Ultrastructural changes of endothelial cell, pericyte and vascular basement membrane in cisplatin-induced neuropathy in rats: correlation with neuropathic changes after treatments

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การเปลี่ยนแปลงโครงสร้างของเซลล์ endothelial เซลล์ pericyte และ vascular basement membrane ในหนูที่เกิดภาวะ neuropathy จาก cisplatin: ความสัมพันธ์กับการเปลี่ยนแปลงของ neuropathy ภายหลังการรักษา

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Cisplatin เป็นยาเคมีบำบัดที่ใช้รักษามะเร็งของหลายอวัยวะ ภาวะเส้นประสาทผิดปกติเป็นผลข้างเคียงหนึ่งที่พบได้และอาจทำให้ต้องลดขนาดยาหรือหยุดยา กลไกที่ทำให้เกิด neuropathy นี้ยังไม่ทราบแน่ชัดแต่ปัจจุบันมีหลักฐานบ่งชี้ว่าการทำงานผิดปกติของหลอดเลือดอาจจะเกี่ยวข้องด้วย Curcumin เป็นสารโพลีฟีนอลในรากของขมิ้นชัน ซึ่งมีฤทธิ์ต้าน oxidative stress และช่วยป้องกันระบบประสาท โดยแสดงผลดีต่อโรคทางระบบประสาทส่วนปลายที่เกิดจาก cisplatin ด้วย ส่วนวิตามิน B เป็นวิตามินที่ละลายในน้ำ มีบทบาทช่วยในกระบวนการเผาผลาญและเกี่ยวข้องกับการทำงานของระบบประสาท มีหลักฐานพบว่าวิตามินบี โดยเฉพาะ B1-6-12 มีประสิทธิภาพในการรักษาโรคทางระบบประสาทหลายโรค ดังนั้นในการศึกษานี้มีวัตถุประสงค์เพื่อดูผลของ cisplatin ต่อ blood-nerve barrier ในหนูและต่อ pericyte ในสภาวะเพาะเลี้ยง โดยใช้ rat brain vascular pericyte (RBVP) เป็นตัวอย่างในการศึกษา นอกจากนี้ยังได้ศึกษาผลของ curcumin และ B1-6-12 อีกด้วย โดยหนูทดลองจะได้รับ cisplatin ฉีดเข้าช่องท้อง ขนาด 2 mg/kg จำนวน 2 ครั้งต่อสัปดาห์ ต่อเนื่องกันเป็นระยะ 5 สัปดาห์ ส่งผลให้น้ำหนักตัวหนู การรับรู้ต่อการรับความรู้สึกร้อน และความเร็วในการนำกระแสประสาทลดลง ซึ่งแสดงถึงภาวะ neuropathy การรักษาร่วมกับ curcumin และ B1-6-12 โดยป้อนทางปากวันละครั้ง จะให้ curcumin 200 mg/kg และ B1-6-12 จำนวน 100, 300 และ 600 mg/kg พบว่าสามารถทำให้ภาวะ neuropathy ดีขึ้น ซึ่งจากผลการศึกษาภายใต้กล้องจุลทรรศน์อิเล็กตรอนแบบส่องผ่าน ผลการทดลองพบว่า cisplatin เพิ่มความถี่และระยะทางของการแยกตัวของ pericyte ในผนังหลอดเลือดฝอยของเส้นประสาทของหนูและปมประสาทไขสันหลังเมื่อเทียบกับกลุ่มควบคุม และ cisplatin ยังลด viability ของ pericyte ด้วย ซึ่ง curcumin และ B1-6-12 (โดยเฉพาะขนาด 100 และ 300 mg/kg/day) สามารถลดความผิดปกติที่เกิดจาก cisplatin ดังกล่าวในผนังหลอดเลือดและเซลล์เพาะเลี้ยงได้ ข้อมูลเหล่านี้แสดงว่า cisplatin ทำให้เกิดความเป็นพิษต่อ pericyte ในสภาวะเพาะเลี้ยงและการแยกตัวของ pericyte ที่ผนังหลอดเลือดในระบบประสาทของหนู curcumin และ B1-6-12 มีประสิทธิภาพในการรักษาความผิดปกติเหล่านี้และควรมีการศึกษาเพิ่มเติมเพื่อพัฒนาเป็นยารักษาภาวะ neuropathy จาก cisplatin ต่อไป

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Ultrastructural changes of endothelial cell, pericyte and vascular basement membrane in cisplatin-induced neuropathy in rats: correlation with neuropathic changes after treatments. Advisor: Prof. SITHIPORN AGTHONG, M.D., Ph.D. Co-advisor: Asst. Prof. DEPICHA JINDATIP, Ph.D.

