IN VIVO INHIBITION OF ACETYLCHOLINESTERASE ACTIVITY IN FUNCTIONALLY DIFFERENT TISSUES OF THE FRESHWATER FISH, *Cyprinus carpio*, UNDER CHLORPYRIFOS EXPOSURE

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SUMMARY

The inhibitory effect of chlorpyrifos on acetylcholinesterase (AChE) activity in different concentrations and exposure periods was investigated in the fish, *Cyprinus carpio*. Sublethal concentrations 14% (0.0224 mg/l) and 7% (0.0112 mg/l) of the lethal concentration (0.160 mg/l) of chlorpyrifos were used in the present study. Carp were exposed to both toxicant concentrations for 1, 7, and 14 days and were allowed to recover in toxicant-free medium for 7 days after 14 days of exposure. AChE activity was determined spectrophotometrically using acetylthiocholine iodide as substrate in the tissues of brain, gill, liver, and muscle. The present study showed time and concentration dependent inhibition of AChE activity by chlorpyrifos in the tissues of the fish, *C. carpio*. The highest decrease in AChE activity was recorded in brain followed by muscle, gill, and liver on day 14 in both sublethal concentrations. AChE activity increased during the recovery period, but was still lower than the control group after both sublethal

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concentrations. Carp in toxic media exhibited erratic and darting swimming movements, hyperexcitability, and loss of equilibrium. These symptoms persisted even after the recovery period and were due to inhibition of brain AChE activity. Depression of AChE activity suggested decreased cholinergic transmission and consequent accumulation of acetylcholine (ACh). Excess accumulation of ACh led to prolonged excitatory postsynaptic potentials resulting in repeated, uncontrolled firing of neurons and cessation of nerve impulses. This led to behavioural and morphological changes due to impaired neurophysiology of the fish. Greater elimination half-life and biotransformation of sequestered chlorpyrifos in the storage tissues may be attributed to the fact that AChE activity failed to reach control levels even after the recovery period.

KEY WORDS

organophosphate, teleost, neurobehavioural toxicity, AChE activity, caudal bending, recovery

INTRODUCTION

Organophosphorus pesticides (OPs) are one of the most important classes of synthetic compounds used for protecting crops, livestock, and human health during the past 60 years /1-3/. Approximately 200 different OPs are currently used commercially worldwide /4/. The main advantage of the OPs is their low accumulation and short-term persistence in the environment /5/.

OPs are esters, amides, or thiol derivatives of either phosphoric acid or thiophosphoric acid. The majority now in use, such as azinphosmethyl, chlorpyrifos, and malathion, contain the thiono moiety (=S). The substitution of (=S) for (=O) on the phosphorus atom increases the toxicity of the insecticide, such as is the case with malathion and its oxygen analogue, malaoxon /6/.

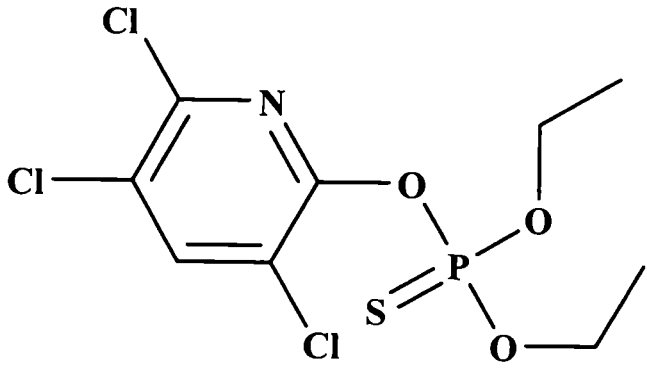
Many organophosphates are potent neurotoxins, functioning by inhibiting the action of acetylcholinesterase (AChE) in nerve cells. The primary effect of chlorpyrifos and other OPs on vertebrate and invertebrate organisms is the inhibition of AChE activity, the enzyme that degrades the neurotransmitter acetylcholine (ACh) in cholinergic

