Changes of mitochondrial ultrastructures and function in central nervous tissue of hens treated with tri-ortho-cresyl phosphate (TOCP)

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Abstract
Tri-ortho-cresyl phosphate (TOCP), an organophosphorus ester, is capable of producing organophosphorus ester-induced delayed neurotoxicity (OPIDN) in humans and sensitive animals. The mechanism of OPIDN has not been fully understood. The present study has been designed to evaluate the role of mitochondrial dysfunctions in the development of OPIDN. Adult hens were treated with 750 mg/kg bw TOCP by gavage and control hens were given an equivalent volume of corn oil. On day 1, 5, 15, 21 post-dosing, respectively, hens were anesthetized by intraperitoneal injection of sodium pentobarbital and perfused with 4% paraformaldehyde. The cerebral cortex cinerea and the ventral horn of lumbar spinal cord were dissected for electron microscopy. Another batch of hens were randomly divided into three experimental groups and control group. Hens in experimental groups were, respectively, given 185, 375, 750 mg/kg bw TOCP orally and control group received solvent. After 1, 5, 15, 21 days of administration, they were sacrificed and the cerebrum and spinal cord dissected for the determination of the mitochondrial permeability transition (MPT), membrane potential (ΔΨm) and the activity of succinate dehydrogenase. Structural changes of mitochondria were observed in hens’ nervous tissues, including vacuolation and fission, which increased with time post-dosing. MPT was increased in both the cerebrum and spinal cord, with the most noticeable increase in the spinal cord. ΔΨm was decreased in both the cerebrum and spinal cord, although there was no significant difference in the three treated groups and control group. The activity of mitochondrial succinate dehydrogenase assayed by methyl thiazolyl tetrazolium (MTT) reduction also confirmed mitochondrial dysfunctions following development of OPIDN. The results suggested mitochondrial dysfunction might partly account for the development of OPIDN induced by TOCP.

Keywords
tri-ortho-cresyl phosphate (TOCP), organophosphorus ester-induced delayed neurotoxicity (OPIDN), morphological changes, mitochondrial permeability transition (MPT), methyl thiazolyl tetrazolium (MTT) reduction

Introduction
Tri-ortho-cresyl phosphate (TOCP), one of the organophosphorus compounds (OPs), was used as oil additives, dye intermediate, solvents, and plasticizer in industry. It is capable of inducing a delayed neurodegenerative condition known as organophosphorus ester-induced delayed neurotoxicity (OPIDN) in human beings and sensitive animals.¹ Signs and symptoms include tingling of the hands and feet, followed by sensory loss, progressive muscle weakness and
flaccidity of the distal skeletal muscles of the lower and upper extremities, and ataxia.2,3 OPIDN is recognized as a distal sensorimotor axonopathy, which is characterized by distal axonal swellings and degeneration and secondary Wallerian-type degeneration of myelin in the most distal portion of large diameter, long axons in the peripheral nerves and in the spinal cord motor and sensory tracts of animals with OPIDN before 7–14 days onset of clinical symptoms.1,4

Although OPIDN has been documented for almost a century, no effective treatment exists and recovery from OPIDN is poor.2,5 Ultrastructural studies showed that axonal swelling containing aggregations of neurofilament (NF) subunits, microtubules, multivesicular vesicles, and proliferation of smooth endoplasmic reticulum were represented in early stage, followed by partial matting, and disappearance of NF subunits from swollen axons.5,7 The mechanism of OPs-induced OPIDN is poorly understood, despite many studies. Recently, increasing evidence suggested that mitochondrial dysfunction might be involved in many neurodegenerative diseases, such as Parkinson’s disease, Alzheimer’s disease, and Huntington’s disease.8–10 In addition, in vitro biochemical and pathological studies indicated that mitochondrial dysfunction was responsible for the neurotoxicity of OPs. Massicotte et al. observed that ATP production decreased and mitochondrial integrity was altered in neuron exposed to neurotoxic OPs.11 Decreased activities of mitochondrial complex I-IV, reduction in mitochondrial transmembrane potential ($\Delta \psi_m$), depleted intracellular ATP content, and damaged cell membrane integrity were also represented in PC 12 cells treated with OPs.12 Changes in ATP concentration and nerve conduction velocity were also found in hens administered with the delayed neurotoxicant phenyl saligenin phosphate (PSP).13

Mitochondria play vital role in maintaining the function of nervous system. To this day, little is known about the correlations between the mitochondrial dysfunction and OPIDN induced by TOCP. The present study examined morphological and functional alterations of the mitochondria in hens’ nervous tissue in a dose- and time-dependent manner following treatment with TOCP to illustrate whether mitochondrial dysfunction account for the development of OPIDN.