Cisplatin is an antineoplastic agent used to treat cancers of several organs. Peripheral neuropathy is one of its major side effects leading to dose reduction or cessation. Underlying mechanisms of cisplatin-induced neuropathy are not fully understood. According to current evidence, vascular dysfunction may play a role. Curcumin is a polyphenol found in the root of Curcuma longa with the anti-oxidant property and neuroprotection. Curcumin has shown effectiveness against experimental cisplatin neuropathy. B vitamins are a class of water-soluble vitamins that play important roles in cell metabolism and maintaining nervous system functions. Increasing evidence suggests the efficacy of vitamin B1-6-12 for several neurological diseases. Therefore, the objective of this study was to examine the ultrastructural changes of blood-nerve barrier (BNB) in the peripheral nerve and dorsal root ganglion (DRG) of rats treated with cisplatin and in vitro effects on rat brain vascular pericytes. Furthermore, the effects of curcumin and B vitamins were examined. Cisplatin neuropathy was induced by intraperitoneal injection of cisplatin 2 mg/kg twice a week for 5 consecutive weeks. The cisplatin-treated rats had reduced body weight, decreased heat sensitivity and slow nerve conduction velocity, indicating neuropathy. In the co-treatment group, curcumin or B vitamins was also given by gavage during the cisplatin treatment. Curcumin 200 mg/kg/day or B1-6-12 (100:100:1) 100, 300 and 600 mg/kg/day was given once daily. Curcumin and all doses of B1-6-12 could significantly improve the severity of neuropathy. Transmission electron microscope (TEM) study of capillaries in the sciatic nerve and DRG found that the cisplatin group had significantly higher frequency of separation and wider separation distance between endothelial cells and pericytes than the control group. Moreover, cisplatin significantly reduced viability of cultured pericytes. Curcumin and B1-6-12, especially low (100 mg/kg/day) and medium doses (300 mg/kg/day), could alleviate the cisplatin-induced pericyte detachment. These two agents also significantly improved pericyte viability. Taken together, cisplatin causes pericyte cytotoxicity in vitro and pericyte detachment in vivo. Curcumin and B1-6-12 are effective against these abnormalities and thus potential therapeutic agents for cisplatin-induced neuropathy. More investigations are needed to elucidate the underlying mechanisms and clinical applications.

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LIST OF ABBREVIATIONS

ALC	Acetyl-L-Carnitine
Ang1	Angiopoietin-1
BBB	Blood-brain barrier
BDNF	Brain-derived neurotrophic factor
bFGF	Basic fibroblast growth factor
BNB	Blood-nerve barrier
CIDP	Chronic inflammatory demyelinating polyradiculoneuropathy
Cis	Cisplatin
CNS	Central nervous system
CTR1	Copper transporter-1
Cur	Curcumin
DPBS	Dulbecco's phosphate-buffered saline
DRG	Dorsal root ganglion
FBS	Fetal bovine serum
GDNF	Glial cell line-derived neurotrophic factor
GSH	Glutathione
ICAM-1	Intercellular adhesion molecule 1
JAM	Junctional adhesion molecule
MTT	Micro-culture tetrazolium assay
NCV	Nerve conduction velocity
NER	Nuclear excision repair
NG2	Nerve-glial antigen-2
NGF	Nerve growth factor
PBS	Phosphate buffered saline
PDGFR-β	Platelet-derived growth factor receptor-beta
PNS	Peripheral nervous system
RBVP	Rat brain vascular pericyte
ROS	Reactive oxygen species
RTK	Receptor-tyrosine kinase

SOD	Superoxide dismutase
TGF-β	Transforming growth factor beta
TEER	Trans-endothelial electrical resistance
TEM	Transmission electron microscope
TRP	Transient receptor potential
VBM	Vascular basement membrane
VEGF	Vascular endothelial growth factor
ZO	Zonula occludens
α-SMA	alpha-smooth muscle actin

CHAPTER I

INTRODUCTION

1.1 Introduction

Cisplatin is an antineoplastic agent used to treat various cancers, such as testicular, ovarian and lung cancers (1). However, cisplatin possesses potentially adverse side effects e.g. nephrotoxicity and peripheral neuropathy (2, 3). Peripheral neuropathy can lead to the cessation of chemotherapy thereby diminishing the effectiveness of cancer treatments. Previous studies have found that cisplatin is highly accumulated in dorsal root ganglia (DRG) compared with brain and spinal cord (4, 5). This can explain the predominant sensory abnormalities observed in cisplatin-induced neuropathy. Morphometry confirms the pathology in the DRG by showing the shrinkage of neuronal cell body, nucleus and nucleolus in the rats treated with cisplatin (6-8). Moreover, loss of large myelinated fibers, is also evident in the peripheral nerve (6, 8). Until now, there is no effective prevention or treatment for this side effect of cisplatin.

Blood-nerve barrier (BNB) is important for the normal function of nerve and changes in the barrier components have been shown in various neuropathies (9, 10). Endothelial cells play a wide variety of crucial roles in the control of vascular function (11). Cisplatin may alter the property of BNB. Previous studies showed that cisplatin caused abnormal function of blood vessels, such as reduced nerve blood flow, reduced number of vasa nervorum, including endothelial cell damage and apoptosis (12, 13). According to these data, cisplatin may negatively affect endothelial cells and impair BNB causing neuropathy. Pericyte might also be affected by cisplatin. It shares the vascular basement membrane with the endothelial cell and co-operate in the control of blood flow, vessel stabilization, angiogenesis and integrity of BNB (14). Our pilot study showed that there were reduced number of pericyte, pericyte migration and disrupted vascular basement membrane in the nerves of rats treated with cisplatin. In addition, expression of protein component of BNB, zonula occludens-2 (ZO-2) tended to decrease in the cisplatin group.