synapses /7/. Duration of exposure, type of OP, as well as species of fish has an effect on the extent of AChE expression. Neurotransmitters such as ACh are profoundly important in the development of the brain. Many OPs have neurotoxic effects on developing organisms even at low levels of exposure. ACh has a much higher turnover rate *in vivo* than any other transmitter including catecholamines and amino acids /8/. AChE was identified as the enzyme responsible for termination of cholinergic transmission by cleavage of the ester linkage of ACh to acetate and choline; AChE is found in cholinergic synapses in the brain as well as in autonomic ganglia, the neuromuscular junction, and the target tissues of the parasympathetic system /9,10/. AChE activity is vital to normal behaviour and muscular function and represents a prime target on which some toxicants can exert a detrimental effect. Inhibition of AChE activity results in a build-up of ACh causing prolonged excitatory postsynaptic potentials. This results in repeated, uncontrolled firing of neurons leading to hyperstimulation of the nerve/muscle fibers, which leads to tetany, paralysis, and eventual death.

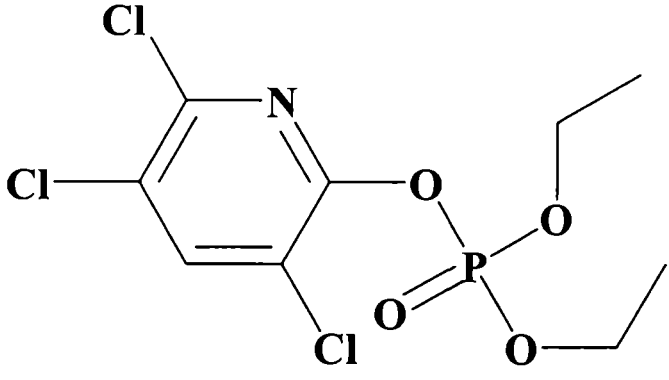
Chlorpyrifos [*O,O*-diethyl-*O*-(3,5,6-trichloro-2-pyridyl)phosphorothioate] is a member of the organophosphate class of insecticides that displays broad spectrum insecticidal activity. Chlorpyrifos protects crops against a wide variety of pests, such as aphids, corn borers, and cutworms.

Chlorpyrifos is transformed into its active analogue chlorpyrifos-oxon (Fig. 1) /11,12/ in animals, which is about 3,000 times as potent against the nervous system as chlorpyrifos itself /13/. Like all organophosphates, chlorpyrifos and chlorpyrifos-oxon kill insects and other animals, including humans, because of their toxicity to the nervous system. They inhibit AChE, which breaks down ACh, involved in neurotransmission across the synaptic junctions. Without a functioning AChE, acetylcholine accumulates, producing rapid twitching of involuntary muscles, convulsions, paralysis, and ultimately death /14/.

AChE activity is a biomarker often used in aquatic ecotoxicology studies /15/, and is a sensitive enzyme to low environmental concentrations of organophosphorous compounds. Chlorpyrifos is an extensively used organophosphate in agricultural fields of our region. The environmental concentrations of chlorpyrifos ranged between 0.011 and 0.022 mg/l, which persisted for several weeks. Contamination of aquatic ecosystems by sublethal levels of chlorpyrifos is



a



b

Fig. 1: Molecular structure of chlorpyrifos (a) and its active analogue chlorpyrifos-oxon (b).

common and has serious impact on non-target fish, including *Cyprinus carpio* (Cyprinidae).

The objective of the present investigation was to determine the effect of sublethal concentrations of chlorpyrifos on AChE activity of the brain, gill, liver, and muscle in *C carpio* Linnaeus and consequent behavioural anomalies in the test species.

