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Lack of Delayed Neurotoxic Effect after Tri-*o*-cresyl Phosphate Treatment in Male Fischer 344 Rats: Biochemical, Neurobehavioral, and Neuropathological Studies

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*Department of Pharmacology, Duke University Medical Center, P.O. Box 3813, Durham, North Carolina 27710; *Neurobehavioral Section, National Institute of Environmental Health Sciences, P.O. Box 12233, Research Triangle Park, North Carolina 27709; and †Experimental Pathology Laboratory, Research Triangle Park, North Carolina 27709*

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Lack of Delayed Neurotoxic Effect after Tri-*o*-cresyl Phosphate Treatment in Male Fischer 344 Rats: Biochemical, Neurobehavioral, and Neuropathological Studies. SOMKUTI, S. G., TILSON, H. A., BROWN, H. R., CAMPBELL, G. A., LAPADULA, D. M., AND ABOU-DONIA, M. B. (1988). *Fundam. Appl. Toxicol.* **10,** 199–205. Tri-*o*-cresyl phosphate (TOCP), which produces a delayed neurotoxic syndrome in humans and some animal species, was given to Fischer 344 (F344) male (18 week old) rats to determine if it causes biochemical, sensorimotor, and neuropathological effects. Animals were given TOCP by gavage in doses ranging from 10 to 100 mg of TOCP/kg daily for a period of 63 days. The rats were subjected to a series of neurobehavioral tests including fore- and hindlimb grip strength, motor activity, tremor, and latency to respond to a thermal stimulus. Central and peripheral nervous tissues were examined for damage characteristic of organophosphorous compound-induced delayed neurotoxicity (OPIDN). Brain neurotoxic esterase and acetylcholinesterase activities were inhibited in a dose-dependent fashion. A group of three chickens treated with 100 mg of TOCP/kg/day for 18 days was included as the positive control for enzymatic and histopathological alterations associated with OPIDN. Rats showed no consistent neurobehavioral changes or evidence of neuropathological damage in nervous tissues associated with treatment. In contrast, chickens treated with TOCP developed delayed neurotoxicity characterized by ataxia, which progressed to paralysis. These neurological changes included swelling, fragmentation, and degeneration of the axon and myelin in both central and peripheral nervous tissues. This study concludes that the F344 rat is not sensitive to the delayed neurotoxic effects of TOCP. When studying OPIDN in rats, care must be exercised in choosing the experimental animal since some strains, e.g., F344, are not sensitive.

Organophosphorus compound-induced delayed neurotoxicity (OPIDN) has been well documented in both humans and various animal species (Smith *et al.*, 1930; Abou-Donia, 1981, 1983). Although many animal species are susceptible to OPIDN, rodents and Japanese quail do not readily exhibit clinical signs of delayed neurotoxicity (Abou-Donia, 1981). At present, the test animal of choice is the chicken since it appears to approximate closely the human clinical condition (Abou-Donia, 1981; U.S. Environmental Protection Agency, 1985). The morphology of OPIDN neuronal lesions has been well characterized (Cavanagh, 1973). It consists of Wallerian-type degeneration of the axon and myelin in the central and peripheral nervous systems.

Recently, the rat has been suggested as an alternative test species for OPIDN even though no objective neurobehavioral alterations, which would be expected to occur concurrent with the development of OPIDN,

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have been demonstrated in the rat (Veronesi, 1984). Other investigators (Smith *et al.*, 1930; Abou-Donia, 1981) have found that the rat is not sensitive to delayed neurotoxicity. Up to this point other investigators have not simultaneously compared the response of the adult chicken to tri-*o*-cresyl phosphate (TOCP)-induced OPIDN to that of the rat. This study makes the comparison.

We initiated biochemical, neurobehavioral, and neuropathological studies to assess the effects of a known OPIDN-inducing compound, TOCP, in the male Fischer 344 rat to examine its possible sensitivity to OPIDN.

