MODULATION OF LASALOCID-INDUCED NEUROTOXICITY BY PHENYLMETHYLSULFONYL FLUORIDE

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PREFACE

This dissertation evaluates the neurotoxicity of lasalocid and the effect of phenylmethylsulfonyl fluoride on the modulation of lasalocid neurotoxicity in chickens and rats. Neurotoxicity of chemicals has been a challenging problem in modern society since a continuous exposure to environmental and man-made chemicals is an ever-present problem. Use of toxicant specific neurotoxicity models and certain chemicals which have the ability of inducing modulation on specific neuropathies has been a valuable tool to investigate different neurotoxicities. Lasalocid, an ionophore antibiotic, has been related to a number of outbreaks of neurotoxicity cases in animal species. Phenylmethylsulfonyl fluoride is a serine protease inhibitor which has been widely utilized in the elucidation of organophosphate induced delayed neuropathy (OPIDN). Chapter I is a review of the literature of ionophores and OPIDN including the role of phenylmethylsulfonyl fluoride in the expression OPIDN. Chapter II deals with lasalocid neurotoxicity and modulation of lasalocid toxicity by PMSF in broiler chickens. The findings of these studies identify and characterize neuropathy with clinical symptoms, electrophysiology and histopathology in broiler chickens. Chapter III is concerned with dose-response studies with respect to lasalocid and describes lasalocid neuropathy in a rat model as well as the effect of PMSF on lasalocid neurotoxicity.

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CHAPTER I

INTRODUCTION AND LITERATURE REVIEW

Introduction

Neuropathies are common features of many diseases resulting from different etiologies. Among these, neuropathies associated with human immounodeficiency virus (HIV), diabetes mellitus, and chronic alcoholism are of special concern because of increasing incidence and some of the most frequent causes of neuropathies in human. In addition, there are numerous causes of peripheral neuropathies including hereditary, toxic, drug induced, inflammatory, ischemic and paraneoplastic (Ludoph and Spencer, 1995; Tan et al., 1995; Srinivasan, et al., 2000).

Lasalocid (LA) is a polyether antibiotic which is used to prevent coccidiosis in poultry and to increase feed efficiency in ruminants. When administered above therapeutic levels, L A can induce a peripheral neuropathy in broiler chickens. Clinical manifestation of this effect can cause ataxia known as duck walking syndrome (Gregory et al., 1995; Roder, 1996).

Organophosphorus (OP) toxicants are another group of neuropathy inducing agents, used for many years as insecticides and related to several outbreaks of a

syndrome known as organophosphorus induced delayed neuropathy (OPIDN). In spite of extensive research and finding putative target enzyme for the initiation of OPIDN, current mechanistic explanations for OPIDN still lack convincing evidence. Methods to elucidate mechanism of the toxicity may include use of other chemicals which can potentiate or prevent toxicity (Lotti, 1992). Phenylmethylsulfonyl fluoride (PMSF) is one such agent which has been widely utilized in mechanistic studies of OPIDN. Neuropathies of different mechanism can be promoted by PMSF (Moretto et al., 1992).

IONOPHORE ANTIBIOTICS

Classification of Ionophores

Ionophores as a class are divided into two general groups based on mode of ion transfer across membranes. These include channel formers and ion carriers.

Channel formers

Channel forming ionophores arrange themselves inside the membrane structure, creating a hydrophilic channel for the ions. By this means, ions from outside the cell pass through the provided hydrophilic channel into the cell. This mode of ion transport is analogous to that of transport proteins found in cell membranes. A well-known example of this type of ion transport is carried out by gramicidin. To form a channel within the membrane, two gramicidin molecules are required to line up across the membrane. When

two gramicidin molecules dimerize within the membrane, a hydrophilic channel is formed with outside consisting of hydrophobic residues (Becker, 1996; Pressman, 1976).

Ion carriers

Ion carriers can be subdivided into neutral ionophores and carboxylic ionophores. Regardless of subdivisions, both neutral and carboxylic ionophores move the ions across lipid bilayer by diffusing together with ions. These ion carriers act in a way that they bind the ions on one side of the cell membrane and allow the ion to sit within the ion carrier. The resulting complex moves across the lipid bilayer and releases the ion on the other side. Valinomycin, lasalocid, nigericin and A23187 are examples of ionophores with this mode of action (Becker, 1996; Pressman, 1976). In order to bind a metal cation, a carboxylic ionophore is required to be in deprotonated anionic form, and it must be in the protonated form (ionophore-H^+) or electrically neutral zwitterion form (I-Metal Ion) to diffuse across the membrane (Bergen and Bates, 1984). The movement of ions across the membranes depends on the concentration gradient of that ion.

Chemistry of Lasalocid

Lasalocid belongs to the subdivision of carboxylic ionophores. The molecular structure of lasalocid contains a tetrahydropyran and a tetrahydrofuran ring which give the name polyether antibiotic. It contains a terminal carboxyl group. All carboxylic ionophores posses linear backbones which contain heterocylic rings with oxygen

residues. For ion complexation, cyclic conformation of structure with oxygen residues inside and alkyl groups outside is required. When the resulting structure binds with the cation a lipid soluble complex is formed (Galitzer and Oehme, 1984). While the head of molecule contains a carboxyl group, the other end of the backbone carries one or two hydroxyl groups (at the tail). Among the carboxylic ionophores, lasalocid has the smallest structural backbone and is thought to form a shell-like structure. This structural orientation allows ions to sit on the molecule instead of placement inside the structure. It is thought that this structural property of lasalocid allows it to complex with a broad spectrum of cations and some organic amines as well (Pressman, 1976). The chemical structure of Lasalocid is shown in figure 1.

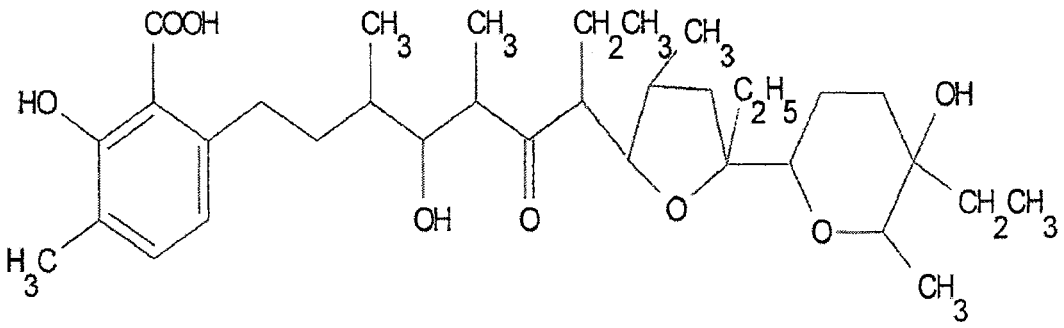

Figure 1. Chemical Structure of Lasalocid

Binding and Transport Selectiviy of Ionophores

Different ionophores have varying degrees of affinity for different cations. For example, while monensin has much more affinity (10 times) for binding Na^+ compared to K^+, lasalocid shows greater affinity for K^+ and equal affinity for both Na^+ and Ca^{++}. The relative affinity of monensin is $Na^+ > K^+ > Li^+ > Rb^+ > Cs^+$ and for lasalocid $K^+ > Rb^+ > Na^+ > Cs^+ > Li^+$. As noted above, lasalocid also binds divalent cations such as Ca^{++}, Mg^{++}, Ba^{++} (Elsasser, 1984; Bergen and Bates, 1984). The oxygens in the chemical structure of ionophores bind to cations through ion-dipole interaction much like a solvation of ions in high-dielectric solvents. The ion selectivity of ionophores is determined by the energy required for desolvation of the ion and the liganding energy attained upon complexation (Pressman, 1976). Interestingly, the transport rate of these ionophores may differ in their selective transfer of ions. For example, the order of relative selectivities for enhacement of membrane permeability of lasalocid is $Ca^{++} > Mg^{++} > Na^+ > K^+$ (Gad et al., 1985).

Use of Ionophores in Food Animal Production

Improvement of feed efficiency and producing more lean protein with rapid growth at a lower cost has been primary objectives in animal husbandry. The most commonly used ionophores (Monensin, Lasalocid and Salinomycin) are incorporated into rations as anticoccidials in poultry and as growth promoters in ruminant feeding. The broad spectrum of antibacterial activity along with the ability of these compounds to passively transport cations across cell membranes is thought to be the basis of their

mechanism of action in the prevention of coccidiosis and improving feed efficiency of ruminants (Elsasser, 1984).

Increase in feed efficiency can be achieved by ionophores via three different ways in ruminants. These include increased efficiency in energy metabolism, improved nitrogen metabolism in the rumen and prevention of lactic acidosis. Increase in feed efficiency is thought to be associated with increasing ruminal propionic acid and decreasing acetate and butyrate with resultant improvement of energy and carbon retention in the rumen. Improved nitrogen metabolism is attributed to increased quantity of dietary protein (Bergen and Bates, 1984). Use of lasalocid and monensin in cattle efficiently prevents lactic acidosis (Nagaraja et al., 1981). In the prevention of lactic acidosis, the effect of ionophores is observed by reducing the gram positive bacteria (responsible for producing ruminal lactate) and increasing the gram negatives (Bergen and Bates, 1984).

Treatment of monensin, salinomycin, and lasalocid in chickens and turkeys significantly inhibited invasion of Eimeria sporozoites in cecal tissues (Augustine et al., 1987). The protective effect of ionophores against coccidia is associated with the ability of these drugs to transport ions across biological membranes and altering ionic balance in coccidia (Augustine et al. 1992).

Toxicity of Ionophores in Animals

The toxicity of ionophores has been widely studied in a number of animal species. A number of toxicity cases have been reported in horses, cattle, sheep, cat, pig and avian

species (Galitzer, 1984, 1986; Hanson, 1981; Oehme and Pickrell, 1999; Van der Linde-Sipman, 1999; Halvorson, 1982; Novilla, 1992). Ionophores are generally safe and effective if used at recommended levels. However, ionophore toxicity might occur due to accidental overdose, misuse and mixing errors in the ration (Novilla, 1992).

Clinical and Pathological Aspects of Ionophore Toxicosis:

Signs of ionophore toxicity comprise undefinite general symptoms. Most common signs of ionophore toxicity include anorexia, hypoactivity, leg weakness, ataxia , dyspnea and diarrhea.

Cattle exhibited anorexia, muscle tremors, incoordination, weakness and slower rumen motility when given feed containing lasalocid or monensin (Galitzer, 1986). Severely affected animals show signs of toxicity within 24 hours of intoxication. Diarrhea and ataxia are also observed in cattle exposed to very high levels of monensin. Death may occur without signs (Novilla, 1992). Newborn calves dosed with 5 mg/kg of lasalocid showed respiratory distress and incoordination (Benson, 1998). In addition, the calves developed muscle weakness, ataxia and increased heart rate. In gross pathologic examination, pulmonary edema and congestion and ecchymotic hemorrhage in gluteal muscle were observed. Microscopic lesions included myocardial necrosis, coagulative necrosis and hemorrhage in skeletal muscle and swelling of vascular endothelial cells of brain. Slight changes in serum and blood chemistry were also seen with increased AST and CK levels (Benson, 1998).

Horses are considered more sensitive to ionophore toxicity than cattle. Oral LD_{50} of monensin is reported to be 2-3 mg/kg in horses (Galitzer, 1995). Lasalocid appears to be less toxic than monensin in horses since the estimated LD_{50} of lasalocid is 21.5 mg/kg. Similar to cattle, clinical signs include anorexia, ataxia, paresis, reduced activity and death (Hanson, 1981). Cardiac muscle is especially sensitive to Monensin and Lasalocid toxicity in horses. Tachycardia and cardiac muscle damage are a result of this sensitivity. In terms of cardiac toxicity, salinomycin is less toxic than monensin and lasalocid (Oehme and Pickrell, 1999).

In avian species, toxicity cases were reported in hens and turkeys. Hens and turkeys developed dyspnea, wing dropping, reduced egg production, reluctance to move and gait abnormalities due to ionophore toxicosis (Halvorson, 1982). In addition, reduced body weight gain and growth rate were observed in chickens fed ration containing monensin (Wagner, 1983). Small pale spleen, congestion of liver and lungs were observed in intoxicated hens. Cardiac and skeletal muscle damage were also observed in affected birds. Birds show myodegeneration in skeletal muscle with hyalinization and subepicardial hemorrhage, and congestion in the ventricle walls (Halvorson, 1982; Wagner, 1983).

Mechanism of Toxicity of Ionophores

The normal ionic gradient of the cell is maintained and tightly controlled by specialized transport complexes found in cell membranes. Examples of these complexes are Na^{+}-K^{+}-ATPase, Ca^{++}-Mg^{++}- ATPase, and Na^{+}-Ca^{++} counter transport systems. If the

cell membrane becomes permeable to ions which are normally controlled by these systems through ionophore mediated transport, the cells lose their ability to control and maintain physiologic ion gradients. Most of toxic effects of ionophores are thought to be mediated by disrupting the normal ionic gradients of cells (Elsasser, 1984). Alterations in cellular Ca^{++} concentration by lasalocid have been associated with disturbances in normal physiological function of the cardiac tissue due to dynamic regulation of cardiac muscle contractility by Ca^{++}. Ionophore cytotoxicity is thought to involve an influx of Na^{+} and Ca^{++} ions with simultaneous efflux of K^{+} ions leading to excess Ca^{++} overload within mitochondria, mitochondrial damage, lack of cellular energy and ultimately muscle necrosis (Novilla, 1992). Another mechanism of Ca^{++}-mediated cell death occurs via apoptosis. It is thought that ionophores lead to apoptosis via calcium-activated endonucleases (Ojcius, 1991). Another mechanism leading to cell death is reported to be associated with activation of influx of Ca^{++} through NMDA receptor leading to activation of phospholipase A_2 and release of arachidonic acid (Safran et al., 1996). Toxic effects on cardiac cells were also observed with monensin which typically affects Na^{+} transport across the membranes. The basis for the cardiac effect of monensin is that movement of Na^{+} ions inside the cell triggers Na^{+}/Ca^{++} exchange system leading to influx of Ca^{++} (Mollenhauer, 1990).

The cardiac toxicity of some ionophores by this manner was studied in neonatal rat cardiac myocyte. Monensin induced cardiac myocyte cytotoxicity at concentrations $\geq$ 10 mM in vitro. The effect caused by monensin on cardiac myocyte included "blebbing" of cell membranes and cellular swelling (Sihier and DuBourdieu, 1992). The elevated levels of Ca^{++} may affect numerous other cellular processes. The cellular processes

activated by Ca^{++} include mostly intracellular signaling pathways and inference with important cellular enzyme systems including auto-oxidation of macromolecules, activation of phospholipase A_2, endonucleases and proteases with an end result of cytotoxicity (Tymianski and Tator, 1996).

Another effect of ionophores is seen on energy metabolism. Alteration of the cellular ionic gradient by ionophores can deplete intracellular ATP levels. This effect is much more detrimental in prokaryotic cells and accounts for its somewhat selective action. For example, monensin causes an influx of Na^+ ions leading to increased intracellular Na^+ concentration. The cell reacts by expending ATP to maintain normal Na^+ balance inside the cell. Subsequently, cells cannot meet the demand for ATP and lyse (Bergen, 1984). The reason for the selective action of ionophores on prokaryotes is based on differences on mode of energy usage and ability for osmoregulation. Coccidia are intracellular parasites that rely on the host cell for energy. Ionophores stimulate coccidia sporozoite's Na^+-K^+-ATPase as a consequence of ionic disturbance. However, rate of ion influx exceeds the capability of Na^+-K^+-ATPase pump to remove excess Na^+ ion because of depletion of energy sources. Increased intracellular Na^+ is followed by an influx of Cl to maintain electroneutrality. This in turn brings water from exterior causing swelling of the parasite. Since the coccidia have no osmoregulatory organelles, coccidia swell and burst (Smith and Galloway, 1983).

Effects of Ionophore Toxicity on Nervous System

Alterations of nervous and muscle tissues were observed in ionophore induced toxicity. Lasalocid caused membrane depolarization in rat muscle fibre membranes and induced spontaneous release of acetylcholine in phrenic nerve-diagram muscle preparation (Jansson, 1976). Lasalocid also increased acetylcholine release from rat brain in vitro by an unknown mechanism (Richter, 1977).

It has been reported that lasalocid, monensin, and ionomycin stimulate release of catecholamines from rat pheochromocytoma and this effect may be dependent on increasing intracellular Ca^{++} (Perlman, et al., 1980). Furthermore, lasalocid and ionophore A 23187 caused inhibition of fast axonal transport and decreased axonal microtubules with a concomitant increase in total calcium content of the nerve (Kanje, et al., 1981). One study showed that lasalocid caused selective degeneration of nerve cells in vitro by a mechanism dependent on increased Ca^{++} influx. This effect of lasalocid was blocked by the NMDA receptor blocker MK-801, suggesting possible involvement of excitatory amino acid in the neurotoxicity of Lasalocid (Safran, et al., 1996).

Alterations in nerve tissues and related clinical signs were reported in broiler chickens fed with Lasalocid. For example, lasalocid caused a dose dependent neurotoxicity in broiler chickens at doses greater than 11.25 mg/kg. The clinical manifestation of neurotoxicity resulted in ataxia. Nerve tissues from affected birds showed a number changes in histopathologic examinations. These included myelin disruption, degeneration and vacuole formation in myelin (Gregory et al., 1995; Roder, 1996). Salinomycin and monensin toxicity was reported in turkey breeders. The clinical

condition in affected birds included paralysis of legs leading to gait disturbances and abnormal positioning of head (Halvorson, 1982). Contamination of cat food with salinomycin resulted in an outbreak of salinomycin neurotoxicity in cats. Toxicated cats developed paresis and paralysis of hindlimbs and lameness. Postmortem examination indicated a distal polyneuropathy including sensory and motor neurons. Although both central and peripheral nerves were found to be affected, peripheral nerve injury was more severe in affected cats. Lesions were localized in axons, myelin sheath, and Schwann cells. These included destruction of myelin sheath with formation of digestion chambers, collapsed axonal sheath filled with foamy macrophages, and swollen Schwann cells (Van der Linde-Sipman, 1999). Rodent species can also be affected by ionophore neurotoxicity. Gad et al (1985) reported that some ionophores including lasalocid cause neurobehavioral signs in mice and rats. Rats dosed with oral ionophores developed tremors, tonic-clonic convulsions and aggressive behaviors.

These reports clearly indicate that one of the toxic effects of ionophores is related to nervous tissues and these effects are capable of inducing clinical, pathological changes in nerves which can be called ionophore induced neurotoxicity. Although the cellular effect of ionophores is attributable to their ability to facilitate ion movements across cell membranes, the mechanism in neurotoxicity is not extensively studied.

One way to study neurotoxicants is to use chemical agents which have the ability to modulate neurotoxicity. Phenylmethylsulfonyl fluoride (PMSF), a serine protease inhibitor, is such an agent which has been used for decades in the mechanistic evaluation of organophosphate induced delayed neuropathy (OPIDN). Neuropathies of different etiology can be promoted by PMSF (Moretto, 1993). Xu et al., (1999) reported that

PMSF significantly increased ataxia induced by p-bromophenylacetylurea in F-344 rats. Phenylmethylsulfonyl fluoride increased the concentrations of p-bromophenylacetylurea and its toxic metabolite suggesting that PMSF alters the toxickinetics of p-bromophenylacetylurea, leading to increased concentration of the toxicant in tissues.

2,5- Hexanedione neuropathy was also reported to be promoted by PMSF. Mechanism of 2,5-Hexanedione toxicity (crosslinking of neurofilaments) differs from OPIDN (Moretto, 1992).

These results show that ionophore induced neurotoxicity can be modulated by PMSF. However, the role of PMSF and some other modulators in OPIDN first need to be reviewed to gain insight into the mechanism of OPIDN. Therefore the rest of the discussion will be on OPIDN.

Overview of Organophosphate Induced Delayed Neurotoxicity (OPIDN)

Many organophosphorus (OP) toxicants irreversibly inhibit the enzyme acetylcholinesterase. Acetylcholinesterase normally degrades acetylcholine into choline and acetic acid at cholinergic synapses. Organophosphate poisoning thus causes the accumulation of acetylcholine, allowing it to react with nicotinic and muscarinic receptors on the postsynaptic cells. The resultant effect is manifested by excess stimulation of cholinergic nerves with related symptoms such as excessive secretions and convulsions (Hayes and Laws, 1991). The cholinergic effect of OP is a well-known and documented aspect of OP poisoning. However, neuropathy is another possible consequence of OP exposure. A single dose of some OPs is enough to cause irreversible

neuropathy 2-3 weeks after exposure (Johnson, 1990). This effect of OP poisoning is referred to organophosphate induced delayed neuropathy (OPIDN). Earlier in the elucidation of mechanism of the OPIDN, it was thought that brain acetylcholinesterase and butyrylcholinesterases might be involved in the mechanism of OPIDN. Later it was found that organophosphate induced delayed neuropathy has no relation to the inhibition of acetylcholinesterase (Carrington, 1989). The neurotoxic effect was then found to be associated with phosphorylation of a brain protein called neuropathy target esterase (NTE) by certain OPs (Johnson, 1969, 1974). Interestingly, while some OPs are able to inhibit NTE and cause OPIDN, some OPs, carbamates and phenylmethylsulfonyl fluoride have the ability to inhibit the enzyme, but they are unable to produce OPIDN (Johnson, 1970). Furthermore, studies showed that the neurotoxic effect of neuropathic OPs can be prevented or potentiated with the administration of these non-neuropathic NTE inhibitors in a sequence-dependent manner (Pope and Padilla, 1990; Massicotte, 1999). To gain insight into the potentiation, protection and other aspects of OPIDN by certain chemicals, it is necessary to review the literature of OPs.

Chemistry of Organophosphorus Compounds

Although organophosphorus compounds show great diversity in details of their chemical structure, they have common backbone. Organophosphorus are aliphatic carbon, cylic, or heterocylic phosphodiesters (Osweiler, 1996; Hayes and Laws, 1991).The general formula of OPs is shown in figure 2.

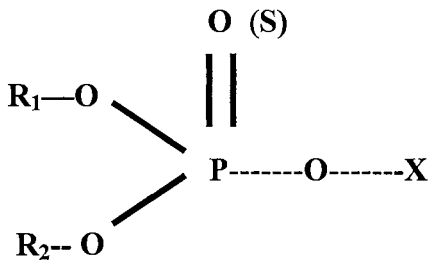

Figure 2. Chemical Structure of organophosphorus compounds.

Pentavalent phosphorus posses double bond with either a sulfur or an oxygen (P=O is capable of inhibiting esterases). "R" groups can vary and bind to the phosphorus either directly (example of this is phosphinates), via an oxygen (example of this phosphates) or via nitrogen (phosphoroamidates) (Lotti, 1992).

Interactions of Organophosphorus Compounds with Target Esterases

Esterases can be divided into two groups which are A esterases and B esterases. A esterases detoxify OPs while B esterases are inhibited by OPs and the latter comprises target esterases (Example: Acetylcholinesterase) in the body. The active site of the esterase, serine hydroxyl group, is phosphorylated, and the active site is then slowly

reactivated by hydrolysis. A subsequent reaction might take place after phosphorylation called aging, however which leaves a negatively charged phosphoryl residue leading to irreversible inhibition of the enzyme (Lotti, 1992).

Role of Neuropathy Target Esterase Enzyme (NTE) in Organophosphorus Induced Delayed Neuropathy

After the discovery of NTE, extensive research has focused on the role of NTE in the initiation of OPIDN. Involvement of NTE in OPIDN was first discovered with the use of non-neuropathic NTE inhibitors. Some chemicals such as PMSF (serine protease inhibitor) and carbamates showed strong inhibitory effect on NTE in vivo, but without producing neuropathy. However, when animals were co-exposed to these chemicals were applied with neuropathic OPs, it was noted that neuropathy induced by OPs can be either protected or potentiated depending on the sequence of application (Pope, 1993). Johnson (1970) showed that certain non-neuropathic NTE inhibitors (PMSF) given prior to neuropathic OPs have protective effect from OPIDN in spite of its extensive inhibitory effect on NTE. Interestingly, given after a neuropathic OP, PMSF promotes the neuropathy (Pope and Padilla, 1990). It was postulated that initiation occurs by a two step process. The first step includes phosphorylation of active site of NTE, and the second step includes "aging" leading to alteration in membrane micro environment and disturbance of homeostasis causing degeneration of long axons. In the protection it was postulated that the second step does not occur because PMSF and carbamates are not capable of aging. Secondly, as a result of occupation of NTE's active site by these

compounds the enzyme is protected from neuropathic agent (Johnson, 1974; Pope 1993). Young chickens are resistant to OPIDN (Johnson and Barnes, 1970), but PMSF can increase sensitivity to OPIDN (Johnson and Lauwerys, 1969; Pope and Padilla 1990; Pope et al., 1993). It was postulated that NTE may not be involved in promotion of OPIDN. Higher doses of neurotoxic OPs show no effect in young chickens despite the inhibition of NTE which is enough to induce OPIDN in adult chickens. However, young chicks become sensitive with PMSF following the neuropathic agent. These studies suggested that NTE may not be the target for promotion (Pope et al, 1992; Harp et al, 1997).

Neuropathy Target Esterase

Neuropathy target esterase is a protein which is found in many tissues. The highest concentration of the enzyme was reported to be in the brain, whereas most of the esterase activity is membrane bound mainly in the endoplasmic reticulum and plasma membrane. Neuropathy target esterase can also be found in different tissues other than nerve tissues. For example, NTE was found in spleen and lymphocytes in higher concentrations (Carrignton, 1989). Despite extensive research, little is known about the physiological role of NTE. Even with long term inhibition by phosphinates and sulfonyl halides (PMSF) NTE inhibition did not cause OPIDN. Therefore, no correlation has been found between the NTE inhibition and well-being of a neuron (Lotti and Moretto, 1993). Primary sequence of human NTE and mouse NTE has high similarity to swiss cheese protein (SWS) in nerves of Drosophila melonagaster. Flies without this protein are affected by glial hyperwrapping and neurodegeneration. A recent article showed for the

first time NTE is essential for embryonic development in mice. Genetic ablation of NTE in mice resulted in hyperactivity, and it was suggested that NTE may play a role in motor activity in mammals (Winrow et al., 2003).

Clinical and Histopathological Presentation of OPIDN

Clinical manifestation of OPIDN is characterized by a delay period which is seen between 8 to 14 days after dosing. The hen has been the animal model in studies of OPIDN to characterize the clinical expression of OPIDN in animal species. Hens given a single dose of 1 mg/kg diisopropyl flurophosphate start to show first sign of ataxia after 8 days following dosing with slight incoordination and leg weakness. Then ataxia progresses with time, and complete ataxia and marked paralysis of the legs develop by day 13 (Sprague and Bickford, 1981). The delayed neurotoxic effect of some OP compounds can be altered by the time dependent administration of PMSF. It has been shown that PMSF intensifies the ataxia when given following a neuropathic OP dosing. In addition, age is another factor in the clinical expression of OPIDN in response to OP treatment. Young chicks are known to be resistant to OPIDN. For instance, while 5 week-old chicks appeared normal in response to OP treatment, chickens receiving same treatment and dose of OP at 8 weeks age developed ataxia. Interestingly, young chicks (for example at 5 weeks of age) become sensitive to the same OP treatment with PMSF post treatment (Pope et al., 1992; Harp et al., 1997).

Lesions in affected animals are distributed in central and peripheral nerves. Within the CNS, the spinal cord is mainly affected with axonal degeneration. Axons appear fragmented and swollen and myelin around the axon is lost. In peripheral nerves,

large and long axons are more susceptible to the damage induced by OPIDN. Changes in peripheral nerves include focal nerve fiber varicosities and paranodal demyelination in the distal part of the nerves. The changes in central and peripheral nerves are defined as a central-peripheral distal sensory-motor axonopathy since distal parts of long axons are mainly affected (Soliman, 1983; Lotti, 1992).

Statement of Dissertation and Hypotheses

The role of PMSF in the modulation of some neuropathies has been reported in several studies. While PMSF has been utilized mainly in the elucidation of mechanism of the OPIDN, several researchers suggested that PMSF can also modulate neuropathies induced by some other neurotoxicants. The neurotoxic effect of lasalocid is believed to be associated with the unique action of this antibiotic on alteration of ionic homeostasis of cells by facilitating ion movement across membranes. In contrast to the mechanism of lasalocid toxicity, OPs toxicity is attributed to inhibition of NTE. While the initiation of OPIDN requires inhibition of NTE, current mechanistic explanations for OPIDN still lack conclusive evidence, and little is known regarding the modulatory effect of PMSF. The purpose of the first part of this study was to evaluate and characterize lasalocid induced neuropathy in 3 and 6 week-old chickens by oral gavage of lasalocid with or without PMSF. The neurotoxicity was characterized using clinical scoring system, light and electron microscopy and electrophysiology. The hypothesis for this phase of the study was that lasalocid induces neuropathy in broiler chickens, and PMSF potentiates lasalocid

induced neuropathy. Several reports demonstrated that PMSF can modulate different types of neuropathies besides OPIDN. It is believed that potentiation of OPIDN by PMSF is not associated with the inhibition of NTE. Therefore, it can be expected that neuropathies of different origin may be potentiated by PMSF. Results from potentiation studies of several different neuropathic agents with PMSF showed that 2-5-hexanedione was promoted by PMSF in hens (Moretto et al., 1992). Furthermore, it was shown that p-Bromophenylacetylurea neurotoxicity was also potentiated by PMSF (Xu,, et al., 1999). There is no plausible explanation for exact mechanism of potentiation of different neuropathies by PMSF. However, it can be speculated that PMSF can modulate LA-neurotoxicity by interfering with Ca^{++} homeostasis. It was reported that TOCP treated hens showed decreased Ca^{++} in scitic nerve homogenates (Lutrell, et al., 1993). Furthermore, it was shown that during the protection of OPIDN by PMSF, PMSF prevented reduction of serum Ca^{++} level which was increased during PMSF potentiation of leptophos (Piao, et al., 2003). The toxicity of LA is linked to increased cellular Ca^{++} overload through facilitation of Ca^{++} influx. A recent study also demonstrated that NTE may have lysophospho lipase activity (LysoPLA) which leads to accumulation of lysolecithin. Lysolecithin is a membrane protein whose accumulation has been associated with myelin degredation (Quisdat, 2003). Since PMSF is an NTE inhibitor, it can inhibit LysoPLA and can be involved in the potentiation in this manner. Therefore, an interaction between PMSF and LA could be possible in this manner. The second part of the thesis deals with developing a rat model of lasalocid neuropathy and examines the effect of dietary salt on lasalocid induced neuropathy. Dietary levels of K^{+} and some other ions might influence ionophore toxicity (Halvorson, 1982). In poultry industry, it is

known that incidence of LA toxicosis with increased water salinity is higher during hot summer period. With the combination of environmental stress factors, increased temperature and water deprivation may play a role in a higher rate of LA neurotoxicity and cardiotoxicity (Gregory et al., 1995). The hypothesis for this phase of the study was that lasalocid neurotoxicity is increased by the addition of salt to the diet. To determine the dose given by the oral route that causes the neurotoxic syndrome in rats by LA, a dose-response study was performed. This helps to characterize and describe the neurotoxic syndrome and develop a rodent model for LA induced neuropathy. The final phase of the second part examined the effect of PMSF posttreatment on lasalocid neuropathy in rats. Although promotion of 2,5-hexanedione by PMSF was shown in hens, same effect was not achieved in rats. Similar resistance to potentiation of acrylamide neuropathy by PMSF was also observed in rats (Moretto and Lotti, 1993).

