ผลของจีเอ็ม 1 ต่อพยาธิสภาพของเส้นประสาทที่เกิดจากยาพาคลิแทคเซลในหนู

นางสาวณัฐธรา ดวงมาตย์พล

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EFFECT OF GM1 ON PACLITAXEL-INDUCED NEUROPATHY IN RATS

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ยาพาคลิแทคเซล (Paclitaxel) หรือชื่อทางการค้าคือแทคซอล (Taxol) เป็นยาที่ใช้อย่างแพร่หลายในการรักษาโรคมะเร็ง แต่ผลข้างเคียงที่เกิดขึ้นคือการเกิดพยาธิสภาพของเส้นประสาททั้งในมนุษย์และสัตว์ทดลอง ซึ่งกลไกการเกิดยังไม่สามารถอธิบายได้ชัดเจนและยังไม่มียาตัวใดที่มีศักยภาพในการป้องกันหรือลดความเป็นพิษต่อเส้นประสาทได้ ดังนั้น ในการศึกษาครั้งนี้มีวัตถุประสงค์ เพื่อศึกษาผลของจีเอ็ม 1 ในการลดการเกิดพยาธิสภาพของเส้นประสาทที่เกิดจากยาพาคลิแทคเซลในหนู วิสตาร์ เพศผู้จำนวน 40 ตัว โดยแบ่งหนูวิสตาร์ออกเป็น 4 กลุ่ม ในกลุม C ไม่ได้รับการฉีดยาใดๆ กลุ่ม P ฉีดยาพาคลิแทคเซล 16 มก./กก./ครั้ง/สัปดาห์ กลุ่ม V ฉีดครีโมฟอร์ (Cremophor) ผสมกับเอทานอล ซึ่งเป็นตัวทำละลายยา สัปดาห์ละ1 ครั้ง และกลุ่ม PG ฉีดยาพาคลิแทคเซลเหมือนกันกลุ่ม P ร่วมกับฉีดจีเอ็ม 1 30 มก./กก./วัน เป็นเวลา 5 สัปดาห์ และทดสอบการตอบสนองต่อการรับความรู้สึกเจ็บปวด และการนำกระแสประสาท พบว่าค่าเวลาในการตอบสนองต่อการรับความรู้สึกเจ็บปวดจากความร้อนทั้งที่หางและที่เท้าหลังของหนูในกลุ่ม P ยาวนานขึ้น รวมทั้งการนำกระแสประสาทที่หางก็ลดลงเมื่อเทียบกับกลุ่ม C ในขณะที่กลุ่ม C และ กลุ่ม PG ไม่ต่างกันจากนั้นเก็บเส้นประสาท sciatic มาศึกษาลักษณะทางโครงสร้างด้วยกล้องจุลทรรศน์ธรรมดา พบว่า เส้นประสาทในกลุ่ม P กลุ่ม PG และกลุ่ม V มี minimal axonal degeneration ส่วนในปมประสาทไขสันหลังไม่พบมีการเปลี่ยนแปลงของเซลล์ การวัดเชิงโครงสร้างของเส้นใยประสาท sciatic พบว่าเส้นใยประสาทขนาด 8 ถึง 9 ไมโครเมตรในกลุ่ม P กลุ่ม PG และกลุ่ม V มีจำนวนเพิ่มขึ้นเมื่อเทียบกับกลุ่ม C ในขณะที่การศึกษาลักษณะทางโครงสร้างด้วยกล้องจุลทรรศน์อิเล็คตรอน พบว่าในกลุ่ม P และกลุ่ม PG ปรากฏ microtubule มี accumulation และอยู่ล้อมรอบ mitochondria นอกจากนี้ยังพบ atypical mitochondria และพบ Schwann cell มีลักษณะเป็น activated cell โดยมีโครงสร้างที่คล้าย nucleolus ใน nucleus ซึ่งในกลุ่ม P ปรากฏให้เห็นเด่นชัดมากกว่าในกลุ่ม PG และกลุ่ม V จากผลการศึกษาข้างต้นแสดงให้เห็นว่า จีเอ็ม 1 น่าจะมีความสามารถในการช่วยลดความเป็นพิษต่อเส้นประสาทที่เกิดจากยาพาคลิแทคเซล ในเรื่องของการรับความรู้สึก ความเร็วในการนำกระแสประสาท และการเปลี่ยนแปลงลักษณะทางโครงสร้างของเส้นประสาท

สาขาวิชา วิทยาศาสตร์การแพทย์ ลายมือชื่อนิสิต ณัฐธรา ดวงมาตย์พล

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KEY WORD: PACLITAXEL / GM1 / PERIPHERAL NEUROPATHY / MORPHOLOGICAL STUDY

NATTHARA DUANGMARDPHON : EFFECT OF GM1 ON PACLITAXEL-INDUCED NEUROPATHY IN RATS. THESIS ADVISOR: ASSOC. PROF. VILAI CHENTANEZ M.D., Ph.D. 57 pp. ISBN 974-14-3455-3.

Paclitaxel (Taxol®) is an anti-cancer drug effective against a wide range of solid tumors. It is known to be neurotoxic in humans. Several studies have demonstrated paclitaxel-induced neurodegeneration and sensory loss in animal models. The mechanisms underlying this dose-limiting side effect are currently unknown and there is no evidence to show the effectiveness of any drugs that can prevent or control these severe side effects. This study reports the use of a rat model of toxic neuropathy to evaluate the role of GM1 as a possible neuroprotectant for paclitaxel. Forty male Wistar rats were equally divided into 4 groups. The C group did not receive any injection. The V and P groups were given a weekly intraperitoneal injection of vehicle (Cremorphor+ethanol) and 16 mg/kg paclitaxel, respectively. The PG group received 30 mg/kg/day GM1 intraperitoneally with paclitaxel in the same dose as the P group, for 5 consecutive weeks. Quantitative sensory tests, nerve conduction velocity (NCV), morphological and morphometrical studies were performed. Prolonged withdrawal time of tail flick and hot plate analgesic tests including reduced tail NCV were observed in the P and V groups. Light-microscopic inspection of the sciatic nerve in the P, PG and V groups showed minimal degenerative axonal changes. The dorsal root ganglia neurons were unaffected in all groups. The morphometric examination presented a significant increase in the percentage of myelinated fibres with a diameter between 8 to 9 μm in the P, V, and PG groups when compared to the C group. There were no significant differences between the groups in other parameters. At the electron microscopic examination, axons of the sciatic nerve in the P and PG groups revealed microtubule accumulation and the tendency to surround mitochondria, while there was no such evidence in the other groups. Furthermore, abnormality in axonal mitochondria and activated Schwann cells with nucleolus like structure were found in the P group. This was also found in the PG and V groups but with less frequency. The results of this study demonstrated that GM1 had some protective role on sensory modality and NCV tests, and nerve pathological changes in paclitaxel-induced neuropathy.

Field of Study : Medical Science Student's Signature: Natthara Duangmardphon

Academic Year : 2006 Advisor's Signature: Vilai Chentanez

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LIST OF ABBREVIATIONS

ANOVA	One-way analysis of variance
CMAP	Compound muscle action potential
ChAT	Choline acetyltransferase
CNS	Central nervous system
Da	Axonal diameter
DMF	Density of myelinated axon fiber
DRG	Dorsal root ganglia
Ds	Myelinated fiber diameter
FA	Fascicle area
G-CSF	Granulocyte colony stimulating factor
GM1	Monosiaic acid ganglioside
INM	Inner nuclear membrane
MAP2	Microtubule associated protein
mPTP	Mitochondrail permeability transition pore
MT	Microtubule
NCV	Nerve conduction velocity
NCX	Sodium/Calcium exchanger
NE	Nuclear envelope
NGF	Nerve growth factor
NTF	Neurotrophic factor
ONM	Outer nuclear membrane
PNS	Periphreral nervous system
RDLN	Retrodorsal lateral nucleus
Trk	Tyrosine kinase

CHAPTER I

INTRODUCTION

1. Background and Rationale

Paclitaxel (Taxol®), crude extract from the bark of the Taxus brevifolia (Pacific yew tree), was discovered in 1963 (Wani et al., 1971). It is an anti-cancer drug effective against a wide range of solid tumors such as ovarian cancer, breast cancer, lung cancer and head and neck cancer (Mross et al., 2000; Tai et al., 2001; Skvortsora et al., 2004).

Since its isolation and characterization in 1971 (Wani et al., 1971), it has been known that paclitaxel has a cytostatic action. It is believed that the antineoplastic properties of paclitaxel are due to its ability to greatly increase the stability of tubulin polymers (Wani et al., 1971) thus inhibiting cellular replication, thereby causing the death of the cell by disrupting the normal microtubule dynamics required for cell division and vital interphase process (Burkhart et al., 1994). The toxic effects of paclitaxel include bone marrow suppression, hypersensitivity reactions, cutaneous reactions, and peripheral neurotoxicity (Rowinsky et al., 1993). Peripheral neurotoxicity is a serious dose-limiting side effect of paclitaxel therapy (Rowinsky EK., 1994), because of the current lack of treatments and knowledge of the mechanism responsible.

Paclitaxel evokes an array of symptoms: numbness and tingling, mechanical allodynia, cold allodynia and on-going burning pain in a stocking and glove-type distribution, an elevation of vibratory thresholds and loss of deep tendon reflexes (Rowinsky et al., 1993; Rowinsky EK., 1994; Forsyth et al., 1997; Dougherly et a., 2004). Sensory conduction velocities and amplitudes of sensory nerve action potentials are reduced (Rowinsky EK., 1994; Forastiere et al., 1993). Histopathologically, severe paclitaxel-induced neuropathy is characterized by nerve fiber loss, axonal atrophy, and demyelination in the sural nerve (Forastiere et al., 1993). Small-fiber sensory modalities or motor system are sometimes affected (McGuire et al., 1989; Forastiere et al., 1993).

At present, there is no evidence showing the effectiveness of any drugs that can prevent or reverse the neurotoxic effects of paclitaxel. Only a few drugs have been tested in clinical trials such as the ACTH analogue 4-9 (Hamers and Sunada., 2004), amifostine (Cavaletti and Sanna., 2002) and glutamine (Openshaw et al., 2004). They had shown their potential to reduce the toxic neuropathy. Preliminary studies of

glutamine showed that this agent may reduce the severity of peripheral neuropathy after high dose paclitaxel (Stubblefield et al., 2005). Furthermore, a neuroprotective effect of nerve growth effect (NGF) was suggested by several in vitro studies on paclitaxel neurotoxicity (Apfel et al., 1991; Hayakawa et al., 1994).

The possibility of reducing the toxic effects of paclitaxel on peripheral nerve would therefore be of great clinical interest. There are several studies of the effects of gangliosides on the nervous tissues. Several experiments revealed the prevention effects of ganglioside treatment in diabetic neuropathy (Figliomeni et al., 1992; Bianchi et al., 1990). The study with experimental diabetic mice had also shown an improvement of reduced nerve conduction velocity after ganglioside treatment ((Figliomeni et al., 1992). Ganglioside can enhance peripheral nerve regenerative capacity after traumatic lesion (Gorio et al., 1980). Furthermore, ganglioside especially monosialic acid ganglioside (GM1), is able to promote survival of neurons (Ferrari et al., 1995). In an animal model of paclitaxel-induced neuropathy, the previous study describes the beneficial effects of ganglioside mixture treatment (Chentanez et al., 2003).

The neuroprotective actions of GM1 in vitro may suggest it as a potential treatment option in paclitaxel-induced neuropathy. Moreover, several morphological changes in the peripheral nerve from animals treated with paclitaxel have been repeated (rats). In this regard, the present study carried out an animal model investigation to find out whether GM1 can alter abnormal neuronal morphology in paclitaxel-induced neuropathy in the rats.

2. Research Question

Can GM1 alter paclitaxel-induced neuropathy in rats ?

3. Objectives of the Study

1. To evaluate the effect of GM1 on paclitaxel-induced neuropathy on sensory behavioral test and electrophysiological study.

2. To perform quantitative morphometric evaluation of sciatic nerve in the paclitaxel and paclitaxel- GM1 rats in comparison to the untreated groups.

3. To evaluate ultrastructural changes of the axon and the Schwann cell.

4. To evaluate morphological changes of sensory neurons of the dorsal root ganglion.

4. Hypothesis

GM1 can reduce abnormality in paclitaxel-induced neuropathy in rats.

5. Key Words

Paclitaxel, Taxol, Ganglioside, GM1, Peripheral neuropathy, Morphological study

6. Expected Benefits and Applications

The results of this study will be a preclinical trial of using GM1 as a protective agent for paclitaxel-induced neuropathy.

CHAPTER II

LITERATURE REVIEW

1. Paclitaxel (Taxol®)

Paclitaxel (Taxol®), the first taxane in clinical use, is active against a broad range of cancers that are generally considered to be refractory to conventional chemotherapy. This agent was discovered as part of a National Cancer Institute Program in which extracts of thousands of plants were screened for anti-cancer activity. In 1963, the crude extract from the bark of Pacific yew (Taxus brevifolia), a scarce and slow-growing evergreen tree in the Pacific Northwest, was found in preclinical studies to have cytotoxic activity against many tumors (Wani et al., 1971). Paclitaxel was identified as the active constituent of this extract in 1971. This drug is a complex ester, shown to be a taxane derivative containing rare oxetan ring (Figure 1.), and is the first compound of this type to have antileukemic and tumor inhibitory properties.

