*J Toxicol Pathol 2004; **17**: 85–94*

Review

Ultrastructural Morphology of Motor Endplate Neurotoxicities in Rats

Kunitoshi Mitsumori[1], Mitsuyoshi Moto[1], and Takayoshi Imazawa[2]

[1]Laboratory of Veterinary Pathology, Division of Animal Science, Institute of Symbiotic Science and Technology, Tokyo University of Agriculture and Technology, 3–5–8 Saiwai-cho, Fuchu-shi, Tokyo 183–8509, Japan
[2]Division of Pathology, National Institute of Health Sciences, 1–18–1 Setagaya-ku, Tokyo 158–8501, Japan

Abstract: Neurotoxic signs suggested by abnormalities of the posterior extremities are sometimes observed in rats and mice given high doses of certain test substances in acute toxicity studies, but abnormal morphological changes are not always detected in the nervous system and skeletal muscles of these animals during routine histopathological examinations. In such cases, toxicities are attributable to the onset of motor endplate neuropathy. In this article, we focus on the ultrastructural changes in neuromuscular junctions induced by several neurotoxic substances, based on the results of a search in the literature and data obtained in our laboratory. The literature survey indicated that nerve endings of the peripheral motor nerves are initially damaged in the neurotoxicity of venoms of snakes such as β-bungarotoxin, notexin and taipoxin, as well as chemically synthesized substances such as DTB, sarin, Vacor and DTBHQ, and various kinds of morphological changes as a result of the destruction of motor endplates could be observed in neuromuscular junctions of the animals exposed to these toxic substances. When paralysis of posterior extremities occurs in rats treated with certain test substances, a part of the lumbrical muscles in the foot pad should be fixed and subjected to electron microscopic examinations to eliminate misjudgments concerning neurotoxicity. (J Toxicol Pathol 2004; **17**: 85–94)

Key words: motor endplate, neuropathy, neurotoxicity, peripheral nerves, ultrastructure

Introduction

Single dose administration studies are usually performed in the early phase of the development of pharmaceuticals, pesticides and industrial chemicals to characterize their toxicities. Neurotoxic signs such as abnormal gait and/or paralysis of posterior extremities are sometimes observed in rats and mice given high doses of test chemicals in these studies. However, abnormal morphological changes are not always detected in the nervous system and skeletal muscles of these animals during routine histopathological examinations of sections stained with hematoxylin and eosin. In addition, the absence of morphological changes in the peripheral nervous system (PNS) is also encountered even in animals showing abnormal gait which have been subjected to short-term repeated dose toxicity studies using certain chemicals. These findings are probably attributable to the fact that it is very difficult to detect subcellular changes in the peripheral nerve-endings such as motor-endplates with light microscopic examinations. Neuromuscular junctions are the specific anatomical sites where toxic changes in "distal axonopathy" and "motor endplate neuropathy" are first observed after the treatment with certain neurotoxic chemicals, but sometimes such neurotoxic changes are missed in routine light microscopic observations. In these cases, it may be misjudged that only dysfunction of the nervous system is induced in these animals, by the treatment with these chemicals, in the absence of evidences of altered cellular structures in neuromuscular junctions. Such a conclusion would result in a false evaluation of the neurotoxicity of newly developed chemicals.

In this article, we classify the neurotoxic substances inducing peripheral neurotoxicities accompanied by morphological damages in the neuromuscular junctions and skeletal muscles, and focus on the ultrastructural changes in neuromuscular junctions induced by several neurotoxic substances. In addition, diagnostic problems of these neurotoxicities are examined and some improvements to procedures to minimize the overlooking of neurotoxic changes in routine light microscopic observations are discussed.

Received: 1 April 2004, Accepted: 12 May 2004
Mailing address: Kunitoshi Mitsumori, Laboratory of Veterinary Pathology, Division of Animal Science, Institute of Symbiotic Science and Technology, Tokyo University of Agriculture and Technology, 3–5–8 Saiwai-cho, Fuchu-shi, Tokyo 183–8509, Japan
TEL & FAX: 81-42-367-5771
E-mail: mitsumor@cc.tuat.ac.jp

Types of Peripheral Neurotoxicities Causing Muscular Atrophy

Peripheral neurotoxicities causing muscular atrophy are classified into three different neuropathies: 1) nerve fiber degeneration secondary to neuronal necrosis, 2) distal axonopathy, and 3) motor endplate neuropathy (Fig. 1). Morphological features of these neuropathies are as described below:

Neuronal death or loss of neuronal cells induced by treatment with neurotoxic chemicals results in degeneration of all of the neuronal cytoplasmic extensions, dendrites and axons, and of the myelin ensheathing the axon. This nerve fiber degeneration secondary to neuronal necrosis is generally identical to that of Wallerian degeneration occurring after nerve crushing. Various stages of degenerative changes consisting of fragmented axons and separation into a discontinuous series of myelin ovoids with macrophage invasion are seen in these nerve fibers. Finally, such nerve fiber degenerations lead to neurogenic muscular atrophy (Fig. 2).

Distal axonopathy is the most frequent pattern of neuronal dysfunction in peripheral neuropathies, and was previously called "dying-back neuropathy", since it was considered in classical neuropathological observations that the distal regions of long nerves underwent degeneration before more proximal regions (Fig. 1). However, detailed studies of the spatial and temporal evolution of nerve fiber degeneration in small animal models of toxic neuropathies have demonstrated that the degenerative process does not progress in a smooth fashion from nerve terminal back toward cell body, but appears to present a sequence of multifocal early pathological changes in the distal axons followed by episodes of Wallerian-like degeneration of a portion of the distal axon[1]. Subsequently the term, "distal axonopathy", has been used instead of "dying-back neuropathy". The early stage of this neuropathy is characterized by multifocal accumulation of neurofilaments in the distal portion of axons and motor nerve terminals, and this neurofilamentous swelling leads to destruction of axoplasm and myelin sheaths. Finally, such nerve fiber degenerations result in neurogenic muscular atrophy.

Motor endplate neuropathy is a degenerative disease in the axon terminals of neuromuscular junctions. This neuropathy is characterized by decreased synaptic vesicles or increased tubular endoplasmic reticulum in the axon terminals (Fig. 3), and can be easily induced in experimental animals by a single intraperitoneal injection of 2,4-dithiobiuret (DTB)[2,3]. It is very difficult to identify the toxic lesions of the neuromuscular junctions and skeletal muscles in light microscopy of animals subjected to a single i.p. injection of DTB, however, when animals receive multiple doses of this chemical, destruction of motor endplates is induced, resulting in severe atrophy of the skeletal muscle (Fig. 3). Therefore, we must be very careful about the onset of this type of neurotoxicity at the early phase of toxication.

