.08 ^b	3.91 ± 0.4 ^c	1.15 ± 0.01 ^d	3.87 ± 0.2 ^c	2.61 ± 0.01 ^d	3.35 ± 0.2 ^c

Results are given as mean±SD (n = 3). Values that have a different superscripts (a,b,c) differ significantly ($p < 0.05$, Duncan's multiple range test).

Unit: LPO; nmol of malonaldehyde formed (mg of protein)⁻¹, GSH; µmol (g wet tissue)⁻¹

Table 2. Effect of sub-lethal concentrations of methyl parathion on the liver specific activity of CAT and SOD in *Labeo rohita*

Conc. (mg L ⁻¹)	15 days		Duration 30 days		45 days	
	CAT	SOD	CAT	SOD	CAT	SOD
Control	8.53 ± 0.8 ^a	1.02 ± 0.2 ^a	7.21 ± 0.8 ^a	1.28 ± 0.3 ^a	8.61 ± 0.7 ^a	1.35 ± 0.2 ^a
0.25	14.48 ± 2.5 ^a	2.45 ± 0.1 ^b	10.4 ± 1.9 ^b	1.81 ± 0.1 ^a	9.63 ± 0.7 ^a	2.25 ± 0.4 ^b
0.50	9.36 ± 1.7 ^a	3.13 ± 0.6 ^b	7.62 ± 1.2 ^a	3.39 ± 0.8 ^b	6.73 ± 0.8 ^b	2.36 ± 0.2 ^b
1.00	7.61 ± 0.4 ^b	6.41 ± 1.2 ^c	7.54 ± 0.7 ^a	6.39 ± 0.1 ^c	5.54 ± 0.9 ^b	3.35 ± 0.2 ^c

Results are given as mean±SD (n = 3). Values that have a different superscripts (a,b,c) differ significantly ($p < 0.05$, Duncan's multiple range test). *Unit: SOD :- one unit of the activity is the amount of protein required to give 50% inhibition of epinephrine auto oxidation, CAT:- nmol of H_2O_2 decomposed min⁻¹ mg protein⁻¹

triggering a number of deleterious reactions. There was a significant ($p < 0.05$) decrease in GPx activity with increase in the concentration of MP compared to the control. At 0.25 mg L^{-1} concentration, a 36% decrease in the activity was noticed on 15 days of exposure. At 1.0 mg L^{-1} MP the GPx activity reduced by 76% compared to the control. Almost similar trend was noticed in the case of 30 and 45 days exposed fish (Table 3). The decreased activity of GPx in liver observed in the present study could be related to the excess O_2 production (Bagnasco et al., 1991) or to the direct action of pesticides on the enzyme synthesis (Bainy et al., 1993). Similar decrease in GPx activity was noticed in mice erythrocytes by the organophosphorus insecticide malathion (Yarsan et al., 1999). GPx inhibition was reported after combined treatment with the pesticides 2, 4-D and azinphosmethyl in the brain of carp, *C. carpio* (Oruc et al., 2004), and in the liver of Nile tilapia, *Oreochromis niloticus* (Oruc & Uner, 2000). GPx plays an important role against LPO, since it is mainly involved in the removal of organic compounds and hydrogen peroxides. Thus, GPx is considered to play an important role in protecting membranes from damage due to LPO (Oost et al., 2003). This observation suggested that the major detoxification function of GPx is the termination of the radical chain propagation (Oost et al., 2003). In this context, GPx inhibition observed in the present study might reflect a possible antioxidant defense failure responsible for the observed increase in LPO levels.

GSH activity in liver of *L. rohita* after MP exposure increased compared to the control. There was a

significant ($p < 0.05$) increase in the GSH activity with increase in the concentration of MP. With increase in the duration of exposure to MP, the GSH level decreased after 30 days irrespective of the concentration of MP (Table 1). The GSH plays an important role in the detoxification of electrophiles and prevention of cellular oxidative stress (Hasspieler et al., 1994; Sies, 1999). Considerable decline in the GSH content in tissue during exposure to MP may be due to an increased utilization of GSH, leading to the formation of oxidized glutathione and an inefficient GSH regeneration. During a moderate oxidative stress, the GSH levels can increase as an adaptive mechanism by means of an increased synthesis. However, a severe oxidative stress may suppress GSH levels due to the impairment of the adaptive mechanisms (Zhang et al., 2004). GSH depletion as seen in the case of fishes exposed to 1.0 mg L^{-1} MP may reduce the cellular ability to scavenge free radicals raising the general oxidative potential in the cell. Cells try to remove the xenobiotics by direct conjugation with GSH or by means of GST, which decrease GSH levels.