MATERIALS AND METHODS

Collection of carp and their maintenance

The State Fisheries Department, Dharwad, India, provided healthy and active *C. carpio* fingerlings (2 ± 0.21 g, 4 ± 0.25 cm). Large aerated crates were used to transport fish to the laboratory. Before investigation, fish were maintained for 30 days in large cement tanks (22 x 12 x 5 feet). Further carp (30 fingerlings) were conditioned (acclimatized) to laboratory conditions for 20 d at 24 ± 1°C in each 100 l glass aquarium (120 x 45 x 80 cm) containing dechlorinated tap water of the quality used in the test. Analysis of physical and chemical water quality parameters was determined by APHA methods /16/, and found as follows: temperature 24 ± 2°C; pH 7.1 ± 0.2 at 24°C; dissolved oxygen 9.6 ± 0.8 mg/l; carbon dioxide 6.3 ± 0.4 mg/l; total hardness 23.4 ± 3.4 mg as $CaCO_3$/l; phosphate 0.39 ± 0.002 µg/l; salinity, nil; specific gravity 1.001; and conductivity less than 10 µS/cm. Water was renewed every day and a 12-12 h photoperiod was maintained during acclimatization and test periods. Fish were fed (*ad libitum*) daily with commercial dry feed pellets (Nova, Aquatic P. Feed) during acclimatization and test periods but for acute toxicity test, feeding was stopped two days prior to exposure to the test medium.

Acute toxicity test

Chlorpyrifos (20% EC [emulsifiable concentrate]) was procured from the local market of Dharwad, Karnataka, India, under the trade name Hyban, 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 chlorpyrifos was drawn directly from this 20% EC using a micropipette.

The carp (10 fingerlings in 20 l of test medium in each replicate) were exposed to varying concentrations of chlorpyrifos with six replicates for each concentration along with the control sets. Concentrations of the test compound used in short-term definitive tests were between the highest concentration at which there was 0% mortality (0.120 mg/l) and the lowest concentration at which there was 100% mortality (0.200 mg/l). Replacement of the water medium was followed by the addition of the desired concentration of the test compound every 24 h.

Mortality was recorded every 24 h and the dead fish were removed when observed, each time noting the number of dead fish at each concentration up to 96 h for estimation of acute toxicity (LC_{50}). Duncan's multiple range test /17/ was employed for comparing mean mortality values after estimating the residual variance by repeated measures ANOVA /18/ for arc sine transformed mortality data (dead individuals/initial number of individuals). Time of exposure was the repeated measure factor while treatment (concentration and control) was the second factor. In addition, LC_{50}s were compared by the method of APHA /16/. The LC_{50} with 95% confidence limits for chlorpyrifos were estimated for 96 h by probit analysis /19/.

Experimental design and toxicant concentrations

Sublethal concentrations of 14% (0.0224 mg/l) and 7% (0.0112 mg/l) of the lethal concentration (96 h LC_{50}) were selected for subacute study. Test medium (20 l) consisted of 10 fish and six replicates were maintained for each sublethal concentration. Carp were exposed to both test concentrations for 1, 7, and 14 days and were allowed to recover in toxicant-free medium for 7 days after 14 days of exposure (designated as R) along with the control sets. In experimental periods test medium was renewed daily followed by addition of the respective test concentrations of chlorpyrifos up to day 14. Exposure concentrations of chlorpyrifos in the test medium were confirmed by GC-MS analysis. AChE activities were determined in brain, gill, liver, and muscle tissues including the controls during the experimental periods. Comparative behavioural responses were studied in both control (toxicant-free medium) and treated fish.

Estimation of AChE (E.C. 3.1.1.7) activity

Tissues were excised in physiological saline (0.9% NaCl). Homogenates (4%) of brain, gill, liver, and muscle were prepared in cold 50 mM Tris-HCl (pH 6.8) extraction buffer using a glass-teflon homogenizer (Remi Motors Ltd., Mumbai, India) and then centrifuged at 3,000 rpm for 15 min. All processes were carried out at 4°C and supernatants were used for enzymatic assay. AChE activity was assayed by the method of Ellman *et al.* /20/ by measuring the increase in extinction at 412 nm in a spectrophotometer (Systronics, model no. 169) and expressed as nM of acetylthiocholine iodide hydrolyzed/mg protein/ min.

