ROLE OF MAPKs IN CISPLATIN-INDUCED NEUROPATHY IN RATS

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บทบาทของ MAPKs ในภาวะเส้นประสาทเสื่อมที่เกิดจาก cisplatin ในหนูแรท

นางสาว ตุลาภรณ์ ว่องธวัชชัย

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
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ตุลาภรณ์ ว่องธวัชชัย : บทบาทของ MAPKs ในภาวะเส้นประสาทเสื่อมที่เกิดจาก cisplatin ในหนูแรท. (Role of MAPKs in cisplatin-induced neuropathy in rats) อ. ที่ปรึกษาวิทยานิพนธ์หลัก: รองศาสตราจารย์ แพทย์หญิง วิไล ชินธเนศ, อ. ที่ปรึกษาวิทยานิพนธ์ร่วม: รองศาสตราจารย์ นายแพทย์ สิทธิพร แอกทอง, 95 หน้า.

Cisplatin (cis-diaminodichloroplatinum) เป็นยาที่นิยมนำมาใช้ในการรักษาโรคมะเร็งชนิดต่างๆ กันอย่างกว้างขวาง ผลข้างเคียงที่สำคัญอย่างหนึ่งของยาชนิดนี้คือ การเกิดพยาธิสภาพต่อระบบประสาทส่วนปลายที่มีผลต่อระบบประสาทรับความรู้สึก (sensory neuropathy) ดังนั้นจึงมีความพยายามในการศึกษาเพื่อลดผลข้างเคียงดังกล่าว โดยที่ Mitogen-activated protein kinases (MAPKs) เป็นกระบวนการหนึ่งที่ถูกกระตุ้นมาจากสิ่งเร้าภายนอกเซลล์และส่งผลให้เกิดการเปลี่ยนแปลงการแสดงออกของยีนภายในเซลล์ประสาท ในการศึกษาทางห้องปฏิบัติการพบว่า cisplatin กระตุ้นกระบวนการของ MAPK cascades ทำให้เกิดการตายของเซลล์ในปมประสาท (DRG neuron) ซึ่งปัจจุบันยังไม่มีการศึกษาในสัตว์ทดลองเกี่ยวกับการเปลี่ยนแปลงของ MAPKs จากการได้รับยา cisplatin ที่มีผลต่อภาวะ neuropathy ดังนั้นวัตถุประสงค์ในการศึกษาครั้งนี้เพื่อศึกษาการเปลี่ยนแปลงของ ERK, p38 และ JNK phosphorylation ในเส้นประสาท sciatic และ DRG ในหนูที่ได้รับยา cisplatin ในช่วงเวลาที่แตกต่างกัน โดยการฉีดยา cisplatin 2 mg/kg สัปดาห์ละ2 ครั้ง เป็นเวลา 5 สัปดาห์ ในหนูกลุ่มทดลอง และ ฉีด normal saline ในปริมาณเดียวกันเป็นกลุ่มควบคุม ทำการทดสอบการเปลี่ยนแปลงทางพฤติกรรม เช่น hot plate test, Von Frey hair test และ motor nerve conduction velocity (MNCV) เป็นระยะเวลา 12 สัปดาห์ พบว่าในหนูกลุ่มที่ได้รับยา cisplatin เกิดภาวะ thermal hypoalgesia ในสัปดาห์ที่ 5 และพบ mechanical allodynia ในสัปดาห์ที่ 3 และ 5 และพบความเร็วในการนำกระแสประสาทในเส้นประสาท sciatic nerve ลดลงตั้งแต่สัปดาห์ที่ 5 จนถึงสัปดาห์ที่12 เมื่อศึกษาทางด้าน morphometric study ยังพบว่าในหนูกลุ่มที่ได้รับยา cisplatin มีค่าเฉลี่ยของความหนาของเนื้อเยื่อ myelin ลดลงในสัปดาห์ที่ 8 และ 12 ค่าเฉลี่ยเส้นผ่านศูนย์กลางของ axon ลดลงในสัปดาห์ที่ 8 และพบลดลงค่าเฉลี่ยเส้นผ่านศูนย์กลางของเส้นใยประสาทในสัปดาห์ที่ 12 และค่า g-ratio เพิ่มขึ้น ในสัปดาห์ที่ 8 เมื่อเปรียบเทียบกับกลุ่มควบคุม ในขณะที่ความหนาแน่นของเส้นใยประสาท ลดลงในสัปดาห์ที่ 12 และไม่พบความแตกต่างของจำนวนเส้นใยประสาทในสัปดาห์ที่ 8 และ 12 การศึกษาใน DRG พบค่าเฉลี่ยของขนาดเซลล์ ขนาดนิวเคลียส และ ขนาดนิวคลีโอลัส ลดลงในสัปดาห์ที่ 5, 8 และ 12 ในกลุ่มที่ได้รับยา cisplatin เปรียบเทียบกับกลุ่มควบคุม นอกจากนี้จากการศึกษาโดยวิธี Western blot ในเส้นประสาท sciatic พบการเกิด phosphorylation ของ ERK ในสัปดาห์ที่ 1และ 8 พบ phosphorylation ของ p38 ในสัปดาห์ที่ 8 เมื่อเปรียบเทียบกับกลุ่มควบคุม ส่วนใน DRG มีการเกิด down-regulation ของ p38ในสัปดาห์ที่ 1 และ up-regulation ของ ERK ในสัปดาห์ที่ 12 ในขณะที่ phosphorylation ของ JNK เพิ่มขึ้นทั้งใน DRG และ เส้นประสาท sciatic ในสัปดาห์ที่ 1 และเมื่อศึกษาโดยวิธี Immunohistochemistry พบว่ามี activation ทั้งใน axons และ Schwann cells ของเส้นประสาท sciatic ส่วนใน DRG พบว่าใน P-ERK พบได้ทั้งใน Satellite cells และ DRG neuron ขนาดเล็ก ซึ่งแตกต่างกับ P-p38 และ P-JNK ที่พบใน DRG neurons ขนาดเล็ก และนิวเคลียสของเซลล์ขนาดใหญ่ จากการศึกษาในครั้งนี้แสดงให้เห็นว่า phosphorylation ของ ERK และ p38 ในเส้นประสาท sciatic ไม่พบความเกี่ยวข้องอย่างชัดเจนกับการเกิด mechanical allodynia, thermal hypoalgesia และ slow MNCV จากการได้รับยา cisplain ในหนูแรท จำเป็นต้องได้รับการศึกษาเพิ่มเติมต่อไป

สาขาวิชา ชีวเวชศาสตร์ ลายมือชื่อนิสิต ตุลาภรณ์ ว่องธวัชชัย

ปีการศึกษา 2553 ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์หลัก .....

ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์ร่วม .....

4989721520 : MAJOR BIOMEDICAL SCIENCES
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TULAPORN WONGTAWATCHAI: ROLE OF MAPKs IN CISPLATIN-INDUCED NEUROPATHY IN RATS. ADVISOR: ASSOCIATE PROFESSOR VILAI CHENTANEZ, M.D., Ph.D., CO-ADVISOR: ASSOCIATE PROFESSOR SITHIPORN AGTHONG, M.D., Ph.D., 95 pp.

Cisplatin (cis-diaminodichloroplatinum) is widely used as an antineoplastic agent. One of the major side effects of cisplatin is sensory neuropathy. Mitogen-activated protein kinases (MAPKs) transduce extracellular stimuli to altered gene expression in neuron. *In vitro*, cisplatin activates the MAPK cascades leading to apoptosis of DRG neurons. To date, no such study has been done in the animal model of cisplatin-induced neuropathy. Therefore, the aim of this study was to investigate the alteration of ERK, JNK and p38 phosphorylation in the sciatic nerve and DRG of cisplatin-treated rats in different time points. Cisplatin was given intraperitoneally 2 mg/kg twice a week for ten doses. Normal saline was used as a vehicle. The hot plate test, Von Frey hair test and motor nerve conduction velocity (MNCV) were examined on the hind paw of rats. Thermal hypoalgesia was observed from the 5th week. In addition, the mechanical allodynia was observed from the 3rd to 5th weeks, whereas MNCV was decreased from the 5th to 12th weeks. In histomorphometric study, sciatic nerve from the cisplatin group showed significant decrease in the mean myelin thickness in the 8th and 12th weeks. The axonal diameter was increased in the 8th week in the cisplatin group. In the 12th week, the reduction of mean nerve fibers diameter was observed. G-ratio in cisplatin-treated group was increased in the 8th week. Fiber density was decreased in the 12th week. No significant difference in the number of total fiber in the 8th and 12th weeks. DRG from cisplatin rats showed significant decrease in mean somatic area, nuclear area, nucleolar area and total cell count in the 5th, 8th and 12th weeks compared with the controls. Western blot study showed that cisplatin increased the phosphorylation of ERK in the 1st and 8th weeks in sciatic nerve and of p38 in the 8th week compared with the controls. In DRG, down-regulation of p38 in the 1st week and up-regulation of ERK in the 12th week have been found. On the other hand, increased phosphorylation of JNK was revealed only in the 1st week in DRG and sciatic nerve. Immunohistochemistry of these has shown that these activations occurred in the axons and Schwann cells. P-ERK in DRG has been found in both satellite cells and small DRG neurons. In contrast, P-p38 and P-JNK were marked in the small DRG neurons and the nuclei of large cells. Accordingly, the data suggested that after comparing changes in ERK and p38 phosphorylation with functional and structural abnormalities, no clear relationship has been observed in cisplatin-induced neuropathy in rats. The further study using inhibitors of MAPKs should be done to clarify the role of MAPKs in with cisplatin-induced neuropathy which could be developed into effective treatment of this neuropathy.

Field of Study : Biomedical Science ..... Student's Signature Tulaporn Wongtawatchai

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LIST OF ABBREVIATIONS

ABCC2	ATP-binding cassette, sub-family C (CFTR/MRP), member 2
APS	Ammonium persulphate
ARF	Acute renal failure
ASK1	Apoptosis signal-regulating kinase
ATP7B	copper transporting P type adenosine triphosphate
ATF-2	Activating transcription factor 2
BDNF	Brain-derived neurotrophic factor
BSA	Bovine serum albumin
CGRP	Calcitonin-gene related peptide
CMAP	Compound muscle action potential
CNS	Central nervous system
DNA	Deoxyribonucleic acid
DRG	Dorsal root ganglion
EMG	Electromyography
ERK	Extracellular-regulated protein kinase
FasL	Fas ligand
FDA	Food and drug Administration
FITC	Fluorescein isothiocyanate
GSH	Glutathione
HMG	High mobility group domain proteins
JNK	c-Jun NH_2-terminal protein kinase
NAC	N-acetylcystein
MAPKs	Mitogen-activated protein kinases
MAPKKK or MEKK	MAPK kinase kinase
MKK or MAPKK or MEK	MAPK kinase
MKP	MAPK phosphatase
MNCV	Motor NCV
MNi	Micronuclei
mtDNA	Mitochrondrial Deoxyribonucleic acid

NaCl	Sodium chloride
NCV	Nerve conduction velocity
NGF	Nerve growth factor
NMDA	*N*-methyl-D-aspartate
PBS	Phosphate buffer saline
PC12 cell	Pheochromocytoma cells
PFA	Paraformaldehyde
PKC	Protein kinase C
PNS	Peripheral nervous system
RA	Retinoic acid
RNA	Ribonucleic acid
RTK	Tyrosine kinase
SAPK	Stress-activated protein kinases
SEM	Standard error of mean
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SNCV	Sensory NCV
STAT	Signal transducer and activator of transcription
STZ	Streptozotocin
TEMED	N,N,N',N'-tetramethylethylene diamine
TNF-α	Tumor necrosis factor-α
TRPA1	Transient receptor potential cation channel, subfamily A, member 1
TRPV1	Transient receptor potential cation channel subfamily V member 1
TUNEL	dUTP nick-end-labeling assay
UVC	Ultraviolet irradiation
VIP	Vasoactive intestinal peptide

CHAPTER I

INTRODUCTION

1. INTRODUCTION

The platinum compound, cisplatin was the first line anti-cancer drug introduced in clinical treatment (Dorr, 1994). It is one of the most effective and commonly used chemotherapeutics for the treatment of many solid tumors, including ovarian, testicular, bladder, lung and head and neck tumors (Boulikas and Vougiouka, 2003). Cytotoxicity produced by cisplatin has been shown to be consequence of the DNA damage caused by the formation of cisplatin-DNA adducts. Intra and inter strand of DNA adducted form interrupt the function of the cellular response including cell cycle arrest, DNA repair, survival, or apoptosis. In animal treated with cisplatin for a long period, the cisplatin concentrations were high in the spinal ganglion and peripheral nerves but a low concentration in the brain and spinal cord (Thompson et al., 1984). Neurotoxicity is a dose limiting side effect of cisplatin. Accumulation of cisplatin in dorsal root ganglion neuron (DRG neuron) produced sensory neuropathy which was determined by the general behavior, pain and temperature sensation and nerve conduction velocity (Muller et al., 1990; Tredici et al., 1999). Hence, modification of the soma, nucleus and nucleolus area were observed in the DRG neuron. The protein and RNA are also the targets of cisplatin (Fischer et al., 2001; Hamers et al., 1996). Therefore, neurotoxic effect of cisplatin may be due to reduction of neuronal protein synthesis as a result of nucleolus change.

It is well established that mitogen-activated protein kinases (MAPKs) were observed in the DRG neuron regulated the intracellular signaling network that ultimately regulated gene expression in responded to a variety of extracellular stimuli. In eukaryotic cells, multiple MAPK pathways normally actively regulated diverse vital processes: metabolism, survival, mitosis and apoptosis(Roux and Blenis, 2004; Wada and Penninger, 2004). Generally, SAPK/JNK and p38 are key mediators of stress and inflammation responses evoked by a variety of physical, chemical and biological stress stimuli, while ERK 1/2 cascade is mostly induced by growth factors. Despite differences in type of stimulation, these pathways are in many cases concurrently active (Roux and Blenis, 2004). In animal study, cisplatin-induced nephrotoxicity activated the MAPK cascade and molecular responses typical of the

stress response in the kidney (Arany and Safirstein, 2003). In renal cell , cisplatin increased the phosphorylation of ERK, JNK and p38 (Arany et al., 2004). Pretreatment with MEK inhibitor, u0126, decreased ERK1/2 phosphorylation following cisplatin administration in functional and histological protection (Ramesh and Reeves, 2002). In addition, mechanical hyperalgesia in diabetic-induced neuropathy was present to be correlated with an increase in ERK, JNK and p38 phosphorylation in the DRG neuron (Daulhac et al., 2006). Inhibitory MAPK kinase substance such as U0126, SB203580, and SP600125 suppressed mechanical hyperalgesia and decrease phosphorylation of the kinases in STZ-induced diabetic rat (Pabbidi et al., 2008). Taken together, it might be proposed that alteration of MAPKs activity in the DRG neuron or the nerve may be one of the causes of cisplatin-induced neuropathy.

Therefore, the aim of this study is to investigate the role of MAPKs in cisplatin-induced neuropathy. The alteration of ERK1/2, JNK and p38 kinase activity in the nerve and DRG of cisplatin treated rats will be determined.

2. RESEARCH QUESTION

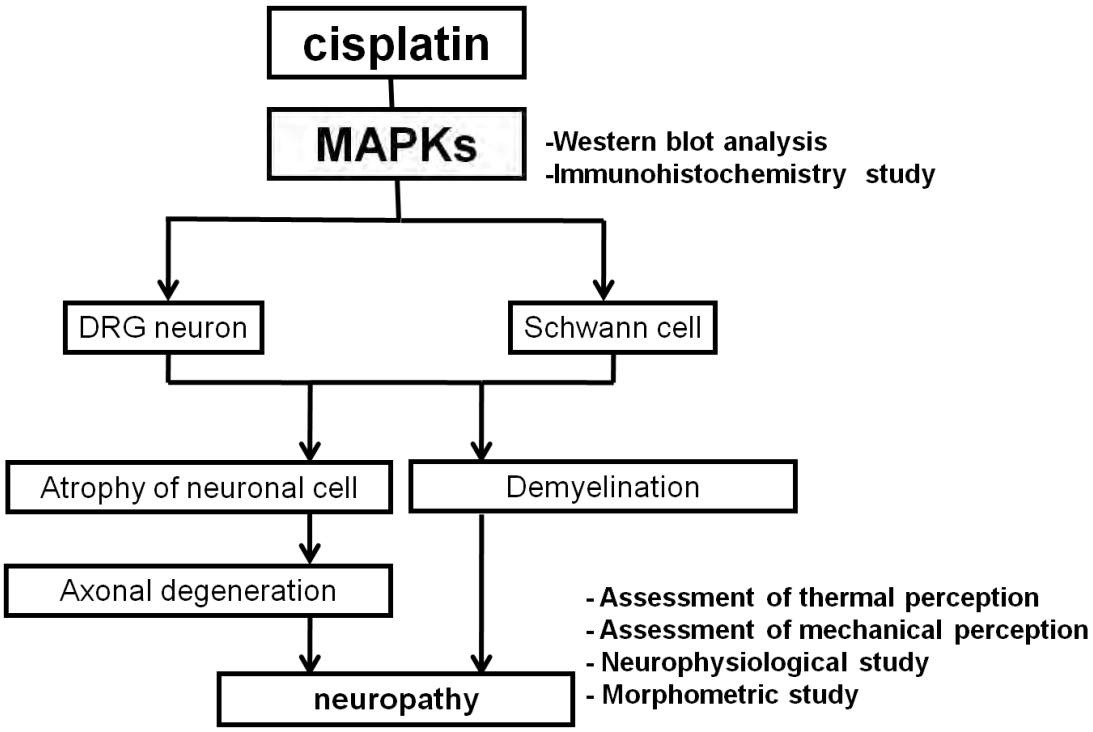
What is the alteration of MAPK phosphorylation in DRG and sciatic nerve in cisplatin-induced neuropathy?

3. OBJECTIVE

To determine the alteration of MAPK phosphorylation in DRG and sciatic nerve of rats with cisplatin-induced neuropathy.

4. CONCEPTUAL FRAMEWORK

Conceptual framework



CHAPTER II

LITERATURE REVIEW

1. CISPLATIN

Cisplatin (*cis*-diaminodichloroplatinum) was the first heavy metal used as an antineoplastic agent. This agent was discovered in 1965 by B. Rosenberg (Rosenberg et al, 1965), who found that the discharge of an electric current from a platinum electrode through nutrition broth inhibited the replication of *Escherichia coli.* Since early 1970s, cisplatin has been used to treat several kinds of solid tumors, including lung, ovary, testis, bladder, head and neck, and endometrium (Prestayko et al., 1979; Mollman, 1990). Cisplatin undergoes rapid distribution to nearly all organs after intravenous administration with the highest levels in kidney, liver, ovary, uterus, skin and bone. In the nervous system, high concentration of cisplatin was found in the DRG (McDonald et al., 2005). This correlates with several side effects of cisplatin such as nephrotoxicity (Arany and Safirstein, 2003) ototoxicity (Brock and Bellman, 1991), nausea and vomiting, alopecia and neurotoxicity. Therefore, the treatment is limited by side effects including nephrotoxicity, emetogenesis and neurotoxicity. The nephrotoxicity and emetogenesis can be overcome by hydration combined with using diuretics and serotonin receptor antagonist (Wong and Giandomenico, 1999). However, neurotoxicity is still an important dose-limiting toxic effect of cisplatin, which can result in hearing loss, tinnitus and peripheral neuropathy (Decatris et al., 2004).

1.1 **Mechanism of action**

Cisplatin (*cis*-diaminodichloroplatinum, or *cis*-DDP) contains NH_3 groups and Cl groups (Fig. 1.). The corresponding *trans* isomer of cisplatin (*trans*-DDP) also forms a coordination complex with DNA. However, unlike cisplatin, trans-DDP is not an effective chemotherapeutic agent.

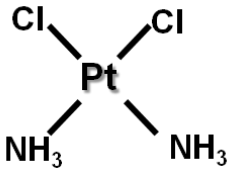


Figure 1. Structure of cisplatin.

Early studies suggested that cisplatin enters the cell mainly by passive diffusion because the concentration of chloride ions outside the cell is higher. However, recent studies have revealed a direct link between the uptake of copper and platinum. These two metals have the same transporter, the yeast protein Ctrl. (Ishida et al., 2002). In addition, the efflux of cisplatin needs the exporter proteins, the ATP7B (copper transporting P type adenosine triphosphate) and ABCC2 or MRP2 (ATP-binding cassette, sub-family C2 or Multidrug resistance protein). These exporter proteins play a role in cisplatin resistance (Komatsu et al., 2000; Cui et al., 1999; Kool et al., 1997; Koike et al., 1997). Once inside the cell, cisplatin is converted to aqueous form. This is due to the lower intra-cellular concentration of chloride ions which facilitates ligand exchange of chloride for water (el-Khateeb, 1999; Jamieson and Lippard, 1999). This aqueous form of cisplatin is very reactive toward RNA and DNA molecules in the nucleus. It is now widely accepted that DNA is the primary target of cisplatin. This mechanism is believed to be the major contribution to its cytotoxicity. We have previously shown that cisplatin induces apoptosis in DRG sensory neurons by covalently binding to nuclear DNA (nDNA), resulting in DNA damage, subsequent p53 activation and Bax-mediated apoptosis via the mitochondria (Jamieson and Lippard, 1999). Recent evidence suggests that direct mitochrondrial (mtDNA) damage may provide a novel, distinct mechanism for cisplatin-induced neurotoxicity separate from the established nDNA damage pathway (Podratz J. et al., 2011).

The platinum atom of cisplatin forms covalent bonds to N7 positions of purines to form1, 2-or 1, 3-intrastrand crosslink and a lower number of interstrand cross-links. The most common purine bases involved in these adduct formation are guanines. The formation of these adducts causes the bending of the helix for 60°C toward the major groove. Cisplatin forms covalent bonds with nucleophilic sites on guanine which induces widening of the minor groove. The formation of cisplatin-DNA cross link distorts the structure of DNA and normal transcription and replication (Jamieson and Lippard, 1999).