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CHAPTER II

MODULATION OF LASALOCID INDUCED NEUROPATHY BY PHENYL METHYL SULFONYL FLUORIDE IN 3 AND 6 WEEK-OLD BROILER CHICKENS

Abstract

Phenyl Methyl Sulfonyl fluoride (PMSF) can potentiate the neuropathy caused by some organophosphorus (OP) and other types of neurotoxicants (e.g., p-bromophenylacetyl urea). Lasalocid is a feed additive used in chickens that is known to cause a peripheral neuropathy. We investigated the ability of PMSF to exacerbate the clinical signs and morphological lesions of lasalocid-induced neuropathy in broiler chickens. Ultrastructural changes, electrophysiologic characteristics before and after lasalocid exposure in different age groups of broiler chickens, and neurotoxic esterase activity (NTE, an enzyme whose inhibition is highly correlated with induction of OP-induced neuropathy) were evaluated. LA (30 mg/kg t.i.d. for 2 days) was given to two different age groups (3 and 6 week-old of chickens), with or without a single dose of PMSF (90 mg/kg, sc) before or after the last LA treatment. All birds were observed daily for 8 days for clinical signs of neuropathy and tissues (brain, spinal cord and sciatic nerve) were removed for histopathologic examination at the end of each study. PMSF

increased ataxia induced by LA in 3 and 6 weeks of age broiler chickens. While LA did not affect NTE enzyme activity, PMSF decreased NTE activity of spinal cord, brain and sciatic nerve in both age groups. LA markedly reduced Motor Nerve Conduction Velocity (MNCV) in the sciatic nerve, but PMSF had no effect alone or in combination with LA. Histopathologically, LA caused lesions only in sciatic nerve. Spinal cord degeneration was noted in birds treated with both LA and PMSF. However, PMSF alone had no effect. These results suggest that PMSF can potentiate the neurotoxicity induced by LA. The mechanism for this modulation requires further investigation.

Introduction

Neuropathy is a term which describes pathological changes in motor, sensory and autonomic neurons as evidenced by derangement in both structure and function. Depending on the nature of the insult, neuropathies can be localized to axon, myelin or to the whole neuron body (Riaz and Tomlinson, 1996). Many toxicants including some organophosphates (OP) and Lasalocid (LA), a carboxylic ionophore, are capable of producing neuropathy (Soliman, 1983; Roder, 1996).

OPs are known to produce a delayed neuropathy in man and domestic animals. Toxic signs induce flaccid paralysis of lower and upper limbs and changes in neurons defined as a central-peripheral distal sensory-motor axonopathy. Although the mechanism is not clearly understood, it is believed that inhibition of Neuropathy Target Esterase (NTE) initiates organophosphate induced delayed neuropathy (OPIDN) (Lotti, 1992). It is proposed that induction of OPIDN occurs by a two step process. NTE is

phosphorylated, and subsequently the OP compound undergoes "aging" leading to alteration in membrane micro environment and disturbance of homeostasis (Pope, et al. 1993). Several studies indicated that NTE plays a role in lipid metabolism and signaling pathway and NTE was shown to be an integral membrane protein (Van Tienhoven, M., et al., 2002; Atkins, J., and Glynn, P., 2000). A recent report suggests that NTE may be involved in control of motor activity (Winrow, et al., 2003). NTE is an enzyme whose exact physiological function is still to be determined, nevertheless inhibition of NTE by OPs has been related to their ability to induce OPIDN (Johnson, 1988).While NTE inhibition appears to initiate OPIDN, other chemicals such as carbamates, phoshinates and sulfonates also have NTE inhibitory effect, but they are unable to produce OPIDN.

Phenyl methyl sulfonyl floride (PMSF) is one such agent. Studies in chickens have shown that when given prior to a neuropathic OP, PMSF can prevent OPIDN. In contrast if PMSF is given after OP administration, delayed neuropathy is exacerbated. Some studies also indicate that PMSF can potentiate p-Bromophenylacetylurea induced neuropathy in rats (Xu, et al., 1999) and delay the recovery from nerve crush (Moretto, et al., 1993). Young chickens are resistant to OPIDN (Johnson and Barnes, 1970), but PMSF can increase sensitivity in young animals(Harp et al, 1996; Pope et al., 1993).

Lasalocid is capable of complexing with mono and divalent cations and forming a lipid soluble structure in membranes, thus allowing passive diffusion of cations across cellular membranes. Ionophores are useful as feed additives to improve feed efficiency and as coccidiostat in the poultry. Ionophores have caused a number of toxic effects in animals including cardiotoxicity, muscle damage, and hepatotoxicity (Galitzer and Oehme, 1984). Furthermore, in vitro incubation of Ca ++ with LA resulted in severe

intramyelinic splitting suggesting a role for Ca++ in the mechanism of LA induced peripheral neuropathy (Roder, 1996). We investigated whether PMSF may alter LA-induced neurotoxicity.

Materials

Lasalocid A Na Salt (Purity ≥ 97 %) and PMSF were purchased from Sigma Chemical Company (St. Louis, MO). Lasalocid was placed into gelatin capsules (Lilly Company®). Phenyl valerate and Phenyl saligenin phosphate (PSP) was purchased from Oryza Laboratories, Inc. (Chelmsford, MA). Paraoxon (diethyl 4-nitrophenyl phosphate) was purchased from Chem Service (West Chester, PA). Mipafox, Trizma® base, EDTA, SDS, 4-aminoantipyrine, and potassium ferri cyanide and dimethyl sulfoxide (DMSO) and glycerol formal were purchased from Sigma Chemical Company (St. Louis, MO).

Animals and Treatments

One day old broiler chickens (Cobb-Cross) were obtained from a commercial source. Chicks were housed in floor pens and maintained at 30 °C under a 23:1hr light: dark illumination cycle. Feed and water were provided ad libitum. At three and six weeks of age, the chickens were randomly divided into eight treatment groups containing 11 birds each. Controls were treated with empty gelatin capsules. Chickens were treated with LA (30 mg/kg t.i.d for 2 days) with or without PMSF (90 mg/kg, sc) in glycerol formal

four hours before initiating LA treatments, or four hours after the last LA treatment. Table 1 shows the groups with their respective treatments in 3 and 6 week-old chickens.

Table I. Treatment groups in 3 and 6 week-old chickens

Group	n	Age (week)	Treatment
			PMSF Pre-treatment
1	11, 11	3, 6	GF (0.5ml/kg, sc) + LA (0 mg/kg) t.i.d. 2 days
2	11, 11	3, 6	PMSF (90 mg/kg, sc) + LA (0 mg/kg) t.i.d 2 days
3	11, 11	3, 6	GF(0.5ml/kg, sc) + LA (30 mg/kg) t.i.d. 2 days
4	11, 11	3, 6	PMSF (90 mg/kg, sc) + LA (30 mg/kg) t.i.d 2 days
			PMSF Post-treatment
5	11, 11	3, 6	LA (0 mg/kg) t.i.d. 2 days + GF (0.5ml/kg, sc)
6	11, 11	3, 6	LA (0 mg/kg) t.i.d 2 days + PMSF (90 mg/kg, sc)
7	11, 11	3, 6	LA (30 mg/kg) t.i.d. 2 days + GF (0.5ml/kg, sc)
8	11, 11	3, 6	LA (30 mg/kg) t.i.d 2 days + PMSF (90 mg/kg, sc)

Twenty four hours after the last treatment, three birds were randomly selected from each group for NTE determination and killed by CO_2 asphyxiation. For motor nerve conduction velocity (MNCV) studies, three birds from each treatment group were randomly selected two days after the onset of clinical ataxia. The remaining chickens were observed for 8 days and daily ataxia scores were assigned to each bird. At day 8, three birds from each group were randomly chosen for histopathologic examination.

Clinical Signs and Observations

All chickens were observed "blind" daily throughout dosing and for 8 days for clinical signs of ataxia. Initially, birds were observed without disturbing for behavioral changes, and then the birds were forced to walk for several minutes to determine the degree of ataxia and to identify if ataxia worsened after exercise. Ataxia scores were given according to clinical ataxia scoring system (Table 2).

Table II. Clinical Ataxia Scoring System

Score	Definition
0	Normal Birds
1, 1+	Slight Ataxia after exercise
2, 2+	Slight Ataxia, Ataxia worsens after exercise
3, 3+	Ataxic birds
4, 4+	Non-ambulatory, alert
5, 5+	Non-ambulatory, not alert, depressed

Neurotoxic Esterase Assay

Neurotoxic esterase activity of nerve tissues can be measured by a differential assay based on relative sensitivity to inhibition of by mipafox and insensitivity to

inhibition by paraoxon. The hen brain shows at least 4 different esterase activity other than NTE which hydrolyse phenyl valerate. Neurotoxic esterase can be measured by the preincubation of two samples with paraoxon or paraoxon plus mipafox before the addition of substrate. The difference between the two activities gives NTE activity (Johnson, 1977). Brain, spinal cord and sciatic nerve of randomly selected birds from each treatment group were dissected and stored at -70 °C until the day of assay. NTE activity was measured by the method of Johnson (1977), as modified by Solimon et al (1982), with slight changes in the incubation time.

Briefly, tissues were homogenized on ice in 9 volumes of 50 mM Tris (hydroxymethyl) amino methane buffer, pH 8.0 containing 0.2 mM EDTA (Tris-EDTA). After homogenization, tissues were centrifuged at 9000×g for 15 minutes. Supernatants of brain (25 µl), spinal cord (25 µl), and sciatic nerve (40 µl) were added to paired tubes (all samples were analyzed in duplicate). Subsequently, paraoxon (50 µl, 0.6 mM) was added to all tubes with or without mipafox (50 µl, 0.75 mM). After 20 minutes of preincubation in a 37 °C water bath, 5 µl of a 25 mg/ml phenyl valerate in water was added to the reaction tubes. Samples of brain, spinal cord and sciatic nerve were incubated in a 37 ° C water bath for 10, 20 and 60 minutes, respectively. The reaction was stopped with 0.5 ml of 50 mM Tris/EDTA containing 1 % sodium dodecyl sulfate and 0.025 % 4-aminoantipyrine. Potassium ferricyanate (0.4 %, 0.25 ml) in water was added to each reaction tube to develop a stable color. After waiting at least 5 minutes, absorbance at 510 nm was recorded. Absorbance values were used to calculate NTE activity as the difference in hydrolysis of phenyl valerate in the absence and presence of mipafox. The extinction coefficient for a µM solution is 0.0139, and this was used to convert to mole

equivalents phenyl valerate hydrolyzed. NTE activity was reported as nmol phenol/minute.mg tissue.

Motor Nerve Conduction Velocity Studies

Motor nerve conduction velocity (MNCV) was evaluated in three birds from each treatment group in 3 and 6 week-old chickens. Chickens were anesthetized (i.m.) with the combination of xylazine (5 mg/kg) and ketamine (15 mg/kg). Birds were positioned in lateral recumbency, and recordings were taken from the left sciatic nerve. Stimulating and recording electrodes were placed caudal to the greater trochanter (gluteal fold) and disto-lateral tarsometotarsal joint (popliteal fossa), respectively. The nerve was stimulated with supramaximal stimuli of 1ms duration at a rate of 1/second using Biosound Genesis 3000-AP.

Histopathologic Examinations

Light Microscopy: Three birds from each group were randomly selected for necropsy and subsequent histopathologic and ultrastructural studies. The chickens were euthanized in a CO2 chamber. The Sciatic and tibial nerves were dissected and fixed in 2.5 % buffered glutaraldehyde. The entire spinal cord was dissected by complete removal of the dorsal and lateral portions of the vertebrae and fixed in 2.5 % glutaraldehyde. The brain and sections of heart and skeletal muscle were removed and placed in fixative. Sections for histopathologic examination were embedded in paraffin, cut 4-6 μm thick

and stained with hematoxylin and eosin (H&E). Selected sections of sciatic nerves and spinal cord were also stained with Severe-Munger and counterstained with Luxol Fast Blue. All sections of tissue were examined by light microscopy and lesions were scored "blind". Lesions in the sciatic nerve and spinal cord were graded according to the following scale:

Nerve Lesions:

0 - No lesions

1 - Swollen axons, foamy vacuolation of myelin

2 - Small distinct random vacuoles (Voids) in myelin

3 - Distinct vacuoles in myelin with atrophy and/or fragmentation of axons

4 - Large distinct myelin vacuoles (Voids), digestion chambers (axon-myelin debris), or loss of axons.

Spinal Cord Lesions:

0 – No lesions

1 – Small number of degenerating axons

2 – Moderate number of degenerating axons

3 – Numerous degenerating axons

Electron Microscopy: Sciatic nerves from affected birds were fixed in a glutaraldehyde/formalin mixture. Nerve pieces were washed with 0.1 M cacodylate buffer three times for 20 minutes. Gross longitudinal and transverse slices of the nerve segments were cut by razor blade. The tissues were post-fixed in 1 % cacodylate buffered

osmium for two hours then washed three times in 0.1 M cacodylate buffer. Following post-fixation, the tissues were dehydrated through graded ethanol series (50, 70, 90, 95, 100) and washed three times with propylene oxide for 20 minutes. The tissues were placed into 1:1 propylene oxide/hard polybed and polymerized a 60 ° C for 48 hours. Blocks of the nerve pieces were thick-sectioned and areas of interest were subsequently processed for thin sectioning. Thin sections of samples were examined using a JEOL 100 CX scanning electron microscope at 80 KV.

Data Analysis:

Analysis of variance procedures were utilized to compare treatment groups in 3 and 6 week-old chickens and for each experiment. For ataxia scores, since data is not continuous, group data are presented as medians±interquartile range (interquartile range: 75^{th} percentile minus 25^{th} percentile of data). For all other endpoints, group data are presented as means. For all analyses, PROC MIXED in PC SAS Version 8.2 was used to perform the analyses of variance, and a Least Significant Difference multiple comparison test was used to assess the significance of individual group comparisons. Significance level of 0.05 was used for comparisons of treatments.

Results

Clinical Signs and Ataxia

Figure 5 shows the effects of PMSF pretreatment on LA-induced ataxia in 3 week-old chickens. No signs of ataxia were observed in control or PMSF-treated birds. LA-treated birds or chickens treated with PMSF+LA exhibited varying degrees of ataxia. Response ranged from no ataxia with clinically normal appearance to severe ataxia and death. While control birds and PMSF treated birds were alert and clinically normal, affected birds were reluctant to move and appeared to be sluggish. When the affected birds were disturbed or forced to walk, they were slow and walked with frequent resting. Onset of clinical ataxia was observed as early as 16 hours after the first exposure in LA or LA+PMSF treated birds (Groups 3 and 4).

Figure 6 shows effects of PMSF posttreatment on LA-induced ataxia in 3 week-old chickens. Clinically, control and PMSF only group did not show any abnormality. However, groups treated with LA and LA+PMSF (Groups 7 and 8) appeared ataxic. Ataxia was most severe on the 3rd day of post LA application in these groups.

Figure 7 shows effects of PMSF pretreatment on LA-induced ataxia in 6 week-old chickens. No signs of ataxia were observed in control and PMSF only group. Both LA and PMSF+LA groups responded with ataxia. However, based on daily median ataxia scores, PMSF+LA caused higher ataxia throughout the observation period (day 1-7) compared to the LA only group.

Figure 8 shows effects of PMSF post treatment on LA-induced ataxia in 6 week-old chickens. Similarly, control and PMSF only group appeared normal. However, groups treated with LA and LA+PMSF showed ataxia with highest scores on day 3.

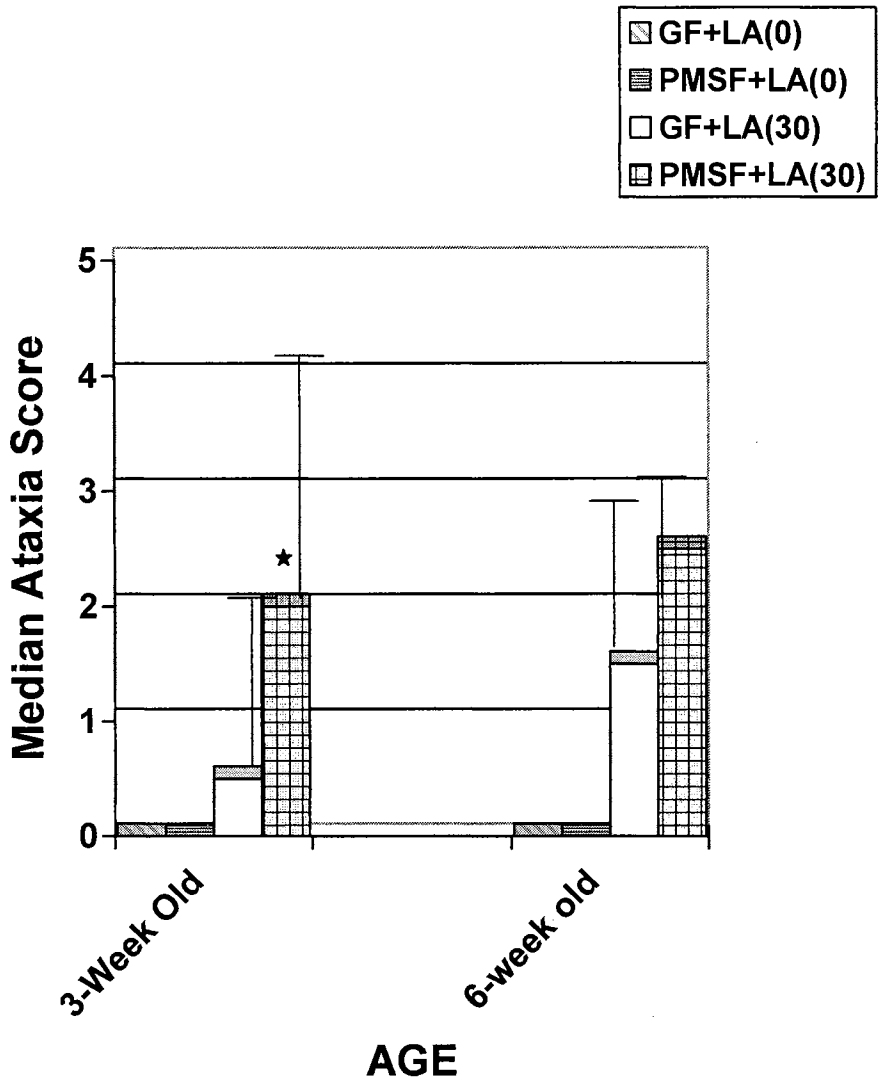

Figure 3. Effects of PMSF pretreatment on LA-induced ataxia in 3 and 6 week-old chickens as cumulative Median Ataxia Scores ± interquartile ranges. Asterisk indicates significant effect of PMSF on LA ataxia, at $p < 0.05$.

GF+LA(0) (Group 1): Control
PMSF+LA(0) (Group 2): Pretreatment with PMSF (90 mg/kg, s.c.) and LA (0 mg/kg)
GF+LA(30) (Group 3): Glycerol formal and LA (30 mg/kg, t.i.d. for 2 days)
PMSF+LA(30) (Group 4): PMSF (90mg/kg,s.c.) and LA (30 mg/kg, t.i.d. for 2 days).

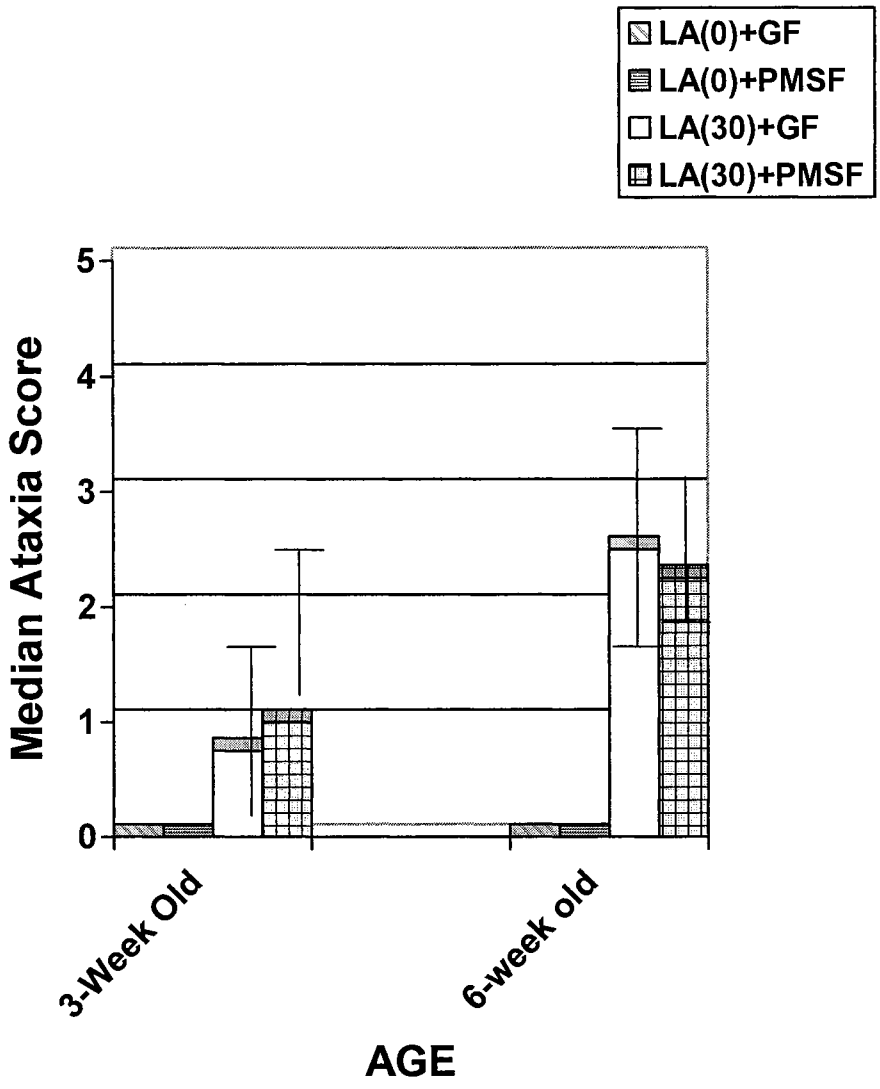

Figure 4. Effects of PMSF posttreatment on LA-induced ataxia in 3 and 6 week-old chickens as cumulative Median Ataxia Scores ± interquartile ranges.

LA(0)+GF (Group 5): Control,
LA(0)+ PMSF (Group 6): LA (0 mg/kg) and post treatment with PMSF (90 mg/kg, s.c.),
LA(30)+GF (Group 7): LA (30 mg/kg, t.i.d. for 2 days) and Glycerol formal,
LA(30)+PMSF (Group 8): LA (30 mg/kg, t.i.d. for 2 days) and PMSF (90mg/kg, s.c.).

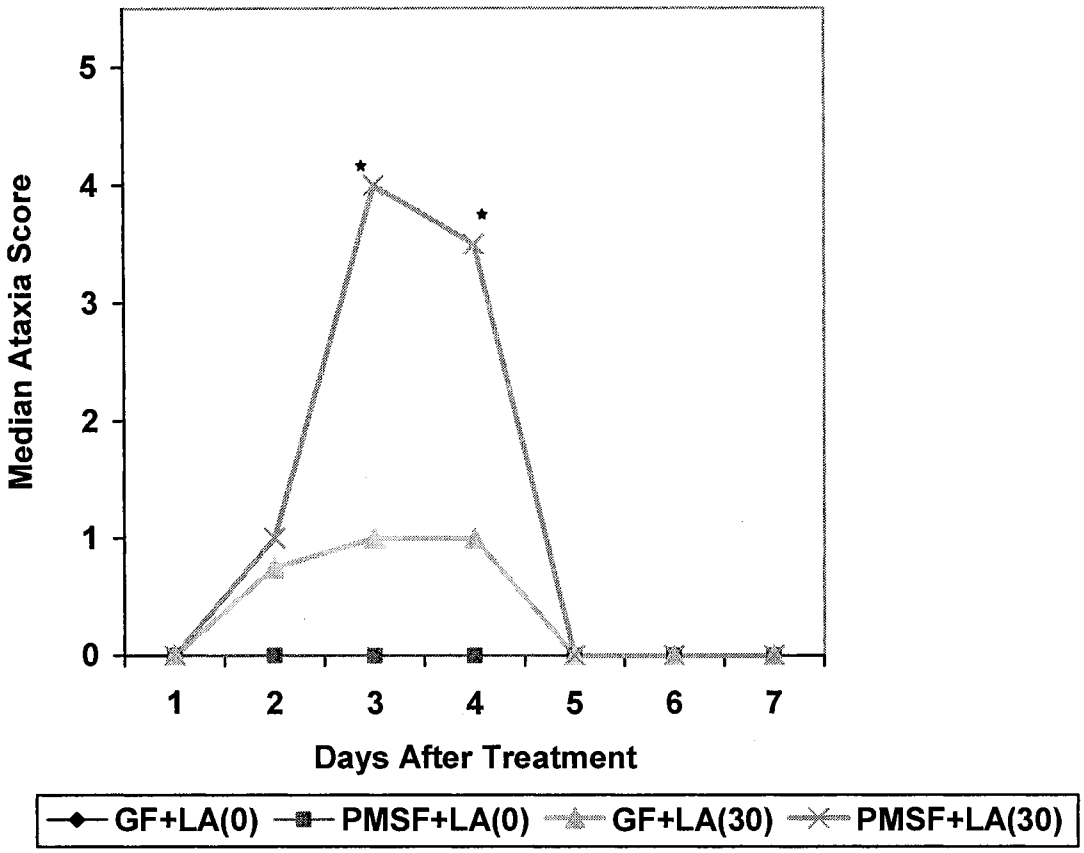

Figure 5. Effects of PMSF pretreatment on LA-induced ataxia in 3 week-old chickens.

GF+LA(0) (Group 1): Control

PMSF+LA(0) (Group 2): Pretreatment with PMSF (90 mg/kg, s.c.) and LA (0 mg/kg)

GF+LA(30) (Group 3): Glycerol formal and LA (30 mg/kg, t.i.d. for 2 days)

PMSF+LA(30) (Group 4): PMSF (90mg/kg,s.c.) and LA (30 mg/kg, t.i.d. for 2 days).

* Indicate significant effect of PMSF compared to GF+LA(30).

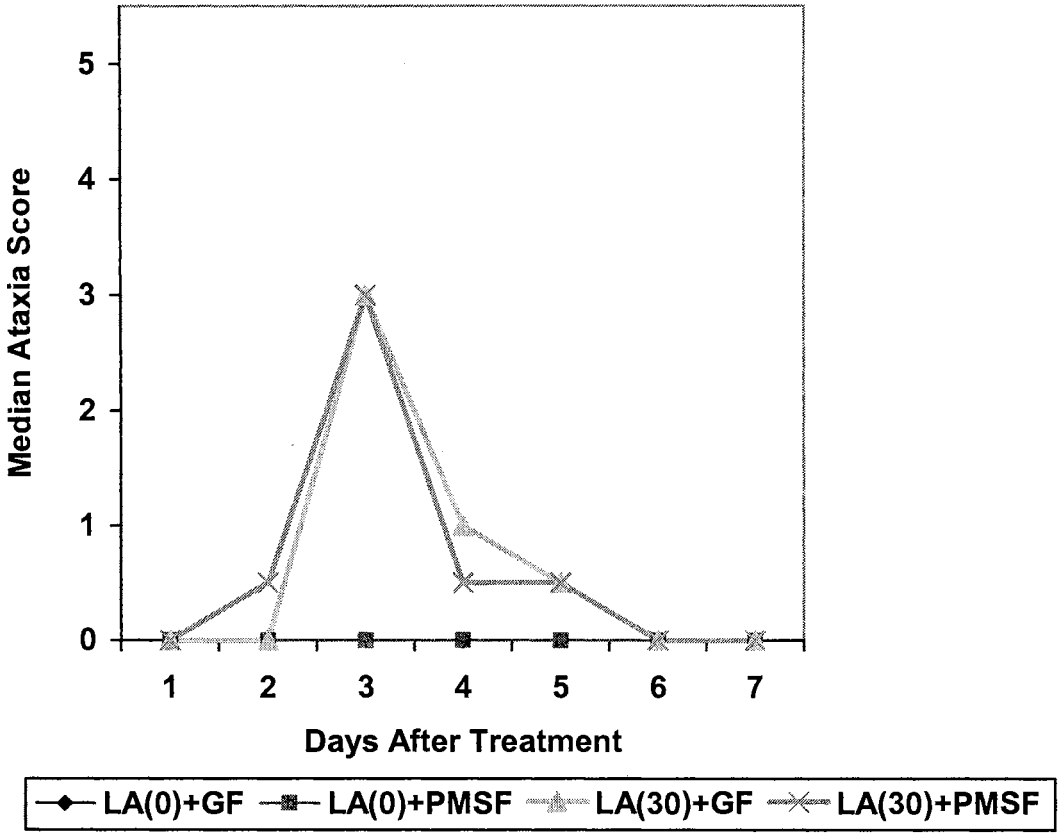

Figure 6. Effects of PMSF posttreatment on LA-induced ataxia in 3 week-old chickens.