Curcumin is a polyphenol found in the root of *Curcuma longa* with the anti-inflammatory, anti-oxidant, and neuroprotective properties (15). It is effective against

several human diseases including neurological diseases (16). Moreover, it has been shown effective against experimental cisplatin neuropathy (6).

B vitamins are a group of water-soluble vitamins that play the main roles in cellular metabolism and maintaining nervous system functions e.g. coenzymes for synthesis of neurotransmitters (17). Due to their essential roles in the nervous system, they have been prescribed to patients with neurological diseases (18). Our pilot data indicated that vitamin B1-6-12 were able to ameliorate cisplatin-induced neuropathy in rats.

However, the effects of curcumin and vitamin B1-6-12 on endothelial cell, pericyte and vascular basement membrane in the peripheral nervous system after cisplatin administration have not been studied. Therefore, the aim of this study is to investigate the effects of curcumin and vitamin B1-6-12 on ultrastructural alterations of endothelial cell, pericyte, and vascular basement membrane in the peripheral nerve and DRG of cisplatin-treated rats.

Moreover, it is unknown whether curcumin and B1-6-12 have any effects on the expression of tight junction proteins synthesized by pericytes treated with cisplatin. Thus, this study also aims to evaluate changes in the expression of tight junction proteins in the cultured pericytes receiving cisplatin only and co-treatment with either curcumin or B1-6-12.

1.2 Keywords

Endothelial cell, Cisplatin, Curcumin, Pericyte, Peripheral neuropathy, Vascular basement membrane, Vitamin B1-6-12

1.3 Research questions

In vivo (animal model)

1. What are the effects of cisplatin on ultrastructural changes in endothelial cells, pericytes and vascular basement membrane in the dorsal root ganglia (DRG) and nerve?
2. What are the effects of curcumin on ultrastructural changes in endothelial cells, pericytes and vascular basement membrane in the DRG and sciatic nerves of rats with cisplatin-induced neuropathy?

3. What are the effects of B1-6-12 on ultrastructural changes in endothelial cells, pericytes and vascular basement membrane in the DRG and sciatic nerves of rats with cisplatin-induced neuropathy?

***In vitro* (rat brain pericyte culture)**

1. What are the effects of cisplatin on the expression of tight junction proteins (ZO-1 and ZO-2) synthesized by pericyte?
2. What are the effects of curcumin on the expression of tight junction proteins (ZO-1 and ZO-2) synthesized by pericyte treated with cisplatin?
3. What are the effects of B1-6-12 on the expression of tight junction proteins (ZO-1 and ZO-2) synthesized by pericyte with treated with cisplatin?

1.4 Objectives

***In vivo* (animal model)**

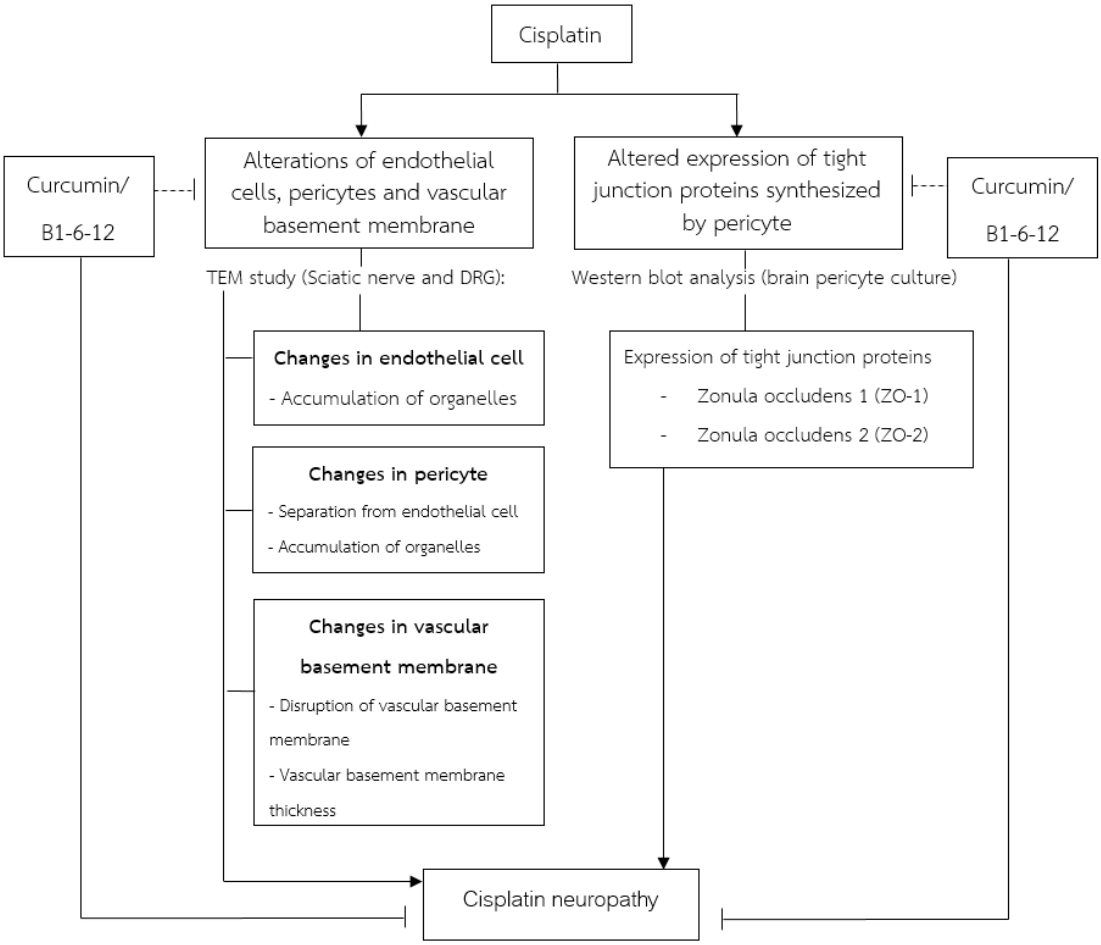
1. To determine the effects of cisplatin on ultrastructural changes in endothelial cells, pericytes and vascular basement membrane in the dorsal root ganglia (DRG) and nerve?
2. To evaluate the effects of curcumin on ultrastructural changes in endothelial cells, pericytes and vascular basement membrane in the DRG and sciatic nerves of rats with cisplatin-induced neuropathy?
3. To evaluate the effects of B1-6-12 on ultrastructural changes in endothelial cells, pericytes and vascular basement membrane in the DRG and sciatic nerves of rats with cisplatin-induced neuropathy?

