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Effect of Diazinon on Acetylcholinesterase Activity and Lipid Peroxidation of *Poecilia reticulata*

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ABSTRACT

The present study was undertaken to evaluate the toxicity and effects of a commercial formulation of the organophosphorous insecticide, Diazinon on acetylcholinesterase and lipid peroxidation activity in the freshwater guppy fish *Poecilia reticulata*. The fish exposed for 96 h to 50, 100 and 150 μg L^{-1} concentrations of diazinon at 24.0±1.0°C. Some characteristics of this aquarium water were dissolved oxygen, 7.2-7.9 mg L^{-1} and conductivity, 0.212-0.260 mS. Each exposure involved 3 treatments with 3 replicates and 1 control (without diazinon). In each aquarium (Volume: 5 L) were 10 fishes. Induction of oxidative stress in various tissues was evidence by increased lipid peroxidation levels. Acetylcholinesterase activity responded positively in a concentration dependent pattern, thus, suggesting the use of these two parameters as potential biomarkers of toxicity associated with contaminations exposure in freshwater fishes.

Key words: Organophosphate insecticide, fish toxicity, biomarkers, oxidative stress, anticholinesterase potential

INTRODUCTION

Pesticides are an economical means to control growth of unwanted pests. Excessive use of these chemicals results in environmental pollution and toxicity to non target organisms. Thus, the use of pesticides has gained worldwide concern (Rao, 2004). The injuries caused by insecticides to aquatic environment are clear and fish are found to bioaccumulate due to the direct exposure to chemicals and ingestion of contaminated preys and food (Livingstone, 2001; Matsumoto *et al*., 2006). Many of these compounds or their metabolites have shown toxic effects related to oxidative stress (Winston and Di Giulio, 1991).

Diazinon is an organophosphorous insecticide (OPI) and acaricide developed in the early 1950s. It is also used throughout the world in the control measure in public health services especially applied to control ectoparasites in veterinary medicine (Watterson, 1999; EPA, 2005). The major source of Diazinon residues in edible crops are from its use as agricultural pesticide while those in meat, offal and other animal products arise from its use as a veterinary drug containing active ingredient. It is mobile and moderately persistent in the environment and does not bioaccumulation (Pehkonen and Zhang, 2002). Due to its chemical properties and widespread use, diazinon is frequently found in point sources and non-point sources in urban and agricultural areas (EPA, 2003).

Although Diazinon is well known to have nuerotoxic, hematotoxic, hepatotoxic, genotoxic and renal effects and influence the reproductive, developmental, respiratory, cardiovascular systems little is known of how it contributes to the oxidative stress on higher animals. OPIs are shown to

exert their action by inhibiting activity of acetyl cholinesterase (AChE) which plays an important role in neurotransmission at cholinergic synapses by rapid hydrolysis of neurotransmitter acetylcholine to choline and acetate (Kwong, 2002) resulting in accumulation of acetylcholine (Fulton and Key, 2001). This leads to tremors, convulsions and finally the death of the aquatic organism. Several factors seem to be involved in affecting the AChE activity caused by OPIs such as length of time and exposure concentration. Diazinon is commonly used for pest control in the agricultural fields surrounding freshwater reservoirs and contaminating aquatic ecosystems. The toxicity of diazinon depends on the inhibition of acetylcholine esterase activity (AChE, EC 3.1.1.7) like other OPIs (Chambers and Carr, 1995). Therefore, measurement of AChE activity in the fish has been described as a method for diagnosing anticholinesterase pesticides in aquatic solutions (Dellali *et al*., 2001). The knowledge of the major factors responsible for the species selective toxicity of this compound among fish may help to improve the classification of OP compounds according to the regulations devoted to the environmental protection (Keizer *et al*., 2001).