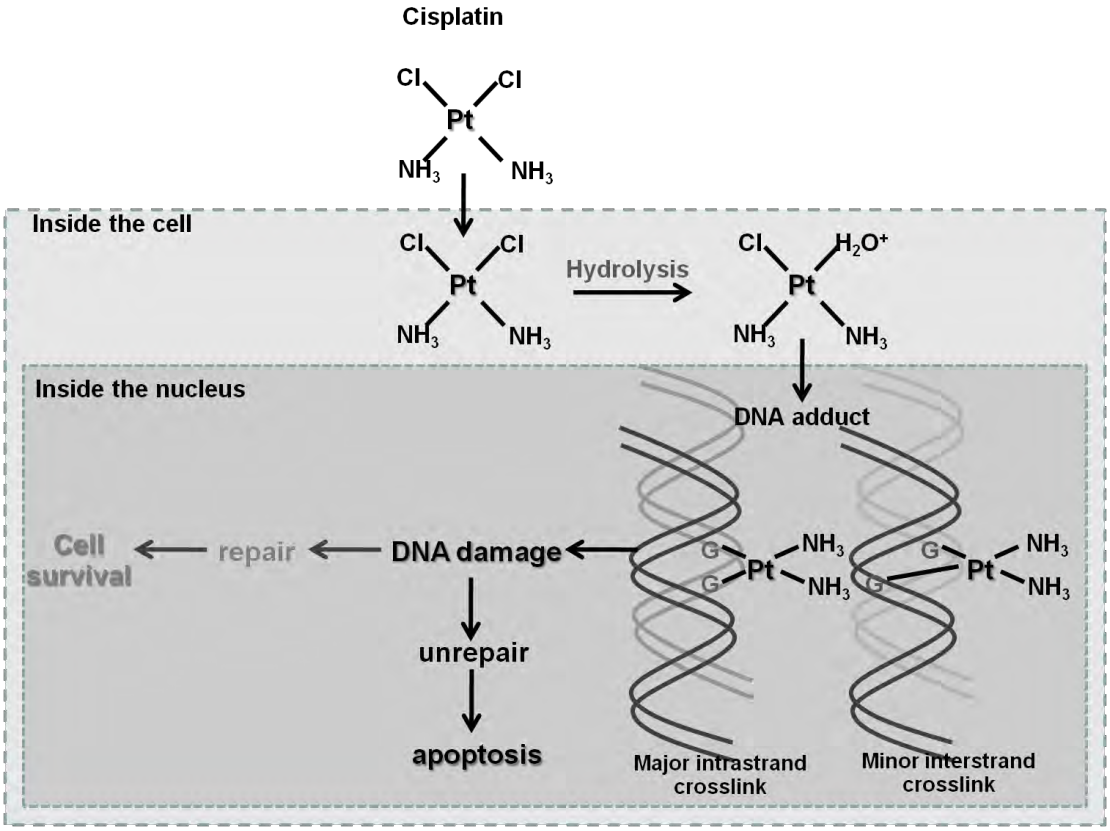


Figure 2. Mechanism of cisplatin.

The cisplatin-DNA adducts detect the protein which has identical sequences of amino acids, a high mobility group (HMG)-domain proteins. HMG-domain proteins bind cisplatin-DNA adducts both in vitro and in vivo. This binding plays an important role in cisplatin activity in killing cells (Wong et al., 2002). The role of HMG-domain proteins in cisplatin cytotoxic activity is that HMG-domain proteins are transcription factors disrupt transcription machinery leading to cell death. Moreover, when the HMG-domain proteins bind to cisplatin- DNA adducts, the adducts are not recognized by the repair machinery. The inhibition of replication by cisplatin suggests that it might kill cancer cells by blocking their ability to synthesize new DNA required for division. The result of the experimental study shows that cisplatin cytotoxicity might be due not only to inhibition of DNA synthesis but also impaired DNA transcription. When the regulatory mechanisms detect the abnormal DNA, they will activate a chain of responses in order to correct it. Intrastrand crosslink undergo repair by nucleotide excision repair, while interstrand crosslink are repaired by homologous recombination. If the amount of DNA damage is more severe than the ability to repair, this ultimately causes apoptosis.

The cellular death started after 18 hr of treatment and became more evident at 22-24 hr. Immunofluorescent analysis demonstrated that cisplatin was able to activate by phosphorylation the anti-apoptotic protein Bcl-2 and to increase with respect to control cells the total amount of the pro-apoptotic protein Bax (Scuteri A et al., 2009).

The action benefit of cisplatin over other antitumor agents is its exhibition of antitumor activity against several tumors without the species specificity.

1.2 **Side effects of cisplatin**

Cisplatin causes several side effects including nephrotoxicity (Arany and Safirstein, 2003) ototoxicity (Brock and Bellman, 1991), nausea, vomiting, alopecia and peripheral neuropathy (Mollman, 1990) which is the most common cause of withdrawal from the treatment.

The pathological changes in cisplatin induced nephrotoxicity are most severe by three to five days following cisplatin treatment (6 mg/kg body weight) and are characterized by widespread tubular necrosis of the corticomedullary area, predominantly in the third segment (S3) or straight portion of the proximal tubule (pars recta). Electron microscopic studies reveal several ultrastructural changes in the pars recta, including severe thinning or focal loss of the brush border, cellular swelling, condensation of nuclear chromatin, cytoplasmic vacuolization, rounded mitochondria with swollen crystals, dissociation of mitochondria from basal enfolding, loss of basal enfolding, increased number and size of pinocytotic vesicles and lysosomal bodies in the apical region. Chronic treatment with cisplatin may further result in cyst formation, interstitial fibrosis and thickening of tubular basement membranes, inducing irreversible renal damage (Dobyan and Levi et al., 1980). In human, disturbance of the electrolyte such as hypomagnesemia; hypocalcemia and hypokalemia have been noted in patients receiving cisplatin.

2. Cisplatin-induced neuropathy

Peripheral neuropathy (PN) is one of the most important side effects of the cisplatin treatment. PN is the dysfunction of peripheral neurons including motor, sensory and autonomic (Windebank and Grisold, 2008). The sensory symptoms

include paresthesia, dysesthesia, hypoesthesia, hyperesthesia, loss of proprioception, loss of pain/temperature discrimination, areflexia and the weakness in the motor symptom. Cisplatin accumulates in the different parts of the body. The major area of the cisplatin accumulation is DRG (McDonald et al., 2005).

Cisplatin is known to be toxic to the nervous system (Mollman, 1990; van der Hoop et al., 1990) and exhibits preferential uptake in the DRG. This preference of cisplatin toward primary sensory neuron may well be explained from the fact that, in contrast to the motor neurons, the spinal ganglia are located outside the blood-brain barrier. Primary sensory neurons are supplied by capillaries with fenestrated wall that allow free passage of the molecules between the circulation and the extracellular fluid of the ganglion. The cisplatin concentrations were high in spinal ganglia and peripheral nerves, whereas they were low in the brain and spinal cord in rats treated with cisplatin for a long period (Thompson et al., 1984).

In DRG, a reduction in the nucleus, cytoplasm, and nucleolus areas of primary sensory neurons was evident and accompanied by damage of nucleolus (Barajon et al., 1996). Rearrangement of nucleolar constituents (pars fibrosa and pale regions) took place (Tomiwa et al., 1986). Cisplatin-induced neuropathy can not be solely explained by the intra-and inter- strand cross links of DNA. This is because neurons are not dividing cells. The protein and RNA are also the targets of cisplatin as well. Therefore, it can be suggested that neurotoxic effect of the cisplatin may be due to reduction of neuronal protein synthesis as a result of nucleolus change in RNA binding and/or extensive binding of cisplatin with cytoplasmic structure or the protein binding (Muller et al., 1992).

However, cisplatin-induced neuropathy is shown to be associated with the impairment of the axonal transport which results in reduction of both the number and velocity of organelles moving in the anterograde and retrograde directions (Russell et al., 1995). This leads to the increased concentration of pain-related peptide in the soma of the DRG neurons, but decreased in the peripheral nerve (Barajon et al., 1996). Other cytoplasmic change includes the occurrence of lipid-like inclusions in

the lysosomes which suggests that cisplatin particularly affects lipid metabolism (Muller et al., 1990).

In peripheral nerves, the pathologic changes showed a decreased in the number of large- and medium-size myelinated nerve fiber and advanced Wallerian degeneration of some large myelinated fibers (Cavaletti et al., 1992; Barajon et al., 1996). Electrophysiological and behavioral studies evidenced a reduction in nerve conduction velocities and impairment in pain detection and coordination. Some of the nerve fibers presented axonal and adaxonal accumulations, showing the presence of an axonopathy (Barajon et al., 1996).

Furthermore, it has been supported that cisplatin acts upon Schwann cells, resulting in the granular condensation of nuclear chromatin. The number of apoptotic cells increased while determined by the dUTP nick-end-labeling assay (TUNEL) method. The number of micronuclei (MNi) which is a sign of cisplatin-induced genotoxicity was increased (Jirsova et al., 2006).

2.1 **Cisplatin-induced neuropathy in human**

Full course of cisplatin chemotherapy always develop a symptomatic and clinically detectable sensory neuropathy (Albers et al., 2007). The symptoms of cisplatin-induced neuropathy include distal paresthesia (tingling in the extremities) and numbness appeared as soon as one month after initiating treatment. The entire platinum compounds produce “coasting” effect in which the neuropathy can progress for a week or several months after the cessation of treatment. Associated signs of large fiber loss were the reduction of vibration, joint position sensations and the diminish or absence of muscle stretch reflexes (Daugaard et al., 1987; Cavaletti et al., 1992). Cisplatin does not usually affect motor and autonomic functions. Loss of motor function can result in loss of strength of distal muscle groups with varying severity (Windebank and Grisold, 2008). Autonomic disturbance includes signs including orthostatic hypotension, constipation, dysfunction of sexual organs and urinary bladder (Windebank and Grisold, 2008). The reflexes are often lost, especially at the ankles which is an early sign (Thompson et al., 1984)

Sensory ataxia may be observed in those patients with severe neuropathy. Small fiber sensation e.g. pain is spared or mildly diminished. Symptoms and signs are symmetric and usually worse distally. Despite the high frequency of neuropathy among patients treated with cisplatin, relatively few patients develop functional impairment sufficient to interfere with activities of daily living. This neuropathy is generally reversible, but recovery may take months to years (Quasthoff and Hartung, 2002). The severity of neuropathy is divided into five grades according to the National Cancer Institute Common Toxicity Criteria in 2006 (Windebank and Grisold, 2008). In brief, neuropathy grade 1-2 shows loss of sensory function but it does not interfere with the daily activity while grade 3-4 disrupts the daily activity. Grade 5 is death. Nevertheless, neurotoxicity is a major reason that cisplatin is discontinued and the cumulative dosage limited, reducing its chemotherapeutic efficacy.

2.2 **Cisplatin-induced neuropathy in animal**

The cisplatin-induced neuropathy in experimental animal is relatively similar to that observed in human. It is characterized by involvement of the sensory nerve fibers secondary to DRG neuronopathy. Administration of cisplatin 2 mg/kg twice a week for five weeks in the rat resulted in the decrease of somatic area, nuclear area and nucleolar area of the DRG neuron (Muller et al., 1990; Cavaletti et al., 1992; Schmidt et al., 1995; Barajon et al., 1996; Tredici et al., 1998; Tredici et al., 1999; Ozturk et al., 2004; Ozturk et al., 2005; Pisano et al., 2003; Carozzi V. et al., 2010). Axonal degeneration and loss of large myelinated nerve fibers were also observed (Barajon et al., 1996; Tredici et al., 1999; Cavaletti et al., 1992).

Behavioral assessments revealed sensory (thermal hypoalgesia and mechanical hyperalgesia)(Cata et al., 2008) and motor loss of coordination and motor force disorders after repeated injections of cisplatin in rodents. For pain sensation (tail-flick test), the latency of the response to a fixed pain stimulus was increased (Tredici et al., 1999; Tredici et al., 1998).

Neurophysiologic studies have shown that cisplatin decreased sensory nerve conduction velocities and reduced the amplitude of nerve action potentials, with

minimal or no motor involvement (De Koning et al., 1987; Boyle Wheeler et al., 1999; Carozzi V. et al., 2010).

Motor and proprioceptive functions were evaluated using rota-rod test. The rota-rod test showed impairment in coordination (Barajon et al., 1996; Tredici et al., 1998; Boyle et al., 1999; Verdu et al., 1999; Authier et al., 2000; Ozturk et al., 2004). This pattern of rota-rod abnormality was attributed to loss of large proprioceptive fiber function, and correlated with a reduction in calcitonin gene-related peptide in large sensory neurons in cervical DRG and an elevation of the distal sensory latency in the caudal nerve of the tail. Gait disturbance in animals treated with cisplatin resulted in toe-walking with arched back (Boyle et al., 1999)

2.3 **Sex related differences in cisplatin-induced neuropathy in rats**

Sexual dimorphism in peripheral nerve abnormalities was reported by Joseph and Levine in 2003. In case of nerve injury, the rate of regeneration was different between sexes (Kujawa K, Emeric E. and Jones K. 1991; Jones K. 1993; Kovacic et al., 2004.). Both sexes have been used to investigate the effects of various treatments on cisplatin neuropathy (Schmidt et al., 1995; Barajon, et al., 1996; Tredici et al., 1999; Authier et al., 2003; Bardos et al., 2003; Pisano et al., 2003). However, there were no comparisons in the functional and pathological abnormalities between the two sexes. Vincristine has been shown to induce neuropathy with greater mechanical hyperalgesia in female than in male rats (Joseph and Levine, 2003). Moreover, in cancer patients treated with cisplatin-based chemotherapy, the worse neurological outcome was found with higher frequency in males than in females (Argyriou et al., 2005.). However, an extensive comparison in the cisplatin-induced neuropathy between sexes has not been performed.

2.4 Prevention and treatment of the cisplatin-induced neuropathy

Since neurotoxicity is a dose-limiting side effect of cisplatin, several trials using neurotrophic factors, antioxidants and herbs for the prevention and treatment were conducted.

Nerve growth factor (NGF) plays an important role in the course of cisplatin-induced neuropathy. Reduction in the circulating levels of nerve growth factor (NGF).

Suggests the deficient neurotrophic support to the dorsal root ganglia sensory neuron induced by cisplatin (Cavaletti et al., 2002). The DRG neurons receive this growth factor mostly by retrograde transport from the periphery. Russel (1995) demonstrated that cisplatin administration reduced axonal transport, a situation which might further reduce the NGF supply to DRG neuron cell body. The NGF circulating level decreased during chronic cisplatin administration in close accordance with the course of neuropathy and returned to normal level after recovery from the neurotoxic damage (Cavaletti et al., 2002). Exogenous NGF administration is able to restore biochemical, structural and functional changes induced by cisplatin (Aloe et al., 2000). These findings suggest that the reduction of NGF availability could be a cause of cisplatin-induced peripheral neuropathy and exogenous NGF administration could prevent or reduce cisplatin neurotoxicity.

Brain-derived neurotrophic factor (BDNF) has been shown to promote survival of differentiated neuroblastoma (NB1643) cells after cisplatin treatment(Middlemas et al., 1999). BDNF stimulates both autophosphorylation of trkB and induction of the immediate early gene, fos, in these cells.

Acetyl-L-Carnitine (ALC), the acetyl ester of L-carnitine, has neuroprotective and neurotrophic action. These effects may be exerted via its antioxidant activity, including the stabilization of intracellular membranes. In animal model, ALC was able to restore the mechanical nociceptive threshold following cisplatin treatment (Ghirardi et al., 2005). The ALC co-treatment was able to significantly reduce the neurotoxicity of both cisplatin and paclitaxel. This effect was correlated with a modulation of the plasma levels of NGF in the cisplatin-treated animals (Pisano et al., 2003). ALC administration promotes the recovery of nerve conduction velocity, restores the mechanical nociceptive threshold by up-regulating the expression of type-2 metabotropic glutamate receptors in DRG (De Grandis et al, 2007). In human study, ALC prevents progression or reverses symptoms during cisplatin treatment(Bianchi et al., 2005; Maestri et al., 2005).

Vitamin E deficiency might be the cause of cisplatin-induced neuropathy. Decrease in plasma vitamin E level was found in human with severe cisplatin neuropathy (Bove et al., 2001). At present, the data are still insufficient to conclude that vitamin E can prevent or limit the neurotoxicity of cisplatin in human patients(Albers et al., 2007).

Glutathione (GSH) is also effective for the prevention of cisplatin-induced neuropathy. It does not reduce the clinical activity of chemotherapeutic drugs. Based on the evaluation of the median, ulnar, and sural sensory nerve conduction, GSH treatment was associated with a statistically significant improvement in reduction of these values (Hamers et al., 1993). The neurophysiological and pathological changes induced by cisplatin administration were less severe in rats co-treated with GSH (Pirovano et al., 1992; Hamers et al., 1993; Tredici et al., 1994).

N-acetylcysteine (NAC) is a precursor of glutathione which can completely block cisplatin-induced apoptosis. It has inhibitory effect on cisplatin induced p53 but not Fas/Fas-L accumulation(Park et al., 2000).

Herbs with the potential to significantly modulate the activity of drug-metabolizing enzymes (cytochrome p450) and/or the drug transporter P-glycoprotein include garlic (Allium sativum), ginkgo (Ginkgo biloba), echinacea (Echinacea purpurea), ginseng (Panax ginseng), St John's wort (Hypericum perforatum), and kava (Piper methysticum) have been explored for their potential effect on cisplatin toxicity. The famous one is Ginkgo (Ginkgo biloba) which protects against cisplatin-induced ototoxicity (Huang et al., 2007) and also cisplatin-induced nephrotoxicity (Gulec et al., 2006). Ginkgo biloba extract is effective in preventing the reduction in NCV, decrease in the number of migrating cells and the length of outgrowing axons caused by cisplatin in DRG culture. In morphological assessment, Ginkgo biloba extract co-administration reverses the shrinkage of nucleus and cell body of DRG neurons induced by cisplatin (Ozturk et al., 2004).

Radicicol, an inhibitor of protein tyrosine kinases has therapeutic potential for neurodegenerative diseases, especially for chemotherapeutic drug-induced sensory neuropathy. It was found to rescue cultured sensory neurons from apoptotic cell death associated with cisplatin (Sano, 2001).

Retinoic acid (RA, vitamin A acid) which is the active metabolite of retinol (vitamin A), has been shown to have neurotrophic effects is able to induce up-regulation of nerve growth factor (NGF). Retinoids also possess antioxidant properties (Chaplan et al., 1994). In the model of cisplatin-induced neuropathy, RA failed to prevent electrophysiological abnormalities and morphometric changes in DRG neurons but it induced a mild generalized protective effect (Tredici et al., 1998).

3. MITOGEN ACTIVATED PROTEIN KINASE (MAPKs) AND CISPLATIN

The MAPKs, are one of the most extensively studied signal transduction molecules (Avruch et al., 2007). They play roles in cell proliferation, differentiation and cell death (Herskowitz et al.,1995; Marshall et al., 1995; Kyriakis and Avruch, 1996). MAPK pathways include a set of three evolutionary conserved, sequentially acting kinases: MAPKK kinases (MAPKKKs), MAPK kinases (MAPKKs) and MAPKs. The MAPKKKs are activated by dual phosphorylation in response to activation of membrane receptors. Then, they phosphorylate the down stream kinases, MAPKKs. The MAPKKs phosphorylate threonine and tyrosine residues on their substrates MAPKs. MAPKs are the last in this signaling cascade. Once activated, they phosphorylate the wide range functions mediated through diverse substrates, including phospholipases, cytoskeletal proteins, several protein kinases (MAPK-activated protein kinases; MKs) and transcription factors. In this way MAPKs can directly regulate the function of various cellular processes by controlling the expression of different genes and activities of various proteins.

MAPK family consists of 3 subfamilies: the extracellular signal-regulated kinases (ERK 1,2,3,4 and 5), the c-Jun N-terminal kinases (JNK 1, 2 and 3, also called stress-activated protein kinase), and the p38 (α,β,γ and δ). Each MAPK is activated through a specific phosphorylation cascade (Fig. 3). The ERK pathway plays a major role in regulating cell growth and differentiation in response to growth factors, cytokines, and phorbol esters (Johnson and Vaillancourt, 1994; Robinson and Cobb, 1997; He et al, 1999). It is also activated by some conditions of stress, particularly oxidant injury, and in such circumstances, is believed to promote cell survival (Guyton et al., 1996; Aikawa et al., 1997; Wang et al., 1998). In contrast, JNK and p38 are generally activated in response to a variety of stress signals including tumor necrosis factor, ionizing and short wave length ultraviolet irradiation (UVC), and hyperosmotic stress. Their activation is frequently associated with induction of apoptosis (Xia et al., 1995; Chen et al., 1996; Brenner et al., 1997; Wang et al., 1998).

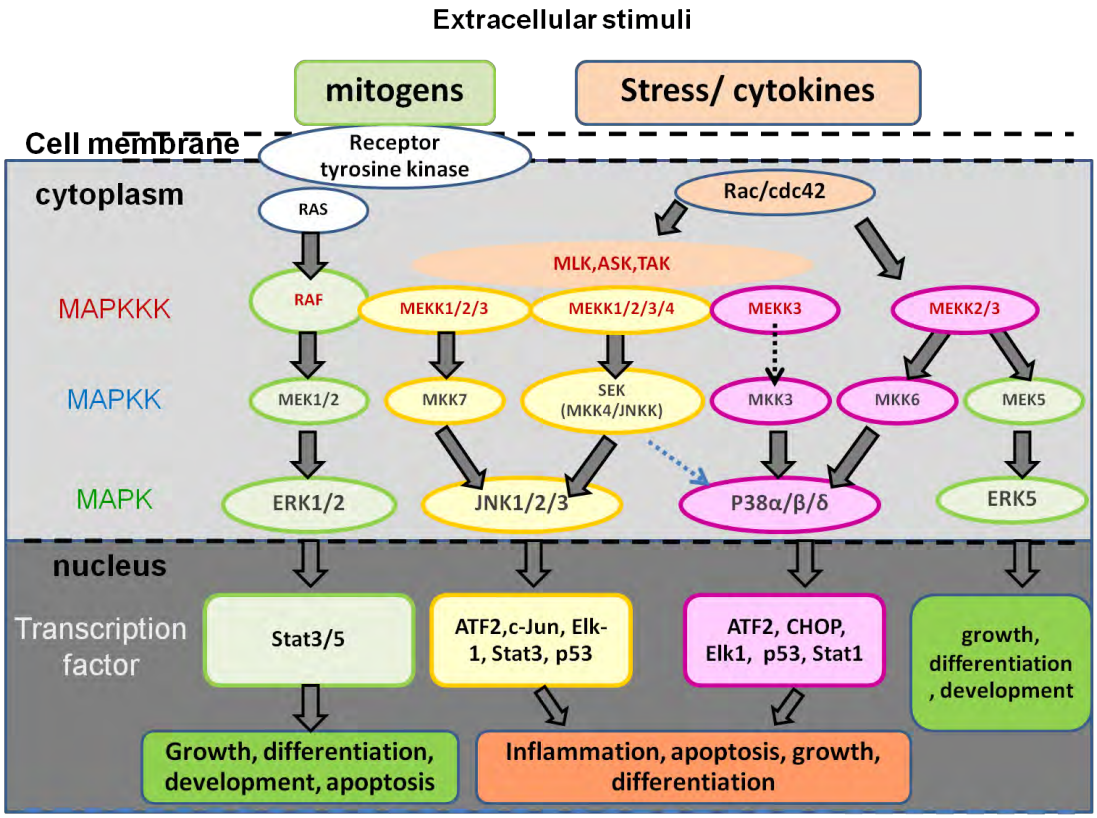


Figure. 3 The mitogen-activated protein kinase (MAPK) pathway.

