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IN VIVO INHIBITION AND RECOVERY OF BRAIN ACETYLCHOLINESTERASE IN TOPMOUTH GUDGEON (*PSEUDORASOBORA PARVA*) FOLLOWING EXPOSURE TO FENITROTHION

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Abstract: Freshwater fish, topmouth gudgeon (*Pseudorasobora parva*), were pretreated with piperronyl butoxide (PBO) or triphenyl phosphate (TPP) and then exposed to different concentrations of fenitrothion (FNT) in a static system. Evaluation of brain acetylcholinesterase (AchE) activity after 24, 48 and 96 h pesticide exposure indicated that AchE activity decreased as the concentration increased. Fish pretreated with TPP exhibited significantly decreased AchE activity whilst in the PBO pretreated group, increased activity was observed as compared with those exposed to FNT alone. The pattern of AchE recovery was also assessed in fish previously exposed for 96h and then transferred to clean (chemical free) water. Following 8 days of recovery period, the AchE activity of those exposed to FNT alone and pretreated with TPP was still lower than that of the control. This study showed that FNT may cause hazard to fish after field application.

Key words: acetylcholinesterase, fenitrothion, fish, inhibition, recovery
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INTRODUCTION

The active ingredient in the formulated product Sumithion, fenitrothion [0,0-dimethyl 0-(3-methyl - 4-nitrophenol) phos-phorothioate], is an organophosphate (OP) insecticide used in agriculture for crop protection and control of vector born diseases of public health and veterinary importance in various countries (Self et al., 1973; Volpe and Mallet, 1981; Gandahusada et al., 1984; Ernst et al., 1991; 1994). Although OP compounds tend to undergo fairly rapid degradation in the environment, with repeated input aquatic organisms may be exposed to sublethal concentrations for an extended period of time (Sancho et al., 1997).

Exposure to OP insecticides is known to decrease the activity of brain AchE in animals, birds, fish, etc. (Fleming & and Grue, 1981; Roberts et al., 1988; Morgan et al., 1990). Several studies successfully used the inhibition of AchE activity as a tool to diagnose Ops, including fenitrothion (FNT) poisoning, in fish (Zinkl et al., 1987; Galgani et al., 1992; Beyers & Sikoski, 1994; Escartin and Porte, 1996). In field studies, inhibition of AchE has been used as a tool in the monitoring of exposure of organisms during forestry spray programs involving OPs such as fenitrothion (Fancey et al., 1987). Also, analysis of brain AchE provides a method for diagnosing poisoning and detecting contamination of the water by anticholinesterase insecticides (Habig et al., 1986; Galgani & Bcoquene, 1989; Day and Scott, 1990; Sancho et al., 1997). In general, AchE has the potential for serving both as biochemical indicator of toxic stress and a sensitive parameter for testing water for the presence of toxicants (Devi and Fingerman, 1995). According to Qifa et.al. (1995), FNT is used in paddy field (in a so called 'rice-fish system') to control the rice stem borer, *Chilo suppressals* (Walker), in China. Although these authors reported that when used at 375 - 750g a.i. per ha (50% EC 500 - 800 × dilution), FNT is safe to fish, its sublethal effect on common species of fish has not been studied.

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Piperonyl butoxide (PBO) is a synthetic methy-lenedioxyphenol (MDP) compound used as a synergist in pesticidal formulations including FNT. Its inhibitory effect on cytochrome P-450-dependent mixed function oxidase (MFO) has been exploited in various studies involving metabolism and toxicity in different species of organisms including fish (Epstein et al., 1967; Reinbold, 1976; Glikman et al., 1977; Levin & Morphy, 1977; Melanocon et al., 1977; Erickson et al., 1988; Qifa et al., 1995). According to Plapp et.al., (1963) and Plapp and Tong (1966), triphenyl phosphate (TPP) was found to be one of the most active OP compounds used as synergist against some resistant strains of insects. In another research work, Oppenoorth and Welling (1976) stated that TPP is a known specific inhibitor of carboxylesterase. Therefore, the knowledge of susceptibility of fish AchE to these synergists as well as combined with FNT would be useful from a toxicological point of view.

The goals of the present study were therefore to: (1) examine the dose-response relationship of brain AchE activity in the topmouth gudgeon (Pseudorasobora parva) exposed to FNT; (2) assess the effect of synergists (PBO and TPP) as well as their respective combinations with FNT on the susceptibility of fish AchE; and (3) determine the pattern of brain AchE activity recovery in fish previously exposed to FNT, synergists and their combinations.