LA(0)+GF (Group 5): Control,

LA(0)+ PMSF (Group 6): LA (0 mg/kg) and post treatment with PMSF (90 mg/kg, s.c.),

LA(30)+GF (Group 7): LA (30 mg/kg, t.i.d. for 2 days) and Glycerol formal,

LA(30)+PMSF (Group 8): LA (30 mg/kg, t.i.d. for 2 days) and PMSF (90mg/kg, s.c.).

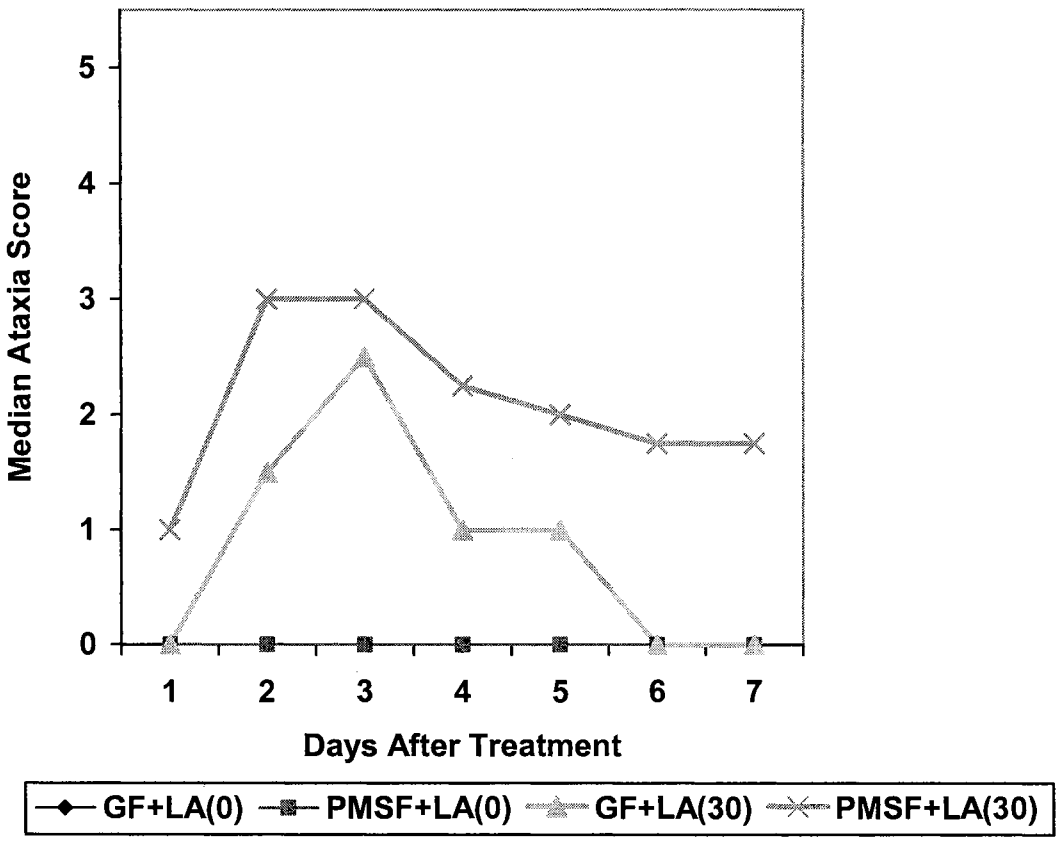

Figure 7. Effects of PMSF pretreatment on LA-induced ataxia in 6 week-old chickens.

LA(0)+GF (Group 5): Control,

LA(0)+ PMSF (Group 6): LA (0 mg/kg) and post treatment with PMSF (90 mg/kg, s.c.),

LA(30)+GF (Group 7): LA (30 mg/kg, t.i.d. for 2 days) and Glycerol formal,

LA(30)+PMSF (Group 8): LA (30 mg/kg, t.i.d. for 2 days) and PMSF (90mg/kg, s.c.).

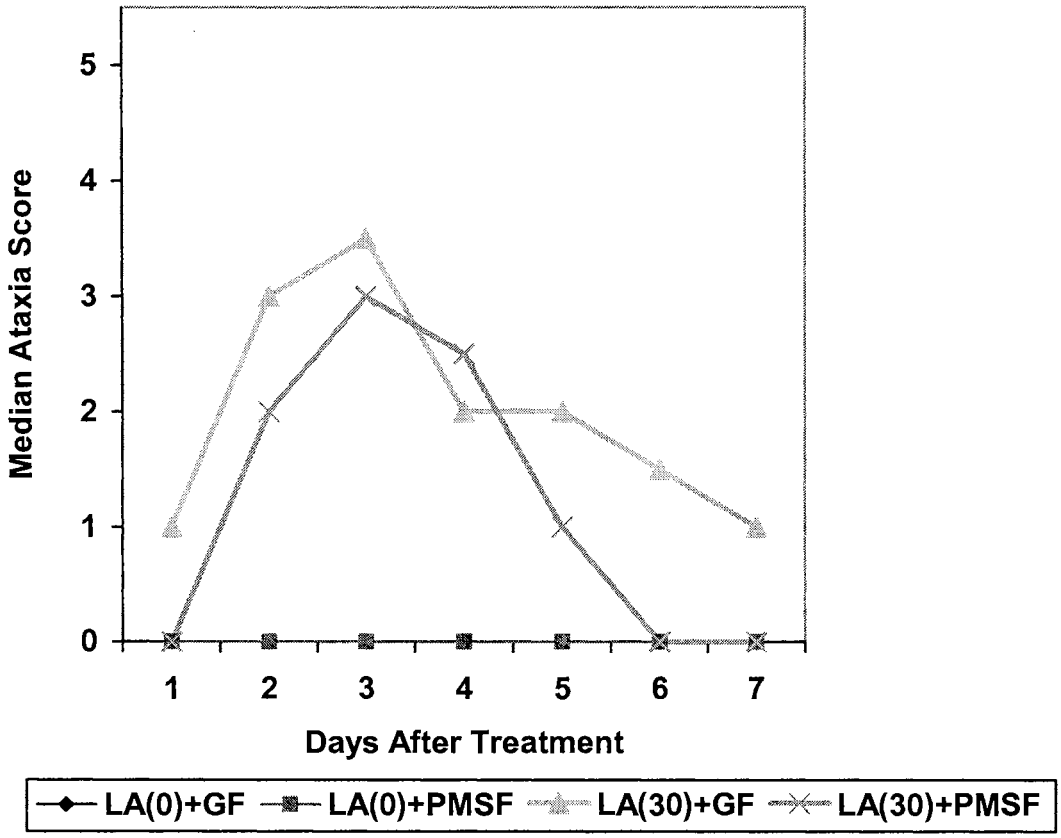

Figure 8. Effects of PMSF posttreatment on LA-induced ataxia in 6 week-old chickens.

LA(0)+GF (Group 5): Control,

LA(0)+ PMSF (Group 6): LA (0 mg/kg) and post treatment with PMSF (90 mg/kg, s.c.),

LA(30)+GF (Group 7): LA (30 mg/kg, t.i.d. for 2 days) and Glycerol formal,

LA(30)+PMSF (Group 8): LA (30 mg/kg, t.i.d. for 2 days) and PMSF (90mg/kg, s.c.).

NTE Activity

Figure 9 shows NTE activities of 3 week-old chickens in PMSF pretreatment group. Brain, spinal cord and sciatic nerve of birds in PMSF and PMSF+LA treatment groups had a significant reduction of NTE activity compared to control and LA only group. However, there was no difference between PMSF only and PMSF+LA groups. Birds treated with LA only did not show any difference from control.

Figure 10 shows NTE activities of 3 week-old chickens in PMSF postreatment group. Similarly, birds treated with PMSF and LA+PMSF had a significant reduction in NTE activity compared to control. LA only group of NTE activities was not different from control.

Figure 11 shows NTE activities of 6 week-old chickens in PMSF pretreatment group. Similar to 3 week-old chickens only PMSF and PMSF+LA treated birds had reduced NTE activity.

Figure 12 shows NTE activities of 6 week-old chickens in PMSF postreatment group. Similar to 3 week-old chickens, while groups treated with PMSF only and LA+PMSF had reduced NTE activity, no difference was found in LA treated birds compared to control.

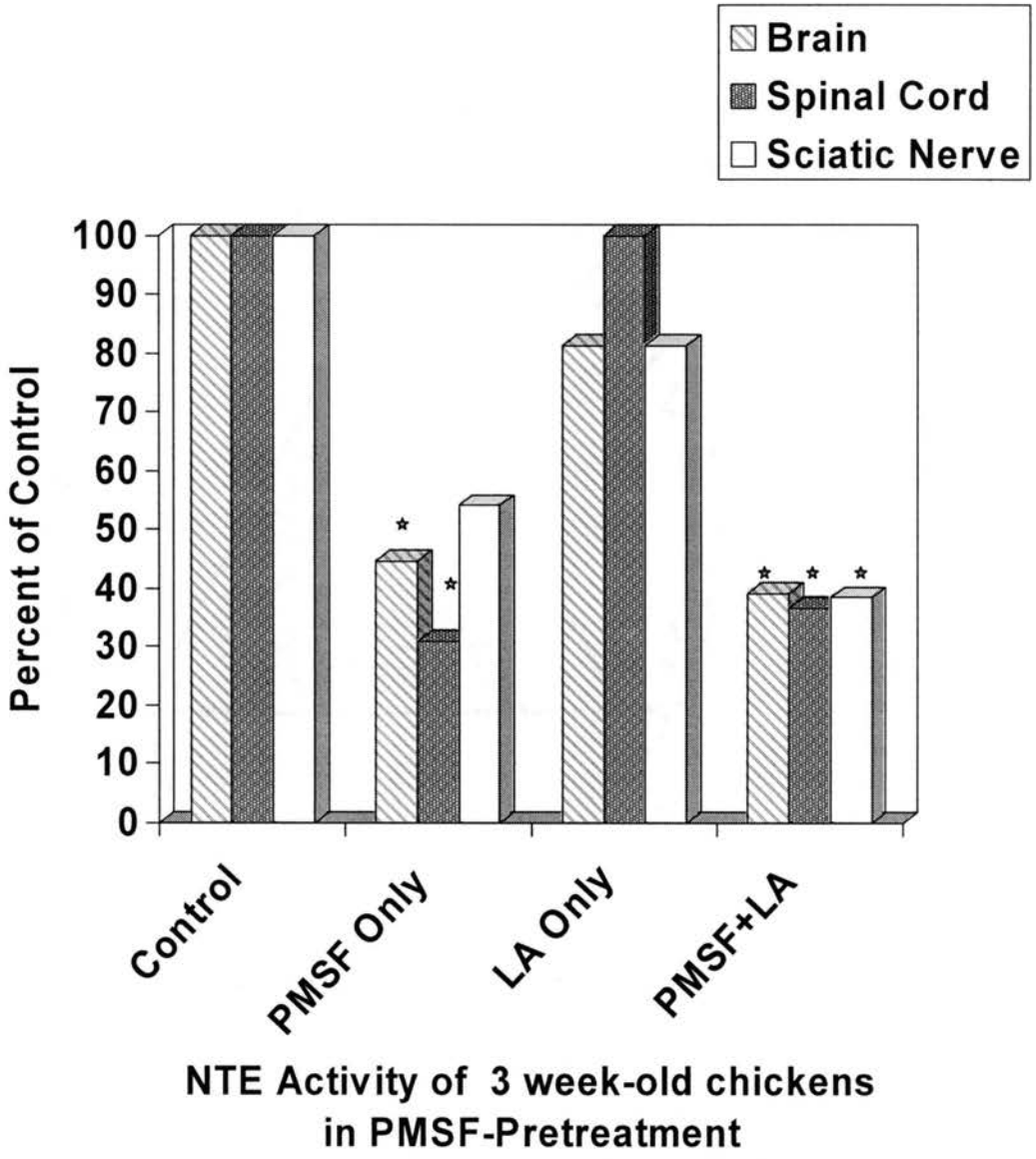

Figure 9. Inhibition of neurotoxic esterase (NTE) activity in Brain, Spinal Cord and Sciatic Nerve as percent of control in PMSF pretreatment in 3 week-old chickens.
* Indicates significant effect of PMSF compared to control, $p < 0.05$.

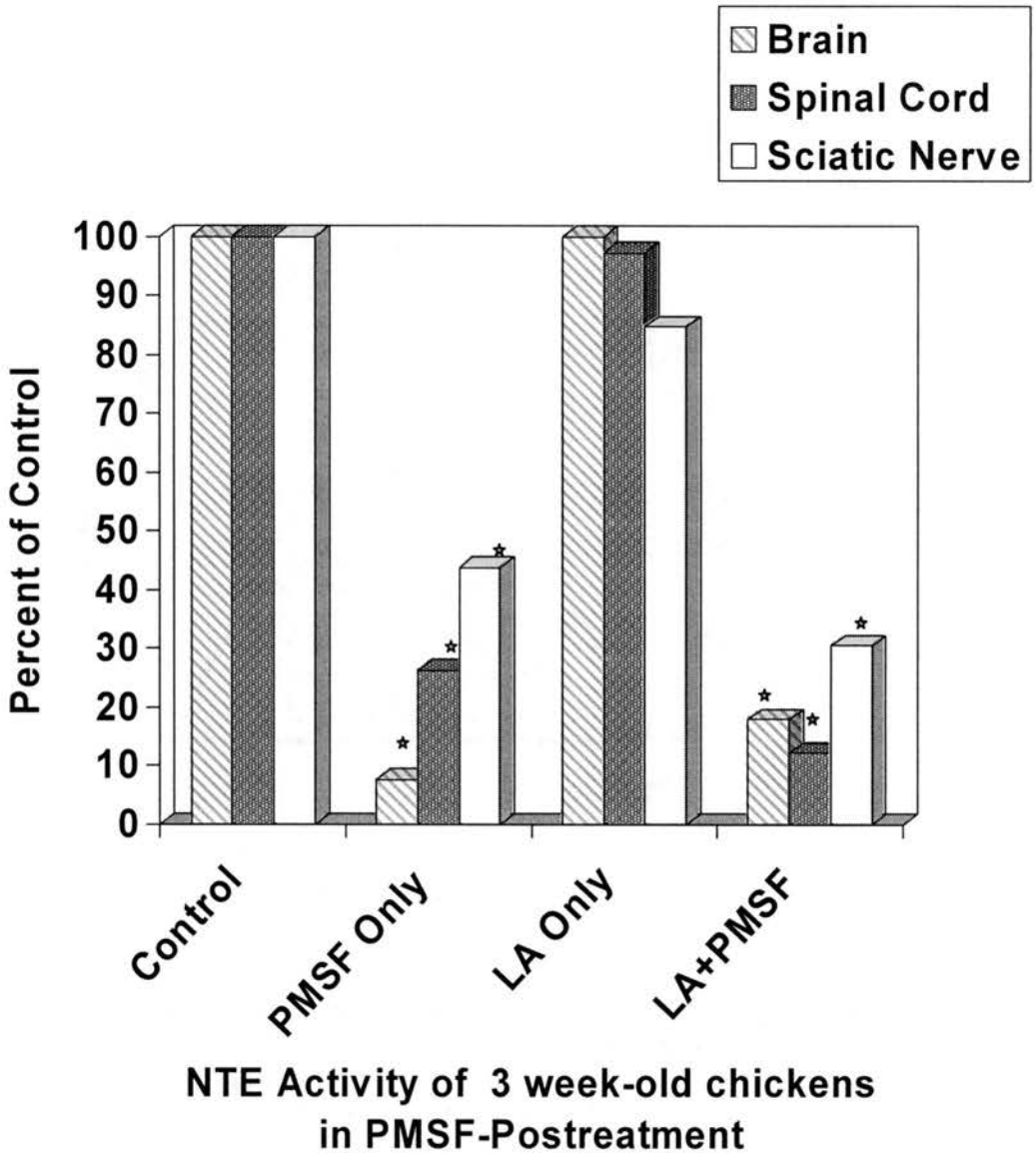

Figure 10. Inhibition of neurotoxic esterase (NTE) activity in Brain, Spinal Cord and Sciatic Nerve as percent of control in PMSF postreatment in 3 week-old chickens.
* Indicates significant effect of PMSF compared to control, $p < 0.05$.

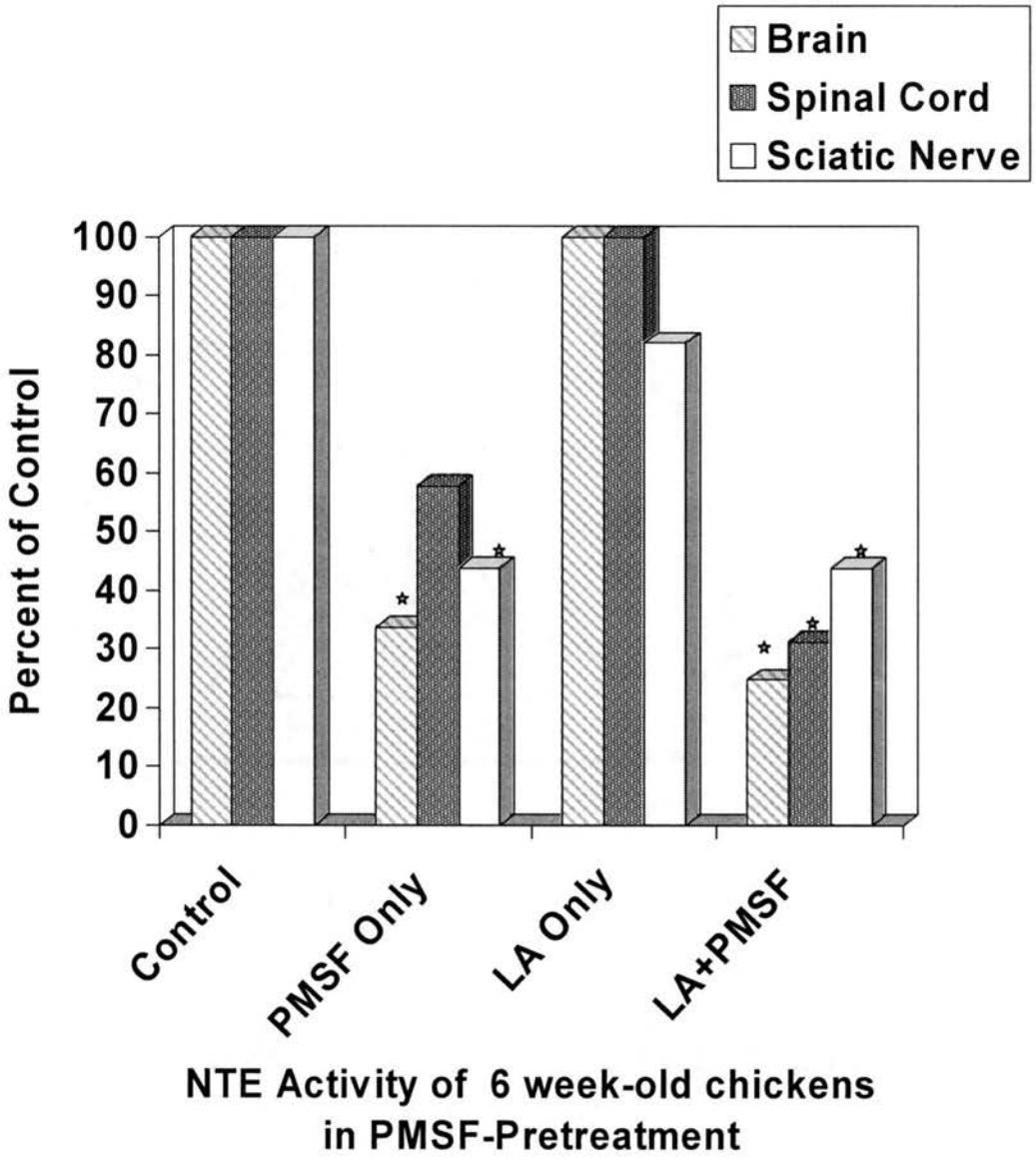

Figure 11. Inhibition of neurotoxic esterase (NTE) activity in Brain, Spinal Cord and Sciatic Nerve as percent of control in PMSF pretreatment in 6 week-old chickens.
* Indicates significant effect of PMSF compared to control, $p < 0.05$.

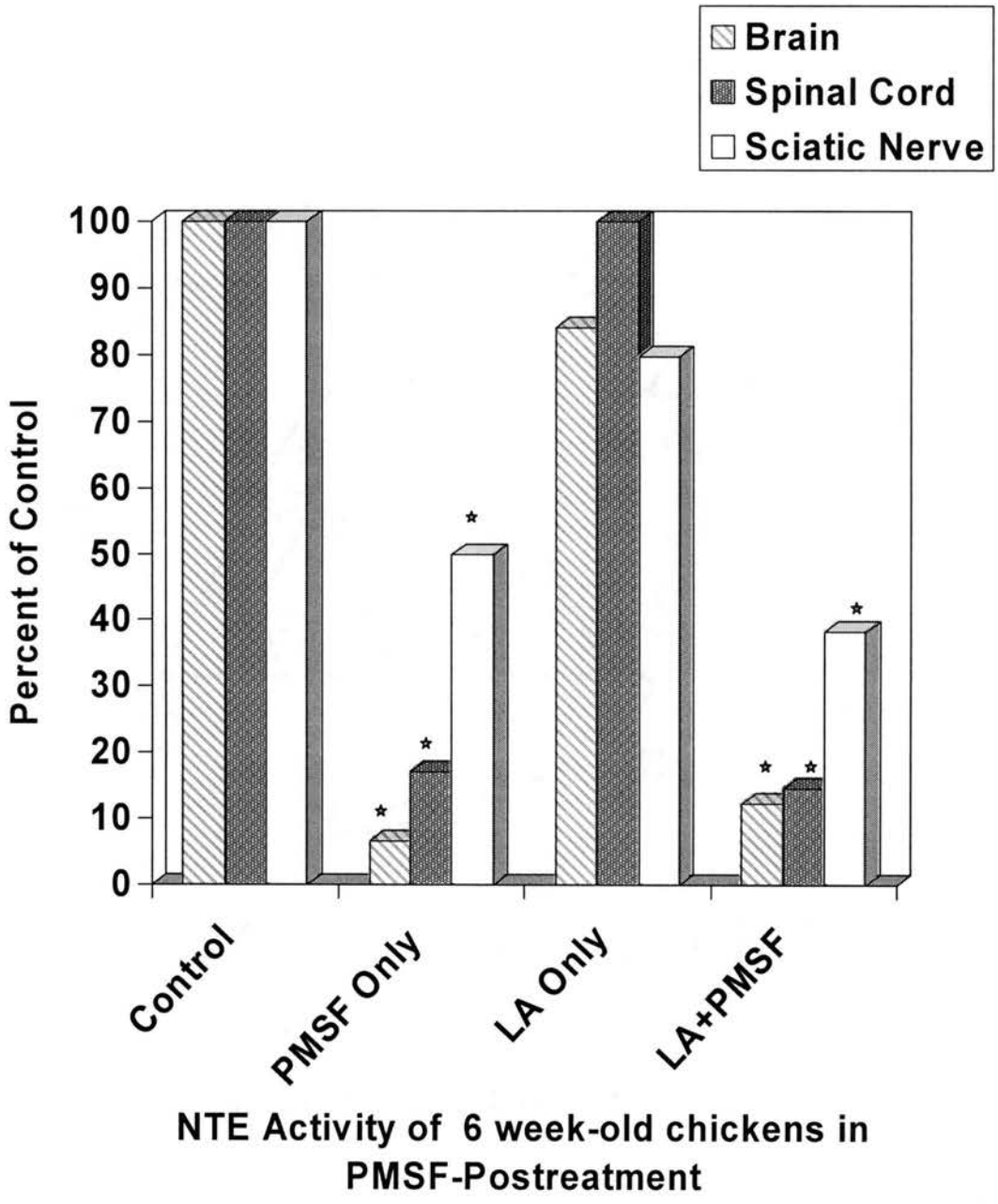

Figure 12. Inhibition of neurotoxic esterase (NTE) activity in Brain, Spinal Cord and Sciatic Nerve as percent of control in PMSF postreatment in 6 week-old chickens.
* Indicates significant effect of PMSFcompared to control, $p < 0.05$.

Motor Nerve Conduction Velocity

Figure 13 and 14 show average motor nerve conduction velocity of pre- and post PMSF treated birds at 3 weeks old age. Mean MNCVs of control birds were 30.7 ± 2.2 m/sec (Group 1) and 36.0 ± 4.4 m/sec (Group 5) in 3 week-old birds, respectively. MNCVs of groups receiving only PMSF were not different from that of the controls. LA treated birds and birds receiving combination of LA and PMSF had decreased MNCVs compared to their respective control groups. However, PMSF did not affect MNCVs of in LA-treated birds.

Figure 13 and 14 show average motor nerve conduction velocities of pre- and post PMSF treated birds at 6 weeks old age. Six week-old chickens treated with LA or PMSF+LA had significantly reduced MNCV in comparison to the control groups, but PMSF did not appear to influence MNCV alone or in combination with LA. Control birds at 6 weeks of age had average MNCVs of 34.4 ± 0.7 m/sec and 35.4 ± 2.3 m/sec, respectively. Similar to 3 week-old chickens, 6 week-old chickens given only PMSF did not show any difference in MNCV.

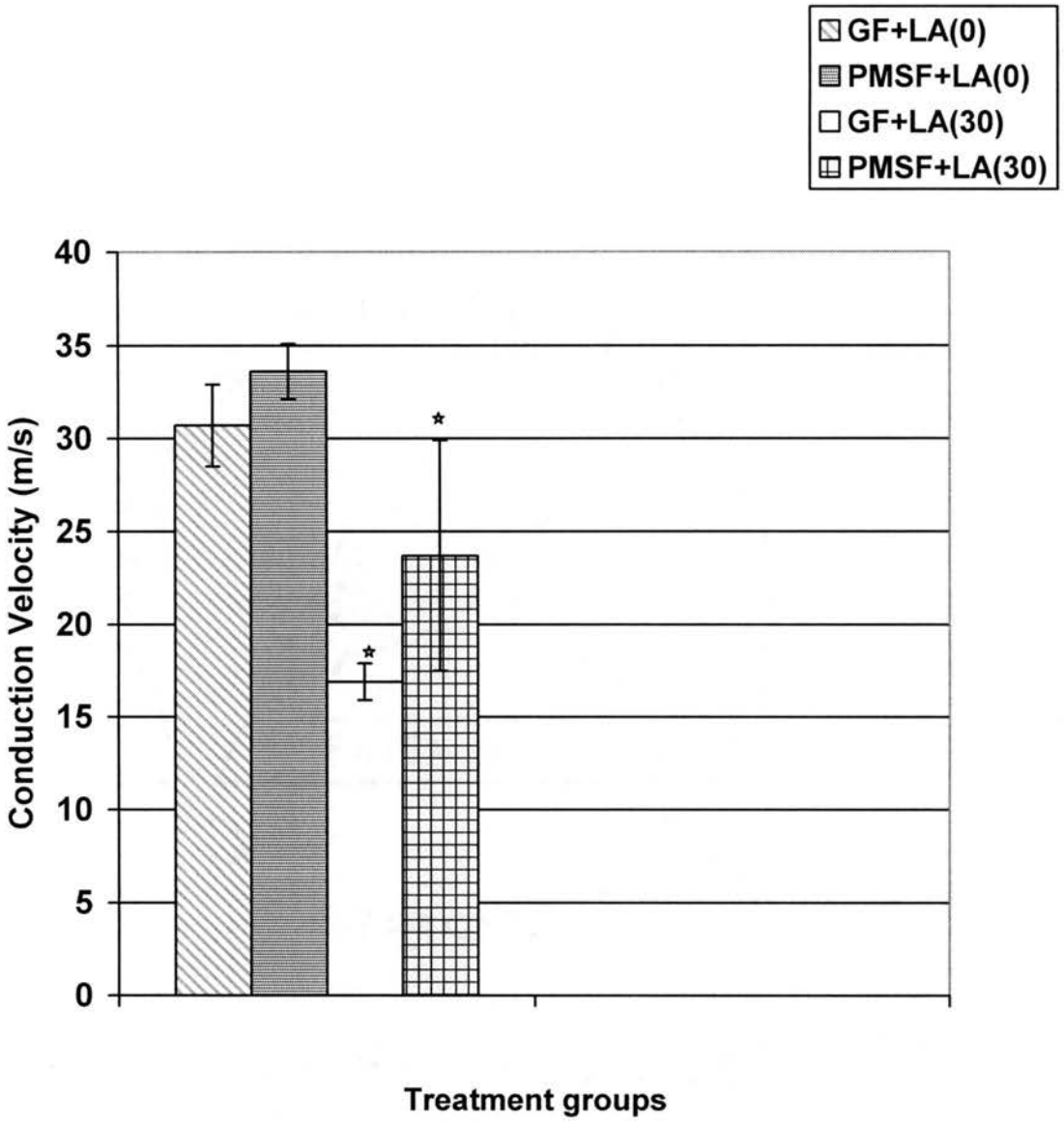

Figure 13. Effects of PMSF pretreatment on MNCVs in 3 week-old chickens.
Mean conduction velocity (m/s) ± sd * Indicates significant decrease in MNCV compared to the control, $p < 0.05$.