The importance of MAPK activation in cell response to cisplatin and resistance to this anti-cancer drug has been gradually appreciated in recent years. Today it is believed that MAPK activation is a major component deciding the cell fate in response to cisplatin. It is well known that cisplatin can induce apoptosis by mitochondrial- or receptor-regulated cell death (Crighton and Ryan, 2004). Depending on the type of induced apoptotic pathway, different signaling molecules can be activated connecting downstream events of MAPK pathways directly or indirectly with pro- or anti-apoptotic proteins. Current knowledge concerning the role of MAPK family members in cell response to cisplatin, as well as their role in cisplatin-resistance is summarized in Brozovic and Osmak, 2007. In vitro study, Scuteri and the co-worker study the role of MAPKs in platinum-induced neuronal apoptosis using specific inhibitors of the various MAPKs it was demonstrated that cisplatin induced neuronal apoptosis is mediated by early p38 and ERK1/2 activation, while JNK has a neuroprotective role. In DRG neuron cisplatin induced the phosphorylation of p38 up to 80%, with a concomitant increase in total amount of this

protein until 8 hr up to 30%. At 8 hr cisplatin-treated cells showed a peak on the phosphorylation of ERK1/2 (100%). The total amount unchanged until 16 hr of drug exposure, was reduced by nearly 80%. The evidence suggesting that the P-ERK was preserved despite the reduction in the total protein amount in response to the toxic stimulus. JNK was strongly activated in cisplatin treatment. After 24 hr, the amount of P-JNK decreased up to 50%. Cisplatin induced a weak reduction in total amount of JNK (30%) (Scuteri A et al., 2009).

Nephrotoxicity is one of the major side effects of cisplatin. The role of MAPK in cisplatin-induced acute renal injury is hardly known. Among the earliest reactions of the kidney to cisplatin is the activation of the MAPK cascade and molecular responses typical of the stress response (Arany, 2003). Cisplatin nephrotoxicity is clearly dose-related and used to be considered dose limiting (Ries, 1986). Cisplatin increased the phosphorylation of ERK, JNK and p38 in the mouse kidney after a single intraperitoneal injection of 20 mg/kg (Arany, 2004). The ERK1/2 pathway mediates cisplatin-induced caspase activation and apoptosis in cultured renal tubular cells. Pretreatment with MEK inhibitor, U0126, decreased ERK1/2 phosphorylation following cisplatin administration with significant functional and histological protection. This beneficial effect was accompanied by decrease in TNF-alpha gene expression level and inflammation, as well as caspase 3 activity and apoptosis. These data provide evidence that ERK1/2 pathway functions as an upstream signal of TNF-alpha-mediated inflammation and caspase 3-mediated apoptosis in cisplatin-induced acute renal failure in mice. Thus, ERK1/2 can be a novel therapeutic target in cisplatin nephrotoxicity(Ramesh, 2002).

However, whether cisplatin stimulate MAPK pathways especially in peripheral neurons is still unknown. Moreover, if MAPKs are activated by cisplatin, what is the role of this activation? Therefore, the objective of this study is to determine the status and role of MAPKs in cisplatin-induced neuropathy.

CHAPTER III

MATERIALS AND METHODS

Study 1: Characterization of the animal model of cisplatin-induced neuropathy

This study was done to compare cisplatin-induced neuropathy in male and female rats in order to select the better model for use in subsequent experiment. Both sexes have been used to investigate the effects of various treatments on cisplatin neuropathy (Schmidt et al., 1995; Barajon, et al., 1996; Tredici et al., 1999; Authier et al., 2003; Bardos et al., 2003; Pisano et al., 2003). However, there were no comparisons in the functional and pathological abnormalities between the two sexes.

EXPERIMENTAL PROCEDURES

1. DRUG ADMINISTRATION

Cisplatin was diluted in normal saline (NSS) and given intraperitoneally 2 mg/kg twice a week for five consecutive weeks (20 mg/kg accumulative dose). Dose of cisplatin used in the present study had been shown to induce both physiological and structural abnormalities of peripheral nerve (Authier et al., 2003).

2. EXPERIMENTAL DESIGN (Fig. 4)

Thirty male and female Wistar rats (from the National Laboratory Animal Center, Mahidol University, Thailand) weighing 200 to 250 g each were used for this study. They were divided into 4 groups which are male +cisplatin group (MC), female +cisplatin group (FC), male + NSS group (MN) and female + NSS group (FN). The rats were kept in aluminium cages on a 12-h light / 12-h dark cycle with access to water and food ad libitum. Room temperature was maintained at 25±2°C. Animals were allowed a 1-week acclimatization period before use in the experiment.

The duration of the experiment was five weeks. The hot plate test was examined at baseline before the injection, the 1^{st} , 2^{nd} , 3^{rd}, 4^{th} and 5^{th} weeks. In addition, sciatic nerve conduction velocity was determined prior to the first treatment as baseline and the end of the experiment.

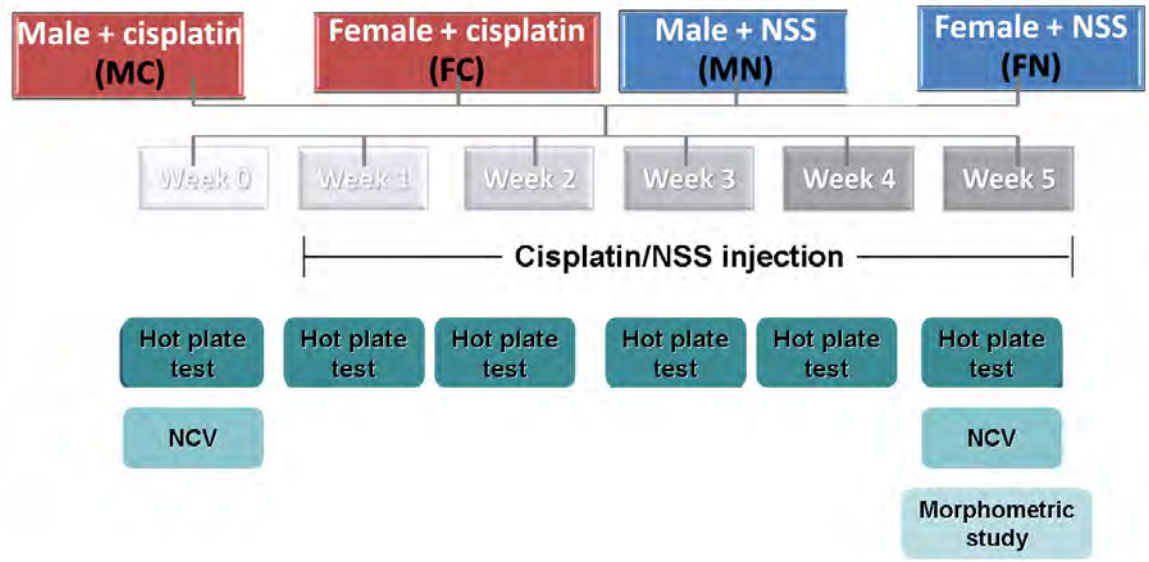


Figure 4. Flow chart of the experimental design of study 1

3. ASSESSMENT OF GENERAL TOXICITY

All rats were examined everyday to detect abnormal clinical signs such as piloerection, gait disturbance (toe-walking with arched back) (Fig. 5) and reduction of general motor activity. Body weight was recorded before each injection twice a week for 5 weeks.

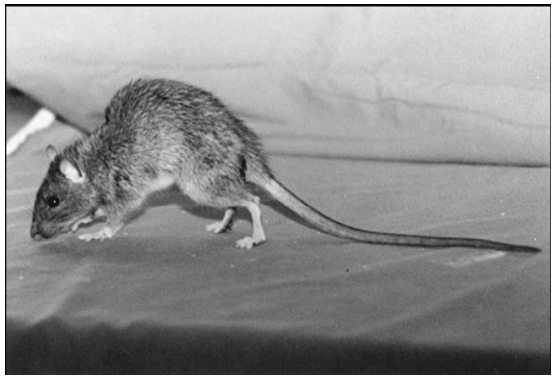


Figure 5. The rat with typical features of gait disturbance in general health determination (Boyle, 1999).

4. EVALUATION OF NEUROPATHY INDUCED BY CISPLATIN

4.1 Assessment of hindpaw thermal perception using hot plate test

Introduction to the hot plate test

The hot plate test is used to assess the thermal perception of the rat hindpaw. Thermal withdrawal latency which is the duration from the start of heat stimulation until the

withdrawal or licking the hindpaw is obtained (Manner, Katz, and Askanazi, 1996; Hashimoto, Ohgami and Yonemasu, 1991).

Method

Before the test, each rat was placed on a hot plate without heat for 3 minutes for acclimatization. For the experiment, the temperature was set at 55°C. The rat was put on the hot plate and then covered with the plexiglass cage. Time from the start when the hindpaws of the rat touch the hot plate until lifted to lick was recorded. This time was defined as the withdrawal latency. The cut-off time of 35 seconds was used to avoid skin injury. The three trials were conducted on each rat with 15 minute intervals (Manner et al., 1996).

4.2 Neurophysiological assessment: motor nerve conduction velocity test

Introduction to nerve conduction velocity test

A nerve conduction velocity (NCV) test is used to determine the conductive function of the group of largest nerve fibers. In motor fiber assessment, the compound muscle action potential (CMAP) is recorded from muscles and analyzed for motor nerve conduction velocity (MNCV). Normal body temperature must be maintained during the NCV test because nerve conduction velocity is temperature-dependent.

Method

The rat was anesthetized using 4% halothane with 1000ml/min oxygen for induction. After that the concentration of the halothane was decreased to 2.5% with 500ml/min oxygen to maintain the anesthesia. Rectal temperature was maintained at 37°C. The rat was in the prone position on the heat pad with the blanket. The stimulating electrode was applied at the sciatic notch close to the sciatic nerve (point 1 Fig. 6) and the recording electrodes were applied in the second and the third interosseous spaces of the same leg. The ground electrode was inserted at the lateral side of the foot. All the electrodes were connected to the EMG machine. Supramaximal stimulus was applied at the stimulation point (point 1) and the CMAP was recorded at point 3. This was repeated at least 4 times. The mean latency from the stimulation to M wave peak of CMAP was determined. Then, the electrodes were moved from the sciatic notch to Achilles' tendon (point 2 Fig. 6) closed to the tibial division of the sciatic nerve and the procedures were repeated. Latency difference

between the stimulation at point 1 and point 2 was determined (Fig. 7). Finally, MNCV was calculated by dividing the distance between point 1 and point 2 by the latency difference as shown below.

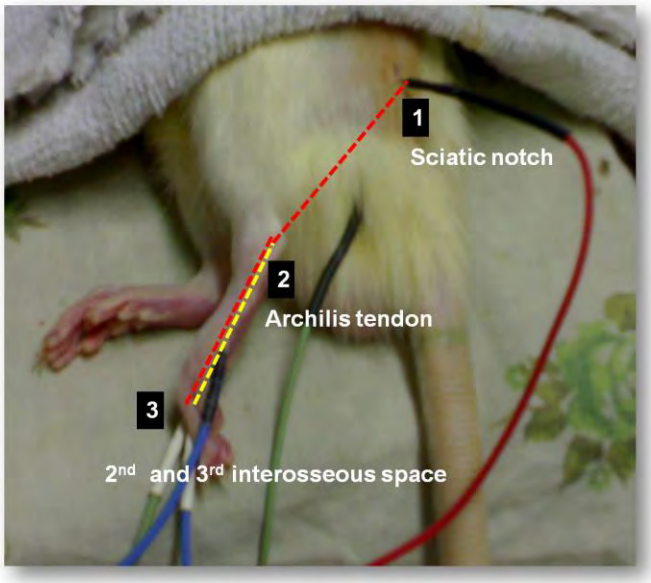


Figure 6. The positions of the stimulating and recording electrodes in the NCV study.

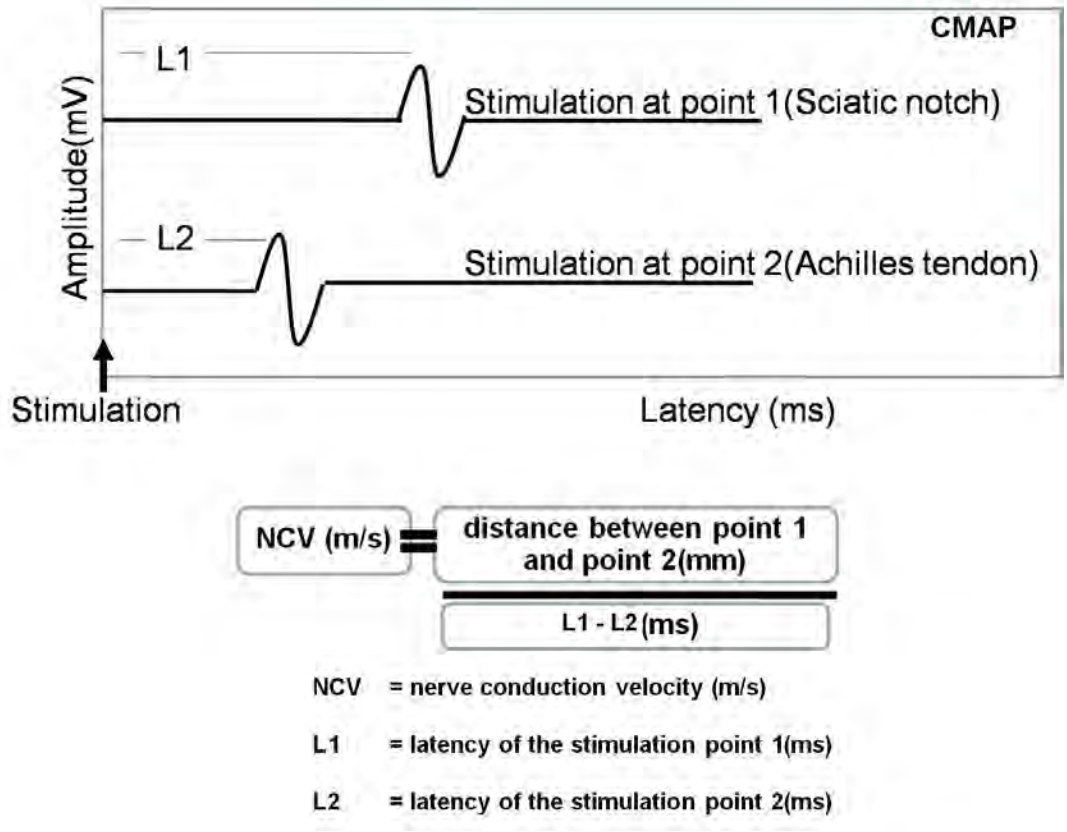


Figure 7. Calculation of motor NCV from the CMAP recording

5. SACRIFICE

Under halothane anesthesia, the animals were undergo intracardiac perfusion with normal saline 200 ml for 8 min and followed by 4% paraformaldehyde (PFA) 400 ml for 15 min. The sciatic nerve and L4 DRG from both right and left sides were obtained.

6. MORPHOMETRY OF SCIATIC NERVE AND DRG

6.1 Tissue collection and sample preparation

L4 DRG and sciatic nerve stretched on one side by the pendulum were suspended in 3% glutaraldehyde for 6 hr. After that, the sciatic nerve was divided into 2 mm segments for the good penetration. The sciatic nerve and L4 DRG were continuously fixed in 3% glutaraldehyde for 24 hr and were incubated with 0.1 M phosphate buffer (PBS) pH 7.4 overnight at 4°C. For the next step, the specimens were washed with PBS for 3 times 10min each. Fixation was done with 1 % osmium tetroxide for 2 hr. The samples were then dehydrated with 70%, 80%, 95%, and 100% alcohols followed by clearing with propylene oxide 15 min twice. Then, the tissues were infiltrated with propylene oxide: Epon (resin) 1:1 for 1 hr., 1:2 overnight and 100 % Epon for 1 hr. Embedding with 100 % Epon in capsule block was left at 60°C for 3 days. The sections were cut at 0.5µm with ultramicrotome for sciatic nerve and 2 µm for DRG. Finally, the sciatic nerve sections were stained with 1% para-Phenylenediamine and the DRG sections were stained with toluidine blue. The sections were screened for artifacts under light microscope.

6.2 Morphometric assessment of sciatic nerve

The sciatic nerve sections were examined under the light microscope connected to the computer with the Image Pro-Plus software. Under 4x objective len, images of the fascicles in the section were imported to the computer and total fascicular area (mm^2) was calculated. For the three window sampling technique (Chentanez et al., 2006), only fascicles large enough to contain three windows of 0.012 mm^2 were further analyzed (Fig. 8A). Under 40x objective len, three window areas were randomly placed, one in the middle and the other two in the periphery of fascicle as shown in Fig.8A. The window frame must not be in contact with the

perineurium or adjacent window. Only nerve fibers completely located in the window were counted. Each window was analyzed for the number of myelinated fiber, myelinated fiber diameter, axon diameter, myelin thickness, and g-ratio using the Images Pro-Plus software. The total fiber in three windows must be used to estimate the total number of myelinated fibers in the whole nerve by comparing with the total fascicular area. The analysis was done by the same investigator to prevent inter-observer variations.

g-ratio = axonal diameter/fiber diameter

fiber density = the total number of myelinated fiber /total fascicular area

myelin thickness = (fiber diameter-axonal diameter)/2

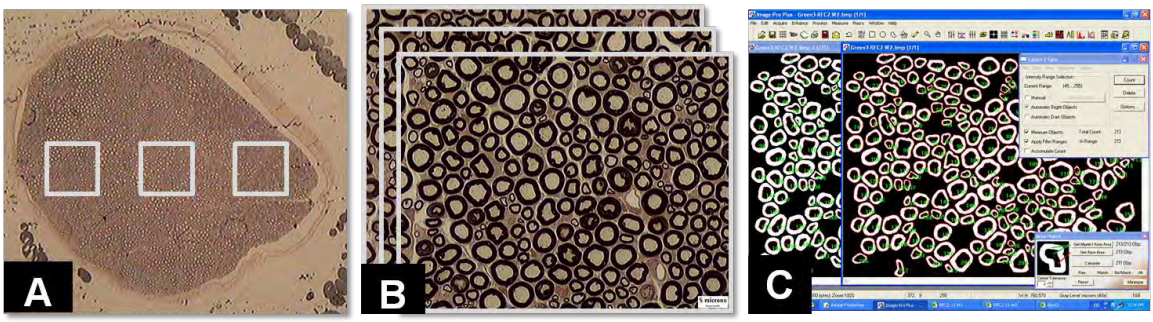


Figure 8. Morphometry of the nerve. A: the three window sampling from the fascicle; B: enlargement of the three windows; C: the three windows were analyzed using the Images Pro-Plus software.

6.3 MORPHOMETRIC ASSESSMENT OF DRG

L4 DRG was serially cut into 2 µm thick sections. One in every 20th section was chosen (40µm apart) in order to avoid counting the same neuron. The selected sections were included for analyses. For estimation of the total number of neurons per DRG (Fig.9A), all neurons with prominent nucleus and nucleolus in the section were counted using Adobe Photoshop CS3 program and this was done for all selected sections (Fig.9B). Then, the number of counted neurons was used to estimate the total number of neurons in the whole DRG.

Total number of DRG neuron = (number of counted neuron/ number of the selected sections) × the number of the whole DRG sections

For the determination of somatic, nuclear and nucleolar areas, images of 300 randomly selected neurons were imported into the computer under 40x objective len. Using Image Pro-Plus software, the pen cursor tablet (G-pen 340) was used to mark around the outline of soma, nucleus and nucleolus in the selected neurons. The areas within the outline were automatically calculated.

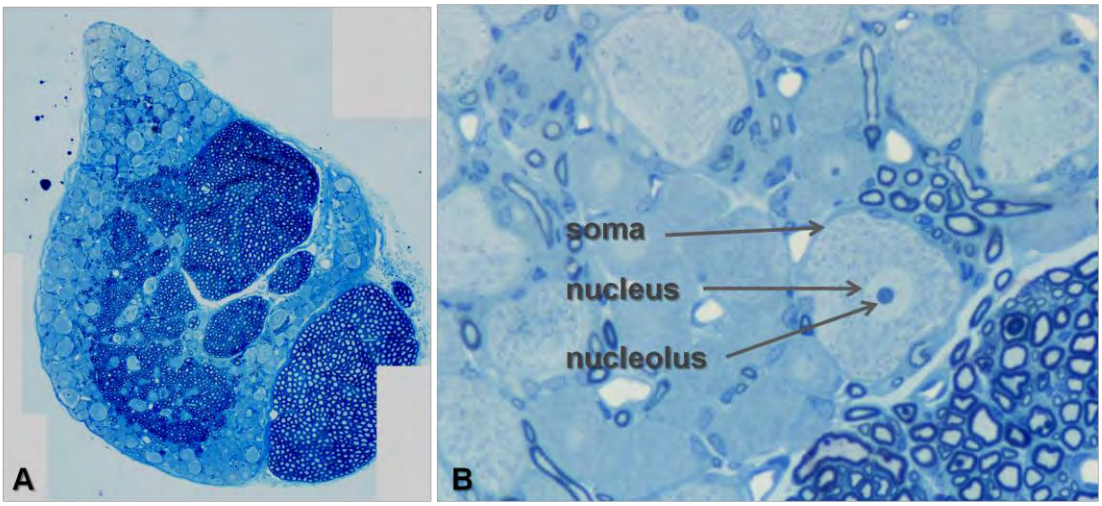


Figure 9. Morphometry of the DRG. A: low power of DRG section; B: high magnification of DRG section showing soma, nucleus and nucleolus.