As described above, neurogenic muscular atrophy of the skeletal muscles is induced as a result of severe damage by neuropathies. However, there are some cases in which muscular atrophy is seldom seen in the early stage of motor endplate neuropathies, because of the difficulty of detecting toxic changes of neuromuscular junctions in light microscopic examinations due to the limitations of light microscopy. In this respect, we must be very careful about

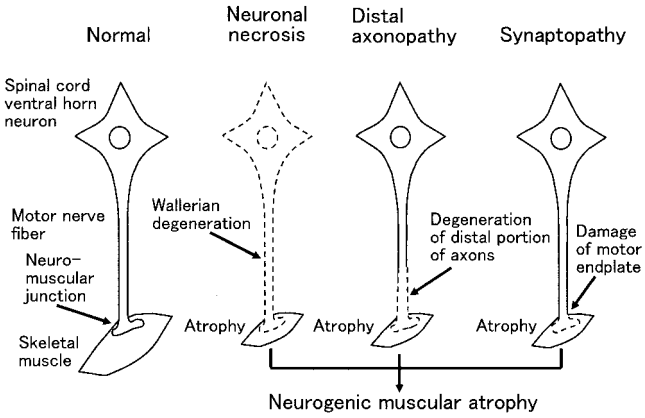

Fig. 1. Types of neurotoxicities in the peripheral nervous system.

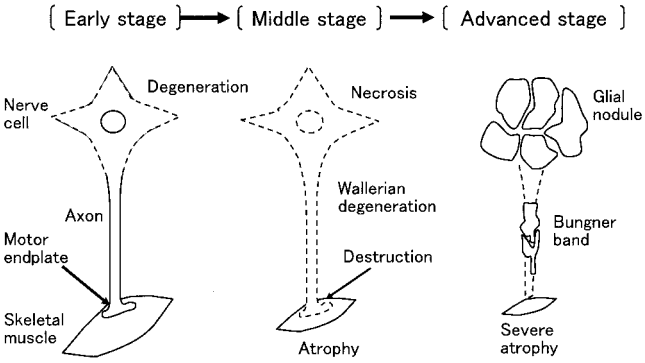

Fig. 2. Time-course changes in peripheral nerves and skeletal muscles of drug-induced neuronal necrosis in motor nerves.

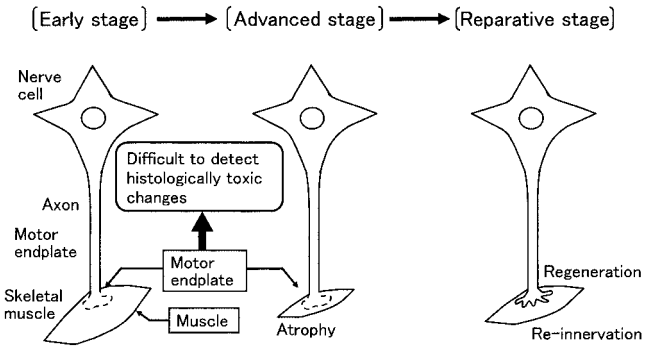

Fig. 3. Time-course changes in peripheral nerves and skeletal muscles of drug-induced motor endplate neuropathy.

the onset of motor endplate neuropathies in routine histopathological examinations.

Neurotoxic Substances Inducing Neurological Signs with Morphological Damage to Motor Endplates

Neurotoxic substances causing motor endplate neurotoxicities in mammals are subdivided into natural toxins originating from bacterial toxins and venoms of snakes, and chemically synthesized substances. As venoms of snakes, β-bungarotoxin, notexin and taipoxin are known to cause damage to motor endplates. Chemically synthesized substances causing motor endplate neuropathies include DTB, sarin (o-isopropyl methylphosphonofluoridate), Vacor (N-3-pyridylmethyl-N'-p-nitrophenyl urea), 2,5-di(tert-butyl)-1,4-hydroquinone (DTBHQ). The neurotoxic and morphological features of these substances are summarized below.

Venoms of snakes

The venoms of snakes are rich sources of phospholipases A_2. Many of the phospholipases appear to have a primary digestive function, but some of them are potent presynaptically active neurotoxins. The most important toxic phospholipases of snake venoms are the neurotoxic/myotoxic phospholipases of elapid snakes. These toxins are the dominant toxic fractions of the venoms of a number of snakes, including the kraits of Southeast Asia and the taipans of Australia, Papua-New Guinea and Irian Jaya[4,5].

It is considered that the most likely toxic components causing long-lasting paralysis are the neurotoxic phospholipases A_2. As a venoms containing phospholipases A_2, β-bungarotoxin, notexin and taipoxin are known as the principal neurotoxins in the venom of kraits, Australian tiger snakes and taipans, respectively. Acute investigation in vivo had suggested that the primary cause of death, following the inoculation of the neurotoxic phospholipases A_2, was either a blockade of transmitter (acetylcholine) release or the depletion of transmitter from motor nerve terminals[6]. In a more recent study of the neuropathological consequences of exposure to toxic phospholipases, Dixon and Harris[7] inoculated the hind limb of rats with sublethal doses of β-bungarotoxin, and showed that the toxin caused the depletion of transmitter from the nerve terminals and the degeneration of nerve terminal and intramascular axons.

2,4-Dithiobiuret (DTB)

DTB is a thiourea derivative, which has been used commercially as a plant root growth promoter, and has been known to produce a quick, reversible flaccid paralysis resulting from accumulation of synaptic vesicles and smooth endoplasmic reticulum (SER) in the neuromuscular junctions of rats after repeated administration[2,3]. DTB was given intraperitoneally to Sprague-Dawley rats twice over 2 to 3 days. Ataxic gait of hind limbs and paralysis of the posterior extremity occurred in these animals immediately after the second administration. In light microscopic examinations, there were no marked changes in the lumbrical muscles of these treated rats. In electron microscopic examinations, most motor endplates were distended by accumulations of dense-cored synaptic vesicles, abnormally swollen mitochondria, intermediate filaments and branching, tubular SER. In the animals that showed prolonged paralysis, motor endplates were destroyed, atrophy/degeneration of muscle fibers characterized by disarrangement of actin and myosin filaments as well as streaming of Z-bands being observed in the lumbrical muscles.

Sarin (O-isopropyl methylphosphonofluoridate)

Sarin is a highly toxic acetylcholinesterase (AChE) inhibitor, and administration at near LD50 dose causes severe signs of toxic cholinergic hyperactivity in both the peripheral and central nervous systems. A single subcutaneous injection of a sublethal dose of sarin (0.08 mg/kg) in rats induced a non-Wallerian-type axonal degeneration of the neuromuscular synapse in the slow twitch, soleus muscle[8]. The degeneration of the neuromuscular synapse was characterized by bead or balloon-like varicosities of the distal and terminal nerve fibers and a retraction of terminal axons. As a possible mechanism of the damage to the neuromuscular synapse, it has been suggested that sarin inactivates the enzyme AChE which is responsible for the breakdown of the neurotransmitter acetylcholine (ACh), leading to its accumulation at ACh receptors and overstimulation of the cholinergic system[9].

