

EFFECTS OF VARIOUS POST-TREATMENT BY PHENYLMETHYLSULFONYL FLUORIDE ON DELAYED NEUROTOXICITY INDUCED BY LEPTOPHOS

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ABSTRACT—Delayed neurotoxicity induced by leptophos, an organophosphorus insecticide, was intensified in hens when phenylmethylsulfonyl fluoride (PMSF) at dose of 30, 60, and 120 mg/kg body weight was administered at different time intervals (24 hr, 3 days, and 5 days) for each dose of PMSF after the hens were exposed to 30 mg/kg (i.v.) of leptophos. The scores for organophosphorus-induced delayed neuropathy (OPIDN) in all groups treated with 120 mg/kg PMSF were significantly higher than those in the group treated with leptophos only ($P < 0.05$ or $P < 0.01$) and the initial signs of OPIDN appeared 2 or 3 days earlier in the former groups than in the latter group. Further, the greater the PMSF post-treatment dose, the more severe were the signs of OPIDN. These findings indicate that post-treatment with PMSF promotes leptophos-induced OPIDN and reduces the period to OPIDN onset. We also examined the effects of various time intervals between PMSF administration and exposure to leptophos on the development of OPIDN. The OPIDN scores in the two groups of hen treated with PMSF on days 3 and 5 after leptophos exposure were high, especially the score of the 5 days treated group became significantly higher on the 18th and 19th day after leptophos administration than even that of the 24 hr treated group with PMSF ($P < 0.05$). These findings suggest that variations in both the dose of PMSF and the time intervals of PMSF post-treatment may affect the delayed neurotoxicity induced by leptophos. Moreover, these results also indicate that PMSF should not be used for either the treatment or the prevention of OPIDN.

KEY WORDS : Delayed neurotoxicity, Leptophos, Phenylmethylsulfonyl fluoride (PMSF), Post-treatment.

INTRODUCTION

Some organophosphorus compounds, such as leptophos (Abou-Donia *et al.*, 1974), triorthocresyl phosphate (Smith *et al.*, 1930), EPN (Abou-Donia and Graham, 1978; Yamauchi *et*

al., 1985), and triphenyl phosphite (Smith *et al.*, 1933) induce delayed neuropathy, in which specific syndromes are exhibited 8–14 days after a single exposure in susceptible species, e.g., humans, cows, goats, cats, and hens: these agents cause degeneration of some long axons in the spinal cord and the peripheral nervous system. Several of these delayed neurotoxic compounds are still widely used as pesticides or antioxidants

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and stabilizers in the polymer industries (Protivova *et al.*, 1973). It has been reported, however, that certain carbamates protected exposed animals from the organophosphorus-induced delayed neuropathy (OPIDN) caused by diisopropyl phosphorofluoridate (DFP) (Johnson and Lauwerys, 1969; Drakontides and Baker, 1983). Other biochemically related chemicals, such as phenylmethylsulfonyl fluoride (PMSF) and some phosphinates, were also found to be protective when given prior to DFP and other organophosphorus esters causing delayed neuropathy (Johnson, 1970, 1974; Carrington, 1989). It was therefore considered that PMSF could be used as to prevent or to treat delayed neurotoxic sign. However, in recent years, it has been reported that PMSF, when given after the ingestion of certain compounds inducing delayed neurotoxicity, promoted OPIDN (Lotti *et al.*, 1991), but, this effect is still controversial.

In the present study, to elucidate the relationship between the effects of PMSF and leptophos, and to confirm whether PMSF is a suitable preventive or therapeutic agent against OPIDN, we examined the effects of various doses and intervals of PMSF post-treatment on OPIDN induced by leptophos in hens.

MATERIALS AND METHODS

Chemicals : Crystallized leptophos was prepared from Phosvel[®] emulsifiable concentrate (Nippon Noyaku Co., Tokyo.), containing 34% active leptophos, by a procedure described previously (Konno *et al.*, 1977). The purity of the crystals was estimated at 96% or more by gas chromatography. Leptophos solution for intravenous (i.v.) injection was prepared just before each injection by dissolving the crystallizing leptophos in dimethyl sulfoxide (DMSO), Tween-80 and saline. PMSF, glycerol formal, DMSO, and Tween-80 were purchased from Wako Pure Chemical Industries (Osaka, Japan). PMSF was dissolved in glycerol formal just before use and injected subcutaneously (s.c.) in the anterothoracic region.