7. STATISTICAL ANALYSIS

Student's unpaired t-test was used to compare mean of experimental groups with the same sex. This was done using SPSS for Windows version 11. Statistically significant difference was noted when $p < 0.05$, 0.01 and 0.001.

8. ETHICAL CONSIDERATION

All protocols involving the animal use in this study were approved by Animal Ethics Committee, Faculty of Medicine, Chulalongkorn University. Moreover, the animal experiments were conformed to the guidelines issued by the Research Council of Thailand.

Study 2 Determination of MAPK phosphorylation in DRG and sciatic nerve of rats with cisplatin-induced neuropathy

ANIMALS

Eighty nine female Wistar rats (from the National Laboratory Animal Center, Mahidol University, Thailand) weighing 200 to 250 g each were used for this study. Females were used as they were more tolerable to the general toxicity of cisplatin while still developed peripheral neuropathy (results of Study 1). They were divided into 2 groups which are cisplatin-treated group (P) and the control group (C). In each group, the animals were subdivided into five subgroups according to the experimental design in figure 10 (1^{st}, 3^{rd}, 5^{th}, 8^{th} and 12^{th}). The rats were kept in aluminium cages on a 12-h light / 12-h dark cycle with access to water and food ad libitum. Room temperature was maintained at 25±2°C. Animals were allowed a 1-week acclimatization period before use in the experiment.

EXPERIMENTAL PROCEDURES

1. DRUG ADMINISTRATION

Cisplatin was diluted in normal saline(NSS) and given to the P group (n=46) intraperitoneally 2 mg/kg twice a week for five consecutive weeks(same as in Study 1). Injected volume of normal saline in the C group (n=43) was equivalent to those of P group with equal body weight (same as in Study 1).

2. EXPERIMENTAL DESIGN (Fig. 10)

The duration of the experiment was 12 weeks. This duration was used as evidence of neuropathy was still observed (Mizisin. et al., 1999).The rats were injected intraperitoneally 2 mg/kg with cisplatin and normal saline in P group and C group respectively twice a week from the 1^{st} to the 5^{th} week. The hot plate test, Von Frey hair test and sciatic nerve conduction velocity were examined at baseline and the 1^{st} , 3^{rd} , 5^{th} , 8^{th} and 12^{th} weeks. In addition, immunohistochemistry and Western blot analysis of ERK, p38 and JNK were examined on the same time points. The schedule of the experiment is summarized in Fig. 10.

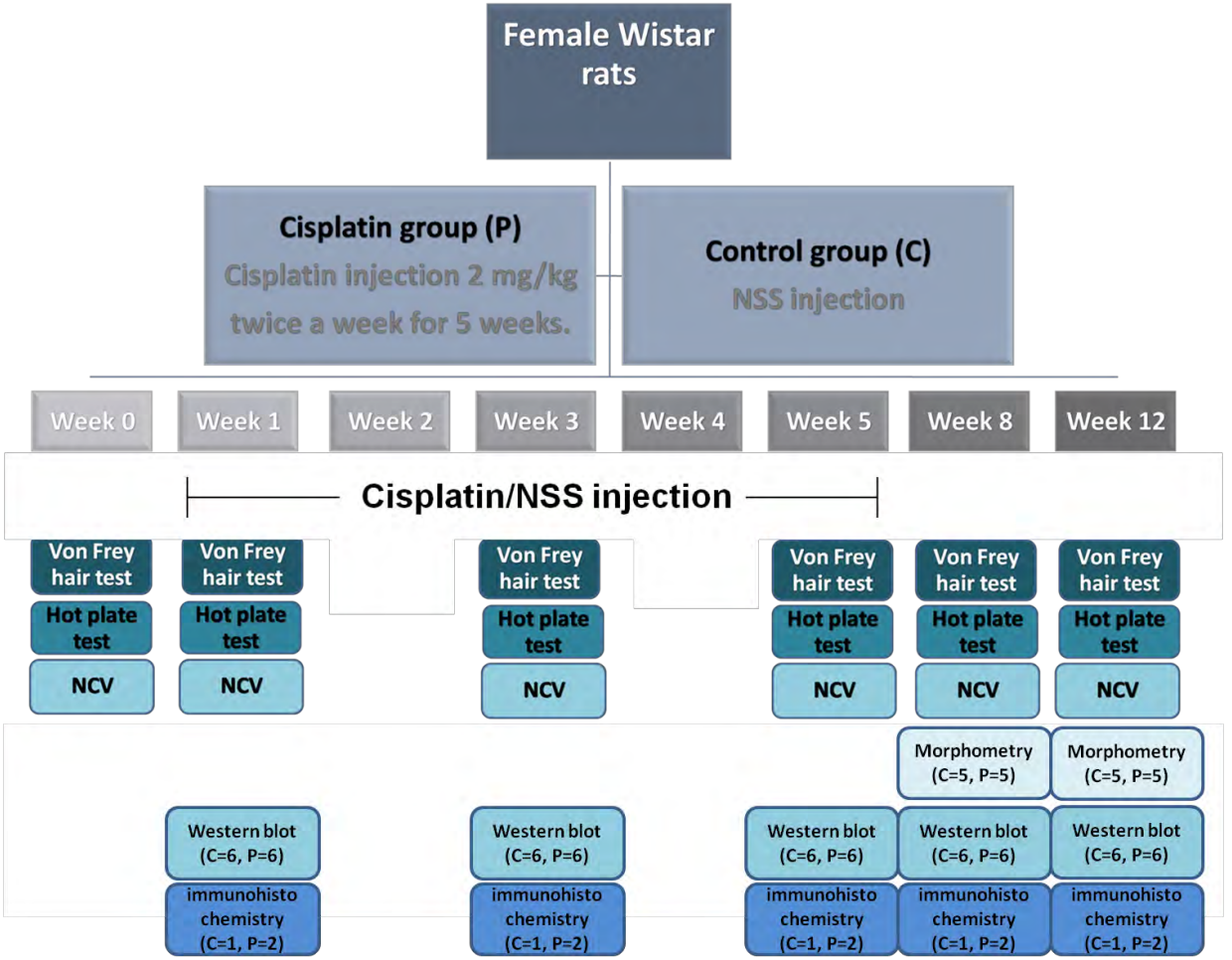


Figure 10. Flow chart of the experimental design of Study 2

3. ASSESSMENT OF GENERAL TOXICITY

All rats will be examined everyday to detect abnormal clinical signs same as Study 1.

4. EVALUATION OF NEUROPATHY INDUCED BY CISPLATIN

4.1 Assessment of hindpaw thermal perception using hot plate test

(same as 4.1 in Study 1)

4.2 Assessment of mechanical allodynia using Von Frey test

Introduction to Von Frey test of mechanical allodynia

The mechanical force is exerted via application of Von Frey hair with known weight to the skin. The elevated wire mesh floor supports a rat while the hair is applied from below, through the wire mesh, to the plantar surface of the hindpaw. The application of a series of hair force is done to determine the paw withdrawal threshold. With this test, mechanical allodynia was observed in cisplatin-induced neuropathy (Chaplan et al., 1994; Takaishi et al., 1996; Authier et al., 2000; Authier et al., 2003; De Grandis et al., 2007; Cata et al., 2008; Joseph and Levine, 2009)

Method

The rat was placed on an elevated plastic mesh surrounded by a clear plastic cage. The Electron Von Frey Anesthesiometer IITC2390 (with calibrated bending forces range from 0.1 to 40 g) was used to stimulate the plantar surface of right hindpaw. The stimulation was started with the minimal hair force. Each stimulus was applied for approximately 1s with an interstimulus interval of at least 5 s (Fig. 11.). If hindpaw withdrawal did not occur, the next larger hair in the series was applied in a similar manner. When the hind paw was withdrawn from a particular hair at least four out of five applications, the force value of that hair in g was considered the withdrawal threshold. In the second and third testing sessions, if the withdrawal threshold did not match the withdrawal threshold of the previous testing session(s) in the same rat, the next larger hair in the series was tested. This was done until the withdrawal thresholds in three successive trials were the same (Takaishi et al, 1996)

Figure 11. Application of the Von Frey filament at the rat hindpaw.

4.3 Neurophysiological assessment :motor nerve conduction velocity test (same as 4.2 in Study 1)

5. SACRIFICE

5.1 Perfusion

Under halothane anesthesia, the twenty nine rats (C=13; P=16) underwent intracardiac perfusion with normal saline 200 ml for 8 minutes followed by 4% paraformaldehyde (PFA) 400 ml for 15 minutes. The sciatic nerve and L4, L5 DRG from both right and left sides were obtained for morphometric and immunohistochemistry studies.

5.2 Fresh removal

The sixty rats (C=30; P=30) underwent overdose anesthesia with halothane and then the chest wall was opened for removing the blood from the right ventricle. Then, the right atrium was cut. This was done to ensure the death. For Western blot study, the sciatic nerve and the DRG (L4-L6) of both sides were removed immediately.

6. MORPHOMETRY OF SCIATIC NERVE AND DRG

6.1 Tissue collection and sample preparation (same as 6.1 in Study 1)

6.2 Morphometric assessment of sciatic nerve (same as 6.2 in Study 1)

6.3 Morphometric assessment of DRG (same as 6.3 in Study 1)

7. WESTERN BLOT ANALYSIS

7.1 Tissue collection and sample preparation

After fresh removal, the specimens were kept in the plastic tube and put immediately onto the dry ice. For the long term preservation, the specimens were kept at -70°C until use. The samples kept at -70°C were homogenized in homogenization buffer. The homogenization buffer was composed of 0.1 mmol/l PIPES pH6.9, 5 mmol/l magnesium chloride, 5mmol/l EGTA, 0.5% Triston X-100, 20%glycerol,10 mmol/l sodium fluoride plus 1mmol/l PMSF, 2 mmol/l sodium orthovanadate and protease inhibitor cocktail. Then, the sample buffer (Trizma HCl pH 6.8, bromphenol blue sodium salt, glycerol, 99% DL-Dithiothreitol, SDS and B-mercapo) was added to each sample.

7.2 Western blot analysis

Bramhall protein assay

This technique employs the Coomassie Blue which binds to the protein in an acidic medium. The amount of dye bound to the sample is dependent to the amount of protein. So, the protein concentration in the sample is determined by comparing the amount of the dye bound to the sample and that bound to the standards. The concentration of protein in each sample is used to calculate the volume loaded to the gel.

Gel electrophoresis

Gel electrophoresis is a technique used to separate proteins according to molecular weight and electric charge**.** SDS-PAGE (SDS polyacrylamide gel electrophoresis) was performed on 30-50 µg protein in acrylamide (30% Acrylamide/Bis solution 37.5:1) gel and the separated proteins were transferred to nitrocellulose membrane using semi-dye electroblotter.

Immunological detection

The membrane was incubated in the primary antibodies to total [(anti-T-ERK (1:5000, Santa Cruz),anti-T-p38 (1:1000, Cell signaling), or anti-T-JNK(1:500, Santa Cruz)] or phosphospecific MAPKs [(anti-P-ERK (1:500, Santa Cruz),anti-P-p38 (1:500, Cell Signaling Technology), or anti-P-JNK(1:200, Santa Cruz)]. The

membrane was washed 5 min in 0.5% casein solution buffer (wash buffer) and incubated in a solution of biotinylated secondary antibodies (Biotinylated Goat Anti-Rabbit IgG antibody 1:150, Vector) for 30 minutes. The membrane was washed again with 5 min wash buffer. AB complex (Vectastain ABC kit standard 1:1,000, Vector) was applied to the membrane for 30 min. The membrane was washed again with 5 min wash buffer. Then, DAB substrate (DAB Peroxidase Substrate kit, vector) was applied on the membrane until dark-brown color developed. Then, the membrane was rinsed with tap water for 5 minutes and left until dry.

Densitometric analysis

The membrane was scanned with the scanner and the images of bands were imported to a microcomputer in tiff format. The densities of specific bands were analyzed using the Image Pro Plus software. Finally, the ratio of phosphorylated to total (P/T) MAPKs was calculated to represent the activities of MAPKs. The data were normalized such that mean control values were equal to 1.

8. STATISTICAL ANALYSIS

The data of experimental groups were compared using Student's unpaired t-test in case of normal distribution. Otherwise, the Wilcoxon Mann-Whitney non-parametric test was used. Significance level was set at $p<0.001$, $p<0.01$ and $p<0.05$.

9. IMMUNOHISTOCHEMISTRY OF DRG AND SCIATIC NERVE

Introduction to immunohistochemistry study

Immunohistochemistry is used to localize specific proteins in tissue sections. The 1° antibody will be applied in the section and followed by the 2° antibody tagged with the enzyme or fluorescent dye. The signal can be amplified with application of Avidin-Biotin complex (ABC).

9.1 Tissue collection and sample preparation

After intracardiac perfusion, L4-L5 DRG were collected and fixed in 10% formalin overnight. The specimens were dehydrated with 70%, 80%, 95%, and 100% alcohol and infiltrated with paraplast for paraffin sections. The sections were cut at 5μm and then deparaffinized and dehydrated through xylenes and graded alcohol series. Immunofluorescence study of ERK, JNK and p38 was done.

The sciatic nerve was stretched on one side by the pendulum and then suspended in 4% PFA overnight. After that, sciatic nerve was fixed in 30% sucrose. Sciatic nerves were then divided into 2 mm segments and embedded in OCT medium. The longitudinal sections were cut at 10μm using the cryostat. Immunofluorescence study of ERK,p38 and JNK was done.

9.2 Immunohistochemistry of DRG

The antigen retrieval technique was used. In brief, the sections were boiled in citrate buffer pH 6.0 (citric acid monohydrate 2.1g in 1 l distilled water) using the pressure cooker for 2 minutes. The sections were then rinsed in tap water for 5 minutes. Incubation of sections for 20 minutes with diluted 10% normal goat serum was done for the next step. The sections were left 48 hr at 4°C in anti-phospho-MAPK antibodies (anti-P-ERK (1:50, Santa Cruz), anti-P-JNK (1:100, Santa Cruz) and anti-P-p38 (1:100, Cell Signaling Technology)). This step was followed by incubation with the biotinylated secondary antibodies (Biotinylated Goat Anti-Rabbit IgG antibody 1:200, Vector) for 45 min at room temperature. Then, avidin-conjugated fluorescein isothiocyanate (FITC) (anti-rabbit-FITC 1:200, Santa Cruz Biotechnology) was added for 40 min. The slides were then mounted with antifading mounting medium (Vectashield, Vector Laboratories), coverslipped and examined under the fluorescence microscope.

9.3 Immunohistochemiatry of sciatic nerve

The nerve sections were blocked with diluted 10% normal horse serum for 20 min. Anti-S-100 (1:100, Chemicon) antibody was incubated overnight at 4°C followed by biotinylated secondary antibody and avidin conjugated with rhodamine (1:200, Santa Cruz Biotechnology) to locate Schwann cells. Then, the slides were incubated in the primary antibody to P-ERK, P-p38 or P-JNK for 48 hours at 4°C. After washing, the slides were incubated in the secondary antibody for 45 min. Then, avidin-conjugated FITC was added for 40 min. The slides were then mounted with antifading mounting medium, coverslipped and examined under the fluorescence microscope.

CHAPTER IV

RESULTS

Study 1: Characterization of the animal model of cisplatin-induced neuropathy

1. ASSESSMENT OF GENERAL TOXICITY

The mean body weight (BW) of the control and treated groups in each sex was similar at the beginning with that of males higher than that of females (Fig. 12). Two week after the start of cisplatin administration, the BW of the cisplatin-treated groups became significantly lower than that of the control groups in both sexes ($p < 0.001$). This decrease was increasingly prominent at later time points with higher severity in males. At the end of the experiment, the average weight loss of males and females was 41% and 23%, respectively. It is worth noting that four rats in the male+cisplatin group (MC), but none in the female+cisplatin group (FC), died in the 5th week.

Table 1. Average body weight of rats throughout the experiment

	BW (g)			
	MN	**MC**	**FN**	**FC**
week 0	231.67 ± 1.64	230.93 ± 2.40	182.87 ± 1.88	178.47 ± 4.72
week 1	268.40 ± 2.52	263.20 ± 2.28	201.20 ± 1.73	196.13 ± 1.86
week 2	304.27 ± 3.67	247.33 ± 4.63[a]	216.53 ± 1.80	192.60 ± 2.19[a]
week 3	322.13 ± 4.40	243.07 ± 6.47[a]	228.27 ± 2.54	197.27 ± 2.76[a]
week 4	338.93 ± 5.34	227.87 ± 7.99[a]	231.87 ± 2.64	192.60 ± 4.68[a]
week 5	359.87 ± 6.17	209.43 ± 7.77[a]	241.80 ± 3.22	185.93 ± 6.00[a]

Data are means ± SEM, [a] $p < 0.001$ MC vs. MN and FC vs. FN, MC=male+cisplatin group; MN=male + NSS group; FC=female+cisplatin group; FN=female+NSS group

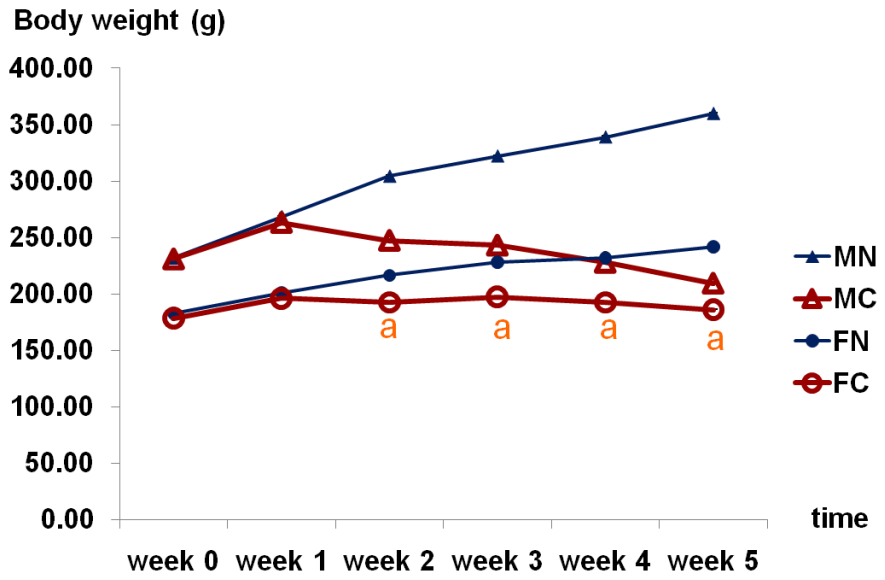


Figure. 12 Changes in the average body weight of rats in each group. Data are means ± SEM, [a] $p < 0.001$ MC vs. MN and FC vs. FN, MC=male+cisplatin group; MN=male + NSS group; FC=female+cisplatin group; FN=female+cisplatin +NSS

2. EVALUATION OF NEUROPATHY INDUCED BY CISPLATIN

2.1 Assessment of hindpaw thermal perception using hot plate test

The average heat latencies were not significantly different among the groups at baseline (Fig. 13). The latencies started to be significantly prolonged in the cisplatin-treated groups since the second week with the higher difference in males ($p < 0.01$) than in females ($p < 0.05$). The maximal change was observed in the fifth week with the latencies over the cut-off value (35 s) in all rats in the MC group and nine of 15 rats in the FC group. Therefore, 35 s was used as the representative latency of the MC group in the last week.

Table 2. Average withdrawal latency of rats throughout the experiment

	Withdrawal latency (s)			
	MN	MC	FN	FC
week 0	19.90 ± 1.43	19.81 ± 1.22	17.83 ± 1.28	22.29 ± 1.22
week 1	14.64 ± 1.13	16.75 ± 1.49	18.40 ± 1.54	20.60 ± 1.10
week 2	14.22 ± 1.30	20.50 ± 1.40#	18.58 ± 1.09	22.59 ± 1.53*
week 3	17.62 ± 1.14	20.28 ± 1.87	20.03 ± 1.51	23.69 ± 1.38*
week 4	17.25 ± 1.85	22.85 ± 1.08	17.73 ± 1.28	23.31 ± 0.06#
week 5	16.13 ± 1.08	35.00 ± 0.00@	19.44 ± 1.23	22.56 ± 0.55@

Data are means ± SEM, # $p < 0.01$ MC vs. MN, FC vs. FN, and * $p < 0.05$ FC vs. FN , @ All rats in the MC group and 9 of 15 rats in the FC group had heat latencies more than 35 s; therefore, 35 s was used for calculation of means

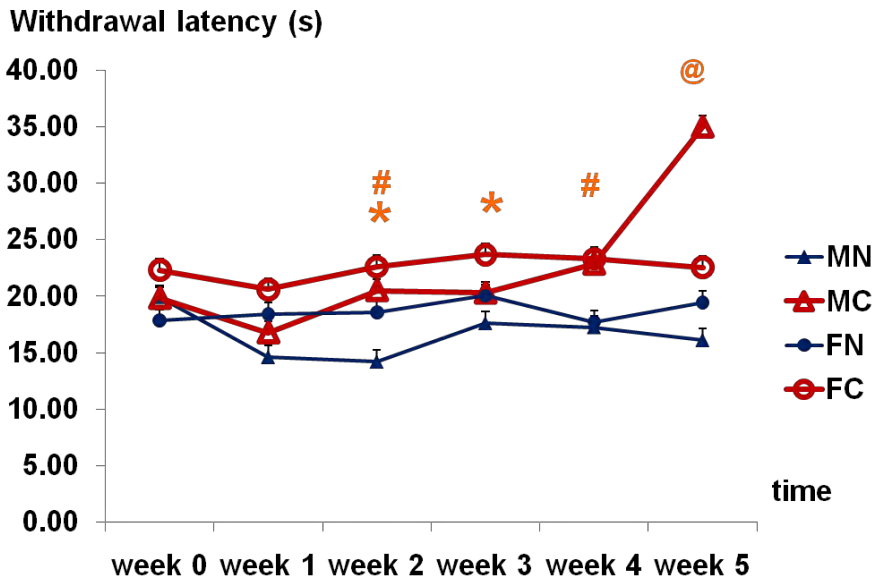


Figure 13. Changes in the heat withdrawal latency of rats in each group. Data are means ± SEM, # $p < 0.01$ MC vs. MN, # $p < 0.01$ FC vs. FN, and * $p < 0.05$ FC vs. FN , @ All rats in the MC group and 9 of 15 rats in the FC group had heat latencies more than 35 s; therefore, 35 s was used for calculation of means

2.2 Neurophysiological assessment: motor nerve conduction velocity test

The sciatic MNCV were similar among the groups at the beginning (Fig. 14). However, at the end of the study, decreased MNCV was found in the MC and FC groups compared with the corresponding controls. The change was more striking in males.