MATERIALS AND METHODS

1. Test chemicals

Fenitrothion (FNT) of technical grade (93% w/w) was obtained from Ningbo pesticide factory (P. R. China). Piperonyl butoxide (PBO) (90%) and triphenyl phosphate (TPP) (> 99%) were products of Aldrich Chemical Company Inc. Acetylthiocholine (≥99%) and eserine (99%) were products of Fluka Chemical Company (Switzerland). 5.5'- Dithobis (2-nitrobenzoic acid) (DTNB) was obtained from Sigma Chemical Company and bovine serum albumin was product of Boehringer Company. All other chemicals were of analytical grade and obtained from local commercial sources.

2. Fish species

The topmouth gudgeon (*Pseudorasobora parva*) was used in this study. The fish (0.6 – 1.0g) was procured from a local pet market and acclimatized to the laboratory conditions for at least seven days prior to use. The fish selected for use in the exposure study were starved for at least 24 hours before use and during the experiment period. During the recovery period (192 h), the fish were provided with food every 24 h and immediately after feeding the water was changed.

3. Experimental conditions

Exposure of the fish to the test chemicals was carried out in 40 liters glass aquaria and the test diluent consisted of 20 liters aerated tap water. Each test aquarium contained 20 – 25 fish (the mass/volume ratio did not exceed 1g fish/L). The test was conducted at twater temperature of 23 ± 1 ℃.

The test concentrations of FNT used were based on the 96 h LC50 values, 1.64 mg/L, of topmouth gudgeon (Solomon et al., 2000). The concentration of PBO (1mg/L) used was based on recommendations of previous researchers (Glikman et al., 1977; Melanocon et al., 1977). The concentration of TPP (0.2 mg/L) used was determined to be the highest concentration that did not cause mortality in fish used in this study.

Two sets of experiments were conducted in this study. The first experiment was carried out in a 96 h static exposure system. In this experiment the fish were exposed to (1) different concentrations of FNT alone and, (2) 0.4 mg/L of FNT after 24 h pretreatment with either PBO (1 mg/L) or TPP (0.2 mg/L) and, (3) PBO or TPP alone and AchE activity in these exposed group was compared with that of the control. In the second experiment, fish exposed for 96 h in the first experiment were then transferred to clean water to monitor the rate of recovery of AchE activity. In all cases fish that were not exposed to chemicals served as control group.

4. Tissue preparation and AchE analysis

From each test group six fish were used for analysis of AchE activity at exposure time of 24, 48 and 96 h, and recovery times of 96 and 192

h. After a sharp blow to the head followed by cervical dislocation killed the test fish, the brain was carefully removed intact and homogenized in 0.05M Tris-HCl buffer (pH 8.1) for about 45 seconds with a Teflon coated manual glass tissue homogenizer and pestle. The homogenate was centrifuged at 2000 rev/min for 15 min., the sediment was discarded and the supernatant used as an enzyme source.

AchE assays were performed spectroph – otometrically using the method of Ellman et. al, (1961). Acetylthiocholine (ATCI) was used as a substrate and DTNB as the thiol indicator. The total protein content of brain tissue from test fish was determined by the folin phenol reagent method of Lowry et al. (1951) using bovine serum albumin as standard. The AchE activity was expressed as nmol·min^{-1}·mg protein^{-1}.

5. Statistical analysis

A two-way analysis of variance (ANOVA) was used to determine treatment effect and Duncan's significant difference test was used for mean separation. The significance level was set at 0.05.

RESULTS AND DISCUSSIONS

1. AchE response

At test concentrations selected for topmouth gudgeon (0.2, 0.4 and 0.6 mg/L), no death of fish was observed during the experiment. Since AchE inhibition observed in this study was less than 50%, the above observation agrees with the statement of Day and Scott (1990) that a 50% or greater depression of AchE activity is indicative of a life-threatening situation in fish. However, few of them exhibited sign of hyperactivity and loss of equilibrium at the highest concentrations. This observation seems to be consistent with that of Grue et al. (1992) that before lethal effects are seen, OP compounds cause a variety of sublethal effects, including physiological changes when inhibition of brain AchE activity exceeds 40%. An experiment conducted to test if acetone (15 μL/L) used with test chemicals had any effect on brain AchE activity indicated that AchE activity did not exhibit any significant difference from their respective controls (data not indicated). Similar result was reported by Sancho et al. (1997) for European eel (*Anguilla anguilla*) exposed to 17 μL/L of acetone.

