STUDIES ON THE DELAYED NEUROTOXICITY OF ORGANOPHOSPHORUS COMPOUNDS- (I)

Hirotoshi ITOH, Mamoru TADOKO and Kiyoshi OIKAWA
First Department of Pathology,
St. Marianna University, School of Medicine
2095, Sugao, Takatsu-ku, Kawasaki 213, Japan
Accepted July 17, 1981

Abstracts—Delayed neurotoxicity experiments of an organophosphorus compound, TOCP, on hens and quails were carried out. The animals were orally exposed with TOCP in dose of 400 mg/kg, and maintained for 25 and 50 days under observation, respectively. They were sacrificed and dissected at different periods after the exposure, and the histopathological examinations were made on those animals.

During the periods of 50 days, no abnormal symptoms except for acute poisoning were noted in the quails. As to the hens, neurological disorders were observed from around 12 days of the experiment.

In hens, the morphological alteration in the earlier stage of the experiment was perivascular cuffing of small round cells seen in the cerebrum, cerebellum and spinal cord. The major alteration was degeneration of axons and myelin in the white matter of the spinal cord. A minimal degree of these changes was found even in the hens in which the clinical signs of neurotoxicity had not yet been observed. Later the process expanded to the whole spinal cord as well as the sciatic nerve. The electron microscopy performed after 15 days revealed moderate change of myelin and axons. From these results, it was concluded that the clinical signs as well as the morphological changes were closely related to the exposure of the compound, TOCP.

Key words: Delayed neurotoxicity, TOCP. Delayed neurotoxicity of TOCP

INTRODUCTION

It has been known that some organophosphorus compounds produce a specific neurotoxicity as well as anticholinesterase toxicity on animals. While a great number of experiments on that neurotoxicity have been studied over many years in Europe and America, only few literatures have been reviewed in Japan.
The organophosphorus compounds that have been said to be neurotoxic agents are listed in Table 1, and TOCP (tri-o-cresyl phosphate) has produced the greatest number of intoxications in human and animals.

TOCP is an organophosphorus ester with a m. w. of 368.36 and the structural formula is given in Fig. 1. Since this compound produced human neurological injury in America during the 1930s, many intoxications due to its polution have been reported in various countries; Holland, England, and Morocco. The acute oral LD50 of this compound is estimated to be 1.000 to 2.000 mg/kg in rats, and because of its physical characters, TOCP has a wide variety of use, particularly as a plasticizer, flame retardant, and solvent.

![Structural formula of TOCP](image)

**Fig. 1.** The structural formula of TOCP.

The studies on the neurotoxicity of TOCP have been carried out for over 60 years. Since Smith (1931) reviewed the toxicological properties of this compound, TOCP poisoning has been extensively reviewed by e.g. Aldridge (1961, 1969) and Baron (1962). However, the nature of this toxicity, especially the mechanism of action and primary site of action, has not yet been conclusively established.

Smith (1931) has reviewed the details of TOCP intoxication in hens, dogs, cats, cows, and primates. Aldridge (1969) has generalized the correlation between the neurotoxic compounds and symptoms of tested animals, and he has also reported TOCP intoxication in humans, hens, dogs, cats, cows, and non-human primates.

These two literatures have clearly shown that TOCP produces neurotoxicity among various kinds of animals, and that the hen is the most sensitive species to TOCP intoxication.

**Table 1.** The list of organophosphorus compounds which demonstrate the delayed neurotoxicity in the animals.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Tested animal</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>TOCP</td>
<td>Hen, Dog, Cat, Calf, Monkey, Quail</td>
<td>Smith (1931), Baron (1962), Aldridge (1961), Shirasu (1979)</td>
</tr>
<tr>
<td>DFP</td>
<td>Hen</td>
<td>Davis (1960, 1961)</td>
</tr>
<tr>
<td>Mipaflox</td>
<td>Hen, Rat</td>
<td>Majno (1961)</td>
</tr>
<tr>
<td>Leptophos</td>
<td>Hen</td>
<td>Abou-Donia (1974, 1976)</td>
</tr>
<tr>
<td>EPN</td>
<td>Hen</td>
<td>Abou-Donia (1979)</td>
</tr>
<tr>
<td>EPBP</td>
<td>Hen</td>
<td>Abou-Donia (1979)</td>
</tr>
</tbody>
</table>
Dilayed neurotoxicity of organophosphorus

The present study gives further confirmation of the relationship between the clinical signs of intoxication and the histopathology in hens and quails exposed to TOCP.