Animals and administration : Adult laying hens (*Gallus gallus domesticus*), weighing 1.53–1.96 kg, were kept in cages and observed for behavior and gait for one week; after no abnormalities were detected, they were randomly divided into 12 groups (4–5 hens each) and allowed food and water *ad libitum* during the experimental period. Nine groups received a sin-

Table 1. Experimental protocol of post-treatment with PMSF after a single dose of leptophos.

Group	Leptophos (i.v.) mg/kg	Time interval [#]	PMSF (s.c.) mg/kg	No. of hens
1	30	24 hr	30	5
2	30	3 days	30	5
3	30	5 days	30	5
4	30	24 hr	60	5
5	30	3 days	60	5
6	30	5 days	60	5
7	30	24 hr	120	5
8	30	3 days	120	5
9	30	5 days	120	4
Leptophos only	30	—	—	5
PMSF only	—	—	120	5
Vehicle only	—	—	—	5

[#]: Time interval between leptophos injection and post-treatment with PMSF.

gle i.v. dose of leptophos and a single dose of PMSF given as post-treatment, according to the experimental protocol shown in Table 1. There were three control groups; leptophos only without post-treatment, 120 mg/kg of PMSF only, and vehicle only. The body weight of the hens was measured periodically and their behavior and gait were observed daily for 20 days.

Assessment of delayed neurotoxicity: A 7-point scale (Yamauchi, 1980) was used to evaluate the progress of clinical signs of delayed neurotoxicity; the scores were: 1) normal (—, 0 point), no defects in posture and working performance; 2) suspected ataxia (—+, 1 point), the bird appeared to lack strength in the leg and tottered; 3) mild ataxia (+, 2 points), the bird was slow, clumsy and had an unsteady gait; 4) gross ataxia (++, 3 points), the gait was always abnormal with waddling, and although the bird was still active, it would often stagger and fall; 5) mild paralysis (+++, 4 points), typical paralytic posture and the appearance of leg drop; 6) severe paralysis (++++, 5 points), the bird was unable to maintain its posture, to stand up, or to move at all; 7) neuropathic death (6 points).

Statistical analysis: Differences in quantitative parameters between groups were examined

for statistical significance using Student's *t*-test, and differences in scores for clinical signs were examined by the Mann-Whitney test; probability values of 5% or less were considered significant.

RESULTS

No clinical signs of organophosphorus-induced delayed neuropathy (OPIDN) were observed in the vehicle only or the PMSF only groups.

1. Effect of post-treatment dose of PMSF on delayed neurotoxicity

The OPIDN scores in groups 1, 4, 7, and the leptophos only are shown in Fig. 1. Clinical signs of OPIDN in group 7 appeared on the 8th day and its OPIDN score increased very rapidly. Compared with the leptophos only group, there was a significant difference in the scores from the 11th to the 20th day after leptophos administration ($P < 0.01$). The OPIDN scores in groups 1 and 4 increased slowly in the same manner as those in the leptophos only group.

The OPIDN scores in groups 2, 5, and 8 are shown in Fig. 2. In groups 5 and 8, OPIDN appeared on the 9th day and the OPIDN scores increased rapidly. Compared with the leptophos

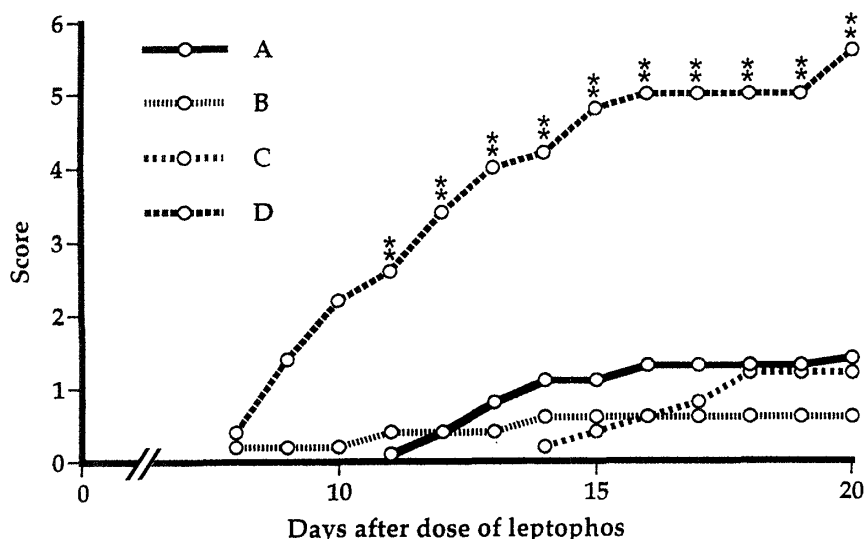


Fig. 1. Changes in OPIDN scores in the three groups that received PMSF 24 hr after leptophos administration (groups 1, 4, 7) and the group that received leptophos only. A: leptophos only; B: group 1; C: group 4; D: group 7. * $P < 0.05$; ** $P < 0.01$, compared with leptophos only group by Mann-Whitney test.