Table 3. Average MNCV of rats throughout the experiment

	MNCV (m/s)			
	MN	MC	FN	FC
week 0	48.53 ± 1.91	48.44 ± 2.65	49.94 ± 3.12	50.10 ± 3.34
week 5	69.74 ± 3.17	49.03 ± 2.63[a]	67.74 ± 2.63	57.01 ± 4.37[#]

Data are means ± SEM, [a] $p < 0.001$ MC vs. MN, # $p < 0.01$ FC vs. FN

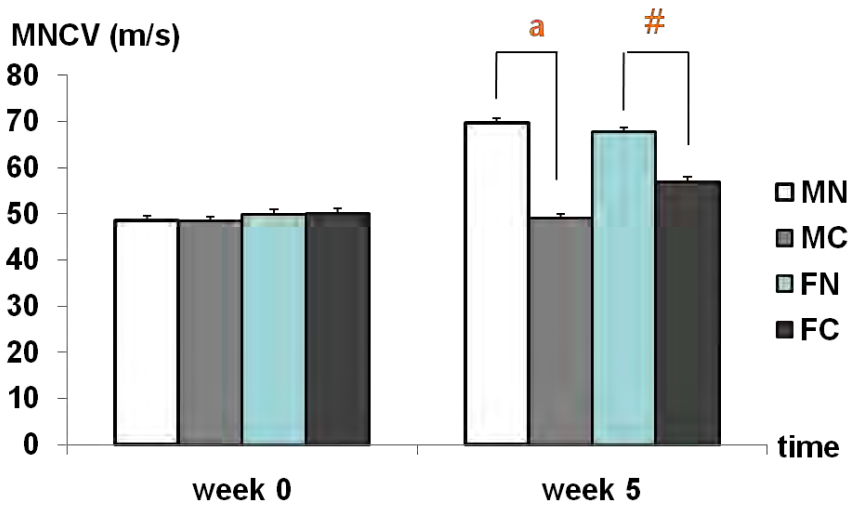


Figure 14. Sciatic motor nerve conduction velocity of rats in each group at baseline and in the 5th week. Data are means ± SEM, [a] $p < 0.001$ MC vs. MN, # $p < 0.01$ FC vs. FN

3. MORPHOMETRIC ASSESSMENT

3.1 Nerve morphometric study

The morphometric data of sciatic nerve are summarized in Table 1. The myelinated fiber and axon diameters were significantly decreased after five weeks of cisplatin treatment relative to the untreated controls in both sexes. The myelinated fiber density was significantly increased in the cisplatin-treated groups of both sexes. However, there was only a trend toward reduced myelin thickness in the cisplatin-

treated compared with the control groups with no significant change in the number of total fiber or g-ratio. Higher severity was observed in females in the reduced fiber and axon diameters (13.3% vs. 8.9% and 16.2% vs. 8.6%, respectively) but similar changes in the myelin thickness and fiber density when compared with males.

Table 4. Morphometric data of sciatic nerve

group	nerve fiber diameter (μm)	Axon diameter (μm)	myelin thickness (μm)	g-ratio	Fiber density (/mm^2)	total fiber
MN	6.62 ± 0.19	3.73 ± 0.10	1.45 ± 0.05	0.56	13,804.17 ± 800.90	8,815 ± 237
MC	6.03 ± 0.10*	3.41 ± 0.04*	1.31 ± 0.04	0.55	18,516.67 ± 465.40$^\#$	9,242 ± 205
FN	7.00 ± 0.11	4.21 ± 0.13	1.40 ± 0.04	0.59	13,657.41 ± 523.20	8,455 ± 1058
FC	6.07 ± 0.10^a	3.53 ± 0.06$^\#$	1.27 ± 0.03	0.57	18,310.18 ± 798.10$^\#$	7,578 ± 549

Data are means ± SEM * = $p < 0.05$, # = $p < 0.01$, a = $p < 0.001$ FC vs. FN or MC vs. MN

3.2 Morphometric study of DRG neurons

The morphometric data of DRG neurons were summarized in Table 5. The somatic and nucleolar areas were significantly difference in both sexes of cisplatin-treated group, but the nuclear area was significantly decreased in FC group compared with FN only in females. In the total number of neuron was not significant decrease in the degree of reduction in these parameters was seemed to be higher in males than in females.

Table 5. Morphometric data of DRG neurons

feature	Total number of neuron	somatic area (μm^2)	nuclear area (μm^2)	nucleolar area (μm^2)
MN	15,693 ± 1,602	1,258.9 ± 109.0	188.6 ± 28.3	14.1 ± 1.7
MC	11,966 ± 1,941	806.2 ± 68.3$^\#$	144.5 ± 8.8	7.9 ± 0.2*
FN	17,343 ± 1,692	1,142.8 ± 19.5	181.3 ± 1.9	13.4 ± 0.9
FC	13,474 ± 1,397	906.1 ± 35.9^a	151.4 ± 5.2^a	10.2 ± 0.4$^\#$

Data are means ± SEM, n = 8 per group, * = $p < 0.05$, # = $p < 0.01$, a = $p < 0.001$ FC vs. FN or MC vs. MN

Cisplatin treatment (2mg/kg twice a week for 5 weeks) was associated with general toxicity, prolonged heat latency, and slow MNCV, which were more severe in male rats. Four males died before the end of experiment. However, sex-related differences in the structural alterations in the myelinated fibers and DRG neurons were variable. This sexual dimorphism should be verified in clinical cisplatin associated neuropathy and its underlying mechanism remains to be clarified. Nevertheless, female rats were used in subsequent study due mainly to their better tolerability to the general toxicity of cisplatin.

Study 2 Determination of MAPK phosphorylation in DRG and sciatic nerve of rats with cisplatin-induced neuropathy

Part 1 Evaluation of cisplatin-induced neuropathy in rats in different time points

1. ASSESSMENT OF GENERAL TOXICITY

The body weight (BW) of cisplatin-treated groups was significantly lower than the control group from the 2nd week throughout the experiment ($p<0.01$) (Fig. 15). One rats in the cisplatin-treated group died after the last injection (5th week). Most cisplatin-treated rats showed a definite change in motor activity characterized by toe walking with arched back from the 4th week.

Table 6. Average body weight of rats throughout the experiment

	BW(g)	
	control	**cisplatin**
week 0	188.58 ± 1.37	191.64 ± 1.68
week 1	219.68 ± 1.36	221.96 ± 1.87
week 2	231.86 ± 1.66	226.28 ± 2.40#
week 3	240.63 ± 1.62	226.45 ± 2.76a
week 4	246.05 ± 1.82	220.35 ± 3.62a
week 5	253.00 ± 1.99	217.37 ± 4.30a
week 6	256.77 ± 1.93	204.37 ± 5.11a
week 7	262.90 ± 2.66	208.00 ± 6.78a
week 8	265.15 ± 2.53	219.80 ± 7.62a
week 10	251.66 ± 3.23	215.81 ± 11.27#
week 12	254.57 ± 3.54	214.66 ± 9.18a

Data are means ± SEM, a $p<0.001$, #$p<0.01$

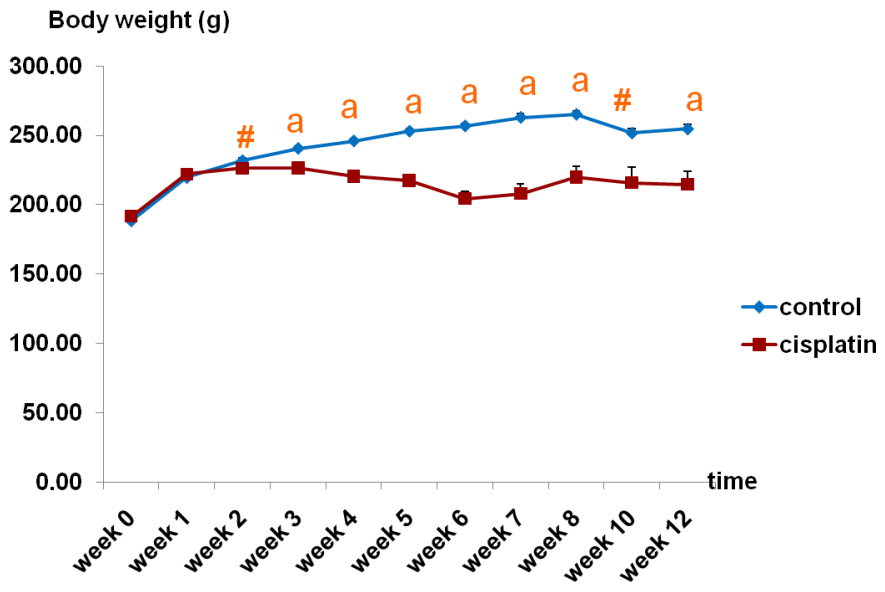


Figure 15. Average body weight of rats in various time points. Data are means ± SEM, [a] p<0.001, #p<0.01

1

2. EVALUATION OF NEUROPATHY INDUCED BY CISPLATIN

2.1 Assessment of hindpaw thermal perception using hot plate test

The baseline of the mean withdrawal latencies in both group were not significantly different (Fig. 16). Cisplatin-treated group showed a significant increase in withdrawal latency in the 5th week (p<0.001).

Table 7 Average withdrawal latency of rats throughout the experiment

	Withdrawal latency (s)	
	control	Cisplatin
week 0	14.58 ± 0.58	13.52 ± 0.45
week 1	13.90 ± 1.41	11.68 ± 0.99
week 3	11.70 ± 0.54	11.40 ± 0.43
week 5	10.51 ± 0.49	14.56 ± 0.81[a]
week 8	12.11 ± 0.69	10.49 ± 0.51
week 10	11.92 ± 0.73	18.66 ± 2.22
week 12	9.73 ± 0.60	11.81 ± 1.13

Data are means ± SEM. [a] p<0.001.

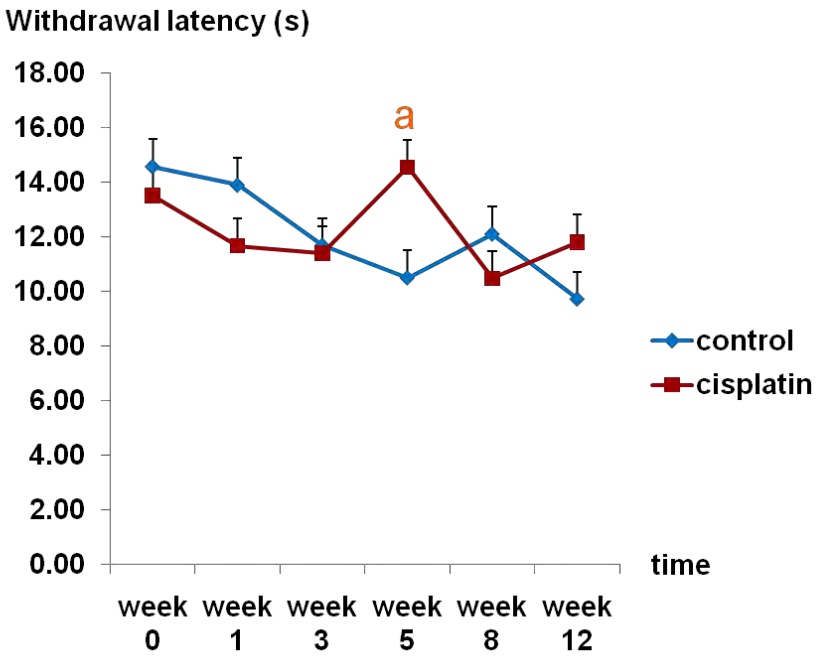


Figure 16. Average withdrawal latency of the hindpaw in various time points. Data are means ± SEM. [a] $p<0.001$.

2.2 Assessment of mechanical allodynia using Von Frey hair test

At baseline, mean withdrawal thresholds were not significantly different between the control and cisplatin-treated groups (Fig. 17). In the cisplatin-treated group, withdrawal thresholds were slightly decreased in the 3rd and 5th weeks. A maximum reduction in the withdrawal threshold of the cisplatin-treated group was noted in the 3rd week. Then, the thresholds of both groups were similar in the 8th and 12th weeks.

Table 8 Average withdrawal threshold of rats throughout the experiment

	Withdrawal threshold (g)	
	control	cisplatin
week 0	26.22 ± 1.88	29.38 ± 1.58
week 1	27.37 ± 6.47	31.75 ± 4.25
week 3	29.21 ± 2.14	25.90 ± 2.08
week 5	33.20 ± 2.30	29.98 ± 2.36
week 8	39.60 ± 0.36	40.00 ± 0.00
week 12	40.00 ± 0.00	40.00 ± 0.00

Data are means ± SEM.

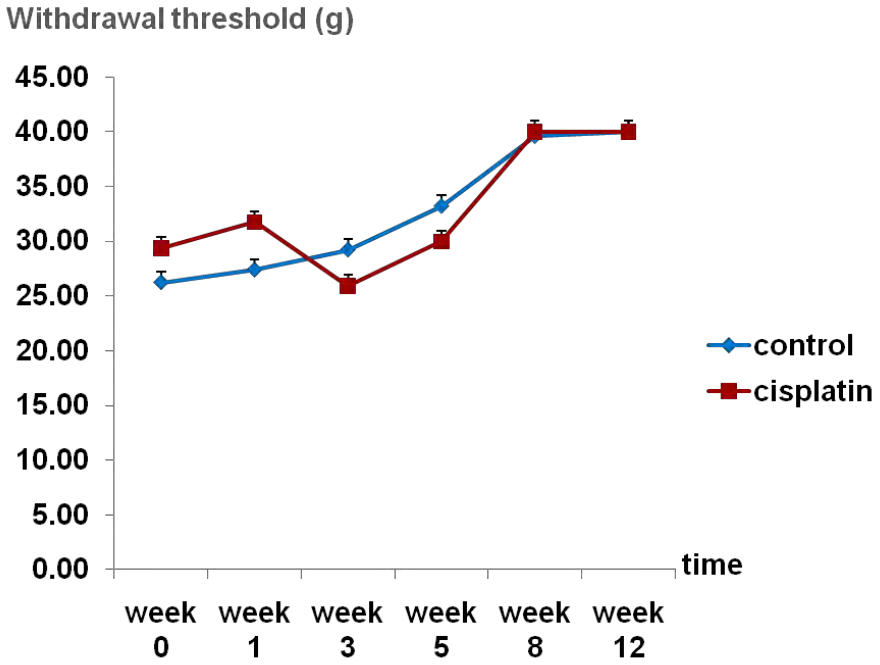


Figure 17. Diagram showing the mean withdrawal threshold of the hindpaw in various time points. Data are means ± SEM.

2.3 Neurophysiological assessment : nerve conduction velocity test

In the 5th and 8th weeks (Fig. 18), the cisplatin-treated group had a significant decrease ($p<0.05$) in the motor nerve conduction velocity (59.95±0.99 m/s and 59.97±1.35 m/s), compared to the control group (67.52±1.10 m/s and 70.65±1.68 m/s), respectively. The maximum change of the cisplatin-treated group was observed in the 12th week (60.72±2.38 m/s) compared with the control (70.48±1.43 m/s) ($p<0.01$).

Table 9 Average MNCV of rats throughout the experiment

	MNCV (m/s)	
	control	**cisplatin**
week 0	54.24 ± 0.87	53.81 ± 0.84
week 3	59.69 ± 0.84	58.79 ± 1.01
week 5	67.52 ± 1.10	59.95 ± 0.99*
week 8	70.65 ± 1.68	59.97 ± 1.35*
week 12	70.48 ± 1.43	60.72 ± 2.38#

Data are means ± SEM. #p<0.01, * p<0.05

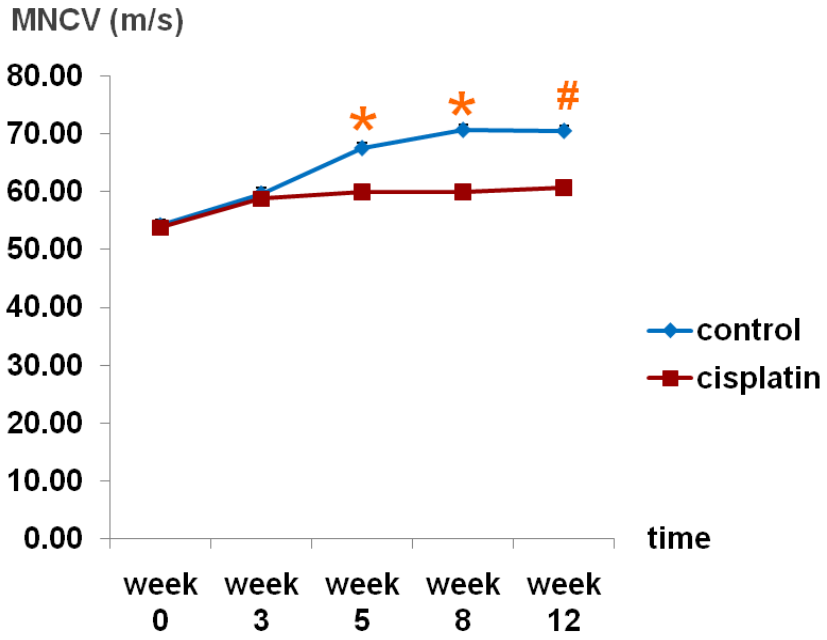


Figure 18. Diagram showing the motor nerve conduction velocity (MNCV) of the sciatic nerve in various time points. Data are means ± SEM. #p<0.01, * p<0.05

3. MORPHOMETRIC ASSESSMENT

3.1 Nerve morphometric study

In the 12th week, the reduction of mean nerve fiber diameter (6.31±0.07 μm) in cisplatin-treated group compared with the controls (3.73±0.14 μm) (p<0.05) was observed. However, in cisplatin-treated group, the axonal diameter (3.89±0.04) was increased in the 8th week compared with the control group (3.49±0.10) (p<0.01). The mean myelin thickness of cisplatin-treated group (1.29±0.020 μm and 1.31±0.17 μm)

was significantly decreased in both 8th and 12th week compare with the control (1.41±0.04 μm and 1.42±0.04 μm) ($p<0.05$), respectively. The g-ratio in cisplatin-treated group (0.60±0.01) was increased in the 8th week compared with the control group (0.55±0.00). The fiber density of cisplatin-treated group (17395.83±519.85 mm^2) was increased in the 12th week compared with the control group (15188.89±618.17 mm^2). No significant differences in the number of total fiber were found in the 8th and 12th weeks (Table 10).

Table 10 The morphometric study of the sciatic nerve in the 8th and 12th weeks.

feature	nerve fiber diameter (μm)	Axon diameter (μm)	myelin thickness (μm)	g-ratio	Fiber density (/mm^2)	total fiber
control week 8	6.30 ± 0.16	3.49 ± 0.10	1.41 ± 0.04	0.55 ± 0.00	16,680.6 ± 814.1	8,263 ± 194
cisplatin week 8	6.47 ± 0.07	3.89 ± 0.04#	1.29 ± 0.02*	0.60 ± 0.01*	15,500.0 ± 295.9	7,958 ± 103
control week 12	6.73 ± 0.14	3.90 ±0.07	1.42 ± 0.04	0.58 ± 0.00	15,188.9 ± 618.2	8,277 ± 279
cisplatin week 12	6.31 ± 0.07*	3.76 ± 0.04	1.31 ± 0.17*	0.58 ± 0.01	17,395.8 ± 519.9*	8,299 ± 194

Data are means ± SEM, * $p<0.05$, # $p<0.01$

Based on the percent distribution of the myelinated nerve fiber diameter (Fig. 19), the number of myelinated fibers larger than 10 μm was reduced by 1.39% in cisplatin-treated compared with the control groups in the 12th week. Accordingly, the graph was shifted to the left. However, the distribution was similar between the control and cisplatin-treated groups in the 8th week.

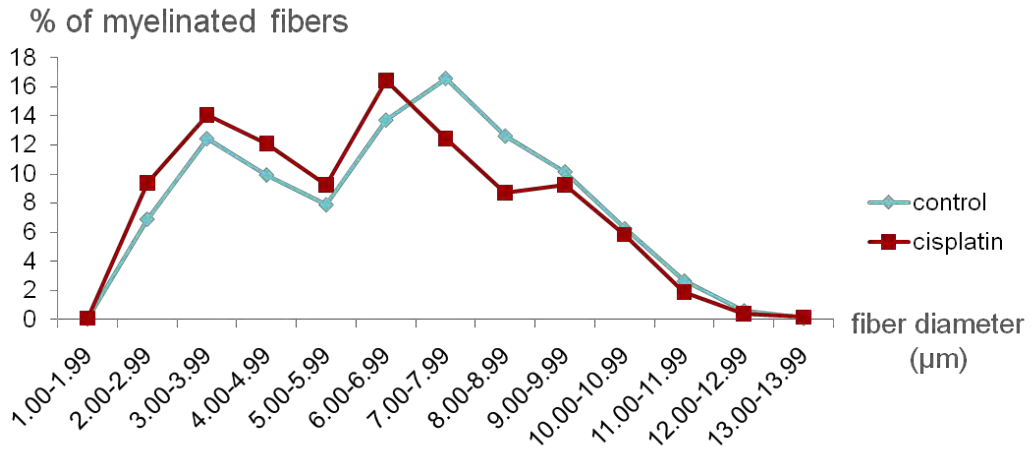


Figure 19. Histogram of the percent distribution of the myelinated nerve fiber diameter of the control and cisplatin groups in the 12th week.

3.2 Morphometric study of DRG neurons

In cisplatin-treated rats, the mean sizes of the soma, nucleus, and nucleolus were significantly decreased compared with those of controls (Table 4). There was a trend toward less number of DRG neurons in the cisplatin compared with control groups, but no significant difference was observed.

Table 11. The morphometric study of the DRG in the 8th and 12th weeks.