Among the potentials, mechanisms of OPI toxicity are the induction of oxidative stress. Oxidative stress is able to compromise many vital functions and lipid peroxidation is a major mechanism reported to be involved in the oxidative cell injury (Bassi *et al*., 2000). Lipid peroxidation (LPO) is a complex process in biological membranes which are rich in polyunsaturated fatty acids. Lipid hydroperoxides decomposing double bonds of unsaturated fatty acids and destructing membrane lipids causing lipid peroxidation (Ribera *et al*., 1991). Some studies reported that OPIs caused lipid peroxidation (Sharma *et al*., 2005; Rajeswary *et al*., 2007; Kehrer, 1993; Kalender *et al*., 2007) in vertebrates. Estimation of LPO in particular has been reported to have high predictive importance describing its use as biomarker (Lackner, 1998; Elia *et al*., 2002).

The guppy (*Poecilia reticulata*) has long been used as a popular animal model in ecological and evolutionary research as well as in behavioral studies. Recently, it is also widely applied as a test organism both in situ and laboratory bioassays, because it is a readily available and easily handled small body-sized species with a short life cycle (Castro *et al*., 2004; Araujo *et al*., 2006). In addition, several studies combining various biochemical measurements in guppies were very useful for studying environmental pollution problems (Larsson *et al*., 2002; Castro *et al*., 2004).

Therefore, the objective of the peresent study was to evaluate the influence of the sublethal concentration of Diazinon on Acetylcholinesterase and lipid peroxidation activity in various tissues of *P. reticulata,* as biomarker of exposure to pollutants and to study their potential interest in predicting toxicity.

MATERIALS AND METHODS

Animals and study design

Fish preparation and adaptation: This study is a part of M. Sc. Project which is carried out at May 2010 in Pune, India. Adult guppy fish (*P. reticulata*, order: Cyprinodontiformes , family: Poecilidae), of both sexes 3.0±0.5 cm in length and 0.8±0.2 g in weight were procured from a local supplier. The fish are stocked in bags containing water and oxygen and transferred to Laboratory. In Laboratory for adaptation of fish with environmental conditions, fish stocked at plastic tubs/aquarium (with volume 20 L) equipped with de-chlorinated water at 24±2°C and under natural photoperiod (~12:12 h) and continuous supply of aeration with air pumps 24 h before experiment. During this period, fish were fed *ad libitum* with commercial fish pellets (food E-JET Micro-Pellet-35% protein). After this adaptation period, fish with similar mean weight separation and survival test was performed later with three replication: 10 fish in each replication, aeration

constant at all time, 96 h experiment period, photo period 12-16 h, stop feeding 48 h before experiment, not exchange water test (water static) and recorded fish mortality in each 24 h, if the above conditions, mortality rate is less than 5%, therefore fish were suitable for the experiment.

Experimental design: The commercial preparations of Basudin 60EM (O, O-diethyl O-[6-methyl-2-(1-methylethyl)-4-pyrimidinyl] phosphorothioate, Syngenta, Diazinon emulsifiable solution, 630 g L^{-1} were used in the experiments. Stock solutions of the test substance were prepared by dissolving the insecticide in tap water. These solutions were further diluted to obtain the experimental concentrations in aquaria. Sublethal concentrations were selected according to 96 h LC_{50} value for Guppy (*P. reticulata*) reported as 8 mg L^{-1} by Keizer *et al.* (2001). After acclimatization, fish were divided into four experimental opaque plastic tubs (5 L): one control group (n = 10) and three test group-animals treated with Diazinon (n = 10 each). The fish were starved for 24 h prior to experimentation to avoid prandial effects and to prevent the deposition of feces in the course of the assay. After 24 h, the water was renewed and test group was submitted to a concentration of 50, 100 and 150 μg L^{-1} of Diazinon (sub-lethal doses of 96 h-LC_{50}). Opaque experimental tanks were used to avoid external disturbances and they were sealed with a cover to prevent sample volatilization. Dissolved oxygen, temperature and photoperiod were maintained as described for the acclimatization period. The fish remained under a semi-static system for 96 h where the experimental solutions were renewed every 24 h to maintain water quality and adjust the concentration of Diazinon. The control group was submitted to the same protocol but without adding Diazinon. During this period, sub-lethal effects like level of activity, swimming performance and color changes were monitored. Three replicates per test concentration were used to avoid test repetition due to system failure and to provide a stronger statistical baseline.

