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Comparison of Effects of Verapamil and Quercetin on Delayed Polyneuropathy Induced by Tri-o-Cresyl Phosphate in Hens

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Organophosphorus compounds (OPs) as a group of toxic chemicals are known to cause acute effects in humans and other susceptible avian and mammalian species due to the inhibition of acetylcholinesterase. Some of these compounds are also known to cause organophosphate-induced delayed polyneuropathy (OPIDP), characterized by pathological lesions in peripheral axons 7-14 days before onset of clinical pathologies (Abou-Donia 1981). The first large scale outbreak of OPIDP was caused by tri-o-cresyl phosphate (TOCP) in the 1920s (Smith *et al.* 1930), which is now widely used in the study of OPIDP. Adult hen is usually the animal model of choice (Abou-Donia 1981).

Although the inhibition and "aging" of a carboxylesterase (neuropathy target esterase, NTE) has been proposed as the initial event of poisoning by Johnson (1970), events occurring between exposure and clinical signs have not been precisely defined (Abou-Donia 1981; Richardson 1992). Investigations of other neuropathies have suggested that breakdown of axonal cytoskeletal elements associated with increased levels of free calcium might be involved (Schlaepfer 1987). Calcium channel blockers have been found to ameliorate the clinical response to phenyl saligenin phosphate (El-Fawal 1989; 1990) and an increase of total sciatic nerve calcium concentration during the development of OPIDP has been observed (El-Fawal 1990). The inhibition of Ca^{2+}-ATPase activity by some OPs has also been observed (Sharma and Bhattacharya 1995). It is known that the inhibition of Ca^{2+}-ATPase leads to a high level of intracellular free calcium, which is contributed to the cell injury (Farber 1981). These studies suggested that the increase of intracellular calcium concentration may be involved in the mechanism of development of OPIDP. Verapamil, a calcium channel blocker can alter neuro-muscular disorders (Dretcher *et al.* 1986) and quercetin, an ATPase inhibitor can inhibit activity of ATPase from membrane (Lang and Racker 1974), the former just has a reverse effect of the latter on the concentration of intracellular free calcium. The present study was performed to determine if verapamil and quercetin can affect the OPIDP in different ways and if they result in reverse effects to testify indirectly to the role of calcium during the development of OPIDP.

MATERIALS AND METHODS

TOCP and physostigmine sulfate were purchased from BDH Chemicals Co. Ltd. (Poole, England). Ouabain was purchased from Merck Co. (Darmstadt, Germany). Adenosin-5' -

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triphosphate disodium salt (5' ATP-Na_2) was purchased from Fluka Chemia-Biochemika (Buchs, Switzerland). Bovine serum album (BSA), tris[hydroxymethyl]methylamine (Tris), ethylene glycol-bis[ß-aminoethyl ether] N,N,N',N'-tetraacetic acid (EGTA), verapamil, and quercetin were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Phenyl valerate and mipafox were synthesized in our laboratory according to the method of Johnson (1977). All other chemicals are domestic products.

Adult white Leghorn laying hens *(Gallus gallus* domesticus) (12 months old and weighing 1.2- 1.4 kg) used in this study were purchased from Yongfeng Poultry Farm (Haidian, Beijing, China). They were individually housed in cages (30 X 40 X 50 cm). The birds were acclimatized for at least 1 week prior to use. Food (Standard layer's diet) purchased from the Xibeiwang Foodstuff Factory (Haidian, Beijing, China) and water were available *ad libitum.* Hens were divided into six groups of 3-5 according to the treatment (control, TOCP, verapamil, quercetin, verapamil + TOCP, and quercetin + TOCP). During the experiment, the temperature in the hen house was controlled at about 20 °C, with 12 hours light each day.