L4 DRG morphometric study				
feature	total number of neuron	somatic area (μm^2)	nuclear area (μm^2)	nucleolar area (μm^2)
control week 8	20,264 ± 1,598	1035.0 ± 30.0	162.3 ± 1.4	12.6 ± 0.1
cisplatin week 8	16,530 ± 1,696	911.0 ± 32.5*	148.3 ± 5.3*	10.5 ± 0.4[a]
control week 12	19,784 ± 1,870	1,115.8 ± 31.7	161.1 ± 3.0	14.9 ± 0.2
cisplatin week 12	16,345 ± 1,642	842.6 ± 39.3[a]	133.8 ± 5.1[#]	10.9 ± 0.9[#]

Data are means ± SEM, [a] $p<0.001$, [#] $p<0.01$, * $p<0.05$

Part 2 MAPK phosphorylation in DRG and sciatic nerve of rats with cisplatin-induced neuropathy

1. MAPK phosphorylation in DRG

Phosphorylation of MAPKs was examined using Western blot analysis in various time points (1st, 3rd, 5th, 8th and 12th weeks) (Fig. 20-22).

The ERK phosphorylation was not significantly different in the control and cisplatin groups in any time points. In the 5th weeks, cisplatin group had trend toward down-regulation of ERK compare with the control group. In contrast, the cisplatin group had a trend toward increase in ERK phosphorylation in the 12th week (Fig. 20). ERK expression (total ERK) was significantly increased in the cisplatin compared with control groups only in the 1st week ($p<0.05$).

A significant decrease in p38 phosphorylation was seen in the 1st week in the cisplatin compared with the control groups ($p<0.01$) (Fig. 21). There was no significant difference in the 3rd , 5th ,8th and 12th weeks. p38 expression (total p38) was significantly increased in the cisplatin compared with the control groups only in the 1st week ($p<0.05$).

In the cisplatin group, the immunoblots JNK phosphorylation showed the upregulated in the 1st week (Fig. 22). No significant difference of JNK phosphorylation in the 3rd, 5th, 8th and 12th weeks. No significant differences in the expression of JNK (total JNK) were found in any time points.

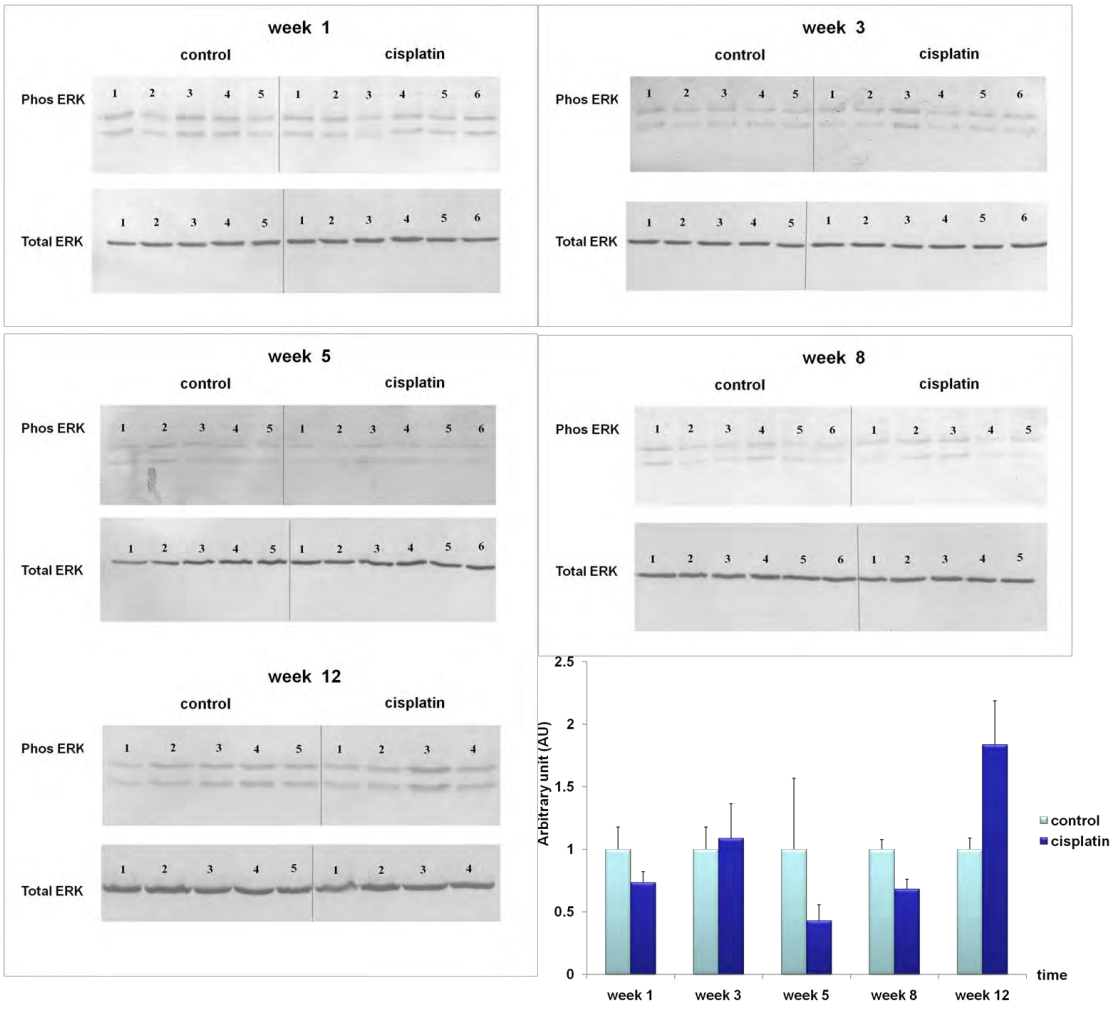


Figure 20. Phosphorylation of ERK in DRG in the control and cisplatin groups in various time points. The immunoblots show the bands of phosphorylated ERK (phos ERK) and total ERK. The graph shows the average ratio of phos ERK to total ERK with the error bars representing SEM.

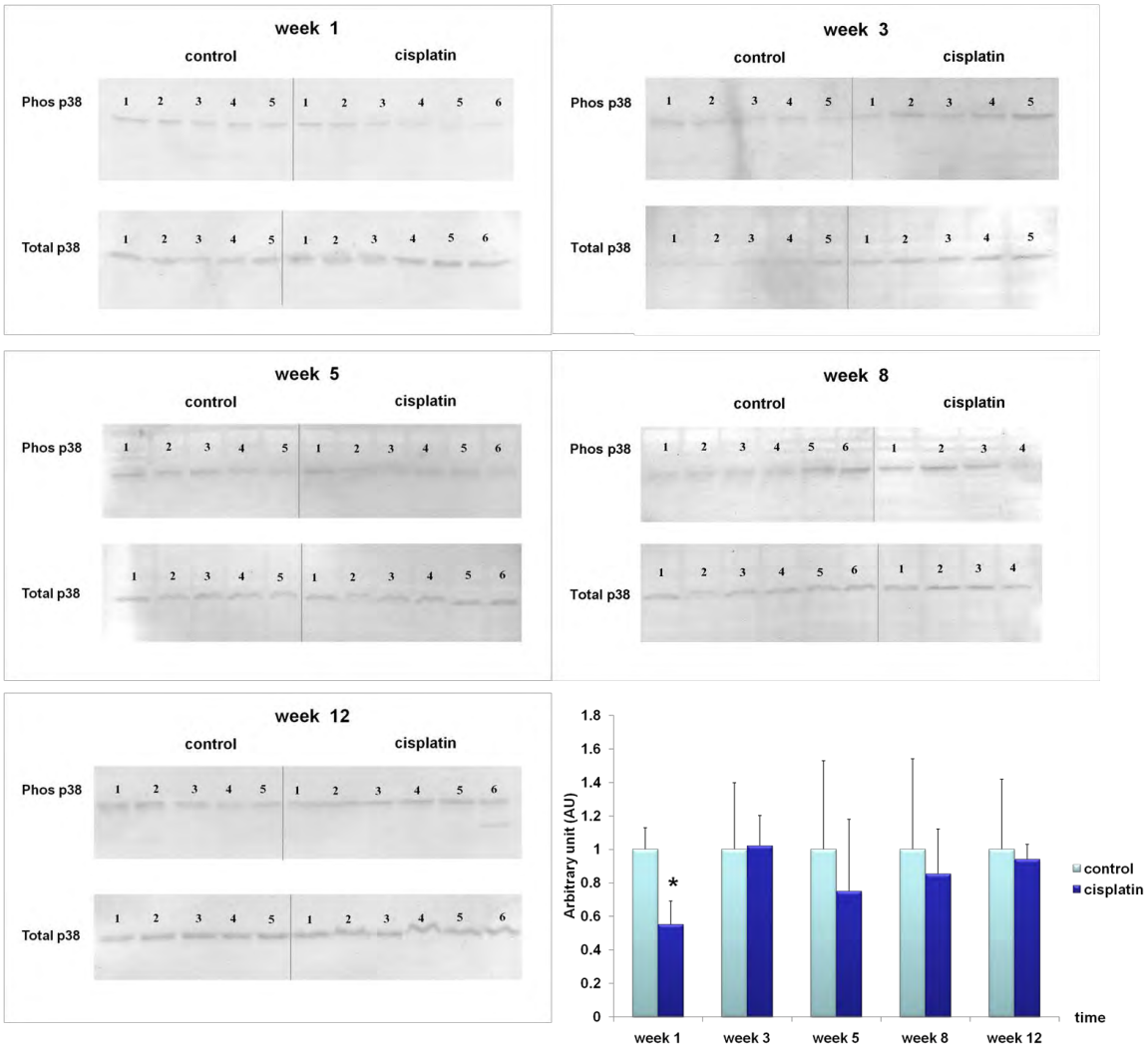


Figure 21. Phosphorylation of p38 in DRG in the control and cisplatin groups in various time points. The immunoblots show the bands of phosphorylated p38 (phos p38) and total p38. The graph shows the average ratio of phos p38 to total p38 with the error bars representing SEM.*$p<0.05$

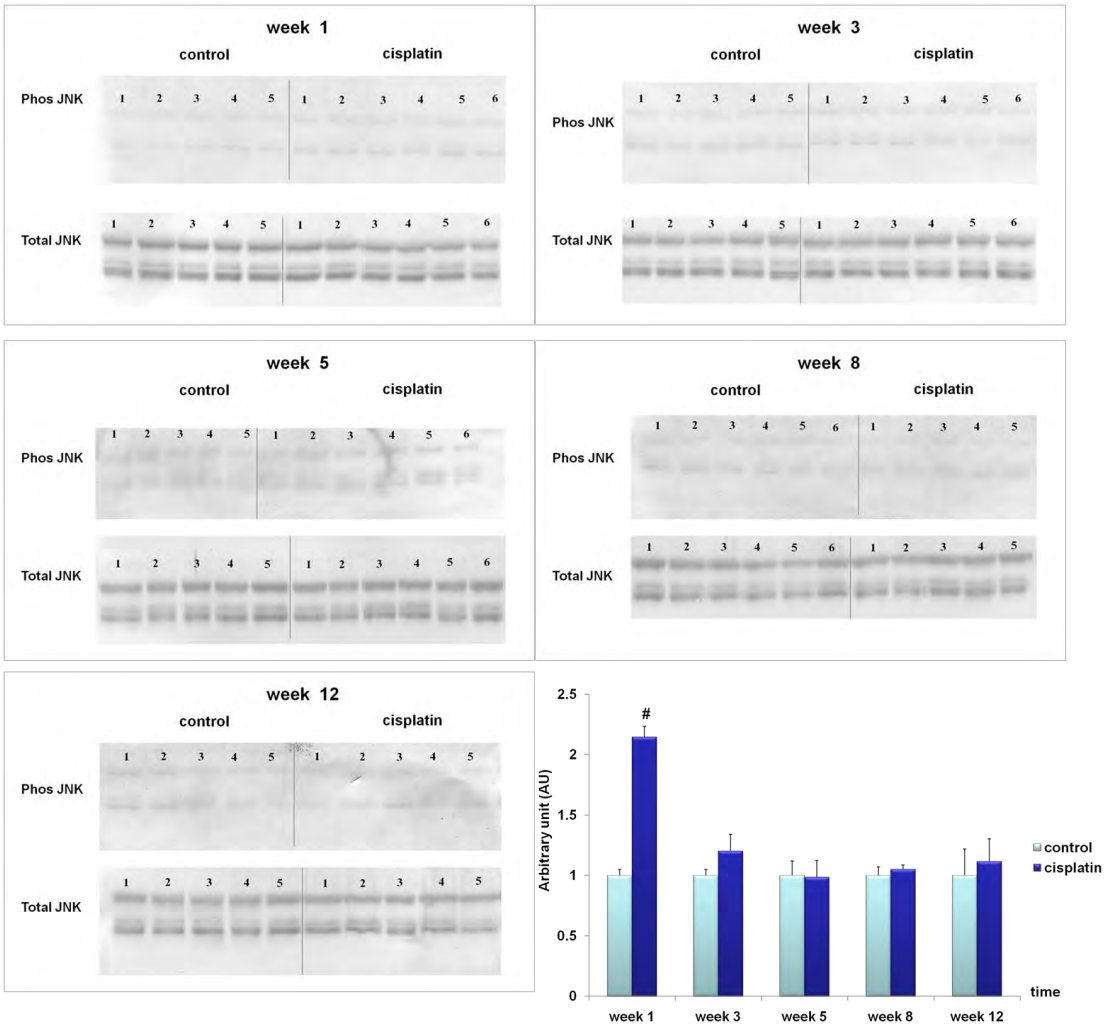


Figure 22. Phosphorylation of JNK in DRG in the control and cisplatin groups in various time points. The immunoblots show the bands of phosphorylated JNK (phos JNK) and total JNK. The graph shows the average ratio of phos JNK to total JNK with the error bars representing SEM. # $p<0.01$

2. MAPK phorylation in sciatic nerve

Phosphorylation of MAPKs was examined using Western blot analysis in various time points (the 1st and 8th weeks) (Fig. 23-25). MAPK phosphorylation was not significantly different in the control and cisplatin groups in the 3rd , 5th 8th and 12th weeks in DRG.

In the 1st and 8th weeks, the ERK phosphorylation was significantly up-regulated in cisplatin groups ($p<0.05$) (Fig. 23). Total ERK was not significantly different in any time points.

A significant increase in p38 phosphorylation was seen only in the 8th week in cisplatin compared with control groups ($p<0.05$) (Fig. 24). Total p38 was not significantly different in any time points.

In cisplatin group, JNK phosphorylation had a significant increase in the 1st week compared with the control group ($p<0.01$). Additionally, no significant differences in the expression of JNK (total JNK) were found in any time points.

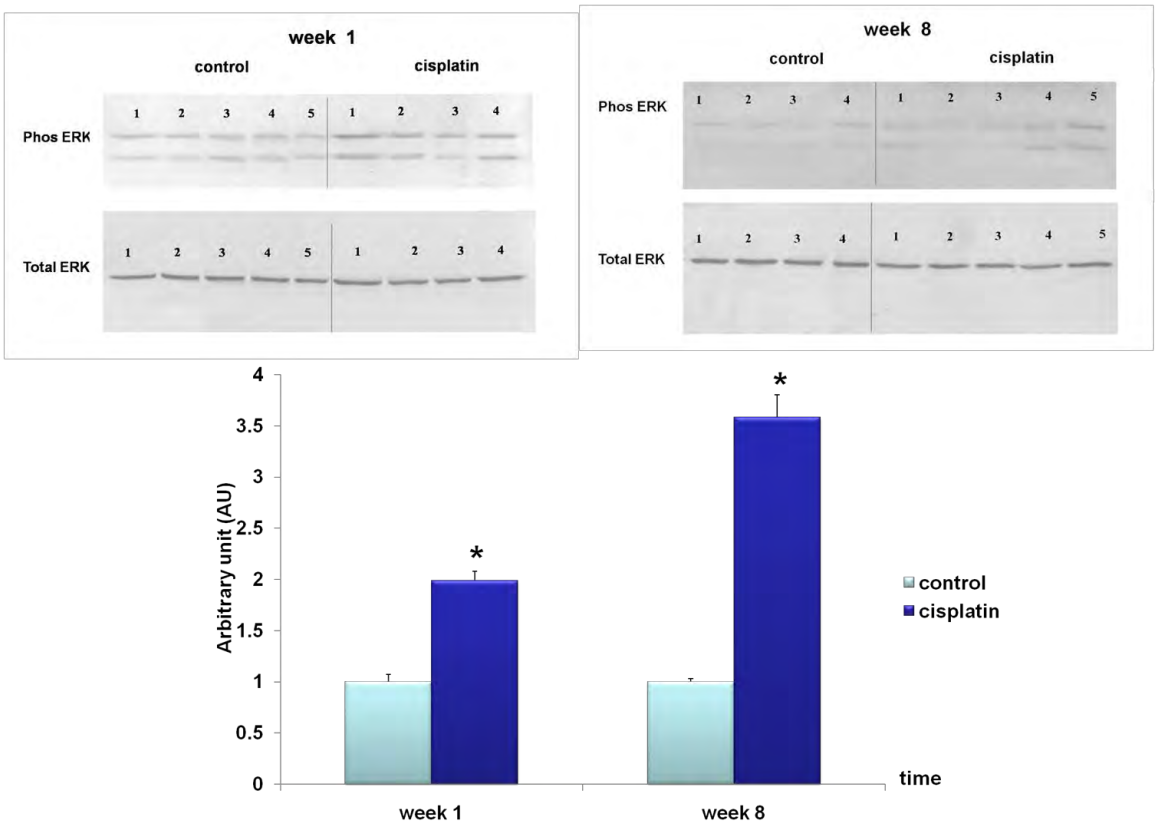


Figure 23. Phosphorylation of ERK in sciatic nerve in the control and cisplatin groups in various time points. The graph shows the average ratio of phos ERK and total ERK with the error bars representing SEM. *$p<0.05$

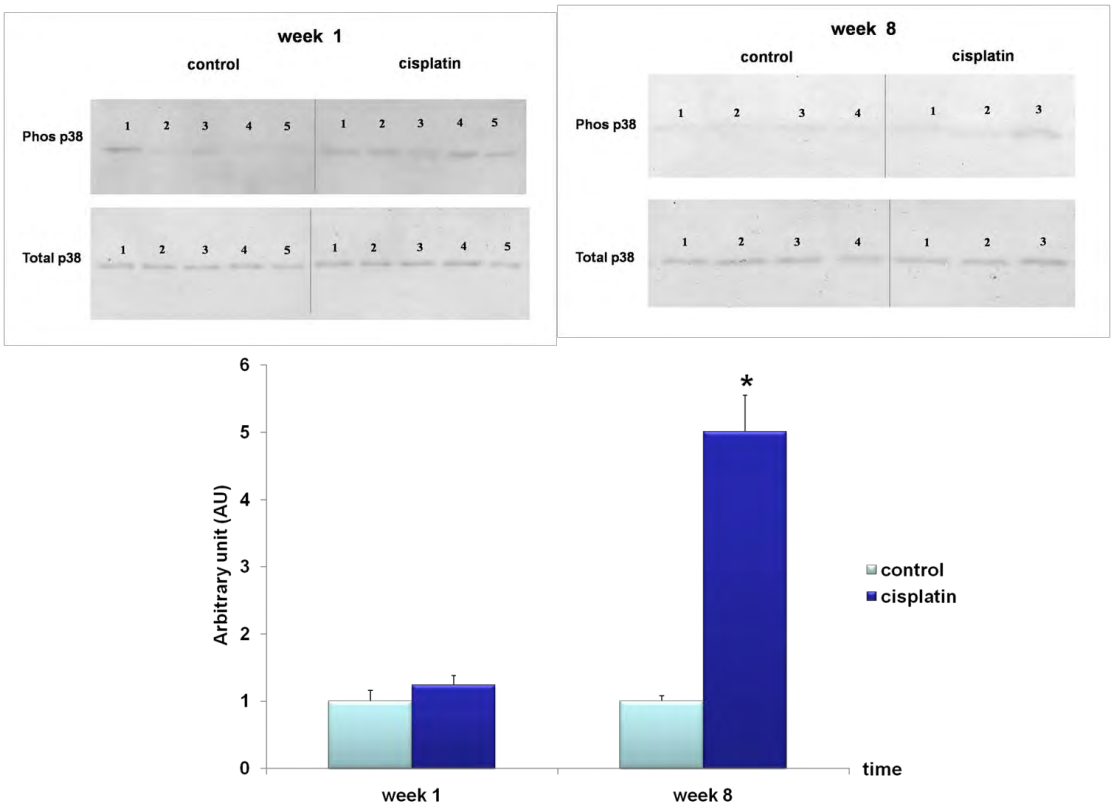


Figure 24. Phosphorylation of p38 in sciatic nerve in the control and cisplatin groups in various time points. The graph shows the average ratio of phos p38 and total p38 with the error bars representing SEM. *p<0.05

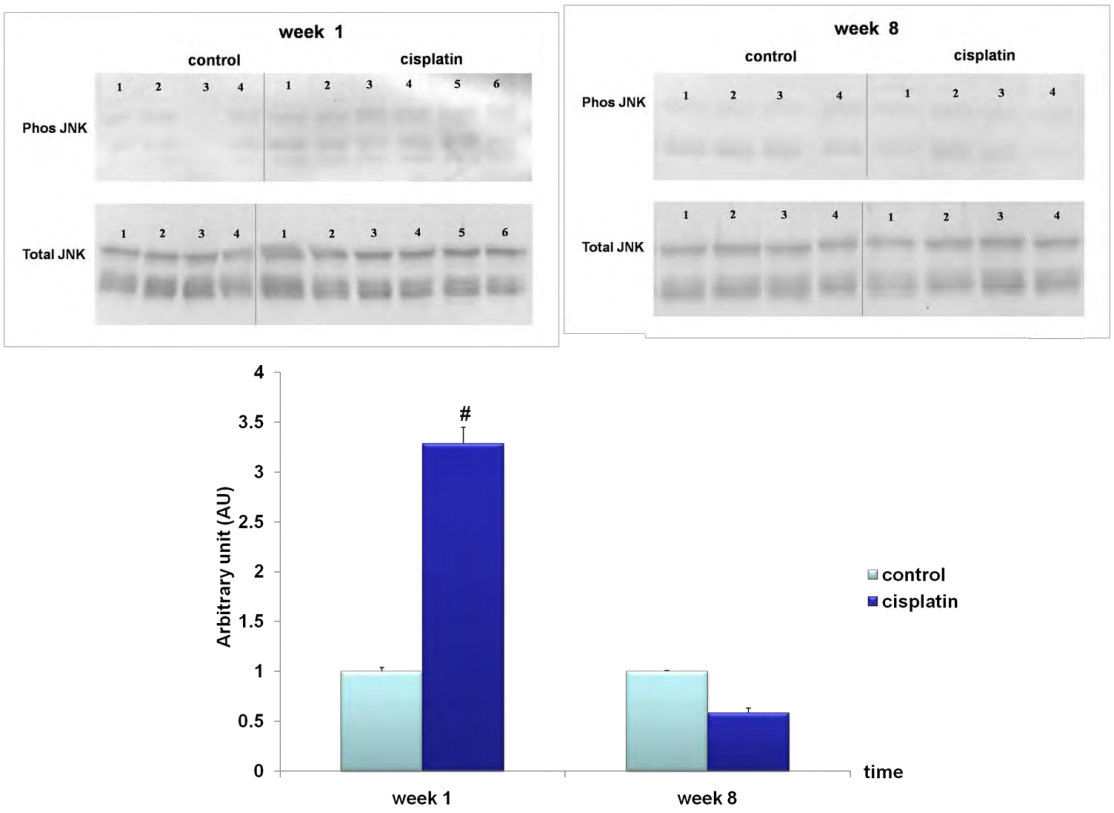


Figure 25. Phosphorylation of JNK in sciatic nerve in the control and cisplatin groups in various time points. The graph shows the average ratio of phos JNK and total JNK with the error bars representing SEM. # $p<0.01$

3. Immunohistochemistry study

3.1 Immunohistochemistry of MAPKs in DRG

Immunoreactivities of P-JNK, P-p38 and P-ERK were examined in cisplatin groups in the 1st, 8th and 12th week (Fig. 26). MAPK phosphorylation was not significantly different in the control and cisplatin groups in the 3rd and the 5th weeks. The P-JNK and P-p38 were located in cytoplasm of small DRG neurons (arrow heads) and nuclei of large DRG neurons (arrows) in the 1st and 8th weeks. P-ERK was expressed in both satellite cells (blank arrows), nuclei of large DRG neuron and cytoplasm of small DRG neurons in the 12th week.