Vacor (N-3-pyridylmethyl-N'-p-nitrophenyl urea)

Vacor is a rodenticide that antagonizes nicotinamide metabolism, and has been known to cause a severe peripheral neuropathy in humans. Ahn *et al.*[10] reported that neuromuscular junctions within the interosseous muscles of the hind foot were observed in time after oral administration of a single dose of Vacor (80 mg/kg of body weight). Remarkable loss of presynaptic vesicles and swollen endoplasmic reticulum in the axon terminal developed at 3 days after Vacor treatment. Progressive degenerative changes consisting of marked loss of presynaptic vesicles, focal disruption of membrane in the axon terminal with disappearance of the number of the damaged axon terminal appeared, and flattening of postsynaptic folds was also seen. These results strongly suggest that degenerative changes in axon terminals at neuromuscular junctions contribute to the peripheral neuropathy developed in the early phase of Vacor poisoning.

2,5-Di(tert-butyl)-1,4-hydroquinone (DTBHQ)

DTBHQ is one of the hydroquinone antioxidants that have been extensively used in the rubber and plastic industries. It has been suggested that DTBHQ elevates cytosolic Ca^{2+} levels via emptying the endoplasmic

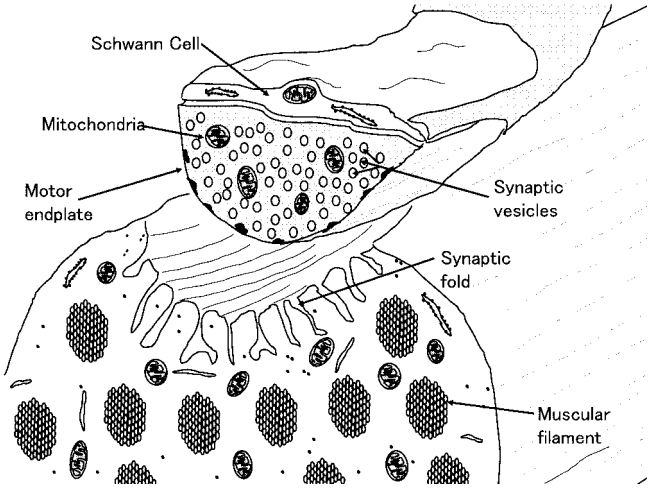

Fig. 4. Illustration of the stereoscopic structure of the neuromuscular junction.

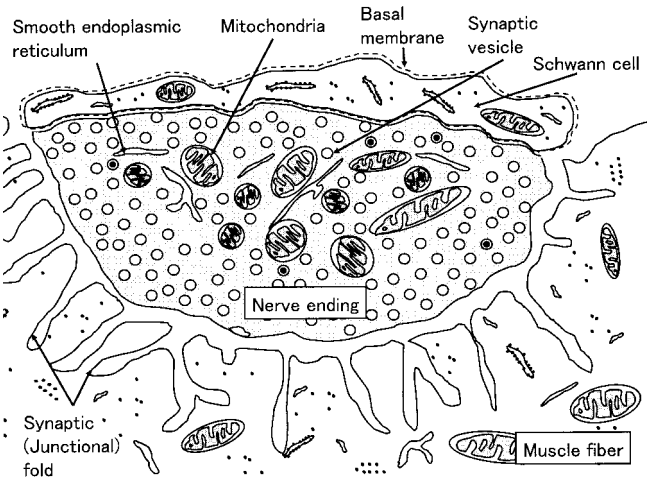

Fig. 5. Illustration of the two dimensional structure of the motor endplate.

reticulum pool and preventing Ca^{2+} reuptake, and also by increasing the Ca^{2+} influx from the extracellular medium by activating the Ca^{2+}-release-activated channel[11]. Since it has been reported that a single oral administration of DTBHQ caused ataxic gait of hind limbs of rats, DTBHQ was expected to exert toxic effects on the peripheral nervous system[12]. We examined motor endplates of the lumbrical muscles of Wistar female rats treated orally for 5 days with 80 mg/kg body weight of DTBHQ by light and electron microscopy[13]. There was a decrease in body weight in the treated rats from the first day after administration, and toxic signs such as adoption of a prone position, salivation, lacrimation, and an abnormal gait and/or muscle weakness (appeared after the third day). No remarkable macroscopic or light microscopic changes were noted in the lumbrical muscles of the treated rats sacrificed one day after the last DTBHQ treatment. Ultrastructurally, neurotoxicity was

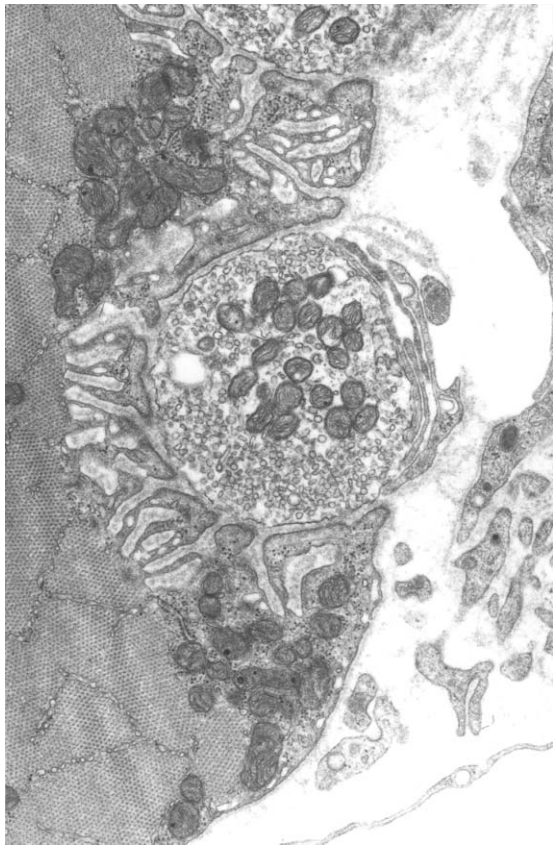

Fig. 6. Electron micrograph of a motor endplate from a control rat. The nerve ending is filled with round synaptic vesicles and mitochondria. × 16,800.

characterized by loss of synaptic vesicles and mitochondria in the motor endplates, and by destruction of the motor terminals. In our other time-course ultrastructural study on motor endplates of the lumbrical muscles of female Wistar rats given a single oral administration of DTBHQ at a dose of 120 mg/kg, neurotoxicity characterized by decreases or loss of synaptic vesicles and mitochondria was observed after 24 hours[14]. At the 1 week time point, nerve endings had disappeared in some of the motor endplates, while many neurite nerve endings suggestive of early stage regeneration were apparent. After 6 weeks, newly-formed re-innervated endplates were observed. The results strongly indicate that DTBHQ targets the motor endplates in the rat lumbrical muscles, and suggest that the resultant damage is responsible for the appearance of neurological signs, such as an abnormal gait and loss of muscle control.