MATERIALS AND METHODS

White leghorn hens (more than 20 months of age) averaging 1.8 kg in weight and laying quails (age of them unknown) averaging 160 g in weight were used throughout the experiment. Tri-o-cresyl phosphate (TOCP) was diluted with polyethylene glycol-400 and administered in a single oral dose of 400 mg/kg in active ingredient (1.0 ml/kg of preparation). Three hens were sacrificed at 0, 5, 10, 15, and 20 days, and 5 hens were sacrificed at 25 days after administration. The control group consisted of 5 hens treated with polyethylene glycol-400, and was sacrificed after 25 days. Each hen was individually housed in a cage and supplied with food and water ad libitum. The general appearances and onset and severity of neurological signs of intoxication were daily observed.

The quails composed of 25 treated and 5 control animals were examined under observation during 50 days. The dosage and the housing conditions were the same as described as those of hens.

At the respective dates of autopsy, the animals were dissected and the liver, kidneys, heart, spleen, lung, digestive tracts, cerebrum, cerebellum, spinal cords, and sciatic nerve were macroscopically examined.

The histopathological preparations were standardized in all animals. With the animals, the brain, spinal cords, and sciatic nerve were excised immediately and placed in the following fixatives;

a) in 10% buffered formalin for histologic preparations and light microscopic studies.

b) for 2 hours in 2% glutaraldehyde solution, kept at 4°C, for electron microscopic studies.

As to light microscopy, the tissues were stained with hematoxylin and eosin (H & E), Klüver-Barrera, Bodian, Luxol fast blue (LFB) combined with PAS, Nauta, Masson and PTAH. For electron microscopy, tissues were post-fixed in 2% OsO₄ solution for 2 hours, and were embedded in Epon 812, and ultrathin sections were doubly stained with uranyl acetate and lead nitrate and observed by a Hitachi HS-9 Electron Microscope.

RESULTS

1) Clinical Signs and Mortality

After 5 to 24 hours of TOCP administration at the dosage of 400 mg/kg body weight, slight grade of salivation, ataxia, and poor feeding considered to be acute cholinergic toxic signs were observed in several of the hens and quails. These symptoms, however, improved with time and disappeared within two days. Thereafter, no neurological signs were observed in the quails during the observation periods of 50 days. As to the hens, no abnormal symptoms were observed from two to around 10 days after
administration. The initial neurological manifestations appeared around 12 days after the exposure to TOCP, were slight unsteadiness of gait and swaying while walking. Several days after the onset of symptoms, the hens were able to walk only a few steps before losing their balance. After that, they developed weakness in the legs, especially in the extensor muscles, and sometimes they fell down on their side sprawling out their legs in front. The results are given in Fig. 2 and Table 2.

Fig. 2. The clinical signs of the hen, 13 days after exposure of TOCP. The animal was incapable of standing and sat with both limbs stretched to one side. The weakness of extensor of the fingers was seen.

Table 2. The incidence of neurological abnormalities in the hens and quails exposed to TOCP.

<table>
<thead>
<tr>
<th>Species</th>
<th>Day after exposure</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>Hen</td>
<td>0*/20**</td>
</tr>
<tr>
<td>Quail</td>
<td>0/25</td>
</tr>
</tbody>
</table>

*: No. of animals exhibited neurological abnormalities
**: No. of animals examined

Food consumption appeared to be normal from two to around 20 days of experiment. Around 20 days after administration, the hens gradually lost appetite associated with the neurological symptoms, and finally, they could not consume their food because they had their difficulty in rising to a standing position. On the other hand, the hens laid immature-soft eggs from around 20 days to the end of the experiment, and diarrhea with greenish colour was observed in most of the animals from around 15 days of the experiment.
Dilayed neurotoxicity of organophosphorus

No mortalities were recorded in this experiment during the observation period of 25 days.

2) Pathology

Gross examinations of all hens and quails revealed essentially unremarkable changes in the brain, spinal cords, sciatic nerve, kidneys, heart, spleen, lung, and digestive tracts. Minimal degree of congestion was observed occasionally in the liver of hens.

3) Light Microscopy

Major histopathological alterations of the hens were observed in the spinal cords including pia matter and dorsal root ganglion and sciatic nerve. The results are summarized in Table 3.

Table 3. The morphological alterations in the hens exposed to TOCP.