In this study experimental group was exposed to three doses (50, 100 and 150 μg L^{-1}) of Diazinon. AChE and LPO and were used as important biomarkers for detection of toxic nature of this pesticide. AChE was evaluated to determine the level of neurotoxicity whereas LPO was evaluated in terms of MDA for oxidative stress.

Biochemical estimations

Tissue homogenate preparation: After 96 h exposure, fish from control as well as test sets were removed from the tubs. They were immobilized on ice for few seconds and immediately dissected on ice. After the sacrifice of the animals by decapitation, tissues like brain, muscles and gills were separated and washed with 0.9% NaCl. Samples were weighed and homogenized in 0.1M ice-cold Potassium Phosphate Buffer (1:10 w/v) using a glass homogenizer. Then the homogenates were centrifuged at 13,000 rpm for 10 min at KUBOTA (3700) refrigerated centrifuge. All processes were carried out at 4°C. Suprernatants were used as enzyme source. Each parameter was measured in triplicate. Enzyme activities were determined by triplicate using a spectrophotometer (JASCO V-630).

Measuring AChE and LPO: Acetylcholinesterase activity is measured according to the method suggested by Ellman *et al.* (1961).

The Thiobarbituric acid reactive substances (TBARS) Assay method was used to evaluate the peroxidation of lipids (LPO) as described by Esterbauer and Cheeseman (1990).

Protein determination: The protein concentrations of the brain, muscle and gill samples were measured using Lowry's method (Lowry *et al.*, 1951), with bovine serum albumin as the standard. All protein measurements were performed in triplicate. The enzymatic activity was calculated in terms of the protein content of the sample.

Statistical analysis: All the values were expressed as Mean±Standard Error (SE). The experiments were repeated on three different occasions in triplicate and that data were analyzed by one-way ANOVA analysis ($p<0.05$), followed by Tukey's test (means comparison) using a statistical software SPSS 18.0. Statistical comparisons were done between control and exposure data from the same species.

RESULTS

Anticholinesterase potential of diazinon: The results showed that Diazinon has a general anticholinesterase potential. Acetylcholinesterase activity measurement in different Diazinon concentrations (50, 100 and 150 μg L^{-1}) are shown in Fig. 1. Exposure of Diazinon resulted in a general dose-dependent inhibition of AChE in brain, muscle and gills of fish compared with control in all experimental doses. When the mean values of all three organs are compared it is found that the difference of inhibition level between all the three treatments of brain was highly significant ($p<0.05$), indicating that the inhibition in AChE was correlated with the concentration of the toxicants. In Muscle and gills there is general inhibition in AChE activity but these values are found to be not significant.

Level of Lipid peroxidation (LPO): The results indicated that Diazinon causes a significant increment in LPO values. Lipid peroxdation activity measurement in different Diazinon concentrations (50, 100 and 150 μg L^{-1}) are shown in Fig. 2. Exposure of Diazinon resulted in a significant induction of LPO in brain, muscle and gills of fish compared with control. Increase in