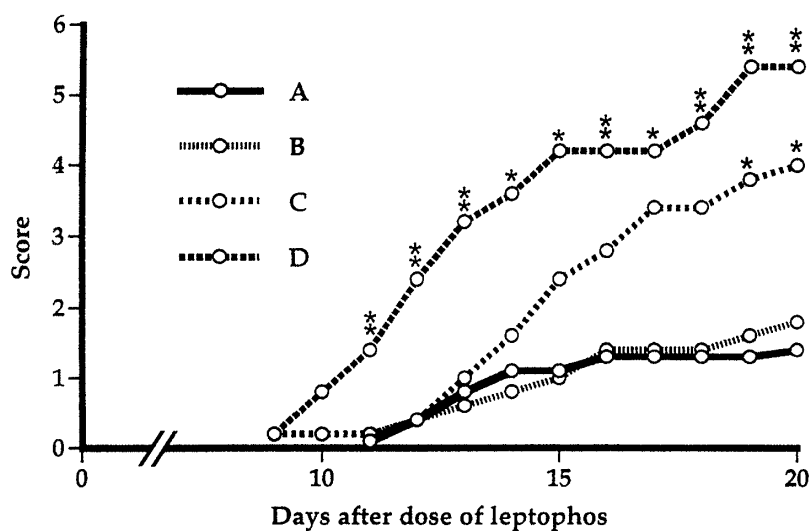


Fig. 2. Changes in OPIDN scores in the three groups that received PMSF 3 days after leptophos administration (groups 2, 5, 8) and the group that received leptophos only. A : leptophos only ; B : group 2 ; C : group 5 ; D : group 8. * $P < 0.05$; ** $P < 0.01$, compared with leptophos only group by Mann-Whitney test.

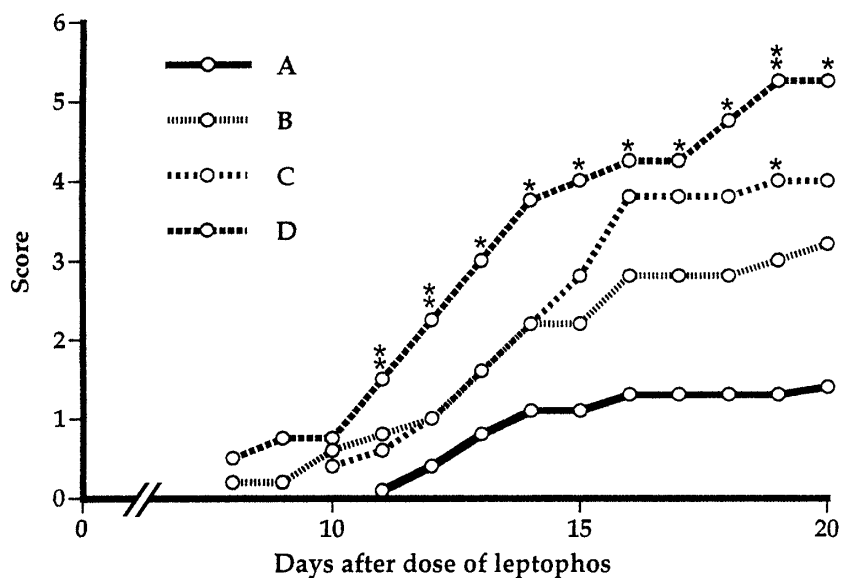


Fig. 3. Changes in OPIDN scores in the three groups that received PMSF 5 days after leptophos administration (groups 3, 6, 9) and the group that received leptophos only. A : leptophos only ; B : group 3 ; C : group 6 ; D : group 9. * $P < 0.05$; ** $P < 0.01$, compared with leptophos only group by Mann-Whitney test.

Table 2. Effect of post-treatment with PMSF at different time intervals after leptophos administration on leptophos-induced OPIDN.

