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TESTICULAR TOXICITY FOLLOWING ORAL ADMINISTRATION OF TRI-*O*-CRESYL PHOSPHATE (TOCP) IN ROOSTERS

(Organophosphorus compound; delayed neurotoxicity; neurotoxic esterase activity)

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SUMMARY

Tri-*o*-cresyl phosphate (TOCP) is a neurotoxic organophosphorus compound that induces a characteristic central-peripheral distal axonopathy and Wallerian-type degeneration, 6–14 days after exposure. This organophosphorus compound-induced delayed neurotoxicity (OPIDN) has been extensively studied in the chicken, the standard test model. Reports of neurotoxic agents causing adverse effects on the male reproductive system initiated the present study which was designed to examine the effects of TOCP on the rooster. Previous work from this laboratory has demonstrated 100 mg TOCP/kg/day to be an OPIDN-inducing dose with minimal mortality in roosters. This dose level was administered to adult leghorn roosters (p.o., $n = 10$) for 18 consecutive days. By days 7–10 of the study, TOCP-treated birds exhibited limb paralysis characteristic of OPIDN. Analysis at termination revealed significant inhibition of neurotoxic esterase activity (NTE) in both brain and testis. There was also a slight decrease in brain acetylcholinesterase (AChe) activity. Sperm motility was shown to be greatly decreased. In addition, sections of formalin-fixed, methacrylate-embedded testes from TOCP-treated birds showed vacuolation of, and disorganization in the seminiferous epithelium. The marginal body weight decreases (17%) in treated animals were not considered to contribute to the testicular toxicity induced by TOCP. Parathion (*O,O*-

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Abbreviations: TOCP, tri-*o*-cresyl phosphate; OPIDN, organophosphorus ester-induced delayed neurotoxicity; AChE, acetylcholinesterase; NSE, nonspecific esterase; BuChE, butyrylcholinesterase; ATCh, acetylthiocholine; BuTCh, butyrylthiocholine; NTE, neurotoxic esterase; H and E, hematoxylin and eosin; LFB, Luxol fast blue; PAS, periodic acid-Schiff's.

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diethyl-*O*-4-nitrophenyl phosphorothioate, 0.1 mg/kg/day, p.o., $n = 3$) was used as a positive control for AChE inhibition and a negative control for inducing OPIDN. Roosters treated continuously with parathion showed a decrease in brain AChE activity, but no changes in NTE, testicular histology, or limb function. These studies demonstrate the testicular toxicity of TOCP in roosters and suggest that this effect is not related to the chemical's anticholinergic action.

INTRODUCTION

Tri-*o*-cresyl phosphate (TOCP) is an organophosphorus compound which is used in industry mainly as an additive to lubricating oil and a softener in the manufacture of plastic products [1]. TOCP causes a delayed neurotoxicity, characterized by a Wallerian-type degeneration and secondary demyelination of the axonal sheath in the central and peripheral nervous systems, resulting in flaccid paresis and paralysis. These effects have been extensively studied and documented and termed organophosphorus ester-induced delayed neurotoxicity (OPIDN) [2,3]. Not all animal species are equally susceptible to OPIDN. While rats display no outward clinical signs and appear relatively insensitive [4], and mice develop characteristic OPIDN after prolonged high dose exposure [5], the adult chicken is especially sensitive and is used as the test animal of choice to study OPIDN [3].

Recent reports indicate that various neurotoxic compounds which are capable of producing Wallerian-type degeneration (including carbon disulfide, 2,5-hexanedione, and acrylamide), cause adverse effects on male reproductive function [6–8]. These observations led us to examine the delayed-neurotoxicant TOCP, and its effects on the rooster testis. We have studied changes in testis histopathology, biochemistry, and sperm motility associated with daily TOCP treatment in relation to neurotoxicity.

METHODS

Chemicals

Tri-*o*-cresyl phosphate (TOCP, 99%) and *O,O*-diethyl *O*-4-nitrophenyl phosphorothioate (parathion, 99%) were obtained from Eastman Kodak Co. (Rochester, NY) and Pfaltz and Bauer (Stamford, CT), respectively. *O,O*-Diethyl *O*-4-nitrophenyl phosphate (paraoxon) was obtained from Sigma Chemical Co. (St. Louis, MO). *N,N*-Diisopropyldiaminophosphorofluoridate (mipafox), was synthesized by the Midwestern Research Institute (Kansas City, MO). The following enzyme substrates were purchased from Sigma Chemical Co. (St. Louis, MO): acetylthiocholine, butyrylthiocholine, and 1-naphthyl acetate. Phenylvalerate was kindly provided by Dr. A. Nomeir.