MATERIALS AND METHODS

Chemicals

TOCP (99%) was purchased from Eastman–Kodak Co. (Rochester, NY). *O,O*-diethyl *O*-4-nitrophenyl phosphate (paraoxon) was obtained from Sigma Chemical Co. (St. Louis, MO). *N,N'*-diisopropyl phosphorodiamidic fluoride (mipafox) was synthesized by the Midwestern Research Institute (Kansas City, MO). Acetylthiocholine iodide was purchased from Sigma Chemical Co. Phenylvalerate was synthesized.

Animals

Fischer 344 male rats (190–210 g and 9–10 weeks old at onset of study) were obtained from Hilltop Lab Animals, Inc. (Scottdale, PA). All rats were housed two per cage in a 21–23°C controlled environment with a 12-hr light cycle (on 0600 until 1800) and provided with NIH-07 open formula diet feed pellets (Purina, St. Louis) and tap water *ad libitum*. Chickens (2.0–2.4 kg range and 24 weeks old) were obtained from Featherdown Farms (Raleigh, NC). Animals were allowed to acclimatize for 1 week prior to experimentation.

Treatments

Groups of 10 rats each were treated with either 10, 50, or 100 mg of TOCP/kg daily at 0800 (p.o. in corn oil) for 63 days. A group of 10 vehicle-treated (corn oil) rats served as the control. Five animals from each group were used in biochemical assays. The remaining five animals were perfused for neuropathological studies. One group of three chickens was treated with 100 mg of TOCP/kg (p.o. in corn oil) daily for 18 days. Another group of three hens was used as control. Animals were observed daily (during dosing) for signs of acute (excess salivation, diarrhea, ocular discharge, tremor) or delayed (disturbances of gait, hindlimb splay, paralysis) neurotoxicity.

Sensorimotor Tasks

Testing procedure and apparatus. Upon completion of dosing, the sensorimotor function of TOCP-treated rats was assessed via four different tests. Testing was done in a sequential fashion by experimenters unaware of the treatment condition of the animals. Potential tremor produced by TOCP was first assessed over a 1.3-min test period by placing rats individually on a platform connected to a load-cell transducer (Gerhart *et al.*, 1982). Tremor occurs in the chicken suffering from OPIDN (Abou-Donia, 1981). Analog signals from the transducer were processed by a Hewlett–Packard spectral analyzer (Model 3582A), which performed a fast Fourier transformation of the signal. Movement was quantified as power (−dBV) occurring in successive 2.5-Hz intervals ranging from 2.5 to 20 Hz. Previous studies have shown that intensity of movement is associated with less negative power values (i.e., increases in power; Gerhart *et al.*, 1982, 1983). The peak power (i.e., highest power reading in the spectrum obtained during the sample period) and most prevalent frequency were recorded.

Motor activity. Vertical and horizontal components of motor activity were independently assessed during 3-min test sessions using a commercially available apparatus (Optovarimex, Columbus Instruments, Columbus, OH). Arrays of equally spaced infrared photodetector/emitter pairs were located 5.5 cm from the floor to measure horizontal activity and 13 cm from the floor to measure vertical activity. The motor activity apparatus was cleaned between individual animal's tests.

Fore- and hindlimb grip strength. Grip strengths were measured using an apparatus and according to procedures described by Meyer *et al.* (1979). Briefly, two push–pull strain gauges attached with either a triangular front grip bar or a hindlimb T-bar were horizontally mounted at opposite ends of a Plexiglas platform. During testing the rat was placed on the apparatus with both forepaws inside the triangular grip and then gently pulled backwards until the grip was broken (terminal measure = forelimb grip strength). The rat continued to be pulled until it gripped the rear T-bar; the pulling continued until its hindlimb grip was broken (hindlimb grip strength). The mean of three determinations was used.

Thermal sensitivity. A commercially available hot-plate apparatus (TechniLab Instruments, Pequannock, NJ) was used to assess reactivity to a thermal stimulus. The hot-plate was set at a nominal 60°C, with an actual surface temperature reading of 57.5 ± 0.2°C. During the test, each rat was picked up by the tail and gently placed onto the surface of the apparatus. The latency to lick one of the hindpaws, which was recorded to an arbitrary maximum of 45 sec, served as the dependent measure.

Chickens were not assessed in behavioral tests.