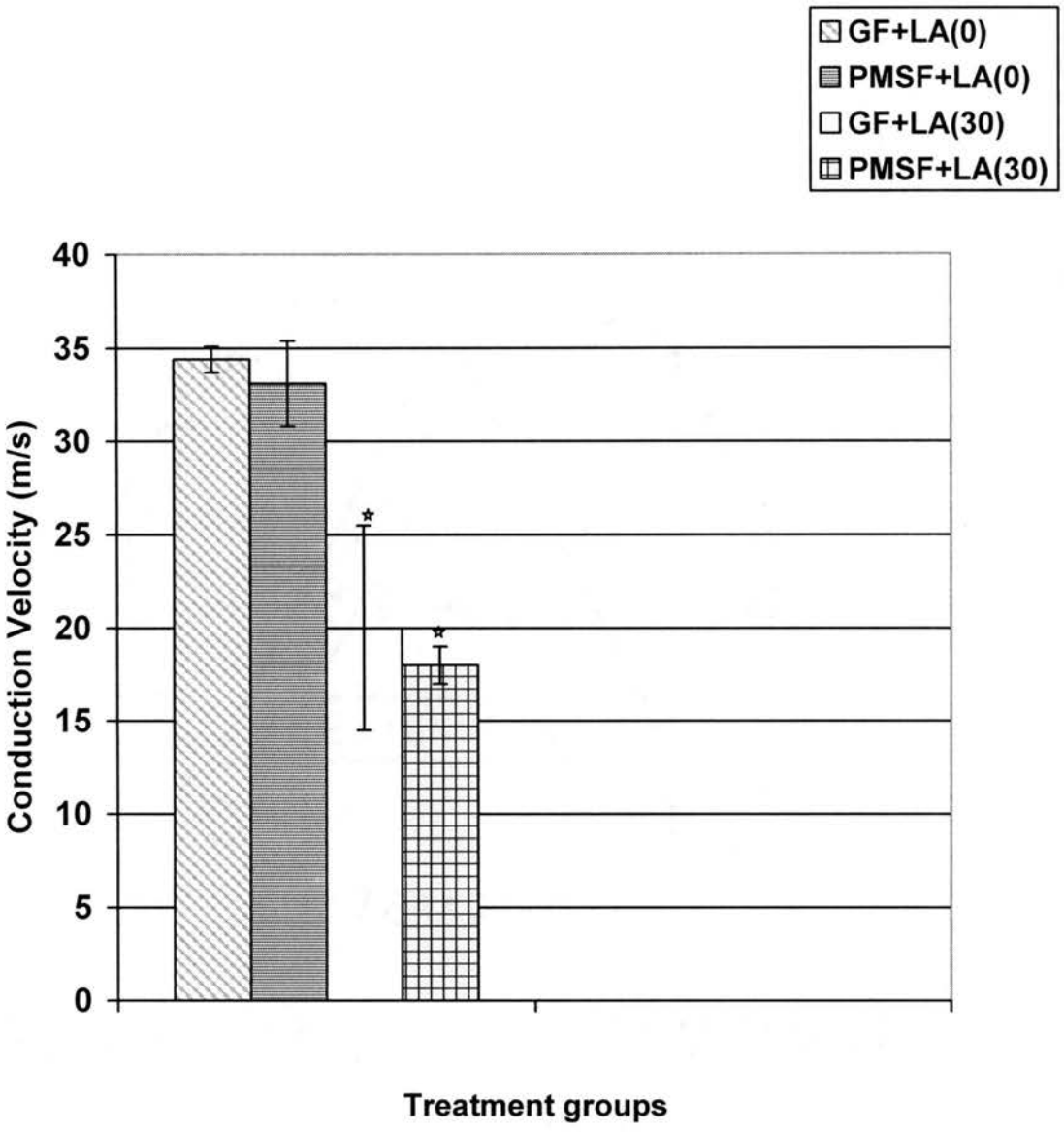

Figure 14. Effects of PMSF pretreatment on MNCVs in 6 week-old chickens. Mean conduction velocity (m/s) ± sd * Indicates significant decrease in MNCV compared to the control, $p < 0.05$.

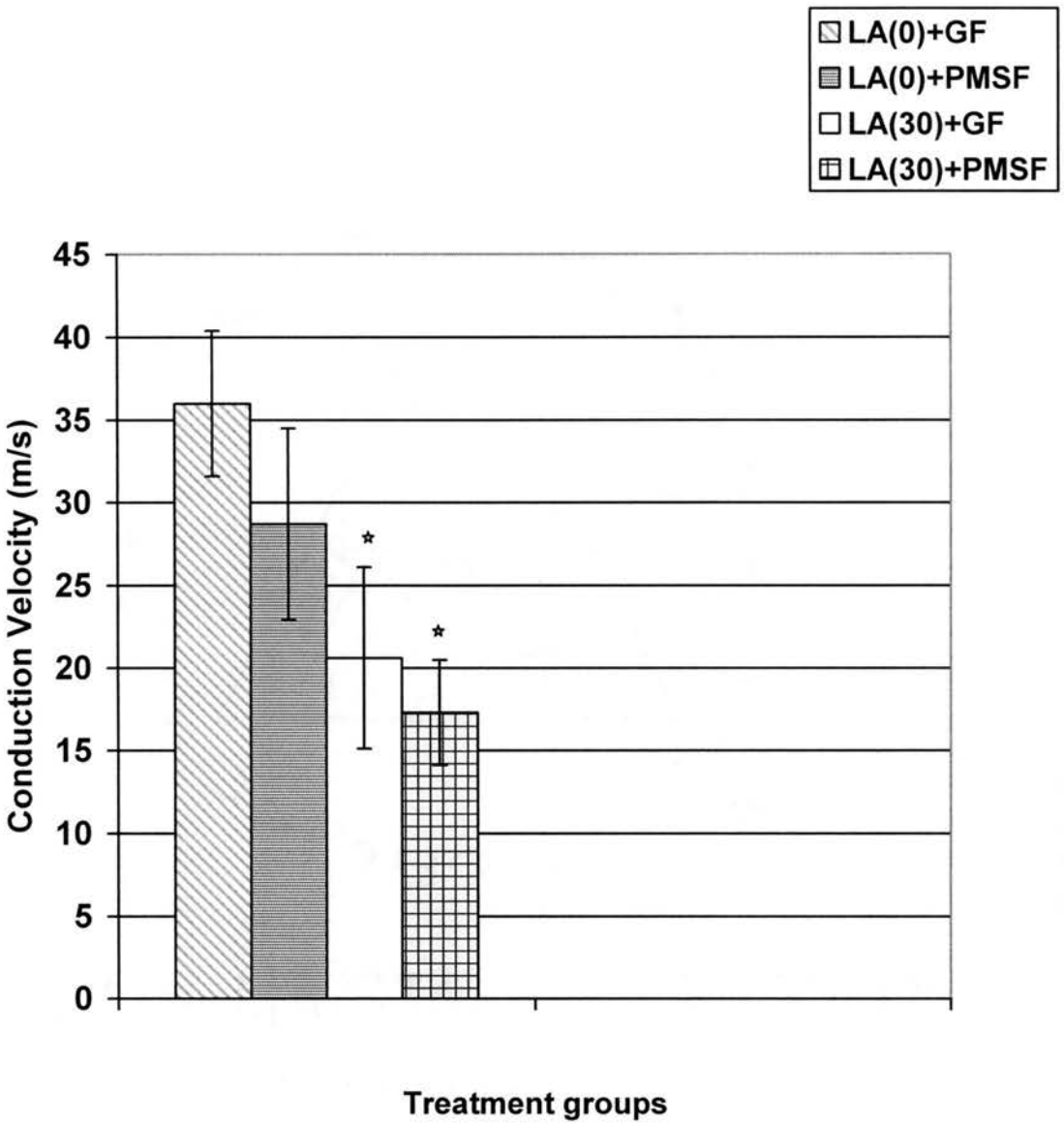

Figure 15. Effects of PMSF posttreatment on MNCVs in 3 week-old chickens. Mean conduction velocity (m/s) ± sd * Indicates significant decrease in MNCV compared to the control, $p < 0.05$.

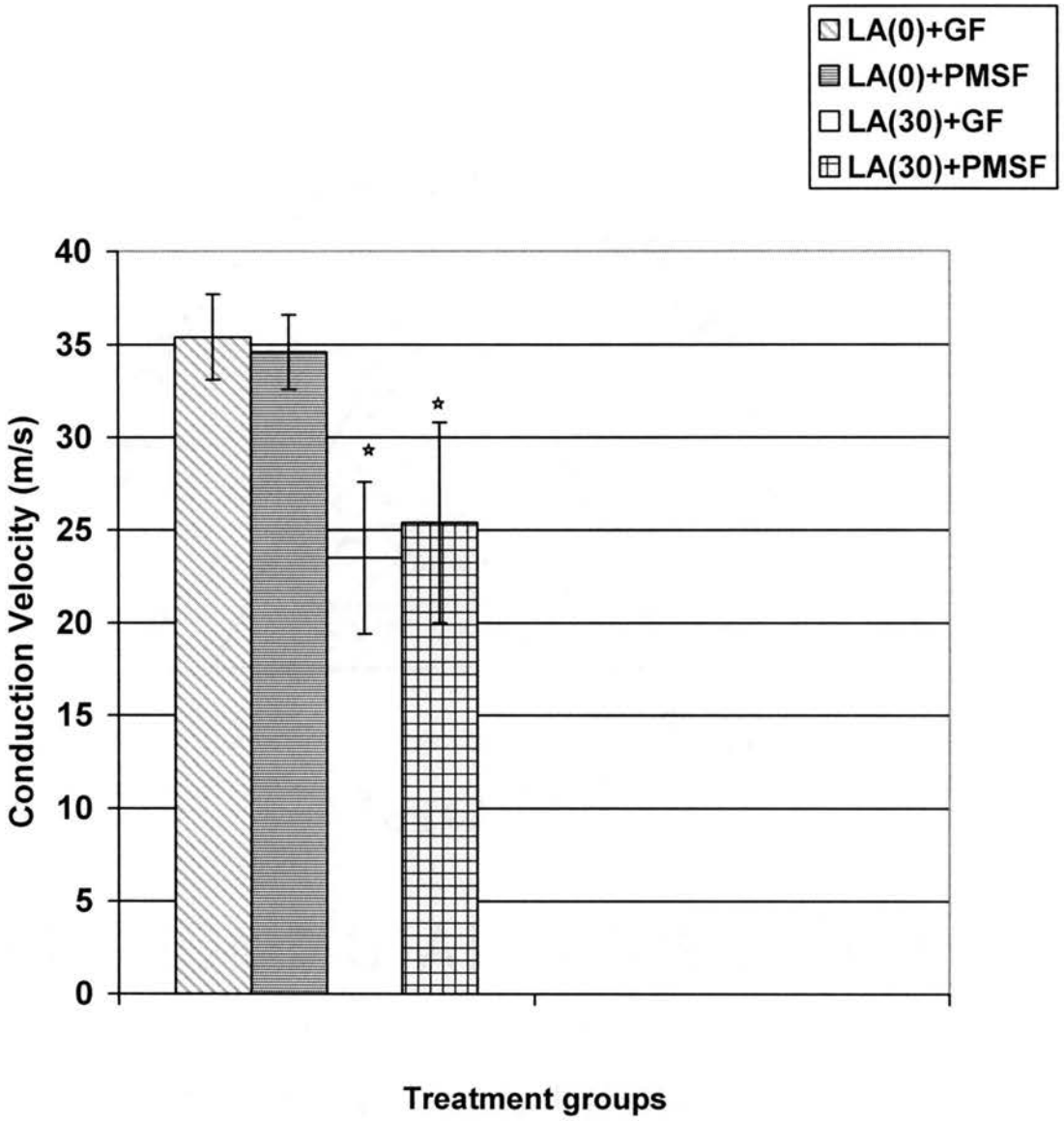

Figure 16. Effects of PMSF posttreatment on MNCVs in 6 week-old chickens. Mean conduction velocity (m/s) ± sd * Indicates significant decrease in MNCV compared to the control, $p < 0.05$.

Light and Electron Microscopic Changes

Light Microscopy

Figure 17 and 18 show sciatic nerve from groups treated with LA. Similar to birds receiving combination of LA and PMSF, these birds showed vacuolated myelin (voids), fragmented axons and digestion chambers.

Figure 19 shows spinal cord of an ataxic bird treated with LA+PMSF. Lesions in the spinal cord included fragmented axons with areas of myelin vacuolations. Three week-old birds receiving LA and post treatment of PMSF had lesions in the spinal cord. Mild lesions were distributed primarily in the dorsal spinocerebellar tracts, fasiculi gracilus and rarely the ventral corticospinal tract. No spinal cord lesions were observed in any other groups of LA or LA with PMSF treated birds in either 3 or 6 week-old birds.

Figure 20 shows sciatic nerve of an ataxic bird treated with LA and PMSF. Lesions included vacuolated myelin (voids), fragmented axons and digestion chambers.

Mean degeneration scores of the sciatic nerve in groups receiving LA and combination of LA and PMSF were significantly higher than those of control in 3 and 6 week-old chickens (Table 3 and 4). No lesions were noted in control or groups receiving only PMSF either in the spinal cord or sciatic nerve.

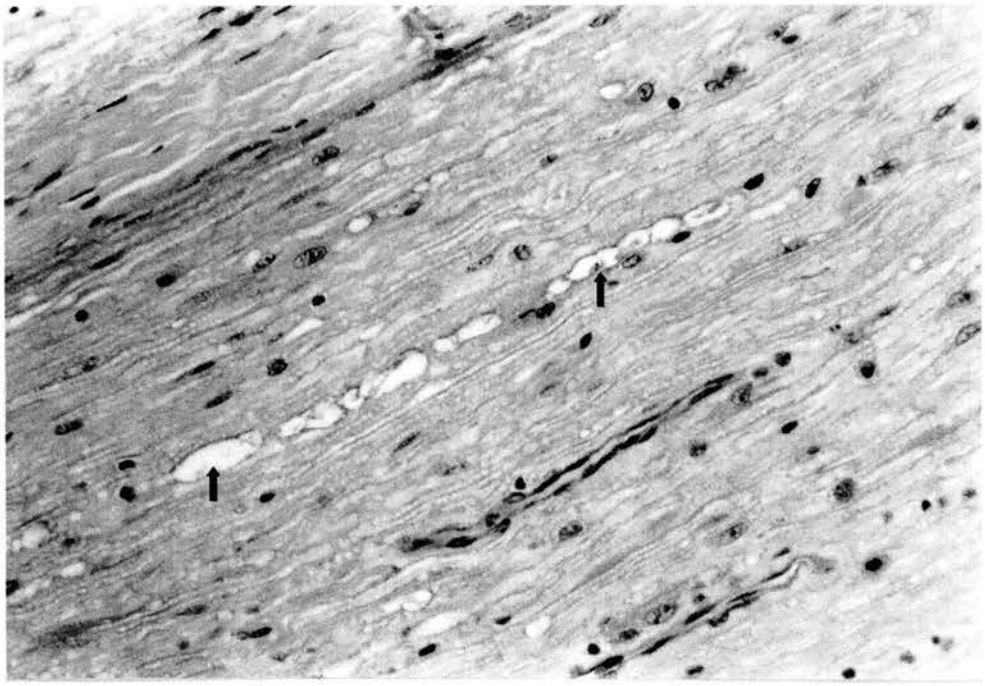

Figure 17. Light micrograph of a sciatic nerve from an ataxic bird treated with lasalocid. Areas of myelin vacuolation (arrows). (H&E), (Magnification 400x)

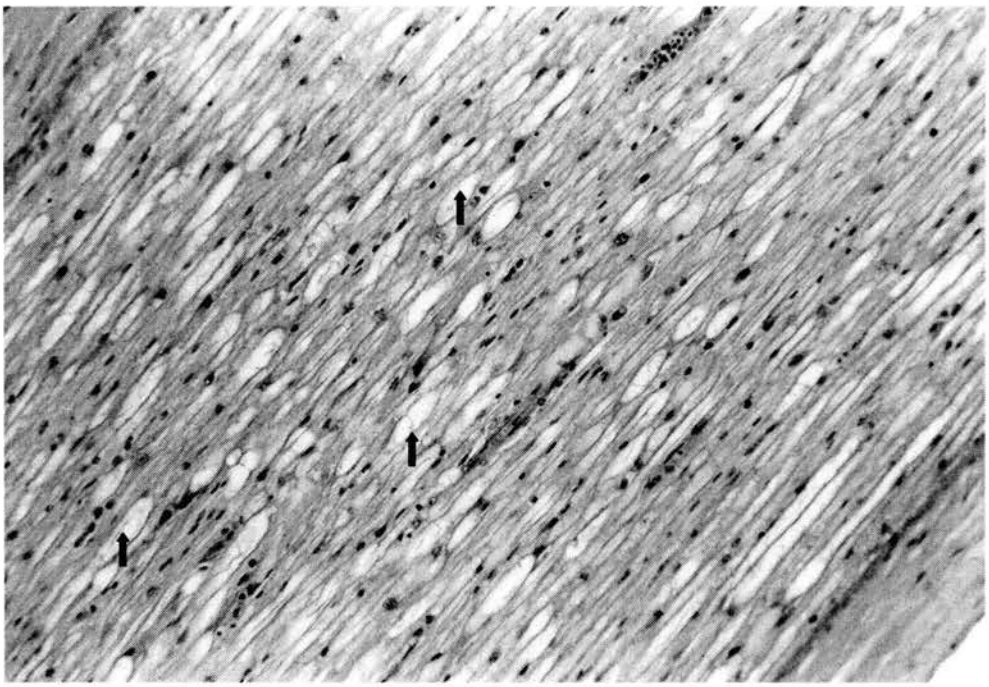

Figure 18. Light micrograph of a sciatic nerve from an ataxic bird treated with lasalocid. Foamy myelin vacuoles with axonal fragments (arrows). (H&E), (Magnification, 200x)

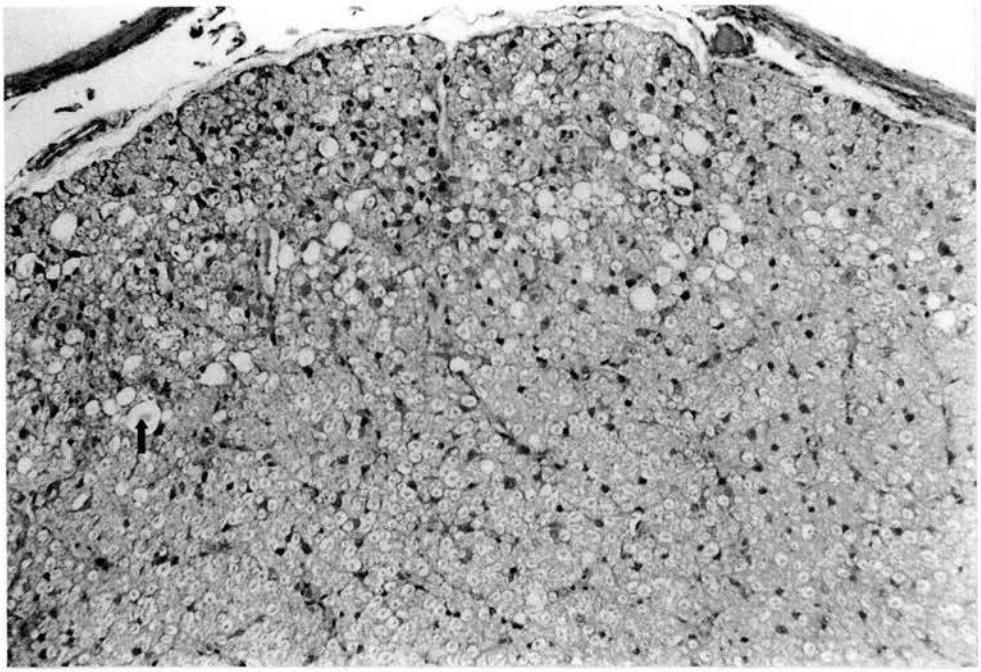

Figure 19. Light micrograph of a spinal cord from an ataxic bird treated with lasalocid and PMSF. Fragmented axon (Arrow). (H&E), (Magnification, 200x)

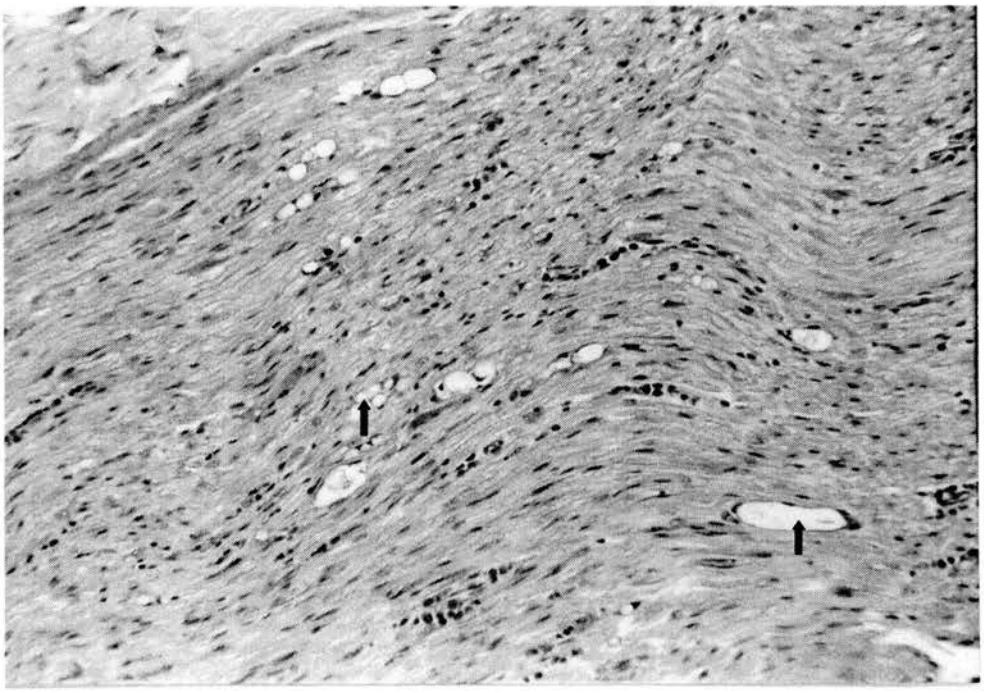

Figure 20. Light micrograph of a sciatic nerve from an ataxic bird treated with lasalocid and PMSF. Multiple areas of myelin vacuolation (arrows). (H&E), (Magnification, 200x)

Table III. Mean degeneration scores in the Sciatic Nerve, Spinal Cord, Brain and Muscle of 3 week-old birds. * Indicates significant difference compared to the controls.
† Indicates significant difference compared to PMSF+LA (30)

PMSF Pre-Treatment	Tissue	Score (Mean)	PMSF Post-Treatment	Tissue	Score (Mean)
1. GF 2. LA (0mg/kg)	Sciatic N.	0	1. LA (0 mg/kg) 2. GF	Sciatic N.	0
	Spinal C.	0		Spinal C.	0
	Brain	0		Brain	0
	Muscle	0		Muscle	0
1. PMSF 2. LA (0mg/kg)	Sciatic N.	0	1. LA (0mg/kg) 2. PMSF	Sciatic N.	0
	Spinal C.	0		Spinal C.	0
	Brain	0		Brain	0
	Muscle	0		Muscle	0
1. GF 2. LA (30mg/kg)	Sciatic N.	3* †	1. LA (30mg/kg) 2. GF	Sciatic N.	2.6*
	Spinal C.	0		Spinal C.	0
	Brain	0		Brain	0
	Muscle	0		Muscle	0
1. PMSF 2. LA (30mg/kg)	Sciatic N.	1.3*	1. LA (30mg/kg) 2. PMSF	Sciatic N.	2.6*
	Spinal C.	0		Spinal C.	0.6*
	Brain	0		Brain	0
	Muscle	0		Muscle	0.3

Table IV. Mean degeneration scores in the Sciatic Nerve (Sciatic N.), Spinal Cord (Spinal C.), Brain and Muscle of 6 week-old birds. * Indicates significant difference compared to the controls. † Indicates significant difference compared to PMSF+LA (30)

PMSF Pre-Treatment	Tissue	Score (Mean)	PMSF Post-Treatment	Tissue	Score (Mean)
1. GF 2. LA (0mg/kg)	Sciatic N.	0	1. LA (0 mg/kg) 2. GF	Sciatic N.	0
	Spinal C.	0		Spinal C.	0
	Brain	0		Brain	0
	Muscle	0		Muscle	0
1. PMSF 2. LA (0mg/kg)	Sciatic N.	0	1. LA (0mg/kg) 2. PMSF	Sciatic N.	0
	Spinal C.	0		Spinal C.	0
	Brain	0		Brain	0
	Muscle	0		Muscle	0
1. GF 2. LA (30mg/kg)	Sciatic N.	2.6* †	1. LA (30mg/kg) 2. GF	Sciatic N.	2.0*
	Spinal C.	0		Spinal C.	0
	Brain	0		Brain	0
	Muscle	0		Muscle	0
1. PMSF 2. LA (30mg/kg)	Sciatic N.	2.0*	1. LA (30mg/kg) 2. PMSF	Sciatic N.	2.3*
	Spinal C.	0		Spinal C.	0
	Brain	0		Brain	0
	Muscle	0		Muscle	0

Electron Microscopy

Figure 21 shows an electron micrograph of a sciatic nerve from the control group. Cross sections of sciatic nerve from control birds appeared normal. Nerve fibers had ovoid or round shape with evenly arranged myelin lamellae. Myelin layers were compact, and borders between myelin structure and axon were tightly attached. The axons had normal appearance with homogeneous distribution of neurofilaments and microtubules.

Figure 22 is a section of sciatic nerve taken from an ataxic bird treated with LA and PMSF. As figure 22 shows, separation of myelin layers and extensive intramyelinic edema were evident. There was also myelin derangement and myelin fragmentation.

Figure 23 and 24 show electron micrographs from the groups treated with LA and PMSF. Prominent vacuolation within myelin layers was observed and the vacuolation were in close proximity to the axonal region. The axon was compressed and distorted as a result of edema and myelin disruption. The Schwann cell looked normal. In addition, tissues surrounding the nerve and Schwann cell appeared normal with evenly distributed collagen fibers.

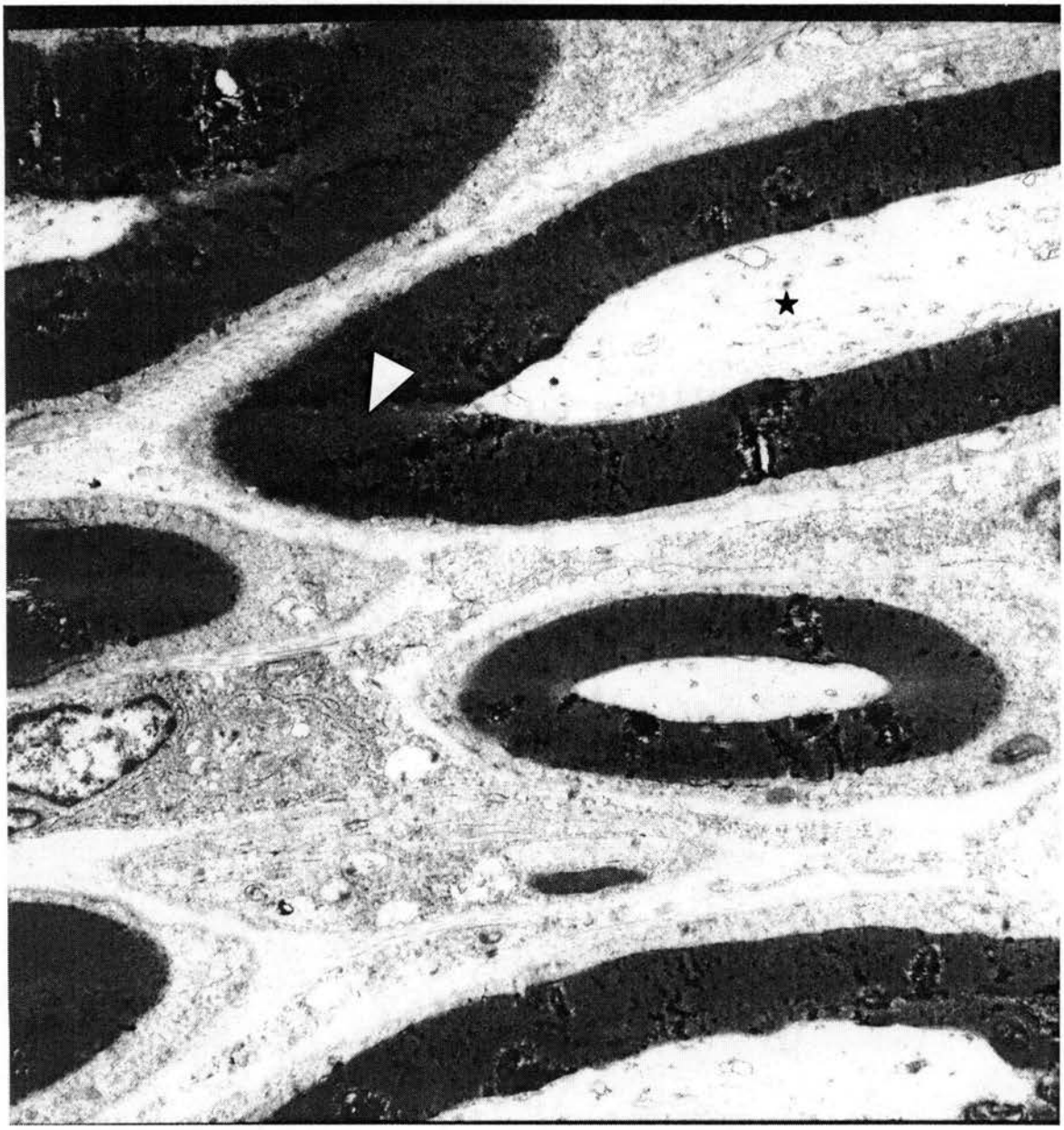

Figure 21. Electron Micrograph of a Sciatic Nerve from the control group. Myelin sheath (diamond), axon (star) (x11,500)

