ISSN 0976 – 3333

Available Online at www.ijpba.info

International Journal of Pharmaceutical & Biological Archives 2011; 2(4):1183-1189

ORIGINAL RESEARCH ARTICLE

Modulation in the Protein Metabolism under Sublethal Concentration of Quinalphos Intoxication in the Freshwater Common Carp, *Cyprinus carpio* (Linnaeus, 1758)

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Received 11 May 2011; Revised 01 Aug 2011; Accepted 06 Aug 2011

ABSTRACT

The fresh water field fish, *Cyprinus carpio* is an important human food source in parts of South India and the fish is constantly exposed to pesticides, which are used extensively to control agricultural pests. Freshwater edible fish, *Cyprinus carpio* were exposed to sublethal concentration (0.75 µl/L) of commercial grade quinalphos (25% Emulsified Concentration) for 5, 10 and 15 days. Liver being the main site of metabolic activity in body and highly active in both toxifying and detoxifying insecticides, was selected for the study purpose. After each exposure periods, liver was taken to study biochemical alterations. Increase in free amino acid, protease activity and Ach levels, in contrast to decrement in total, structural and soluble proteins and acetylcholinesterase (AchE) activity were observed in 5 and l0 day of exposure, but on 15 day of exposure all the values reached nearer to normal condition. Restoration of protein fractions, free amino acid, protease activity, acetylcholine (Ach) and acetylcholinesterase (AchE) levels to normal, implies that after 10 day of exposure there seems to exist an oscillatory phase in protein turnover towards a more synthetic phase leading to the establishment of recuperation and adaptation phenomena. Thus it is inferred that exposure to quinalphos in sublethal doses affect protein metabolism and normal neural physiology of the liver. But recovery in later periods may be revitalization phenomenon as every organism strives to overcome the stress to prove its existence. Recovery phenomenon may be adaptive and even strategic.

Key words: Acetyl cholinesterase, chronic exposure, fish, liver toxic, organophosphate pesticide, protein profile

INTRODUCTION

Pesticides have been widely used all over the world to control insects, pests and disease vectors and they are one of the most potentially harmful chemicals introduced into the environment, though they have contributed considerably to human welfare, their adverse effects on non-target organisms are significant[29] . Pesticides ultimately finds their way into aquatic habitats such as rivers, lakes and ponds, and have been found to be toxic to organisms, which found to be contributing substantially to the food chain and in the water bodies, where fish encounter with them and develop various metabolic abnormalities[15]. They accumulate in fish and affect human health too via ecological cycling and biological magnification.

All of the organophosphates (OP) are nerve poisons. They block the active site of an enzyme acetyl cholinesterase (AchE) that breaks down and removes a neurotransmitter substance acetylcholine (Ach) from the nerve synapse and the excessive build up of acetylcholine results in symptoms of hyperactivity, including tremors, convulsions, and eventually death [23].

Extensive use of OP compounds has resulted in a wide spread distribution of these chemicals in the environment. They are much less persistent than the organochlorines and do not accumulate in fatty tissues. Due to their rapid biodegradability and lesser persistency in the environment, the OP compounds replaced the more persistent organochlorine compounds. Organophosphorous pesticides (OPs) to a large extent replaced the persistent chlorinated pesticides in the 1970s and at the beginning of 1980s. The main advantage of the OPs is their low cumulative ability and short-term persistence in the environment [25]. These

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pesticides leave residues in the soil and water for several days after their application, and pose a constant threat to the non-target organisms especially fishes [17]. Therefore, the usage of OP pesticides has an impact on environment leading to the development of several adaptations such as morphological, physiological, biochemical and behavioral ones at various levels of organization in the organisms to suit their environment [13].

The liver plays an important role in several vital functions of basic metabolism and it is also the major organ of accumulation, biotransformation and excretion of contaminants in fish, including degradation and bioactivation of pesticides and the evaluation of biochemical changes in fish liver has become an important tool for monitoring environmental exposure of fish to contaminants in experimental studies [19] . Therefore, assessment of the protein metabolism [22] and neural physiology can be considered as a diagnostic tool to determine the physiological process of the cell. Hence, an attempt has been made in the present study (study conducted from July 2006 to December 2006, in the Karnatak University's Research Laboratory, Molecular and Environmental Toxicology Division, Department of Zoology, Karnatak Science College, Dharwad) to evaluate the effect of quinalphos on some biochemical aspects of liver in the freshwater fish, *Cyprinus carpio* exposed sublethal concentration.