**Material and methods**

**Materials**

Tri-ortho-cresyl phosphate (TOCP, purity > 99%) was purchased from Shanghai Chemical Reagent Co. (Shanghai, China). Rhodamine 123, rotenone, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) were purchased from Sigma Chemical Co. (St Louis, Missouri, USA). BCA™ protein assay kit was purchased from Pierce Biotechnology, Inc. (Rockford, Illinois, USA). All other chemicals were of highest quality commercially available.

**Observation of mitochondrial ultrastructures**

Roman hens, 10 months old and weighing 1.5–2.0 kg, were obtained from Institute of Poultry, Academy of Agriculture of Shandong (Jinan, China). Drinking water and complete-value hen powder food were available ad libitum. The animal room was maintained at approximately 22°C and 50% humidity with a 12-h light/dark cycle. All experiments were carried out in accordance with the NIH Guide for Care and Use of Laboratory Animals and followed the principles in the ‘Use of Animals in Toxicology.’ After 7 days acclimatization, hens were randomly divided into 2 groups: TOCP-treated group ($n = 12$) received TOCP by gavage at dosage of 750 mg/kg-bw and control group ($n = 4$) received an equivalent volume of corn oil. On days 1, 5, 15, 21 post-dosing, the hens from TOCP and control groups were respectively anesthetized by intraperitoneal injection of an overdose of sodium pentobarbital (100 mg/kg-bw), perfused transcardially with 100 mL 0.9% saline, and 1000 mL of 0.1 M phosphate buffer (PB; pH 7.4) containing 4% (w/v) paraformaldehyde.14 Small pieces of tissue were dissected from the cerebral cortex cinerea and the ventral horn of lumbar spinal cord and cut into approximately 1 mm cubes. The cubes were post-fixed with 2% osmium tetroxide for 2 h at 4°C and then dehydrated in an ethanol series. The samples were embedded in Epoxy resin and were cut into 60–70 nm thick sections using LKB III ultramicrotome (Sweden). Then the sections were double-stained with uranyl acetate and lead citrate and examined by a JEM-1200EX transmission electron microscopy (JEOL Co., Japan) and photographed.

**Neurological behavior and mitochondrial functions testing**

**Animals’ treatment.** Another batch of animals were randomly divided into four groups, i.e. three experimental groups (low-dosage, medium-dosage, and high-dosage, $n = 24$ in each group) and the corresponding time-matched control group ($n = 24$). The hens in...
Experimental groups were treated with TOCP by gavage at single dosages of 185, 375, 750 mg/kg-bw, respectively. TOCP was dissolved in corn oil and administered at 0.65 mL/kg-bw, while hens in control group received an equivalent volume of corn oil. After exposure to TOCP, each hen was examined daily for the delayed neurotoxicity. Neurological evaluation was performed in five min by a trained and blinded observer who was not involved in animal care and administration. OPIDN neurological signs were assessed by an eight-point graded scale (0, normal ambulation; 1–2, slight and infrequent hindlimb incoordination; 3–4, moderate but definite hindlimb incoordination; 5–6, severe and frequent difficulty in walking and standing erect; 7–8, virtual to complete hindlimb paralysis). Six hens in each group were randomly selected and sacrificed on the corresponding time points of 1, 5, 15, 21 days post-exposure, respectively. The cerebrum and spinal cord were quickly dissected and prepared for mitochondrial functional assay.

**Isolation and purification of the mitochondria.** The preparation of mitochondria was proceeded as previously described by Clark and Nicklas. Briefly, the cerebrum and spinal cord samples were rapidly removed into ice-cold isolation medium (0.25 M sucrose, 10 mM Tris-HCl, 0.5 mM K₂EDTA, 0.06 M sucrose, 0.24 M mannitol, 0.03 M Ficoll, 0.02 M CaCl₂, 5 mM Tris-HCl, pH 7.4) and chopped finely with scissors while being washed frequently with ice-cold isolation medium. The tissues were homogenized (2,000 rev/min, 5 min) in 9-volume cold isolation medium with a glass pestle. Then the homogenate was centrifuged for 3 min at 2000 g to obtain the homogenate with a clear supernatant. The homogenate was again centrifuged for 8 min at 12,500 g to get the crude mitochondrial pellet. The crude mitochondrial pellet was resuspended in a 3% Ficoll medium (3% Ficoll, 0.12 M mannitol, 0.03 M sucrose, 25 μM K⁺-EDTA, 5 mM Tris-HCl, pH 7.4). This suspension was carefully layered onto a 6% Ficoll medium (6% Ficoll, 0.24 M mannitol, 0.06 M sucrose, 50 μM K⁺-EDTA, 10 mM Tris-HCl, pH 7.4) and centrifuged for 30 min at 11,500 × g. The supernatant was decanted and the mitochondrial pellet was resuspended in isolation medium and recentrifuged for 10 min at 12,500 × g to get the purified mitochondria. The final pellet was reconstituted in the isolation medium. The protein concentration of the mitochondrial suspension was quantified using BCA™ protein assay kit. All procedures were conducted at 4°C.