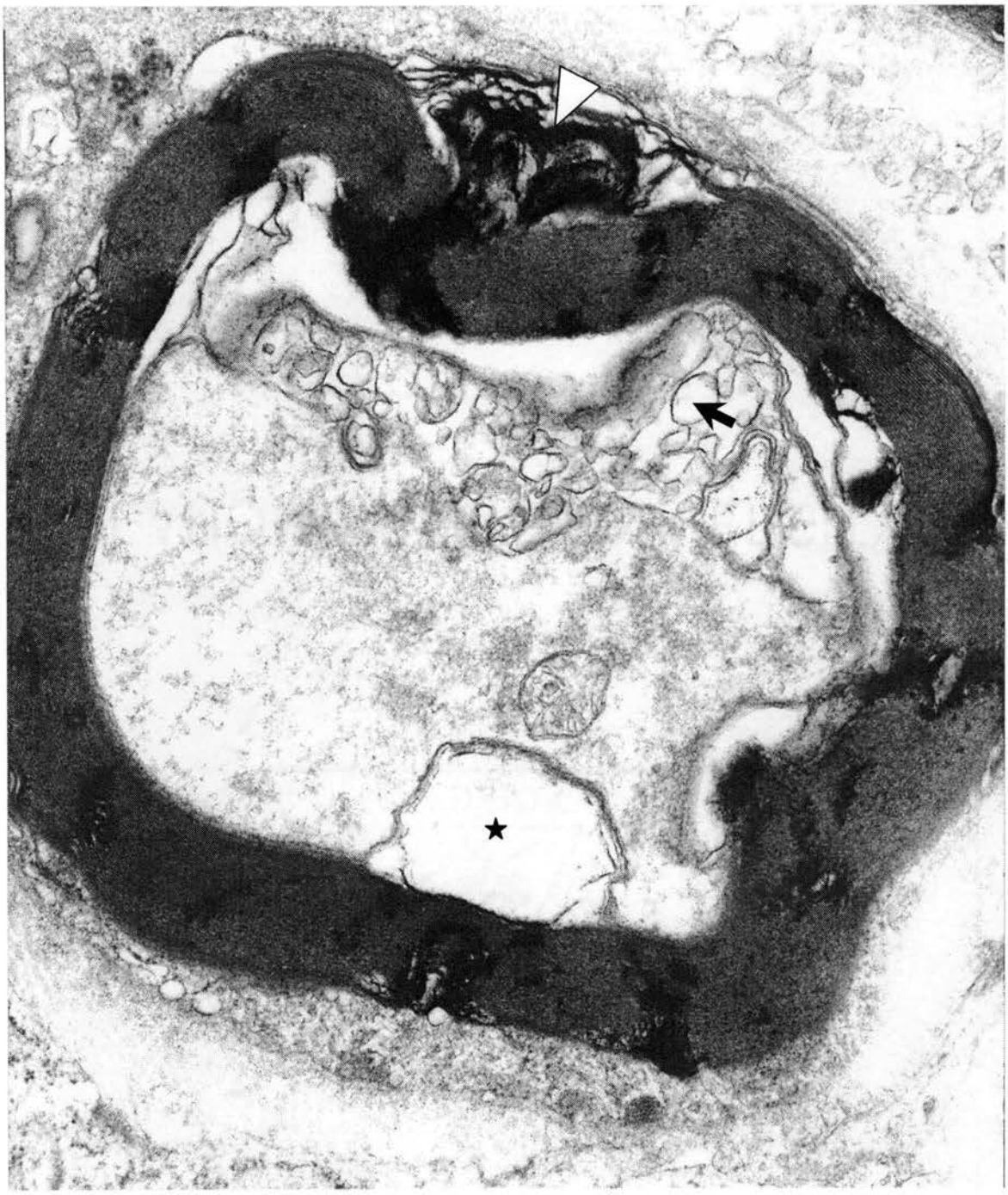

Figure 22. Electron Micrograph of a Sciatic Nerve from LA and PMSF treated chicken. Vacuoles within myelin (Thick arrow), . Separation of myelin layers and formation of edema (Star), and myelin fragmentation (diamond). (x46,000)

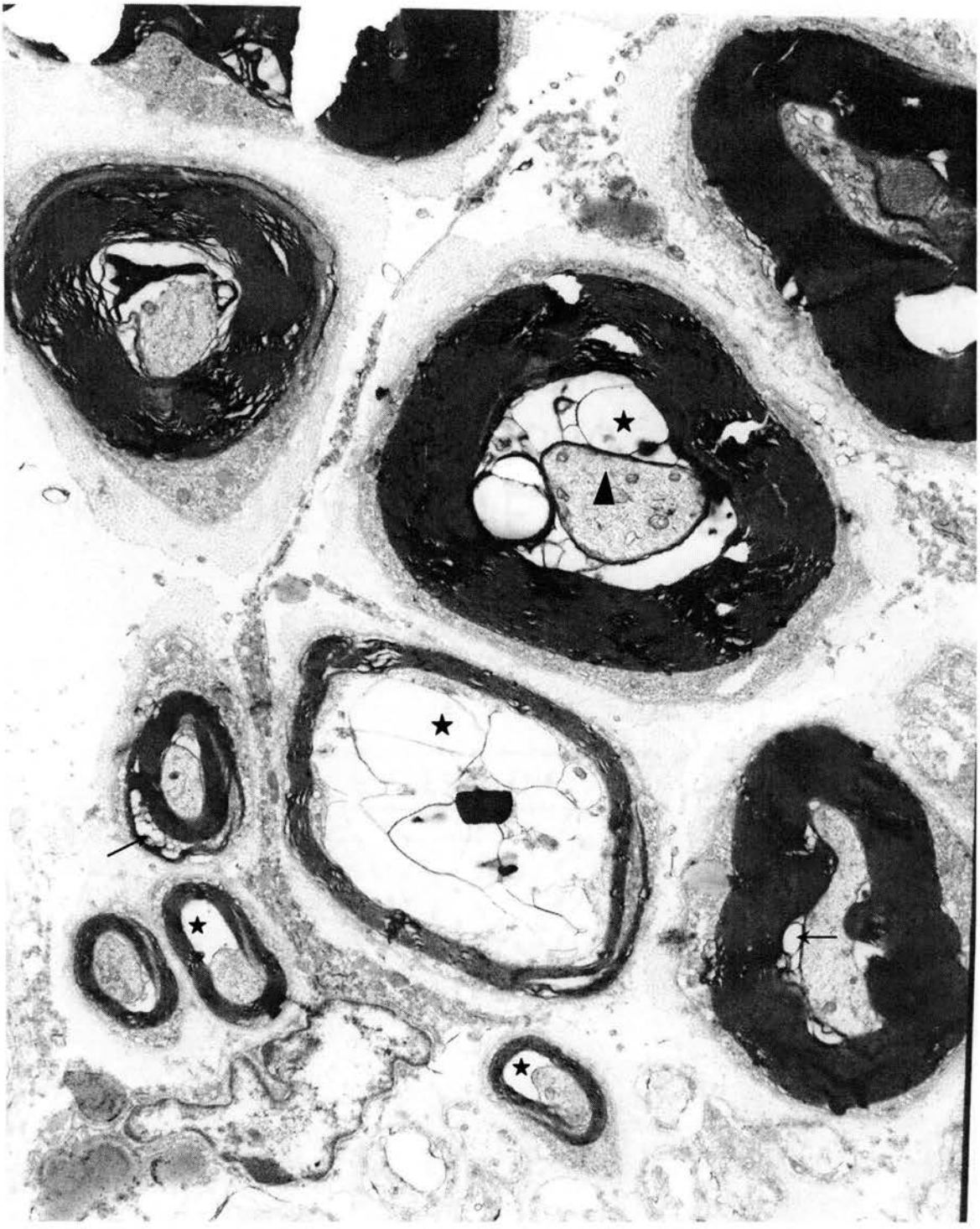

Figure 23. Electron Micrograph of a Sciatic Nerve from LA and PMSF treated bird. Extensive edema (star), myelin vacuolation (arrow), intact compressed axon (Arrow head). (x10,000)

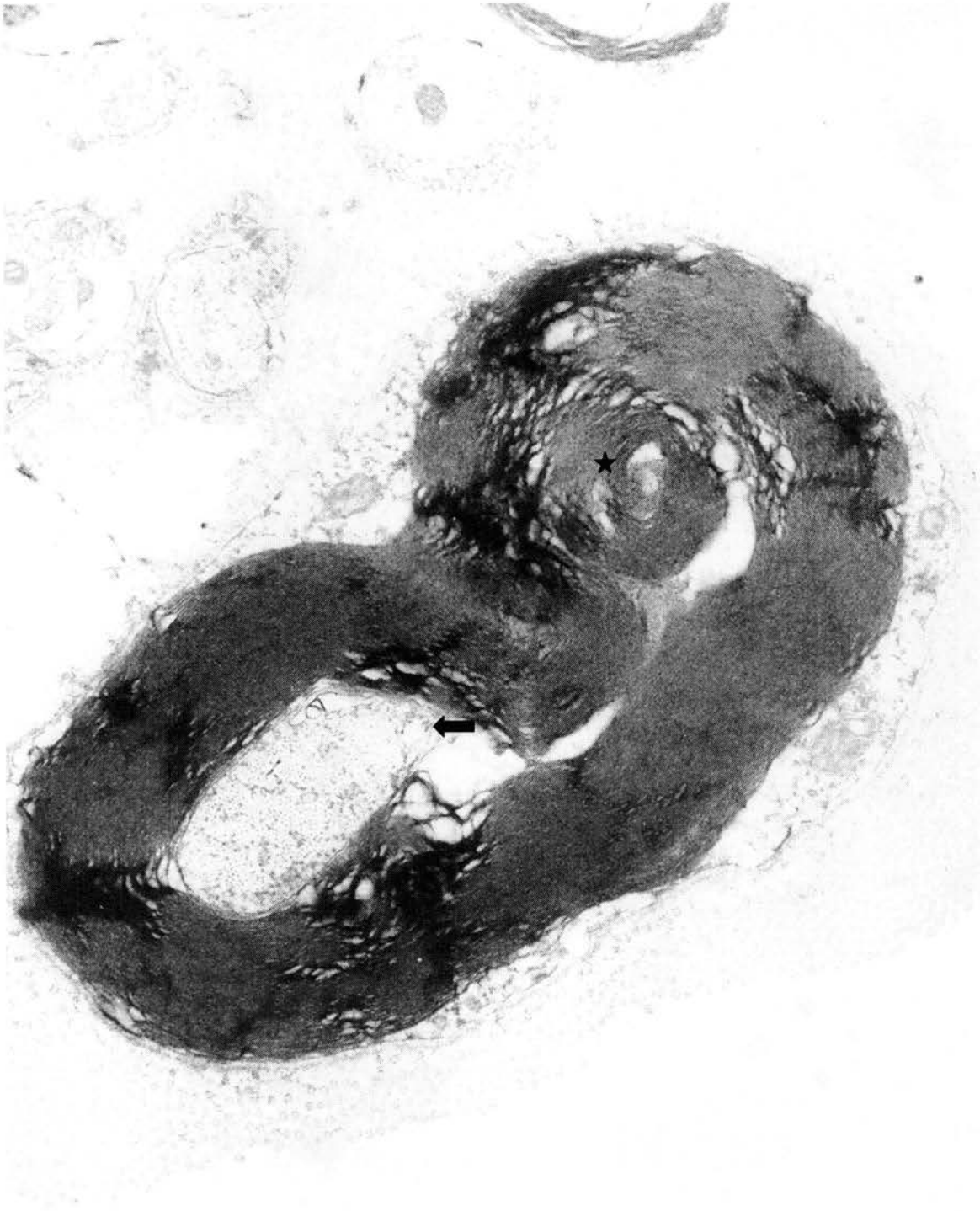

Figure 24. Electron Micrograph of the Sciatic Nerve from a LA and PMSF treated birds. Myelin fragment (star), intact-compressed axon (Arrow). (x32,000)

Discussion:

A wide range of xenobiotics have been shown to cause neuropathies in man and experimental animals. Some organophosphorus toxicants have been known for decades to cause neuropathy. Other toxicants including hexacarbons and acrlyamide can cause neuropathy through poorly understood mechanisms. Previous studies showed that lasalocid (LA) and some other ionophores can cause a neuropathy in chickens through facilitation of mono and divalent cation transport across cell membranes (Halvorson et al., 1982; Gregory et al., 1995; Roder, 1996; Van der Linde-Sipman et al., 1999).

PMSF, a serine protease inhibitor, can modify the progression of OPIDN (Johnson, 1969; Pope and Padilla, 1990; Harp et al 1997) and other forms of neurodegeneration (Moretto et al, 1992). The studies presented here show that PMSF may also modulate the neuropathy induced by LA. In our studies, LA (30 mg/kg, p.o., t.i.d. for 2 days) induced moderate to severe ataxia and death in 3 and 6 week old chickens. Neurotoxic signs of LA alone were ataxia, loss of righting reflex, paralysis of hindlimbs and death. Signs of neuropathy noted following LA were very similar to those reported by Roder (1996).

While PMSF pretreatment increased the severity of ataxia in 3 week-old birds, PMSF post-treatment did not substantially affect ataxia. The results obtained here are not comparable to potentiation of OPIDN by PMSF, because of different time dependent effects of PMSF on OPIDN. It was shown that PMSF either prevents or potentiates OPIDN depending on the sequence of administration of PMSF. When given prior to OP exposure, PMSF protected animals from neurotoxic challenge (Carrington and Abou-Donia, 1983; Pope and Padilla, 1990; Massicotte et al, 1999), while when given after OP exposure, PMSF potentiated OPIDN (Harp et al, 1997; Pope and Padilla, 1990). It was

proposed that the protective effect of PMSF is related to prior occupation of the active site of NTE thus preventing the neuropathic OP from binding NTE. In the case of potentiation, Pope and Padilla (1990) suggested involvement of another PMSF binding site which results in modification of subsequent steps in OPIDN or disruption of cellular repair process.

Xu et al., (1999) reported that PMSF significantly increased ataxia induced by p-bromophenylacetylurea in F-344 rats. Increased toxicity was observed in PMSF pretreated rats but not in PMSF post-treated rats. Phenylmethylsulfonyl fluoride increased the concentrations of p-bromophenylacetylurea and its toxic metabolite suggesting that PMSF alters the toxickinetics of p-bromophenylacetylurea, leading to increased concentration of the toxicant in tissues. Similarly, we observed more toxicity in LA-treated birds with PMSF pretreatment, but lesser or no effect with posttreatment. Accordingly, a toxicokinetic interaction between PMSF and LA may be responsible. Moretto et al. (1993) reported that following a severe nerve crush in chickens, PMSF pre and post treatment delayed the recovery period. The authors speculated that PMSF might interfere with repair machinery in the axons since PMSF did not increase the severity of nerve crush but delayed the recovery. Disruption of a repair mechanism by PMSF in the case of potentiation of OPIDN and other chemically-induced neuropathies was also suggested (Pope and Padilla, 1990: Moretto et al., 1993). 2,5- Hexanedione neuropathy was reported to be promoted by PMSF. The putative mechanism of 2,5-Hexanedione toxicity (i.e., crosslinking of neurofilaments) differs from that of OPIDN, suggesting neuropathies caused by different mechanisms can be promoted by PMSF (Moretto et al., 1992).

Pathologic changes in neuronal tissues in response to LA treatment with or without PMSF were interesting in that LA alone did not cause any change in spinal cord or brain, but effects were confined to the sciatic nerve only. Effects included vacuole formation in myelin, empty spaces filled with fluid due to sequestration and breakdown of cytoplasmic component, axon fragmentation and digestion chambers. Such changes are in agreement with the findings reported by Gregory et al. (1995) and Roder (1996). While there was no change in spinal cord of LA treated birds, PMSF pretreatment led to slight but significant cord degeneration in LA-treated birds.

One of the proposed mechanisms of OPIDN is associated with alteration of neurofilament (NF) subunits by OP compounds. It was demonstrated that disopropyl phosphofluoride led to the elevation of NF subunit proteins and mRNAs (Xie, et al., 2001; Xie et al., 2002) and PMSF treatment can modify this effect. It was further suggested that PMSF pretreatment could also modifies the expression of some other cytoskeletal protein mRNA. This effect was shown in transgenic animals that devoid of NFs in their axon (Xie, et al., 2001). This findings indicates that other cytoskeletal proteins can be affected by PMSF pretreatment. Therefore, a possible interference with cytoskeletal proteins and NF by PMSF might have occurred in the pretreatment of LA-neurotoxicity leading to increased response as ataxia and increased lesions in the nerve fibers.

Another possible mechanism might be associated with alteration of lysolecithin. It was shown that NTE may have lysophospho pholipase activity. Lysophospho pholipase hydrolyses lysolecithin as its physiologic substrate which is known to cause myelin degredation in nerves. Since PMSF is an NTE inhibitor, inhibition of lysophospho

pholipase by PMSF can subsequently lead to accumulation of lysolecithin causing an increased response in LA-induced neurotoxicity when given together. However, we observed increased response in PMSF pretreatment not in postreated birds. One explanation for this effect might be that LA may have already caused damage in the nerve fibers so that no additional detrimental effect is caused by PMSF in the progression of nerve damage. In contrast, in pretreatment of PMSF, there might be a chance for an interaction between PMSF and LA to produce potentiating effect.

It is also suggested that PMSF might inhibit some cellular events triggered by the activation of phospholipase C (PLC), such as activation of phospholipase A_2 in ischemia (Wang et al., 2001). One of the pathways in LA neurotoxicity is caused by activation phospholipase A_2 via increased Ca^{++} overload. In the PMSF postreatment, PMSF somehow may have acted somehow on this pathway leading to relatively decreased response compared to PMSF pretreatment.

One can also speculate on toxicokinetic interaction and alteration of repair process. PMSF may alter the toxicokinetics of LA so that more LA can reach the tissues including spinal cord, with higher LA concentration eliciting spinal cord lesions. PMSF was shown to promote p-bromophenylacetylurea neuropathy through toxicokinetic alterations (Xu, et al., 1999). Another explanation is that PMSF can alter cellular repair process of subclinically affected nerve tissue in LA toxicated birds. Interestingly, the distribution of lesions was localized primarily in the dorsal spinocerebellar tracts, fasiculi gracilus and the ventral corticospinal tract in the spinal cord which was similar to lesions seen in OPIDN (Bouldin and Cavanagh, 1979).

Ultrastructural changes included intramyelinic edema, myelin separation and vacuolation. These results are similar to previous reports of LA toxicity (Gregory et al., 1995; Roder, 1996).

NTE activity of spinal cord, brain and sciatic nerve of LA treated birds showed no difference from that of control birds. This is not surprising since LA exerts its toxic effect through a mechanism unrelated to NTE inhibition. However, PMSF significantly inhibited spinal cord, brain and sciatic nerve NTE activity.

Motor Nerve Conduction Velocity findings showed that PMSF does not affect sciatic nerve conduction velocity in LA treated birds; nevertheless LA causes significant reduction of conduction velocity in the sciatic nerve. MNCV values obtained herein are very close to Roder's (1996) observations. Robertson and Anderson (1986) reported normal nerve conduction velocities of adult hens. The values presented by the authors were similar to those of control birds in our study.

In conclusion, PMSF may potentiate LA-induced peripheral neuropathy and induce spinal cord lesions when given with LA in broiler chickens. The mechanism for spinal cord lesions and exacerbation of ataxia warrants further investigation.

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CHAPTER III

NEUROTOXICITY OF LASALOCID AND EFFECTS OF PHENYL METHYL SULFONYL FLUORIDE IN MALE SPRAGUE DAWLEY RATS

Abstract

Lasalocid toxicity has been associated with several neurotoxicity cases in animal species. Unpublished field reports indicated that incidence of lasalocid neurotoxiciy shows a higher rate in chickens during heat stress with increased water salinity. Male Sprague Dawley rats were given 0, 10, 20, 40 mg/kg of lasalocid two times a day for 3 days with or without 0.9 % saline in the drinking water. Additional rats were given 20 mg/kg lasalocid two times a day for 3 days before phenylmethylsulfonyl fluoride (PMSF) treatment. Lasalocid neuropathy and modulation by PMSF were evaluated using an ataxia scoring system and histopathology. Rats showed ataxia in the 20 and 40 mg/kg dose groups with higher death rate in group receiving 40 mg/kg of lasalocid. Dietary salt did not affect ataxia induced by lasalocid. Rats responded to lasalocid+PMSF treatment with a higher ataxia than the lasalocid only group. PMSF may potentiate neuropathy induced by lasalocid. While the optimum dose to produce ataxia (without high mortality) appears to be 20 mg/kg of lasalocid, increasing salinity of water has no effect on the neurotoxicity of lasalocid.

Introduction

Ingestion of cat food which was accidentally contaminated with the ionophore salinomycin resulted in outbreak of acute polyneuropathy in the Netherlands in 1996 (Van der Linde-Sipman et al., 1999). Clinical symptoms of the affected cats were acute lameness and paralysis of hindlimbs followed by forelimbs. Histopathologic examination of the affected cats showed that peripheral nerves of both hind- and forelimbs were affected. The changes included swelling, fragmentation and loss of axon and digestion chambers in myelin (Van der Linde-Sipman et al., 1999). Furthermore, Gregory et al (1995) reported that chickens fed a ration containing LA under heat-stressed conditions had lesions in the sciatic nerve including swollen and shrunken axons and also vacuole formation in the myelin sheath.

Several studies reported toxic effect of ionophores in rodent species. Gad et al (1985) reported that some ionophores including LA caused central and peripheral nervous system effects leading to tonic and clonic convulsions and aggressive behaviors in CD-1 mice and Fisher 344 rats.

Phenylmethylsulfonyl floride (PMSF) can modulate certain neuropathies. A well known example of this effect is seen in organophosphate induced delayed neuropathy (OPIDN). Phenylmethylsulfonyl floride is a serine protease inhibitor which has been utilized to elucidate organophosphate induced delayed neuropathy (OPIDN) (Lotti, 1992). Besides OPIDN, PMSF can also potentiate p-Bromophenylacetylurea induced neuropathy in rats (Xu, et al., 1999) and delays the recovery from nerve crush (Moretto,

et al., 1993). Therefore, we investigated a possible interaction between LA and PMSF in rats.

The main hypothesis of this study was that LA induces peripheral neuropathy, and PMSF exacerbates the neurotoxic effect of LA in rats. To determine the dose given by oral route that causes the neurotoxic syndrome in rats by LA, dose response study was performed. In addition, there are some reports that indicate dietary levels of K+ and some other ions might influence ionophore toxicity (Halvorson, 1982). In the poultry industry, it is known that incidence of LA toxicosis is higher during the hot summer period with increased water salinity (VanHooser personal observations). With the combination of environmental stress factors, increased temperature and water deprivation may play a role for higher rate of LA neurotoxicity and cardiotoxicity (Gregory et al., 1995). Therefore, we investigated also the effect of 0.9 % NaCl in drinking water on LA toxicity in rats.

Materials and Methods

Materials:

All materials including Lasalocid A Sodium salt (purity ≥97 %), PMSF, dimethyl sulfoxide (DMSO), glycerol formal (GF) were purchased from Sigma Chemical Company, St. Louis, MO. Lasalocid was dissolved in DMSO (vehicle for LA) at a concentration of 10 mg/ml. Phenylmethylsulfonyl fluoride was dissolved in GF (vehicle for PMSF) at a concentration of 90 mg/ml. Saline solution (0.9 %) was prepared with deionized water.

Animals and Treatment:

Characterization of Oral Dose Response and Effect of Dietary Salt on Lasalocid Toxicity in Sprague Dawley Rats

Thirty two male Sprague Dawley rats with approximate body weight 175-200 g. (46-49 day-old) were obtained from a commercial source (Harlan, Oregon, WI). All rats upon arrival were acclimatized 7 days before the start of experiment and food and water were provided ad libitum. Following the acclimatization period, the rats were divided into 8 groups containing 4 rats each. Two days before the experiment, water was discontinued and 0.9 % NaCl solution was given to groups 2, 4, 6, and 8 until the end of experiment. On the day of dosing, all rats were weighed and doses of LA were given orally according to the design below (Table V).

Four different doses of LA including 0, 10, 20, and 40 mg/kg 2 times a day for 3 days, with or without 0.9 % NaCl in the drinking water were used.

Table V. Experimental design: Treatment groups in Characterization of Oral Dose Response and Effect of Dietary Salt on lasalocid toxicity in Sprague Dawley Rats in male Sprague Dawley rats

Group	# of rats	Treatment
1	4	LA (0 mg/kg) in DMSO 2 times for 3 days + normal water
2	4	LA (0 mg/kg) in DMSO 2 times for 3 days + 0.9 % NaCl
3	4	LA (10 mg/kg) in DMSO 2 times for 3 days + normal water
4	4	LA (10 mg/kg) in DMSO 2 times for 3 days + 0.9 % NaCl
5	4	LA (20 mg/kg) in DMSO 2 times for 3 days + normal water
6	4	LA (20 mg/kg) in DMSO 2 times for 3 days + 0.9 % NaCl
7	4	LA (40 mg/kg) in DMSO 2 times for 3 days + normal water
8	4	LA (40 mg/kg) in DMSO 2 times for 3 days + 0.9 % NaCl

LA-induced neuropathy and effects of PMSF in male Sprague Dawley rats

Twenty four male Sprague-Dawley rats with approximate body weight of 175-210 grams (46-48 day-old) were obtained from Harlan (Oregon, WI). The rats were housed in groups of 2 and allowed to adapt to the environment for at least 1 week prior to dosing. Feed and water were provided ad libitum. Following the acclimatization period, the rats were randomly divided into 4 groups containing 6 rats each. Table VI shows the treatment groups.

Table VI. Treatment groups in LA-induced neuropathy and effects of PMSF in male Sprague Dawley rats.

Group	n	Treatment Groups
1 (Control)	6	LA* (0 mg/kg, oral gavage) + PMSF* (0 mg/kg, s.c.)
2 (PMSF only)	6	LA* (0 mg/kg, oral gavage) + PMSF (90 mg/kg, s.c.)
3 (LA only)	6	LA (20 mg/kg, oral gavage) + PMSF* (0 mg/ml, s.c.)
4 (LA+PMSF)	6	LA (20 mg/kg, oral gavage) + PMSF (90 mg/kg, s.c.)

* DMSO and GF (vehicles) were used as LA (0 mg/kg) and PMSF (0 mg/kg), respectively.

Group 4 and 3 received 20 mg/kg of LA with or without PMSF, respectively. These groups were treated with 20 mg/kg of LA (in DMSO) by oral gavage 2 times a day for 3 days. Four hours following the final LA application, a single dose of PMSF (90mg/kg, s.c., in GF) or GF only was administered, respectively. Group 2 received DMSO (0.1 ml per 100 g. of body weight) by oral gavage 2 times a day for 3 days. Four hours following last DMSO application, a single dose of PMSF (90mg/kg, s.c., in GF) was administered. Group 1 was treated with vehicles only and served as control.

Clinical Observations

Starting from day 1, all rats were observed daily 2 times a day until they were sacrificed (day 8). All treatment groups were evaluated for the development of ataxia and behavioral changes. Individual rats were graded "blind" to assign an ataxia score. Each

rat was placed on the ground and allowed to acclimate to the environment for a couple of minutes. Then the rats were observed without further disturbance for behavioral changes. To determine gait abnormalities and degree of ataxia, the rats were allowed to move freely. Then they were forced to walk to detect slight ataxia and to determine if the degree of ataxia increased. Ataxia scores were given according to clinical ataxia scoring system (Table VII).

Table VII. Clinical Ataxia Scoring System

Score	Definition
0	Normal rats
1	Slight ataxia after exercise
2	Slight ataxia, ataxia worsens after exercise
3	Ataxic rats
4	Non-ambulatory, alert
5	Non-ambulatory, not alert, depressed

Determination of Foot Splay

Spreading of hind limbs was measured in all rats in each treatment group. Determination of foot splay was carried out by the method of Edwards and Parker (1977). Briefly, both feet were pressed onto an inkpad, and the rat was held horizontally

in dorso-ventral position with the body 32 cm above the surface of a table. The rat was then released from the top, and the distance between the ink marks was measured upon landing. The procedure was repeated 3 times for each rat and the average of 3 readings was used.

Histopathologic Examinations

Light Microscopy: Three rats from each group were randomly selected for necropsy and histopathologic and ultra structural studies. The rats were killed in a CO_2 chamber. The sciatic nerve and the entire spinal cord were dissected and fixed in 2.5 % buffered glutaraldehyde. The brain and sections of skeletal muscle were taken and placed in fixative. Sections for histopathologic examination were embedded in paraffin, cut 4-6 μm thick and stained with hematoxylin and eosin (H&E). Selected sections of sciatic nerves and spinal cord were also stained with Severe-Munger and counterstained with Luxol Fast Blue. All sections of tissue were examined by light microscopy and lesions scored "blind". Lesions in the sciatic nerve and spinal cord were graded according to the following scale:

Nerve Lesions:

0 - No lesions

1 - Swollen axons, foamy vacuolation of myelin

2 - Small distinct random vacuoles (Voids) in myelin

3 - Distinct vacuoles in myelin with atrophy and/or fragmentation of axons

4 - Large distinct myelin vacuoles (Voids), digestion chambers (axon-myelin debris), or loss of axons.

Spinal Cord Lesions:

0 – No lesions

1 – Small number of degenerating axons

2 – Moderate number of degenerating axons

3 – Numerous degenerating axons

Electron Microscopy: Sciatic nerves from affected rats were fixed in a glutaraldehyde/formalin mixture. Nerve pieces were washed with 0.1 M cacodylate buffer three times for 20 minutes. Gross transverse slices of the nerve segments were cut . The tissues were post-fixed in 1 % cacodylate buffered osmium for two hours then washed three times in 0.1 M cacodylate buffer. After post-fixation, the tissues were dehydrated by graded ethanol series (50, 70, 90, 95, 100) and washed three times with propylene oxide for 20 minutes. The tissues were put into 1:1 propylene oxide/hard polybed and polymerized a 60 ° C for 48 hours. Blocks of the nerve pieces were thick-sectioned and areas of interest were subsequently processed for thin sectioning. Thin

sections of samples were examined using a JEOL 100 CX transmission electron microscope at 80 KV.

Data Analysis:

Data were analyzed using one-way analysis of variance (ANOVA) with a general linear model (GLM) using SAS. For ataxia scores, group data are presented as medians±inter quartile range calculated as 75^{th} percentile minus 25^{th} percentile of data. For all other endpoints, group data are presented as means. Differences among experimental groups were attained using LSD multiple comparison test. Significance level of 0.05 was used for comparisons of treatments.

Results

Clinical Signs and Ataxia in Characterization of Oral Dose Response and Effect of Dietary Salt in Sprague Dawley Rats

Figure 25 depicts the median ataxia scores in the characterization of oral dose response and effect of dietary salt on lasalocid toxicity in male Sprague Dawley rats. Rats in control groups (0 mg/kg of LA with or without 0.9 % NaCl solution) appeared normal clinically. All rats in these groups were active and alert, and showed no behavioral changes or gait abnormalities. No mortality was observed. While only one rat (LA (10 mg/kg in DMSO 2 times for 3 days + 0.9 % NaCl) in 10 mg/kg LA dose-group showed ataxia with the score of 2, remaining rats in this dose group appeared normal (Figure 25).