<table>
<thead>
<tr>
<th>Day after dosing</th>
<th>No. of hens examined</th>
<th>Cerebrum/Cerebellum</th>
<th>Cervical</th>
<th>Spinal cord Thoracic</th>
<th>Lumber</th>
<th>Sciatic nerve</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>3</td>
<td>PVC±</td>
<td>PVC±</td>
<td>PVC±</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>3</td>
<td>PVC±</td>
<td>MD(a,1)+</td>
<td>AD(a,1)+</td>
<td>PVC±</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>MD(a,1)±</td>
<td>AD(a,1)±</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>3</td>
<td>PVC±</td>
<td>MD(a,1)+</td>
<td>AD(a,1)+</td>
<td>PVC±</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>MD(a,1)±</td>
<td>AD(a,1)±</td>
<td></td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>3</td>
<td>PVC±</td>
<td>MD(a,1)+</td>
<td>AD(a,1)+</td>
<td>MD+</td>
<td>ME+</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>MD(a,1)±</td>
<td>AD(a,1)±</td>
<td>AD+</td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>3</td>
<td>MD(a,1)+</td>
<td>MD(a,1)+</td>
<td>MD(a,1)+</td>
<td>MD+</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>AD(a,1)+</td>
<td>AD(a,1)+</td>
<td>AD+</td>
<td></td>
</tr>
<tr>
<td>25</td>
<td>5</td>
<td>MD(a,1,p)+</td>
<td>MD(a,1,p)+</td>
<td>MD(a,1,++)</td>
<td>ME+</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>AD(a,1,p)+</td>
<td>AD(a,1,++)</td>
<td>AD+</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>G(1)+</td>
<td>G(1)+</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

ABBREVIATIONS

PVC: Perivascular cuffing
AD: Axonal degeneration
a: anterior funiculus
p: posterior funiculus
±: minimal
++: moderate
MD: Myelin degeneration
G: Glial reaction
l: lateral funiculus
+ : mild
+++: severe

As shown in Table 3, perivascular cuffing of small round cells (PVC) was demonstrated in the cortex and medulla of the cerebrum and cerebellum after 5 days of TOCP administration. Minimal change of degenerative myelin and axons was observed in the anterior funiculus of the cervical spinal cord. After 10 days, PVC was also dominant in the cortex and medulla of the cerebrum and cerebellum as well as in the whole length of the spinal cords (Fig. 3), associated with minimal degenerative changes involving swelling and occasional degeneration of axons and degeneration and demyelination of myelin. After 15 days, PVC was also observed in the cortex and medulla of the cerebrum and cerebellum as well as in spinal cords, and moderate changes of myelin and axons were observed in the whole spinal cords and sciatic nerve (Figs. 4, 5, 6).
Fig. 3. Lumber spinal cord from hen, 10 days after exposure to TOCP. Perivascular cuffing of small round cells in the gray matter. H. E., ×200.

Fig. 4. Upper thoracic spinal cord from hen, 15 days after exposure to TOCP. Moderate demyelination is seen in the anterior and lateral funiculus. K. & B. ×2.

At twenty days of the experiment, marked changes of myelin and axons were seen in the marginal area of anterior and lateral funiculus of the whole spinal cords as well as in the sciatic nerve (Fig. 7). At the terminal sacrifice of 25 days, the above changes became severe, and extended from the margin to deeper areas of the white matter of the spinal cords. Furthermore, the following changes were observed in some animals;
Fig. 5. Lower thoracic spinal cord from hen, 15 days after exposure to TOCP. Moderate demyelination is observed in the anterior and lateral funiculus. K. & B. ×2.

Fig. 6. Cervical spinal cord from hen, 15 days after exposure to TOCP. Degeneration of myelin and axons is seen in the marginal zone of lateral funiculus. H. E., ×200.

change in the posterior funiculus of the spinal cord; glial proliferation in the marginal lateral funiculus of the cervical and thoracic spinal cord; change in the white matter of the cerebellum. These alterations in the spinal cord are diagrammed in Fig. 8.
Fig. 7. Sciatic nerve from hen, 20 days after exposure to TOCP. There is degeneration of myelin and axons. H.E. ×200.

Fig. 8. Diagram of the degeneration of myelin and axons in the spinal cord of hens exposed to TOCP.
Dilayed neurotoxicity of organophosphorus

Fig. 9. The anterior column of thoracic spinal cord from hen, 15 days after exposure to TOCP.
The cross-section of a myelinated nerve fiber showing moderate myelin degeneration. It was marked by a striking structural anomaly with regard to the regular spacing of the myelin. Small alveolated membrane compartments are seen.