**Measurement of mitochondrial permeability transition (MPT).** Mitochondrial swelling was assayed by decrease of A540 nm. The isolated mitochondria were modulated to 0.5 mg/mL and incubated in the assay buffer (125 mM sucrose, 65 mM KCl, 5 mM succinate, 5 μM rotenone, 10 mM Tris-HCl, pH 7.4). MPT was initiated by adding 50 μM calcium chloride, and monitored by measuring the decrease of A540 nm (ΔA540 nm) at 37°C using an ultraviolet spectrophotometer in five min.

**Detection of mitochondrial membrane potential (ΔΨm).** ΔΨm was detected by monitoring the fluorescence quenching of Rh123 dynamically. Fluorescence with excitation at 503 nm and emission at 527 nm was detected in a reaction buffer (250 mM sucrose, 2 mM HEPES, 0.5 mM KH₂PO₄, 4.2 mM sodium succinate, pH 7.4, and 0.3 μM Rh123) using F-4500FL Spectrophotometer (Hitachi High-Technologies Co., Japan). The mitochondria were diluted to 0.5 mg/mL in the buffer and incubated for 3 min. The fluorescence was recorded again, and the alteration of the ΔΨm was detected by the decrease of the fluorescence.

**Methyl thiazolyl tetrazolium (MTT) Reduction.** MTT reduction was used to assess the activity of the mitochondrial respiratory chain by the method of Masoud et al. The dye is converted to water-insoluble purple formazan on the reductive cleavage of its tetrazolium ring by the succinate dehydrogenase system of the active mitochondria. The reaction mixture containing mitochondrial preparation (80–100 μg protein) and 0.02 mL of MTT (0.1 mg/mL) was incubated at 37°C for 30 min and then centrifuged at 1000 × g for 5 min at room temperature to obtain the formazan pellet. The pellet was dissolved in 1 mL of acidic isopropanol and the mixture was recentrifuged at 1000 × g for 5 min at room temperature. Then the absorbance of the supernatant was measured at 595 nm. Results were expressed as A595 nm/mg protein.

**Statistical analyses**

The data were expressed as mean ± SD. SPSS16.0 statistical software was employed for statistical analysis. All the data were analyzed with one-way analysis of variance (ANOVA), followed by LSD’s post hoc tests. The differences were considered significantly at p < 0.05 level.

**Results**

**Neurological behavior scores**

Exposure to TOCP produced obvious progressive gait abnormalities and there were observable differences...
in the temporal onset, progression, or severity of these clinical signs among hens exposed to 185, 375, and 750 mg/kg/bw TOCP, respectively. Hens in 750 mg/kg/bw TOCP-treated group began to show slightly abnormal gait on day 6 post-dosing (mean clinical score $= 0.80 \pm 0.56$). Clinical signs progressed to disturbances in gait and unsteadiness, and signs were severe on day 10 post-dosing (mean clinical score $= 3.80 \pm 0.42$). All of the hens reached total hindlimb paralysis on day 16 (mean clinical score $= 7.60 \pm 0.89$). Hens in 375 mg/kg/bw TOCP-treated group began to show slightly abnormal gait on day 7 and reached total paralysis on day 17 (mean clinical score $= 7.50 \pm 0.58$). Hens in 185 mg/kg/bw TOCP-treated group had mildly abnormal gait on day 8, but not all hens in this group reached total paralysis on day 21.

The mitochondrial structures had no change on day 1 post-dosing, while on day 5, there were slight abnormalities, exhibited as mild swelling and the slight decrease of mitochondrial cristae. On day 15, most of the mitochondria were swollen, and the mitochondrial cristae ruptured, decreased, and even disappeared. Several numbers of the mitochondria were vacuolated as observed under the electron microscopy. On day 21, as shown in Figure 2E, vacuolation of mitochondria, decrease or disappearance of mitochondria cristae, emptying of mitochondrial matrix, and swollen endoplasmic reticulum were much more obvious.