Some rats treated with 20 and 40 mg/kg of LA with or without 0.9 % NaCl solution (Group 5, 6, 7 and 8) developed ataxia. Ataxia was more prominent in groups treated with 40mg/kg LA with or without 0.9 % NaCl solution. Some rats in these groups developed diarrhea following the next day (day 2) of LA treatment. Clinically affected rats were reluctant to move and less active compared to controls and the rats given 10 mg/kg LA with or without NaCl. Rats with ataxia scores 4 had difficulty to stay erect side of body with no righting reflex. Rats in this condition were unable to move hind limbs; however they attempted to move with front legs. While no mortality was seen in groups receiving 10 or 20 mg/kg LA with or without 0.9 % NaCl solution, groups receiving highest dose of LA with or without NaCl showed high mortality (3/4, 2/4 respectively).

Figure 26 shows the results from the evaluation of foot-splay after treatment with LA in different dose groups with or without NaCl. No difference was noted between control rats and the treatment groups. Mean foot-splay values of ataxic birds were similar to the controls and were not affected by the treatment.

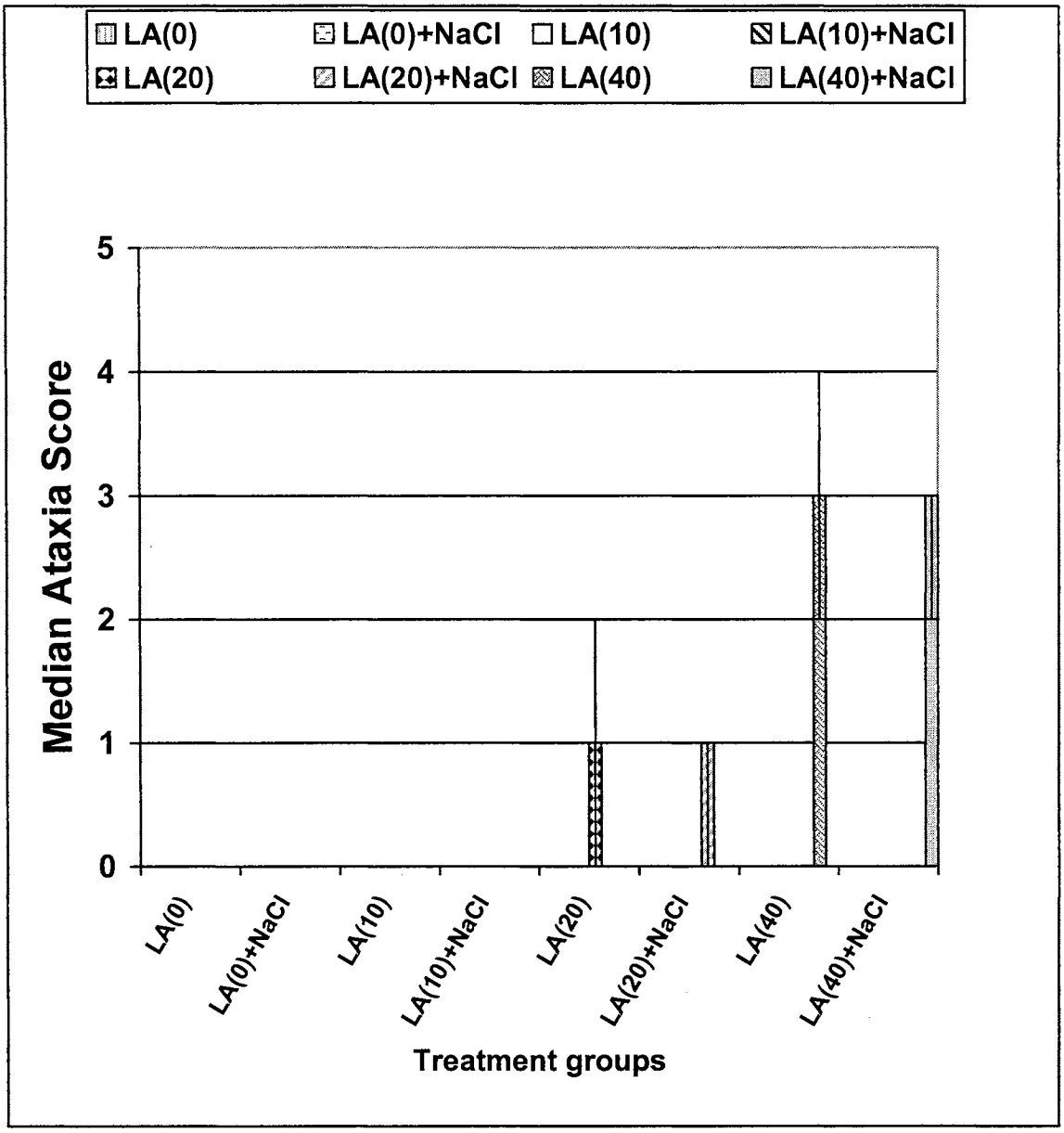

Figure 25. Median Ataxia Scores in Characterization of Oral Dose Response and Effect of Dietary Salt on Lasalocid toxicity in male Sprague Dawley Rats, (n=4). Data are shown as median ataxia scores ± interquartile ranges (calculated as 75^{th} percentile minus 25^{th} percentile of the data).

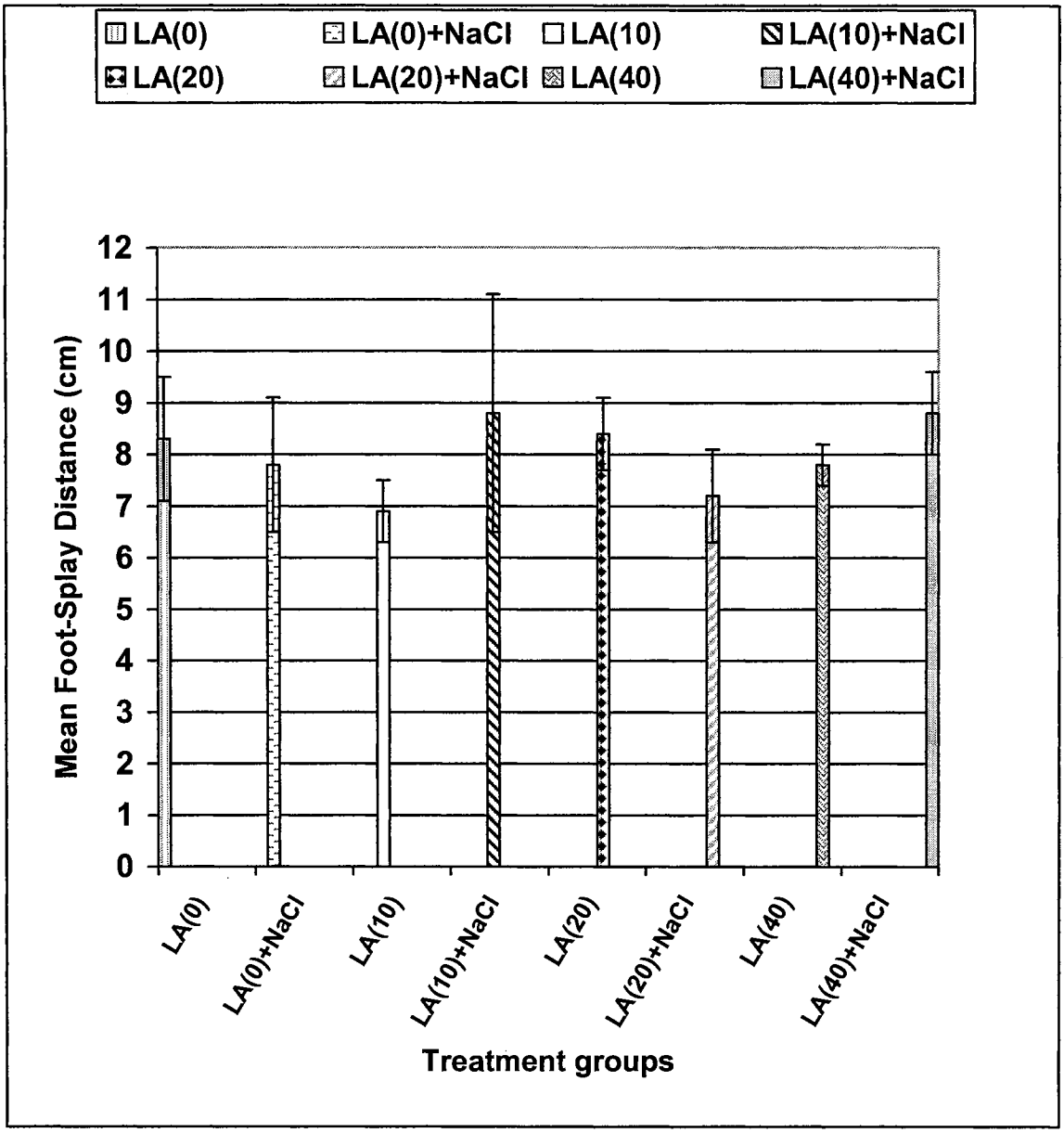

Figure 26. Mean Foot-Splay values in Characterization of Oral Dose Response and Effect of Dietary Salt on lasalocid toxicity in male Sprague Dawley Rats, (n=4).

Clinical Signs and Ataxia in La-induced neuropathy and effects of PMSF on Lasalocid Toxicity in male Sprague Dawley rats

Figure 27 shows the results from LA and LA+PMSF study. Rats in control and PMSF only treatment were clinically normal, and the rats in these groups showed no signs of ataxia (one rat in PMSF only group was scored as slightly ataxic at the beginning of study). Figure 28 shows the number of rats showing ataxia in each treatment group. Some rats in groups treated with LA+PMSF and LA only responded to the drug treatment by showing slight to moderate ataxia (ataxia scores ranging from 0 to 2) (Figure 27). None of the rats in these groups showed full-blown ataxia (ataxia scores of 3–5). Ataxic rats exhibited weakness, decreased righting reflex, very slow movement and loss of grip in hind limbs. Based on median ataxia scores, group receiving LA+PMSF treatment had statistically higher median ataxia scores compared to the other groups. Rats with clinical ataxia score 1 were able to walk and right themselves when placed on dorsal position. When they were forced to walk for a while they showed slight ataxia with imbalance in their walking pattern. These rats also were able to react pain stimuli. Likewise rats with ataxia score 2 were able to walk and right themselves when placed on their dorsum, but they had weakness and slight difficulty to right themselves. When they were placed on the ground, they were reluctant to move and had slight disturbance in their walking behavior. The ataxia was worsened when the rats in this category were forced to walk for several minutes. In addition, when the rats were tested for grip strength a decrease in hind limps was observed. Grip strength was normal in front legs. These rats

also responded painful stimuli like control rats. In addition, rats treated with LA and LA+PMSF showed slight diarrhea, loss of appetite and decreased body weight.

Figure 29 depicts number of deaths in treatment groups. There was no mortality in PMSF only group while 1 out of 6 rats in control was lost during the experiment. Two rats out of 6 from both groups treated with LA+PMSF and LA only died towards the end of study.

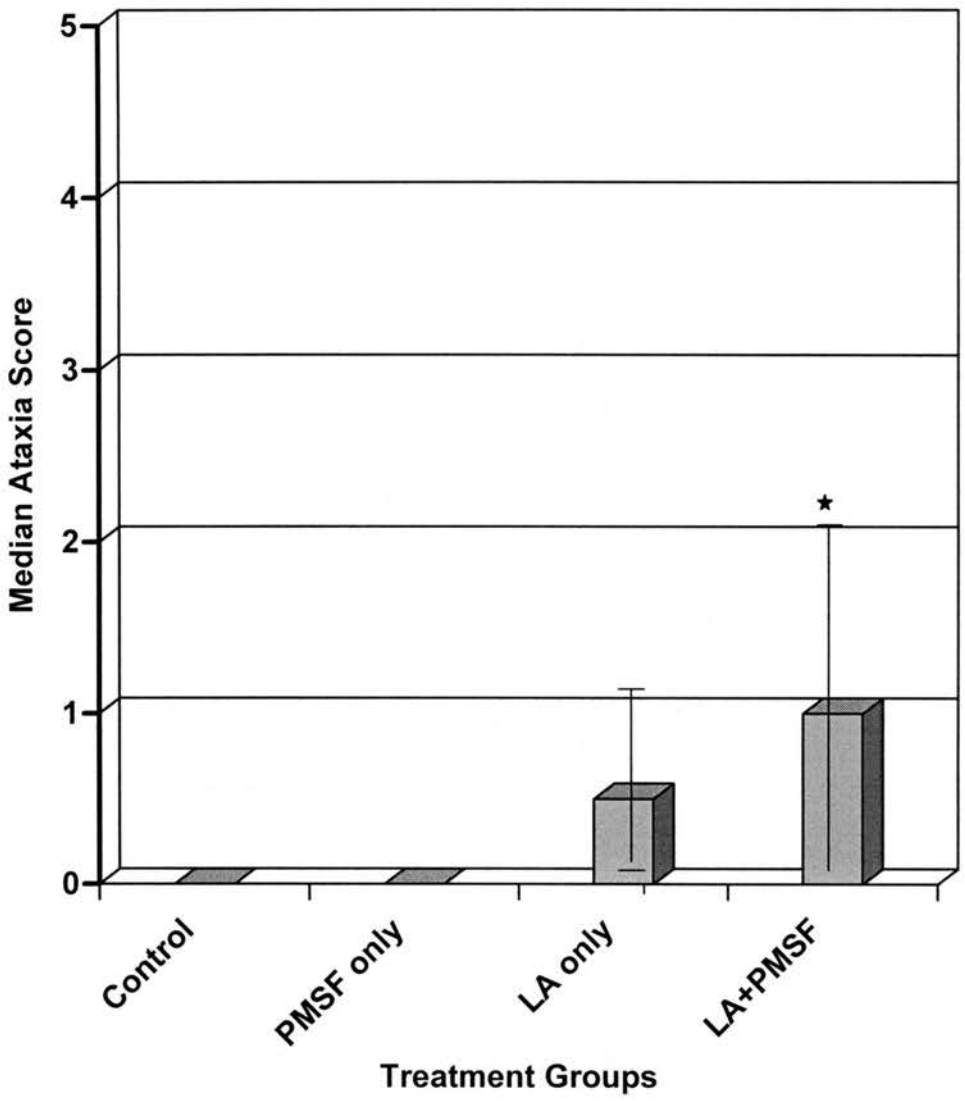

Figure 27. Median Ataxia Score in LA-induced neuropathy and effects of PMSF in male Sprague Dawley. (LA Only: 20 mg/kg LA 2 times a day for 3 days, LA+PMSF: 20 mg/kg LA 2 times a day for 3 days plus 90 mg/kg single dose of PMSF 4 hours after LA dosing). Data are shown as median ataxia scores ± interquartile ranges (calculated as 75^{th} percentile minus 25^{th} percentile of the data). Asterisk indicates significant effect, at $p < 0.05$

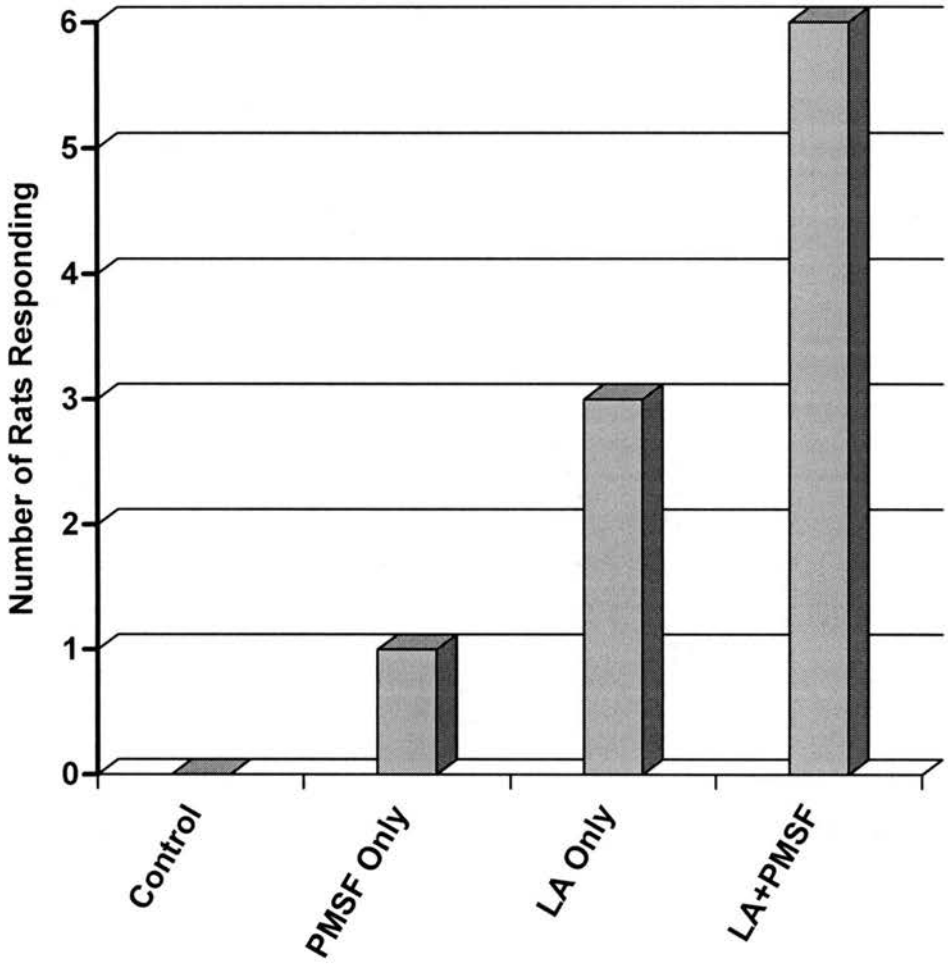

Figure 28. Frequency Distribution of Rats Showing Ataxia in LA-induced neuropathy and effects of PMSF in male Sprague Dawley rats.

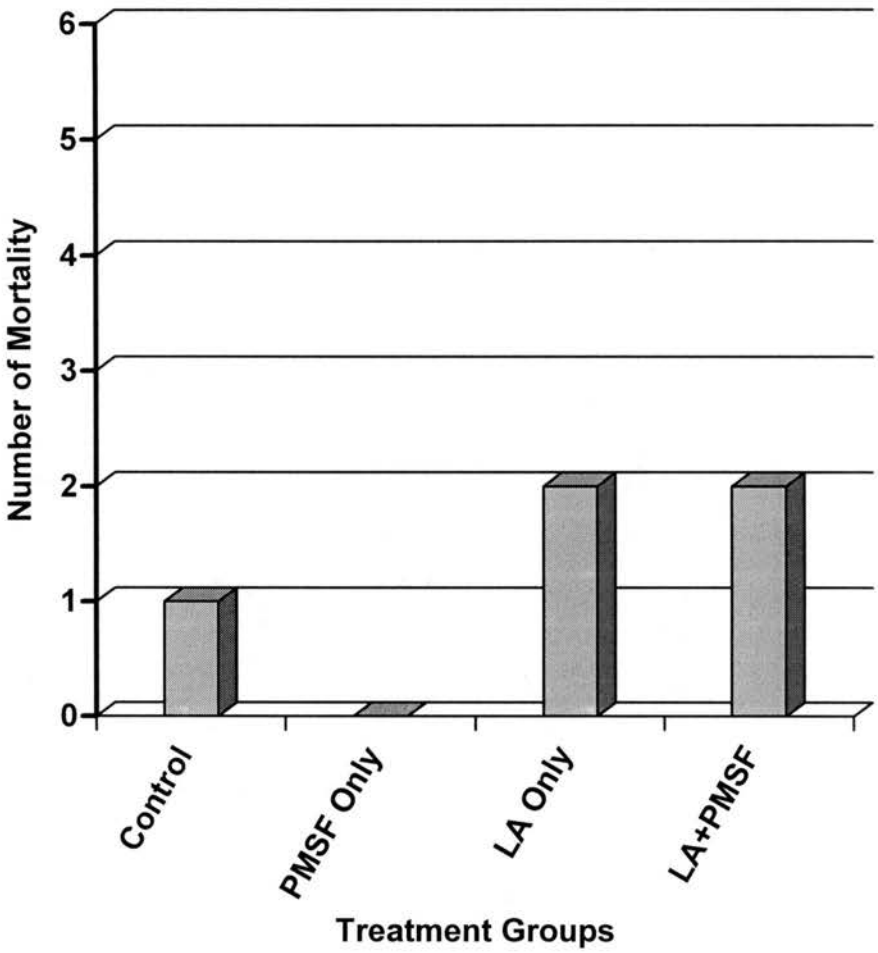

Figure 29. Number of deaths in treatment groups in LA-induced neuropathy and effects of PMSF in male Sprague Dawley rats (n=6 per group).

Foot Splay Study

Figure 30 shows mean foot-splay distance in LA-induced neuropathy and effects of PMSF on lasalocid toxicity in male Sprague Dawley rats. Mean foot-splay technique did not discriminate degree of neuropathy and also did not reveal clear indication of neuropathy when the values of foot-splay assay were compared to those of control. Based on mean distance between the hindlimbs upon landing, there was no significant difference among the treatment groups. Although there was a decrease in mean foot-splay in rats treated with LA and PMSF compared to control rats, the difference between group 4 and control group was not statistically significant.

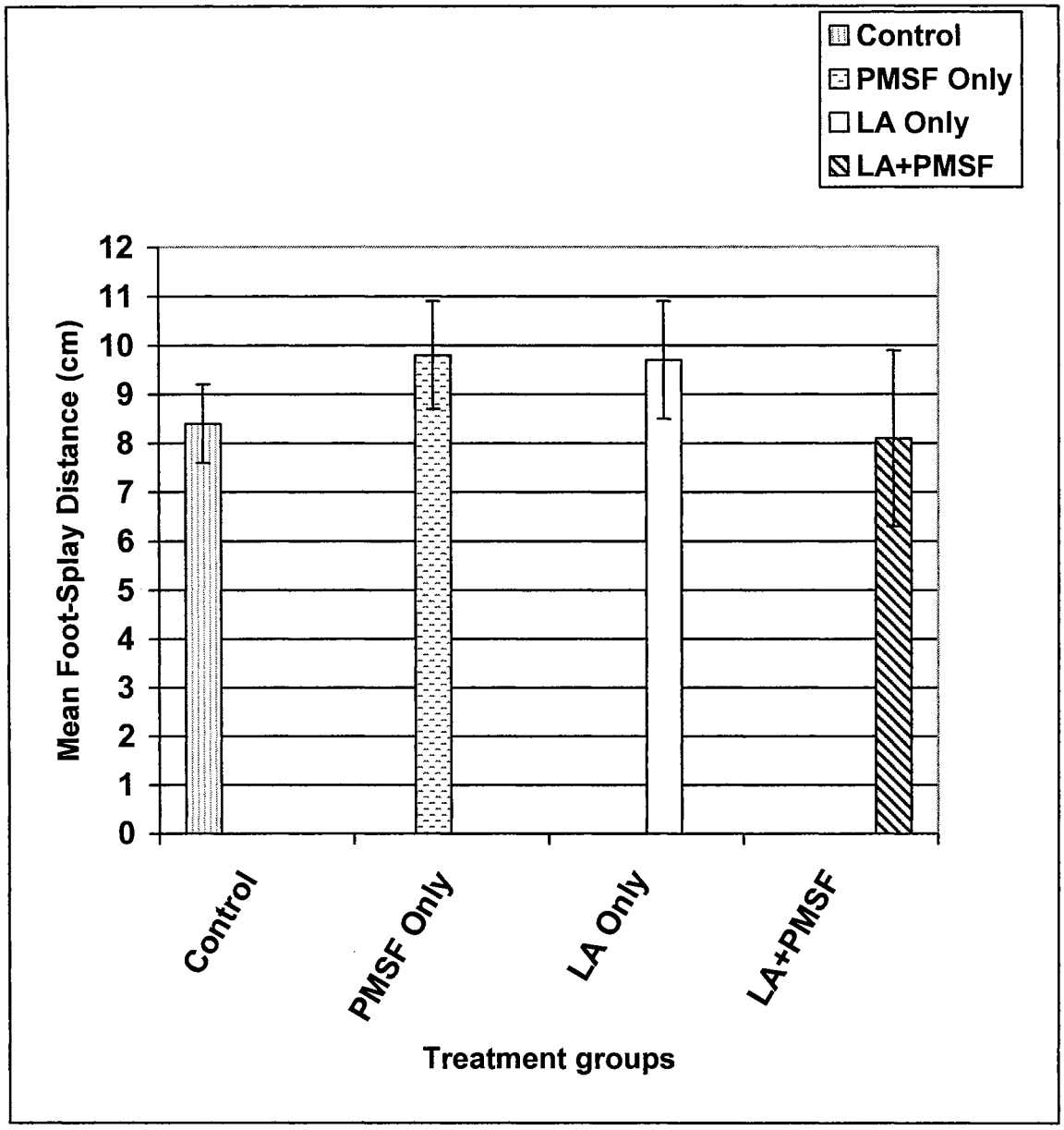

Figure 30. Mean foot splay distance in LA-induced neuropathy and effects of PMSF on LA toxicity in male Sprague Dawley rats. Mean±sd, (n=6 per group).

HISTOPATHOLOGY

Light Microscopy:

Figure 31 shows a light micrograph of a sciatic nerve from an ataxic rat treated with lasalocid (20mg/kg 2 times a day for 3 days). Lesions were minimal and consisted of random vacuolation of myelin sheets. No spinal cord lesions were noted in any treatment groups at the light microscopic level.

Figure 32 shows a light micrograph of muscle tissue from a rat treated with lasalocid (40 mg/kg 2times a day for 3 days) and 0.9 % NaCl in drinking water. Mild necrosis of skeletal muscle was observed. In the affected areas, loss of striation, fragmentation, hypertrophic satellite cells and edema between myofibers were present. Muscle lesions were only present in groups treated with the highest dose group of lasalocid (40 mg/kg) with 0.9 % NaCl in drinking water. No muscle lesions were observed in any other groups.

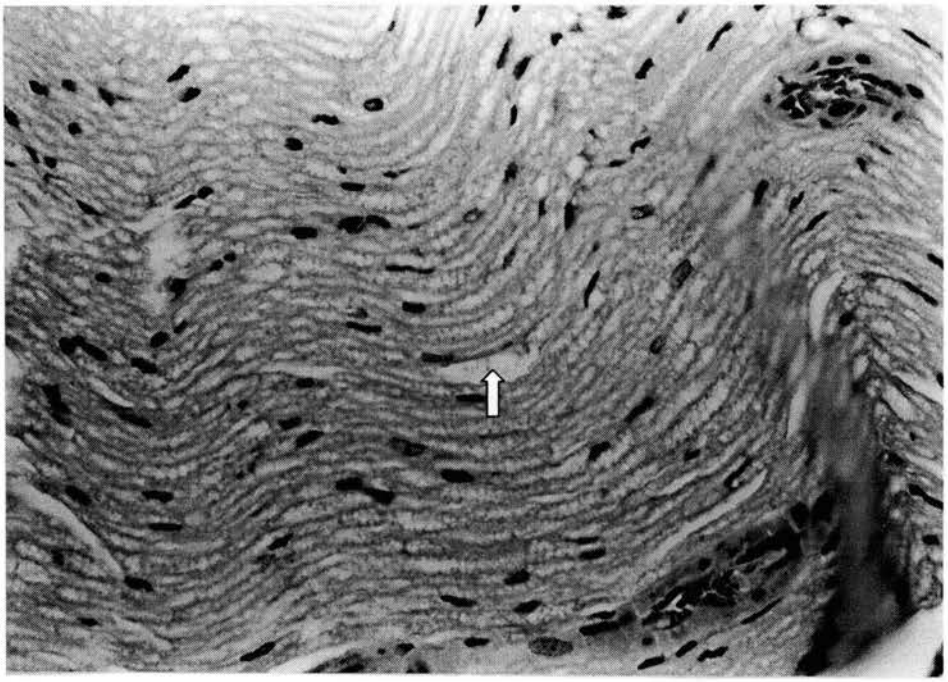

Figure 31. Light micrograph of a sciatic nerve from an ataxic rat treated with lasalocid. Vacuolation of myelin sheath (Arrow). (H&E), (x 200)

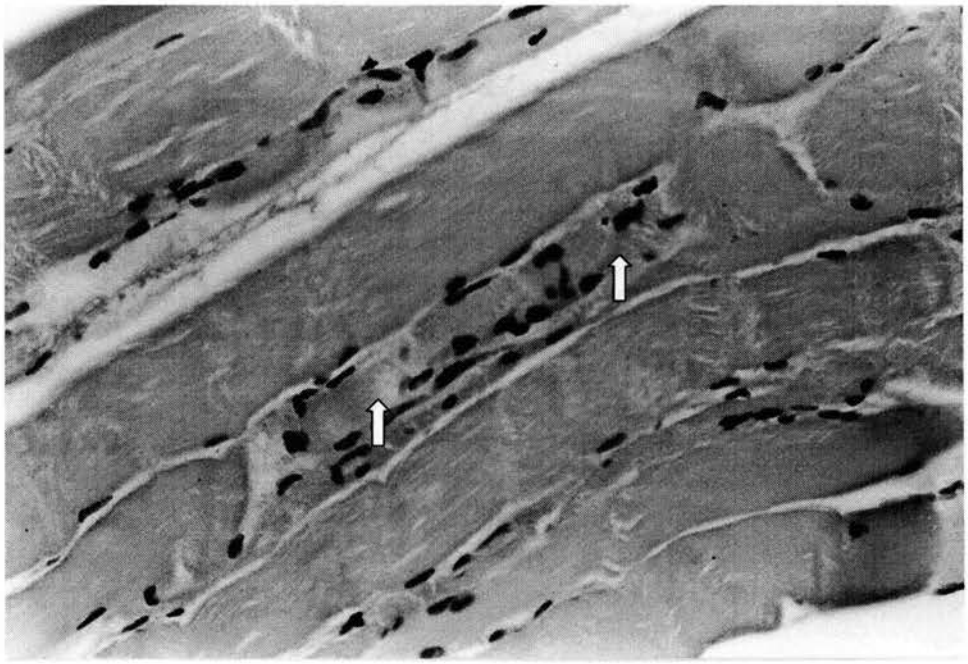

Figure 32. Light micrograph of a muscle tissue from a rat treated with lasalocid (40 mg/kg 2times a day for 3 days) and 0.9 % NaCl in drinking water. Necrosis of the tissue with loss of striation and fragmentation of myofibers (Arrows). (H&E), (x 200).