Roosters

Adult leghorn roosters (3 years old, 4–5 kg) were obtained from Featherdown Farms (Raleigh, NC). Animals were housed individually in stainless-steel cages, in humidity (40–60%) and temperature (21–23°C)-controlled rooms with a 12-h light cycle. Filtered tap water and feed (chicken feed, Ralston-Purina) were provided ad libitum.

Treatment protocols

A group of 10 vehicle (corn oil)-treated roosters served as the control population for the 5 treatment groups. Administration of doses was staggered such that all animals were sacrificed on the same day.

Effect of single dose

Two groups of 3 roosters each received either a single oral dose of 750 mg/kg TOCP or 5 mg/kg parathion in corn oil by gavage. All animals were then killed by a lethal i.p. injection of 100 mg/kg sodium pentobarbital after 24 h. An additional 5 animals received a single 750 mg/kg dose of TOCP and were killed after 18 days.

Effect of 18 daily doses

Eighteen daily doses of 100 mg/kg TOCP ($n = 10$ animals) or 0.1 mg/kg parathion ($n = 3$) in corn oil were administered orally. Body weights were monitored weekly and at the end of the experiment. Animals were examined daily for neurological dysfunction and graded according to a system established by Abou-Donia [9].

Enzymatic analysis

Immediately after decapitation of the animal, brain, plasma, and left testis were removed quickly and held at 4°C until assayed. Brain acetylcholinesterase (AChE), testis nonspecific esterase (NSE), and plasma butyrylcholinesterase (BuChE) were measured as described previously and expressed as micromoles of acetylthiocholine (AThC), nanomoles α-naphthyl acetate hydrolyzed per minute per milligram protein, or butyrylthiocholine (BuTCh) hydrolyzed per minute per milligram protein, respectively [10,11]. Neurotoxic esterase (NTE) was determined by the method of Johnson [12] and expressed as micromoles of phenyl valerate hydrolyzed per minute per milligram protein.

Proteins were determined in brain, testis and plasma by the method of Lowry et al. [13] with bovine serum albumin (Sigma, St. Louis, MO) as standard.

Histological methods

Nervous tissues were taken for histopathological studies shortly after sacrifice at the end of the experiment. Sciatic, tibial, and peroneal nerves along with the spinal cord were fixed for 3 weeks in neutral phosphate-buffered 10% formalin solution.

Cross- and parasagittal 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 ethanols 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 or H and E with LFB.

A section (1–1.5 g) of each testis was removed and placed for 24 h in 10% neutral buffered formalin. The remainder of the testis was used for biochemical analyses described above. A 2 mm thick cross-section was postfixed in 5% glutaraldehyde, 4% paraformaldehyde, in 0.2 M cacodylate buffer (pH 7.4) for 24 h. The sections were rinsed in 10 ml of 0.1 M phosphate buffer, pH 7.4 for 15 min, dehydrated in graded ethanols for 15 min each and embedded in 2-hydroxyethyl methacrylate (Dupont, Sorvall). Sections were cut at 2 μm, stained with periodic acid-Schiff's (PAS) and counterstained with Harris-Mayer's hematoxylin [14].

Evaluation of sperm motility

A 2.5 cm segment of the vas deferens from all animals was removed. A small sample of sperm was expressed from the vas deferens into egg yolk buffer at 37°C [15]. A sample of this sperm preparation was placed on a 37° slide and the motility was calculated by counting all the sperm in 10 fields (magnification 40×) and categorized as either motile or nonmotile (any movement vs. no movement). Sperm motility was determined within 5 min after the animal had been sacrificed.

Statistics

Significance of the difference between control and treated animals was assessed by a 2-tailed Student *t*-test. A *P* value of 0.05 or less was considered significant.

RESULTS

Clinical and necropsy observations

Roosters treated with a single oral dose of 750 mg/kg TOCP 24 h prior to killing showed no outward clinical signs. Animals administered a single oral dose of 5 mg/kg parathion exhibited signs of acute cholinergic toxicity (excess salivation and diarrhea). Roosters treated with either a single oral 750 mg/kg dose of TOCP then kept for 18 days, or given daily oral doses of 100 mg/kg TOCP for 18 days, developed the characteristic signs of OPIDN at approximately day 12 (ataxia, diminished leg movement), which progressed to paralysis by day 18 (inability to stand or walk). Animals given 18 daily oral doses of 0.1 mg/kg parathion exhibited no demonstrable clinical signs. Vehicle-treated control roosters remained normal.