Ultrastructural Morphology of Motor Endplate Neurotoxicity

As described above, neurotoxic changes are first observed in the axon terminal at the neuromuscular junctions only in transmission electron microscopic examinations, while it is difficult to detect such changes in light microscopic examinations. In this section, we summarize

the ultrastructural changes in the neuromuscular junctions that are induced by the above neurotoxic chemicals, and propose a scheme of the time course toxic changes in the neuromuscular junctions.

Normal structure of the neuromuscular junction

The neuromuscular junction is located at the end of the peripheral motor nerve that connects with skeletal muscle fibers, and is composed of the motor endplate, secondary junctional fold (synaptic fold) and skeletal muscle fibers (Figs. 4–6). The motor endplate is the expanded terminal of a motor axon in synaptic contact with a striated muscle fiber, together with the subneural apparatus of the muscle fibers. Many rounded synaptic vesicles, with a narrow range mean size, are observed packed close together within the normal motor endplates. Numerous elongated and round mitochondria are found interspersed among the packed synaptic vesicles. Synaptic folds are located between the motor endplate and skeletal muscles. Acetylcholine released from the synaptic vesicles is transmitted to skeletal muscle fibers via this synaptic fold.

Early toxic changes in the neuromuscular junction

There are two different morphological changes in the motor endplates as an early toxic change: 1) decreases of synaptic vesicles (Figs. 7 and 8) and 2) accumulation of branching smooth endoplasmic reticulum (Figs. 9 and 10). Decreases of synaptic vesicles in the motor endplates are induced by the treatment of venoms such as β-bungarotoxin[7,15], notexin and taipoxin[16], Vacor[10,17] and DTBHQ[13,14]. Accumulations of smooth endoplasmic reticulum and bead or balloon-like varicosities are observed in the animals toxicated with sarin[8] and DTB[2,3]. In this stage, no abnormal changes such as streaming of the Z-line and the degeneration of myofibrils are observed in the skeletal muscles of treated animals (Fig. 11).

Advanced toxic changes in the neuromuscular junction

Advanced change of the motor endplate is characterized by loss of synaptic vesicles and mitochondria in the synaptic terminals or destruction of motor endplates (Figs. 12 and 13). In this stage, some of the endplates appear mostly naked, because of a complete loss of nerve endings (Fig. 14). In these endplates, several phylopodia of Schwann cells as well as foot processes of macrophages and fibroblasts are seen to extend over the post-synaptic membranes (Fig. 15). Such a complete loss of motor endplates results in streaming of the Z-line, degeneration and atrophy of myofibrils (Fig. 16). Therefore, skeletal muscular degeneration and atrophy can be detected also in light microscopic observations.

Regenerative changes in the neuromuscular junction

After withdrawals of the neurotoxic substances, various sizes of multiple neurite growth cones surrounded by proliferated phylopodia are observed in damaged endplates where junctional folds are markedly distorted (Fig. 17). Some of the growth cones differentiate into newly-formed nerve endings that contain a few synaptic vesicles and mitochondria (Fig. 18). New ectopic endings, originating from the same endplate, are discovered adjacent to the terminal axon and also distant from the parent endplate. The

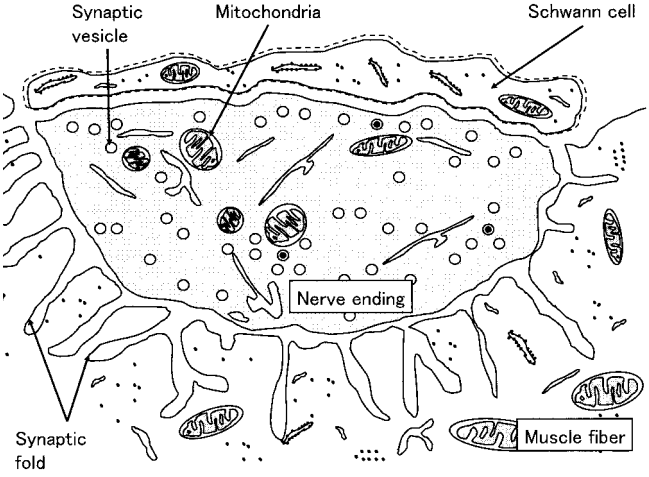

Fig. 7. Illustration of the two dimensional structure of the motor endplate. Early toxic change showing decrease of synaptic vesicles.

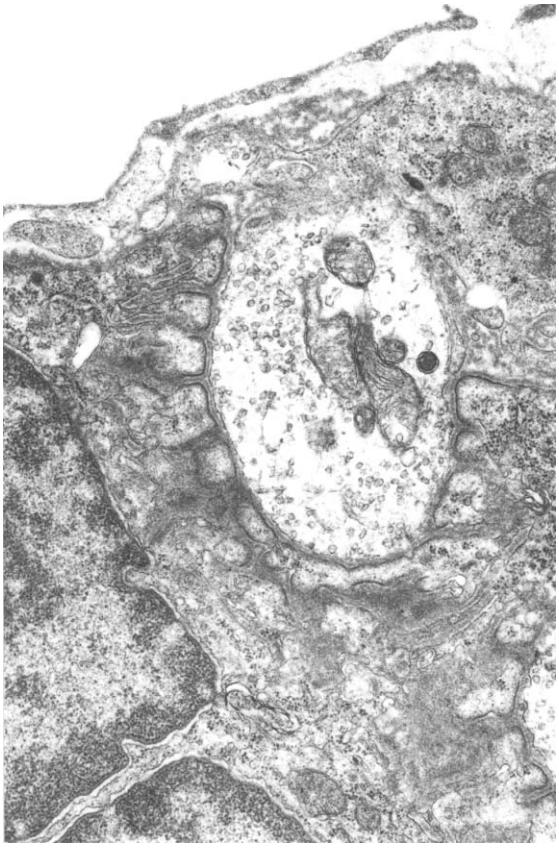

Fig. 8. Electron micrograph of the motor endplate in a rat given DTBHQ. Synaptic vesicles and mitochondria are strikingly decreased in the terminal axon. × 18,000.

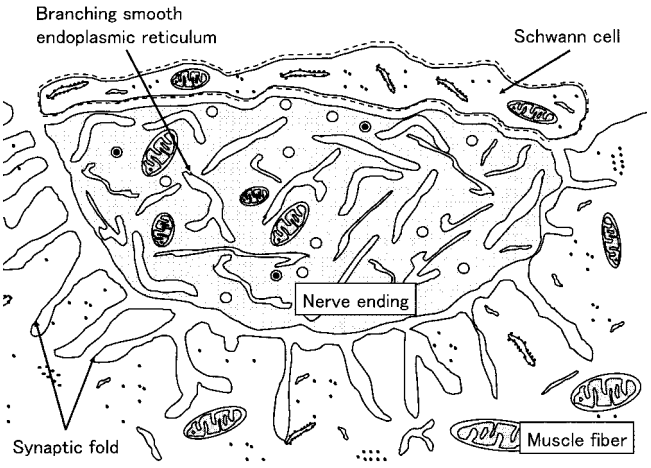

Fig. 9. Illustration of the two dimensional structure of the motor endplate. Early toxic change showing accumulation of branching smooth endoplasmic reticulum in the nerve ending.