Protein content was estimated according to the method of Lowry *et al.* /21/ using bovine serum albumin as standard.

Statistical analysis

Data correspond to the average of six replicates. The data obtained were analyzed statistically using Duncan's multiple range test /17/.

RESULTS AND DISCUSSION

Chlorpyrifos toxicity

Acute toxicity (96 h LC_{50}) of chlorpyrifos for the freshwater fish, *C. carpio*, was found to be 0.160 mg/l. The upper and lower 95% confidence limits were found to be 0.168 and 0.151 mg/l, respectively. No significant mortality was observed during the experimental periods, but the fish showed dullness, loss of equilibrium, loss of feeding, and erratic swimming, which indicated symptoms of stress.

Inhibition of AChE activity

Fish exposed to sublethal concentration (7% of 96 h LC_{50}) of chlorpyrifos recorded maximum depression in AChE activity in the brain (-54.48%) followed by muscle (-52.68%), gill (-43.54%), and liver (-35.88%) on day 14 of exposure. Recovery for 7 days showed an increase in AChE activity compared to day 7 and 14, but it remained low in the liver (-8.15%) and gill (-10.51%) compared to day 1. AChE activity was lower in brain (-23.22%) and muscle (-19.87%) in the

recovery period compared to day 1 and controls. Overall decline in AChE activity significantly differed in comparison with the control group even after the recovery period (Table 1, Fig. 2).

The decrease in AChE activity in the tissues of the fish exposed to sublethal concentration (14% of 96 h LC_{50}) of chlorpyrifos was highest in the brain (-75.27%) followed by muscle (-72.45%), gill (-58.28%), and liver (-51.15%) on day 14 of exposure (Table 1). Recovery for 7 days showed an increase in AChE activity compared to 7 and 14 days, but remained lower compared to day 1 and controls (Fig. 2). The brain (-39.80%) showed the highest decline in AChE activity followed by muscle (-34.61%), gill (-23.70%), and liver (-17.49%) in the recovery period.

Time and concentration dependent inhibition of AChE activity by chlorpyrifos in the tissues of *C. carpio* was found in the present study. At sublethal concentrations, chlorpyrifos caused greater inhibition of AChE activity in brain, muscle, gill, and liver tissues. Inhibition of AChE activity in functionally vital organs, such as brain, gill, and liver, leads to impaired critical neurophysiological, neurochemical, and neurobehavioural processes in the fish. Further, these effects were most clearly seen following acute exposure, but they were also observed in subacute cases as well. The inhibition of AChE activity results in build-up of ACh within the nerve synapses leading to a variety of neurotoxic effects and decreased cholinergic transmission /22/. Results obtained by different workers independently of tissues and species used are quite similar in the AChE inhibitory effects by OPs; our results are in accordance with earlier observations made by Rao /23/, Chawanrat *et al.* /24/ and Elif and Demet /25/.

Depression of AChE activity in the brain is more sensitive to chlorpyrifos exposure than that in muscle, gill, and liver of the carp. The data reflect that inhibition of this magnitude may not be lethal to all species, but it may pose a deleterious impact on important neurobehavioural functions, such as swimming. The behavioural changes observed in the exposed fish, such as erratic, darting, and burst swimming, are due to impaired neuronal function of the central nervous system because of inhibition of brain AChE activity.