Dose of leptophos	Groups of hens		OPIDN score (Mean \pm S.D.) each day after leptophos injection																
	Time-interval between leptophos and treatment with PMSF	No. of hens ^{a)}	14th day	15th day	16th day	17th day	18th day	19th day	20th day										
30 mg/kg	24 hr	15	1.7 \pm 1.9	1.9 \pm 2.2	2.1 \pm 2.3	2.1 \pm 2.2	2.3 \pm 2.2	2.3 \pm 2.2	2.5 \pm 2.5										
30 mg/kg	3 days	15	2.0 \pm 1.7	2.5 \pm 1.8	2.8 \pm 1.6	3.0 \pm 1.7	3.1 \pm 1.8	3.6 \pm 1.9	3.7 \pm 2.0										
30 mg/kg	5 days	14	2.6 \pm 1.4	2.9 \pm 1.5	3.6 \pm 1.6	3.6 \pm 1.6	3.8 \pm 1.6 ^{b)}	4.1 \pm 1.8 ^{b)}	4.1 \pm 1.8										

a) : Includes all hens given 30, 60, and 120 mg/kg PMSF post-treatment.

b) : Significant difference from the group received PMSF 24 hr after leptophos administration by Mann-Whitney test ($P < 0.05$).

only group, the differences were significant ($P < 0.05$, $P < 0.01$).

The OPIDN scores in groups 3, 6, and 9 are shown in Fig. 3. OPIDN in these three groups appeared on the 8th and 10th days, and the OPIDN scores were higher than that in the leptophos only group, the OPIDN score in group 9 being significantly higher ($P < 0.01$).

These results show that for all time intervals after leptophos administration, the greater the dose of post-treatment PMSF, the greater was the severity of OPIDN.

2. Effect of time-interval between dose of leptophos and post-treatment with PMSF on delayed neurotoxicity

We observed no significant difference in the OPIDN among the 3 time interval groups (24 hr, 3 days, and 5 days), compared the OPIDN score within each dose of post-treated with PMSF. Therefore, to investigate the effect of the time interval on OPIDN we compared the OPIDN scores among 3 time interval groups with summing 3 dose (30, 60, and 120 mg/kg) of post-treated with PMSF.

The results are shown in Table 2. The OPIDN scores in the two groups post-treated with PMSF on 3 and 5 days after leptophos administration were higher than that in the group post-treated with PMSF 24 hr after leptophos, the OPIDN score in the group post-treated with PMSF on 5 days after leptophos being significantly higher on the 18th and 19th experimental day than ($P < 0.05$).

These results showed that OPIDN was markedly intensified when the interval between leptophos dosing and post-treatment with PMSF was 5 days compared to intervals of 24 hr and 3 days.

3. Changes in Body Weight

Changes in the average body weight of the hens in each group are shown in Table 3. Despite development of OPIDN, the body weights of hens not only in the vehicle group but also in the leptophos only group and group 1 had increased on the 13th day by 5% or 11% of their initial weight. In contrast, however, in groups 4, 7, and 9, the average body weight decreased by 2% to 5% of their initial weight. The percent in body weight in these 3 groups was significantly different from that in the vehicle only group ($P <$

Table 3. Body weight changes.

Group	No. of hens	percent change in body weight ^{a)}
1	5	11.00 ± 6.72
2	5	0.61 ± 11.72
3	5	-0.98 ± 17.61
4	5	-4.74 ± 6.59 ^{b)}
5	5	0.26 ± 4.71
6	5	-0.45 ± 3.68 ^{b)}
7	5	-3.20 ± 3.42 ^{b)}
8	5	0.30 ± 5.65
9	4	-1.93 ± 4.97 ^{b)}
Leptophos only	5	4.67 ± 12.22
Vehicle only	5	11.63 ± 7.75

a) : The percent change in body weight was the rate of the difference between the body weight on the 1st day and on the 13th day to the body weight on the 1st day (%).

b) : Significant differences from the vehicle only group by Student's *t*-test ($P < 0.05$).

0.05) but was not significantly different from that of the leptophos only group.

DISCUSSION

It has been reported that some carbamates, phosphinates, and sulfonates, such as PMSF, protected hens from delayed neuropathy when given prior to the administration of certain organophosphorus compounds that induced OPIDN, for example, leptophos (Baker *et al.*, 1980; Carrington *et al.*, 1988). However, when these compounds were administered after exposure to such organophosphorus compounds, they promoted OPIDN (Lotti *et al.*, 1991; Moretto *et al.*, 1992; Peraica *et al.*, 1993). The results of our previous study showed that PMSF protected hens from leptophos-induced OPIDN when given 12 hr subsequent to leptophos exposure (Piao *et al.*, 1993).