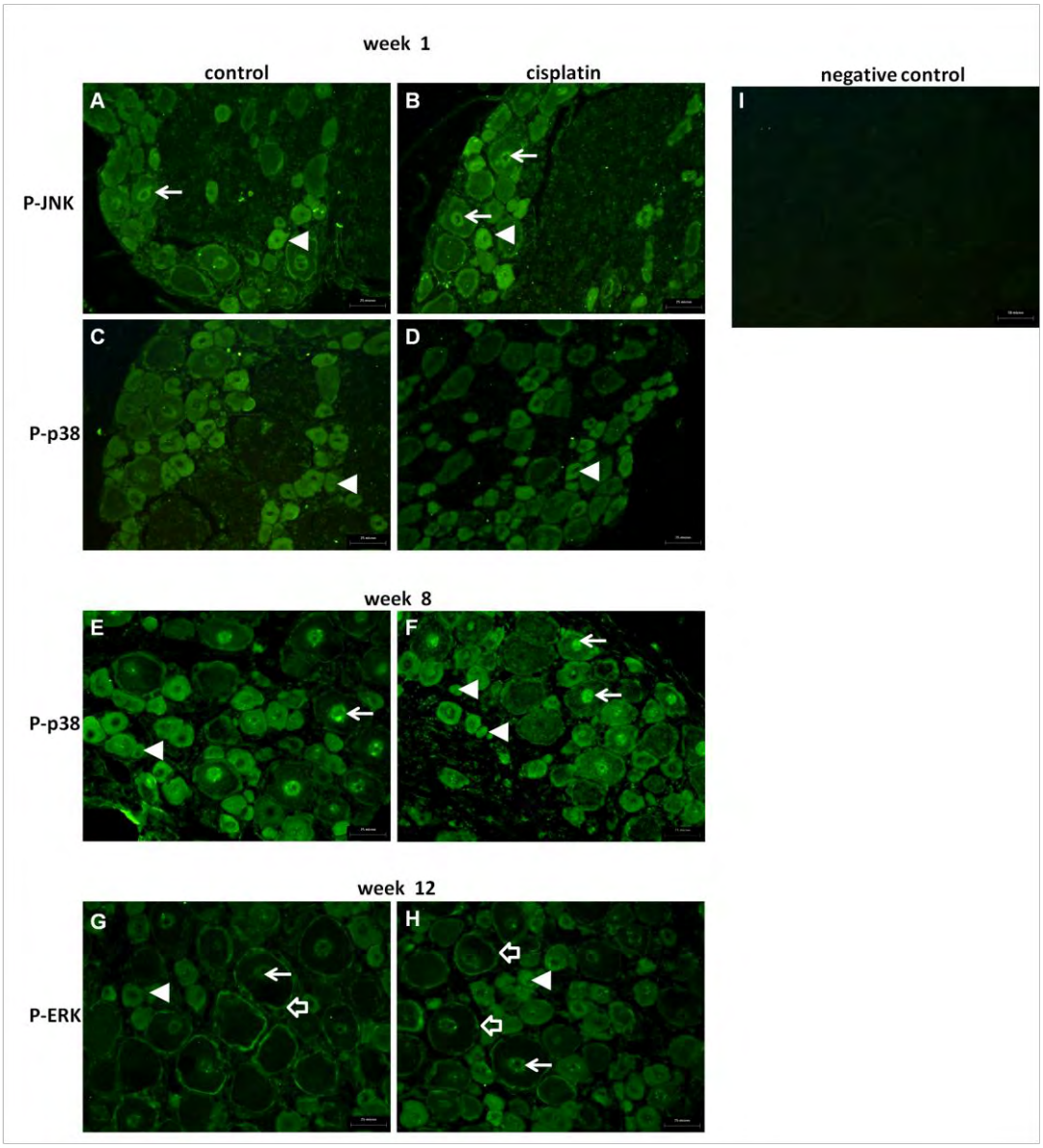


Figure 26. Immunoreactivity of MAPKs in DRG from control and cisplatin groups in the 1st, 8th and 12th weeks. The negative control in which the section was exposed to secondary antibody conjugated with FITC without primary antibody is shown in I. The P-JNK and P-p38 were located in cytoplasm of small DRG neurons (arrow heads) and nuclei of the large DRG neuron (arrows). P-ERK was expressed in both Satellite cells (blank arrows), nuclei of large DRG neuron and cytoplasm of small DRG neurons. Scale bars represent 25 μm.

3.2 Immunohistochemistry study of the sciatic nerve

Longitudinal sections of the sciatic nerve in the 1st week are shown in Figure 27. P-JNK and P-ERK were located in both axons and Schwann cells (co-localization of MAPKs and S100). Similarly, in the 8th week, P-ERK and P-p38 were also localized in both axons and Schwann cells (Fig. 28).

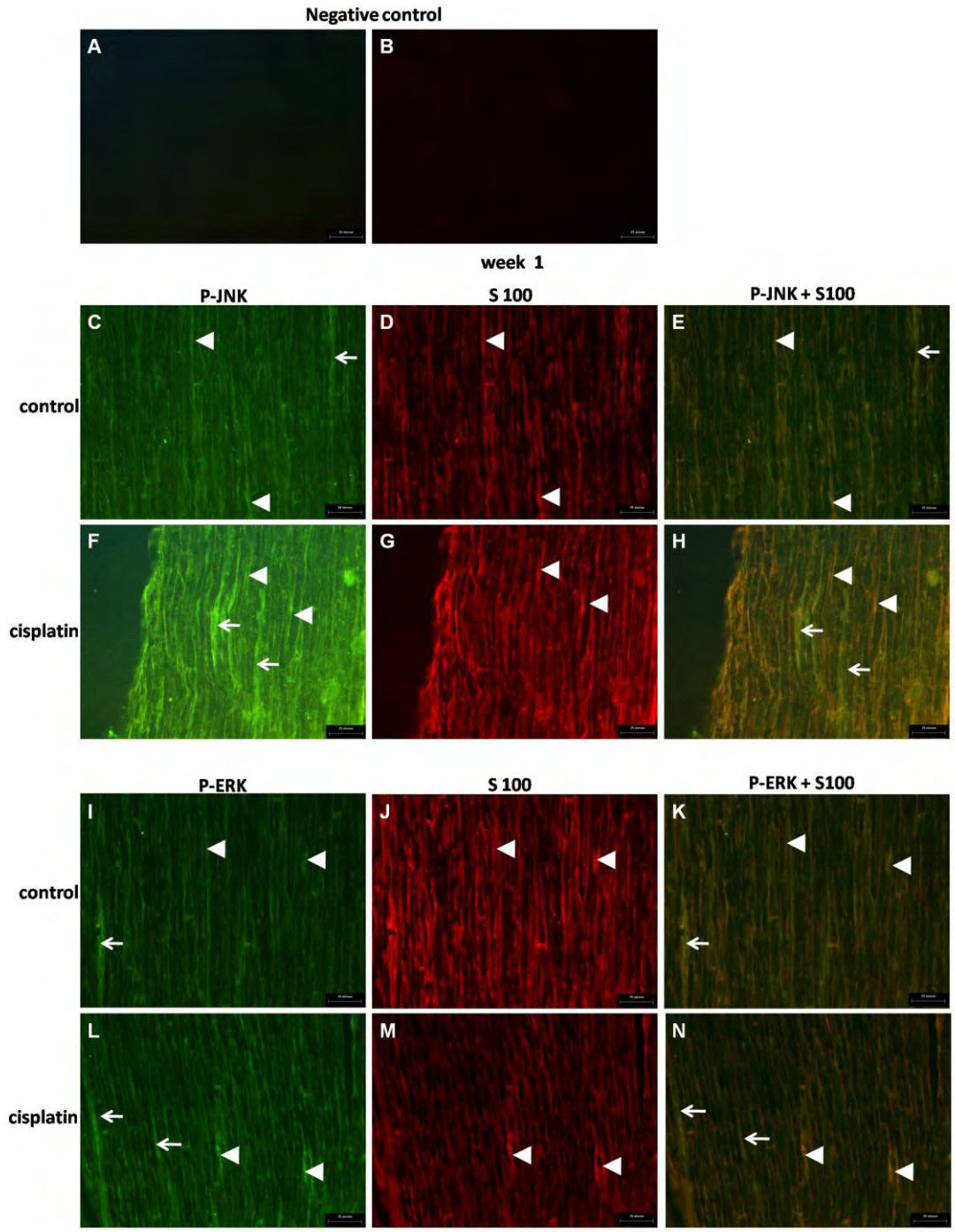


Figure 27. Immunoreactivity of MAPKs and S100 in sciatic nerve from control and cisplatin groups in the 1st week. Longitudinal sections of sciatic nerve are shown. JNK-P and ERK-P are shown in the 1st column. S100 is shown in the 2nd column. The co-localization of MAPKs and S100 is shown in the 3rd column. The negative control in which sections were exposed to secondary antibody conjugated with FITC or rhodamine without primary antibodies is shown in A and B, respectively. The P-JNK and P-ERK were located in both Schwann cells (arrows head) and axons (arrows). Co-localization was also determined (open arrows). Scale bars represent 25 μm.

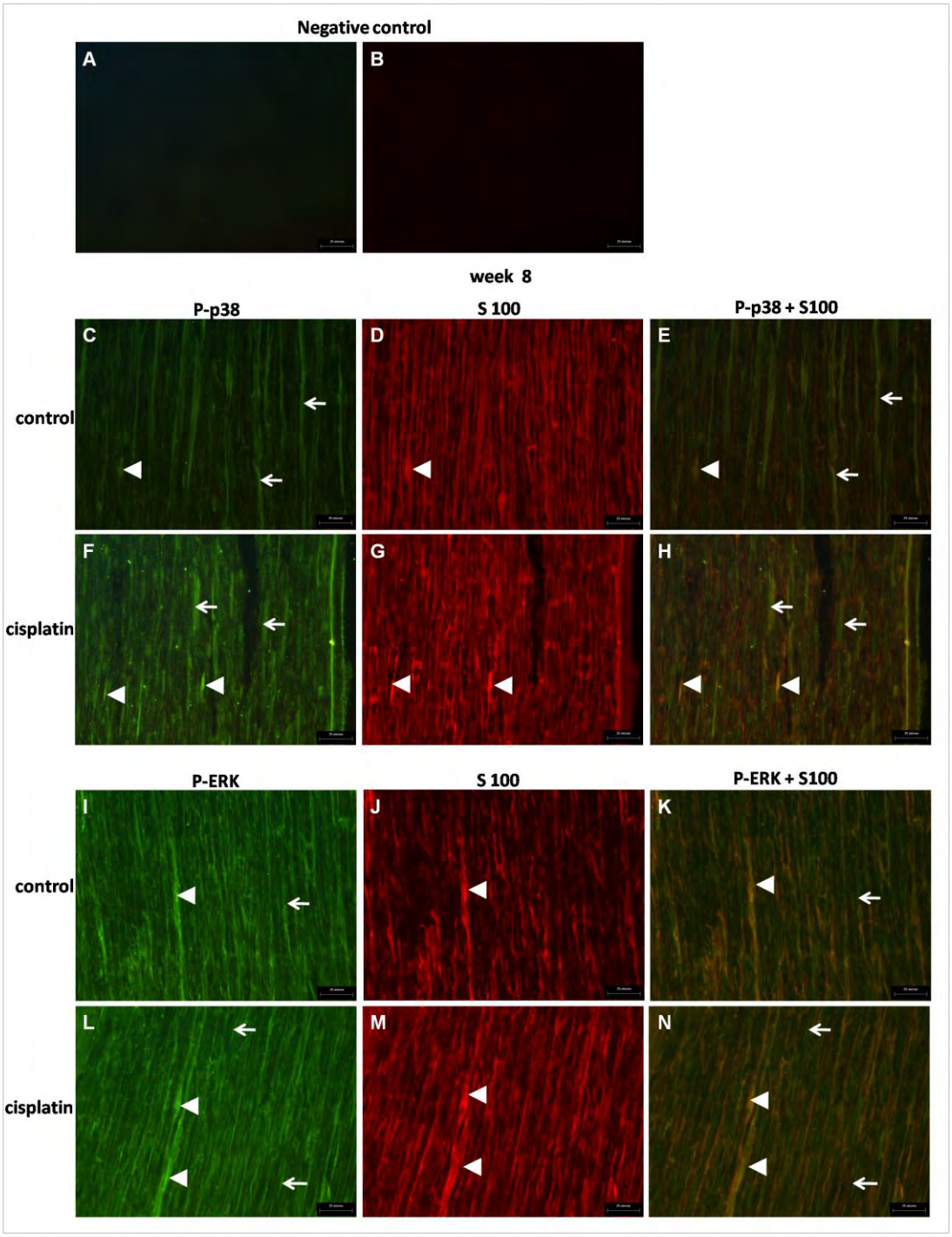


Figure 28. Immunoreactivity of MAPKs and S100 in sciatic nerve from control and cisplatin groups in the 8th week. Longitudinal sections of sciatic nerve are shown. P38 and ERK-P are shown in the 1st column. S100 is shown in the 2nd column. The co-localization of MAPKs and S100 is shown in the 3rd column. The negative control in which sections were exposed to secondary antibody conjugated with FITC or rhodamine without primary antibodies is shown in A and B, respectively. The P-p38 and P-ERK were located in both Schwann cells (arrows head) and axons (arrows). Co-localization was also determined (open arrows). Scale bars represent 25 µm.

The results of both part 1 and 2 of Study 2 were summarized in Table 12

Table 12 The summary of results from morphometric and western blot studies of DRG and sciatic nerve in Study 2

	week 1	week 3	week 5	week 8	week 12
general toxicity					
mortality	-	-	-	1	-
body weight	-	↓	↓	↓	↓
functional test					
hot plate test	↔	↔	hypoalgesia	↔	↔
Von Frey hair test	↔	allodynia	allodynia	↔	↔
MNCV	↔	↔	↓	↓	↓
morphometric study of DRG					
total number of neuron	-	-	-	⇣	⇣
somatic area	-	-	-	↓	↓
nuclear area	-	-	-	↓	↓
nucleolar area	-	-	-	↓	↓
morphometric study of sciatic nerve					
nerve fiber diameter	-	-	-	↔	↓
axon diameter	-	-	-	↑	⇣
myelin thickness	-	-	-	↓	↓
g-ratio	-	-	-	↑	↔
fiber density	-	-	-	↔	↑
total number of fiber	-	-	-	↔	↔
MAPKs in DRG					
p-ERK	↔	↔	⇣	↔	⇡
p-p38	↓	↔	↔	↔	↔
p-JNK	↑	↔	↔	↔	↔
MAPKs in sciatic nerve					
p-ERK	↑	-	-	↑	-
p-p38	↔	-	-	↑	-
p-JNK	↑	-	-	↔	-

↑= significant increase in cisplatin VS control groups, ⇡ = trend toward increase in cisplatin VS control groups, ↓= significant decrease in cisplatin VS control groups, ⇣ = trend toward decrease in cisplatin VS control groups, ↔= no difference

CHAPTER V
DISCUSSION

The main objective was to determine the alteration of MAPK phosphorylation in DRG and sciatic nerve of rats with cisplatin-induced neuropathy. Evaluation of the functional and morphometric changes was performed to confirm the neuropathy. However, many factors, such as the pattern of cisplatin administration, experimental conditions, rat strain and sex may also influence the development and severity of cisplatin neuropathy. Sex difference has been shown to influence mechanical hyperalgesia in a model of vincristine-induced neuropathy (Joseph and Levine, 2003). Regarding cisplatin administration, the protocol varies among different studies (Schmidt et al., 1995; Barajon, et al., 1996; Tredici et al., 1999; Authier et al., 2003; Bardos et al., 2003; Pisano et al., 2003). Therefore, the first study was done to determine the appropriate model for cisplatin-induced neuropathy with comparison between male and female rats. The schedule and dosage of cisplatin used was based on the previous study (Authier et al., 2003).

The results from the first study showed that cisplatin caused significant weight loss and prolonged heat latency (thermal hypoalgesia) in both sexes. These findings were similar to the previous studies (Barajon et al., 1996; Tredici et al., 1998; Boyle et al., 1999; Bardos et al., 2003; Ozturk et al., 2005). Thermal hypoalgesia may be explained by altered levels of peptide neurotransmitters. Alteration in the levels of calcitonin gene-related peptide (CGRP) and vasoactive intestinal peptide (VIP) have been shown in DRG and sciatic nerve following cisplatin treatment (Verdu et al., 1999). The reduction in some of these neuropeptides in the peripheral nerve innervating cutaneous structure in foot pads could result in thermal hypoalgesia (Verdu et al., 1999). Therefore, whether reduced levels of the above neurotransmitters are responsible for hypoalgesia in cisplatin neuropathy remains to be examined. It is worth nothing that changes in thermal sensation are likely dependent to dose of cisplatin. High doses of cisplatin produced hypoalgesia whereas lower doses produced hyperalgesia (Cata et al., 2008). The electrophysiological study revealed the reduction in MNCV in the 5^{th} week after cisplatin administration. However, this is in contrast to the previous studies which found that only sensory NCV (SNCV) was affected (Terheggen et al., 1989; Muller et al., 1990; Bardos et al., 2003). The inconsistency

may be due to the higher dose used in this study. The previous studies have shown that the reduction in NCV was dose-dependent (Verdu et al., 1998; Authier et al., 2003).

Atrophy of cell body, nucleus, and nucleolus of DRG neurons observed in the present study was consistent with other reports (Cavaletti et al., 1992b; Barajon et al., 1996; Schmidt et al., 1995; Tredici et al., 1998; Tredici et al., 1999; Ozturk et al., 2005; Carozzi et al., 2010). This and another study (Tredici et al., 1999) also found the trend toward reduced number of DRG neurons in the cisplatin-treated rats compared with the controls. This specific action of cisplatin to DRG may be explained by the fact that DRG are located outside the blood-brain barrier. In rats treated for prolonged periods with cisplatin, cisplatin concentrations are higher in DRG and peripheral nerve than in the brain and spinal cord (Thompson, 1984). In vivo, DNA-Platinum binding in rat DRG was greater than in other tissues (McDonald et al., 2005). For this reason, the higher accumulation of cisplatin in DRG may explain why cisplatin predominantly causes sensory neurotoxicity (Dzagnidze et al., 2007). It is well known that cisplatin induces DNA damage leading to transcription inhibition and cell cycle arrest. However, in terminally differentiated cells such as neurons, cell cycle arrest may not explain the degeneration of DRG neurons. Instead, impaired transcription may be one of the causes. Moreover, since cisplatin can bind with proteins and RNA, it is also suggested that the neurotoxic action of cisplatin may be due to reduction in neuronal protein synthesis as a result of RNA binding and protein dysfunctions due to protein binding (Terheggen 1989; Tredici et al., 1999).

As for the nerve, the present results demonstrated a significant decrease in myelinated fiber and axon diameters including lower fiber density with preserved g-ratio. Similar findings have been previously reported (Barajon et al., 1996; Cavaletti et al., 1992; Tredici et al., 1999). The axon atrophy might be explained by the similar processes responsible for atrophy of DRG cell bodies. The reduction in myelin thickness was also found but not significantly different. This change may be explained by the accumulation of platinum DNA-adduct in Schwann cells (Jirsova et al., 2006). The atrophy of axon together with the myelin degeneration may explain the reduction in MNCV describes earlier. Similarly, the sural nerve biopsies from patients

treated with high doses of cisplatin showed a decrease in the axonal diameter, myelin thickness, and the number of large myelinated nerve fibers (Roelofs et al., 1984; Krarup-Hansen et al., 1993). Furthermore, it has been supposed that cisplatin acts upon Schwann cells, resulting in demyelination of sensory axons (Muller et al., 1990). Loss of large myelinated fiber and abnormalities in the myelin sheath was also observed following cisplatin treatment (Authier et al., 2003).