All tested birds were given 10 mg/kg atropine sulfate and 0.1 mg/kg physostigmine sulfate in saline subcutaneously 15 min before the administration of TOCP to alleviate the cholinergic effects. The hens of TOCP group were given a single oral dose of 750 mg/kg TOCP in gelatin capsule, while the other hens were given equivalent amounts of empty gelatin capsule. Verapamil was dissolved in saline and injected daily into the breast muscle at a rate of 7.0 mg/kg for 6 days. Quercetin in dimethyl sulfoxidc (DMSO) was given at a daily i.m. dose of 5.0 mg/kg for 6 days. The first of the six doses of verapamil or quercetin was given one day prior to TOCP administration.

The hens were observed daily for signs of necrologic dysfunction throughout the 28-day test period. Scores for clinical signs were evaluated according to Johnson and Barnes (1970). The hens were killed by decapitation on day 28 after administration of TOCP or vehicle. Immediately following decapitation, the entire brain was removed by dissection and immersed in ice-cold 0.32 *M* sucrose. Meninges and superficial blood vessels of the brain were carefully stripped off and then homogenized in 7 volumes of fresh ice-cold 0.32 M sucrose using a glass/Teflon homogenizer with 12 up-and-down strokes at a medium speed. The distal segments of sciatic nerves of the hens were obtained from the freshly removed specimen. For light microscopy, the tissue of nerves were routinely fixed in 10% neutral-buffered formalin, trimmed, embedded in paraffin wax, sectioned at 4 µm, stained with hematoxylin and eosin (H & E), and examined under the microscope. For transmission electron microscopy, the nerves were cut into sections with less than 1 mm^3 in size and placed in 3% glutaraldehyde in 0.1 *M* sodium cacotylate at pH 7.4. After 2 hr of fixation at 4 °C, nerve segments were rinsed in buffer, postfixed in 1% osmium tetroxide, rinsed in buffer again, dehydrated in a graded series of ethanol, embedded in Epon 812 epoxy resin, sectioned at 60 nm of thickness, stained with uranyl acetate and lead citrate, and examined in an electron microscope.

Synaptic plasma membrane (SPM) of the hen brain was prepared according to Jones and Matus (1974) with a slight modification. The membrane pellet (SPMs) obtained by the method of combined floatation-sedimentation density centrifugation was resuspended in 5 mM Tris-HCl buffer (pH 8.00) and assayed either immediately or after a 2-day storage at -20 °C.

Ca^{2+}-ATPase activity was measured in 100 µl reaction medium containing 50 mM Tris-HCl (pH 7.40), 5 mM $CaCl_2$, and 0.5 mM Ouabain. Aliquots of SPM (5-10 µg protein) were preincubated in test tubes at 37 °C for 10 min. The reaction was intiated by the addition of 50 mM ATP (final conc. 5 mM) and terminated after 5 min by the addition of 50 µl ice-cold 15% (W/V) trichoroacetic acid (TCA). In control tubes, TCA was directly added to SPM. All incubation tubes were then placed in an ice bath and the total volume was made to 1 ml with distilled water. The released inorganic phosphorus was determined by the calorimetric assay of Muszbek *et al* (1977). NTE activity was measured spectrophotometrically in the brain homogenate using the method of Johnson (1977) with modification of Kayyali *et al* (1991). The protein was quantitated by the method of Bradford (1976) using Coomassie brilliant blue G-250 with bovine serum albumin as a standard.

All data were analyzed by a analysis of variance with Newman-Keuls method of comparisons to determine statistical differences between control and experimental groups, with $p < 0.05$ considered significant. All data are expressed as means ± standard errors, except where otherwise indicated.