MATERIAL AND METHODS

A total of 160 healthy and active *C. carpio* fingerlings weighing 5±2 g and average length of 5.5±0.23 cm were collected from the State Fisheries Department, Dharwad, Karnataka, India and were transferred to large cement tanks. Carps were acclimatized to laboratory conditions for 15 day at 24±1°C and are held in large glass aquaria containing dechlorinated tap water of the quality used in the test and its physico-chemical characteristics were analyzed following the methods mentioned in APHA (2005) and found as follows: water temperature: 24±2°C, pH: 7.1±0.2 at 24°C, dissolved oxygen: 6.3±0.8 mg/L, carbon dioxide: 6.3±0.4 mg/L, total hardness: 23.4±3.4 mg/L as $CaCO_3$, phosphate: 0.39±0.002 µg/L, salinity: 1ppm, specific gravity: 1.0030 and conductivity less than 10 µScm-1. Water was renewed everyday and 12-12 hour of photoperiod was maintained daily during acclimation and test periods. Fish were fed regularly with oil cake and rice bran during acclimation and feeding was stopped three days prior to exposure to test medium.

An acute toxicity (LC_{50}) test by the static renewal bioassay method was conducted to determine the toxicity of quinalphos (O,O-diethyl-O-quinoxalinyl-2-thionophosphate); (25% emulsified concentration, EC) in the freshwater fish, *C. carpio* which were exposed to various concentrations of quinalphos for 96 hour and the pesticide was procured from the supplied by Hyderabad Chemical Supplies Limited, Hyderabad, India The expiry date of the test substance checked prior to initiation of the treatment was found suitable for the exposure. The required quantity of quinalphos was drawn directly from this emulsified concentration using a variable micropipette. After 96 hour of exposure the data obtained was subjected to Finney's probit analysis method [11] and Dragstedt-Beheren's equation [4] as mentioned by[5] to determine LC_{50} value. The concentration at which 50% survival/mortality occurred in quinalphos treated fishes was taken as the median lethal concentration (LC_{50}) for 96 hour, which was 7.5 µl/L. One tenth of the LC_{50} value (0.75 µl/L) was taken for the sublethal studies according to Sprague [28].

Estimation of soluble, structural and total proteins:

The soluble, structural and the total proteins in the liver were estimated using the folin-phenol reagent method as described by Lowry [16]. 1% homogenate (W/V) was prepared in an ice-cold 0.25 M sucrose solution. For soluble and structural proteins, 1.0 mL of the homogenate was taken and centrifuged at 3000 rpm for 10 min. The supernatant was separated and to both the supernatant and residue, 3 mL of 10% trichloroacetic acid (TCA) was added and again centrifuged at 3000 rpm. The supernatants were discarded and the residues were taken for experimentation. For total proteins, 1 mL of homogenate was taken; to it 3 mL of 10% TCA was added and centrifuged at 3000 rpm. The supernatant was discarded and the residue was taken for experimentation. All three residues were dissolved in 5 mL of 0.1N sodium hydroxide and to 1 mL of each of these solutions, 4 mL of reagent -D (mixture of 2% sodium carbonate and 0.5% copper sulphate in 50:1 ratio) was added. The samples were allowed to stand for 10 min, at the end of which 0.4 mL of folin-phenol reagent (diluted with double distilled water in 1:1 ratio before use) was added. Finally, the optical density of the colour developed was measured using a spectrophotometer at a wavelength of 600 nm. A

mixture of 4 mL of reagent-D and 0.4 mL of folin-phenol reagent was used as a blank. Bovine serum albumin was used for the preparation of protein standards. The protein content is expressed as mg/g wet wt. of the liver.