The GST activity in the liver of *L. rohita* after MP exposure increased compared to control. There was a significant ($p < 0.05$) increase in GST activity with increase in the concentration of MP (Table 3). The GST activity is involved in detoxification and excretion of xenobiotics and their metabolites (Jokanovic, 2001). It plays an important role in protecting tissue from oxidative stress (Banerjee et al., 1999). Highly reactive electrophilic components can be removed before they covalently bind to tissue nucleophilic compounds by the action of this

Table 3. Effect of sub-lethal concentrations of methyl parathion on the liver specific activity of GPx and GST in *Labeo rohita*

Conc. (mg L^{-1})	Duration					
	15 days		30 days		45 days	
	GPx	GST	GPx	GST	GPx	GST
Control	4.65 ± 0.37^a	1761.2 ± 72^a	4.39 ± 0.41^a	1741.4 ± 116^a	4.89 ± 0.16^a	1415 ± 376^a
0.25	3.08 ± 0.10^b	1854.2 ± 83^a	2.43 ± 0.07^b	3588.4 ± 261^b	2.67 ± 0.38^b	2216.8 ± 238^b
0.50	2.82 ± 0.20^b	3149.3 ± 36	2.40 ± 0.12^b	3034.3 ± 84^c	1.25 ± 0.18^c	4575.5 ± 402^c
1.00	1.13 ± 0.15^c	5249.6 ± 86	1.61 ± 0.11^c	5275.4 ± 162^d	1.08 ± 0.11^c	5562.1 ± 257^d

Results are given as mean $\pm$ SD ($n = 3$). Values that have a different superscripts (a,b,c) differ significantly ($p < 0.05$, Duncan's multiple range test).

Unit: GPx: $\text{nmol of GSH oxidized min}^{-1} (\text{mg protein})^{-1}$, GST; $\mu\text{mol of chloro-2,4-dinitrobenzene conjugate formed min}^{-1} (\text{mg protein})^{-1}$

enzyme. Toxic effects of pesticides however inhibit the action of this enzyme. Increased GST activity in tissues may indicate the development of a defensive mechanism to counteract the effects of MP and may reflect the possibility of a more efficient protection against pesticide toxicity. The GST activity increases in response to different concentrations of MP for a particular period of exposure signifying the detoxifying mechanism in liver. This compares well with earlier reports of freshwater characid fish (Monterio et al., 2006).

Table 4. Effect of sub-lethal concentrations of methyl parathion on the brain specific activity of AChE (nmol mg protein⁻¹ min⁻¹) in *Labeo rohita*

Conc. (mg L ⁻¹)	Duration		
	15 days	30 days	45 days
Control	211.5 ± 28 ^a	257.1 ± 55 ^a	229.8 ± 24 ^a
0.25	174.3 ± 59 ^{ab}	229.1 ± 11 ^a	228.2 ± 10 ^a
0.50	127.8 ± 04 ^b	214.6 ± 08 ^a	158.4 ± 21 ^b
1.00	118.7 ± 13 ^b	145.9 ± 12 ^b	125.7 ± 06 ^b

Results are given as mean±SD (n = 3). Values that have a different superscripts (a,b) differ significantly (p<0.05, Duncan's multiple range test).

AChE activity of the enzyme decreased with increase in the concentration of MP and also with increase in duration of exposure. During 15 days of exposure at 0.25 mg L⁻¹, about 18% inhibition of AChE activity was noticed, which increased to 40 and 44% at higher concentrations of 0.5 and 1.0 mg L⁻¹ respectively. For the lowest concentration, the activity increased to the level of control and dropped again by 45 days. At higher concentration (0.5 and 1.0 mg L⁻¹) long duration of exposure marginally increased the activities. Longer duration of exposure, however, decreased the activity compared to control (Table 4). An increased AChE activity at lower MP concentration indicates that the tissues try to make up for the loss of activity on prolonged MP exposure, but at higher concentration, the effect was noticeably decreased. AChE activity showed a continuous decrease in brain, gill and muscle of tilapia on exposure to RPR2 (Rao, 2006; Rao et al., 2003a, b). In conclusion, the results clearly indicate *L. rohita*, exposed to sub lethal concentration of MP, showed significant effect on antioxidant enzymes and AChE activity. The significant changes in AChE activity can be used as good biomarkers for MP exposure.

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