Fig. 10. The anterior column of thoracic spinal cord from hen, 15 days after exposure to TOCP.
The cross-section of a myelinated nerve fiber exhibiting moderate proliferation of smooth endoplasmic reticulum. There are many vesicular and tubular elements with some membrane-limited cavities.

— 295 —
As to the quails, because no neurotoxic signs and no macroscopical findings were observed, histopathological examinations were not performed.

4) Electron Microscopy

Electron microscopic examinations were carried out on hens sacrificed at 15 days after TOCP administration.

In the anterior funiculus of the thoracic spinal cord, myelinated nerve fibers exhibited a moderate degree of myelin and axons degeneration. Myelin was observed to be a striking structural anomaly with regard to the regular spacing, associated with many small alveolated membrane compartments (Fig. 9). In the axons, a moderate degree of proliferation of smooth endoplasmic reticulum as well as an increase of vesicular and tubular elements were found. Some membrane limited cavities were distributed among them (Fig. 10).

DISCUSSION

It is well known that the toxicological properties of organophosphorus compounds have an anticholinesterase action. The nature of this mechanism was thought that the organophosphorus compound inhibited acetylcholinesterase, a hydrolyzing enzyme of acetylcholine, and the resulting accumulation of acetylcholine gave rise to the cholinergic symptoms.

Generally, cholinergic poisonings could be acute and are ordinarily seen within 24 hours after exposure to the organophosphorus compound, followed by a fairly rapid and complete recovery. This recovery might be attributable to the facts that the compounds are rapidly decomposed and excreted from the animals.

On the other hand, during the 1930s, it had been introduced that some organophosphorus compounds produced irreversible specific neuropathy. Since intoxications were found in America, many experiments were carried out on TOCP to investigate this specific neuropathy. According to Smith (1930), who reviewed the American human cases with neuropathy, they were said to be related to TOCP polution, based on the results of many investigations on several test animals.

Afterwards, various experiments on Mipafos (Majno, 1962), TOCP (Baron, 1962, Aldridge, 1969), DFP (Davis, 1972), leptophos (Abou-Donia, 1974, 1976), EPB (Abou-Donia, 1979), and EPN (Abou-Donia, 1979) were carried out, and it became known that many organophosphorus compounds should be considered to have potentially specific neuropathy. As there was a latent period of about 8 to 12 days after exposure to organophosphorus compound before neural signs were manifested, the neuropathy was considered as a delayed neurotoxicity. Many experiments were carried out, to investigate the correlation between neuropathy and the compounds, especially their chemical structure. There was, however, no simple relation among them, and the fundamental questions on the nature of the neuropathy had not been answered.

The authors in this report confirmed the initiated nature of the delayed neuro-

— 296 —
Dilayed neurotoxicity of organophosphorus

toxicity in the animals based on the results of the experiments to determine the relationship between the clinical signs of neuropathy and morphology with the course of time.

Shirasu (1979) examined TOCP in quails and confirmed that cases of neuropathy were observed after 12 to 23 days under repeated administration of 4 to 8 ml/kg. On the other hand, single exposure of TOCP at 400 mg/kg, in our experiment, produced no signs of delayed neurotoxicity during the period of 50 days in quails, while acute cholinergic symptoms were found at an earlier period of the experiment. It was considered that the difference of these results could be attributable to the difference of the dose of the compound and the methods of administration. Nevertheless, it was noted that quails should have a rather lower sensitivity than hens to TOCP exposure.

The onset of neuropathy in hens was demonstrated after around 12 days of single TOCP administration at the dosage of 400 mg/kg. This result resembled the observation of Smith (1931) and Baron (1962) for 8 days after 0.4 ml/kg and 11 days after 1.0 ml/kg of a single dose of TOCP, respectively.

From the results of autopsy of hens, congestion was noted in the liver of some animals. This change was considered to be circulatory insufficiency and to be attributable to the secondary effects of the compound.

From the results of histopathological examinations, one predominant change was perivascular cuffing of small round cells observed at an earlier period of the experiment. This change was seen around 5 to 10 days in the cerebrum and cerebellum as well as around 15 days in the spinal cords after administration of TOCP. As it was observed before the onset of clinical signs of neuropathy, this change was considered to be a reactive lesion attributable to the primary stimulation of the compound. On the other hand, the distribution of PVC appeared to be transferred from higher to lower parts of the nervous system, related with the course of time. It was considered that such transference of the location of PVC could be related to the migratory location of the stimulation, and also related to the location of lesions in myelin and axons in the spinal cords. However, this change has not been reported in the previous studies by other authors, and we also could not determine the exact nature of this change.