**Effects of TOCP on morphological changes of mitochondria in the cerebrum.** The ultrastructural alterations of mitochondria in the spinal cord were much more obvious than those in the cerebrum. The structure of mitochondria represented changes on day 5 post-dosing. Several of them were slightly swollen and part of the cristae ruptured. On day 15, the swelling and vacuolation of mitochondria were more obvious and subsequently almost all those in the neurons were vacuolated on day 21 post-dosing (Figure 3D, E). In addition, dissolved nuclear membrane was displayed as well.

**Effects of TOCP on morphological changes of mitochondria in the spinal cord.** The ultrastructural alterations of mitochondria in the spinal cord were much more obvious than those in the cerebrum. The structure of mitochondria represented changes on day 5 post-dosing. Several of them were slightly swollen and part of the cristae ruptured. On day 15, the swelling and vacuolation of mitochondria were more obvious and subsequently almost all those in the neurons were vacuolated on day 21 post-dosing (Figure 3D, E). In addition, dissolved nuclear membrane was displayed as well.

**Effects of TOCP on mitochondrial functions**

**Effects of TOCP on $Ca^{2+}$-induced MPT.** $Ca^{2+}$ resulted in mitochondria swelling, shown as the progressive drop of A540 nm. $Ca^{2+}$-induced MPT in the cerebrum had no significant changes after administration of TOCP (Figure 4A). However, in the spinal cord, $\Delta A540$ nm of the three treated groups was decreased by 36.8%, 43.8%, and 42.1%, respectively, on day 1 post-dosing compared to the control group ($p < 0.05$). On days 5 and 15 post-dosing, the decreased trends of $\Delta A540$ nm were still kept in the 375 and 750 mg/kg/bw TOCP-treated groups hens ($p < 0.05$). Subsequently, on day 21 post-posing, $\Delta A540$ nm of the three experimental groups slightly increased, although there was no significant change compared to the control group (Figure 4B). Additionally, $\Delta A540$ nm of the three treated groups has no significant difference as time went on.

**Effects of TOCP on the $\Delta \psi_m$.** $\Delta \psi_m$ was detected by monitoring the fluorescence quenching of Rh123 dynamically. In this study, although TOCP resulted in the decrease of $\Delta \psi_m$ in hens’ nervous tissues, there was no significant difference between the experimental groups and control group. Also, there was no

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**Figure 1.** Graph of daily clinical sign scores. Hens in 750 mg/kg bw tri-ortho-cresyl phosphate (TOCP) exposure began to represent slightly abnormal gait on day 6 with daily progression, reached total paralysis on day 16. Hens in 375 mg/kg bw TOCP exposure took on slightly abnormal gait on day 7 and reached total paralysis on day 17. Hens in 185 mg/kg bw TOCP exposure had mildly abnormal gait on day 8, but not all hens in this group reached total paralysis on day 21.

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**Morphological changes of mitochondria**

**Effects of TOCP on morphological changes of mitochondria in the cerebrum.** In the cerebrum, the progressive morphological changes of mitochondria were observed.

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**Effects of TOCP on morphological changes of mitochondria in the spinal cord.** The ultrastructural alterations of mitochondria in the spinal cord were much more obvious than those in the cerebrum. The structure of mitochondria represented changes on day 5 post-dosing. Several of them were slightly swollen and part of the cristae ruptured. On day 15, the swelling and vacuolation of mitochondria were more obvious and subsequently almost all those in the neurons were vacuolated on day 21 post-dosing (Figure 3D, E). In addition, dissolved nuclear membrane was displayed as well.

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**Effects of TOCP on the $\Delta \psi_m$.** $\Delta \psi_m$ was detected by monitoring the fluorescence quenching of Rh123 dynamically. In this study, although TOCP resulted in the decrease of $\Delta \psi_m$ in hens’ nervous tissues, there was no significant difference between the experimental groups and control group. Also, there was no
significant difference of $\Delta \psi_m$ in the three treated groups as time went on (shown in Figure 5).