Single treatment with either TOCP or parathion did not result in any biologically significant body weight changes (Table I). The animals treated daily with 100 mg

TABLE I

CHANGES IN BODY AND TESTIS WEIGHT AND SPERM MOTILITY FOLLOWING ORAL ADMINISTRATION OF PARATHION AND TOCP

	Control	TOCP		Parathion
		Single[a] (750 mg/kg)	Daily (100 mg/kg)	daily (0.1 mg/kg)
Percent of initial body weight	110.8 ± 4.3	102.4 ± 2.7	83.2 ± 1.7*	103.2 ± 6.6
Right testis weight (g)	27.42 ± 4.17	33.76 ± 3.15	15.77 ± 1.29*	35.42 ± 3.83
Percent sperm motile	69.33 ± 14.33	74.33 ± 9.73	0.67 ± 0.32*	57.04 ± 2.89

[a] These results are from the 18-day single dose experiment.
* Significantly different from control ($P < 0.05$).
± Standard error.

TOCP/kg lost an average of 17% of their total body weight. In these animals, right testis weights were all significantly decreased compared to controls. Testis to body weight ratios were calculated: control, 0.53%; single dose 750 mg/kg TOCP, 0.68%; daily 100 mg/kg TOCP, 0.38%; parathion, 0.69%. No statistically significant differences were noted.

Sperm motility

Sperm motility was reduced to near 0 in animals treated daily with TOCP whereas the single treated group was unaffected. Sperm motility of roosters receiving daily doses of parathion was not significantly different from controls (Table I).

Histopathology

Testis. In control animals, greater than 90% of all tubules showed the characteristic columnar radiations of maturing germ cells (Fig. 1). Elongated spermatids were located luminal to immature round spermatids and spermatocytes reflecting normal seminiferous epithelial organization and morphology. The vas deferens revealed dense concentrations of spermatozoa. There was no consistent pattern of pathology present in control animals.

In 5 of 10 roosters treated with 100 mg TOCP/kg/day for 18 days, there was a significant disorganization of the seminiferous epithelium which affected 20–80% of the tubules per animal. About 80% of the affected tubules showed exfoliation or vacuolation of spermatocytes and spermatids, with infrequent multinucleated giant cells (Fig. 2). In less than 10% of the affected tubules only a single layer of cells was visible around the periphery of the tubule. The remaining affected tubules were characterized by the absence of round or elongated spermatids. Occasional tubules contained 5–20 malformed elongated spermatids, displaying hooked nuclei and frequently surrounded by small amounts of cytoplasm. These were not seen in

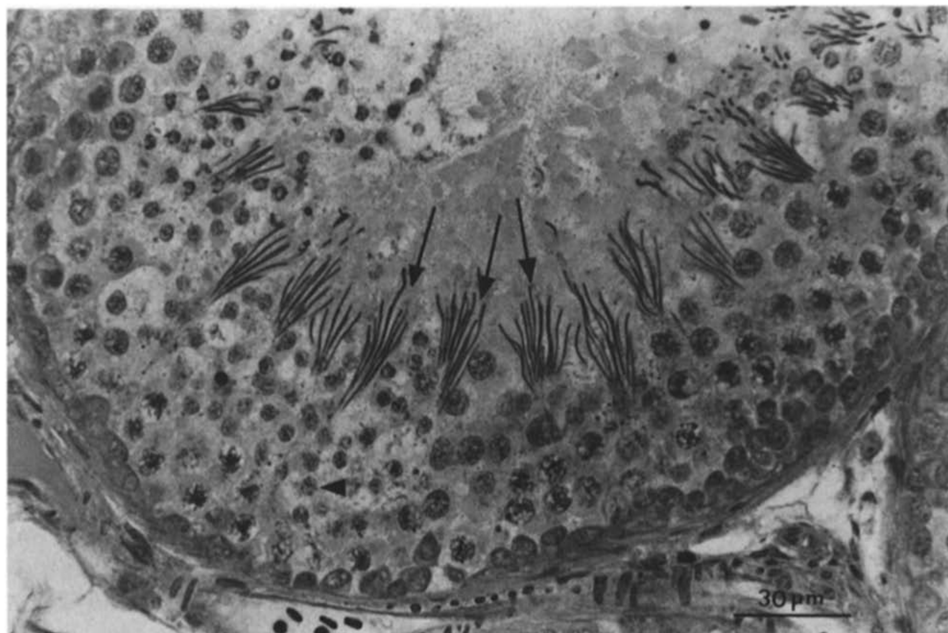

Fig. 1. Seminiferous tubule from control rooster, containing spermatocytes, round spermatids (arrowhead) and elongated spermatids (arrow).