RESULTS AND DISCUSSION

Clinical signs of OPIDP can not be seen until 8 days after administration of TOCP or TOCP + quercetin. No signs of ataxia were evident in hens treated with either TOCP + verapamil, verapamil alone, or quercetin alone at this time. OPIDP progressed in severity over the next several days. By day 10 all hens given TOCP with or without verapamil and TOCP with quercetin showed clinical evidence of OPIDP, but the degree of ataxia varied depending on the treatment. Hens given TOCP alone exhibited more serious ataxia than those given TOCP + verapamil, but were less severely affected than those given TOCP + quercetin (Table 1). Scores of ataxia for TOCP + verapamil-treated hens were consistently lower than the TOCP-treated group during the entire 28-day study, while the scores for the treatment with TOCP alone, particularly after day 21. Hens given verapamil or quercetin only, or the vehicle did not develop ataxia (Fig. 1, Table 1).

On day 28 neither the NTE nor the Ca^{2+}-ATPase was significantly affected in hens treated with verapamil, quercetin, TOCP, verapamil + TOCP, or quercetin + TOCP, although the activities of NTE seem to be slightly decreased ($p > 0.05$) (Table 2).

Microscopic examination of distal portion of sciatic nerve 28 days after treatment with TOCP revealed that myelinatcd axons were severely degenerated accompanied by many “empty’ Schwann cells; few intact myelinatcd axons were seen. Degeneration in the sciatic nerves from hens treated with TOCP + quercetin was more pronounced (Fig. 2). However, the nerves from TOCP + verapamil-treated hens had numerous intact myelinatcd axons, with a small number of fibers undergoing Wallerian degeneration (Fig. 2). In electron microscopy, many regenerating myelinated axons were recognized in the nerves of hens given TOCP + verapamil. In contrast, numerous degenerated axons accompanied by myelinolysis and formation of vacuoles were seen in the nerves of hens given TOCP + quercetin (Fig. 3). Such lesions were seldom seen in sciatic nerves from control hens or hens given either verapamil or quercetin alone (Table 1).

Table 1. Effects of verapamil and quercetin on the clinical signs and histopathological changes of peripheal nerves from hens with delayed polyneuropathy induced by TOCP

Treatment [a]	Clinical score [b]	Histological score [c]
Control	0	0.10 ± 0.05
TOCP	47.65 ± 4.21 [d]	3.11 ± 0.46 [d]
Verapamil	0	0.09 ± 0.05
Quercetin	0.10 ± 0.08	0.08 ± 0.04
Verapamil + TOCP	18.15 ± 2.03 [d, e]	2.04 ± 0.31 [d, e]
Quercetin + TOCP	53.80 ± 5.31 [d]	3.82 ± 0.15 [d, e]

[a] Control given empety capsule. Verapamil and quercetin, 7mg/kg and 5 mg/kg, respectively, for 6 days (im) with TOCP 750 mg/kg (po) administrated on the second day of drag treatment.
[b] Cumulative daily ataxia scores over the 28-day experimental period. Mean ± SE, n = 3-5. Walking performance was evaluated according to the 0-4 point scale of Johnson and Burnes (1970), 0 = no deficit; 1 = slightly abnormal gait: 2 = severely abnormal gait: 3 = animal can stand but frequently collapses; 4 = animal unable to stand or leg paralysis.
[c] Histological scores of the sciatic nerves sampled at 28 days after TOCP administration. Mean ± SE, n = 3-5. The degree of axonal degeneration was scored according to the 0-4 point scale of Moretto *et al.* (1989). 0= no degeneration; 1= occasional degenerating fibers; 2= moderate number of degenerating fibers; 3 = pronounced degenerating; 4 = severe degeneration.
[d] Siginificantly different from control values, $p < 0.05$.
[e] Siginificantly different from TOCP group, $p < 0.05$.