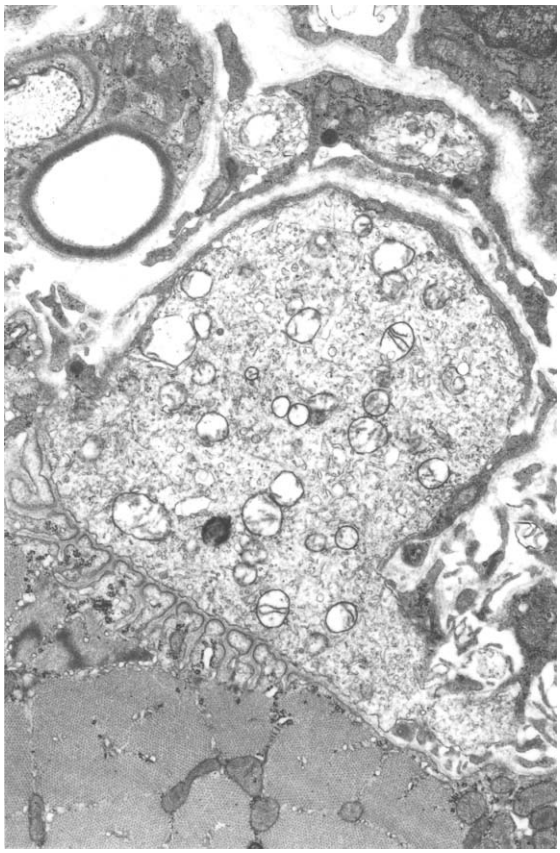

Fig. 10. Electron micrograph of the motor endplate in a rat given DTB, showing accumulation of branching smooth endoplasmic reticulum in the nerve ending. × 10,000.

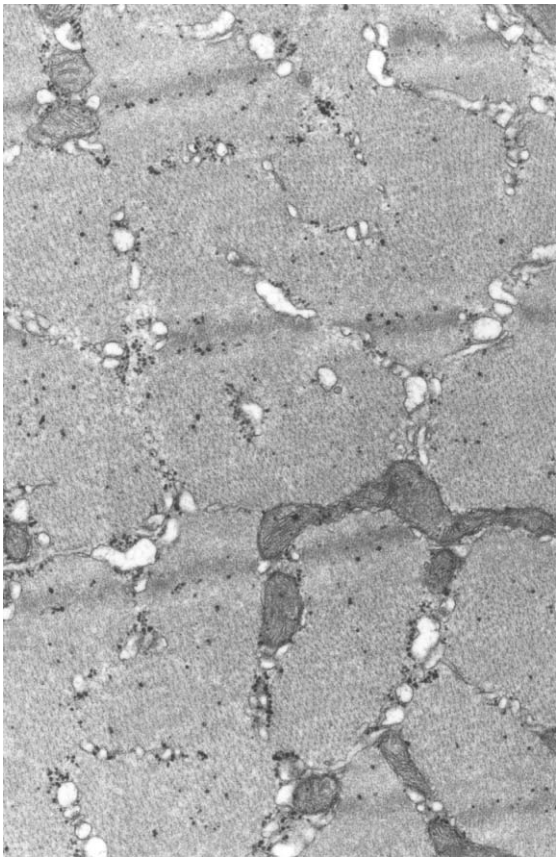

Fig. 11. Electron micrograph of the lumbrical muscle in a rat given a single intraperitoneal injection of DTB. No marked changes are observed, while accumulation of branching smooth endoplasmic reticulum is detected in the nerve ending. × 24,000.

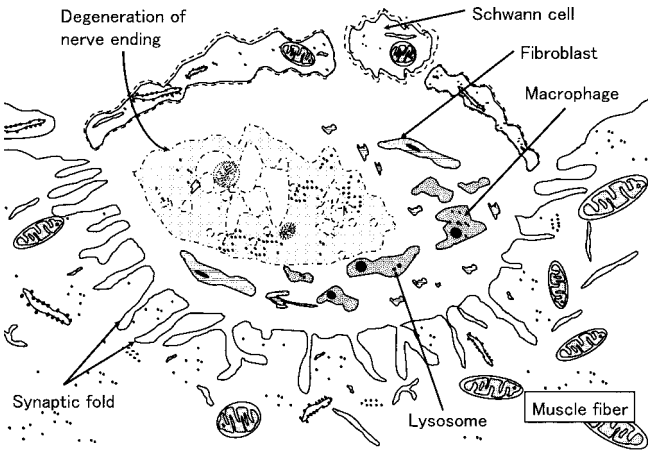

Fig. 12. Illustration of the two dimensional structure of the motor endplate. Advanced toxic change showing destruction of the motor endplate as well as loss of synaptic vesicles and mitochondria in the nerve ending.

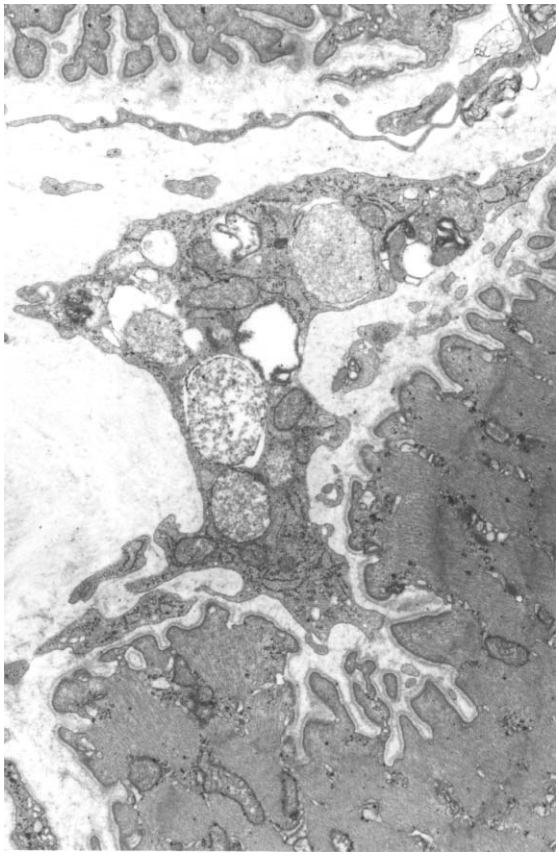

Fig. 13. Electron micrograph of the motor endplate in a rat given DTB. The synaptic vesicles and mitochondria have disappeared from the nerve endings, and have been replaced by amorphous proteinacious material. × 12,000.