control tubules. The remaining 5 TOCP-treated animals showed no consistent pattern of pathology. The 2 single dose TOCP (1- and 18-day) treatment groups as well as the parathion animals demonstrated no histopathological damage in the testis. The vas deferens from daily treated animals had greatly diminished concentrations of spermatozoa. There were also PAS-positive staining droplets present in vas deferens of these animals.

Spinal cord. Examination of cross-sections of spinal cord from vehicle control animals showed no histopathological lesions. TOCP-treated roosters showed characteristic degeneration in the lateral columns below the cervical areas 18 days after a single oral dose of 750 mg/kg or following 18 oral doses of 100 mg/kg TOCP. Degeneration was also seen in the lumbar region in tracts lying in the ventral tissue. Swelling, fragmentation of myelin and axons, and degenerated mitochondria were seen. There were no pathological changes observed in parathion-treated animals, or in animals killed 24 h after a single oral dose of 750 mg/kg TOCP.

Enzymatic activities

Brain. Brain AChE-specific activity was 16% lower than control 1 day after a single 750 mg/kg dose of TOCP (Table II). Parathion, the positive control for in-

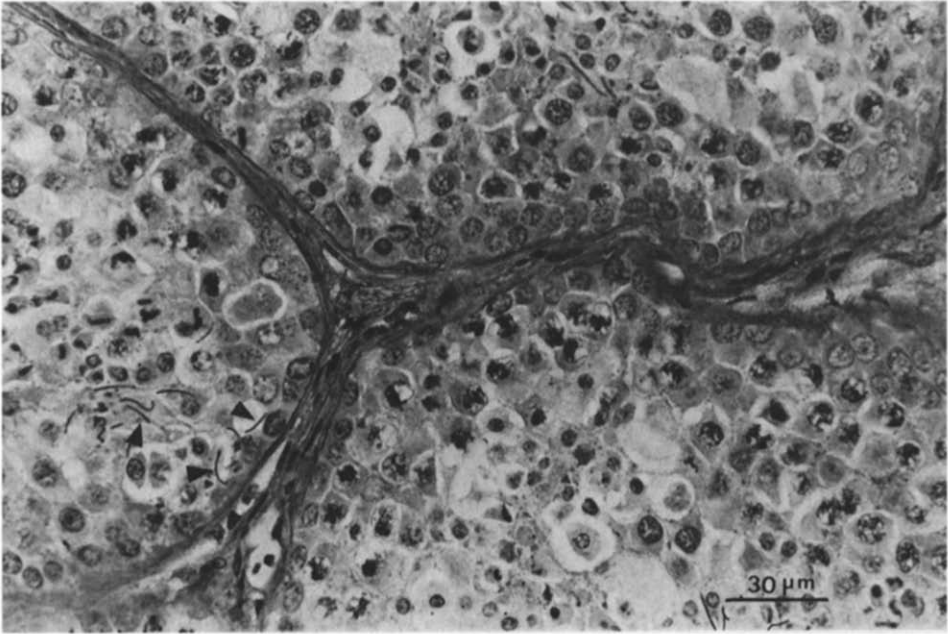

Fig. 2. Seminiferous tubules from rooster treated with 100 mg/kg/day TOCP for 18 days. Note vacuolation of epithelium, and altered arrangement of elongated spermatids in tubule on left (arrowhead).

hibition of AChE activity, resulted in near 100% inhibition following a single 5 mg/kg dose, compared to control values. A significant (80%) inhibition of brain neurotoxic esterase activity was also seen after a single acute dose of TOCP, whereas treatment with parathion resulted in no inhibition.

Esterase (AChE and NTE) activities assayed 18 days following a single oral dose of 750 mg TOCP/kg were 20% and 60% inhibited, respectively.