It is unusual, however, to administer compounds like PMSF or carbamates to human subjects for the chemoprophylaxis of OPIDN. It should be really considered very important risk to exposed PMSF subsequent to OPIDN under the 2 following circumstances: one is to treat patient of poisoning after exposure to organophosphates

with PMSF as a therapy and another is people such as farmers to be exposed to the mixed contamination of carbamates which have the same effect to promote OPIDN as PMSF and organophosphorus pesticides as 'occupational exposure'.

In this study, we examined the effects of PMSF on leptophos-induced OPIDN at longer time intervals between exposure to leptophos and post-treatment with PMSF than the intervals reported in earlier studies. When PMSF was given posterior to leptophos, the delayed neuropathic symptoms caused by leptophos intensified with the manner of dose of PMSF and response relationship, in accordance with the results reported by Lotti *et al.* (1991). Furthermore, the period until onset of the initial signs of OPIDN in the three groups post-treated with PMSF of 120 mg/kg (groups 7, 8, 9) was two or three days shorter than that in the group that received leptophos only (Table 4), indicating that post-treatment with PMSF reduced the time to OPIDN onset.

We also examined the effects of various time intervals between leptophos injection and PMSF post-treatment on delayed neurotoxicity. The OPIDN scores in the two groups post-treated with PMSF on 3 or 5 days were higher than that in the group post-treated with PMSF at 24 hr from the 15th to the 20th experimental days, the score of the group post-treated on 5 days being significantly higher on the 18th and 19th experimental days than that in the 24 hr post-treated group ($P < 0.05$). These findings suggest

that since the PMSF post-treatment interval of 5 days is close to the period of the onset of OPIDN, which occurs at least 8 days after exposure to leptophos, and since the leptophos-induced degeneration of the peripheral nerves develops during this period, the promotive effect of PMSF at this 5 days interval may exert the greatest influence on OPIDN of the 3 time intervals in this experiment. In addition, although PMSF cannot be used as a therapeutic or preventive agent for OPIDN due to the reason mentioned above, in the study of OPIDN, post-treatment with PMSF could be very valuable for preparing an experimentally susceptible animal model of delayed neurotoxicity when the acute toxicity of the organophosphates to be examined, e.g., EPN or cyanofenphos, is very strong and then delayed neurotoxic effect is weak. When post-treatment with PMSF is employed these organophosphorus compounds, even at a small dose, also induce OPIDN.

At present, the mechanisms by which OPIDN is prevented by PMSF pretreatment and promoted by PMSF post-treatment have not yet been completely clarified. We previously examined the effects of PMSF on the degradation of leptophos in the tissues of hens, finding PMSF to have no effect on the clearance of leptophos (Yamauchi *et al.*, 1993). Lotti *et al.* (1991) reported that the protection afforded by PMSF was related to inhibition of the putative target of OPIDN, neuropathy target esterase (NTE). More than 70%–80% of NTE in the nervous system of hens, followed by a molecular rear-

Table 4. Effects of PMSF post-treatment on time of onset of leptophos-induced OPIDN.

Dose of leptophos (mg/kg)	Post-treatment dose of PMSF (mg/kg)	Average time of onset of OPIDN (days)				
		OPIDN score				
		(- +) ^{a)}	(+) ^{b)}	(+ +) ^{c)}	(+ + +) ^{d)}	(+ + + +) ^{e)}
30		11.7	12	13	14.7	16
30	30	9	11.3	12.5	14	16
30	60	11	13	14	15.3	17
30	120	8.3	9	11	12	14

a) : Suspected ataxia.

b) : Mild ataxia.

c) : Gross ataxia.

d) : Mild paralysis.

e) : Severe paralysis.

rangement called 'aging', initiates OPIDN. PMSF and other protective chemicals inhibit NTE, but OPIDN does not develop because aging cannot occur. However, no reasonable explanation has yet been proposed for the different responses of PMSF when employed pre-treatment and post-treatment. The results of recent research indicate that aging may not always be essential in causing neuropathy. The agents, mipafox, methamidophos, and sulfonyl fluoride produce an inhibited NTE which apparently does not age and yet produces neuropathy (1993). The precise physiological function of NTE is not known. Moretto *et al.* (1994) reported that the promotion of OPIDN by PMSF was related to the interaction of this promoter with a target other than NTE, however, this other target has not been identified. In conclusion, results of the present study suggest that the dose of PMSF post-treatment, as well as the time after exposure to leptophos are important factors in the promotion of OPIDN. To explore the mechanism of the effects exerted by PMSF on the delayed neurotoxicity induced by organophosphorus compounds, further research in pathology, molecular biology, and biochemistry is required.

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