***In vitro* (rat brain pericyte culture)**

1. To determine the effects of cisplatin on the expression of tight junction proteins (ZO-1 and ZO-2) synthesized by pericyte?
2. To investigate the effects of curcumin on the expression of tight junction proteins (ZO-1 and ZO-2) synthesized by pericyte treated with cisplatin?
3. To investigate the effects of B1-6-12 on the expression of tight junction proteins (ZO-1 and ZO-2) synthesized by pericyte treated with cisplatin?

1.5 Conceptual Framework

In vivo (animal model) and *In vitro* (rat brain pericyte culture)



1.6 Research design

Experimental and retrospective studies

1.7 Expect Benefits and Applications

The data will suggest if the antioxidant curcumin and B vitamins have beneficial effects on the structural abnormalities of BNB in cisplatin-induced neuropathy. The results of this study will be useful for the development of prevention or treatment of cisplatin-induced neuropathy by targeting the BNB.

CHAPTER II

REVIEW OF THE RELATED LITERATURES

2.1 Nervous system

The nervous system is composed of neurons and various types of glial cells. It has 2 major parts: (19, 20)

2.1.1 Central nervous system (CNS)

2.1.1.1 Brain

2.1.1.2 Spinal cord

2.1.2 Peripheral nervous system (PNS)

- Cranial nerve 12 pairs, leaving the brain and passing through foramina in the skull.
- Spinal nerve 31 pairs, including 8 cervical, 12 thoracic, 5 lumbar, 5 sacral and 1 coccygeal, which leave the spinal cord and pass through intervertebral foramina in the vertebral column.

The cranial and spinal nerves, which contain bundles of axons or nerve fibers with Schwann cells as glial cells. Its main function is to conduct information between targets and the CNS.

- Ganglion (19, 21)

Ganglion is a structure containing nerve cell bodies and supporting cells which are satellite glial cells (SGCs). The ganglion includes sensory ganglion and autonomic ganglion. The sensory ganglion consists of cranial nerve ganglion and spinal or dorsal root ganglion.

Dorsal root ganglion (DRG) contains the cell bodies of sensory neurons that convey the sensory information from the body's periphery to the spinal cord. A layer of connective tissue that is continuous with the perineurium and epineurium of the peripheral nerve surrounds each ganglion. These neurons are pseudo-unipolar and their cell bodies are relatively round (Figure 1).

Autonomic ganglion contains cell bodies of postsynaptic sympathetic and parasympathetic neurons (19). However, cranial nerve ganglia contain cell bodies of either sensory neurons or parasympathetic neurons.

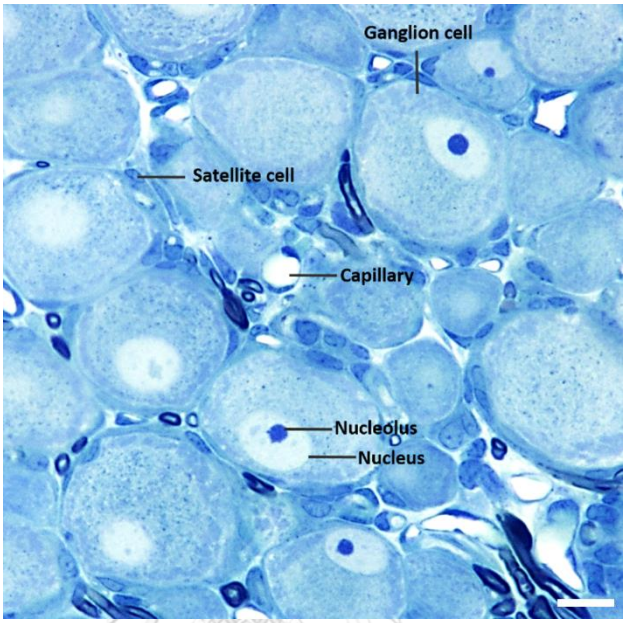


Figure 1 Dorsal root ganglia (scale bar = 10 μm)