**MTT reduction.** Reduction of water-soluble tetrazolium salt MTT to its formazan is regarded as an indicator of mitochondrial respiration, especially the activity of mitochondrial succinate dehydrogenase. The reaction is attributed mainly to mitochondrial enzymes and electron carriers. In the cerebrum, there was no significant decrease in the activity of mitochondrial succinate dehydrogenase on days 1 and 5 post-dosing. On days 15 and 21, the activity of the enzyme in high-dosage group was significantly decreased (16.8% on day 15 and 30.7% on day 21). In the spinal cord, compared with control group, the activity of mitochondrial succinate dehydrogenase in high-dosage group decreased by 28.0% ($p < 0.05$) on day 5 post-dosing. The decrease trend was still kept in three TOCP-treated groups hens on day 15, decreasing by 37.4% ($p < 0.01$) in medium-dosage group and 42.1% ($p < 0.01$) in high-dosage group. At the end of the experiment, day 21 post-dosing, the activity of mitochondrial succinate dehydrogenase was slightly increased, but still decreased compared with the control group. There was no significant difference of its activity in the three treated groups as the experiment progressed (Figure 6).

Figure 2. Electron micrographs of cerebrum of hens in experimental group at 1, 5, 15, 21 days after exposure to tri-ortho-cresyl phosphate (TOCP) and hens in control group showing morphological changes of mitochondria in neuron. A-control, B-day 1, C-day 5, D-day 15, E-day 21. The mitochondria took on slight abnormalities on day 5, and on day 15, most of the mitochondria were swollen, and the mitochondrial cristae ruptured, decreased, and even disappeared. On day 21, vacuolation of mitochondria, decrease or disappearance of mitochondria cristae, emptying of mitochondrial matrix, and swollen endoplasmic reticulum were much more obvious.
Discussion

In spite of extensive research related to the development of OPIDN induced by OPs, its precise mechanism remains unclear. Recently, many lines of evidence suggested that mitochondrial dysfunction might be involved in the neurodegenerative diseases.\textsuperscript{8-10} To investigate whether the mitochondrial dysfunction was responsible for the development of OPIDN, we induced OPIDN in hens with three different dosages of TOCP, observed the morphological changes of mitochondria, and examined the alterations of \( \text{Ca}^{2+} \)-induced MPT, \( \Delta \psi_m \), and MTT reduction. The results showed that there were evident morphological changes under electron microscope, significant increase of \( \text{Ca}^{2+} \)-induced MPT, and obvious decrease in the activity of mitochondrial enzymes before the onset of clinical signs of OPIDN.

Mitochondria, the energy factories of cells, make and supply most of the required energy to cells to carry out all their functions. They make use of 85\%—90\% of oxygen in cells to produce ATP by oxidative phosphorylation. In nervous system, axons represent more than 99\% of the volume of a cell. As protein and lipid synthesis occur almost exclusively in the cell body, anterograde axonal transport is required to supply the axon with newly synthesized material by the microtubule motor kinesin and kinesin-related motor proteins. In return, material targeted for degradation is actively returned to the cell body by retrograde axonal transport.\textsuperscript{22} ATP has been

\begin{figure}
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\caption{Electron micrographs of spinal cord of hens in experimental group at 1, 5, 15, 21 days after exposure to tri-ortho-cresyl phosphate (TOCP) and hens in control group showing morphological changes of mitochondria in neuron. A-control, B-day 1, C-day 5, D-day 15, E-day 21. The structures of mitochondria began to represent changes on day 5 post-dosing. Some mitochondria were slightly swollen and part of the mitochondrial cristae ruptured. On day 15 post-dosing, the swelling and vacuolation of mitochondria were more obvious and subsequently almost all mitochondria in neurons were vacuolated on day 21 post-dosing.}
\end{figure}
Mitochondria are composed of an outer membrane, intermembrane space, an inner membrane, and a matrix. There are large amounts of polyunsaturated fatty acids (PUFA) on the membrane, and the cavity is full of soluble protein and lipid with low electron concentration. In the matrix are enzymes related to tricarboxylic acid cycle, fatty acid oxidation, amino acid decomposition, and protein synthesis. The characteristics of mitochondrial structure and functions make them more vulnerable to oxidative stress than other organelles in cell. Oxidative damage accumulates more in mitochondria than in the rest of the cell because electrons continually leak from the respiratory chain to form damaging reactive oxygen species (ROS). Moreover, mitochondria are of great importance to maintain the function of nervous system, especially peripheral nervous system.