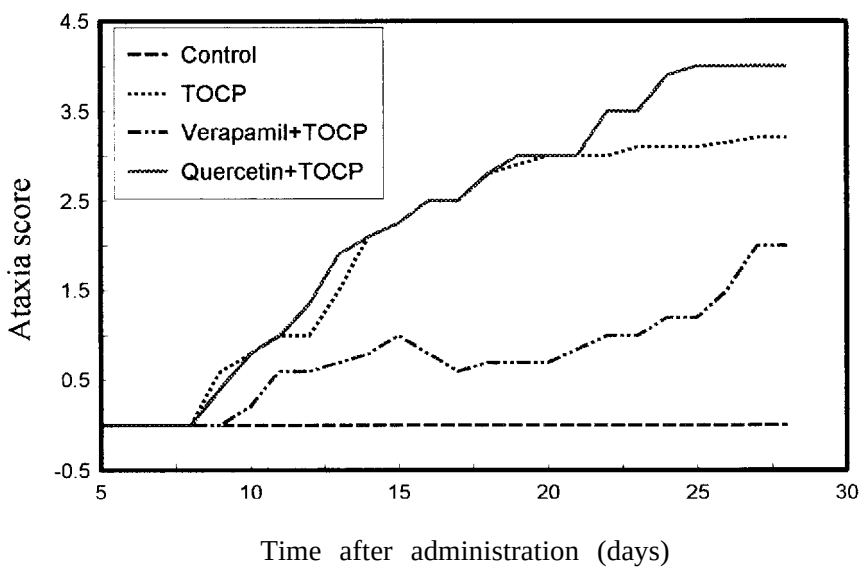

Figure 1. Development of clinical signs in hens after administration of different agents. 3-5 hens each group were tested. Increasing ataxia scores reflect progression in deficits as detailed under footnote "b" for Table 1.