Caudal bending was noticed with time and persisted even under the recovery period in the test population exposed to 14% and 7% of lethal concentration of chlorpyrifos by 30% and 20%, respectively. The extent of caudal bending was more pronounced after exposure to

TABLE 1

AChE activity (nM of acetylthiocholine iodide hydrolyzed/mg protein/min) in the tissues of the fish, *C. carpio*, following exposure to 14% (0.0224 mg/l) and 7% (0.0112 mg/l) of lethal concentration (0.160 mg/l) of chlorpyrifos

Experimental period	Brain	Gill	Liver	Muscle
		7% of 96 h LC_{50}		
Control	$328.62^{a} \pm 3.88$	$197.21^{a} \pm 3.91$	$186.44^{a} \pm 4.56$	$303.28^{a} \pm 4.09$
Day 1	$256.89^{b} \pm 3.74$	$167.15^{c} \pm 5.01$	$165.97^{c} \pm 4.85$	$245.86^{b} \pm 3.65$
Day 7	$198.92^{e} \pm 4.96$	$130.29^{f} \pm 4.56$	$141.71^{f} \pm 3.95$	$197.15^{f} \pm 4.94$
Day 14	$149.57^{h} \pm 3.59$	$111.34^{g} \pm 3.86$	$119.53^{g} \pm 4.64$	$143.51^{h} \pm 4.71$
Recovery	$252.31^{c} \pm 3.67$	$176.47^{b} \pm 4.95$	$171.23^{b} \pm 3.69$	$242.99^{c} \pm 3.86$
		14% of 96 h LC_{50}		
Day 1	$223.55^{d} \pm 4.85$	$153.35^{d} \pm 3.48$	$156.50^{d} \pm 3.85$	$227.46^{d} \pm 3.34$
Day 7	$158.91^{g} \pm 3.78$	$107.61^{h} \pm 4.58$	$113.08^{h} \pm 4.86$	$144.31^{g} \pm 4.81$
Day 14	$81.24^{i} \pm 3.59$	$82.27^{i} \pm 3.65$	$91.06^{i} \pm 4.32$	$83.53^{i} \pm 3.96$
Recovery	$197.82^{f} \pm 4.78$	$150.46^{e} \pm 4.58$	$153.83^{e} \pm 3.76$	$198.29^{e} \pm 5.23$

Data are means ± SD (n = 6). Results followed by a letter are significant ($p < 0.05$) according to Duncan's multiple range test.