- **Peripheral nerve**

Peripheral nerve or nerve contains numerous nerve fibers. Nerve fibers, consisting of axons and Schwann cells, are classified by functions, conduction velocity and fiber size (diameter). Endoneurium is a loose connective tissue surrounding the axon and Schwann cells. The endoneurium contains small blood vessels, fibroblasts and macrophages. The axons are grouped into bundles called nerve fascicles, and each fascicle (bundle) is covered by the connective tissue layer known as perineurium. Likewise, the epineurium is a connective tissue covering all fascicles as the outermost layer of nerve (Figure 2) (19, 21).

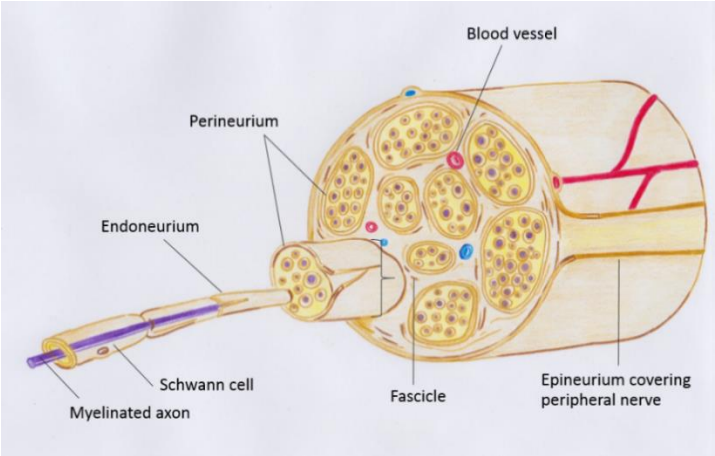


Figure 2 Structure of nerve

Classification of axon/nerve fiber

1. Myelinated axon/nerve fiber

The axon is surrounded by myelin sheath, which is interrupted by the nodes of Ranvier. Myelin sheath, because of its insulating function increases the efficiency and speed of impulse conduction. In the CNS, the myelin sheath is produced by oligodendrocyte, while in the PNS, it is produced by Schwann cell. In the nerve, there is only one Schwann cell for each segment (internode) of one nerve fiber. The thickness of myelin sheath and the length of the internode vary in proportion to the diameter of axon. The myelinated axons are larger than the unmyelinated ones (21).

2. Unmyelinated axon/nerve fiber

It is a group of smaller axons without myelin sheath. In the PNS, one Schwann cell usually supports several unmyelinated axons (21).

2.2 Pericyte

2.2.1 What is pericyte?

Pericyte was first identified by Charles Rouget in 1873 and also called Rouget cell. It was found on the outside of capillaries, both straight portions and branch points of the capillaries (22). The name pericyte was first used by Zimmermann in 1923. He found that the pericytes were around the blood capillaries in various species. They are continuous with the smooth muscle cells of veins and arteries and show numerous processes (23). In addition, pericytes are known as the perivascular cells (24). However,

the other cell types that can inhabit in the perivascular space are macrophages and fibroblasts (25).

Pericytes are found around the pre-capillary arterioles, capillaries, post-capillary venules and collecting venules, particularly abundant at post-capillaries venules. Although, they are related to vascular smooth muscle cells, they can be separated from smooth muscle cells by their location close to the endothelium. In large vessels, these two cell types are separated by connective tissue intima (26).

Pericytes and endothelial cells, share basement membrane and collaborate in many functions, such as control of blood flow, vessel stabilization, angiogenesis, regulation of blood-brain and blood-nerve barriers (23).

2.2.2 Localization and distribution of pericyte

The coverage of microvessels by the pericytes differs with the tissue types and is directly related to the permeability of vessel wall. Pericytes have the highest density in microvessels of the neural tissues, e.g. brain including retina (27). In the brain and retina, the ratio of pericyte-to-endothelial cell is also higher than the other organs (1:3 in the brain vs. 1:100 in the striated muscles) (28). These data are in accordance with the essential role of pericyte in blood–retinal barrier and blood-brain barrier.

Frank et al., 1987 (29) showed that 22–30% of cerebral capillary surface was covered by the pericytes compared with 30-70% in Dalkara et al., 2011 (28) and 99% in Engelhardt et al., 2009 (30).

2.2.3 Morphology of pericyte

The morphology of pericyte may vary in different organs. Typical CNS pericytes are star-shaped cells with round nuclei (compared with elongated cigar-shaped nucleus of endothelial cell) and multiple cytoplasmic processes surrounding the capillary endothelium. Its cytoplasm contains large vacuoles and lysosomes (Figure 3) (24). Moreover, the pericytes in the human brain contain a large amount of acid phosphatase (31).