Table 1 shows that topmouth gudgeon exhibited greater decrease in brain AchE activity at higher concentrations and shorter exposure time. In all concentrations tested, the maximum AchE inhibition was noted at 24 h, the inhibition progressively decreasing over the 96 h exposure. Such a concentration-dependent response was also observed in the *A. anguilla* (Sancho et al., 1997) and in the crayfish, *Procambarus clarkii* (Escartin and Porte., 1996) exposed to FNT. Furthermore, such relationship had also been reported for a variety of other OP insecticides, e.g., dichlorvos (Hoy et al., 1991), methylparathion (Da Silva et al., 1993) and, diazinon (Ceron et al., 1996).

Table 1 Brain AchE activity (nmol·min^{-1}·mg protein^{-1}) of topmouth gudgeon (*P. parva*) exposed to different concentrations of fenitrothion

Exposure period (h)	Concentrations (mg/L)			
	0.00(control)	0.20	0.40	0.60
24	804.4 ± 5.69 (100)	500.59 ± 3.54* (62.2)	477.25 ± 3.37* (59.3)	441.84 ± 3.13* (54.9)
48	809.2 ± 2.59 (100)	630.37 ± 2.02* (77.9)	532.45 ± 1.71* (65.8)	498.46 ± 1.60* (61.9)
96	812.43 ± 2.84 (100)	738.50 ± 2.58* (90.9)	671.07 ± 2.37* (82.6)	557.33 ± 1.94* (68.6)

Activities are mean ± SD of three experiments (in each experiment six fish were used). Values in parenthesis are percentage AchE activities. * Significantly different from the control at $P < 0.05$

Genlin et al. (1991) detected an initial FNT concentration of 2.8 mg/L in paddy field (in rice-fish system) water after applying FNT to the field at the rate of 375-750g a.i. per ha (50% EC 500 – 800 × dilution). They reported that at this application rate, FNT did not cause death in common carp, crucian carp and grass carp, and stated that it is safe to fish. Since the LC_{50} of FNT for topmouth gudgeon (1.64 mg/L) is much lower than the reported concentration in water, it seems obvious that such concentration (2.8 mg/L) can cause death in topmouth gudgeon.

AchE activity of topmouth gudgeon exposed to PBO alone was significantly increased (by 13.7%) compared with the control during the first 24 h exposure, but progressively decreased by 2.4 and 9.9% at the end of 48 and 96 h, respectively. These results suggest that, increased AchE activity may be due to induction by parent PBO. Moreover, it seems reasonable to assume that the decreased activity with exposure time perhaps is due to progressive decrease in the concentration of PBO in the test system. These assumptions seem to be supported by the observations of Erickson et al. (1988) that in the water of static test system PBO concentration decreases as the animal assimilates it, perhaps dropping to a low enough concentration that uptake of PBO no longer occurs. However, the exact mechanism of induction or inhibition of AchE by PBO alone needs further investigation.

Compared with those exposed to FNT alone, AchE activity in those pretreated with PBO and then exposed to FNT was increased by 38.9%, 30.9%, and 5.8% following exposure for 24, 48 and 96 h, respectively, indicating an increase with exposure time (Fig. 1a). According to Kulkarni and Hodgson (1980), the parent phosphorothionates (P = S), such as FNT, parathion, etc., are poor AchE inhibitors, but are metabolically activated *in vivo* by the cytochrome P-450 monoxyge-nase (MO) system to the oxygen analogs (P = O) which are potent AchE inhibitors. Therefore, it is likely that inhibition of MO by PBO could significantly reduce the amount of oxygen analog of FNT (fenitroxon) produced and the net result would be increased AchE activity compared with those exposed to FNT alone.

Furthermore, FNT was shown by Hollingrworth (1969) to be metabolized (detoxified) by a glutathione (GSH) -dependent mechanism and Kamienski and Morphy (1971) suggested that, a large proportion of methyl parathion, a dimethyl phosphorothionate like FNT, undergo a GSH-dependent process during inhibition of MO by PBO. More recent study by Solomon et al. (2000) revealed that topmouth gudgeon pretreated with PBO had higher hepatic glutathione S-transferase (GST) activity than that in those exposed to FNT alone. Therefore, the increased AchE activity in the PBO pretreated group could be due to GST activity.

The AchE activity of fish exposed to TPP alone (0.2 mg/L) was significantly reduced ($P < 0.05$), the lowest inhibition (11.8%) being at the end of 24 h and the highest (32.1%) at 96h (Fig. 1b). These results suggest that TPP may have direct effect on AchE. However, according to Oppenoorth and Welling (1976), TPP is known to be a specific inhibitor of carboxylesterase. Considering this, a direct inhibition observed in the present study needs further investigation.