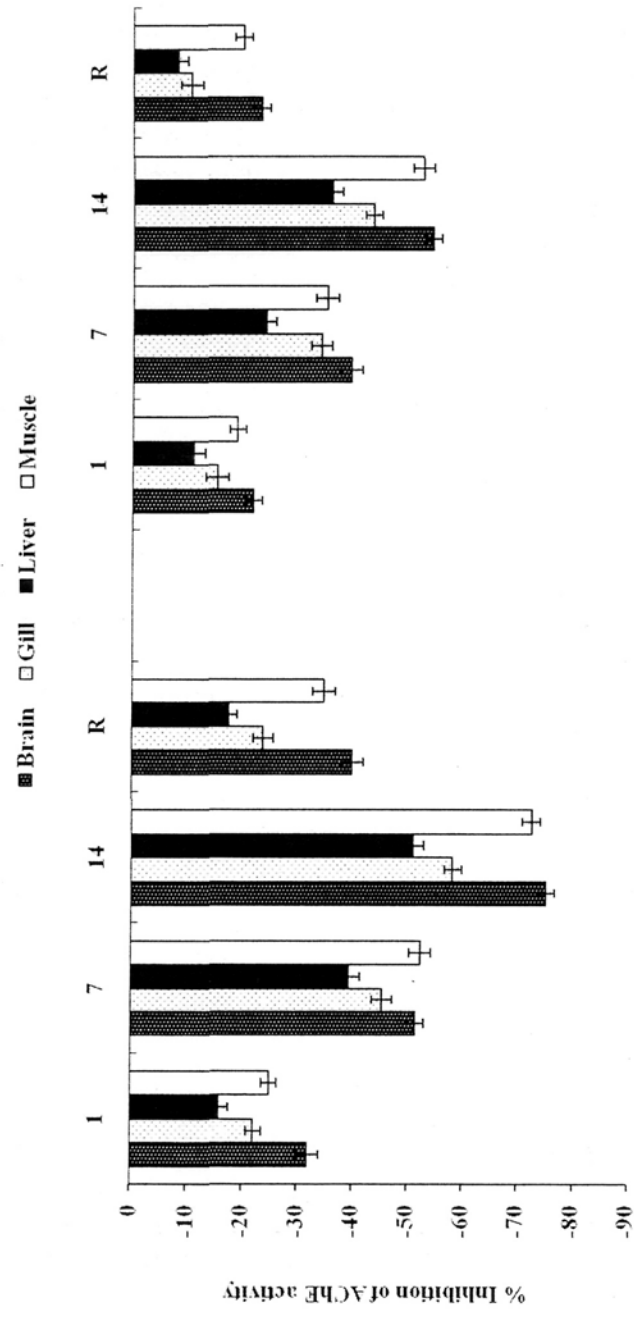


Fig. 2: Inhibition of AChE activity in the tissues of common carp (*C carpio*) fingerlings during exposure to sublethal concentrations (14% and 7% of 96 h LC_{50}) of chlorpyrifos for 1, 7 and 14 days, and recovery over 7 days. Graph represents trend of the data given in Table 1.

the higher toxicant concentration. This greatly retarded the normal swimming pattern. Caudal bending may be a sort of paralysis, which is due to the inhibition of muscular AChE activity resulting in blockage of neural transmission. Bending of the caudal region is due to the fact that the caudal portion is the thinnest structure and hence can be affected due to paralysis of the caudal musculature by inhibition of AChE activity as shown in the present study. Further, inhibition of AChE activity results in a progressive accumulation of ACh, especially during periods of repetitive stimulation, leading to desensitization of nicotinic acetylcholine receptors (nAChRs) and consequent muscular weakness /26/. Thus, chlorpyrifos reduced instinctive behavioural responses and affected morphological features by depression of AChE activity.

Chlorpyrifos (CPF) inhibits AChE activity due to the effects of its active oxygen analog chlorpyrifos-oxon (CPF-oxon) /27/. The parent compound, CPF, is metabolized to the oxon form via a desulfuration reaction initiated by cytochrome P450 (CYP) /28,29/. Competing with formation of the oxon is the detoxification metabolism of CPF to 3,5,6-trichloro-2-pyridinol (TCP) via a dearylation reaction utilizing the same enzymes. A-esterase (PON1) also contributes to the production of TCP through the metabolism of CPF-oxon /29/. The ratio between the toxification/detoxification reactions determines the degree of enzyme inhibition and can be used to evaluate metabolic processes /27/.

AChE activity failed to reach control levels even after the recovery period, which may be due to the greater elimination half-life of chlorpyrifos. Elimination of chlorpyrifos for longer periods may lead to biotransformation of sequestered chlorpyrifos in the storage organs (adipose and muscular tissues) to their active oxygen analog CPF-oxon. Further, in the recovery period, newly synthesized AChE may be inhibited by the active oxygen analog CPF-oxon.

CONCLUSION

Neurotoxicity of chlorpyrifos was shown in the current study by inhibition of AChE activity at sublethal concentrations in the tissues of the fish, *C. carpio*, in a typical time and concentration dependent relationship. Inhibition of AChE activity in the brain appears to be an early process in response to sublethal exposures, and could be a more

sensitive biomarker than inhibition of AChE activity in the muscle, gill, and liver to characterize toxicological impact. Depression of AChE activity had a critical impact on fish neurophysiology, which ultimately led to impaired behavioural responses and morphological deformities. Even after the recovery period, AChE activity failed to reach control levels, which might be due to the large elimination half-life of chlorpyrifos and biotransformation of sequestered chlorpyrifos in the storage organs to its active oxygen analog CPF-oxon. Further, during the recovery period, newly synthesized AChE enzyme may be inhibited by the active oxygen analog CPF-oxon.

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