Esterase Assays

Immediately after decapitation of rats (five from each group), the brain was removed quickly and held at 4°C until assayed (within 1 hr). Brain acetylcholinesterase (AChE) was measured as described previously (Abou-Donia and Preissig, 1976) and expressed as micromoles of acetylthiocholine (AtCh) hydrolyzed per minute per milligram of protein. Neurotoxic esterase (NTE) was determined by the method of Johnson (1977) and expressed as nanomoles of phenylvalerate hydrolyzed per minute per milligram of protein. Mipafox preinhibition (40 μM) was carried out for 20 min at 37°C.

Proteins were determined in brain by the method of Lowry *et al.* (1951) with bovine serum albumin (Sigma) as the standard.

Neuropathology

Nerve tissue was taken following whole animal perfusion (n = 5) for histopathologic studies (Abou-Donia *et al.*, 1986). Sciatic nerves along with the spinal cord were fixed for 3 weeks in neutral phosphate-buffered 10% formalin solution. Cross, parasagittal, and longitudinal sections near the midline were prepared from the cervical, thoracic, and lumbar regions of the spinal cord. Peripheral nerves were prepared in cross and longitudinal sections. Tissues were dehydrated in graded ethanol and embedded in paraffin. Sections (8 μm) from spinal cord were stained with hematoxylin and eosin (H and E) combined with Luxol fast blue (LFB). Sections from peripheral nerves were stained with Holmes' silver stain and H and E with LFB. Sections were examined by two independent neuropathologists in a "blind" fashion.

Statistics

Data were analyzed for overall effects of treatment using a factorial analysis of variance (ANOVA; Winer, 1971). Fisher's least significant difference (LSD) test was used to determine significance between experimental and control groups. The accepted level of significance was set at $p < 0.05$.

RESULTS

General Observations

Over the course of the study, the body weights of control and 10 mg TOCP/kg/day treated rats increased by 52 and 59% from their initial weight, respectively (Table 1). Rats treated with either 50 or 100 mg of TOCP/kg/day gained 52 and 51% of their pretreatment weight, respectively. Despite severe inhibition of brain AChE activity, only occasional signs of acute cholinergic toxicity (diarrhea, salivation, and ocular discharge) were observed and only in the 50 and 100 mg TOCP/kg/day treated animals. The control and 10 mg TOCP/kg/day treated groups did not display any cholinergic signs. At no time during the experiment did treated animals display any clinical signs of delayed neurotoxicity. Analysis of body weights taken before and after dosing indicated that there was no significant treatment-related change (F = 1.01) (Table 1).

TABLE 1

EFFECTS OF TOCP ON RAT BODY WEIGHTS

Treatment (mg/kg)	Average body weight (±SE)[a]		
	Initial weight	End of dosing	Average % change of initial weight
Control	204.6 ± 2.7	311.4 ± 0.1	111.6 ± 1.1
10	194.2 ± 2.2	308.2 ± 6.5	112.9 ± 6.2
50	186.6 ± 3.4	284.2 ± 6.3	95.7 ± 6.5
100	187.7 ± 5.1	283.3 ± 6.9	98.4 ± 9.3

[a] Data are means of 10 rats per group ± standard error.

Chickens treated with TOCP developed the characteristic ataxia (Day 7 of treatment) and paralysis (Day 15 of treatment) associated with OPIDN.

Sensorimotor Studies

Repeated exposure to TOCP appeared to have few consistent effects on sensorimotor function in rats (Table 2). One-way ANOVAs indicated no significant effect of TOCP on responsiveness to the hot plate (F = 0.06), hindlimb grip strength (F = 0.66), or the peak frequency (Hz) from the spectral analysis of