Electron Microscopy:

Figures 33 and 34 show electron micrographs of sciatic nerves from the control. As noted from the micrographs, cross section of the sciatic nerve showed no abnormality in structure. The myelin sheath was compact with evenly arranged structural appearance. Likewise, axons and the Schwann cells were also normal.

Figure 35 shows an electron micrograph of a sciatic nerve from a rat treated with PMSF only. Similar to the control rats, no abnormalities were noted in the sciatic nerve. The myelin sheath, axons and the Schwann cells were intact. They showed no difference from the control.

Figures 36, 37 and 38 are micrographs of the sciatic nerves from rats treated with lasalocid only. Lesions included splitting of myelin sheet with edema, fragmentation and vacuolation of the myelin. As noted from Figures 36 and 37, myelin separation and vacuole formation were generalized across the myelin lamellae. In Figure 38, myelin appears as fragmented and the pieces of these myelin fragments were also filled with vacuolations.

Figures 39, 40 and 41 are micrographs of the sciatic nerve sections taken from rats treated with lasalocid and PMSF. Lesions were similar to the rats treated with lasalocid only. Similarly, vacuolation and myelin fragmentation (Figure 39) were present in sections. However, as seen in Figure 40, areas of pale myelin were noted in this group suggesting loss of myelin and lipid. The axons and Schwann cells did not show an abnormality except for a slightly compressed and distorted appearance. Figure 41 shows slight fragmentation of myelin.

Figures 42 and 43 are from the spinal cord of control rats. No abnormality was seen in nerves of the spinal cord. The myelin sheath appeared as successively wrapped and evenly compacted layers. The axon and the Schwann cells looked normal also.

Figure 44 is from the spinal cord of rats treated with PMSF only. Similar to control no change was seen in the appearance of nerves and the nerve section looked normal.

Figures 45, 46, 47 and 48 show micrographs of spinal cord taken from rats treated with LA only. As noted from the micrographs, extensive myelin splitting was observed, and almost all nerve fibers appeared to be affected by myelin splitting in the sections. The axons and Schwann cells were normal and showed no difference from the control.

Figures 49, 50 and 51 are from the spinal cord of LA+PMSF treated rats. A greater degree of myelin separation was seen in small nerve fibers (Figure 49). There was also extensive edema between myelin layers and some small nerve fibers appeared to lose their axon. The space of axon appeared to be filled by fluid and myelin layers (Figure 50). Similarly, myelin separation and edema between myelin layers are present in figure 51.

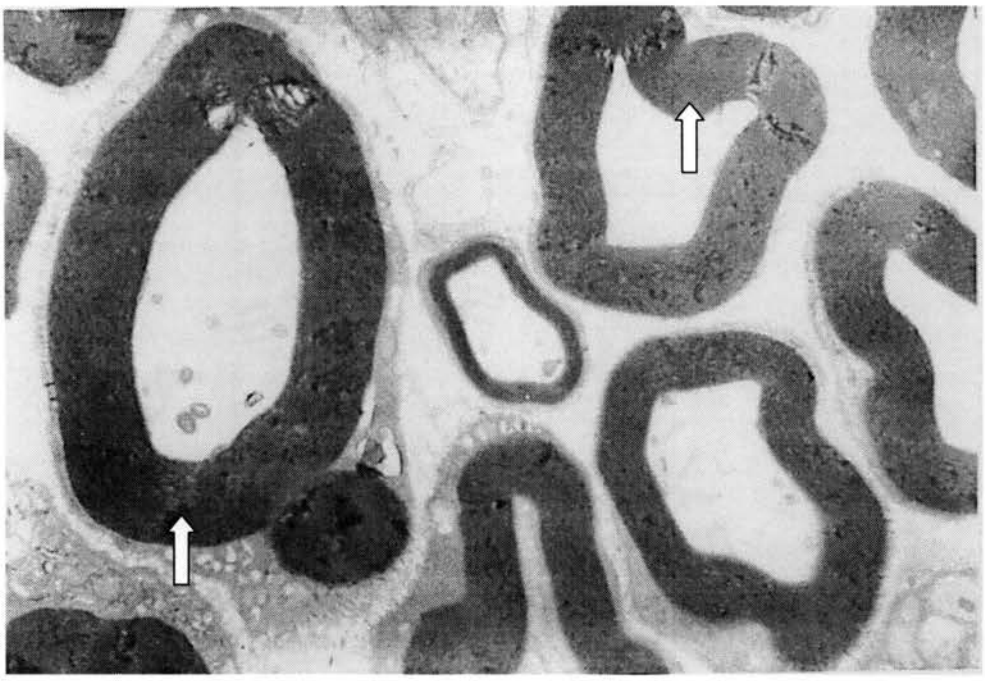

Figure 33. Electron micrograph of a sciatic nerve from a control rat in the study of lasalocid induced neuropathy and effect of PMSF in male Sprague Dawley rats. Note the compact evenly arranged myelin sheath (arrows). (x 9000)

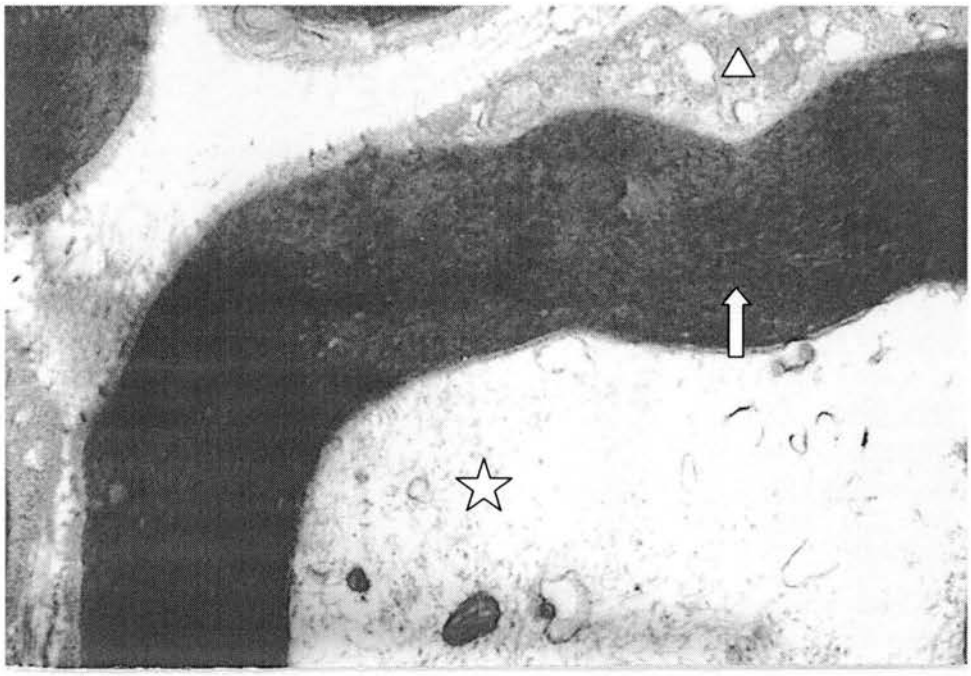

Figure 34. Electron micrograph of a sciatic nerve from a control rat in the study of Lasalocid induced neuropathy and effect of PMSF in male Sprague Dawley rats. Myelin sheath (arrow), axon (star) and the Schwann cell (diamond). (x 24,700)

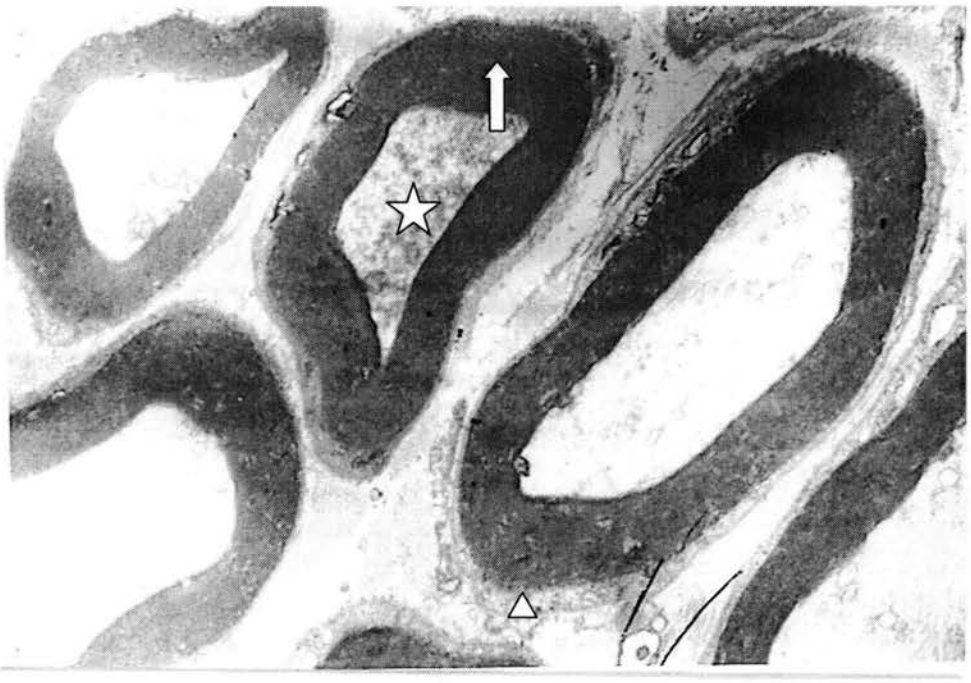

Figure 35. Electron micrograph of a sciatic nerve from PMSF only rat in the study of Lasalocid induced neuropathy and effect of PMSF in male Sprague Dawley rats. Myelin sheath (arrow), axon (star) and the Schwann cell (diamond). (x 10,000).

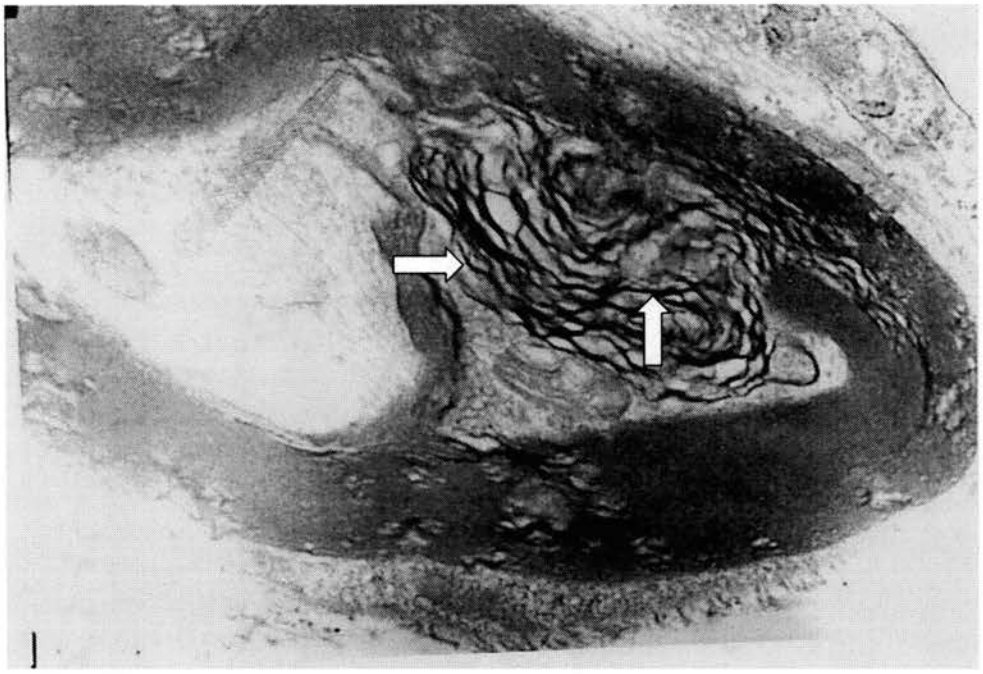

Figure 36. Electron Micrograph of a sciatic nerve from lasalocid only rat in Lasalocid induced neuropathy and effect of PMSF in male Sprague Dawley rats. Note the myelin fragmentation and separation (arrows). (x 28,500).

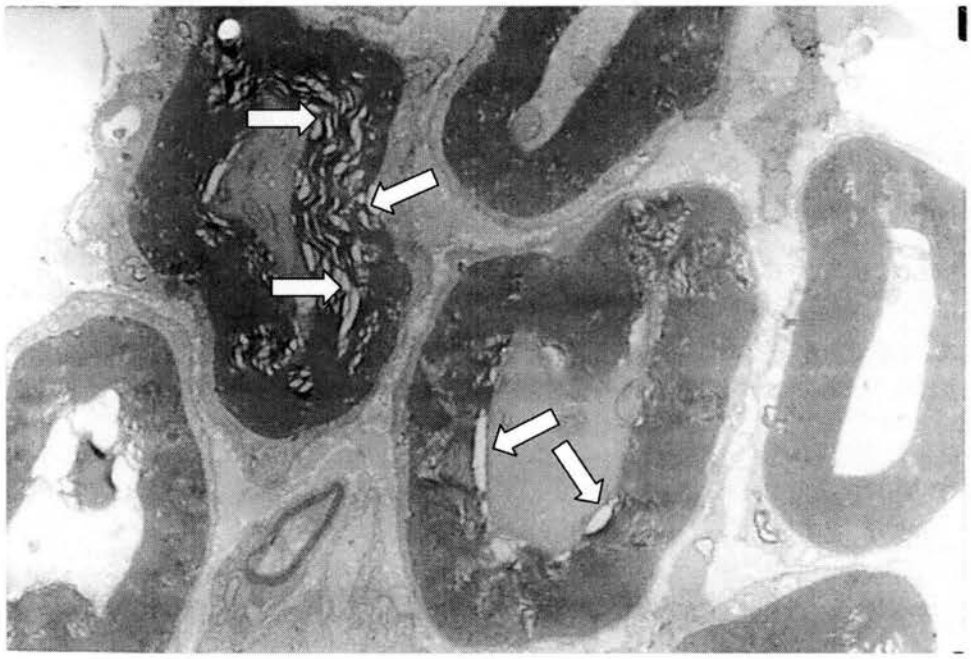

Figure 37. Electron Micrograph of a sciatic nerve from lasalocid only rat in Lasalocid induced neuropathy and effect of PMSF in male Sprague Dawley rats. Myelin vacuolation and separation (arrows). (x 10,000).

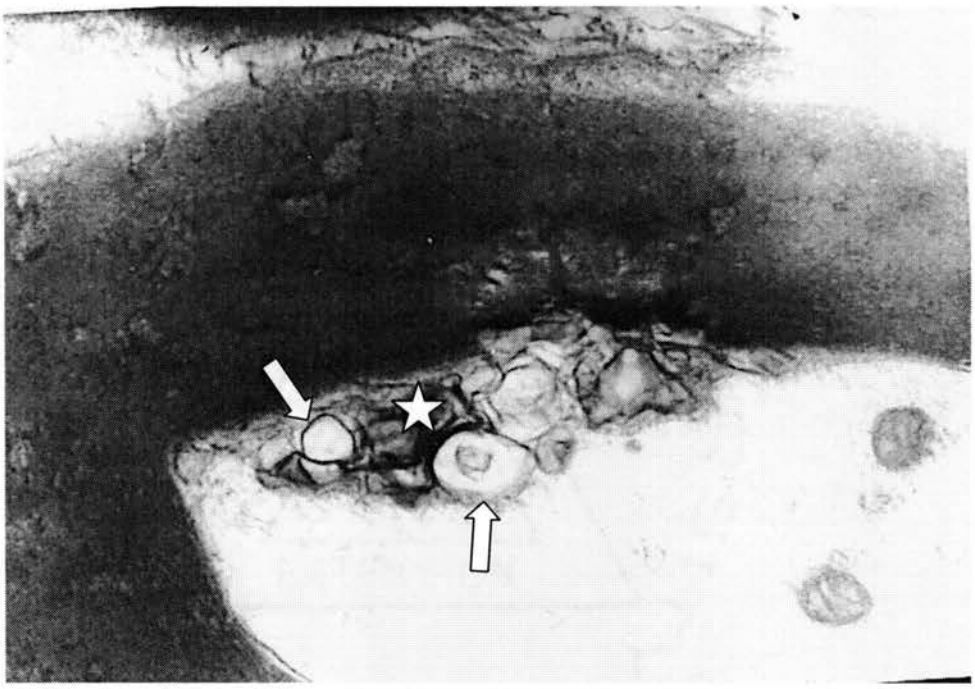

Figure 38. Electron micrograph of a sciatic nerve from a rat treated with LA only in the study of lasalocid induced neuropathy and effect of PMSF in male Sprague Dawley rats. Note the myelin fragmentation (star), and vacuolation (arrows). (x 27,700).

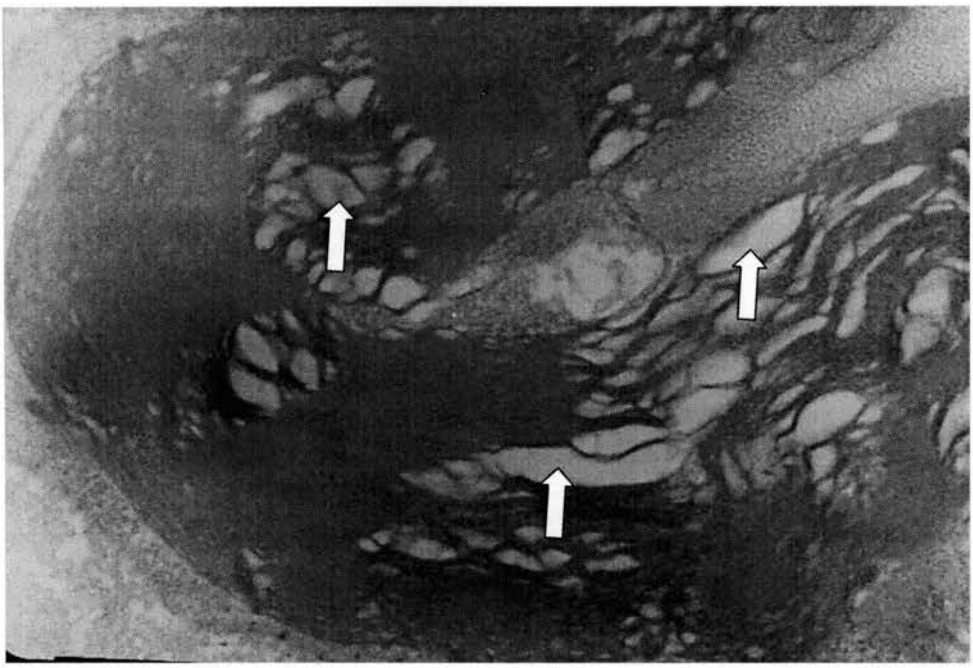

Figure 39. Electron micrograph of a sciatic nerve from a rat treated with LA+PMSF in the study of lasalocid induced neuropathy and effect of PMSF in male Sprague Dawley rats. Note the myelin vacuolation (arrows). (x 41,000).

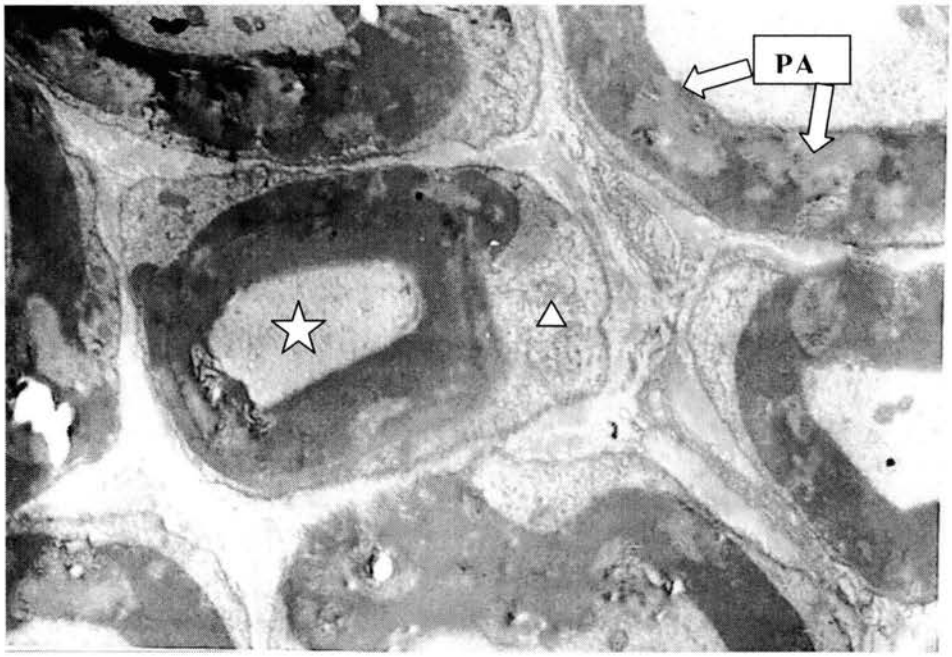

Figure 40. Electron micrograph of a sciatic nerve from a rat treated with LA+PMSF in the study of lasalocid induced neuropathy and effect of PMSF in male Sprague Dawley rats. Note the pale areas on myelin sheath (PA), axon (star), the Schwann cell (diamond). (x 10,400).

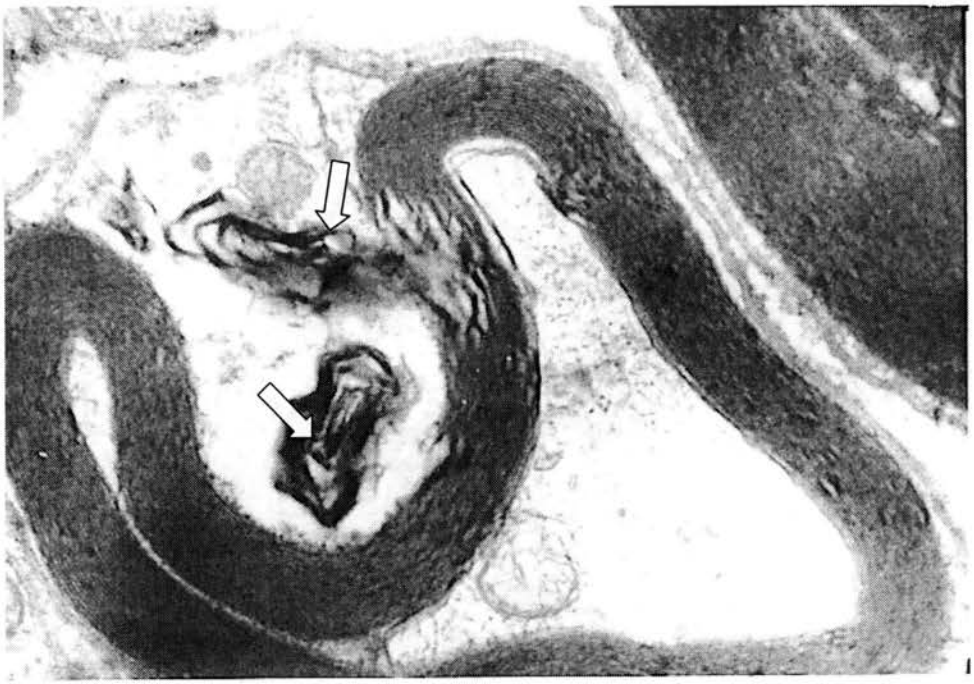

Figure 41. Electron micrograph of a sciatic nerve from a rat treated with LA+PMSF in the study of lasalocid induced neuropathy and effect of PMSF in male Sprague Dawley rats. Slight myelin fragmentation (arrows). (x 34,800)

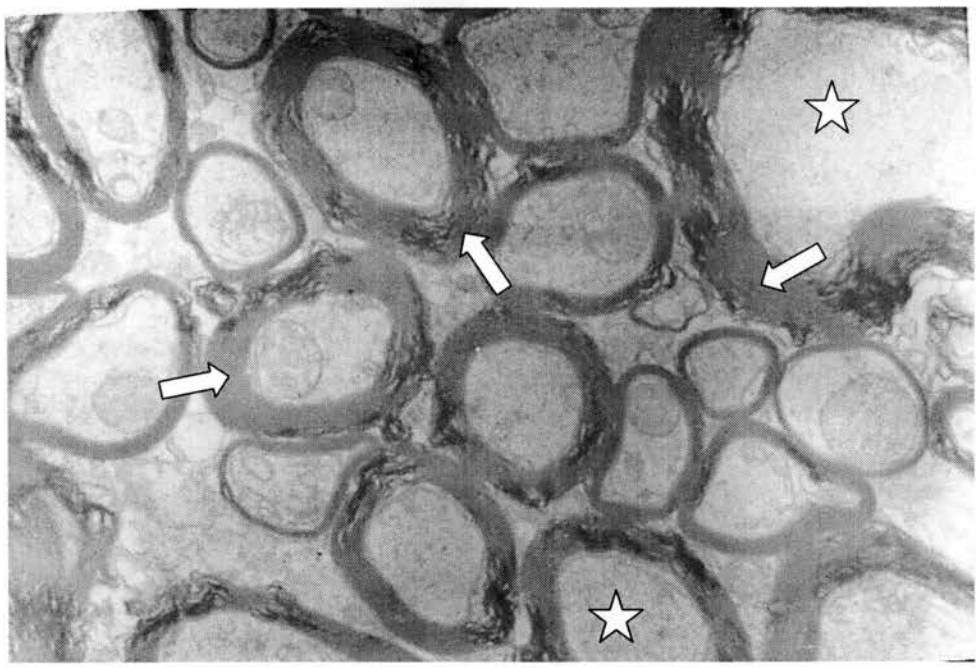

Figure 42. Electron micrograph of the spinal cord (dorsal column) from a control rat in the study of lasalocid induced neuropathy and effect of PMSF in male Sprague Dawley rats. Myelin sheath (arrows), axons (stars). (x 15,400)

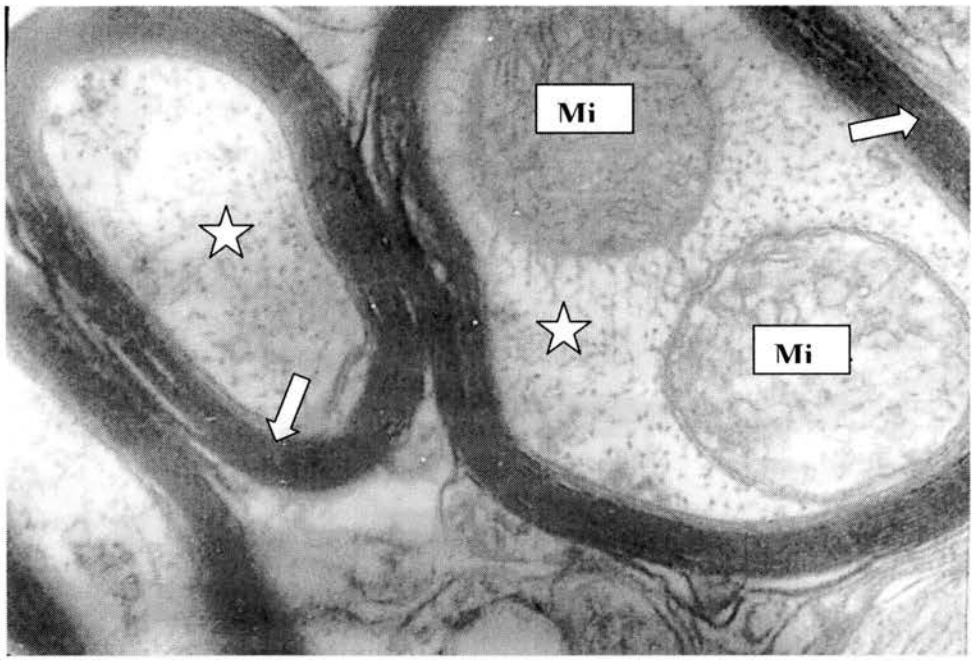

Figure 43. Electron micrograph of the spinal cord (dorsal column) from a control rat in the study of lasalocid induced neuropathy and effect of PMSF in male Sprague Dawley rats. Myelin sheath (arrows), axons (stars), a big mitochondrion in the axon(Mi). (x49,000)

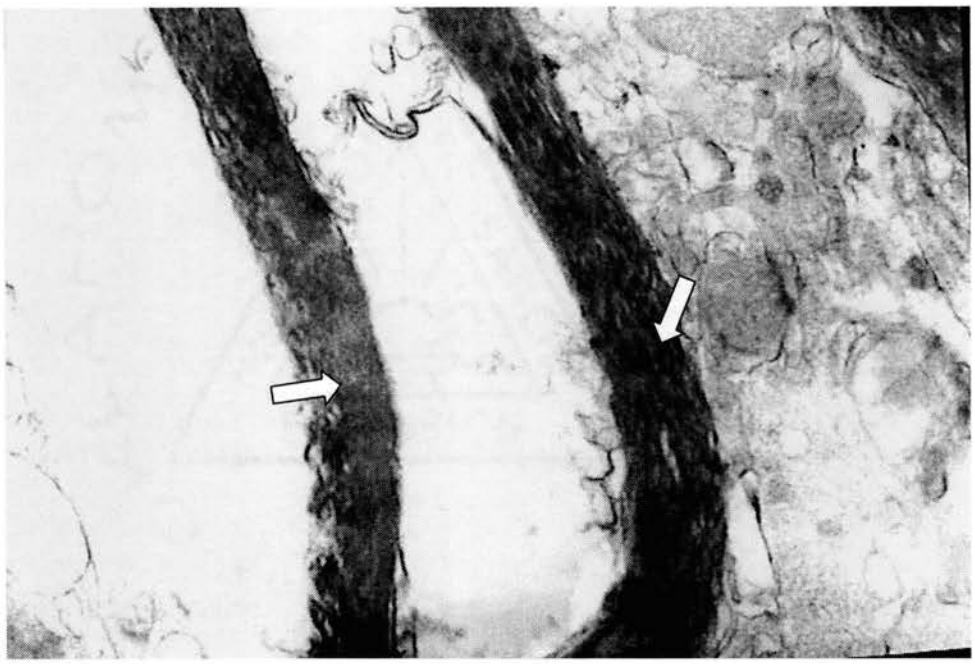

Figure 44. Electron micrograph of the spinal cord (dorsal column) from PMSF only rat in the study of Lasalocid induced neuropathy and effect of PMSF in male Sprague Dawley rats. Myelin sheath (arrows). (x 26,000).