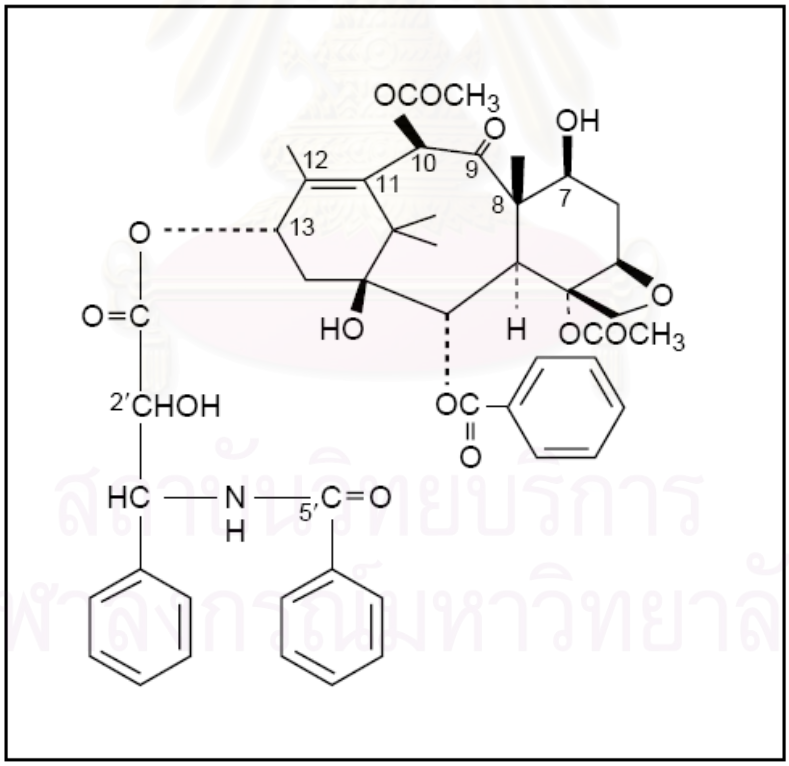

Figure 1. Structure of Paclitaxel (Alastair et al, 1995)

1.1 Mechanism of action of paclitaxel

Paclitaxel is an anticancer agent which kills cancer cells through the induction of apoptosis. There are three hypothetic possible pathways for paclitaxel-induced apoptosis (Figure 2) (Manfredi and Horwitz., 1984; Burkhart et al., 1994; Cheng et al., 1995; Parekh and Simpkins., 1997; Fan., 1999). The cellular target for paclitaxel is the microtubule. Therefore, the first pathway (indicated by solid lines in Figure 2) can be considered as the microtubule pathway in which cell death occurs following mitotic arrest. After paclitaxel crosses the plasma membrane, it binds specifically to the N-terminal 31^{st} amino acids of β-tubulin subunit and promotes polymerization and inhibits disassembly of microtubules by suppressing dynamic changes, thereby resulting in stable and dysfunctional microtubules. Hyperpolymerized and hyperstabilized microtubule lead to GM_2/M phase and finally apoptosis.

It is known that the most toxic side-effect of paclitaxel is a sensory neuropathy. The precise mechanisms underlying neurotoxicity are not yet clear. Previous studies found dysfunctional microtubules in axons, Schwann cells and dorsal root ganglia (Roytta and Raine., 1985; Cavaletti et al., 1995; Cavaletti et al., 1997; Authier et al., 2000; Persohn et al., 2005). According to these findings, neurotoxicity of paclitaxel may be explained as follows. Overdose of paclitaxel promotes excessive microtubule assembly, leading to the formation of large bundles of disordered microtubules. These disorganized microtubules are thought to accumulate in the cells. Abnormal accumulation of microtubules may disturb many critical cell functions. On the other hand, axonal transport in neuron may be disturbed, since it is totally dependent on tubulin-microtubules polymerization-depolymerization dynamics. Schwann cells are also a major target of paclitaxel in the peripheral nerves (Cavaletti et al., 1995; Cavaletti et al., 1997; Authier et al., 2000). Cytoplasmic and nuclear changes in the Schwann cells are considered to reflect a toxic injury by paclitaxel. These clinical and experimental observations support the first hypothetic pathway for the mechanism of neurotoxicity of paclitaxel.

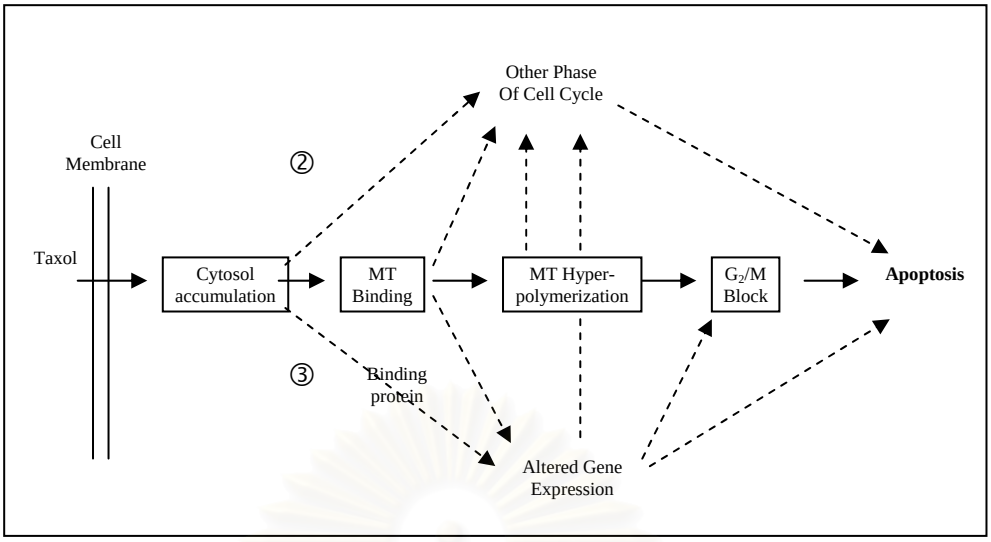

Figure 2. Hypothesized pathways of paclitaxel-induced apoptosis MT=microtubule (Fan., 1999)

The second pathway suggests that cell death induced by paclitaxel is still via the microtubule system, but uncoupled from mitotic arrest. Cells may undergo apoptosis from other phases of the cell cycle. Although microtubules are critical in mitotic function as primary constituents of the mitotic spindle apparatus, they are also important for the performance of many vital interphase functions, such as intracellular vesicular transport, maintenance of cell shape, cellular mobility, and perhaps even transmission of signals from cell-surface receptors to the nucleus (Letourneau et al., 1986; Nogales., 2000). Therefore, when the normal structure and function of the microtubular network are disrupted by paclitaxel, all of the cellular functions associated with the tubulin-microtubule system may be lost or damaged. In cells which this takes place, there may be failure to maintain normal growth and metabolic activities, and, thus, cells might also undergo apoptosis even though the cells are not arrested in the G2/M phase.

The third pathway assumes that paclitaxel might exert its cell-killing activity via a pathway completely independent of microtubules. Paclitaxel might cause cell death through a gene-directed process (Manfredi and Horwitz., 1984; Parekh and Simpkins., 1997).

Morphologically, most cells exposed to a high concentration of paclitaxel seem to undergo apoptosis by the first pathway (Bhalla et al., 1993; Cheng et al., 1995), while apoptotic cell death induced by low concentrations of paclitaxel is more likely to take place via the second or third pathways (Jordan et al., 1996; Fan et al., 1998).

Furthermore, paclitaxel may have a direct action on the mitochondrial membrane. Several studies reported that paclitaxel can alter both mitochondrial function and structure. This has been hypothesized that paclitaxel evokes calcium release from mitochondria via the opening of mitochondrial permeability transition pore (mPTP) (Evtodienko et al., 1996; Kidd et al., 2002). β-tubulin is shown to bind to axonal mitochondria (Carre et al., 2002: Mironov et al., 2005). Stabilization of microtubule by paclitaxel may modify the interaction of microtubule with the outer mitochondrial membrane, thus delivering an adequate signal for the opening of mPTP, and inducing a loss of mitochondrial Ca^{2+}. The consequence of mPTP opening is mitochondrial swelling, which has been linked to painful peripheral neuropathy (Flatter et al., 2006; Dalakas et al., 2001; Mironov et al., 2005). The hypothetical mechanism for paclitaxel-induced neuropathy is susmmarized in Figure 3.

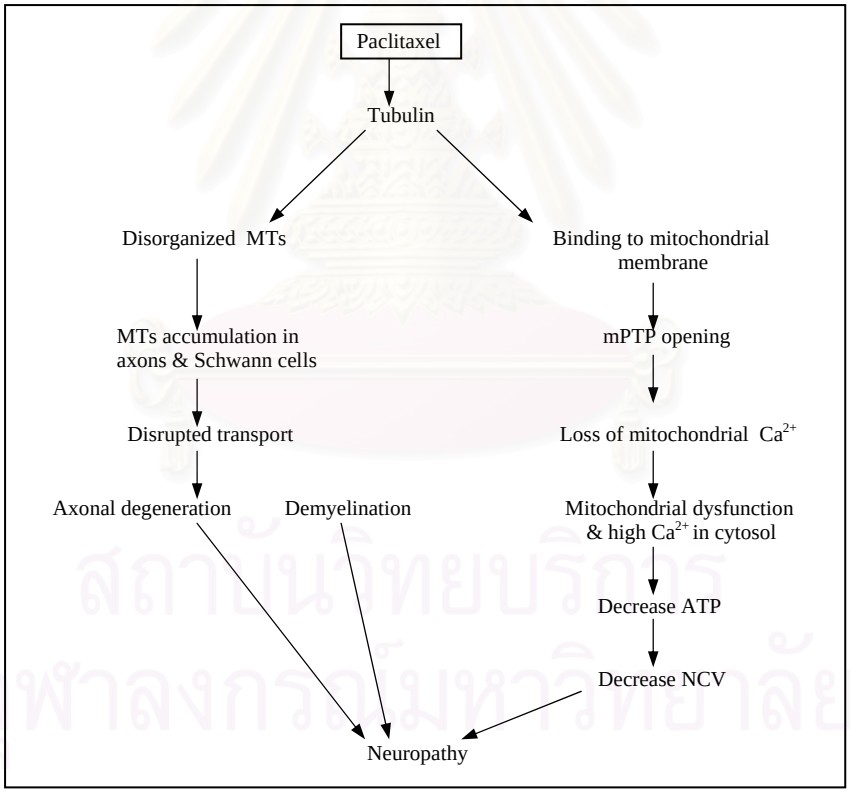

Figure 3. Hypothetical mechanism of paclitaxel-induced neurotoxicity. MTs=microtubules; mPTP= mitochondrial permeability transition pore

1.2 Side effects

Neurotoxicity

Paclitaxel induces a peripheral neuropathy characterized by sensory symptoms such as numbness, tingling, burning pain and paresthesia. Patients describe some or all of these symptoms in a stocking-glove distribution (Rowinsky et al., 1993; Forsyth et al., 1997; Dougherty et al., 2004). Sensory symptoms usually start symmetrically in the feet, but sometimes appear simultaneously in both hands and feet.

On neurological examination, distal loss of sensations mediated by both large (vibratory and position sense) and small fibers (temperature and pain sense) and loss of deep tendon reflexes can be found. The severity of neuropathy is related to both the single and the cumulative dose of paclitaxel. At lower dosages per cycle (135-200 mg/m^2), neuropathy is rarely observed. In contrast, at higher dosages per cycle (> 250 mg/m^2) a more severe neuropathy is more prominent, which often starts following the first cycle (Sahenk et al., 1994; Dougherty et al., 2004).

The distal, symmetric, length-dependent neurologic deficits suggest that paclitaxel causes a sensory and motor axonal loss similar to the “dying-back” neuropathies. A few patients have the simultaneous onset of symptoms in the arms and legs, involvement of the face (perioral numbness), the predominance of large-fiber loss (Persohn et al., 2006), and diffuse areflexia (Rowimsky et al., 1993; Chaudhry et al., 1994). Motor and automatic dysfunction may also occur, especially at high doses and in patients with preexisting neuropathies caused by diabetes mellitus and alcoholism. In addition, optic nerve disturbances characterized by scintillating scotomata, may occur (Capri et al., 1994). Unfortunately, paclitaxel-induced pain and sensory abnormalities can become chronic, persisting for months or years following the termination of paclitaxel therapy (van den Bent et al., 1997; Dougherty et al., 2004).

Previous studies of paclitaxel-induced neuropathy both in animals and patients have shown that paclitaxel reduces sensory and motor nerve conduction velocities, and action potential amplitudes (Chaudhry et al., 1994; Cliffer et al., 1998; Cavaletti et al., 1995; Authier et al., 2000; Chentanez et al., 2003). One study demonstrated the morphological changes induced by paclitaxel in patients treated for cancer (Sahenk et al., 1994). The sural nerve biopsy showed severe nerve fiber loss, axonal atrophy, secondary demyelination and absent axonal regeneration. No

evidence of Schwann cell proliferation or axonal sprouting. No microtubule aggregation in either axons or Schwann cells was encountered.

However, early morphological studies reported axonal regeneration when paclitaxel was injected into the sciatic nerve of the rat (Roytta et al., 1984; Roytta and Raine., 1985, 1986). They have clearly shown that the drug is able to induce marked microtubule accumulation in axons and Schwann cells, in association with demyelination.

Recent rat studies are more clinically relevant as they employed systemic paclitaxel administration, either intravenous (Cavaletti et al., 1997; Cliffer et al., 1998) or intraperitoneal (Cavaletti et al., 1995; Authier et al., 2000; Polomano et al., 2004; Chentanez et al., 2003; Persohn et al., 2005; Flatter et al., 2006) instead of direct nerve injection. The consensus of these studies is that the degree of degeneration observed is positively correlated to the dose of paclitaxel.

Some of these studies used high dose paclitaxel and observed histological changes. Morphological examinations showed mild endoneurial edema (Cavaletti et al., 1995; Chentanez et al., 2003).Moreover, a decreased number of large myelinated fibers was observed (Cavaletti et al., 1995; Authier et al., 2000; Persohn et al., 2005), while the small ones were increased, and some degenerating fibers were also observed (Cavaletti et al., 1995., 1997). The Schwann cell appeared enlarged and in some cases showed a prominent, dark intranuclear structure resembling a nucleolus and dark axonal profiles (Cavaletti et al., 1997). The Schwann cells at the electron microscope level in paclitaxel-treated rats had the features of activated cell.

In addition, fibers in the dorsal and ventral roots had an abnormal mild intraaxonal accumulation of aggregated microtubules. These microtubular aggregations were clearly more evident within the axons of myelinated fibers. The dorsal root ganglia and spinal cord neurons were unaffected (Cavaletti et al., 1995, 1997). Recently, the morphological examination revealed axonal degeneration, collapse and fragmentation of myelin sheath (Authier et al., 2000).

Polomano et al. (2000) reported that low doses of paclitaxel-induced pain without sciatic nerve degeneration, but revealed endoneurial edema. Using low doses of paclitaxel in rats, Flatter et al (2006) suggested that paclitaxel-induced pain was not associated with axonal degeneration and an abnormality of axonal microtubules.

Furthermore, several studies also reported the potential role of mitochondria in paclitaxel-induced neuronal cell death (Nakata et al., 1999; Andre et al., 2000; Kidd et al., 2002; Mironov et al., 2005; Flatter et al., 2006). They showed the paclitaxel-induced mitochondrial swelling might lead to inducing pain and neuronal cell death.