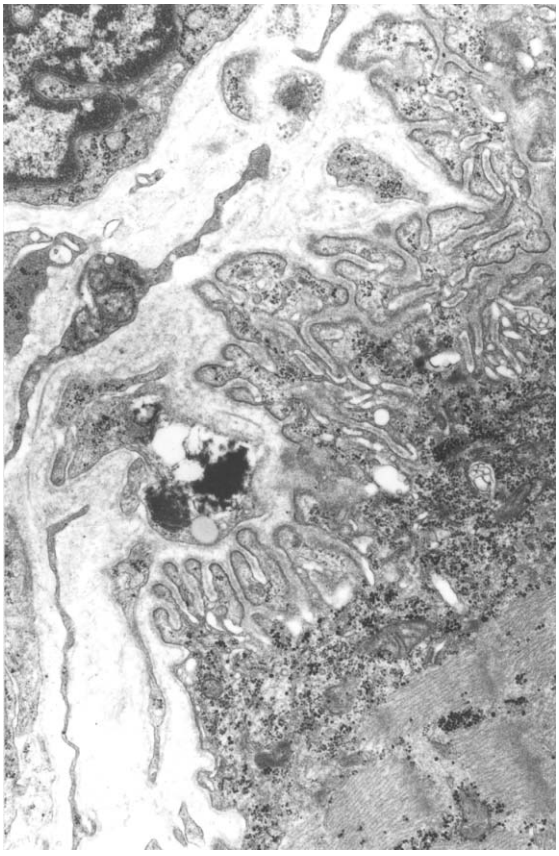

Fig. 14. Electron micrograph of the motor endplate in a rat given DTB, showing a completely denervated motor endplate. Some of the motor endplates appear mostly naked, because of a complete loss of nerve ending. × 15,000.

new sprouting may serve to compensate for the loss of synaptic contact caused by the toxins. With increasing time, very large nerve endings containing many synaptic vesicles and mitochondria with complexed junctional folds are formed in the denervated endplates (Fig. 19). Occasionally, some of the re-innervated motor endplates are indistinguishable from intact endplates, except for distorted and atrophic junctional folds.

As described above, various kinds of morphological changes can be observed in neuromuscular junctions of the animals exposed to toxic substances causing motor endplate neuropathies. Since these changes are different depending on the stage of neurotoxicities, it is important to recognize the stage of the neuropathies in the neuromuscular junction. When complete loss of motor endplates is detected in almost all the neuromuscular junction, we must consider that regeneration of nerve endings will not occur anymore. On the other hand, when various sizes of multiple neurite growth cones surrounded by proliferated phylopodia are seen in the nerve endings, the possibility that these affected nerve endings will be regenerated in the near future is probably very high.

Improved Methods to Minimize the Overlooking of Neurotoxic Changes in Routine Light Microscopic Observations

In the Organization for Economic Co-operation and Development (OECD) test guideline 424 on neurotoxicity tests in rats, it is noted that tissue selection for neuropathology studies is an important consideration[18]. In this guideline, the specific areas to be examined in the PNS include the dorsal root ganglia, dorsal and ventral roots, proximal sciatic nerve, proximal tibial nerve, tibial nerve calf muscle branches and calf muscle. There are no statements indicating that more distal portions of the PNS should be examined more carefully. However, it has been recommended in draft guidance documents[19] that, during initial neurotoxicology studies, tissue sampling should be broad so as to avoid missing an important target site in the nervous system. In this respect, in addition to the tissues identified in the specific guideline, it is important for the pathologist conducting the neuropathological examinations to be aware of any information that may alter the focus of the tissue selection and examination processes on the regions of the nervous system such as neuromuscular junctions that

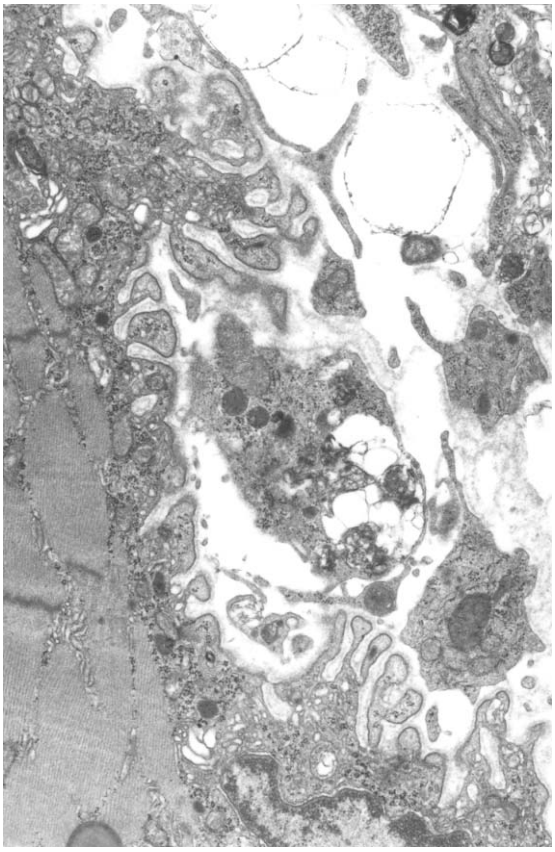

Fig. 15. Electron micrograph of the motor endplate in a rat given DTB, showing a completely denervated motor endplate. Nerve endings are not visible, and some foot processes of macrophages and fibroblasts extend over the post-synaptic membranes. × 10,000.

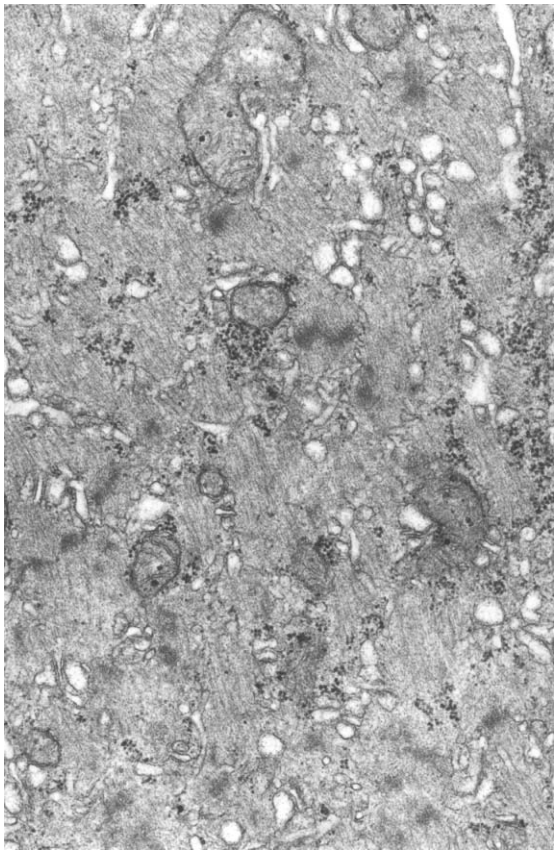

Fig. 16. Electron micrograph of the lumbrical muscle in a rat given DTB, showing streaming of the Z-line, degeneration and atrophy of myofibrils. × 24,000.

may be affected by chemical exposure. Therefore, when neurotoxic signs such as abnormal gait and/or paralysis of posterior extremities suggestive of damages of the neuromuscular junctions are observed, it is recommended that lumbrical muscles in the foot pad of hind limbs, which is collected but not used in the initial phases of the neuropathology examination, be saved in alcoholic or glutaraldehyde fixatives for future immunohistochemistry and electron microscopy.