Estimation of free amino acids:

Free amino acid level in the liver was estimated by the ninhydrin method as described by Moore and Stein [21]. 5% organ homogenates (W/V) were prepared in 10% TCA and centrifuged at 2000 rpm for 15 min. To 0.2 mL of supernatant, 2.0 mL of ninhydrin reagent was added and the contents were boiled for exactly 5 min. They were cooled under tap water and the volume was made to 10 mL with distilled water. The optical density of the colour developed was measured using a spectrophotometer at a wavelength of 570 nm. A blank using distilled water and amino acid standards were also run similarly. The free amino acid levels are expressed as mg amino acid nitrogen released/g wet wt. of the liver.

Estimation of protease activity

Protease activity in the liver was estimated using the ninhydrin method as described by Davis and Smith [8]. 1% homogenate (W/V) was prepared in distilled water. To 2.0 mL of homogenate 0.5 mL of 1% casein and 2.0 mL of 0.1 M phosphate buffer (pH 5.0) were added. The contents were mixed well and incubated at 30^{o}C for 30 min. The reaction was stopped by adding 2 mL of 2% ninhydrin reagent. Again the contents were mixed thoroughly and placed in a boiling water bath for 20 min. The solution was cooled and made to 10 mL with diluents (distilled water and n-propanol in 1:1 ratio). The optical density of the colour developed was measured using spectrophotometer at a wavelength of 570 nm. A blank taking 2.0 mL of distilled water and a control taking 2.0 mL of boiled enzyme were also run similarly. Amino acid standards were prepared alongside for comparison. The protease activity is expressed as µ moles amino acid nitrogen released/mg protein/h.

Estimation of Acetylcholine (ACh) content:

The tissue ACh content was estimated by the method of Hestrin [2]. After isolating and weighing, the liver was teased and transferred into tubes, already kept in the boiling water bath for 10 minutes, to inactivate the enzyme acetyl cholinesterase and to release the bound ACh. The tubes were cooled and the contents were homogenized in 2.0 mL of distilled water, 2.0 mL of alkaline hydroxylamine hydrochloride and 1.0 mL of 1:1 diluted HCl with water was added to the supernatant. The optical density of the sample was measured at 540nm in a spectrophotometer against the blank. The blank consisted of 2.0 mL of distilled water, 2.0 mL of alkaline hydroxylamine hydrochloride, 1.0 mL of diluted HCl and 10 mL of ferric chloride solution. A standard graph was prepared with ACh and the values were expressed as µM of ACh/g wet wt. of liver.

Estimation of Acetyl cholinesterase (AChE, EC 3.1.1.7) activity:

Acetylcholinesterase activity was estimated by the method of Metcalf [20]. 3% homogenate of liver was prepared in cold 0.25 M sucrose solution and the homogenate itself was used for the enzyme assay. The reaction mixture of 3.0 mL contained 12 µM of acetylcholine chloride, 100 µM of sodium phosphate buffer (pH 7.4) and 1.0 mL of homogenate. After incubating at 37 0C for 30 min and the reaction was stopped by adding 2.0 mL of alkaline hydroxylamine hydrochloride solution followed by 1.0 mL of HCl (1:1, HCl:H_2O). The unincubated samples were treated with 2.0 mL of alkaline hydroxylamine hydrochloride followed by 1.0 mL of HCl prior to the addition of the homogenate. The contents were thoroughly mixed and filtered. To the clear filter 1.0 mL of 0.37 M ferric chloride solution was added and the colour was read at 540 nm in a spectrophotometer using the blank. The blank preparation is the same as the homogenate. The experimental except distilled water substitutes as µM of acetylcholine hydrolyzed/mg protein/h.

Statistical Analysis:

The data were subjected to analysis of variance and the means were compared by Duncan's new multiple range to test at 0.05% confidence level[10] to find the mean comparison among the results.

RESULTS AND DISCUSSION

Biochemical alterations in the liver of the fish exposed to quinalphos were presented (**Table.1, 2 & 3**). The total, structural and soluble proteins and AchE activity were decreased, whereas free amino acids, protease activity and Ach were found to increase in fish exposed for 5 and 10 day. But in fish treated with quinalphos 15 day all the values restored nearer to normal.