Until now, there is no effective treatment for severe paclitaxel-induced neuropathies, although a variety of agents have shown potential in vivo and in vitro. These include an ACTH 4-9 fragment with growth factor activity (ORG 2766) (Hamers et al., 1993), prosaptide (Campana et al., 1998), venlafaxin (Durand and Goldwasser., 2002), amifostine (Openshaw et al., 2004), vitamin E (Argyriou et al., 2005), and glutamine (Stubblefield et al., 2005). These drugs had shown their potential to reduce the toxic neuropathy. However, further development of these neuroprotectants has been discarded due to the possibility of impairment the cytotoxic efficacy, intrinsic toxicity, the need for parenteral administration, and prohibitive cost.

Other adverse effects of paclitaxel

Hypersensitivity Reactions

Hypersensitivity reactions typically occur early in the treatment course and sometimes within the first hour of infusion. The majority occurred after the first or second dose. Minor hypersensitivity reactions such as flushing, skin reactions, tachycardia, dyspnea and hypotension, do not require interruption treatment or interruption of therapy. Anaphylaxis and severe hypersensitivity reactions characterized by hypotension, angioedema or generalized urticaria have occurred in approximately 2% of patients receiving paclitaxel. Those reactions are probably histamine-mediated. Rare fatal reactions have occurred in patients despite pre-treatment. Therefore, all patients should be pretreated with corticosteroids, diphenhydramine, and H_2 antagonists. In case of severe hypersensitivity reaction, paclitaxel infusion should be discontinued immediately and the patient should not be retreated with paclitaxel (Weiss et al., 1990; Spencer at al., 1994).

Hematologic Toxicity

Bone marrow suppression is the major dose limiting toxicity of paclitaxel. Neutropenia is the most important hematologic toxicity (Rowinsky et al., 1993). Its onset is usually on day 8 to 10 after treatment, and recovery is usually complete by day 5 to 21. Among patient treated in

the Phase III second-line ovarian study with a 3-hour infusion, neutrophil number declined below 500 cells/mm^3 in 14% of the patients treated with a dose of 135 mg/m^2 compared with 27% at a dose of 175 mg/m^2. In the same study, severe neutropenia (< 500cells/mm^3) was more frequent with the 24-hour than with the 3-hour infusion. Therefore, the infusion duration had a greater impact on myelosuppression than the dose. The use of supportive therapy, including granulocyte colony stimulating factor (G-CSF), is recommended of patients who experience severe neutropenia (Schiller et al., 1994).

2. Gangliosides

The name ganglioside was first applied by the German scientist Ernst Klenk in 1942 to lipids newly isolated from ganglion cells of brain. They were shown to be oligoglycosylceramides containing *N*-acetylneuraminic acid (sialic acid or 'NANA' or 'SA' or Neu5Ac) residues (or less commonly *N*-glycoloyl-neuraminic acid, Neu5Gc). This residue is joined via glycosidic linkages to one or more of the monosaccharide units, i.e. via the hydroxyl group on position 2, or to another sialic acid residue**.** The structure of one of the common monosialo-gangliosides, G_{M1}, is illustrated (Figure 4) (Wu et al, 2005).

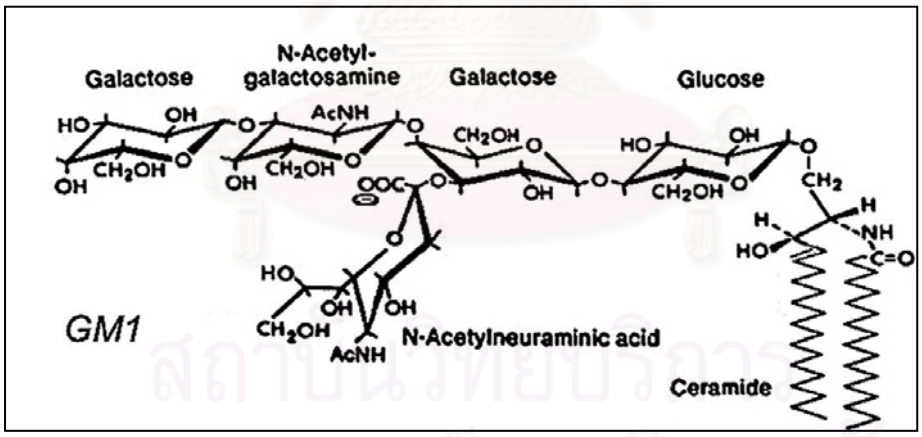

Figure 4. Structure of Ganglioside GM1 (Wu et al, 2005)

Gangliosides are found mainly in virtually all vertebrate cells (Yu and Siato., 1989; Hakomori., 2000) as well as some invertebrate tissues (Zvezdina et al., 1989; Saito et al., 2001). The structural category of gangliosides belonging to the gangliotetraose family constitutes the primary type of sialoglycoconjugate in vertebrate neurons and is

predominantly localized in the plasma membranes of those cells (Ledeen., 1989; Ledeen and Wu., 1992).

There is also considerable evidence for the existence of intracellular pools (Ledeen et al., 1988; Gillard et al., 1993), which, although quantitatively minor, are crucial for important signaling, transport, and regulatory functions. An early study reported gangliosides as being present in every subcellular fraction of bovine mammary gland and rat liver, the level in nuclei from the latter being comparable to that in total homogenate (Keenan et al., 1972). A subsequent report showed ganglioside content of the nuclear membrane from rat liver to be close to 10% of that in the plasma membrane, and GM1 as the dominant species (Matyas and Morre., 1987).

2.1 Effects of GM1 on the nervous system

Both in vitro and in vivo experiments indicated that exogenously supplied GM1could be inserted into the membranes. GM1 promotes neuronal growth and differentiation in cell culture, and enhances phenotypic expression and neuronal repair in animal models of neurotruama and aging. It is a sphingolipid with pleiotropic neurotrophic actions that, in part, acts like a neurotrophic factor (Hadjiconstantinou and Neff., 1998). Reportedly, it improves spinal cord function after an injury and corrects cholinergic (Vogelsberg et al., 1997) neurochemistry of the aged spinal cord. In addition, GM1 treatment can reduce motor neuron death after ventral root avulsion in adult rats (Oliveira et al., 2000)

Goettl et al. (2003) investigated the nummber, size, and staining intensity of choline acetyltransferase (ChAT) immunopositive cells in the retrodorsal lateral nucleus (RDLN) of the spinal cord of rats following left sciatic nerve distal transection (axotomy) and treatment with GM1 ganglioside. They found that GM1 can facilitate the phenotypic recovery of RDLN motor neurons during aging and after axotomy.

Its neuroprotective actions may be due to the potentiation of the effect of nerve growth factor on the axotomized septal cholinergic neurons (Panni et al., 1998). Administration of GM1 in high doses during the critical period of motor neuron death after nerve root avulsion could diminish local inflammation (Oliveira et al., 2000). Bianchi et al. (1986) reported the normal mitochondria of hippocampal slices from the GM1-treated rats compared to swelling mitochondria seen in.

GM1 rescued neuronal cells from apoptotic death (Ferrari et al., 1995) and it can also enhance neuritogenesis by altering the distribution of microtubule associated protein (MAP2) and actin from the perikaryon and subplasmalemmal layer, respectively, to active neuritogenesis sites where they can regulate microtubule and microfilament interactions (Wang et al., 1998). Da-Silva CF. (2001) found that GM1 purified from porcine brain was able to enhance nerve fiber regeneration (Da-Silva., 2001). GM1 has been shown to stimulate neurite outgrowth in cell culture, enhance the activity of nerve growth factor (NGF) in NGF-responsive cells and stimulate neuronal sprouting both in vitro and in vivo (Toffano et al., 1983; Ferrari et al., 1983).

Several studies pointed to an influence of GM1 on neuritogenesis in cells with differentiation potential. The mechanism involved the modulation of Ca^{2+} flux (Wu and Ledeen., 1991; Wu et al., 1995, 2005). The apoptotic effect of dysregulative Ca^{2+} in cultured central nervous system (CNS) neurons, induced by both glutamate and high K^{+}, was partially attenuated by GM1 (Wu et al., 2004). Furthermore, induced and spontaneous neuritogenesis are associated with enhanced expression of ganglioside GM1 in the nuclear membrane (Wu et al., 1995a).

However, excessive accumulation of GM1 causes neuronal apoptosis (Wu et al., 1995). Many studies reported that GM1 fraction induces production of the anti-GM1 antibody which induces Guillain-Barŕe syndrome (GBS) in some patients and animal models (Yuki et al., 1997, 2001; Sheikh et al., 2004; Kaida et al., 2004). GM1 antibody also inhibited neurite formation in the regenerating optic system of goldfish w (Spirman et al., 1984).

2.2 Neuroprotective mechanism of GM1

Exogenously administered ganglioside GM1, provokes regeneration in the peripheral nervous system (Gorio et al., 1983) and prevents degeneration in the CNS following trauma (Cuello et al., 1989; Garofalo et al., 1994). However, the precise mechanism of GM1 function has not been elucidated. NGF was the first identified member of family of neurotrophic factors that function both in vitro and in vivo to promote neuronal survival and differentiation (Levi., 1987). GM1 potentiates the neurotrophic effect of NGF in vivo (Cuello et al., 1989; Fong et al., 1995).

Signal transduction by NGF is mediated through specific cell surface receptors. NGF binds to the high-affinity cell surface receptors

tyrosine kinase A (Trk) A; which is a receptor tyrosine kinase. The NGF signaling cascade is known to be initiated by ligand-induced activation of Trk A (Kaplan et al., 1991; Meakin et al., 1991), which is thought to be activated by the formation of NGF-induced dimerization of Trk A monomers, followed by transautophosphorylation (Hartman et al., 1992).

The potentiation of NGF-mediated activation of tyrosine phosphorylation of Trk A (Ferrari et al., 1995) and potentiation of NGF-mediated neurite outgrowth and neurofilament expression by GM1, appear to involve the autophosphorylation of Trk A. It has also been shown that GM1 augments NGF-induced dimerisation of Trk A monomers (Farooqui et al., 1997), which may indicate that the neurotrophic properties of GM1 may be mediated via the activation of Trk A signal transduction.

Therefore, there are two means by which ganglioside GM1 affects receptor dimerization: one is that they influence membrane dynamics, leading to a local reorganization of membrane architecture that favors receptor interaction and dimerization; the other, which is supported by the report that GM1 binds to glycosylated Trk A (Mutoh et al.,1995), is that ganglioside favors dimerization of receptors by directly interacting with them (Figure 5).

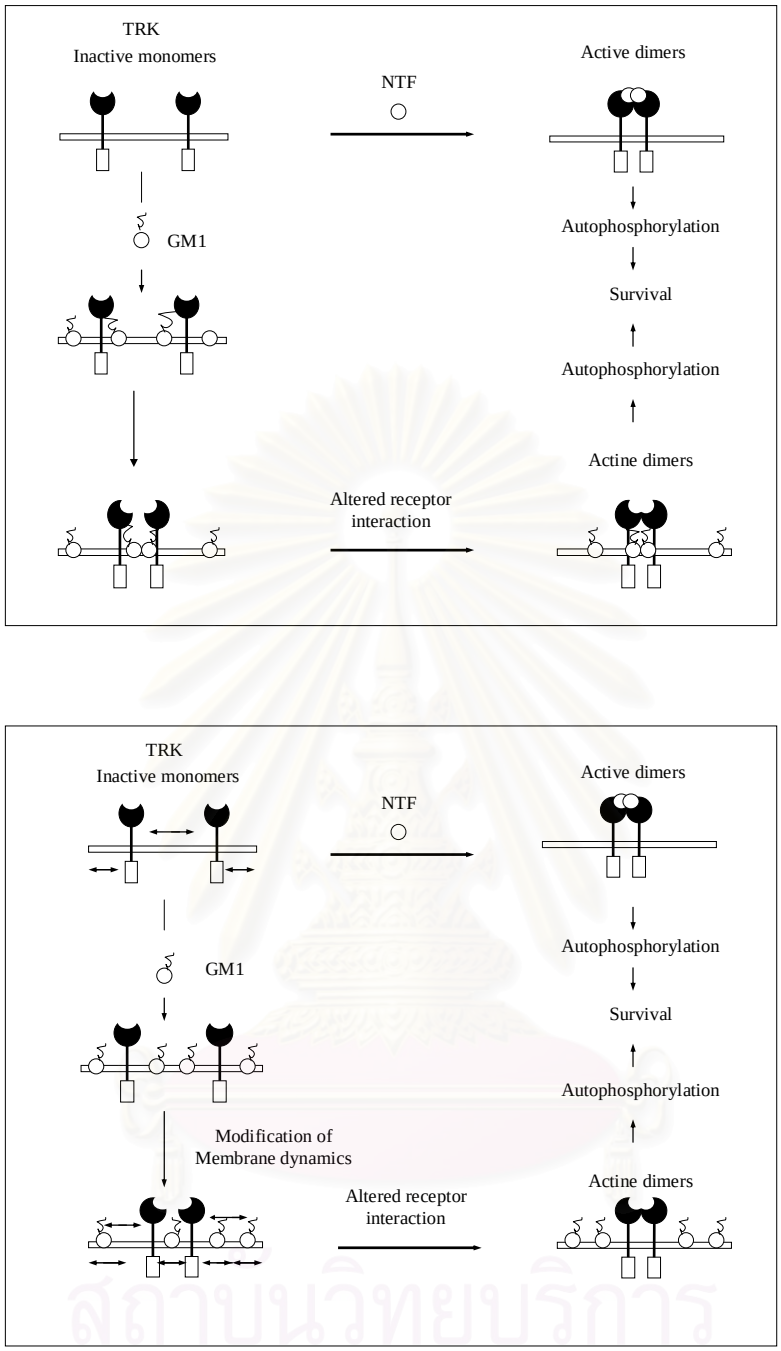

Figure 5. Scheme 1 shows that GM1 may bind directly to TRKs, thereby favouring their dimerization. Scheme 2 suggests that GM1 may alter local membrane dynamics, thereby favoring TRK dimerization. NTF=neurotrophic factor, Trk=tyrosine kinase (Mutoh et al., 1995)

Furthermore, in view of many reports proposing that the side effects of paclitaxel might be due to an action of paclitaxel on mitochondrial membrane and thus interfering with the Ca^{2+} homeostasis (Flatter et al., 2006; Dalakas et al., 2001; Mironov et al., 2005).

Several studies reported that GM1 influences Ca^{2+} flux across both nuclear envelope (NE) and plasma membrane (PM) (Wu et al., 1991, 1995, 1995, 2005; Ledeen and Wu., 2006, 2006). Calcium is known to play role in signaling mechanisms that regulate neuronal differentiation (Ghosh and Greenberg., 1995).