Immunohistochemical methods have been used to better characterize the findings observed in routine histological examinations. Immunohistochemical methods using antibody to acetylcholinesterase can sometimes be used to demonstrate the presence of acetylcholinesterase that precedes observable tissue damage[7,16]. In addition, since synaptophysin and neurofilament are located in the motor endplates and their antibodies are commercially available, such immunohistochemical methods are recommended for the detection of early changes of the neurotoxicity[7,16]. However, since neuromuscular junctions are not always found in paraffin embedded sections, there is a possibility that no positive areas are detected in immunohistochemistry of synaptophysin and acetylcholinesterase. To eliminate such an oversight, the method of nerve-muscle preparations has been introduced for the immunohistochemistry of neuromuscular junctions[7,16].

Transmission electron microscopy is the best morphological examination for detecting motor endplate neuropathies. The techniques used in the preparation of ultrathin sections of these nerve tissues have been described by Spencer and Schaumburg[20] and others. Due to the greater resolution as compared to light microscopic examinations, electron microscopy can be used to identify the subcellular or organellar changes induced by a neurotoxic chemical[20–23]. Although electron microscopy is not a routine requirement for neurotoxicity studies, it is a powerful tool for characterizing the subcellular effects of a neurotoxicant in neuromuscular junctions. However, due to the extremely small tissue sample size used for electron microscopy, proper tissue selection is absolutely essential to ensure that changes which are observed by light microscopy are selected for ultrastructural examinations. In this respect, the confirmation of the presence of neuromuscular junction in semi-thin sections is very important for the conduct of electron microscopy on motor endplate neuropathies. In our experience, the neuromuscular junction can be easily detected in the Epon-embedded sections stained with

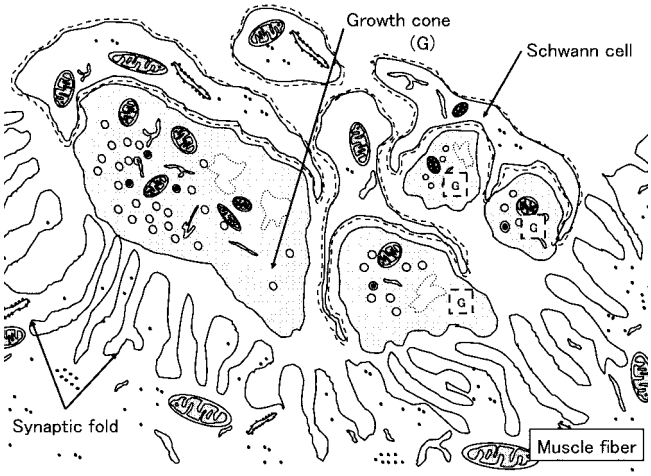

Fig. 17. Illustration of the two dimensional structure of the motor endplate showing regenerative changes. Various sizes of multiple neurite growth cones surrounded by proliferated phylopodia (Schwann cell cytoplasm) are observed in the damaged motor endplates where junctional folds are markedly distorted.

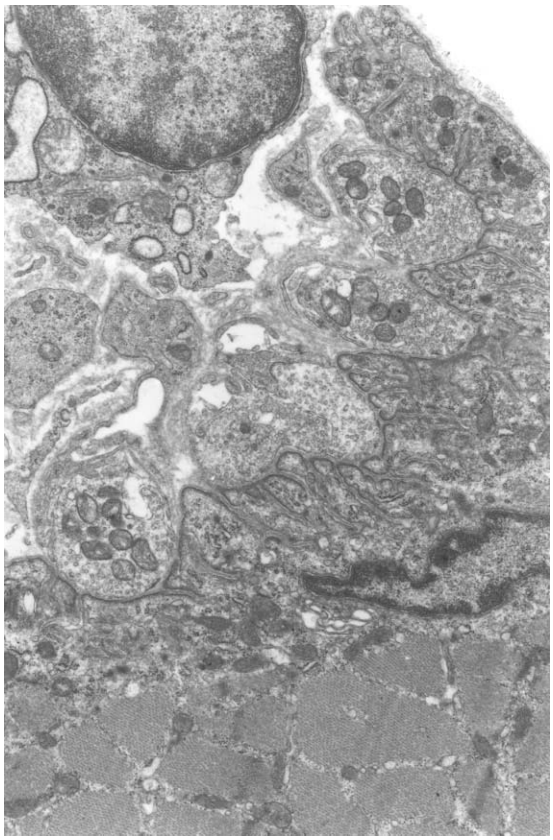

Fig. 18. Electron micrograph of the motor endplate in a rat given DTBHQ, showing four growth cones differentiating into newly-formed nerve endings that contain a few synaptic vesicles and mitochondria. Many immature nerve endings, surrounded by cytoplasmic extensions of Schwann cells, are apparent in a denervated endplate. × 8,000.

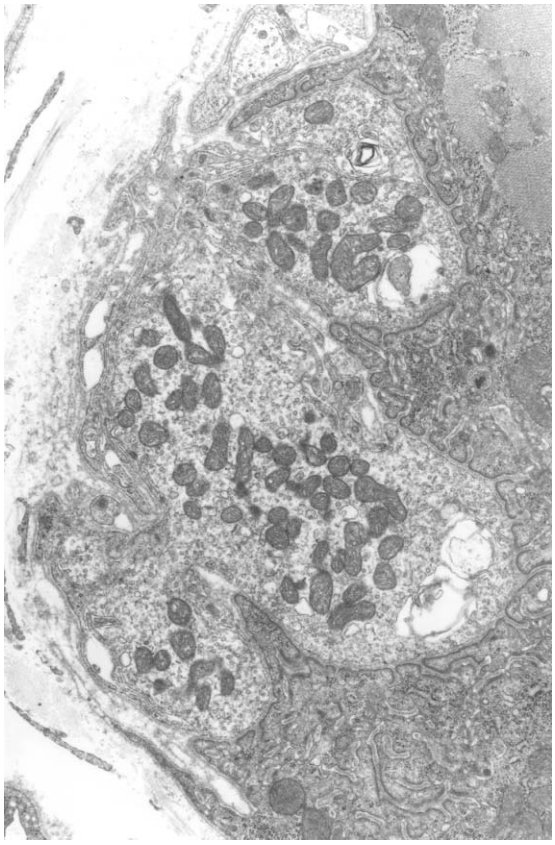

Fig. 19. Electron micrograph of the motor endplate in a rat given DTB, showing more progressed regenerative change. Very large nerve endings containing many synaptic vesicles and mitochondria with complex junctional folds are formed in the denervated endplates. × 8,000.

toluidine blue of the lumbrical muscles in the foot pad from the rat hind limbs.