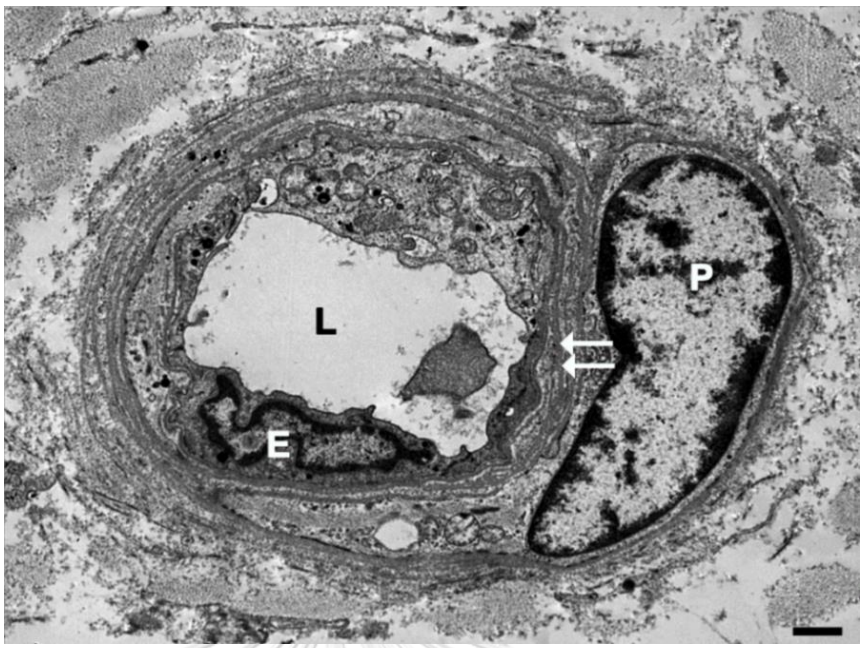


Figure 3 A blood capillary in the human peripheral nerve, showing pericyte lining the abluminal surface of endothelial cell. (P = Pericyte, E = Endothelial cell, L = Capillary lumen, Arrows = Vascular basement membrane) (scale bar = 1 μm)

In the pathological conditions, the pericyte processes may retract from the microvessel wall indicating pericyte migration (24).

2.2.4 Pericyte identification

The study of pericyte is hampered by the lack of pericyte-specific marker. Moreover, expression of markers varies depending on tissues and subpopulations (25). Identification requires a stain series with a combination of negative and positive immunoreactivities. Pericytes can be identified by multiple markers including:

2.2.4.1 Contractile proteins

- **α**-Smooth muscle actin (**α**-SMA is not present in all pericyte e.g. CNS) and not present in endothelial cell. However, vascular smooth muscle cells (VSMA) also express this marker (27).

2.2.4.2 Cytoskeletal proteins (32, 33)

- Desmin
- Vimentin
- Nestin

2.2.4.3 Others (28, 31, 33, 34)

- Platelet-derived growth factor receptor-β (PDGFR-β), a receptor-tyrosine kinase (RTK), located on the cell surface (27), is shown to mark 100% of cultured pericyte (33). It is essential for pericyte recruitment during angiogenesis (35).
- A chondroitin sulfate proteoglycan is also known as neuron-glial antigen 2 (NG2) (27, 33)
- Intercellular adhesion molecule 1 (ICAM-1)
- Vascular cell adhesion molecule 1 (VCAM-1)
- Aminopeptidase A and N
- Regulator of G-protein signaling5 (RGS5): present in the brain pericytes during embryo, but not in adult brain (33).
- Promoter trap transgene xLacZ4
- CD44, CD73, CD90, and CD105 are markers for mesenchymal stem cells.

Nevertheless, there is no ideal specific marker for pericytes in every organ. The expression of markers is also dependent to the stages of development.

2.2.5 Function of pericyte

The endothelial cell form the interior lining of the microvessel wall, while the pericyte encircle and shares the basement membrane with the endothelial cell (26). In addition, one pericyte frequently contacts many endothelial cells, thus may coordinate and integrate responses of the endothelial cells (26).

In the vascular basement membrane between the pericyte and endothelial cell, there are junctional complexes consisting of (Figure 4):

