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Response of Brain and Liver Cholinesterases of Nile Tilapia, *Oreochromis niloticus,* to Single and Multiple Exposures of Chlorpyrifos and Carbosulfan

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The toxicity of organophosphorus and carbamate insecticides is mainly due to the inhibition of acetylcholinesterase (AChE), the enzyme which cleaves the neurotransmitter acetylcholine, thereby interfering with proper neurotransmission in cholinergic synapses and neuromuscular junctions. Although both types of insecticides have a common mode of action, organophosphorus insecticides are irreversible inhibitors of AChE whereas carbamates are often considered as reversible inhibitors of the enzyme as a relatively weak bond is formed between the carbamate and AChE (Ecobichon 1992). In addition to AChE, vertebrates may also contain the related enzyme pseudocholinesterase in several tissues (Massoulié et al. 1993). Both enzymes are inhibited by organophosphorus and carbamate insecticides and referred to as cholinesterses (ChE).

ChE activities of fish have been recognized as a potential biochemical indicator for toxic effects of these insecticides (Gruber and Munn 1998; Dembélé et al. 2000; Fulton and Key 2001). Organophosphorus and carbamate insecticides are widely used in tropical agriculture. Nile tilapia, *Oreochromis niloticus* has been suggested as a bioindicator species for testing pollutant impact especially in tropical waterbodies due to its abundance and hardy nature. With repeated inputs of anticholinesterase chemicals to the aquatic environments, fish may be exposed to acutely lethal to sublethal concentrations. The degradation of the chemical on the other hand would allow the affected fish to recover from the poisoning. An understanding of the patterns of inhibition and recovery of ChE enzymes of Nile tilapia under different exposure regimes would enable better interpretation of ChE data of this fish exposed to environmental applications of anticholinesterase chemicals. The objective of the present study was to evaluate the response of cholinesterase in brain and liver tissues of Nile tilapia following single and multiple exposure to two selected anticholinesterase insecticides viz. chlopyrifos (an organophosphorous insecticide) and carbosulfan (a carbamate).

MATERIALS AND METHODS

Fingerlings of Nile tilapia (*Oreochromis niloticus*) were obtained from Rambadagalla Fish Breeding Station, National Aquaculture Development Authority of Sri Lanka. Fish were allowed to acclimate to laboratory conditions in glass aquaria filled with continuously aerated aged tap water under natural photoperiod for two weeks. Half of the water in each aquarium was exchanged with aged tap water every three days.

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During the acclimation period, fish were fed daily with commercial fish food pellets (Prima, Sri Lanka) at 1% of the body weight. The body size of the fish used in the experiments was 6.2 – 7.8 cm in total length and 4.5 – 6.9 g in body weight.

Commercial preparations of two insecticides viz. Pyrinex® (chlorpyrifos, 400 g L^{-1}, an emulcifiable concentrate from Baur and Co. Ltd., Colombo, Sri Lanka) and Marshal® (carbosulfan 200 g L^{-1}, a soluble concentrate from FMC Corporation, PA, USA) were used for the insecticide exposure. Prior to the introduction of fish, stock solutions of chlorpyrifos or carbosulfan (1 mg L^{-1}) were prepared freshly by diluting commercial formulations of the insecticide with aged tap water. The solutions were further diluted to obtain a concentration of 10 µg L^{-1} of chlorpyrifos or carbosulfan in the glass aquaria (60 X 30 X 30 cm) which contained 40 L of the test water.

The experiments were designed to expose the fish to chlorpyrifos or carbosulfan once or twice during the experimental period. In each exposure, several aquaria each containing six fish in 40 L of the test medium were used. In the first experiment, thirty fish were introduced to the test media containing 10 µg L^{-1} chlorpyrifos (five aquaria each containing six fish in 40 L of the medium). After 24 hours of chlorpyrifos exposure (1^{st} exposure), three fish from two of the aquaria (n=6) were randomly obtained for the ChE assay and the remaining fish were transferred to clean aged tap water for recovery. After 7 days and 14 days of recovery in clean water, samples of these fish (n = 6) were obtained for ChE assay.

Twelve fish which were allowed to recover in aged tap water for 7 days after the 1^{st} exposure were re-exposed to freshly prepared 10 µg L^{-1} chlorpyrifos for an additional 24 hours (2^{nd} exposure). In the 2^{nd} exposure two aquaria were used and each contained six individuals of the recovering fish. After the 2^{nd} exposure, three fish were obtained from each of the aquaria for ChE assay and the remaining fish were transferred to clean aged tap water and allowed to recover for seven days until they were used for ChE assay. Fish maintained continuously in the clean aged tap water served as controls (n = 6 per aquarium) and they were sacrificed along with the insecticide exposed fish at each time period for ChE assay.