Administration of TOCP (100 mg/kg/day) for 18 days resulted in 30% inhibition of brain AChE activity and 87% inhibition of brain NTE activity. To assure survival of the animals receiving parathion, the dose was decreased to 0.1 mg/kg/day when administered on a daily basis. Consequently, the inhibition of brain AChE observed after 18 days was minimal (24%). Parathion did not inhibit brain NTE.

Testis. A single acute dose of 750 mg/kg TOCP resulted in 30% inhibition of NSE specific activity; however, a single dose of parathion had only a minimal inhibitory effect (11%). Both 18-day TOCP treatment paradigms (single and daily) demonstrated weakly inhibited testis NSE (6% and 17%, respectively) indicating a slight recovery of enzymatic activity. Parathion given daily resulted in a 21% inhibition on day 18.

Testis NTE values were substantially reduced (55% inhibition) following the single acute 750 mg/kg TOCP dose. After 18 daily 100 mg/kg doses, NTE activity

was inhibited 85%. No inhibition of NTE activity was observed 18 days after a single 750 mg/kg dose. Parathion given either acutely or daily had no significant inhibitory effect on testicular NTE (12% and 10%, respectively).

TABLE II

ESTERASE-SPECIFIC ACTIVITIES[a]

Enzyme	Duration of treatment (days)	TOCP			Parathion		
		Dose (mg/kg)	Enzyme activity (±SE)	Percent of control	Dose (mg/kg)	Enzyme activity (±SE)	Percent of control
Brain AChE[b]		Control	72.8±4.2	100.0			
	1	Single 750	61.3±5.1	84.2	Single 5.0	1.0±0.4*	1.4
	18	Single 750	58.1±4.8	79.8			
	18	Daily 100	51.5±6.6*	70.7	Daily 0.1	54.9±3.6	75.4
Brain NTE[c]		Control	54.3±4.2	100.0			
	1	Single 750	10.5±2.0*	19.3	Single 5.0	55.9±4.0	102.9
	18	Single 750	21.5±0.9*	39.6			
	18	Daily 100	6.8±3.1*	12.5	Daily 0.1	53.4±1.9	98.3
Plasma BuChE[d]		Control	2.5±0.3	100.0			
	1	Single 750	0.6±0.1*	24.0	Single 5.0	0.4±0.1*	16.0
	18	Single 750	1.5±0.2*	60.0			
	18	Daily 100	0.7±0.1*	28.0	Daily 0.1	1.3±0.1*	52.0
Testis NTE[c]		Control	5.6±1.1	100.0			
	1	Single 750	2.5±0.7*	44.6	Single 5.0	4.9±0.2	87.5
	18	Single 750	5.8±0.9	103.5			
	18	Daily 100	0.8±0.2*	14.3	Daily 0.1	5.0±1.0	89.3
Testis NSE[e]		Control	97.3±15.5	100.0			
	1	Single 750	67.8±10.5*	69.7	Single 5.0	86.5±9.7	88.9
	18	Single 750	91.4±7.2	93.9			
	18	Daily 100	80.2±9.4	82.4	Daily 0.1	76.8±7.1*	78.9

[a] Treatments were staggered such that all groups were sacrificed on the same day and compared to the same control population group. Each value represents the mean ± standard error (SE) for duplicate assays.

[b] Brain acetylcholinesterase (AChE) activity is expressed as micromoles acetylthiocholine hydrolyzed/min/mg protein.

[c] Neurotoxic esterase (NTE) activity is expressed as micromoles of phenylvalerate hydrolyzed/min/mg protein.

[d] Plasma butyrylcholinesterase (BuChE) activity is expressed as micromoles butyrylthiocholine hydrolyzed/min/mg protein.

[e] Nonspecific esterase (NSE) activity is expressed as nanomoles α-naphthyl acetate hydrolyzed/min/mg protein.

* Significantly different from control ($P<0.05$).

Plasma. A single acute dose of 750 mg/kg TOCP dramatically inhibited BuChE (76%). BuChE activity was less inhibited (40%) 18 days following administration of a single 750 mg/kg dose of TOCP. Daily treatment with 100 mg/kg TOCP resulted in 72% BuChE inhibition. Parathion was also effective at inhibiting BuChE activity (84%); however, 18 daily doses at 0.1 mg/kg resulted in inhibition that was less than what was seen after a single dose (48%).