Proteins are mainly involved in the architecture of the cell; which is the chief source of nitrogenous metabolism. During chronic periods of stress, they are also a source of energy. During stress, fish need more energy to detoxify toxicants and to overcome stress the protein is used to meet the increased energy demand. Thus the depletion of

protein fraction in liver may have been due to their degradation and possible utilization for metabolic purposes. The decreases in total protein level and increases in total free amino acid level suggest the high protein hydrolytic activity due to elevation of protease activity and increases in free amino acid levels were the result of breakdown of protein for energy and impaired incorporation of amino acids in protein synthesis [14]. It is also attributed to lesser use of amino acids and their involvement in the maintenance of an acid-base balance. The decrement in the total, structural and soluble proteins suggests the existence of a high proteolytic activity, and impairment in the protein biosynthesis.

The decrease in protein levels may also be due to their degradation. The decreased protein content might also be attributed to the destruction or necrosis of hepatocyte cells and consequent impairment in protein synthesis machinery [24]. The quantity of protein is dependent on the rate of protein synthesis, or on rate of its degradation. The quantity of protein may also be affected due to impaired incorporation of amino acids into polypeptide chains [27]. The degradation may be due to oxidative stress [18] which is a characteristic of OP compounds, besides their inhibitory effect on AchE. Oxidative stress also induce changes in free radical production, when the free radical production overwhelms the endogenous antioxidant levels, they cause considerable cell damage and death. All the major biomolecules like lipids, proteins, and nucleic acids may be attacked by free radicals [6].

The degradation products may in turn be fed into a tricarboxylic acid cycle through the aminotransferase system to cope up with the high energy demands augmented during quinalphos stress [3]. Total, Structural and soluble protein contents were depleted in the liver tissue exposed to the lethal concentration of fenvalerate indicating the breakdown of these proteins due to the acute pesticide toxic stress and generally the breakdown of proteins dominates over synthesis under enhanced proteolytic activity[7]. To have a more precise understanding of the variation among structural, soluble and total proteins, the ratios namely Sop/Tp, Stp/Tp and Sop/Stp were calculated. The Stp/Tp values were correspondingly high over Sop/Tp and Sop/Stp values. These ratios clearly indicate that recycling of soluble proteins was considerably higher compared with structural proteins. However, the net protein budget was balanced at the total proteins level. Hypoprotenemia was observed in the liver of fish exposed to OP pesticides by various investigators, thus supporting the findings of the present study [9]. Decline in the protein content of liver would suggest an intensive proteolysis which in turn contributes the increase of free amino acids to be fed into the TCA cycle as keto acids, thus supporting the view of [12].

The elevation of free amino acids has a functional relevance so as to meet energy demands and is also involved in osmoregulation. The increase in protease activity under stress condition clearly suggests that quinalphos induces high protease activity which leads to the formation of higher free amino acid content causing hepatotoxicity. The liver is found to be affected more than any other tissues [3] and because it is the metabolic centre for detoxification and also the host in absorbing greater OP pesticide residues. The restoration of the protein level to normalcy indicates that after 15 day of exposure there seems to exist an oscillatory phase on protein turnover towards a more synthetic phase or a less degradative phase leading to the establishment of recuperation and adaptive phenomena.

Decrease in AchE activity and increase in Ach levels is due to the inhibition of AchE and consequent accumulation of Ach. Acetyl cholinesterase is an enzyme that modulates the amount of neurotransmitter substance at neuron junctions. The inhibition of AchE and elevation in Ach content may be due to the decreased ionic composition in the liver exposed for 5 and 10 day which is in accordance with the earlier reports, thus supporting the findings of current investigation [26]. Increase in AchE activity and decreased Ach content in 15 day may be due to rapid detoxification of quinalphos as liver is a major site for detoxication, assuming that pesticide concentration is within the threshold limit. This nominal concentration might be rapidly detoxified leaving less for inhibitory activities. These results reflect that organisms are adopting with the sublethal concentration which seems to be strategic and adaptive.