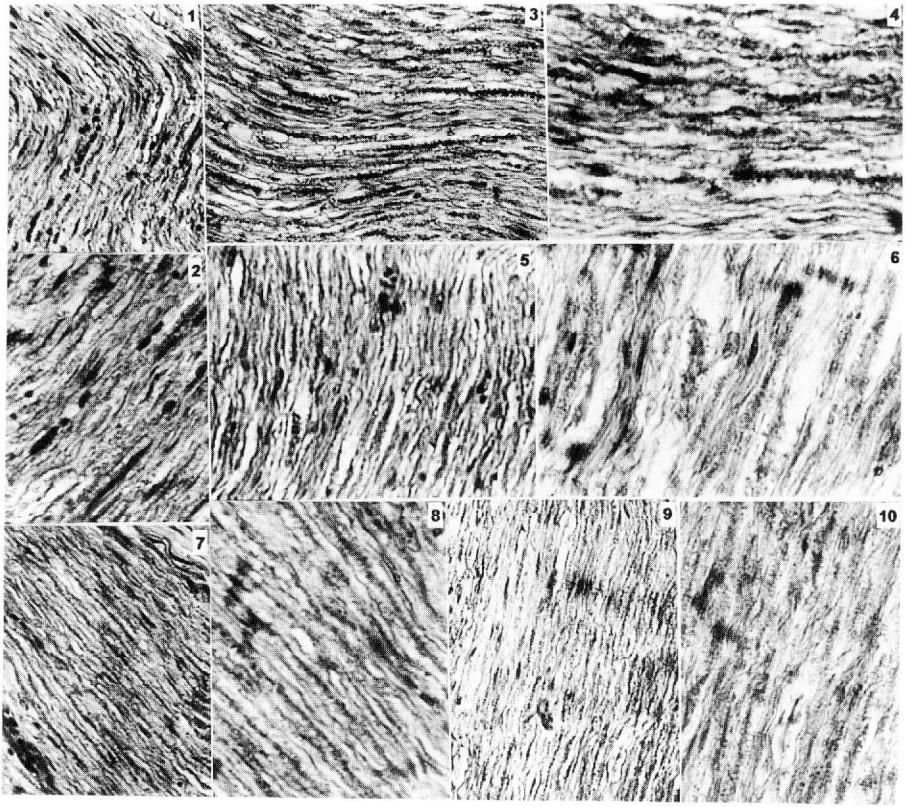

Figure 2. Photomicrographs of longitudinal sections of sciatic nerves from the tested hens (H & E staining). 1, Normal axons from healthy hen, X 120; 2. Higher magnification of Fig. 2.1. X 240; 3. Degeneration of the myelinated fibers from hens treated with TOCP (750 mg/kg, P.O.), X 120: 4. Higher magnification of Fig. 2.3 showing the severe swelling and degeneration of the axons. X 240; 5. Severe degeneration of sciatic nerves and vacuolization, which from a quercetin-treated hen 28 days after TOCP administration. X 120; 6. Higher magnification of Fig. 2.5 showing the vacuolization of the nerve fibers. X 240; 7. Normal sciatic nerves from a hen given quercetin only . X 120; 8. Higher magnification of Fig. 2.7. X 240; 9. The mild axonal degeneration of sciatic nerves from a verapamil-treated hen 28 days after TOCP administration. X 120; 10. Higher magnification of Fig. 2.9. X 240.

Results presented here indicate that the calcium channel blocker verapamil modifies the delayed neuropathy induced by TOCP, which is consistent with the finding of El-Fawal *et al.* (1989). Our experiments demonstrate that verapamil alleviates not only the clinical signs of OPIDP but also the histological lesions of nerves. On the contrary, the ATPase