Susceptibility of AchE to FNT was significantly increased at the end of 96 h exposure period in those fish exposed first to TPP (Fig 1b). This suggests that inhibition of carboxylesterase by TPP might have increased tissue concentration of FNT and the production of its oxygen analog (fenitroxon) which consequently decreased AchE activity. This indirectly indicates the importance of carboxylesterase in detoxification of FNT. This last conclusion seems to be supported by reports from Chambers and Chambers (1990), Chambers et al. (1993) that OP-sensitive aliesterases are alternate phosphorylation sites that can serve as effective protection from the inhibition of the OP to the target enzyme, AchE.

In general, since a 20% decline in AchE activity was used as evidence of exposure to OPs (Fleming and Grue, 1981; Zinkl et al., 1991), topmouth gudgeon that have encountered FNT and TPP concentrations of 0.4 mg/L, 0.2 mg/L and higher, respectively, as well as their combinations, may be highly affected.

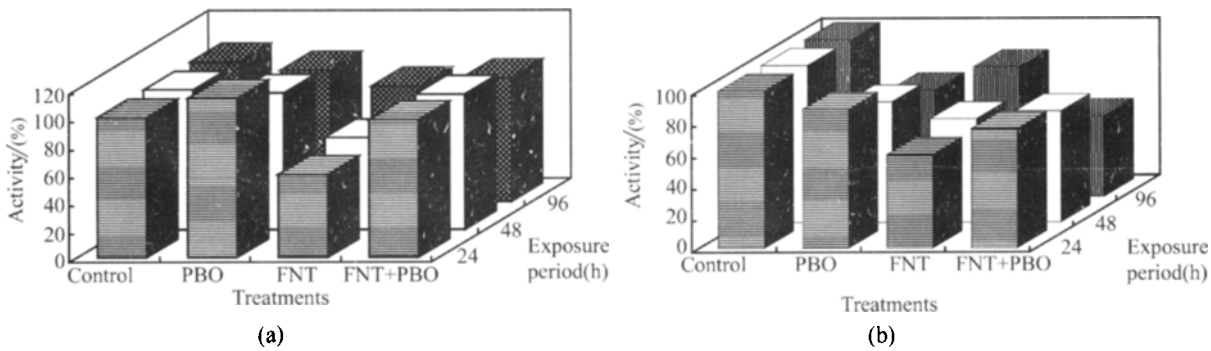

Fig.1 Brain AchE activity (%) of topmouth gudgeon (*P. parva*): (a) pretreated with PBO (1 mg/L) and then exposed to FNT (0.4 mg/L); (b) Pretreated with TPP (0.2 mg/L) and then exposed to FNT (0.4 mg/L). For PBO and TPP, exposure period does not include pretreatment time (24 h)

2. AchE recovery

Our study clearly demonstrated that, brain AchE activity of topmouth gudgeon during the recovery period was influenced by the concentrations of FNT used during exposure time (Table 2). This is consistent with a recent finding of Sancho et al. (1997), who reported that European eel exposed to 0.04 and 0.02 mg/L FNT still had reduced brain AchE activity (by 43 and 29%, respectively) after recovery period of 192 h. Brain AchE activity in the fish, Salmo salar, exposed to FNT concentration of 0.004 mg/L can return to control values in less than a week but concentrations greater than 0.08 mg/L require a recovery period of at least 6 weeks (Morgan et al., 1990).

Several investigators also reported prolonged recovery of AchE in fish following exposure to other OP insecticides. For example, brain AchE activity of brook trout, rainbow trout, and cohon salmo after exposure to malathion completely recovered after 25 days (Sanders et al., 1981). According to Ansari and Kumar (1984), approximately 12 days were required for rainbow trout brain AchE activity to return to normal after exposure to methamidophos, and 15 days were required after exposure to acephate. Seven days after exposure to dichlorvos, brain AchE activity of tilapia had not fully recovered (Zinkl et al., 1991). Similarly, the recovery of channel catfish (*I. punctatus*) and blue crab (*Callinectes sapidus*) brain AchE activity required approximately 4 weeks following a short time sublethal exposure to S, S, S-tri-n-butyl phosphorothioate (DEF) (Habig et al., 1986). Other earlier studies using OP pesticides, i.e., malathion (Post & Leasure, 1974), dicrothopos (Fleming & Grue, 1981), also demonstrated that depression of brain AchE activity requires a long recovery period.