TABLE 2

EFFECTS OF TOCP ON NEUROBEHAVIORAL FUNCTION OF F344 RATS

	Average response ± SE[a]						
		Motor activity (counts/3 min)		Strength (g)		Spectral analysis	
Treatment (mg/kg)	Hot plate (sec)	Horz	Vert	Fore	Hind	pk hz	pk −dbv
Control (0 mg/kg)	11.8 ± 1.0	922 ± 135	8 ± 2	700 ± 20	502 ± 19	8.4 ± 1.2	76.4 ± 2.5
10	11.4 ± 0.3	1033 ± 183	9 ± 2	614 ± 23[b]	462 ± 19	7.8 ± 1.0	72.0 ± 1.5
50	11.4 ± 0.8	1191 ± 107	12 ± 2	610 ± 23[b]	461 ± 31	6.7 ± 0.7	70.4 ± 1.1
100	11.3 ± 0.9	1412 ± 112	16 ± 2	649 ± 33[b]	410 ± 27	6.9 ± 0.7	70.2 ± 1.6

[a] Data are means of 10 rats per group.
[b] Significantly different from control ($p < 0.05$).

movement ($F = 0.70$). Tremor was not evident in any of the rats at the time of testing. Near significant effects were observed for the peak intensity (−dBV) of movement [$F = (3,35) = 2.65, p > 0.0640$], horizontal motor activity [$F(3,35) = 2.27, p > 0.0970$], and vertical motor activity [$F(3,35) = 2.76, p > 0.0565$]. Effects on forelimb grip strength were marginally significant [$F(3,35) = 2.97, p > 0.0449$]. Post hoc Fisher's LSDs indicated that forelimb was decreased in animals that received 10, 50, or 100 mg/kg of TOCP; however, there was no dose–response relationship, so the significance of these data is not clear.

Esterase Assays

Brain acetylcholinesterase activity in treated rats was inhibited in a dose-dependent manner with the maximum inhibition being 80% of control values (Table 3). Neurotoxic esterase activity was likewise inhibited with peak inhibition of 66% following 10 mg of TOCP/kg/day. Chickens exhibited similar inhibitions in both enzymes which showed inhibition of 68 and 84% for brain AChE and NTE, respectively.

Histopathologic Observations

Sections from cervical, thoracic, and lumbosacral areas of the spinal cord and peripheral nerves from all control and treated animals were examined by light microscopy. Tissues from rats were examined for presence of neuropathology (spinal cord and sciatic nerve), and no degenerative changes associated with neurotoxic compounds were seen. Equivocal changes, defined as swollen axons without fragmentation or loss of myelin staining, were observed in the spinal cord of one rat receiving 100 mg/kg/day.

Chickens displayed characteristic degeneration in the dorsal and lateral columns in cervical spinal cord and in the lateral columns below the cervical areas. Degeneration was also seen in the lumbar region in tracts lying in the ventral tissue. Swelling and fragmentation of myelin and axons in peripheral nerves were seen also.

DISCUSSION

This study shows that the F344 rat is not sensitive to delayed neurotoxicity induced by TOCP. This was demonstrated by the absence of unequivocal neuropathological al-

TABLE 3

RAT BRAIN ACETYLCHOLINESTERASE AND NEUROTOXIC ESTERASE SPECIFIC ACTIVITIES[a]

Treatment group[b]	Acetylcholinesterase activity		Neurotoxic esterase activity	
	μm/min/mg	% of control	nm/mg/min	% of control
Control	20.76 ± 1.72	100.0	12.61 ± 1.28	100.0
10 mg TOCP/kg/day	23.33 ± 3.00	112.4	17.33 ± 1.35	137.4
50 mg TOCP/kg/day	10.74 ± 1.33[c]	51.7	11.03 ± 1.04	87.5
100 mg TOCP/kg/day	4.18 ± 1.49[c]	20.1	4.32 ± 1.20[c]	34.2

[a] Mean ± standard error.
[b] $n = 5$ animals/group.
[c] Significantly different from control ($p < 0.05$).

terations or consistent neurobehavioral changes in rats following subchronic oral administration of 100 mg of TOCP/kg/day for 63 days. These results contrast with the finding that daily oral dosing of 10 mg of TOCP/kg produced delayed neurotoxicity in hens (Abou-Donia and Graham, 1979). It is unlikely that higher doses of TOCP would have caused OPIDN in rats since daily oral administration of 150 mg/kg of TOCP produced significant mortality in rats (Somkuti *et al.*, 1987).