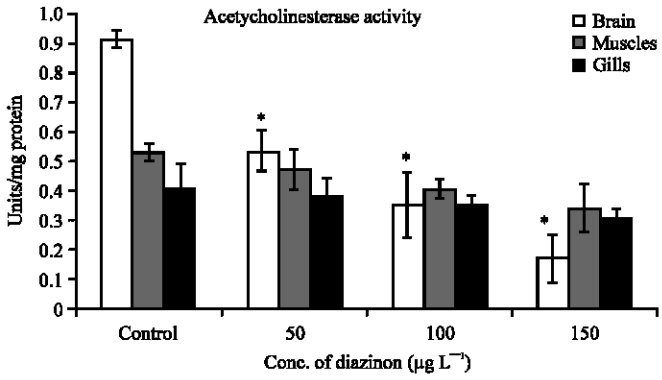

Fig. 1: AChE activity in brain, muscles and gills of control and Diazinon-exposed *P. reticulata*. The values are expressed as Means±SE (n = 10). AChE values are expressed as Units/mg of protein. The significance levels observed are $p<0.05$ when compared with control group values. Asterisks indicate significant difference between treatments ($p<0.05$) Acetylcholinesterase activity measurement in different Diazinon concentrations (50, 100 and 150 μg L^{-1}) are shown in Fig. 1. In Muscle and gills there is general inhibition in AChE activity but these values are found to be not significant

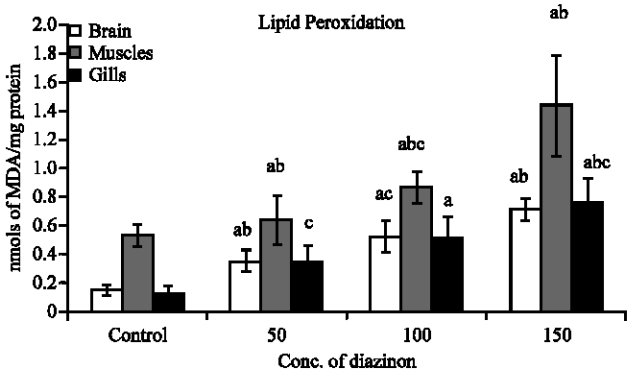

Fig. 2: LPO activity in brain, muscles and gills of control and Diazinon-exposed *P. reticulata*. The values are expressed as Means±SE (n = 10). LPO values are expressed as nanomoles of MDA formed/ mg of protein. The significance levels observed are $p<0.05$ when compared with control group values. Different alphabets (a, b and c) indicate significant difference between treatments ($p<0.05$) Increase in the mean values of MDA for 50 μg L^{-1} exposure was significant at in brain, liver and gills. At 100 and 150μg L^{-1} exposures, mean values of MDA were significant in all the three tissues.

the mean values of MDA for 50 μg L^{-1} exposure was significant at in brain, liver and gills (Fig. 2). At 100 and 150 μg L^{-1} exposures, mean values of MDA were significant in all the three tissues.

After 96 h the fish exposed to different concentrations of diazinon, the highest mean values of MDA of LPO were measured in all organs at 150 μg L^{-1} exposure.

Statistical analysis indicated that the differences between 100 and 150 μg L^{-1} was significance ($p<0.05$). Among the three tissues, the maximum values of MDA were recorded for muscles.

DISCUSSION

The results of the present study have demonstrated that the applied doses of Diazinon could have affected the LPO and AChE level in fish.

OP pesticides have several toxic properties, the most prominent effect of which is AChE inhibition. AChE activity is therefore widely used in biomonitoring studies as a biomarker of OP pesticide exposure. In this study, the reduction of AChE activity is assumed to have been resulted from the direct action of diazinon exposure on active site of this enzyme.

All groups tested with diazinon in this study revealed an inhibition of AChE activity in all treated organs of *P. reticulata*. This accord with the study reporting a positive correlation with insecticide concentration and the time of exposure associated with the degree of AChE inhibition of *Tilapia mossambica* in relation to the interacting effects of sublethal concentrations of dichlorvos (Rath and Misra, 1981). Similar results have also been determined in sunfish, *Lepomis gibbosus* (Benke and Murphy, 1974); in *Danio rerio* (Ansari and Kumar, 1984), *Seriola dumerilli* (Jebali *et al*., 2006) and freshwater catfish *Heteropneutes fossilis* (Chandra, 2008) after malathion exposure. Decrease of AChE activity by Diazinon intoxication has been reported in different animals and fish as *Oreochromis niloticus* (Tridico *et al*., 2010). However, most of the AChE studies are done in fish brains, because the most evident effects are observed in nervous tissue. Therefore, the brain of *P. reticulata* showed the highest AChE inhibition, as expected by Rao and Rao (1984)