Restoring nuclear Ca^{2+} homeostasis is based on the presence of an a Na^{+}/Ca^{+} exchanger (NCX) in the NE that is dependent on GM1 for optimal activity: this was demonstrated in neurons and neuronal cell lines (Xie et al., 2004a). The NE exchanger binds to GM1 with unusually high affinity and to locate at the inner mitochondrial membrane (INM) of the NE (Figure 6). The outer membrane of this double membrane structure is continuous with the endoplasmic reticulum (ER) membrane and hence the NE lumen is continuous with the ER lumen, thereby providing a storage site for a significant portion of nuclear Ca^{2+} (Thomas et al., 1992; Petersen et al., 1998).

The GM1–exchanger complex is thus required to mediates Ca^{2+} transfer between nucleoplasm and NE, which might well provide the basis of its neuroprotective function. The occurrence of Na^{+}/Ca^{2+} exchanger–GM1complexes in the NE of neural cells suggests a protective mechanism of GM1 in vertebrate cells against the potentially harmful effects of excessive intracellular Ca^{2+}. The presence of nuclear pore complexes throughout the NE are thought to permit free passage of Ca^{2+} between cytosol and nucleoplasm, thereby promoting equilibration of Ca^{2+} in the nucleoplasm with that in the cytosol (Bootman et al., 2000: 371-8).

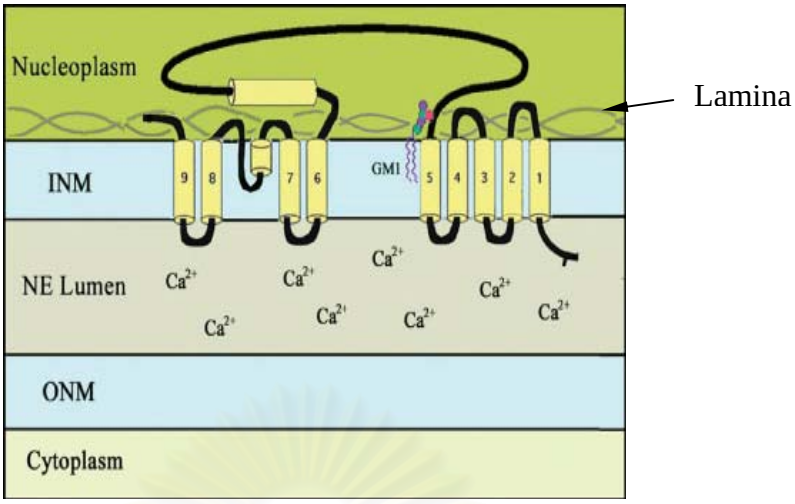

Figure 6. Proposed topology of NCX and GM1 in the nuclear membrane. The outer nuclear membrane (ONM) is continuous with the ER (not shown), while the inner nuclear membrane (INM) is closely associated with the nuclear lamina. The luminal space between the two membranes of the nuclear envelope (NE) is a storage site for Ca^{2+}. The large loop between transmembrane units 5 and 6 is seen as located in the nucleoplasm (low Ca^{2+} side) with GM1 oligosaccharide facing the loop. A positive charged amino acid (e.g., Arg) in the segment produced by B exon interacts with the negative charge of *N*-acetylneuraminic acid toproduce the tightly associated NCX/GM1 complex (Ledeen et al., 2006).

CHAPTER III

MATERIALS AND METHODS

1. Animals and Experimental procedures

1.1 Animals

Forty male Wistar rats (from the National Laboratory Animal Centre, Mahidol University, Thailand) weighing 200 to 250 g each were used for this study. They were equally divided into 4 groups. These were the control group (C group), the vehicle group (V group), the paclitaxel group (P group) and the paclitaxel-GM1 group (PG group). The rats were kept in aluminium cages on a 12-h light / 12-h dark cycle with free access to water and food at a room temperature of 25±2°C. Animals were allowed a 1-week acclimatization period before they were used in the experiment.

The procedures involving animals were approved by the institutional ethical committee and done according to the guideline declared by the national research council of Thailand. All efforts were made to minimize animals suffering and to reduce the number of animals used.

1.2 Experimental procedures

1.2.1 Experimental groups

Taxol-treated group (P group)

Paclitaxel (Taxol®, Bristol-Myers Squibb, France), in vehicle (Cremophor EL, Sigma) (6.0 mg/ml) was diluted with normal saline (NaCl 0.9%) just before administration (to a final concentration of 1.2 mg/ml). Injected volumes of the paclitaxel were calculated according to the body weight of each rat. Paclitaxel 16 mg/kg was administered intraperitoneally once a week for five consecutive weeks, giving a cumulative dose of 80 mg/kg/rat. This treatment protocol has been previously shown to induce neuropathy in the treated rats (Authier et al., 2000).

Control group (C group)

Control group (C group): no injection

Vehicle group (V group)

The vehicle, made up of Cremophor EL (Sigma) and absolute ethanol in equal parts (1:1 v/v), was diluted at the time of injection with saline (NaCl 0.9%). Injected volumes of vehicle were calculated according to the body weight of each rat. The vehicle was injected intraperitoneally once a week for five consecutive weeks.

Paclitaxel-GM1 group (PG group)

The PG group, received 30 mg/kg/day of GM1 (GM1®, TRB Pharma S.A., Argentina) intraperitoneally for three days before the first dose of paclitaxel, and then five times per week for five consecutive weeks. Additional 30 mg/kg/day of GM1 was administered intraperitoneally for three days after the last dose of paclitaxel. The animals in this group received paclitaxel in the same dose and same schedule as in the P group. Injected volumes of the paclitaxel and the GM1 were calculated according to the body weight of each rat.

1.2.2 Assessment of general toxicity

A baseline body weight was obtained for each animal prior to administration of the first dose and then once a week before the injection and after the last dose of paclitaxel. In addition, all rats were examined every day to detect clinical sign such as limb weakness or death.

1.2.3 Quantitative sensory test

1.2.3.1 Tail flick analgesic test

Figure 7. Tail flick analgesic meter

Procedure

Each rat was placed on the tail flick apparatus [Tail Flick Analgesia Meter (Harvard Apparatus, UK)] (Figure 7). The rat's tail was placed on the photocell, then the infrared lamp, was switched on. When the rat flicked its tail from the photocell, the heat was switched off. The time between the start of the heat stimulus and the tail flick was defined as the reaction time.

The test was then repeated 3 times with an interval of 5 minutes between each test. The mean reaction time was then calculated. The cut-off duration was 30 seconds to avoid skin injury. A baseline reaction time was obtained for each animal prior to administration of the first dose and then once a week before the injection and after the last dose. Before testing, the animals were exposed to the tail flick analgesic meter in order to be familiar with the apparatus.

1.2.3.2 Hot plate analgesic test

Figure 8. Hot plate analgesic meter

Procedure

Each rat was placed on a hot plate (Ugo Basile, Italy) (Figure 8) where a constant temperature of 55°C was maintained. This temperature has been tested before and is considered suitable since it is low enough to avoid burning the skin, but is high enough to induce the hind paw licking response. A transparent plastic cage was placed around the hot plate to

prevent the animal from leaving the plate. Then, the START/STOP button was immediately pushed. When the rat lifted its hind paw to lick, the START/STOP button was pressed allowing a measurement of duration between the start of the heat stimulus and the point that animal licked its hind paw. The animal was then immediately removed from the hot plate. This time was defined as the reaction time.

The test was then repeated at least 4 times with an interval of 5 minutes between each test. The mean reaction time was then calculated. The cut-off duration was 30 seconds to avoid skin injury. Generally, if the first reaction time was abnormally long, the value was discarded. There was no selection toward right or left sides since the timer was stopped when any hind paw was licked. A baseline reaction time was obtained for each animal prior to administration of the first dose and then at each week before the injection and after the last dose. Before testing, the animals were exposed to the hot plate in order to be familiar with the apparatus.

1.2.4 Electrophysiological measurement

In this study, motor NCV test was performed to assess peripheral nerve function. The conduction velocity is calculated by dividing the length of the nerve segment between the two stimulation points by the difference between the latencies from two different stimulation points which is the time the nerve impulse takes to travel between those two points. Latency is defined as the time from the stimulation of the nerve to the peak of CMAP (compound muscle action potential) or action potential (Figure 9). The stimulation point must be as close as possible to the nerve.

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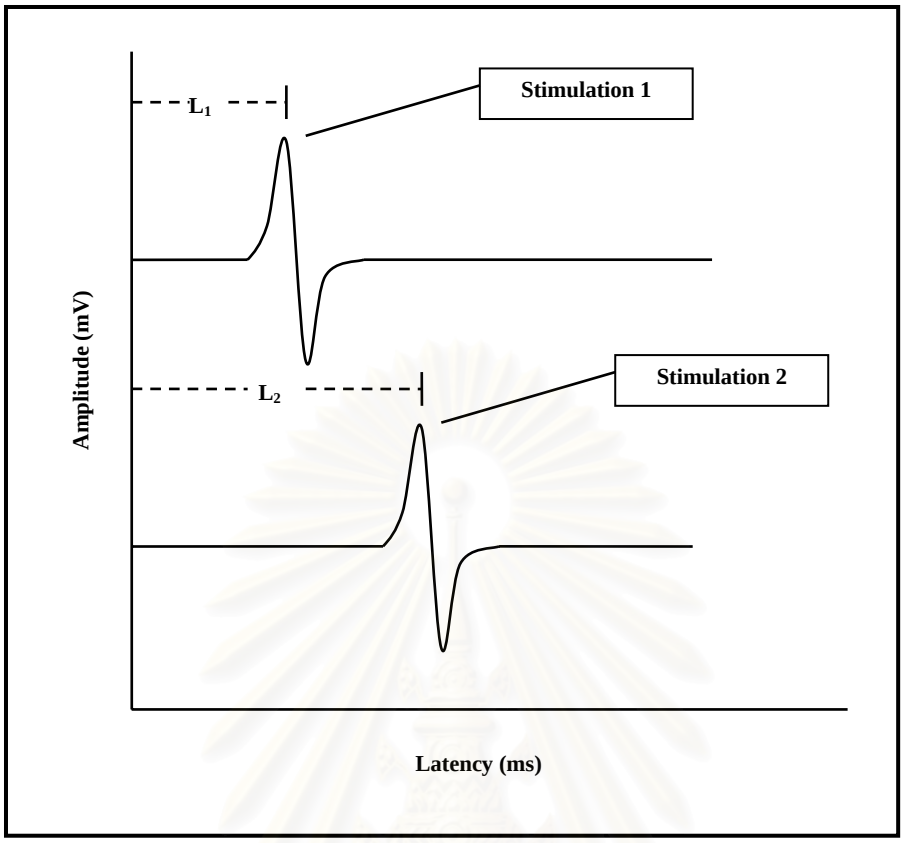

Figure 9. CMAP recording after two-point nerve stimulation where point 1 is closer to the recording position than point 2
L_1 = Latency of the stimulation at point 1, L_2 = Latency of the stimulation at point 2

$$\text{Conduction velocity (m/s)} = \frac{\text{Distance (mm)}}{L_2 - L_1 \text{ (ms)}} = \frac{D}{L_2 - L_1}$$

D is the distance between the two stimulation points (point 1 and point 2) in millimeters and L_1 and L_2 are the latencies of the stimulation at point 1 and 2 in milliseconds, respectively.

Important factors influencing nerve conduction parameters are the body temperature and age of the animals. These factors must be strictly controlled.

Procedure

The rats were anesthetized by 4% halothane with 1 l/min oxygen for 2 to 3 min until the animal was in a completely unconscious state. The concentration of halothane was decreased to 1.5 to 2% with 500 ml/min

oxygen. The animal was placed on the table and was covered by a warm blanket. The stimulating electrode was placed at approximately 3 cm distal to the base of the tail. The active recording electrode was placed at 2 cm distal to the stimulating electrode. Then, the reference recording electrode was placed at 1 cm distal to the active recording electrode (Figure 10).

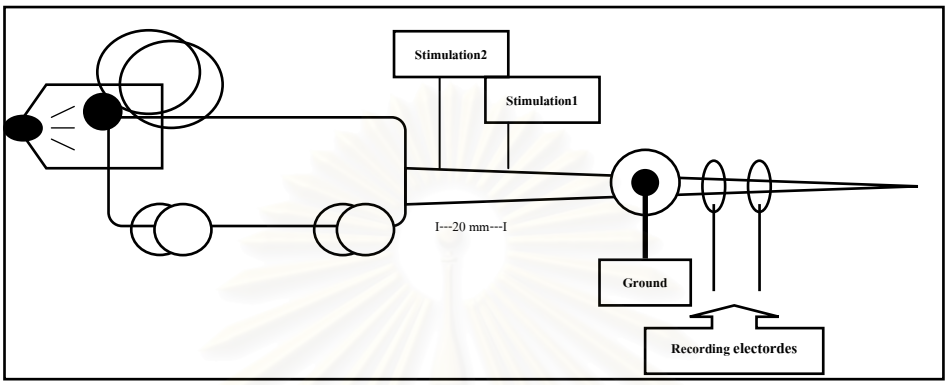

Figure 10. Diagram explaining position of stimulating and recording electrode in NCV study

Finally, the ground electrode was placed between the stimulating and active recording electrodes. The tail nerve was stimulated 5 times with supramaximal stimuli. Then, the stimulating electrode was moved to the proximal part of the tail 2 cm from the previous position. The stimulation was repeated again. The electrical activity was recorded using the oscilloscope. The averaged traces were obtained and used to calculate the conduction velocity. Nerve conduction velocity was measured one week before the beginning of the experiment (base line), then every week before injection and one week after the last injection.

1.2.5 Microscopic examination

1.2.5.1 Tissue Preparation

At the end of the experiment, all animals were deeply anesthetized by an intraperitoneal injection of overdose pentobarbital. After opening the thoracic cavity, the animal was intracardially perfused with 4% paraformaldehyde. Dorsal root ganglia at the levels of L4 and L5 and the sciatic nerve were removed for microscopic examination.