Conclusion

Based on the results of our literature survey and our previous studies, it was concluded that nerve endings of the peripheral motor nerves are initially damaged in the neurotoxicity of venoms of snakes such as β-bungarotoxin, notexin and taipoxin, as well as chemically synthesized substances such as DTB, sarin, Vacor and DTBHQ. However, we have to recognize the fact that it is very difficult to detect toxic changes in such neuromuscular junctions especially at the early stage of toxication under a light microscope. In this respect, electron microscopic examinations are absolutely necessary for the pathological evaluation of neurotoxic chemicals targeting the neuromuscular junctions, although the conduct of such examinations has not been described in the international guidelines for neurotoxicity tests. In addition, because of the extremely small tissue sample size used for electron microscopy, proper tissue selection is essential to ensure that the changes which are observed in light microscopy and

immunohistochemistry are selected for ultrastructural examinations. Therefore, when neurotoxic signs such as paralysis of the posterior extremities, suggestive of damage to the neuromuscular junction occur in rats treated with a test substance, a part of the skeletal muscle in the foot pad of the hind limbs such as lumbrical muscles should be fixed and subjected to electron microscopic examination to eliminate the misjudgment in the absence of evidence of morphological changes in the neuromuscular junctions, that only dysfunction of the nervous system is induced in the animals by the treatment with a test substance.

References

1. Spencer PS and Shaumburg HH. Central-peripheral distal axonopathy; the pathology of dying-back polyneuropathies. Prog Neuropathol 1977; **3**: 253–295.
2. Jones HB. Dithiobiuret neurotoxicity: an ultrastructural investigation of the lesion in preterminal axons and motor endplates in the rat lumbrical muscle. Acta Neuripathol 1989; **78**: 72–85.
3. Sahenk Z. Distal terminal axonopathy produced by 2,4-dithiobiuret: effects of long-term intoxication in rats. Acta Neuropathol 1990; **81**: 141–147.
4. Harris JB. Phospholipases in snake venoms and their effects on nerve and muscle. In: Snake Toxins, AL Harvey (ed), New York: Pergamon Press, 91–124, 1991.
5. Hawgood B and Bon C. Snake venom presynaptic toxins. In: Handbook of Natural Toxins, vol 5: Reptile Venoms and Toxins, AT Tu (ed), New York: Marcel Dekker, 3–52, 1991.
6. Hamilton RC, Broad AJ, and Sutherland SK. Effects of Australian eastern brown snake (*Pseudonaja textilis*) venom on the ultrastructure of nerve terminals on the rat diaphragm. Neurosci Lett 1980; **19**: 45–50.
7. Dixon R and Harris JB. Nerve terminal damage by β-bungarotoxin: its clinical significance. Am J Pathol 1999; **154**: 447–455.
8. Kawabuchi M, Cintra WM, Deshpande SS, and Albuquerque EX. Morphological and electronphysiological study of distal motor nerve fiber degeneration and sprouting after irreversible cholinesterase inhibition. Synapse 1991; **3**: 218–228.
9. Damodaran TV, Jones KH, Patel AG, and Abou-Donia MB. Sarin (nerve agent GB) - induced differential expression of mRNA coding for the acetylcholinesterase gene in the rat central nervous system. Biochem Pharmacol 2003; **65**: 2041–2047.
10. Ahn JS, Lee TH, and Lee Mc. Ultrastructure of neuromuscular junction in vacor-induced diabetic rats. Korean J Intern Med 1998; **13**: 47–50.
11. Thastrup O. Role of Ca^{2+}-ATPases in regulation of cellular Ca^{2+} signalling, as studied with the selective microsomal Ca^{2+}-ATPase inhibitor, thapsigargin. Agents Actions 1990; **29**: 8–15.
12. Kitajima S, Kurokawa Y, and Inoue T. Single-dose toxicities of 2,5-di(tert-butyl)-1,4-hydroquinone in the rat: toxicants as of hydroquinone derivative or a microsomal Ca^{2+} ATPase inhibitor? Toxicol Res 1996; **21**: 201–204.
13. Mitsumori K, Imazawa T, Onodera H, Takahashi M, Kitajima S, Inoue T, and Kurokawa Y. Ultrastructural changes in motor endplates of the lumbrical muscles of rats induced by a microsomal Ca^{2+} ATPase inhibitor, 2,5-di(tert-butyl)-1,4-hydroquinone. Arch Toxicol 1998; **72**: 115–118.
14. Imazawa T, Mitsumori K, Kitajima S, Onodera H, Tamura T, Takahashi M, and Hirose M. Time course of ultrastructural changes and immunoelectron microscopic localization of neurocalcin in motor endplates of the lumbrical muscles of rats given a single administration of 2,5-di(tert-butyl)-1,4-hydroquinone. Acta Neuropathol 2000; **99**: 109–116.
15. Rowan EG. What does β-bungarotoxin do at the neuromuscular junction? Toxicon 2001; **39**: 107–118.
16. Harris JB, Grubb BD, Maltin CA, and Dixon R. The neurotoxicity of the venom phospholipases A_2, notexin and taipoxin. Exptl Neurol 2000; **161**: 517–526.
17. Watson DF and Griffin JW. Vacor neuropathy: ultrastructural and axonal transport studies. J Neuropathol Exp Neurol 1987; **46**: 96–108.
18. OECD. Guideline 424 on Neurotoxicity Study in Rodents. Organization for Economic Co-operation and Development, Paris, 1997.
19. OECD. Revised Draft Guidance Document for Neurotoxicity Testing. Organization for Economic Co-operation and Development, Paris, 2003.
20. Spencer PS and Schaumburg HH. Classification of neurotoxic disease: a morphological approach. In: Experimental and Clinical Neurotoxicology, PS Spencer and HH Schaumburg (eds), Baltimore: Williams & Wilkins, 92–99, 1980.
21. Hirano A and Llena JF. The central nervous system as a target in toxic-metabolic states. In: Experimental and Clinical Neurotoxicology. PS Spencer and HH Schaumburg (eds), Baltimore: Williams & Wilkins, 24–34. 1980.
22. Price DL and Griffin JW. Neurons and ensheathing cells as targets of disease processes. In: Experimental and Clinical Neurotoxicology. PS Spencer and HH Schaumburg (eds), Baltimore: Williams & Wilkins, 2–23, 1980.
23. Thomas PK. The peripherval nervous system as a target for toxic substances. In: Experimental and Clinical Neurotoxicology. PS Spencer and HH Schaumburg (eds), Baltimore: Williams & Wilkins, 35–47, 1980.