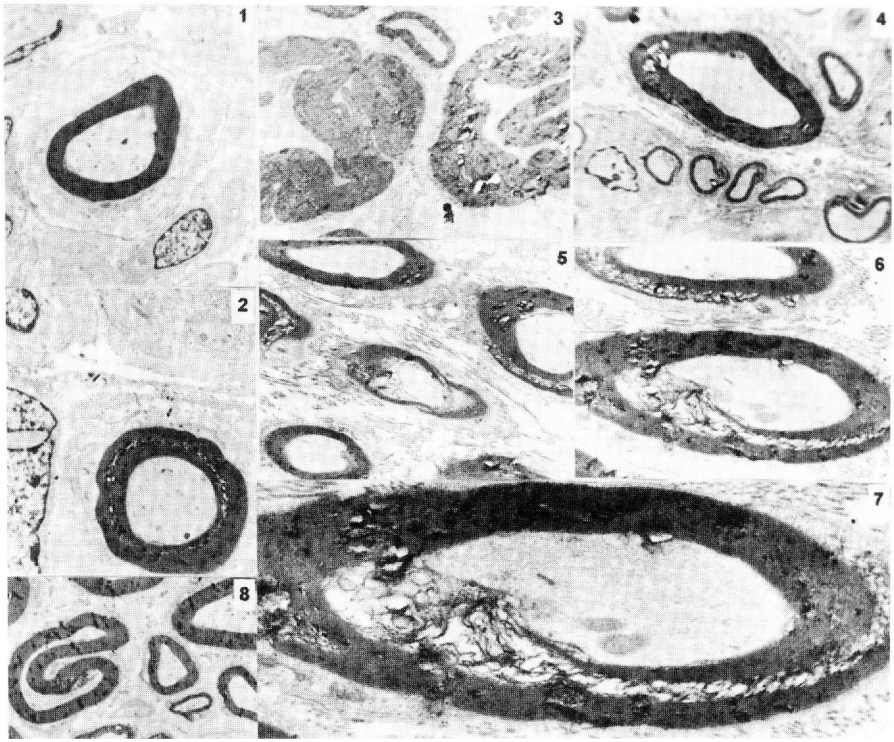

Figure 3. Electron photomicrographs of sciatic nerves of hens with or without delayed polyneuropathy induced by tri-o-cresyl phosphate. 1. Normal myelinatcd fiber from healthy hen, X 5000; 2. Initial axonal degeneration and Schwann cell hyperplasia with swelling of the nucleus of the myelinated fiber from hens treated with TOCP (750 mg/kg, P.O.), X 5000; 3. Severe swelling of the axons and forming monstrous figures, which from the hen treated with TOCP, X 5000; 4. Cluster of thinly myelinated regenerating axons and associated Schwann cells of sciatic nerve from a verapamil-treated hen 28 days after TOCP administration. X 5000; 5. Extensive axonal degeneration of sciatic nerve from a quercetin-treated hen 28 days after TOCP administration. X 3000; 6. Myelinolysis of sciatic nerves from a hen treated with quercetin + TOCP. X 5000; 7. Higher magnification of Fig. 2.6 showing the formation of web and vacuoles derived from the damaged fibers and the neurofilament accumulation within the axon. X 10000; 8. Normal axons from the sciatic nerves of a hen given quercetin only. X 5000.

inhibitor quercetin tended to accentuate the ataxia of OPIDP and the histologic lesions of sciatic nerves. In the distal region of the sciatic nerves of the hens given quercetin + TOCP, degenerated myelinated fibers, myelinolysis and vacuoles were more abundant than in nerves of hens given TOCP only. In contrast, a largc number of intact myelinated fibers remained and more active axonal regeneration was evident in sciatic nerves of hens treated with verapamil + TOCP, compared with those hens treated with TOCP only.

Table 2 Effects of verapamil and quercetin on the activities of brain NTE and SPM Ca^{2+}-ATPase from hen brain 28 days after administration of TOCP *(in vivo)*

Treatment [a]	NTE [b] (nmole phenol/min/mg protein)	Ca^{2+}-ATPase [b] (μmole Pi / hr / mg protein)
Control	33.32 ± 0.22	52.32 ± 6.39
TOCP	31.94 ± 0.28 (96.14)	52.19 ± 7.16 (99.75)
Verapamil	27.85 ± 0.19 (83.85)	54.22 ± 7.41 (103.63)
Quercetin	27.97 ± 0.21 (84.21)	48.77 ± 5.95 (93.21)
Verapamil + TOCP	29.46 ± 0.27 (88.69)	51.21 ± 6.25 (97.88)
Quercetin + TOCP	29.21 ± 0.20 (87.93)	47.95 ± 5.88 (91.64)

[a]Control given empty capsule. Verapamil (7mg/kg) and quercetin (5 mg/kg) were administered for 6 days (im) with TOCP 750 mg/kg (po) administered on the second day of drug treatment.
[b]Mean ± SE, n = 3-5. Percentage of control in parentheses. None of the experimental groups was significantly different from the control group, $p > 0.05$.

The present study demonstrated modification of OPIDP by two drugs which are capable of altering directly or indirectly intracellular Ca^{2+} level. It's known that Ca^{2+} plays a pivotal role as a messenger linking external stimuli to the intracellular environment in the nerve (Miller 1987). Ca^{2+} is involved in the degeneration and death of cells, including nerve cells (Farber 1981). It is also well known that verapamil as a calcium channel blocker can inhibit both the movement of calcium ion into the cell and its mobilization from intracellular storage sites (Needleman *et al.* 1985), which leads to an decrease of intracellular free calcium level. Quercetin, as an inhibitor of ATPase, can reduce the activities of ATPase and increase intracellular sodium concentration, which in turn inhibits the acitivity of Ca^{2+}-ATPase (Gill *et al.* 1981). As Ca^{2+}-ATPase is inhibited, calcium efflux of a cell (e.g., neuron) is decreased and calicium influx is increased, which leads to the high level of intracellular free calcium.

Although verapamil and quercetin modified the ataxia and nervous lesions of OPIDP, neither drug significantly altered the activities of NTE or Ca^{2+}-ATPase of brain from hens 28 days after administration. Perhaps the activities of the enzyme have recovered at the time of assay. Although the present study has not confirmed whether an accumulation of excessive intracellular free calcium is an initial biochemical event of OPIDP, it provides an impetus for further investigations into the role of Ca^{2+} in the development of OPIDP.

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