Neurotoxic esterase (NTE), an uncharacterized protein that has unknown biochemical or physiological functions in hen brain, has been suggested as a useful marker for OPIDN (Johnson, 1977, 1978). An inhibition of at least 70% of hen brain NTE 24 hr after a single administration of an organophosphorus compound strongly suggests that this chemical has the potential to produce OPIDN. Further studies have demonstrated that multiple doses of organophosphorus compounds that produce delayed neurotoxicity only cause an inhibition of approximately 50% of NTE activity. Thus, 20 injections of diisopropyl phosphorofluoridate into hens produced delayed neurotoxicity and resulted in 50% inhibition of hen brain NTE (Sprague *et al.*, 1981). In the present study, subchronic administration of 100 mg of TOCP/kg produced 66% inhibition of rat-brain NTE activity without producing delayed neurotoxicity. These results add more confirming evidence to the neuropathological and neurobehavioral findings that the F344 rat is not sensitive to TOCP-induced delayed neurotoxicity. It is also possible that the rat may require a higher level of inhibition, compared to the hen, to produce delayed neurotoxicity. Furthermore, some organophosphorus compounds such as dichlorvos are able to produce OPIDN without substantial inhibition of hen brain NTE (Johnson, 1978). This previous study and the present results suggest that inhibition of NTE may not be a necessary condition for OPIDN. Since the role of NTE in the mechanisms of OPIDN is not known, one must be cautious in interpreting the significance of its inhibition.

The conclusion that TOCP (at the doses utilized) does not produce OPIDN in the F344 rat after 9 weeks of daily treatment contrasts the findings of Veronesi (1984), who observed spinal cord lesions in Long Evans rats following daily treatment for 6 weeks with similar doses of TOCP (116 mg/kg/day). Strain differences (Fischer vs Long Evans) may account for the underlying sensitivity to the delayed neurotoxicity of TOCP. It was recognized as early as 1930 that not all animal species are equally susceptible to development of OPIDN following organophosphorus ester exposure. It has been found that humans, chickens, cats, cows, lambs, sheep,

ferrets, and water buffalo are susceptible (Smith *et al.*, 1930; Draper *et al.*, 1952; Malone, 1964; Hansen *et al.*, 1968; Abou-Donia *et al.*, 1983, 1986; Kogut *et al.*, 1986). Rodent species (mice, rats, guinea pigs, hamsters, and gerbils) appear to be relatively less sensitive to OPIDN (Smith *et al.*, 1930; Smith and Lillie, 1931; Abou-Donia, 1981; Lapadula *et al.*, 1985). Species differences possibly could be attributed to several explanations. There could be variation in the neurons of different species which would account for the differences between species. Another difference between species (Abou-Donia, 1981) and strains of rats (Waxman and Walsh, 1982) could be in the metabolism and pharmacokinetics of organophosphorus neurotoxicants (Abou-Donia, 1983; Abou-Donia and Nomeir, 1986).

An overview of the literature suggests that the rat may not be a model for studying OPIDN for the following reasons: (1) the long duration of treatment necessary before onset of any neuropathological damage, (2) the extremely high doses required to induce observable neuropathological damage; such doses would be a limiting factor in studying organophosphorus compounds with high anticholinergic activity, (3) the minimal clinical manifestations of OPIDN in the rat, (4) the apparent inconsistency of sensitivity to OPIDN among various rat strains, and (5) complete absence of gross ataxia after a single exposure to TOCP, as seen in the chicken and human (Abou-Donia *et al.*, 1983; Bowden *et al.*, 1930). Long Evans rats exhibited minimal ambulation problems despite severe central and peripheral nervous system degeneration (Veronesi, 1984). The reasons for the differential sensitivity between rats and chickens might be attributed to the sensitivity of the OPIDN target (Patton *et al.*, 1985a,b), regeneration of nervous tissues, or inherent disposition and metabolism capabilities (Abou-Donia, 1983; Abou-Donia and Nomeir, 1986) present among species.

Although the rat is not an appropriate model to study OPIDN, this animal species might be a good model for investigating species and strain selectivity of OPIDN.

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