Liver being the main site of metabolic activity in body and highly active in both toxifying and detoxifying insecticides, was selected for the study purpose. The reduction in protein content may be due to increased utilization of protein to meet out the energy demand when the fish is under stress condition. The pronounced increase in protease activity may be due to the damage caused to the lysosomal membrane, thus

permitting the leakage of lysosomal enzyme into cytosol. Biomarkers are thus of the interest to be able to quickly detect interactions pollutants/organisms after exposure and in certain cases, to predict potential toxic effects. Thus it is inferred that exposure to quinalphos in sublethal doses affect protein metabolism and normal neural physiology of the liver. But recovery in later periods may be revitalization phenomenon as every organism strives to overcome the stress to prove its existence. Recovery phenomenon may be adaptive and even strategic. But an agricultural effort reducing the use of pesticides and implementing natural remedies for pest encroachment can become one solution for pesticide pollution. Globally, studies need to simultaneously consider the benefits to both agricultural and conservation fishes; scientists from both communities should provide input to make realistic and informed decisions about the protection and conservation of aquatic biodiversity within agricultural landscapes.

Table 1: Biochemical parameters in the liver of the fish, *Cyprinus carpio* following exposure to sublethal concentration of quinalphos.

Parameters	Control	Sublethal Exposure periods in days		
		5	10	15
Total proteins (Tp)	135.85 a	104.27 c	76.31 d	105.27 b
±SD	2.76	1.94	1.98	2.33
% Change	----	-23.24	-44.07	-22.54
Structural proteins(Stp)	90.46 a	61.84 c	46.99 d	69.31b
±SD	2.12	1.81	2.37	1.68
% Change	----	-40.02	-51.89	-23.38
Soluble protein (Sop)	44.27 a	39.88 b	28.70 d	34.73 c
±SD	4.39	3.04	1.30	3.07
% Change	----	-9.91	-35.17	-21.54
Sop/Tp	0.325	0.382	0.376	0.329
% Change	----	+17.53	+15.69	+1.23
Stp/Tp	0.665	0.593	0.615	0.658
% Change	----	-10.82	-7.51	-1.05
Sop/Stp	0.484	0.644	0.610	0.50
% Change	----	-31.69	+24.74	+2.451

Means are ± SD (n=6) for a tissue in a column followed by the same letter are not significantly different ($P \leq 0.05$) from each other according to Duncan's multiple range (DMR) test.

Table 2: Protease activity (µM of amino acids/mg protein/h) and free amino acid (mg/g wet wt.) in the liver of the fish, *Cyprinus carpio* following exposure to sublethal concentration of quinalphos.

Parameters	Control	Sublethal Exposure period in days		
		5	10	15
Protease activity	0.553 c	0.569 d	0.595 a	0.529 b
±SD	0.021	0.011	0.019	0.012
% Change	----	+2.89	+6.32	-4.33
Free amino acid	4.937 g	5.034 d	5.259 a	5.687 e
±SD	0.086	0.080	0.072	0.044
% Change	----	+1.98	+6.25	+15.19

Means are ± SD (n=6) for a tissue in a column followed by the same letter are not significantly different ($P \leq 0.05$) from each other according to Duncan's multiple range (DMR) test.

Table 3: AchE activity (µM of acetylcholine hydrolyzed/mg protein/h) and Ach level (µM/g wet wt.) in the liver of the fish, *Cyprinus carpio* following exposure to sublethal concentration of quinalphos.

Parameters	Control	Sublethal Exposure period in days		
		5	10	15
AchE activity	1.952 d	1.631 a	1.726 b	1.871 c
±SD	0.008	0.007	0.004	0.005
% Change	----	-16.44	-11.57	-4.14
Ach	16.891 a	17.092 d	17.387 e	16.901 f
±SD	0.0023	0.003	0.005	0.004
% Change	----	-1.189	-2.93	-0.005

Means are ± SD (n=6) for a tissue in a column followed by the same letter are not significantly different ($P \leq 0.05$) from each other according to Duncan's multiple range (DMR) test.

ACKNOWLEDGMENT

I am thankful to my parents and my family members for their kind support during research work.

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