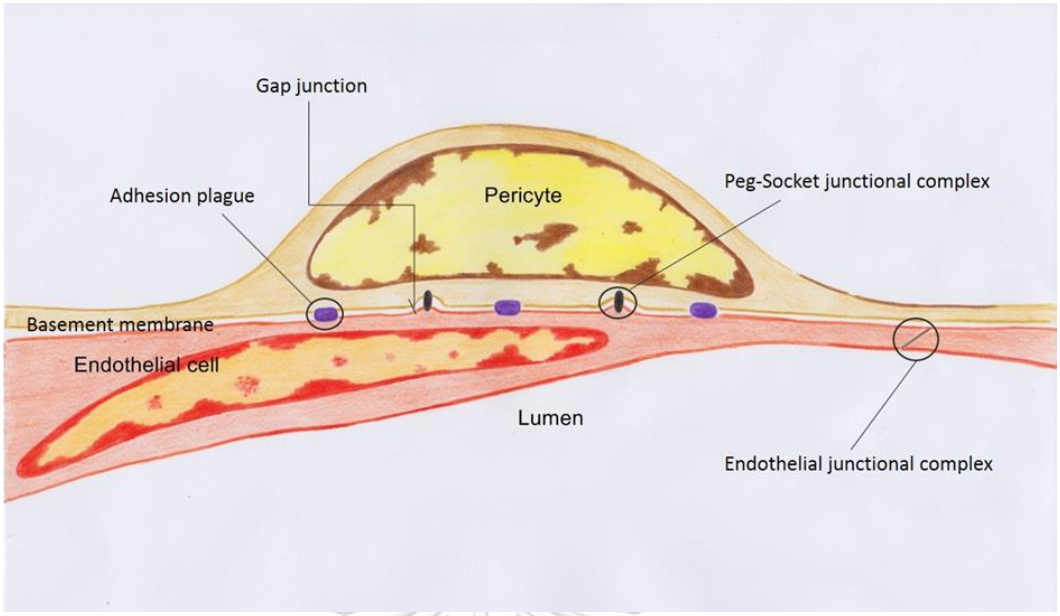


Figure 4 The junctional complexes between the endothelial cell and pericyte in the microvessel.

- Peg-socket junctional complex interactions prevent the pericytes from entering through the basement membrane and contacting other cells and vascular (27).
- Gap junction permits direct connection between the cytoplasm of endothelial cell and pericyte for exchange of small molecules and ions (27).
- Adhesion plaque is responsible for transmission of contractile forces from pericyte to endothelial cell (27) and contains a number of proteins including fibronectin (26).

Therefore, the main functions of pericyte includes (24):

- Contribution to blood-brain barrier (BBB) and blood-nerve barrier (BNB) properties
- Participation in angiogenesis
- Constriction of microvessels: Pericyte can directly control the diameter of capillary, as its presence has been shown to be necessary for constriction (36).
- Immune and phagocytic functions

- Role on hemostasis: After vascular injury, the pericyte may play a role in regulating blood coagulation such as thrombin generation in the microvessel (24). Previous studies show that the pericyte has pro- and anticoagulant activities (31).
- Multipotent cell: Pericyte can differentiate into smooth muscle cells, fibroblasts, macrophage cells, endothelial cells, chondrocytes, and adipocytes, including osteogenic progenitor cells (32, 33, 37, 38). In addition, the multipotentiality of pericyte includes the differentiation into neurons and glial cells (38).

Pericyte may also mediate pathological repair such as, in ischemia. Pericyte constricts microvessels trapping blood cells, which prevents microcirculatory reperfusion after removal of the clot in stroke (39).

Furthermore, when the microvessels lose the pericytes, they hyperdilates and leads to hemorrhage, which finally result in embryonic lethality, diabetic retinopathy and edema (39).

2.3 Barriers in the nervous system

2.3.1 Blood-brain barrier (BBB)

BBB is a specialized system that plays a role in controlling the exchanges between the blood and the CNS. The barrier is comprised of specific physical barrier and transporters which together provide the appropriate extracellular environment of CNS. It protects CNS from toxic materials in the blood and maintains nutrients to neural tissue (24).

The barrier is primarily formed by (Figure 5)

- Endothelial cell
- Astrocyte end-feet
- Vascular basement membrane
- Pericyte

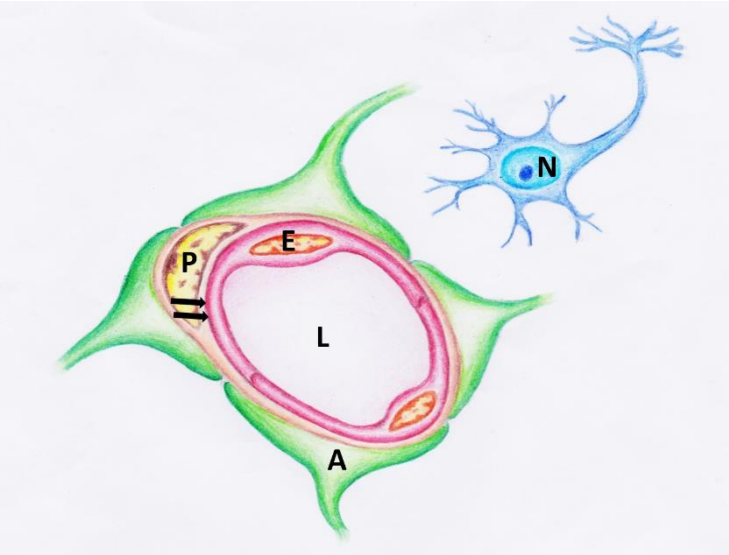


Figure 5 Component of blood-brain barrier (P = Pericyte, E = Endothelial cell, L = Capillary lumen, Arrows = Vascular basement membrane, A = Astrocyte end-feet, N = neuron)

2.3.1.1 Endothelial cell in the BBB

In the brain capillary, endothelial cell is unique from endothelial cell in other organs due to its functional, biochemical and morphological properties (24, 30). In addition, between adjacent endothelial cells, there are tight junctions which limit permeability of the BBB (24).

Claudin-1, -3, -5 and -12 have been reported to be localized at the tight junctions in brain capillary endothelial cells (40). Especially, claudin-5 is the most important component of BBB in terms of function (41).