Sciatic nerve and dorsal root ganglia samples were fixed in 2.5% glutaraldehyde for a period of 24-h at 4°C . The samples were rinsed with 0.1 M cacodylate buffer (pH 7.4, 3X, 10min) followed by osmification with 1% osmium for 2 hours. Following additional rinsed in cacodylate buffer, the nerve samples were dehydrated through ethanol (70-100%) followed by propylene oxide: Epon (1:1, 1:1, 1:2), and finally embedded in epoxy resin. Semithin sections (1µm) of sciatic nerve were cut from the epon blocks using glass knives on an ultramicrotome, and then stained with 1% Para-phenylenediamine for preliminary examination with a light microscope at 40x to assess specimen quality and for morphometric evaluations. Thick sections (3µm) of dorsal root ganglia were obtained using an ultramicrotome as well, and stained by toluidine blue before examination under light microscope. Thin (60-90nm) sections of sciatic nerve were cut on an ultramicrotome with a diamond knife. The sections were mounted on copper mesh grids, double stained with lead and uranyl acetate. Eight fields from every copper mesh grids in each group (n = 6 for each group) were examined in a JEOL JEM. 1210 electron microscope. Ultramicrographs were taken by digital camera attached to the electron microscope. Each axon was searched for microtubular accumulation, atypical mitochondria and activated Schwann cells in both myelinated and unmyelinated axons. The examiner was unaware as to whether the rat had been treated groups or a control group.

1.2.5.2 Morphometric evaluation

Morphometric analysis of sciatic nerves (n = 6 for each group) were performed on 1 µm semithin sections using an Image Pro Plus analysis (Media cybernetics), connected with a light microscope. A microscopic image with an area of approximately 0.012 mm^2 was displayed on video screen. The computer-assisted image analysis allowed for the determination of fiber diameter, axon diameter, total number of myelinated fibers, and fascicle area. At the same time, the g-ratio (i.e. the ratio between the axonal diameter divided by the fiber diameter) was calculated. Myelin thickness was calculated as half the difference between axon diameter and fiber diameter. Axon density was calculated by dividing the total number of myelinated fibers by the fascicle area. The examiner was unaware as to whether the rat had been treated groups or a control group.

$$\text{g-ratio} = \frac{\text{Axonal diameter}}{\text{Fiber diameter}}$$

$$\text{Myelin thickness } (\mu m) = \frac{\text{Fiber diameter - Axon diameter}}{2}$$

$$\text{Axon density (myelinated fibers/mm}^2\text{)} = \frac{\text{Total myelinated fibers}}{\text{Fascicle area}}$$

2. Statistical analysis

All data were analysed using SPSS for Windows version 10 for statistical analysis. Statistically significant differences were shown when p values were less than 0.05, unless otherwise stated.

The data were checked for normal distribution and homogeneity of variances. Comparisons between groups were made by one-way analysis of variance (ANONA) followed by Tukey's post hoc test. However, if the measurements were extremely skewed from normal distribution and/or had a markedly significant difference in variances, a Kruskal-Wallis test (non-parametric test for ANOVA) was used.

CHAPTER IV

RESULTS

1. Assessment of general toxicity

Two rats died, in the vehicle and paclitaxel groups, after the third and fourth injection**,** respectively. Four rats died, in the PG group after the 4th injection. All other animals survived until the end of the study and received five injections as scheduled in the protocol without any dose reduction. The body weight was significant decreased in the paclitaxel treated group and the paclitaxel-GM1 (PG group) when compared to the vehicle and control groups from the second week to the end of the study (Figure 11).

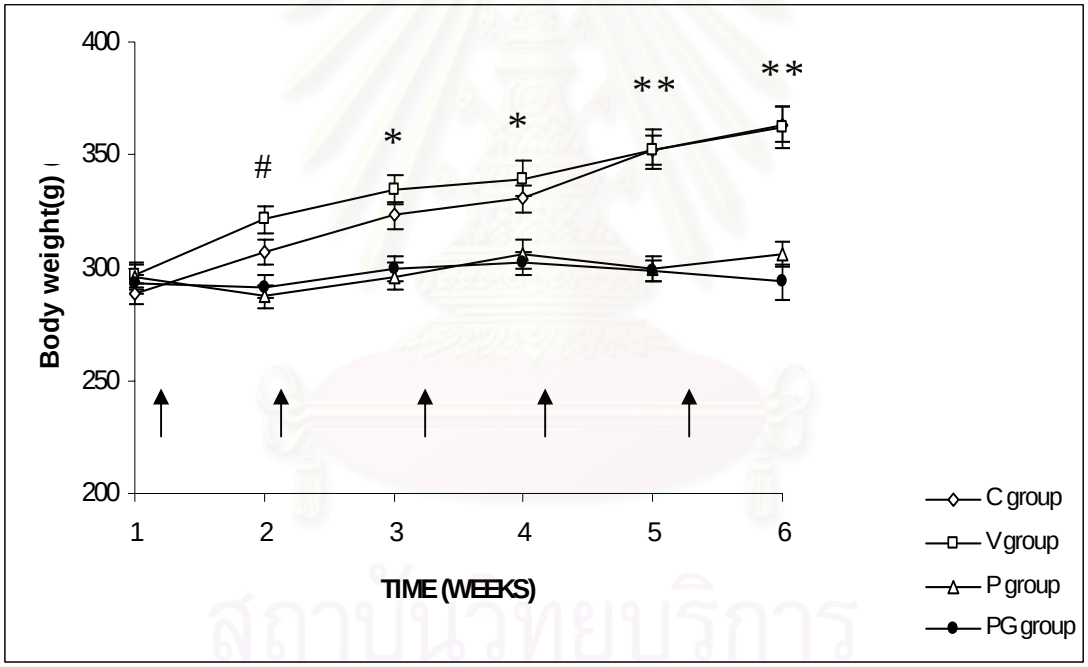

Figure 11. The body weight changes (5 injections) observed in each experimental group (Δ, Taxol® 16 mg/kg) (●, Taxol® 16 mg/kg, GM1 30 mg/kg) (◊, control) (□, vehicle) during the experiment. Each rat received one i.p. injection (arrow). The bars represent SEM. A significant difference (# $p < 0.05$, * $p < 0.01$, ** $p < 0.001$) was observed versus the control group from the 2nd week to the end of the study for the P and PG groups.

2. Quantitative sensory tests

2.1 Tail flick analgesic test

The tail withdrawal time to a radiant heat stimulus applied to the tail of the rat in each experimental group is demonstrated in Figure 12. At base-line, there were no significant differences between groups in the average heat-evoked response reaction times. The significant increase ($p < 0.05$) in the tail withdrawal times were observed the P group compared to the C group from the second to the fourth injection and returned to the similar level seen in other groups in the fifth injection and 7 days after the last injection. The maximal increase was +46% compared to baseline ($p < 0.05$). In V group, reaction time was significantly prolonged relative to the C group in the fourth injection ($p < 0.05$). There were no significant differences between the PG and C groups.

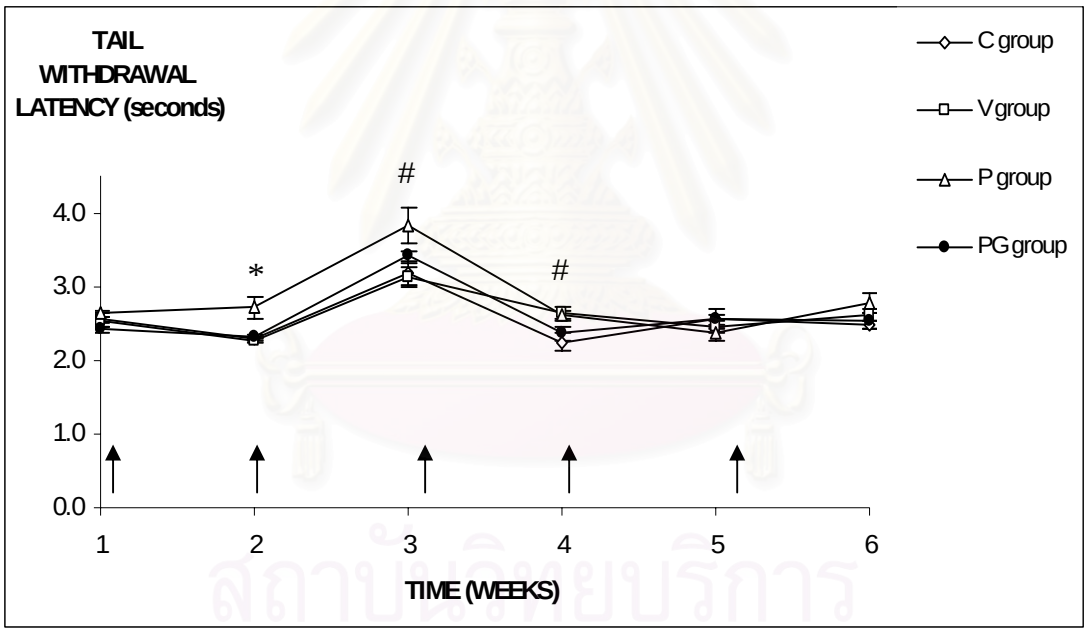

Figure 12. Thermal hypoalgesia (5 injections) – tail withdrawal times to a radiant heat stimulus applied to the tail of the rat in each experimental group (Δ, Taxol® 16 mg/kg) (●, Taxol® 16 mg/kg, GM1 30 mg/kg) (◊, control) (□, vehicle). Each rat received one i.p. injection (arrow) per week for 5 weeks. The bars represent SEM. A significant difference (# $p < 0.05$, * $p < 0.01$) was observed versus the control group from the 2nd to the 4th week for the group.

2.2 Hot plate analgesic test

At base-line (Figure 13), there were no significant differences between the groups in the average heat-evoked response reaction times. In paclitaxel-injected rats, compared to those of other groups, a significant increase in the hind paw withdrawal time in the fourth week was observed ($p < 0.05$). There was no significant difference between the PG and C groups.

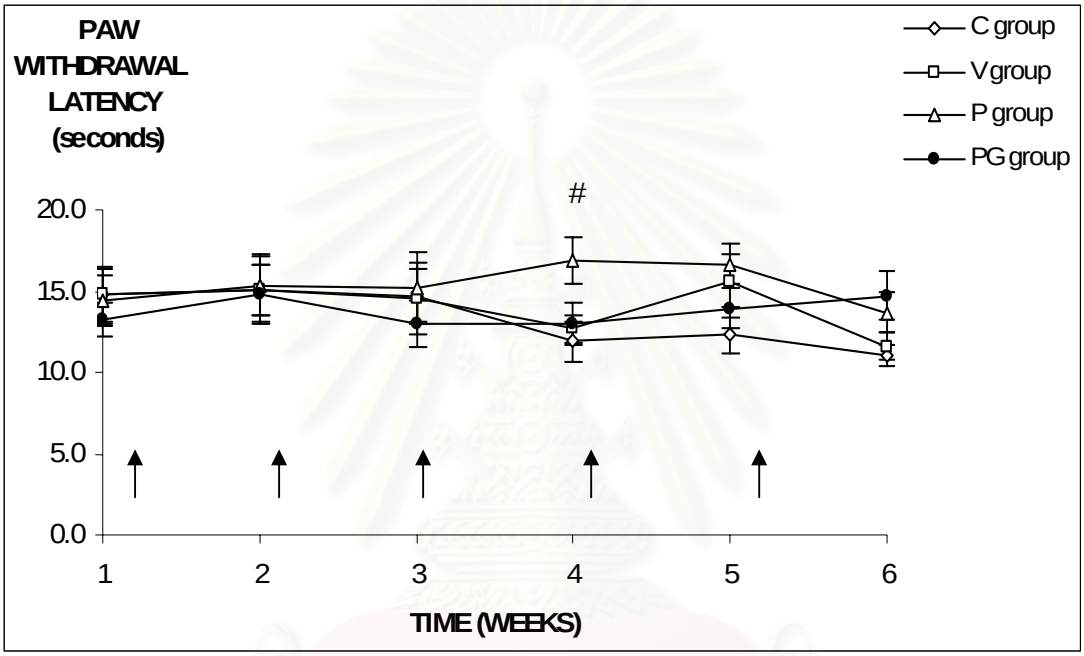

Figure 13. Thermal hypoalgesia (5 injections) – paw withdrawal latencies to a radiant heat stimulus applied to the tail of the rat in each experimental group (Δ, Taxol® 16 mg/kg) (●, Taxol® 16 mg/kg, GM1 30 mg/kg) (◊, control) (□, vehicle). Each rat received one i.p. injection (arrow) per week for 5 weeks. The bars represent SEM. A significant difference (# $p < 0.05$) was observed versus the control group at the 4th week for the P group.

2.3 Electrophysiological measurement

No significant difference between groups was observed at baseline. The administration of paclitaxel induced a significant decrease in tail nerve conduction velocity compared to the control group in the third week ($p < 0.05$) and 7 days after the last injection ($p < 0.05$) (Figure 14). In V group, tail nerve conduction velocity was significantly decreased in the third week compared to the C group. There were no significant differences between the PG and C groups.

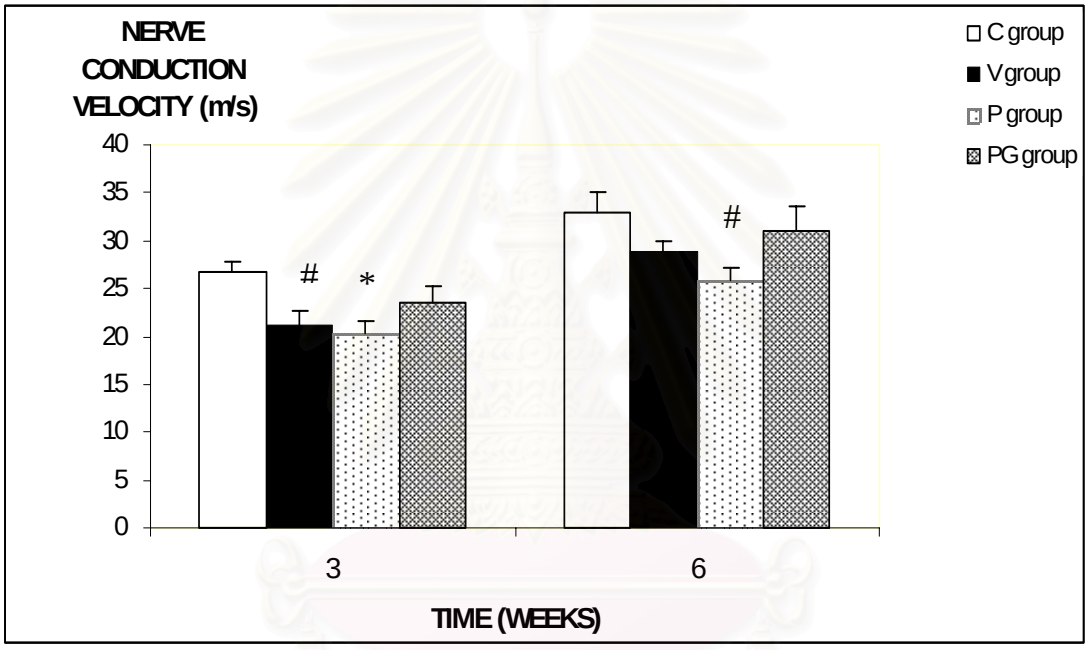

Figure 14. Tail nerve conduction velocity in the control, vehicle, paclitaxel and paclitaxel-GM1 groups. The bars represent SEM. A significant difference (# $p < 0.05$, * $p < 0.01$) was observed versus the control group in the 3rd and one week after the 5th injection for the P. The V group was observed versus the control group in the 3rd week.