According to Bruijin and Hermens (1993), the mode of action of OP compounds is presumed to be based on a direct chemical reaction with the enzyme AchE. This may be an indication that OPs apparently bind irreversibly to AchE. The present results as well as previous studies (Escartin & Porte, 1996; Sancho et al., 1997) also reflected the affinity of FNT for fish AchE. Therefore, the prolonged recovery period of AchE activity observed in fish exposed to FNT as well as other OPs suggest that, when the level of AchE is being depressed, animals must synthesize new enzymes to return the activity to normal.

In topmouth gudgeon treated with PBO alone (1mg/L), the AchE activity was increased above the control with recovery time. An increase of 18.5% was observed compared with the control after the recovery period of 196 h. Similarly, the AchE activity in those previously pretreated with PBO and then exposed to FNT (0.4mg/L) was increased by 14.7% compared with the control (Fig.2a). Comparison of the AchE recovery in fish exposed to FNT alone with those pretreated with PBO and then exposed to FNT revealed that the latter exhibited higher activity than those exposed to FNT alone. That is, an increase of 9.7% was observed following the recovery time of 192 h (Fig.2a). These data indicates that,

AchE recovery is faster in those pretreated with PBO than those exposed to FNT alone. This perhaps was due to induction of AchE by PBO.

In topmouth gudgeon previously treated with TPP alone, the AchE activity decreased by 5.9% compared with the control following the recovery period of 192 h. As can be seen from Fig. 2b, at the end of 96 h and 192 h recovery period, the AchE activity of FNT (TPP was still decreased by 29.2% and 19%, respectively, compared with that of FNT alone. Since TPP is an OP compound, such result perhaps is due to its strong affinity to brain AchE.

Table 2 Recovery of brain AchE activity in topmouth gudgeon (*P. parva*) transferred to clean water after 96h exposure to fenitrothion

Recovery period (h)	Concentrations (mg/L) during exposure period			
	0.00(control)	0.20	0.40	0.60
0[a]	812.43 ± 2.84 (100)	738.50 ± 2.58* (90.9)	671.07 ± 2.35* (82.6)	557.33 ± 1.95* (68.6)
96	808.79 ± 4.43 (100)	854.58 ± 20.15* (105.7)	754.60 ± 4.13* (93.3)	688.28 ± 3.76* (85.1)
192	805.36 ± 4.89 (100)	859.32 ± 16.87* (106.7)	765.09 ± 4.64* (95.0)	728.85 ± 5.99* (90.5)

Activities are mean (SD of three experiments (in each experiment six fish were used). Values in parenthesis are percentage AchE activities.

[a] Recovery time of zero refers to the activity at the end of 96h exposure.

* Significantly different from the control at $P < 0.05$

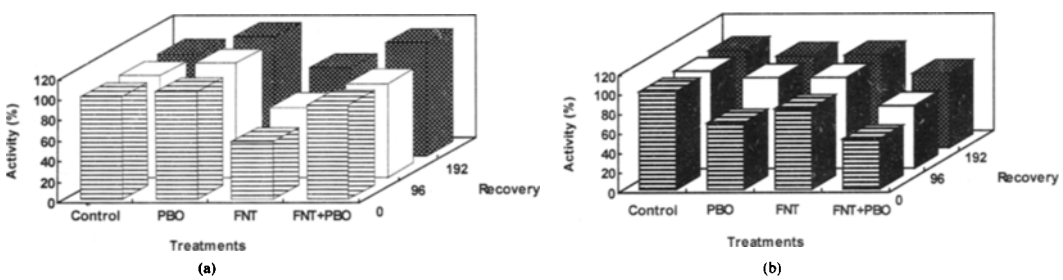

Fig.2 Recovery of brain AchE activity (%) of topmouth gudgeon (*P. parva*): (a) pretreated with PBO (1 mg/L) and then exposed to FNT (0.4 mg/L); (b) Pretreated with TPP(0.2 mg/L) and then exposed to FNT (0.4 mg/L). For PBO and TPP, exposure period does not include pretreatment time (24 h). Recovery time of zero refers to the AchE activity at the end of 96h exposure.

CONCLUSIONS

The present study demonstrated that in topmouth gudgeon exposed to FNT concentrations ranging from 0.4 to 0.6 mg/L and exposed to 0.4 mg/L of FNT after pretreating with TPP, AchE activity was significantly depressed. This implies that topmouth gudgeon that encounter FNT concentrations greater than 0.4 mg/L could be in danger. Therefore, field application (especially in paddy field) of FNT at the rate that can introduce such concentrations could be dangerous for the future survival of topmouth gudgeon and other related fish species. AchE of topmouth gudgeon seems to be sensitive to inhibition by FNT. Therefore, with additional studies in other species, brain AchE of topmouth gudgeon may be used to monitor FNT and other related compounds in the environment.

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