compared the AChE inhibition in different tissues of the teleost, *Tilapia mossambica* exposed to 1/3 of methyl parathion LC_{50} for 48 h. Afterwards, they observed that brain had the highest inhibition levels followed by muscle, gills and liver. Ram *et al.* (2011) reported that Polytrin C which is a combination pesticide significantly inhibits the plasma and brain AChE levels of wistar rats. Srinivasa Rao *et al.* (2008) reported that seven different synthetic compounds of Imidacloprid with various substitutions caused high concentration of AChE which may be due to its inhibitory action in post-synaptic regions of nerves. The AChE inhibition is fairly related to the tissue enervation level. Hence, we can say that the highest AChE concentration the highest inhibition susceptibility. Specific AChE activity significantly ($p<0.05$) decreased in *P. reticulata* in all tissues, after the 96 h of experimental exposure to methyl parathion.

The extent of LPO is determined by the balance between the production of oxidants and the removal and scavenging of those oxidants by antioxidants (Halliwell, 1987; Lopez-Torres *et al.*, 1993; Filho, 1996). Generation of oxidative stress and consequent lipid peroxidation by pesticides is reported in many species. Due to high concentration of polyunsaturated fatty acids in cells, lipid peroxidation is a major outcome of the free radical mediated injury. Two broad outcomes of lipid peroxidation are structural damage of cellular membranes and generation of oxidized products, some of which are chemically reactive and may covalently modify cellular macromolecules. These reactive products are thought to be the major effectors of tissue damage from lipid peroxidation (Mattson, 1998). One of the most damaging effects of free radicals and their products in cells is the peroxidation of membrane lipids of which MDA is an indicator. MDA is the final product of lipid peroxidation and a sensitive diagnostic index of oxidative injury in cells (Shalata and Tal, 1998). Detailed studies have provided evidence that many species exhibit an increased MDA following stress produced by some xenobiotics (Luo *et al.*, 2005; Shi *et al.*, 2005a, b). However, when a low level of stress is applied, an adaptive response takes place in the cells. This adaptation may be associated with de novo protein synthesis, or might be due to the activities of various damage removal and repair enzymes. Ince *et al.* (2010) reported that dorsal skin application of deltamethrin (7.5 g L^{-1}) for 7 days significantly increased blood MDA level. Salama *et al.* (2005) reported that upon 48 h exposure to various pesticides to land snail *Helix aspersa* carbafuran signifantly inhibited the AchE levels while none of the pesticide was found to induce LP level. Sushma *et al.* (2006) reported that exposure to sublethal dose (3.5 mg kg^{-1}) of aluminium acetate significantly increased the LP in all brain regions of albino mice. The results of the present study have demonstrated that the applied dosages of diazinon could have affected the AChE and MDA concentration in the fish. This is evidenced from our observation that, upon diazinon treatment in vivo, the concentration of AChE and MDA in Brain muscle and gills differ from those of controls. MDA is a major oxidation product of and increased MDA content is an important indicator of lipid peroxidation (Freeman and Crapo, 1981). The increased MDA content might have resulted from an increase of free radicals as a result of stress condition in the fishes with diazinon intoxication.

Results of the present study showed that MDA content significantly increased in the all tissues of fish treated with three different doses of pesticide. Similar results are reported in *Unio tumidus* (Doyotte *et al.*, 1997) and rainbowtrout, *Oncorhynchus mykiss* (Isik and Celik, 2008), The findings revealed that diazinon not only changes AChE activity but also affects lipid peroxidation. The findings also indicated that *P. reticulata* has a good antioxidant defense system which can reduce oxidative damages.

CONCLUSION

The present investigation indicated that the pesticide Diazinon is toxic to guppy fish and further validate our observations that upon Diazinon treatment the concentration of AChE and MDA in brain, muscles and gills differ from those of controls. Thus AChE and LPO in fish could be effectively used as biomarkers of pesticide toxicity.

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