The alterations of myelin and axons, that were composed of degeneration of myelin, demyelination, degeneration of axons, and loss of axons were distributed in the anterior and lateral funiculus of the spinal cords. These results were supported by Smith (1931), Barron (1962) and others. Minimal lesions were observed at 5 to 10 days after administration of the compound when the clinical neurotoxic signs had not been recorded, and 15 to 20 days after TOCP exposure, they appeared to have progress towards marked changes. In the hens sacrificed at an earlier period of the experiment, the changes of myelin and axons were located only in the anterior funiculus of cervical spinal cord. However, they were found to spread into the anterior and lateral funiculus of the cervical and thoracic spinal cords with the course of time. At 15 to 20 days, these changes were predominant in the lumbar spinal cord and sciatic nerves. At the termi-
nal examination, the changes were seen to have spread further into deeper areas of the anterior and lateral funiculus as well as, in rare case, to the posterior funiculus.

On the other hand, glial reaction was observed in the lateral funiculus from hens of terminal sacrifice. This change was already reviewed by Baron (1962), who investigated the delayed neurotoxicity on hens with a metabolite of TOCP. Further, in our experiments of terminal examinations, lesions of degeneration in myelin and axons were observed in the white matter of the cerebellum of one hen. It was not clearly determined whether those lesions were related to the effects of the compound or not.

As we mentioned above, myelin and axonal lesions were seen to have expanded from the upper to the whole spinal cords, and to have gradually progressed toward marked injury. However, it could not be clearly confirmed in the former literatures whether those changes should be systemic neuropathy. In our opinion, because marked changes were found in the lower compared with upper levels of the spinal cord, it was considered that the neuropathy should be said to be systemic. It was concluded that these neurotoxic symptoms should be said to be neurogenic, because the morphological changes were found even in the hens, which were sacrificed before onset of neuropathy.

No specific changes were seen in the neurons of the cerebrum, cerebellum, and spinal cords in our experiments. Smith (1931) reviewed that chromatolysis and fatty degeneration were observed in the neurons of anterior horn of the spinal cord from most cases of human, dog, monkey, and cat. Further, the same changes as above mentioned were also demonstrated in a few hens. However, the other authors did not describe such changes, and it was considered that changes of neurons were not specific in the experiments with hens.

Only few literatures have described the electron microscopic findings in the delayed neurotoxicity tests. According to Bischoff (1970), who reviewed the results of electron microscopy on hens treated with TOCP, proliferation of smooth endoplasmic reticulum and increased numbers of vesicular and tubular elements as well as increased numbers of mitochondria were said to be observed in the axons of white matter of the spinal cord. In another experiment on the sciatic nerve (Bischoff, 1967), degenerative changes were demonstrated in the myelin. While his examinations were carried out on hens of 10 days after TOCP exposure, it should be noted that the changes of axons of spinal cord were predominant rather than those of myelin in such an early case of hen.

In our experiments with electron microscopy of the anterior funiculus of thoracic spinal cord from hens sacrificed 15 days after TOCP exposure, the axons revealed proliferation of smooth endoplasmic reticulum, increased numbers of vesicular and tubular elements, and some membrane limited cavities. In the same cases, moderate degeneration of myelin associated with abnormal striking structure from regular spacing of myelin were observed. These results resembled the observation of Bischoff. However, it was not concluded in our experiments whether these changes initiated in the axons or myelin.
SUMMARY

The delayed neurotoxicity experiments were carried out on hens and quails using an organophosphorus compound, TOCP, and the neurological toxic symptoms and histopathological changes were examined in the course of time.
1) With a single oral administration of TOCP at a dose of 400 mg/kg, hens developed the neurotoxic signs after 12 days of administration, while no specific changes were observed in quails.
2) From the results of histopathological examinations in hens, the initial sign of intoxication was perivascular cuffing observed in the cerebrum, cerebellum, spinal cords, and sciatic nerve. This change seemed to shift in its location with the lapse of time, and disappeared at the later period of the experiments. The major lesions were degeneration of myelin and axons found in the white matter of the spinal cords. Minimal degree of these lesions, located in the cervical spinal cord, was observed at an early stage of the experiment, when the neurotoxic signs were not clinically observed. They were considered to be progressive and spread from the upper to the entire spinal cords as well as sciatic nerve.
3) The electron microscopy of anterior funiculus of the thoracic spinal cord revealed marked degeneration of myelin and axons 15 days after administration of TOCP.

REFERENCES

HIROTOSHI ITOH et al.