Comparing between sexes, the general toxicity of cisplatin was more severe in males than in females as indicated by the higher degree of weight loss and mortality. The death found in the MC group but not the MN group suggests that this was likely related to cisplatin treatment. Similarly, a higher incidence of prolonged heat latency over 35 s was found in males (11/11) than in females (9/15) in the last week. In case of MNCV, reduction was more prominent in males. Furthermore, the morphological changes of nerve appeared to be more severe in females, whereas, those of DRG neurons were more prominent in males. The influence of sex on the development of neuropathy and nerve injury has been reported. Joseph and Levine, 2003 reported the higher degree of hyperalgesia in vincristine-induced and diabetic neuropathies in female rats (Joseph and Levine, 2003). They have suggested that these sex-related differences were estrogen dependent and related to certain PKC isoforms. Estrogen dependence may be explained by the estrogen receptor proteins localized in sensory neurons which can serve as binding sites for estrogen (Patrone et al., 1999). Thus, certain aspects of the structure, function and neurochemistry of sensory neurons may be influenced by the estrogen (Papka et al., 1997). The estrogen and estrogen receptor play an important role in the development and survival of DRG neurons (Patrone et al., 1999). Similarly, male patients tended to develop more severe neurological deficits induced by cisplatin (Argyriou et al., 2005). In contrast, two studies observed the faster regeneration and functional recovery after nerve injury in female animals (Jones K., 1993; Kovacic et al., 2004). The more effective cell support of peripheral nerve has been proposed as the responsible mechanism in this case. However, more studies are needed to elucidate the exact underlying mechanism.

Taken together, the female rat receiving 2 mg/kg twice a week for 5 weeks (total accumulated dose of 20 mg/kg) was chosen to be the model of cisplatin-induced

neuropathy since females could survive until the end of the experiment and still developed characteristic cisplatin-induced neuropathy. Therefore, this model was used in Study 2 to examine MAPKs.

In Study 2, the cisplatin-treated group had the weight loss from the 3rd to the 8th weeks and increased withdrawal latency was also observed in the 5th week. This thermal hypoalgesia has been already observed in Study 1. Cisplatin-treated group had the decreased mechanical threshold compared with the control group from the 3rd to the 5th weeks which then fully recovered. A maximum reduction in the threshold of the cisplatin-treated group was noted in the 3rd week. The decrease in mechanical threshold indicates mechanical allodynia. One study has shown that the up-regulation of TRPV1 (transient receptor potential vanilloid 1) and TRPA1 (transient receptor potential cation channel, subfamily A, member 1) mRNA were coupled with mechanical hypersensitivity in cisplatin-treated mice (Ta et al., 2010). TRPV1 and TRPA1 are the primary detectors involved in chemical and thermal evoked pain sensation. According to Lajer and Daugaard (1999), hypomagnesemia is a frequent complication during chemotherapy with cisplatin, affecting 90% of patients (Lajer and Daugaard, 1999). Magnesium is known to bind to *N*-methyl-D-aspartate (NMDA) receptor channels. A decrease in magnesium levels could enhance nociception by increasing binding between glutamate and NMDA receptors (Begon et al., 2000). Taken together, changes in neurotransmission of related receptors are likely responsible for mechanical allodynia in cisplatin neuropathy.

This study also showed the decrease in MNCV from the 5th to the 12th weeks. This, again, confirmed the reduced MNCV in the 5th week shown in Study 1 and also suggested that neuropathy persisted until the 12th week.

In Study 2, the mean myelin thickness of cisplatin-treated group was significantly decreased in the 8th and 12th weeks compared with the control group. This corresponds with the decrease in MNCV. In the 12th week, the reduction of mean nerve fiber diameter in cisplatin-treated group was in agreement with the previous reports indicating that the large myelinated nerve fibers are more affected. The ratio of the axonal diameter and fiber diameter (g-ratio) was increased in the 8th week.

Increase in the g-ratio indicates more severity in reduced myelin thickness than reduced axonal diameter. The fiber density of cisplatin group was increased in the 12th week compared with the control. The percentage of the number of myelinated nerve fibers larger than 10 μm was slightly reduced in the 12th week in cisplatin-treated group compared with the control, indicating predominant loss of large myelinated fibers. The above results were similar with the previous studies (Cavaletti et al., 1992; Barajon et al., 1996; Tredici et al., 1999) which showed peripheral nerve axonopathy mainly involving the large myelinated nerve fibers. The loss of the large myelinated nerve fibers with the reduction in the myelin thickness could explain decrease in MNCV after cisplatin administration in this study.

Because of no effective therapy exists for the cisplatin-induced neuropathy, a better understanding of the mechanisms responsible for this neuropathy is required. Since abnormalities in diabetic neuropathy are related to an increase in ERK and p38 phosphorylation in neurons and Schwann cells (Tomlinson 1999; Purves et al., 2001; Agthong and Tomlinson, 2002; Price et al., 2004; Daulhac et al., 2006). For this reason, it is interesting to examine the relationship between MAPK phosphorylation and cisplatin neuropathy.

In Study 2, JNK phosphorylation was up-regulated only in the 1st week in DRG and sciatic nerve in cisplatin group. However, no evidence of cisplatin-induced neuropathy was observed in this period. Immunohistchemistry study has shown that this activation occurred in the small DRG neurons and nuclei of the large DRG neurons. This JNK activation was localized in both Schwann cells and axons. These findings are consistent with those of Fernyhough (Fernyhough et al., 1999) which showed that immunoreactivity of phosphorylated and total JNK was present in the cytoplasm and nuclei of sensory neurons of STZ-induced diabetic rats. Taken together, increased phosphorylation of JNK was unlikely responsible for functional and structural abnormalities in cisplatin-induced neuropathy. Most studies have related JNK pathway to apoptosis induced by genotoxic stress, UV radiation and chemotherapeutic drugs (Xie et al., 1995; Chen, Meyer, and Tan. 1996; Kyriakis and Avruch, 1996; Yu et al., 1996; Shirakabe et al., 1997; Sánchez-Perez, Murguía, and Perona. 1998). However, a specific inhibitor of JNK caused worse cellular death of

neurons exposed to cisplatin (Scuteri et al., 2009) indicating the role of JNK in neuronal survival. Therefore, despite the uncertain role of JNK in apoptosis, brief and early activation of JNK found in this study suggested that it was not likely responsible for functional and structural abnormalities in cisplatin neuropathy.

Phosphorylation of ERK was up-regulated in sciatic nerve in the 8th week. P38 phosphorylation was also increased in the 8th week. In DRG, ERK phosphorylation tended to decrease in cisplatin-treated rats in the 5th week and tended to increase in the 12th week. For p38, its phosphorylation was reduced in the 1st week. Immunohistochemistry has shown that this activation occurred in the axons and Schwann cells of the sciatic nerve. In DRG, p38 activation occurred in the cytoplasm of small neurons and nuclei of the large neurons whereas ERK phosphorylation was found in the satellite cells and small neurons.

As for behavioral and electrophysiological abnormalities, mechanical allodynia was found in the 3rd to the 5th weeks and thermal hypoalgesia was found in the 5th week and slow MNCV from the 5th to the 12th weeks. After comparing changes in ERK and p38 phosphorylation with functional and structural abnormalities, no clear relationship has been observed. This is because all abnormalities started in the 3rd or 5th week while ERK and p38 phosphorylation was activated in the 8th week. However, the roles of MAPKs in various neuropathies have been demonstrated.

In diabetic neuropathy, mechanical hyperalgesia was correlated with an increase in ERK, p38 and JNK phosphorylation in DRG at 3 weeks after induction of diabetes (Daulhac et al., 2006). Treatment with antioxidants decreases the activation of ERK but increases JNK activation, suggesting that ERK is injurious and JNK is protective (Price et al. 2003). ERK is highly expressed in neurons and its activation has been observed in neurodegeneration (Colucci-D'Amato et al., 2003), and oxidative stress that results in neuronal damage (Purves et al., 2001). In diabetic neuropathy, p38 activation is related to a reduction in NCV (Agthong and Tomlinson, 2002; Price et al., 2004).

A relationship between cisplatin administration and MAPK change in DRG culture has been investigated at different time points (2-24 hr) (Scuteri et al., 2009). Cellular death from cisplatin administration started after 18 hr of treatment and became more evident at 22–24 hr. By using specific inhibitors of MAPKs, they demonstrated that cisplatin induced neuronal apoptosis is mediated by early p38 and ERK activation, while JNK has a neuroprotective role (Scuteri et al., 2009).

Several studies have related ERK phosphorylation to nociceptive pathway. The very rapid phosphorylation of ERK occurred in DRG neurons taking part in the transmission of various noxious signals. The phosphorylation of ERK in DRG occurs in response to noxious stimulation. For example, the electrical stimulation of A delta fibers induced ERK phosphorylation primarily in neurons with myelinated fibers (Dai et al., 2002). C-Fiber activation by capsaicin injection induced ERK phosphorylation in small neurons with unmyelinated fibers containing VR-1 receptor, suggesting that ERK phosphorylation in DRG neurons is modality specific (Dai et al., 2002). The activation of ERK in DRG may occur in different populations of DRG neurons after peripheral inflammation and axotomy, through alterations in the target-derived NGF (Obata et al., 2003). On the other hand, Ji and colleagues showed that p38 activation in the DRG is required for NGF-induced increase in TRPV1 expression and contributes to the maintenance of inflammatory pain hypersensitivity (Ji et al., 2002). Some reports have demonstrated that not only peripheral inflammation but axotomy can induce p38 activation in small DRG neurons as well (Ji et al., 2002; Kim et al., 2002; Jin et al., 2003; Schafers et al., 2003). In addition to ERK and p38, JNK can also be activated by inflammation and/or nerve injury. Peripheral axotomy has been shown to induce long-term JNK activation in DRG neurons (Kenney and Kocsis, 1998; Hou et al., 2003). In DRG, ERK phosphorylation was also upregulated in satellite glial cells and, in particular, the larger diameter neurons after sciatic nerve transection (Obata et al., 2003b). Peripheral axotomy also induces p38 activation in satellite cells surrounding DRG neurons (Jin et al., 2003).

From the above data, MAPKs have the role in diabetic neuropathy, pain due to inflammation and nerve injury including in vitro neurotoxicity of cisplatin. However, in this study, the correlation in phosphorylation of ERK, JNK and p38 with various

abnormalities of cisplatin-induced neuropathy in different time points could not be established. The further study using inhibitors of ERK, JNK and p38 should be done to clarify the roles of MAPKs in cisplatin-induced neuropathy which could be developed into effective treatment of this neuropathy.

CHAPTER VI
CONCLUSIONS

Characterization of the animal model of cisplatin-induced neuropathy

Cisplatin treatment (2mg/kg twice a week for 5 weeks) was associated with general toxicity, prolonged heat latency, and slow MNCV, which were more severe in male rats. Four males died before the end of experiment. However, sex-related differences in the structural alterations in the myelinated fibers and DRG neurons were variable. This sexual dimorphism should be verified in clinical cisplatin associated neuropathy and its underlying mechanism remains to be clarified. Nevertheless, female rats were used in subsequent study due mainly to their better tolerability to the general toxicity of cisplatin.

Evaluation of cisplatin-induced neuropathy in different time points

Behavioral and electrophysiological studies

The rats received cisplatin had thermal hypoalgesia in the 5^{th} week, mechanical allodynia from the 3^{rd} to 5^{th} weeks and decreased MNCV from the 5^{th} to 12^{th} weeks.

Morphometric study,

Sciatic nerve from the cisplatin rats had a significant decrease in the mean myelin thickness in the 8^{th} and 12^{th} weeks. The axonal diameter was increased in the 8^{th} week in cisplatin-treated compared with the control groups. In the 12^{th} week, the reduction of mean nerve fiber diameter was observed. The ratio of the axonal diameter to fiber diameter (g-ratio) in cisplatin-treated group was increased in the 8^{th} week compared with the control group. The fiber density was increased in the 12^{th} week. No significantly difference in the number of total fiber was observed in the 8^{th} and 12^{th} weeks. DRG had a significant decrease in mean somatic area, nuclear area, nucleolar area and total cell count in the 5^{th}, 8^{th} and 12^{th} weeks in the cisplatin compared with the control groups.

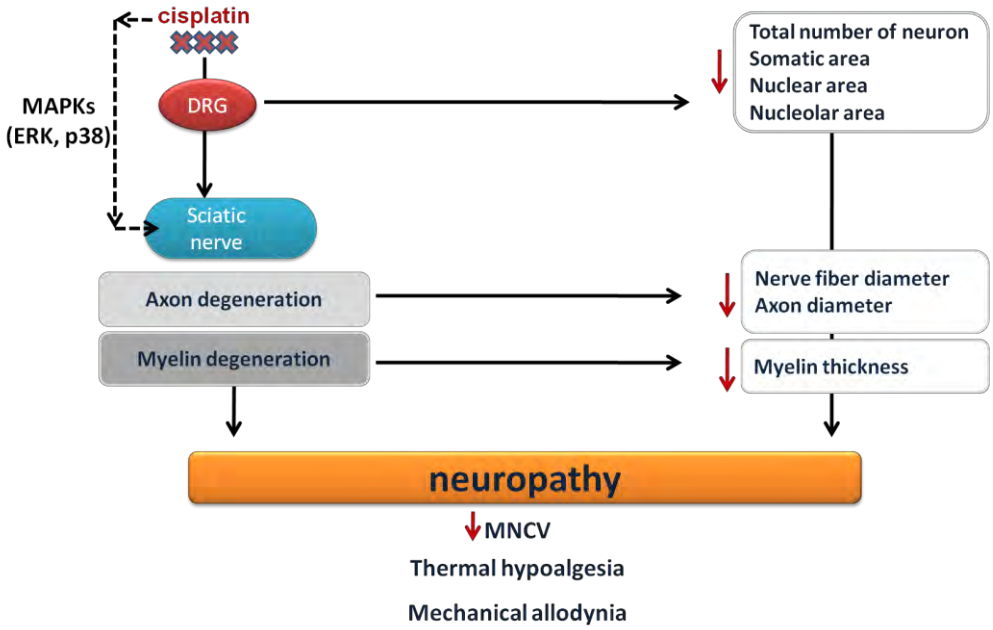
Determination of MAPK phosphorylation in DRG and sciatic nerve of rats with cisplatin-induced neuropathy

Western blot study

Cisplatin enhanced the phosphorylation of ERK in the 1st and the 8th week in sciatic nerve and p38 in the 8th week compared with the controls. In DRG, down-regulation of p38 in the 1st week and up-regulation of ERK in the 12th week have been found. On the other hand, increased phosphorylation of JNK was found only in the 1st week in DRG and sciatic nerve.

Immunohistochemistry of sciatic nerve and DRG neuron

Phosphorylation of MAPKs occurred in the axons and Schwann cells of sciatic nerve. The localization of P-ERK in DRG has been observed in both satellite cells and small DRG neurons. In particular, P-p38 and P-JNK were marked in the small DRG neurons and the nuclei of large cells.



In conclusion, cisplatin induced DNA damage leading to transcription inhibition as a result of protein dysfunctions cause the reduction of total number of neuron, somatic area, nuclear area and nucleolar area. Atrophy of DRG cell bodies might be produced to axon atrophy leading to the reduction of nerve fiber diameter and axon diameter. Furthermore, it has been supported that cisplatin acts upon Schwann cells, resulting in the reduction of myelin thickness. Taken together, these morphometric changes were confirmed the neuropathy resulting in slow MNCV, thermal hypoalgesia and mechanical allodynia. Phosphorylation of MAPKs (ERK and p38) has been found in sciatic nerve. The alteration of MAPKs in sciatic nerve may

involve axon degeneration and myelin degeneration. These finding need the further study using inhibitors to clarify the roles of MAPKs in cisplatin-induced neuropathy.

Accordingly, these data suggested that the alteration of ERK, p38 and JNK phosphorylation in DRG and sciatic nerve occurred in different time points of cisplatin-induced neuropathy.

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APPENDICES

APPENDIX A

Tissue processing for ultramicrotome technigue

DRG and sciatic nerve were fixed with 3% Glutaraldehyde 4ºC

↓

Wash in 0.1 M phosphate buffer pH 7.4 (PBS) overnight

↓

Wash in PBS for 3 times 10 min each

↓

Fix in 1 % osmium tetroxide for 2 hr

↓

Dehydrate with graded alcohol

70% for 2 times and overnight

80% for 2 times

95%, and for 2 times

100% alcohols for 2 times

↓

Clearing with Propylene oxide for 2 in 15 min.

↓

Infiltrate with Propylene oxide:Epon (resin) 1:1 for 1 hr.

1:2 overnight

100 % for 1 hr.

↓

Embedding with 100 % Epon in capsule block was left at 60°C for 3 days

APPENDIX B

Solutions and dyes used in the tissue processing

0.1 M phosphate buffer pH 7.4 (PBS)

Stock solution A: 0.2 M sodium phosphate monobasic

0.2 M sodium phosphate monobasic	27.80 g.
Distilled water	1000 ml.

Stock solution B: 0.2 M sodium phosphate dibasic

Na_2HPO4	28.40 g.
Distilled water	1000 ml.

Working solution 0.2M buffer solution: stock solution A 19 ml. + B 81 ml. for pH 7.4

3% Glutaraldehyde in phosphate buffer pH7.4

30% glutaraldehyde	10 ml.
0.1 M phosphate buffer pH 7.4 (PBS)	30 ml.

1 % osmium tetroxide

Osmium tetraoxide	1 g.
0.1 M phosphate buffer pH 7.4 (PBS)	100 ml.

Epon (resin)

Total volume	50 ml.	100 ml.	150 ml	200 ml.
Epon 812	25 ml.	50 ml.	75 ml.	100 ml.
DDSA (Dodecenyl succinic anhydride)	20 ml.	40 ml.	60 ml.	80 ml.
NMA (Nadic methyl anhydride)	10 ml.	20 ml.	30 ml.	40 ml.
DMP-30	1 ml.	2 ml.	3 ml.	4 ml.

1% para-phenylenediamine

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

Toluidine blue

Sodium borate	5 g.
Distilled water	500 ml.
Toluidine blue	5 g.
Azure II	5 g.

30% sucrose in 0.1 M phosphate buffer

0.1 M Phosphate buffer pH 7.4	100 ml.
Sucrose	30 g.

APPENDIX C

Preparation of sample homogenization for western blot technique

Homogenization buffer

0.1 M PIPES, pH 6.9	12.1 g
5 mM $MgCl_2$ (magnesium chloride)	5ml
5 mM EGTA (ethylene glycol tetraacetic acid)	5 ml
0.5 % Triton X-100	2.5 ml
20 % glycerol	100 ml
10 mM NaF (sodium fluoride)	0.21 g
Deionized water	387.5 ml
total	500 ml

5 ml of homogenization buffer was mixed with 1mmol/l PMSF (phenylmethylsulfonyl fluoride), 0.2 M sodium orthovanadate and 0.5 ml Protease inhibitor cocktail. Then, the sample buffer was added to each sample.

APPENDIX D

Preparation of stacking gel and running gel for western blot technique

Composition	10% Running gel		Stacking gel	
	1 plate	**2 plate**	**1 plate**	**2 plate**
30% Acrylamide mix	3.3 ml	9.9 ml	0.65 ml	1.95 ml
1 M running gel buffer	3.75 ml	11.25 ml		
1 M stacking gel buffer			0.625 ml	1.875 ml
Distilled water	2.75 ml	8.25 ml	3.28 ml	9.84 ml
10% SDS	100 µl	300 µl	50 µl	150 µl
10% APS	100 µl	300 µl	37.5 µl	112.5 µl
TEMED	10 µl	30 µl	5 µl	15 µl
Total volume	**10 ml**	**30 ml**	**4.6 ml**	**13.9 ml**

30% Acrylamide/Bis solution 37.5:1 solution (Sigma, UK)

Running gel buffer: 1M Trizma pre-set crystal, pH 8.8

Stacking gel buffer: 1 M Tris-base, pH 6.8

10% SDS (Sodium dodecyl sulfate, minimum9.85% GC) (Sigma, UK)

10% APS (Ammonium Persulfate) (Sigma,UK)

TEMED (N,N,N',N'-tetramethylethane-1,2-diamine)

APPENDIX E

List of antibodies for western blot technique

Antibody	Supplier	Animal raised in/ poly or monoclonal species cross reactivity	Concentration of primary antibody and incubation time	Concentration of secondary antibody and incubation time	Blocking solution	Apparent molecular weight (kDa)	Protein (µg)
P-ERK	Santa Cruz	Rabbit polyclonal rat	1:500 Overnight 4°C	1:150 30 minutes*	5 % casein solution (Vector)	42, 44	25
T-ERK	Santa Cruz	Rabbit polyclonal rat	1:5000 Overnight 4°C	1:150 30 minutes*	5 % casein solution (Vector)	42, 44	25
P-p38 Detect p38 α,β and γ	Cell Signaling Technology	Rabbit polyclonal rat	1:500 Overnight 4°C	1:150 30 minutes*	5 % casein solution (Vector)	38	35
T-p38	Cell Signaling Technology	Rabbit polyclonal rat	1:1000 Overnight 4°C	1:150 30 minutes*	5 % casein solution (Vector)	38	35

Antibody	Supplier	Animal raised in/ poly or monoclonal species cross reactivity	Concentration of primary antibody and incubation time	Concentration of secondary antibody and incubation time	Blocking solution	Apparent molecular weight (kDa)	Protein (µg)
P-JNK Detect JNK 1,2 and 3	Santa Cruz	Rabbit polyclonal rat	1:200 Overnight 4°C	1:150 30 minutes*	5 % casein solution (Vector)	46, 54/56	25
T-JNK Detect JNK 1,2 and 3	Santa Cruz	Rabbit polyclonal rat	1:500 Overnight 4°C	1:150 30 minutes*	5 % casein solution (Vector)	42, 54/56	25

*= room temperature

APPENDIX F

Substrate solution for immunological detection in western blot analysis

To 10 ml of distilled water, add 4 drops of buffer stock (pH 7.5) solution and mix well

↓

Add 8 drops of DAB stock solution and mix well

↓

Add 4 drops of the Hydrogen Peroxide solution and mix well

↓

Add 4 drops of Nikel solution and mix well

BIOGRAPHY

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Activity: IBRO course in neuroscience national institute of mental health and neuroscience (NIMHANS) Bangalore, India August 24-September 1, 2005

Poster Presentation:

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2. The 29th Annual Meeting of the society of Anatomy of Thailand, May 2006.
3. The 30th Annual Meeting of the society of Anatomy of Thailand, May 2007.
4. IBRO course of neuroscience, Naresuan University, Phitsanulok, Thailand, July 2007.
5. The 40th annual meeting of the society for neuroscience (SfN), San Diego California, USA, November 2010