DISCUSSION

Tri-*o*-cresyl phosphate (TOCP) is an industrial compound known to produce organophosphate-induced delayed neurotoxicity in chickens and humans [2,3]. It also causes a weak anticholinergic activity due to the inhibition of AChE [16]. Other esterases, including carboxylesterase, butyrylcholinesterase, and neurotoxic esterase are also inhibited [11,19]. TOCP is not unique among neurotoxic compounds in causing testicular toxicity. Other organophosphates have been reported (in rats) to induce seminiferous tubule damage including trimethylphosphate and dimethyl methylphosphonate [18–20]. Neurotoxic compounds that induce testicular pathology include carbon disulfide, hexanedione and acrylamide [6–8].

Various parameters of male reproductive system function were affected by daily oral administration of TOCP to the adult leghorn rooster. Testicular enzymatic activities (NSE and NTE) were inhibited 24 h after a single 750 mg TOCP/kg dose. Single administration of mono-*o*-cresyl phosphate has also been shown to inhibit rooster testis NTE by Lotti [17]. Daily administration of TOCP resulted in further inhibition of testis and brain NTE. Previous studies determined that TOCP remained in nervous tissue with a slow elimination rate [21–23]. Brain AChE and NTE activities remained inhibited 18 days following a single dose of TOCP suggesting this to be true. TOCP has also been demonstrated both in vivo and in vitro to undergo metabolic activation to a 5-fold more potent neurotoxic agent: saligenin cyclic-*o*-tolyl phosphate [23,24].

Histopathological examination of testes from animals treated daily with TOCP revealed damage to the seminiferous epithelium in half of the treated roosters. Multinucleated giant cells as well as presence of elongated spermatid nuclei near the basement membrane were also noted.

Daily treatment with TOCP caused near-complete inhibition of sperm motility. The mechanism responsible for sperm motility is currently unknown. A cholinergic-dependent component of motility has been postulated [25,26]. Goodman and Harbison have determined that carnitine acetyltransferase activity plays a major role in maintenance of motility [17]. They have recently suggested that the cholinergic system in sperm motility has been extremely overestimated [28]. Given the possible presence of acetylcholinesterase in sperm, parathion (an anticholinergic agent known to inhibit AChE) would have been expected to inhibit motility. Its ineffectiveness at inhibiting sperm motility cannot alone rule out a cholinergic role in

motility. It is possible that an inhibitory concentration was not attained due to the low doses which were utilized to ensure survival of the animals for the duration of the 18-day study.

Testis weight, which is a valuable index of reproductive toxicity, was significantly decreased in daily TOCP-treated animals [29]. The decrease is consistent with germ cell loss; this was seen histologically in 5 of 10 animals. However, from these data, one cannot conclusively evaluate the effects of lowered body weight on testis weight; 3 of the 5 treated roosters showed decreases in body weight without significant changes in testis weight.

It is known that compounds which produce delayed neurotoxic effects effectively inhibit carboxylesterase, BuChE and NTE [9, 16]. Johnson has determined that inhibition of NTE correlates well with production of OPIDN; however, the mechanistic link (if one exists) between these two phenomena remains unknown [30].

TOCP's ability to cause morphological alterations may be a characteristic of delayed neurotoxic chemicals. Nondelayed, acute neurotoxic organophosphorus compounds including trimethylphosphate, malathion, and hexamethylphosphoramide have reversible and transient inhibitory effects upon sperm motility, serum gonadotropin levels, and testicular histology [18,31–35]. The lesions produced by the acutely active organophosphorus compounds differ markedly from those produced by TOCP, suggesting different mechanisms of toxicity.

Abou-Donia and colleagues have seen an increase in phosphorylation of brain tubulin after TOCP exposure [36–38]. They proposed this results from an alteration of calcium homeostasis causing an increase in Ca^{2+}-calmodulin-dependent phosphorylation of tubulin. This leads to tubulin aggregation, disruption of axoplasmic transport, and, ultimately, to the internodal swelling and structural damage seen in OPIDN lesions. One may postulate a role for NTE in which an active metabolite produced from TOCP by NTE interacts with target molecules and causes disruption of intracellular calcium levels, leading to cytoarchitectural damage. The mechanism by which TOCP produces nervous tissue damage may be similar to that of seminiferous tubule damage.

The OPIDN-causing compound TOCP is shown by these studies to have a toxic effect upon the rooster reproductive tract. The evidence shows TOCP to produce testicular toxicity in roosters, accompanied by inhibition of brain AChE, testicular NTE and carboxylesterase, and plasma BuChE. Studies are being carried out to further elucidate TOCP-induced testicular toxicity in avian and mammalian species.

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