In the second experiment, the same procedure was repeated for single or multiple exposures to 10 µg L^{-1} carbosulfan along with another set of comparable controls. Fish were fed daily with the fish food pellets at 1% of the body weight. Aged tap water in the aquaria was renewed every 2 to 3 days. Temperature, pH, dissolved oxygen (DO) in water in the aquaria were measured once in two days using water quality monitors (HACH company, USA) and were within the favorable limits for fish: temperature 26 – 28 °C; pH 7.1-7.2; DO 4.9-5.1 mg L^{-1}.

The samples of fish were sacrificed and brains and liver were removed. The enzyme source was prepared by homogenizing the tissues with 0.1 M phosphate buffer, pH 8.0. ChE activities of the homogenates were assayed using acetylthiocholine iodide as the substrate following the method of Ellman et al. (1961). The chemicals for the ChE assay were obtained from Sigma, USA. Enzyme activities in the tissues are expressed as mean ± standard deviations of six fish per group. Differences between the ChE activities of insecticide-exposed and comparable control group were determined using Students' t-test. Percentage inhibition of enzyme activity was calculated as differences

in enzyme activity of the insecticide exposed fish in relation to the mean value of the respective control group. Mean differences were considered significant at $P \leq 0.05$ (Zar 1999).

RESULTS AND DISCUSSION

The responses of the cholinesterase enzymes in brain and liver tissues of Nile tilapia to initial chlorpyrifos or carbosulfan exposure and subsequent exposure to the same concentration of the insecticide are presented in Table 1. Results indicate that the enzyme activities of the insecticide exposed fish were significantly lower than those of the respective control fish. Percentages of ChE inhibition were greater in both tissues when the fish were exposed to the organophosphorous insecticide, chlorpyrifos, than to the same concentration of a cabamate insecticide, carbosulfan, indicating comparatively higher toxicity of chlorpyrifos to Nile tilapia at the concentration tested. Chlorpyrifos was also found to be more toxic than carbosulfan to fry of the common carp (De Mel and Pathiratne 2005).

Results indicate that the ChE activities in the liver tissues of the control fish (fish unexposed to the insecticides) were four to five folds lower than the activity of the brain tissues (Table 1). However, the degree of inhibition of ChE activity in the fish exposed to chlorpyrifos or carbosulfan was higher in liver tissues in comparison to the brain tissues. This may be due to several reasons. Fewer ChE molecules were probably present in liver as reflected by the control enzyme activities. Therefore, the concentration of the insecticide used in this study could have yielded an apparently larger effect in the liver tissues. Brain, with a higher level of ChE activity, displayed a proportionally a lower degree of inhibition. Chlorpyrifos is a phosphorothionate insecticide which must be activated by the cytochrome P450 dependent enzyme system to the oxon metabolite to effectively inhibit acetylcholinesterse at the primary target site (Straus and Chambers 1995). Carbosulfan may be metabolized in the liver by hydrolysis to phenols or to dibutylamine or carbofuran and subsequently may be further metabolized. Carbofuran, which is one of the metabolites of carbosulfan, is considered a highly toxic pesticide. Higher inhibition of ChE in the liver of Nile tilapia may also be due to the production of more potent reactive metabolites of the parent insecticide in the liver tissues by cytochrome P450 dependent enzymes. Low levels of cytochrome P450 in brain would lead to little (if any) target site activation. Recently, Rodríguez-Fuentes and Gold-Bouchot (2004) demonstrated that AChE is the enzyme present in brain tissue of Nile tilapia, whereas atypical ChE enzymes are present in both liver and muscle tissues. Presence of different molecular forms of ChE in brain and liver tissues of Nile tilapia may have had different sensitivities to the insecticides used in this study. Straus and Chambers (1995) found that in channel catfish acetylcholinesterase activity in the liver was much lower than that of brain but liver exhibited a higher percentage of enzyme inhibition in comparison to brain and muscle tissues when the fish were exposed to anticholinesterase pesticides.