Figure 4. Effects of tri-ortho-cresyl phosphate (TOCP) on Ca²⁺-induced mitochondrial permeability transition (MPT). A-cerebrum; B-spinal cord. The results were represented as a mean ± SD. Statistical significance was determined by using one-way analysis of variance (ANOVA). * p < 0.05, compared with the control group. Ca²⁺-induced MPT was shown as the progressive drop of A540 nm. It had no significant changes after administration of TOCP in cerebrum. While in spinal cord, ΔA540 nm of the three treated groups was all significantly decreased on day 1 post-dosing compared to control group. ΔA540 nm of 375 and 750 mg/kg bw TOCP-treated groups kept the decrease trend on days 5 and 15 post-dosing. Subsequently, on day 21, ΔA540 nm of three experimental groups slightly increased.

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were involved in the toxic effects of TOCP on nervous tissues. Therefore, mitochondria might be highly related to the development of OPIDN induced by neurotoxic OPs.

The electron micrographs revealed morphological changes in spinal cord mitochondria as early as day 1 post-dosing. More obvious swelling and vacuolation were observed on day 5 post-dosing, and coincided with obvious clinical signs of OPIDN (Figure 3). As symptoms progressed with time, the lesions exacerbated gradually. The morphological changes in the cerebrum were not as evident as those in the spinal cord. These results were in keeping with the alterations of Ca$^{2+}$-induced MPT and $\Delta\psi_m$ in this study. Alterations in Ca$^{2+}$-induced MPT and $\Delta\psi_m$ are now thought to be a central regulatory mechanism for cell death induction. Once mitochondria membrane permeabilization occurs, cells die either by apoptosis or necrosis. In this study, Ca$^{2+}$-induced MPT was increased in the hens treated with TOCP compared to the control group, with the most evident changes on day 1 post-dosing in the spinal cord ($p < 0.05$; Figure 4B). These facts suggested that mitochondrial dysfunction might be partly responsible for the development of OPIDN induced by TOCP. Supportive evidence has also come from study of Massicotte et al. It suggested that OPs could result in the reduction of ATP level in nervous system prior to the onset of delayed neurotoxic symptoms.

MPT is thought to be induced by the opening of a mega channel that is known as the mitochondrial permeability transition pore (MPTP), which putatively consists of the voltage-dependent anion channel (VDAC), the adenine nucleotide translocator (ANT), cyclophilin-D (Cyp-D), and other molecules. Many factors can induce the opening of the MPTP. Of interest is a high NAD$^+/\text{NADH}$ ratio or free radical generation. Any other factors that may induce an oxidative stress are possible to favor the opening of the MPTP, which results in the rapid ion movement followed by extensive mitochondrial swelling and the loss of the mitochondrial membrane potential.
Furthermore, increased intra-axonal calcium entry and active sequestration by mitochondria might be another factor that could induce the opening of the MPTP, resulting in mitochondrial swelling and bioenergetic impairment. The OPs that cause a delayed neuropathy with degeneration of long axons in peripheral nerves and spinal cord can result in calcium entry, elevation of axonal calpain activity, and Wallerian-type degeneration. Lopachin and Lehning’s study reported that axonal Ca\(^{2+}\) entry involved in the pathophysiologic processes induced by chemical neurotoxicants and other types of nerve damage. Kaur et al. also suggested that in response to excessive mitochondrial Ca\(^{2+}\) loads, mitochondria might incur Ca\(^{2+}\)-induced respiratory impairment by inhibition of electron transport chain and oxidative phosphorylation, which may activate key enzymes responsible for increased ROS generation.

In addition, MTT reduction which is a marker of mitochondrial respiration was also markedly reduced in the brain and spinal cord of TOCP-exposed animals. Study by Masoud et al. also suggested that the activity of mitochondrial succinate dehydrogenase was obviously decreased in the brain of monocrotophos (MCP)- or dichlorvos (DDVP)-exposed rats and the activities of mitochondrial complex I (NADH dehydrogenase), II (succinate dehydrogenase), and IV (cytochrome oxidase) in isolated mitochondria from different brain regions were all significantly decreased. As mentioned above, the oxidative stress of nervous system induced by TOCP might damage the mitochondrial...
electron transport chain, which consequently led to inhibition of ATP synthesis.

In our present study, we developed OPIDN hens model with three different dosages of TOCP, and observed mitochondrial dysfunctions in hens’ nervous tissues before the onset of clinical signs, showing as obvious pathological changes, increased Ca\textsuperscript{2+}-induced MPT, decrease of $\Delta \Psi_m$, and mitochondrial succinate dehydrogenase. These results indicated that mitochondrial dysfunction might be partly responsible for the development of OPIDN.

**Conflict of interest statement**
The authors declare that there are no conflicts of interest.

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