3. Microscopic examinations

3.1 Light microscopic examination

In Epon semithin transverse sections of the sciatic nerve, minimal degenerative axonal changes in the V, P and PG groups were observed (Figure 15B, C and D). Moreover, mild endoneurial edema and mild myelinated fiber loss were seen in some cases of paclitaxel-treated rats (Figure 15C). Endoneurial and perineurial vessels were normal. In the dorsal root ganglia, no definite pathological changes in sensory neurons in all groups. The incidence of multinucleolated neurons and eccentric nucleoli have not been detected in the P, V and PG groups.

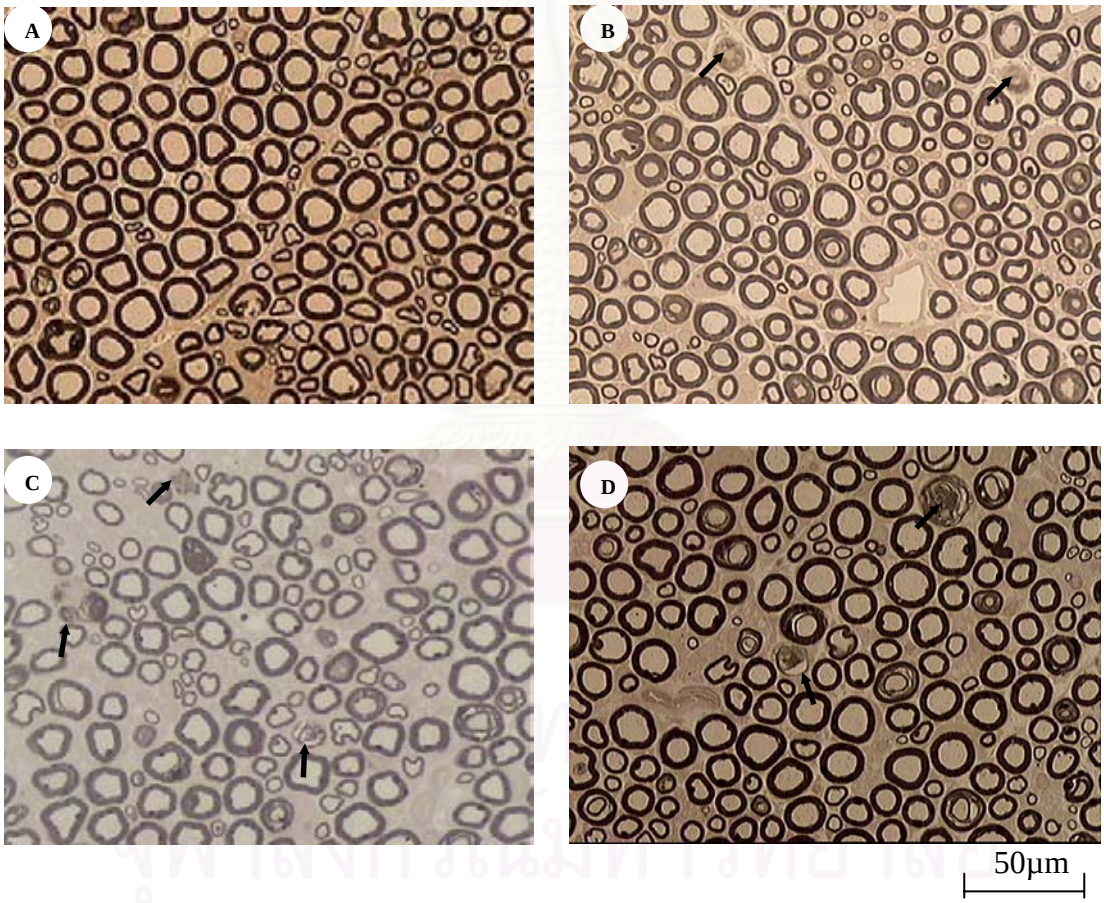

Figure 15. Micrographs obtained from sciatic nerves of (A) control, (B) vehicle-treated rat, (C) paclitaxel-treated rat and (D) paclitaxel-GM1-treated rat. Few degenerating fibers (arrows) are shown (B, C and D). In (C), mild endoneurial edema is presented.
Magnification: ×400

3.2 Morphometric evaluation

Table 1. Summary of the morphometric results obtained from light microscopy

Drug dose (mg/kg i.p.)	Control		Vehicle			Paclitaxel			Paclitaxel-GM1		
	Mean	SD	Mean	SD	P value	Mean	SD	P value	Mean	SD	P value
DMF (Fibers/mm^2)	15226	1737	13708	2032	0.476	14748	1782	0.966	14108	1176	0.705
$\bar{x}$ of Ds (µm)	6.40	0.32	6.67	0.36	0.480	6.49	0.30	0.954	6.58	0.22	0.751
$\bar{x}$ of Myelin thickness(µm)	1.46	0.09	1.49	0.15	0.968	1.46	0.07	0.999	1.48	0.10	0.996
g-ratio	0.53	0.01	0.54	0.04	0.917	0.54	0.02	0.907	0.54	0.04	0.937
Myelin thickness (%)											
(0-1 µm)	24.28	2.82	24.24	6.11	1.000	27.21	3.68	0.724	24.06	4.87	1.000
(> 1-2 µm)	61.60	5.43	58.86	3.52	0.654	58.58	3.00	0.583	60.41	3.49	0.956
(> 2-3 µm)	13.08	5.30	15.61	7.63	0.911	13.42	4.73	1.000	14.52	6.96	0.981
(> 3 µm)	1.03	0.95	1.29	0.94	0.939	0.78	0.74	0.950	0.99	0.29	1.000
MF (%)											
(Ø>1-2 µm)	0.69	0.10	0.48	0.30	0.428	0.55	0.22	0.718	0.48	0.20	0.435
(Ø > 2-3 µm)	8.35	1.30	6.37	1.20	0.076	7.27	1.37	0.504	6.66	1.14	0.155
(Ø > 3-4 µm)	12.14	1.34	11.64	1.23	0.858	12.38	0.91	0.980	11.66	0.62	0.876
(Ø > 4-5 µm)	10.83	0.72	10.38	0.70	0.746	10.85	0.81	1.000	10.74	0.67	0.997
(Ø > 5-6 µm)	10.34	1.37	9.68	1.80	0.876	10.25	1.59	1.000	9.86	0.79	0.946
(Ø > 6-7 µm)	13.05	7.24	15.27	2.52	0.802	14.96	2.70	0.861	14.81	2.49	0.887
(Ø > 7-8 µm)	14.59	0.89	15.56	1.08	0.632	14.77	1.92	0.995	16.08	1.14	0.281
(Ø > 8-9 µm)	10.50	0.29	12.28	1.06	0.005*	12.03	0.71	0.017*	12.23	0.70	0.007*
(Ø > 9-10 µm)	9.10	1.11	9.44	1.02	0.929	9.79	0.92	0.609	9.74	0.56	0.663
(Ø > 10 µm)	7.18	4.53	8.90	5.11	0.921	7.14	4.18	1.000	7.69	4.15	0.998
MF (%)											
(Ø ≤ 5.5 µm)	37.2	3.3	33.9	3.4	0.964	36.3	2.8	0.497	34.6	1.6	0.254
(Ø > 5.5, < 10 µm)	55.4	3.9	56.9	3.6	0.979	56.6	3.1	0.805	57.8	3.7	0.889
(Ø ≥ 10 µm)	7.4	4.5	9.2	5.1	1.000	7.1	4.2	0.997	7.6	4.2	0.917

***** Statistically significant differences: $p < 0.05$
DMF = Density of melinated axon fiber
$\bar{x}$ of Ds = Mean of myelinated fiber diameter
$\bar{x}$ of Myelin thickness = Mean of myelin thickness
MF (%) = Percentage of myelinated fiber with diameter
Myelin thickness (%) = Percentage of myelinated fiber with myelin thickness

The morphometric data are summarized in Table 1. The myelinated fiber density was decreased and the mean fiber diameter was increased in the V, P and PG groups vs. control group, despite insignificant differences. The myelin thickness and g-ratio were unchanged in the V, P and PG groups. There was a significant increase in the percentage of myelinated fibers with diameter between 8 to 9 µm in the P, V, and PG groups when compared to the C group.

3.3 Electron microscopic examination

The axon of the sciatic nerve in the P and PG groups revealed mild microtubule accumulation (Figure 16C and D), while there was no such evidence in the other groups (Figure 16A and B). The microtubules in the P and PG groups also showed an increased tendency to surround mitochondria (Figure 17A, B and C), and the PG group revealed the formation of linear microtubule aggregation (Figure 17D). Furthermore, atypical mitochondria including swelling and vacuolated mitochondria were observed in the P (Figure 18A, B and D), PG (Figure 18C) and V groups, but more frequent in the P group.

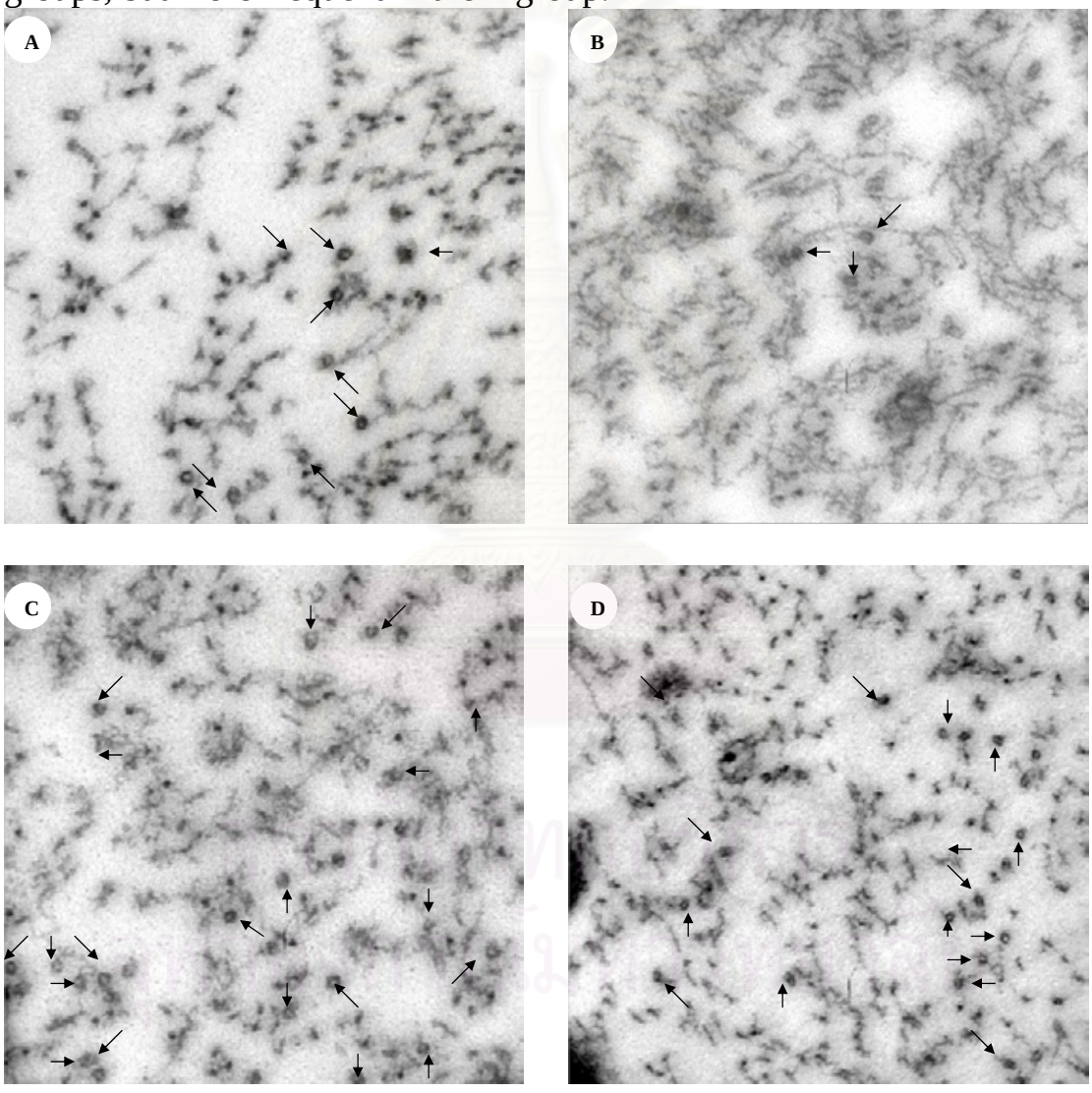

Figure 16. Transmission electron micrograph from sciatic nerve of (A) C group, (B) V group, (C) P group, (D) PG group. (C) The P and (D) PG groups show increased density of microtubules (arrow) Magnification: ×20000