Table 1. Cholinesterase activities* (nmoles min^{-1} mg^{-1}) in brain and liver tissues of fingerlings of Nile tilapia, *Oreochromis niloticus* exposed once or twice to chlorpyrifos or carbosulfan

Fish	1st exposure	7 days recovery after 1st exposure	2nd exposure	7 days recovery after 2nd exposure	14 days recovery after 1st exposure
Brain tissue					
Control	18.09 ± 1.51	17.53 ± 0.61	17.69 ± 1.19	16.45 ± 0.79	16.44 ± 0.79
Chlorpyrifos exposed	8.68 ± 0.66** (52%)	9.71 ± 1.10** (45%)	5.87 ± 0.44** (67%)	6.88 ± 0.56** (58%)	10.43 ± 1.28** (37%)
Control	18.18 ± 0.55	17.09 ±1.03	17.59 ± 0.62	16.82 ± 0.44	16.81 ± 0.42
Carbosulfan exposed	14.47 ± 1.43** (20%)	14.05 ± 2.23** (18%)	11.41 ± 1.58** (35%)	12.44 ± 0.86** (26%)	14.68 ± 0.19** (13%)
Liver tissue					
Control	3.87 ± 0.36	3.39 ± 0.68	3.84 ± 0.39	4.17 ± 0.51	4.16 ± 0.50
Chlorpyrifos exposed	1.02 ± 0.11** (74%)	1.44 ± 0.16** (58%)	0.59 ± 0.08** (85%)	1.39 ± 0.34** (67%)	2.73 ± 0.23** (34%)
Control	3.58 ± 0.39	3.19 ± 0.16	3.18 ± 0.49	3.22 ± 0.12	3.23 ± 0.22
Carbosulfan exposed	2.21 ± 0.13** (38%)	2.33 ± 0.26** (27%)	1.77 ± 0.17** (44%)	1.98 ± 0.25** (39%)	2.45 ± 0.24** (24%)

*Fish were exposed to 10 μg L^{-1} chlorpyrifos or carbosulfan for 24 hours (1st exposure) and were allowed to recover in aged tap water for 7 - 14 days. Another group of fish which had been exposed to chlorpyrifos or carbosulfan (1st exposure) were subsequently re-exposed to freshly prepared insecticide at the same concentration for an additional 24 hours (2nd exposure) and were allowed to recover for 7 days. Results are presented as mean ± standard deviation of six fish. Numbers in parentheses indicate the percent inhibition of cholinesterase activity.

** Significantly different from the respective controls (Students' t-test, $P \leq 0.05$).

ChE activity was greatly inhibited in both tissues when the fish were exposed to the same concentration of the insecticide for the second time. Therefore repeated exposures to anticholinesterse compounds appear to bring about increased depression of ChE activity in Nile tilapia.
In the recovery phase, the ChE activity of the brain and liver tissues of all insecticide exposed fish differed significantly from the control fish and full recovery was not attained at the end of the experimental period. The results show that the recovery of ChE activity in the brain and liver tissue of the fish exposed only once to the insecticide was greater than that in the fish exposed to the insecticide for the second time. The brain ChE of the fish exposed once to chlorpyrifos or carbosulfan recovered up to 63% (37% inhibition) and 87% (13% inhibition) of the controls at the end of the experiment whereas the activity in fish exposed to chlorpyrifos for two times recovered only up to 42% (58% inhibition) and 74% (26% inhibition) of the controls respectively. The same pattern was seen in the recovery of enzyme activity in the liver tissues. The results indicate that the history of prior exposure to anticholinesterse pesticides influences the recovery period of ChE activity in the brain and liver tissues of Nile tilapia if the enzyme had not returned to those of normal levels following the earlier exposure.

As the organophosphorus insecticides irreversibly bind with the AChE forming a stable bond, toxicity will persist until sufficient quantities of new enzyme are synthesized to destroy the excess acetylcholine efficiently at the synapses. Carbamates are considered as reversible inhibitors of AChE. Hence the full complement of AChE may not need to be synthesized because some is released from the carbamate – AChE bonding to continue normal activity (Ecobichon 1992). Morgan et al. (1990) demonstrated that the time for recovery of brain acetylcholinesterase in Atlantic salmon was a function of the degree of initial inhibition by the organophosphorous insecticide, fenitrothione. This is likely because of the recovery of enzyme activity of the fish exposed to anticholinesterase chemicals especially organophosphorous insecticides is mainly as a result of de novo synthesis of enzyme protein. Hence the greater degree of inhibition, the more protein synthesis is required (Fulton and Key 2001). As repeated exposures appear to bring about increased depression of ChE activity in brain and liver tissues of Nile tilapia, intermittent anticholinesterase pesticide applications, especially organophosphorus insecticides, to the agricultural lands during cultivation seasons may manifest a threat to the fish populations inhabiting water bodies adjacent to these lands. Even though the effects on ChE activity may not be perceptible in Nile tilapia initially due to the very low concentrations, significant inhibition of ChE activity may occur upon repeated exposure to even very low levels of the anticholinesterase compounds in the environment during the cultivation seasons.

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