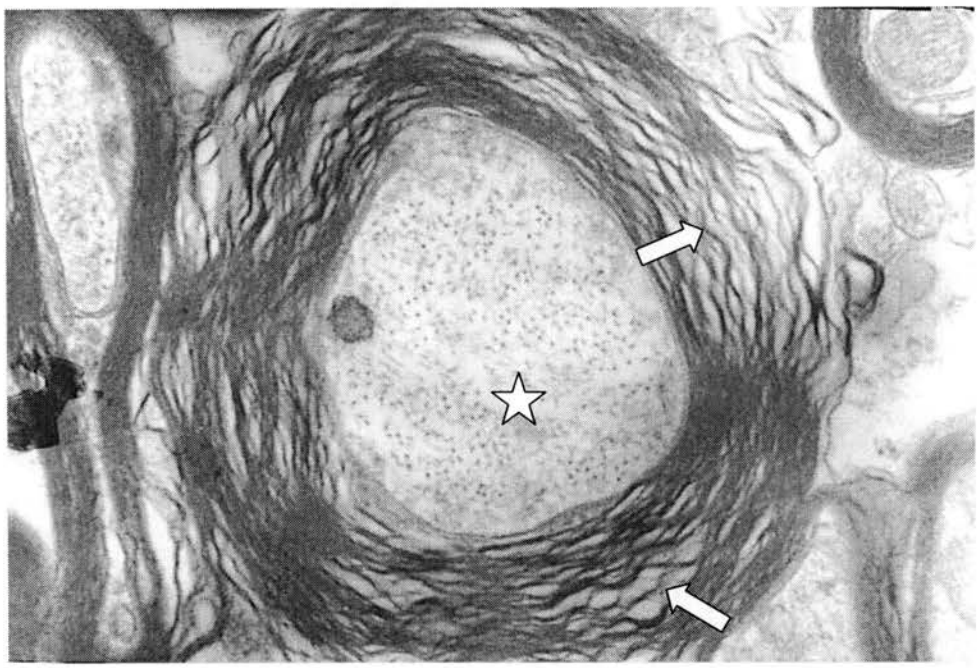

Figure 45. Electron micrograph of the spinal cord (dorsal column) from a rat treated with LA only in the study of lasalocid induced neuropathy and effect of PMSF in male Sprague Dawley rats. Note the extensive myelin splitting (arrows), normal axon (star). (x 40,000)

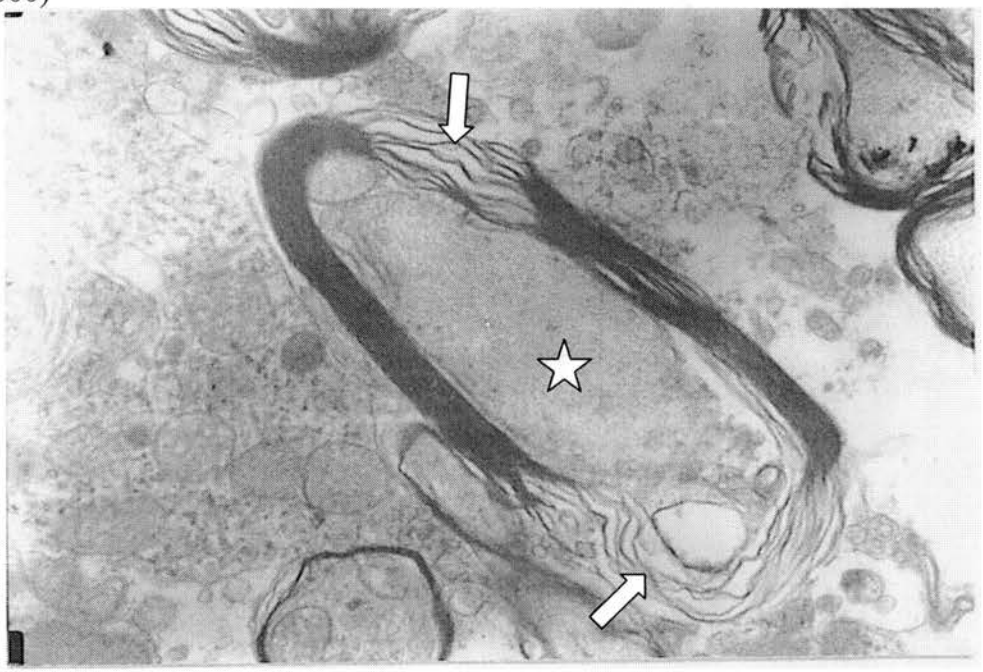

Figure 46. Electron micrograph of the spinal cord (dorsal column) from a rat treated with LA only in the study of lasalocid induced neuropathy and effect of PMSF in male Sprague Dawley rats. Areas of myelin splitting (arrows), axon (star). (x 17,500)

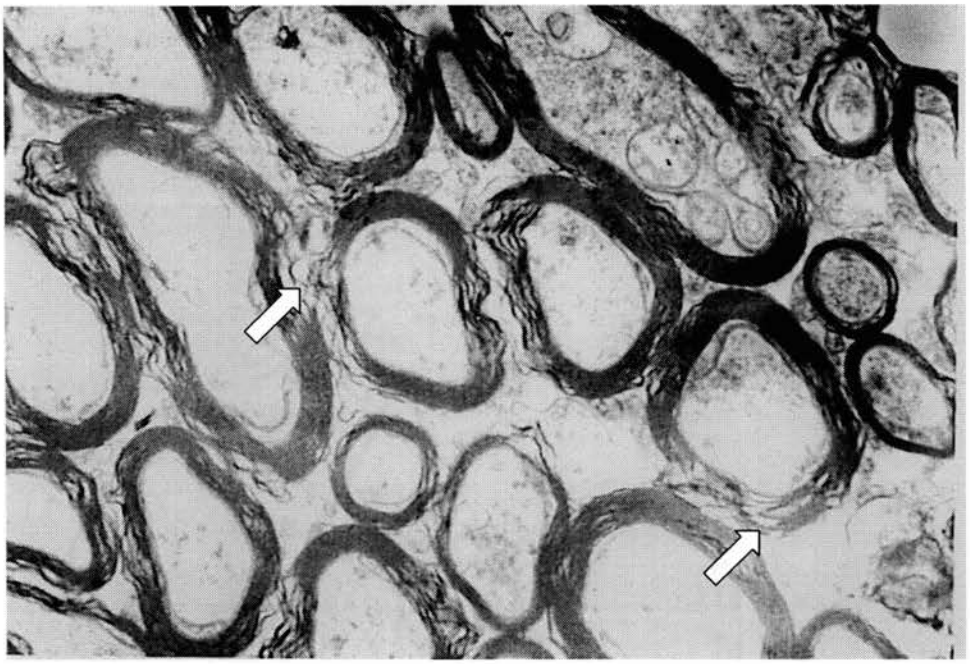

Figure 47. Electron micrograph of the spinal cord (dorsal column) from a rat treated with LA only in the study of lasalocid induced neuropathy and effect of PMSF in male Sprague Dawley rats. Areas of myelin splitting (arrows), (x 18,500).

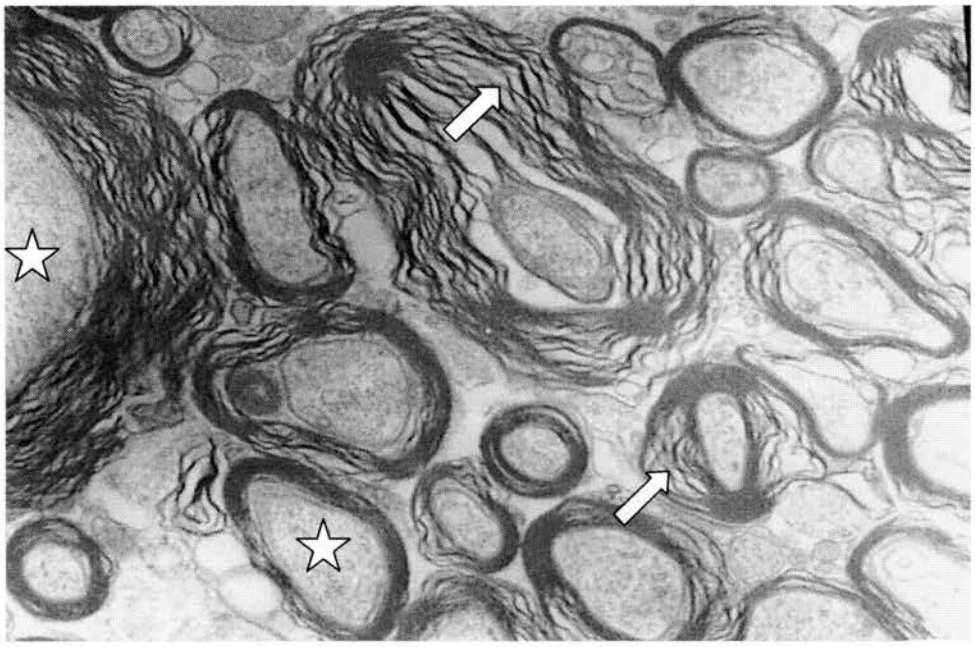

Figure 48. Electron micrograph of the spinal cord (dorsal column) from a rat treated with LA only in the study of lasalocid induced neuropathy and effect of PMSF in male Sprague Dawley rats. Note the extensive myelin splitting (arrows), normal axon (star). (x 20,000)

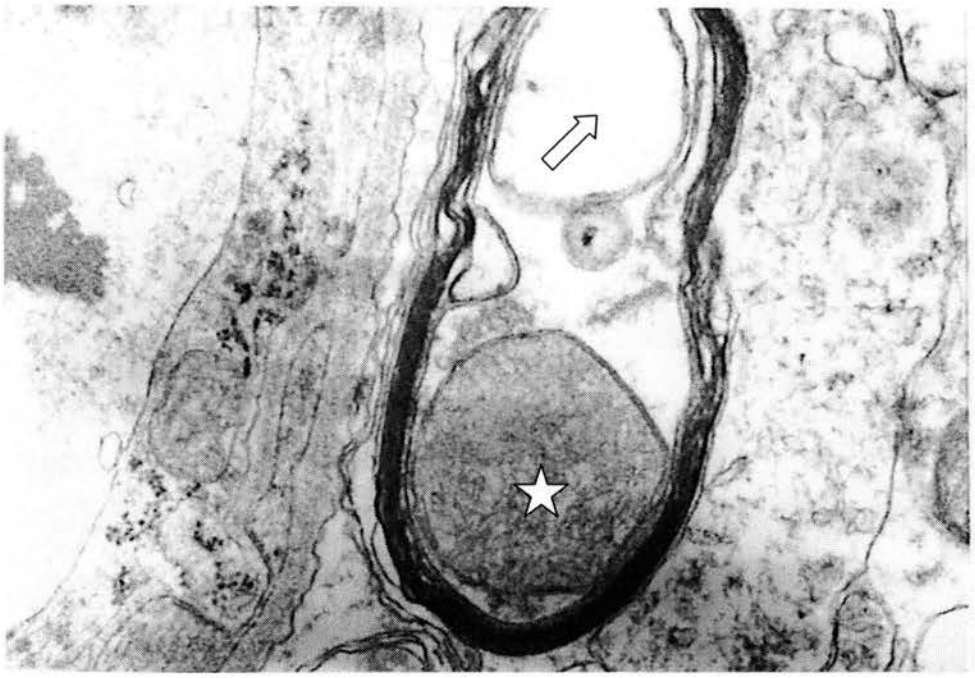

Figure 49. Electron micrograph of the spinal cord (dorsal column) from a rat treated with LA+PMSF in the study of lasalocid induced neuropathy and effect of PMSF in male Sprague Dawley rats. Note myelin separation and edema (arrow), axon (star). (x 32,000)

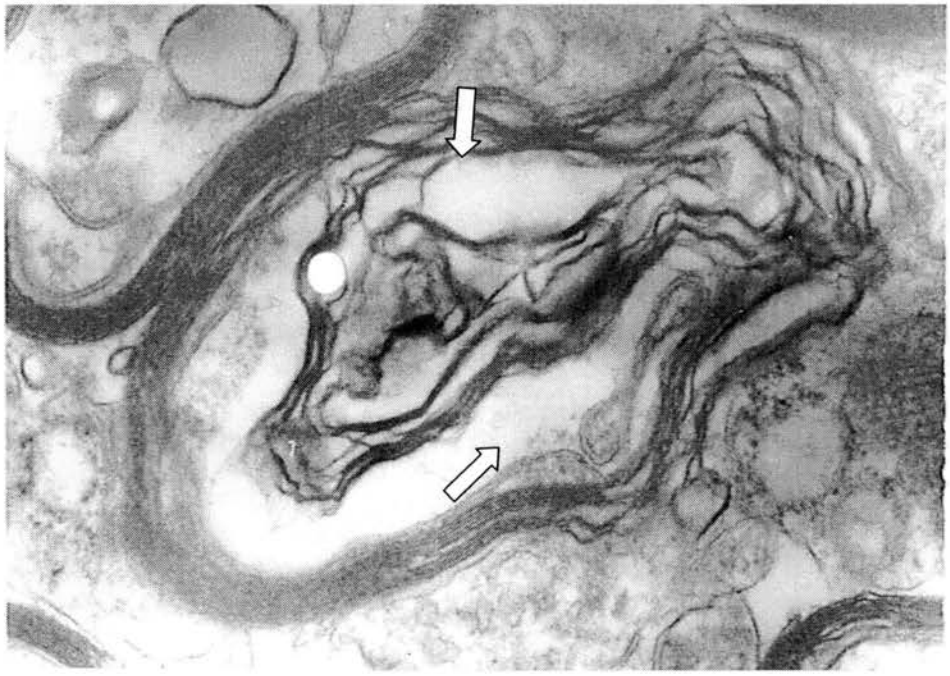

Figure 50. Electron micrograph of the spinal cord (dorsal column) from a rat treated with LA+PMSF in the study of lasalocid induced neuropathy and effect of PMSF in male Sprague Dawley rats. Note myelin separation and edema (arrow), (x 45,000)

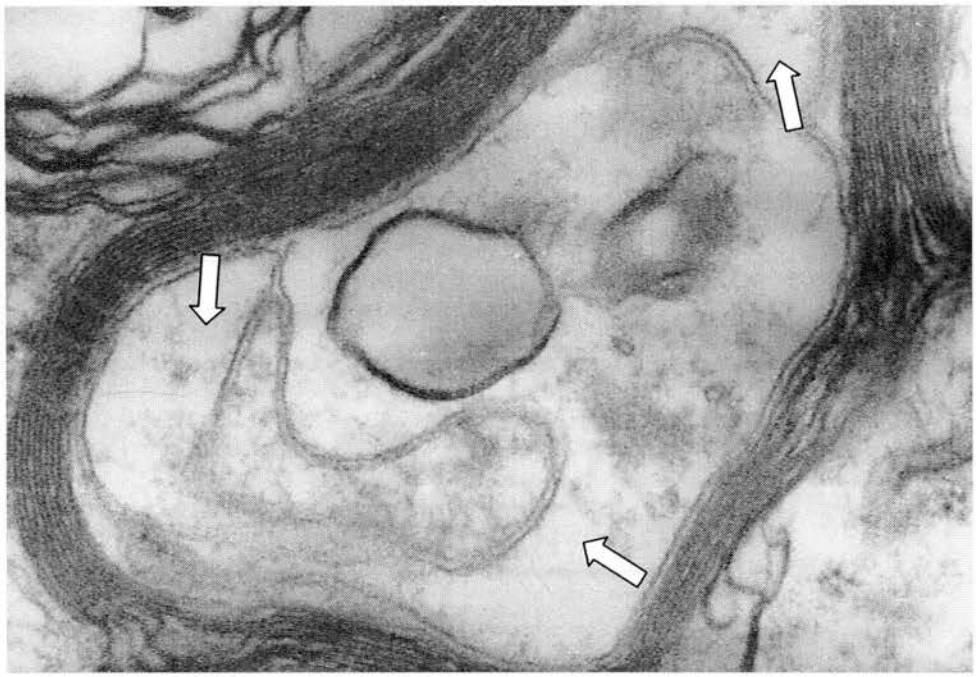

Figure 51. Electron micrograph of the spinal cord (dorsal column) from a rat treated with LA+PMSF in the study of lasalocid induced neuropathy and effect of PMSF in male Sprague Dawley rats. Note myelin separation and edema (arrow), (x 77,000)

Discussion

In our studies, LA (20 mg/kg, p.o., 2 times a day for 3 days) induced slight to moderate ataxia in rats. At this dose, no mortality was observed and it can be suggested that 20 mg/kg of lasalocid given by oral gavage 2 times a day for 3 days is the optimum dose to produce ataxia in male Sprague Dawley rats. It is known that incidence of LA toxicosis is higher with increased water salinity in broiler chickens during hot summer period. With the combination of environmental stress factors, increased temperature and water deprivation may play a vital role for higher rate of LA neurotoxicity (Gregory et al., 1995). Addition of 0.9 % NaCl in drinking water had no effect on neurotoxicity of lasalocid in rats. Although we did not observe any detrimental effect of 0.9 % of NaCl in drinking water, the percentage of NaCl used in this study may not be sufficient to produce additional effect on lasalocid neurotoxicity. Increasing NaCl ratio or water deprivation may be needed to see enhanced toxic response. The field reports indicating the effect of increased water salinity on LA neurotoxicity might be related to disruption of intracellular elemental composition of Na^+ and Ca^{++}. There is increasing evidence to indicate that the structural and functional consequences of variety of injury processes to target cells might be mediated by a disruption of intracellular distribution of Na^+, K^+ and Ca^{++} and water regulation (MacKnight, 1987; Trump, 1989). Using electron probe X-ray microanalysis, it was demonstrated that a correlation exists between cellular morphological structure and elemental composition and water content (Lopachin, et al., 1990). It has been shown that various types of neuropathies caused by drugs, toxins and diabetes can be mediated via disruption of subcellular elemental composition of Na^+, K^+ and Ca^{++} in the peripheral nerves. It was hypothesized that there is an increase in intra-

axonal Na^+, preceded by an inhibition of Na^+-K^+-ATPase activity leading to reduced conduction of nerve impulses. Furthermore, it was also suggested that accumulation of increased axoplasmic Ca^{++} may be responsible for peripheral nerve axon damage and degeneration resulting from several types of injury as it is seen in acrylamide toxicity and transection (Lopachin, et al., 1990; 1992a; 1992b; 1994; Lehning, et al., 1996). The increased intra-axonal Ca^{++} is mediated by intracellular Na^+ and subsequent influx of Ca^{++} ions through reverse operation of Na^+-Ca^{++} exchanger (Lehning, 1996). In addition, it was shown that in transected nerves, ultrastructural changes are mediated by Na^+ and Ca^{++} concentration in the media. Sodium in the media of damaged dendrites causes dilation of the Golgi cisternae and increased mitochondrial electron opacity similar to neurons incubated with monensin (Emery et al., 1991).

Based on ataxia scores, PMSF appeared to slightly increase degree of ataxia compared to the rats treated with LA only. In the mechanism of PMSF-induced modulation of OPIDN, several mechanisms are suggested including inhibition of NTE (Johnson, 1970) and alteration of neurofilament (Xie, 2002) subunits. However, several studies also reported that alterations in Ca^{++} homeostasis can be seen in OPIDN. Luttrell et al (1993) demonstrated that total Ca^{++} in homogenates of the sciatic nerves was decreased significantly in TOTP-treated hens and it was suggested that at fully developed OPIDN the alteration of the Ca^{++} balance is an indicator of axonopathy in a degenerated nerve following injury. Interestingly, two studies showed that when hens were dosed with TOCP and leptophos, these two OP compounds led to decreased serum Ca^{++} levels in OP treated hens with signs of OPIDN. However, prior administration of PMSF protected the animals from delayed neuropathy and blocked the decrease in serum Ca^{++} concentration.

Furthermore, when PMSF administered following the OP compounds to the hens it potentiated OPIDN and caused a decrease in serum Ca^{++} concentration (Piao et al., 1996; Piao et al., 2003). In the same study, it was also shown that Calcicol as calcium tonic alleviated the neuropathy when applied before the onset of delayed neuropathy (Piao et al., 2003). These results suggest that PMSF can alter Ca^{++} homeostasis. Lasalocid is known to act by facilitating Ca^{++} movement across membranes. Increase in serum Ca^{++} induced by PMSF in the postreatment can provide increased availability of Ca^{++} for cells to be transferred across membranes. Therefore a possible interaction between LA and PMSF might occur on Ca^{++} homeostasis leading to potentiation of neurotoxicity as it is seen in our rat study.

Another possible mechanism for the potentiation of LA neurotoxicity by PMSF could be associated with alteration in neurofilament (NF) subunits in nerve fibers. Reduced levels of NF transcripts were found in many neurodegenerative disease conditions caused by neurotoxic chemicals (acrylamide, aluminum), nerve injury, aging and some other diseases (Alzhemer's disease, Parkinson's disease, diabetes). It has been reported that diisopropyl phosphofluoride (DFP), a neuropathic OP, altered NF subunit protein level and mRNA expression in hens. However, there was no alteration in PMSF-protected hens. When applied after DFP, PMSF increased medium molecular weight NF and decreased expression of NF subunits in the spinal cord that did not occur in DFP alone treatment (Xie et al., 2001; Xie et al., 2002). Similary, PMSF can alter cytoskeletal composition of nerve fibers in LA-induced neuropathy leading to increased response as ataxia and increased lesions in spinal cord.

A recent study demonstrated that NTE may have lysophospho lipase (LysoPLA) activity with lysolecithin as its physiological substrate. This effect has been shown in mouse brain by replacing phenyl valerate (non-physiological substrate used in NTE assay) with lysolecithin. The results showed that delayed neurotoxicants applied to the mice inhibited NTE-LysoPLA and NTE same degree, and NTE-LysoPLA inhibition was correlated with delayed neurotoxicty. It was concluded that OP-induced delayed toxicity could be due to NTE-LysoPLA inhibition resulting in accumulation of lysolecithin. Lysolecithin is a membrane lysophospholipid which is hydrolysed by LysoPLA. They play some critical roles in phospholipid metabolism for cell survival and function. They are also involved in receptor-mediated signal transduction via G protein-coupled mechanism. In high concentrations, lysolecithin causes demyelination and axonal lesions in nerves (Quistad, et al., 2003). Effect of PMSF on NTE-LysoPLA is unknown. However, PMSF is known to be one of the inhibitors of NTE. Therefore, it can be expected that inhibiton of NTE-LysoPLA by PMSF is quite possible. We can speculate for the mechanism of potentiating effect of PMSF on LA-induced neuropathy based on these current findings. It is possible that PMSF may have also inhibited NTE-LysoPLA activity in the membranes of nerves leading to membrane disturbance and altered phospholipids composition. This will change membrane permeability characteristics to Ca^{++} with the additional effect of LA which causes Ca^{++} overload. Since NTE is a membrane bound protein, same effect of PMSF on NTE-LysoPLA can be seen in membranous organelles such as in mitochondria. The excessive Ca^{++} overload in mitochondria causes uncoupling of oxidative phosphorylation leading to decreased ATP production and subsequent disturbance in ionic homeostasis.

Pathologic findings in neuronal tissues in response to LA treatment with or without PMSF included intramyelinic edema, myelin separation, splitting and vacuolation. These results are similar to previous reports of LA toxicity (Gregory et al., 1995; Roder, 1996). Myelin splitting can be seen in a variety of toxic and diseases conditions. Hexachlorophene, triethyltin, and ionomycin are several examples of chemicals leading to myelin splitting associated with myelinopathy (Lennart et al., 1978; Cammer, et al., 1975). Mechanism underlying this includes alterations in nerve and supporting cells. Some myelinopathies (myelin splitting) develop due to axonopathy, swannopathy and interstitial edema. Endoneurial edema was suggested in hexachlorophene toxicity due to vascular changes. Increased endoneurial fluid pressure leading to endoneurial edema can cause also edema within myelin layers. This effect may also be due to direct injury to myelin sheets by increasing the permeability of myelin to osmotically active cations (Robert, 1994).

Alterations in composition or expression of myelin basic protein have been associated with myelin splitting in several neuropathies. Myelin basic protein is a structural protein that plays a role in the compaction of myelin lamellae and serves as an adhesive between myelin layers (Veronosi et al., 1992).

In triethyltin and hexachlorophene neurotoxicity, intramyelinic edema and myelin splitting can be related to changes in mitochondrial oxidative phosphorilation. Biochemical changes in mitochondria are due to uncoupling of oxidative phosphorilation (Graham et al, 1973; Cammer, et al., 1975). Ionophores facilitate concentration dependent transport of Na^{+}, K^{+}, and Ca^{++} across membranes by forming lipid soluble structures. This effect accounts for the toxicity of ionophores. Electrically neutral

exchange of cations for protons across cell membranes disturbs electrochemical gradient across mitochondrial membrane leading to decreased ATP production and increased ATP use. Monensin and Salinomycin increase intracellular Na concentration whereas lasalocid increases intracytoplasmic K^+ and Ca^{++} concentrations. Therefore, increased ion concentration may cause influx of extracellular fluid into cells. This fluid can accumulate between myelin layers as edema and vacuole formation.

The ataxia induced by LA in rats was slight to moderate. However, light and ultra structural examination of the sciatic nerve and spinal cord revealed that pathological lesions in myelin sheath of nerve fibers were somewhat severe compared to the degree of clinical ataxia. The reason for this inconsistency between the degree of ataxia and severe pathological lesions might be due to the fact that nuclei of nerve fibers localized in spinal cord controlling sciatic nerve to the hindlimbs might be still intact and not damaged. In addition, although there was an extensive myelin splitting and vacuolation, myelin sheath appeared still intact and no demyelination was p resent. Therefore, the lesioned myelin sheath might have provided some insulation for impulse conduction and the ataxia may be reflected as slight or moderate ataxia. However, this seems not to be the case since MNCV data from chicken study indicates that nerve conduction velocity was impaired due to LA toxicity. Anatomically, movement of avian species depends on two hindlimbs and body balance has to be provided on two legs. In contrast, the rats are able to use all four limbs for body balance and movement. Therefore, body weight and balance are distributed to all four legs. Thus, an even small abnormality in physiological function or anatomical structure in their hindlimbs may be manifested clinically as more severe ataxia in chickens.

In conclusion, the results of our study showed that LA induces ataxia and pathological changes in the sciatic nerve and spinal cord of rats. Addition of 0.9 % NaCl in drinking water has no effect on ataxia induced by LA. The novel finding of this study is that phenylmethylsulfonyl fluoride increases the ataxia in LA-treated rats implying that neuropathies of different origin can be potentiated by PMSF. The results of this study do not provide information on the exact mechanism of this potentiation. Therefore, future studies need to be performed on the mechanism that leads to the potentiating effect of PMSF on different types of neuropathies.

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CHAPTER IV

SUMMARY AND CONCLUSIONS

The results of the studies presented in the previous chapters indicate that lasalocid (LA) is capable of producing a neuropathy in broiler chickens and rats. In chickens, we were able to produce an ataxia by the administration of 30 mg/kg of LA t.i.d. for 2 days. Clinical manifestation of the neuropathy was observed as ataxia, loss of righting reflex, loss of sensitivity in pain perception in hindlimbs. Using clinical ataxia scoring system, the degree of ataxia was evaluated and the ataxia ranged from slight to severe in chickens. Administration of phenylmethylsulfonyl fluoride (PMSF) before or after LA treatment increased the degree of pathologic changes in broiler chickens. The lesions included edema and vacuole formation in myelin sheath, myelin splitting and fragmentation. At light microscopic level, while chickens treated with LA+PMSF at 3 weeks old age showed lesions in the spinal cord, no spinal cord lesions were observed in chickens treated with LA only. Pathogenesis of the spinal cord lesions shown (Chapter 2) in LA+PMSF treated birds requires further investigations. In addition, neurotoxic esterase (NTE) assay showed that although PMSF reduced neurotoxic esterease (NTE) activity LA did not have any effect on the NTE activity of sciatic nerve, spinal cord and brain in chickens. Motor nerve conduction velocity (MNCV) of the sciatic nerve from treatment groups indicated that while LA reduced motor nerve conduction velocity

(MNCV) in the sciatic nerve, PMSF did not affect the MNCV either alone or in combination of LA.

In the rat study, dose response study indicated that administration of 20 mg/kg of LA 2 times a day for 3 days by gavage produced slight ataxia in male Sprague Dawley rats. At this dose, no mortality were observed in the rats, therefore 20 mg/kg of LA is the optimum dose to produce ataxia without causing death. Rat studies demonstrated that lasalocid-induced neurotoxicity may provide a useful mammalian model of drug-induced neuropathy. Addition of 0.9 % NaCl in drinking water did not affect ataxia and nerve pathology. However, high dose of LA (40 mg/kg) with 0.9 % NaCl produced muscle lesions including mild necrosis of skeletal muscle with loss of striation and edema between myofibers.

In the potentiation study, PMSF worsened the ataxia in LA-treated rats. Electron microscopic examination of the sciatic nerve and spinal cord demonstrated that LA causes lesions including myelin splitting, edema between myelin lamellae, vacuole formation in the myelin sheath.

The consistent finding of PMSF effect on LA-toxicity in both chickens and rats is that PMSF potentiates LA-neurotoxicity and produces similar pathological lesions in the sciatic nerve and spinal cord.

The mechanism of lasalocid induced pathologic lesions is unknown. However, role of ionophores in the alteration of cellular ionic balance supposedly is the resultant effect of ionophore toxicity for these changes. Additional mechanistic studies must be performed to determine the pathogenesis of the lesions.

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