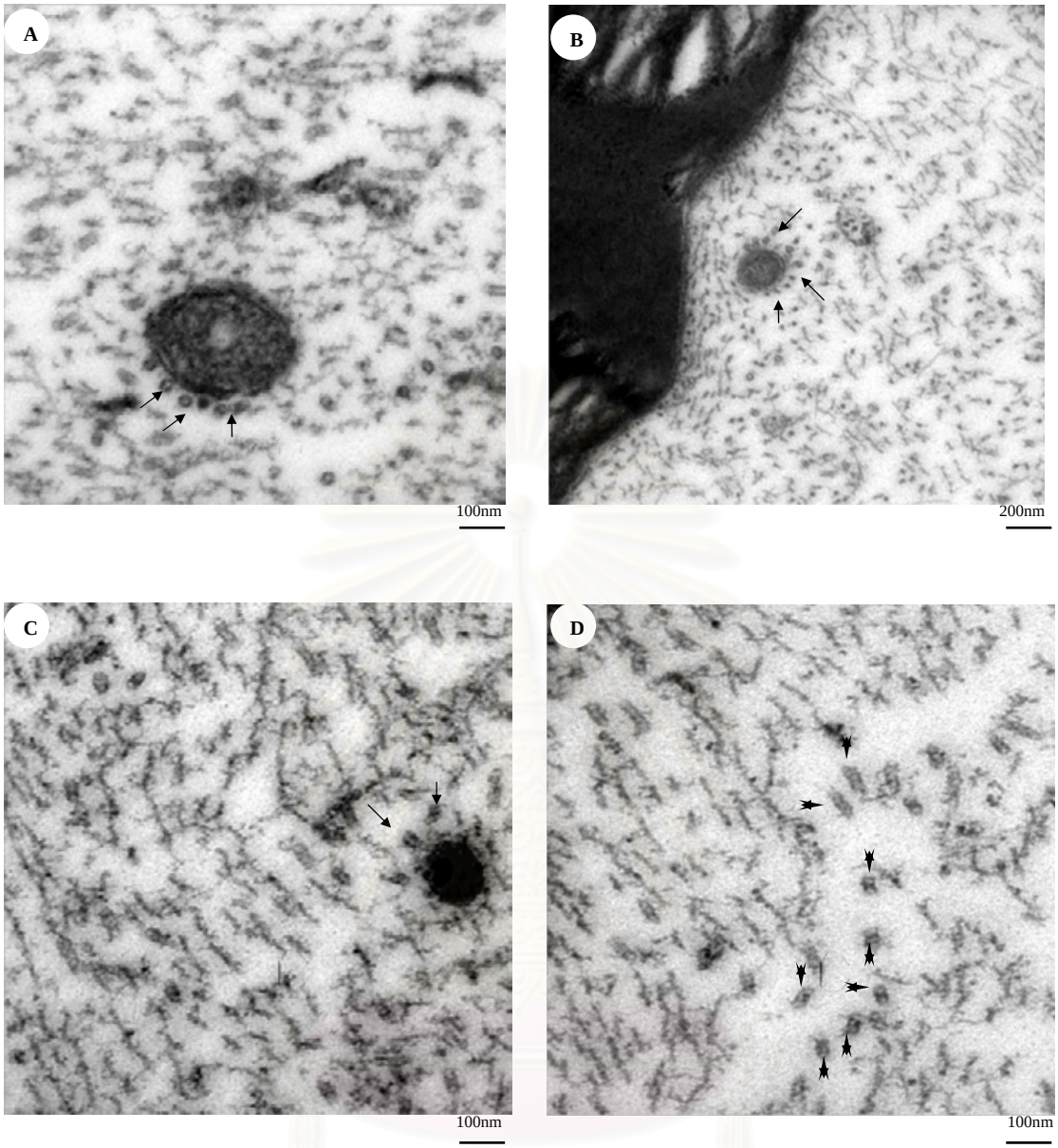

Figure 17. Electron micrographs from sciatic nerve of the P (A and B) and PG (C) groups show the tendency of microtubules to surround mitochondria (arrow) and (D) the PG group shows the formation of linear microtubule aggregates (arrowhead).
Magnification: (B) ×12000, (A, C and D) ×20000

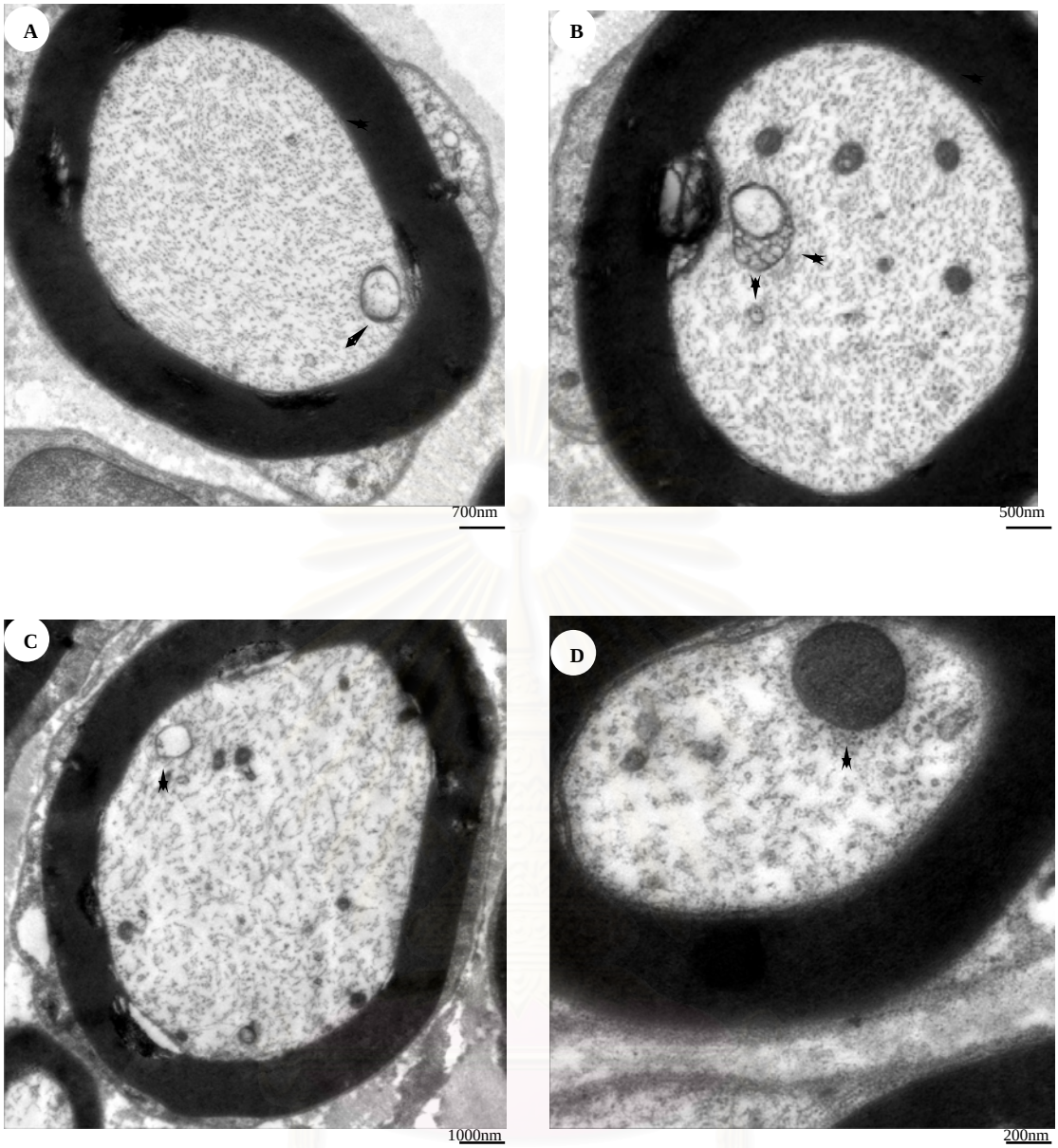

Figure 18. Electron micrographs from sciatic nerve of the P (A, B and D) and the PG (C) groups show atypical mitochondria (arrowhead). Magnification: (C) ×2500, (A) ×4000, (B) ×5000, (D) ×12000

Unmyelinated axons showed the accumulation of microtubule Figure 19A), swelling and vacuolated mitochondria (Figure 19B, C and D) in the P group, however, this was also found in the PG and V groups but with less frequency.

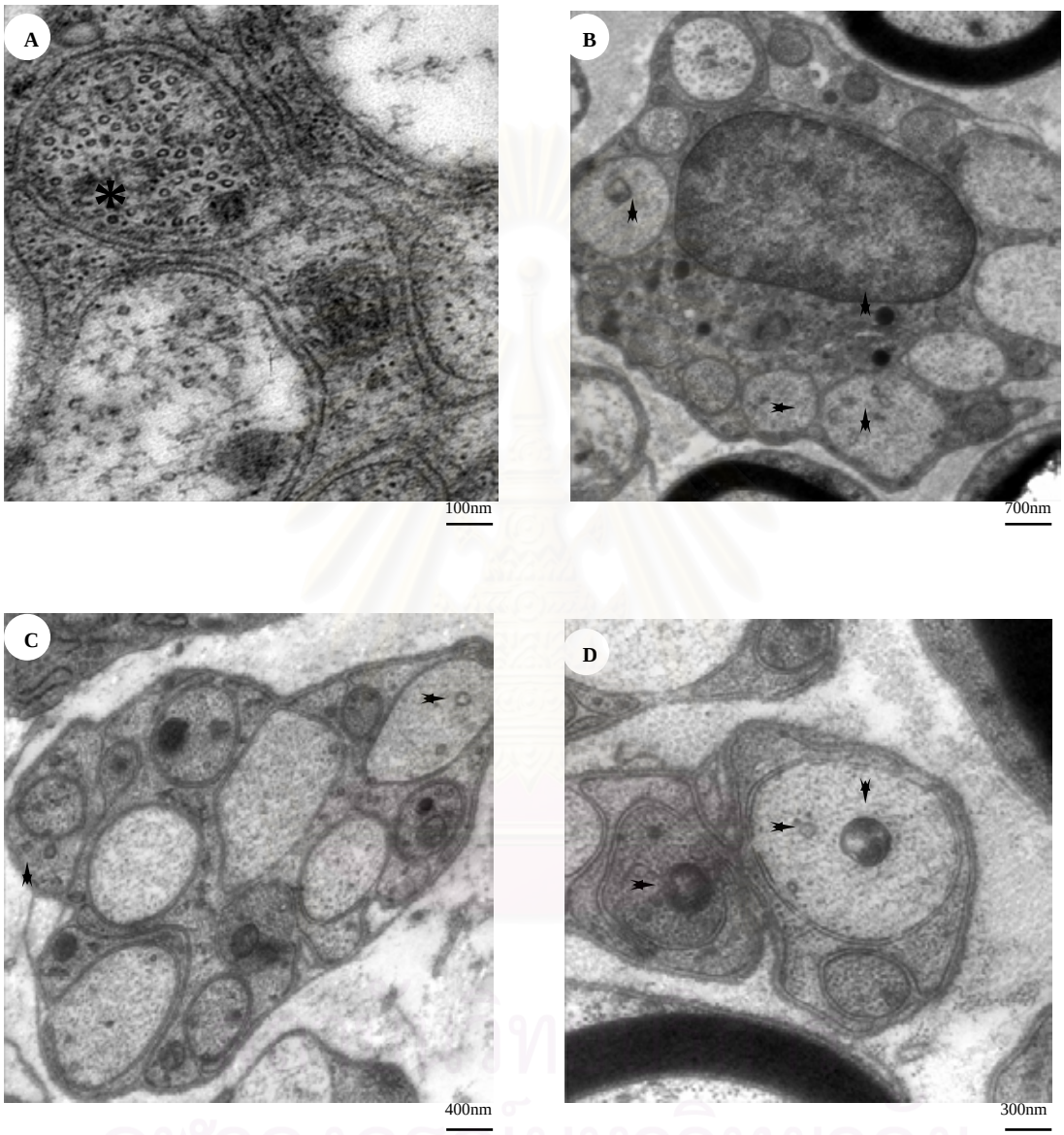

Figure 19. Accumulation of microtubule (mark *) is shown in the figure A. Swelling of mitochondria and vesicle structures (arrowhead) were seen in the figure B, C and D. Magnification: (B) ×4000, (C) ×6000, (D) ×8000, (A) ×20000

The Schwann cells in the P group had the features of activated cells, with an evident accumulation of intracytoplamic organelles (Figure 20A and 20B). These features were also observed in the PG and V groups but less evidence. Frequently, the same activated Schwann cells had intranuclear formations with the morphological appearance of nucleolus. No accumulation of microtubule was seen within Schwann cell cytoplasm in all groups. Vessels and connective tissue were also normal.

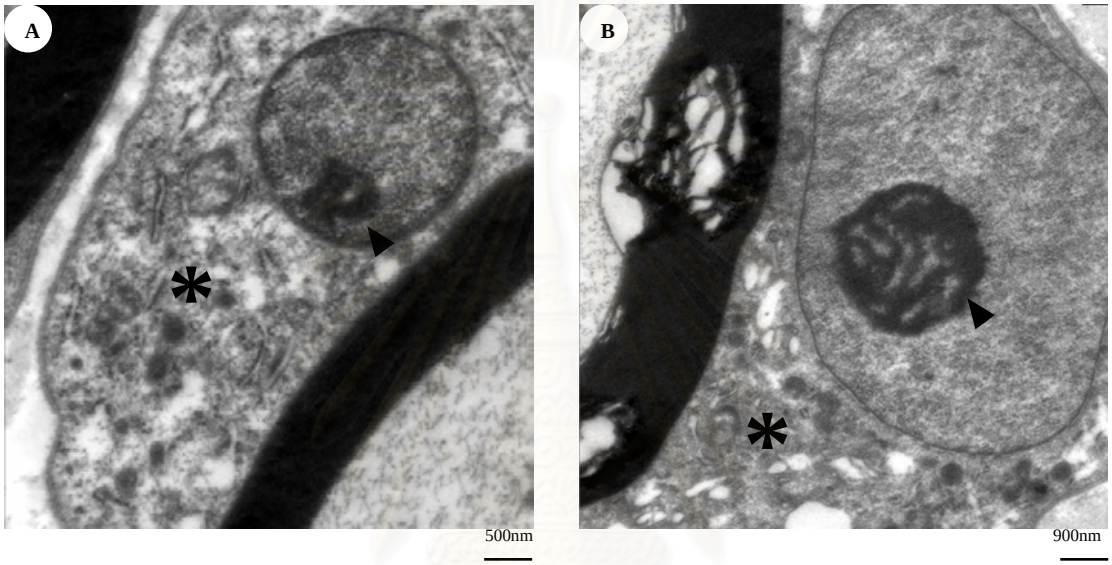

Figure 20. Electron micrographs from sciatic nerve of the P group show (A) activated Schwann cells with accumulation of organelles in the cytoplasm (mark *) (5000×) and (B) chromatin condensation with the features of a nucleolus (arrowhead) (3000×).

CHAPTER V

DISCUSSION

There is currently no effective treatment to prevent or reverse paclitaxel-induced neuropathy (Quasthoff and Hartung., 2002). In addition, the mechanisms responsible for the nerve damage and the pain syndrome are unknown (Polomano et al., 2001). Several studies have reported that GM1 elicits a neuroprotective effect when inserted into membranes both in vivo and in vitro (Hadjiconstantinou and Neff, 1998: Vogelsberg et al., 1997; Oliveira et al., 2000). A previous study reported a neuroprotective properties of gangliosides to prevent toxic neuropathy of paclitaxel (Chentanez et al., 2003). This study has investigated the effect of GM1 on paclitaxel-induced neuropathy in rats in more detail the morphological events in sciatic nerve fibers.

The GM1 dosage (30/mg/kg) used in this study is similar to that used by Goettl and coworkers (Goettl et al, 2003). The schedule of repeated intraperitoneal paclitaxel (16mg/kg), once a week for five weeks, was previously used by Autheir and coworkers (Autheir et al., 2000) in male Wistar rats. This dose is a good compromise between the appearance of nociceptive disorder and the preservation of good clinical status.

The significant decrease in body weight had been observed in the P and PG groups since the second injection until the end of study, when compared to the C group. This result is similar to other study (Autheir et al., 2000). Bardos and coworkers (Bardos et al, 2003) also reported weight loss as a result of low-dose paclitaxel treatment (5mg/kg). This weight loss is likely due to the general toxicity of paclitaxel.

Evaluation of the nociceptive threshold was demonstrated by evaluation of hot plate and tail-flick reaction time. The thermal hypoalgesia in the P group was found from the second to the fourth injection. This finding is similar to other report showing increased reaction time by paclitaxel using the same test (Cavaletti et al, 1997). This study also observed thermal hypoalgesia at the fourth week of the vehicle group. With regard to the hind paw heat analgesic test, significantly increased reaction time in the P group in the fourth week, similar to the tail heat analgesic test, was seen. This finding is consistent with the previously described results, in which paclitaxel was reported to increased the pain threshold (Autheir et al., 2000). No significant change in the pain threshold was observed in the PG group. However, in the last

two weeks of this experiment, both sensory tests did not show any significant abnormality in the P group relative to the other group. This may be due to behavioral learning of each animal in both tests.

Electrophysiological examination in the tail nerve of rats treated with repeated paclitaxel injection showed a significant decrease in the nerve conduction velocity at week 3 and 6 compared to the C group. This result is similar to the study of Autheir at el. (2000) and Pershon et al. (2005). We also found a significant decrease of NCV in the V group at the third week when compared to the C group. No significant difference between the PG and C groups.

The previous studies have reported the neuropathy in the animals induced by the vehicle cremophor (Autheir et al., 2000; Gelderblom et al., 2001). However, recent study has found that exposure to paclitaxel but not cremophor is asscociated with neuropathy in cancer patients (Mielke et al., 2005). The result of this study indicates that vehicle may partly contribute to the development of neuropathy seen in the P group. Therefore, the results from the future studies with paclitaxel dissolved in cremophor should be analyzed with caution and the vehicle treatment group should be included in the studies.

With regard to light microscopic examination, minimal axonal degeneration in the P, PG and V groups were evidenced. We found mild endoneurial edema only in the P group. This is consistent with the other reports of the animals treated with paclitaxel which also detected endoneurial edema in the sciatic nerves of rats (Cavaletti et al., 1995; Polomano et al., 2001; Chentanez et al., 2003). However, there was no significant difference between group in the morphometric evaluation of the g-ratio and the myelinated fiber density in this study. There was a significant increase in the percentage of myelinaed fibers with diameter between 8 to 9 μm in the P, PG and V groups. Paclitaxel-induced dorsal root ganglia neuropathy has been suggested (Roytta et al., 1984; Thompson et al., 1984). In this study normal results were obtained by light microscopy on dorsal root ganglia neurons in all groups and were in agreement with the preliminary morphological data which was obtained by Apfel and coworkers in mice injected intraperitonelly with paclitaxel (Apfel et al., 1991).

At the ultrastructural level, the present findings demonstrated mild intraaxonal microtubule accumulation in the P and PG groups. This finding is consistent with previous studies (Roytta et al., 1984, 1985 and 1986; Cavaletti et al., 1995 and 1997; Autheir et al., 2000; Pershon et al.,

2005), in which paclitaxel was reported to cause an increase in the number of intraaxonal microtubules.

In this study, the Schwann cells revealed the cytoplasmic and nuclear changes in the P, PG and V groups, but more frequent in the P group. This pathological change was reported by Cavaletti et al. (1995 and 1997) in the sciatic nerve which examined in detail in the models of paclitaxel neuropathy in rats as well. No microtubular accumulation was observed within the Schwann cells.

Furthermore, atypical mitochondria were found in the P group. It was also observed in the PG and V groups but with less evidence. Abnormality of Schwann cells and mitochondria may be due to a direct action of paclitaxel on the mitochondrial membranes, causing the mitochondrial permeability transition pore (mPTP) to open and release mitochondrial calcium. β-tubulin is specifically associated with the mPTP (Carre et al, 2002), thus providing a paclitaxel binding site on mitochondria. The morphological consequence of mPTP opening is mitochondrial swelling. The opening of mPTPs have been observed in the neuronal cells and nerves of animal models with paclitaxel (Kodd et al., 2002; Mironov et al., 2005; Flatter et al., 2006).

The concentration of Ca^{2+} in the cytosol should be kept low in resting cells. Nerve cell is the cell which makes extensive use of Ca^{2+} signaling. Mitochondria have an important role in returning the Ca^{2+} concentration to normal after a Ca^{2+} signaling. Therefore, it seems likely that mitochondria have a direct influence to regulate Ca^{2+} homeostasis in cytosol (Alberts et al., 2002). After paclitaxel exerts neuronal cell, it might evoke dysfunction of neuronal mitochondria and abnormality gradient of intracellular calcium, causing morphological change both in axon and Schwann cells, inducing peripheral neuropathy.

Therefore, regulation of intracellular Ca^{2+} is of critical importance in relation to cell viability and signaling processes that govern virtually every aspect of cell behavior. Regulation of Ca^{2+} homeostasis is based on the presence of NCX in the NE and PM that is dependent on GM1, which might well provide the basis of its neuroprotective function. Such results in this study indicated that the PG group had no significant difference when compared to the C group in those behavioral tests and NCV evaluation. These findings are consonant with GM1 being the crucial ganglioside of Ca^{2+} regulation with attendant neuroprotection. Moreover, this study also showed less pathological changes, especially the evidence of mitochondrial dysfunction. Such observations could be hypothesized

that GM1 might act on mitochondrial membrane similar to the mechanism on NE and PM (Wu et al., 1991, 1995, 2005; Ledeen and Wu. 2006). Therefore, GM1 may alter the mitochondrial membrane and promote the function of mitochondria to take calcium ion. These hypotheses need further investigation. The other one may be due to GM1 promote potentiation of NGF to preserve neuronal survival and functions by binding to Trk (Mutohetal., 1995; Ferrari et al., 1995).

CHAPTER VI

CONCLUSION

This study showed a significant difference of withdrawal time of tail flick and hotplate tests, and reduction of NCV in the P and V groups, but not found in the PG group when compared to the C group. At the light microscope of the sciatic nerve in the P, V and PG groups revealed m axonal degeneration. However, morphometric study did not demonstrate significant changes in fiber density, fiber diameter, myelin thickness and g-ratio in the P, V and PG groups when compared to the C group. At the ultrastructural level in axons of the sciatic nerve showed mild microtubule accumulation in the P and PG groups, while there was no such evidence in the other groups. Moreover, atypical mitochondria and activated Schwann cells were found in the P group. However, this was also found in the PG and V groups but with less frequency.

Therefore, this study indicates that administration of GM1 can minimize the severity of hypoalgesia and maintain the normal NCV in the animal model of paclitaxel-induced neuropathy. Morphological protective effect of GM1 is less evident.

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APPENDICES

Appendix 1

Table 2 The result from the body weight

Time (Weeks)		1	2	3	4	5	6
C-group	MEAN(g)±SEM	288.8±4.57	306.9±5.49	323.2±6.04	330.8±6.04	352.4±6.6	363.2±7.77
V-group	MEAN(g)±SEM	296.5±5.24	321.2±5.65	334.4±6.63	339.6±8.25	352.5±8.48	362.2±8.90
P-group	MEAN(g)±SEM	296.3±5.91	*287.1±5.35	*296.1±5.96	*305.9±6.16	*299.4±5.53	*306.0±5.24
PG-group	MEAN(g)±SEM	292.7±4.47	*291.6±5.12	*299.3±5.35	*301.9±5.17	*298.6±4.69	*293.7±7.97

Table 3 The result from Tail analgesic test

Time (Weeks)		1	2	3	4	5	6
C-group	MEAN(ms)±SEM	2.57±0.07	2.28±0.03	3.17±0.14	2.25±0.12	2.55±0.07	2.48±0.06
V-group	MEAN(ms)±SEM	2.52±0.06	2.27±0.04	3.13±0.13	*2.64±0.08	2.46±0.08	2.61±0.08
P-group	MEAN(ms)±SEM	2.63±0.05	*2.71±0.14	*3.83±0.25	*2.61±0.07	2.36±0.09	2.78±0.14
PG-group	MEAN(ms)±SEM	2.42±0.04	2.32±0.03	3.41±0.08	2.36±0.09	2.56±0.12	2.54±0.10

Table 5 The result from Hot plate analgesic test

Time (Weeks)		1	2	3	4	5	6
C-group	MEAN(ms)±SEM	14.80±1.77	15.03±1.55	14.65±1.72	11.89±1.28	12.28±1.08	11.08±0.65
V-group	MEAN(ms)±SEM	14.78±1.63	15.06±2.03	14.54±2.22	12.68±0.88	15.63±1.59	11.62±0.86
P-group	MEAN(ms)±SEM	14.41±1.52	15.39±1.91	15.22±2.12	*16.89±1.43	16.69±1.19	13.68±1.23
PG-group	MEAN(ms)±SEM	13.26±1.09	14.86±1.73	12.95±1.41	12.99±1.25	13.96±1.19	14.72±1.51

Table 6 The result from NCV evaluation

Time (Weeks)		1	2	3	4	5	6
C-group	MEAN(m/s)±SEM	24.71±1.53	28.98±1.69	26.73±1.18	26.51±0.83	30.31±1.73	32.89±2.22
V-group	MEAN(m/s)±SEM	24.22±1.12	25.64±2.41	*21.28±1.34	27.26±2.14	29.41±1.48	28.79±1.08
P-group	MEAN(m/s)±SEM	23.14±1.31	23.83±1.31	*20.30±1.29	27.30±0.82	29.32±1.45	*25.93±1.18
PG-group	MEAN(m/s)±SEM	23.78±1.87	25.92±1.10	23.52±1.62	25.90±1.13	25.14±1.17	31.09±2.45

***** Statistically significant differences

Appendix 2 Tissue Processing

Fixed in 2.5% Glutaraldehyde 4ºC

↓

Wash in 0.1 M Cacodylate Buffer pH 7.4 (10 mins*3 times)

↓

Fixed in 1% Osmium tetraoxide (OsO_4) (2 hours)

↓

Wash in 0.1 M Cacodylate Buffer pH 7.4 (10 mins*3 times)

↓

Dehydration with graded alcohol

70% alcohol (10 mins*2 times) Overnight

80% alcohol (10 mins*2 times)

95% alcohol (10 mins*2 times)

100% alcohol (15 mins*3imes)

↓

Propylene oxide (15 mins*2 times)

↓

Infiltrate with Propylene oxide: Epoxy resin

1:1 (1 hour)

1:2 (12-18 hours)

↓

Infiltrate with 100% Epoxy resin (1-2 hours)

↓

Embeded in block capsule (incubate at 60 ºC 2-3 days)

Appendix 3 Solution

0.1 M Cacodylate buffer

Sodium cacodylate 21.4 g., Trihydrate MW. = 214.02
Cacodylate acid
dH_2O 986 ml.
0.2 M HCl 14 ml.

2% Glutaraldehyde

25% glutaraldehyde 20 ml.
0.1 M cacodylate buffer 230 ml.

1% Osmium

Osmium tetraoxide 1 g.
0.1 M cacodylate buffer 100 ml.

1% para-phenylenediamine

Sodium borate 5 g.
Distilled water 500 ml.: 1% sodium borate
Methylene blue 5 g.
1% sodium borate 500 ml.

Uranyl acetate

10 g Uranyl Acetate
100 ml Methanol

Lead citrate

1 g Lead Citrate
100 ml freshly distilled water
0.4g NaOH per 1 ml

Appendix 4 Morphometric evaluation

Morphometric analysis of sciatic nerves were performed on 1 µm semithin sections using an Image Pro Plus analysis (Media cybernetics), connected with a light microscope. A microscopic image with an area of approximately 0.012 mm^2 was displayed on video screen. The computer-assisted image analysis allowed for the determination of fiber diameter, axon diameter, total number of myelinated fibers, and fascicle area. At the same time, the g-ratio (i.e. the ratio between the axonal diameter divided by the fiber diameter) was calculated. Myelin thickness was calculated as half the difference between axon diameter and fiber diameter. Axon density was calculated by dividing the total number of myelinated fibers by the fascicle area.

$$\text{g-ratio} = \frac{\text{Axonal diameter}}{\text{Fiber diameter}}$$

$$\text{Myelin thickness (µm)} = \frac{\text{Fiber diameter - Axon diameter}}{2}$$

$$\text{Axon density (myelinated fibers/mm}^2\text{)} = \frac{\text{Total myelinated fibers}}{\text{Fascicle area}}$$

Appendix 5 Drug Injection

Paclitaxel

Generic name: Taxol

Composition:

Biosynthetic paclitaxel	6mg
Purified polyoxyethylated castor oil (Cremophor EL^{TM})	527mg
Dehydrated alcohol USP	49.7%V/V

GM1

Generic name: Monosialotetrahexosylganglioside (GM1) Sodium Salt

Composition:

GM-1 20mg	Each 2ml ampoule contains:	
Active ingredient:	Monosialotetrahexosylganglioside sodium salt	20mg
Excipients:	Sodium dibasic phodphate-12H_2O	6mg
	Sodium monobasic phosphate-2H_2O	0.5mg
	Sodium chloride	16mg
	Water for injection q.s. to	2ml
GM-1 100mg	Each 5ml vital contains:	
Active ingredient:	Monosialotetrahexosylganglioside sodium salt	100mg
Excipients:	Sodium dibasic phodphate-12H_2O	15mg
	Sodium monobasic phosphate-2H_2O	1.25mg
	Sodium chloride	40mg
	Water for injection q.s. to	5ml

BIOGRAPHY

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