2.3.1.2 Vascular basement membrane in the BBB

The vascular basement membrane is a crucial part of the BBB. that is located between endothelial cell and pericyte and also pericyte from endothelial cell and astrocyte end-feet. Its maintenance and formation are regulated by endothelial cell, pericyte, and astrocyte. Vascular basement membrane is composed mainly of proteoglycans, elastin, collagen, laminin and fibronectin (24).

2.3.1.3 Pericyte in the BBB

The pericyte has a role in the maintenance and stabilization of BBB (24). Pericyte participates in tightenting the intercellular junctions between brain endothelial

cells by cell-cell interaction. In addition, it also produces the soluble factors including the vascular endothelial growth factor (VEGF) that increases the permeability and the basic fibroblast growth factor (bFGF) that promotes the intercellular junction to tighter (24, 42). Moreover, it expresses protein components of barrier: occludin, zonula occludens 1 & 2 (ZO-1 & ZO-2), junctional adhesion molecule (JAM), claudin-12 (not claudin-5) (43).

2.3.1.4 Blood-brain barrier dysfunction and pericyte involvement

Presently, there are many diseases associated with abnormality of BBB, for example tumors, epilepsy, Alzheimer's and Parkinson's diseases, edema, multiple sclerosis, hypoxia and ischemia (24). Armulik et al., (2010) showed the role of pericyte in BBB by correlating pericyte density with BBB permeability (35). Bell et al., (2010) also presented a correlation between BBB breakdown and loss of pericyte (44).

The permeability of the BBB can be increased due to chemical mediators that are released in pathologic conditions. Moreover, pericyte can release mediators in response to pathology (45).

In neurodegenerative diseases e.g. Alzheimer's disease (AD), the length of capillaries in the brain is reduced which decreases the nutrient transport across the BBB as well as the elimination of waste products from the brain (46, 47). Furthermore, the accumulating β-amyloid in AD is toxic to endothelial cell and pericyte (48).

In traumatic brain injury, Dore-duffy et al., (2000) reported the pericyte migration from the microvascular wall (49). In stroke, Yemisci et al., (2009) demonstrated that, after brain ischemia, pericyte contributed to the blood capillary constriction and accordingly disruption of blood flow (50).

2.3.2 Blood nerve barrier (BNB)

In the peripheral nervous system, BNB is located at the wall of endoneurial blood vessels within the nerve fascicle (Figure 6).

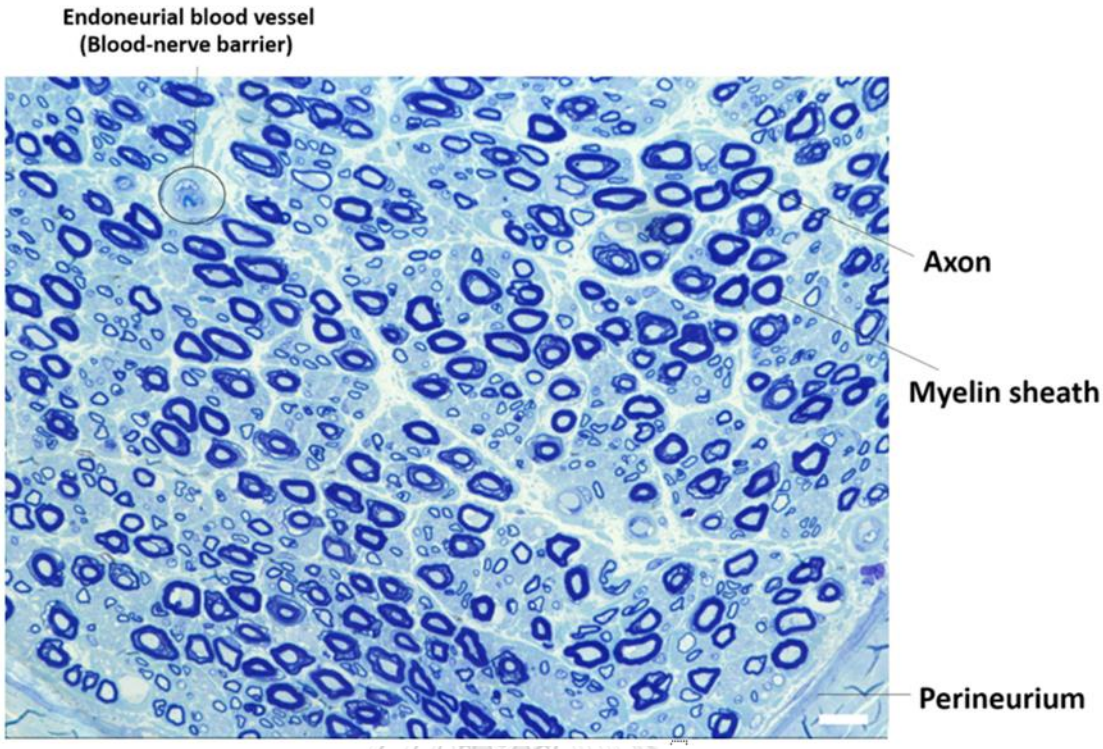


Figure 6 Endoneurial blood vessels (scale bar = 10 μm)

BNB is composed of three main structures (Figure 7):

1. Endothelial cells of endoneurial microvessels
2. Vascular basement membrane shared between endothelial cells and pericyte
3. Pericyte

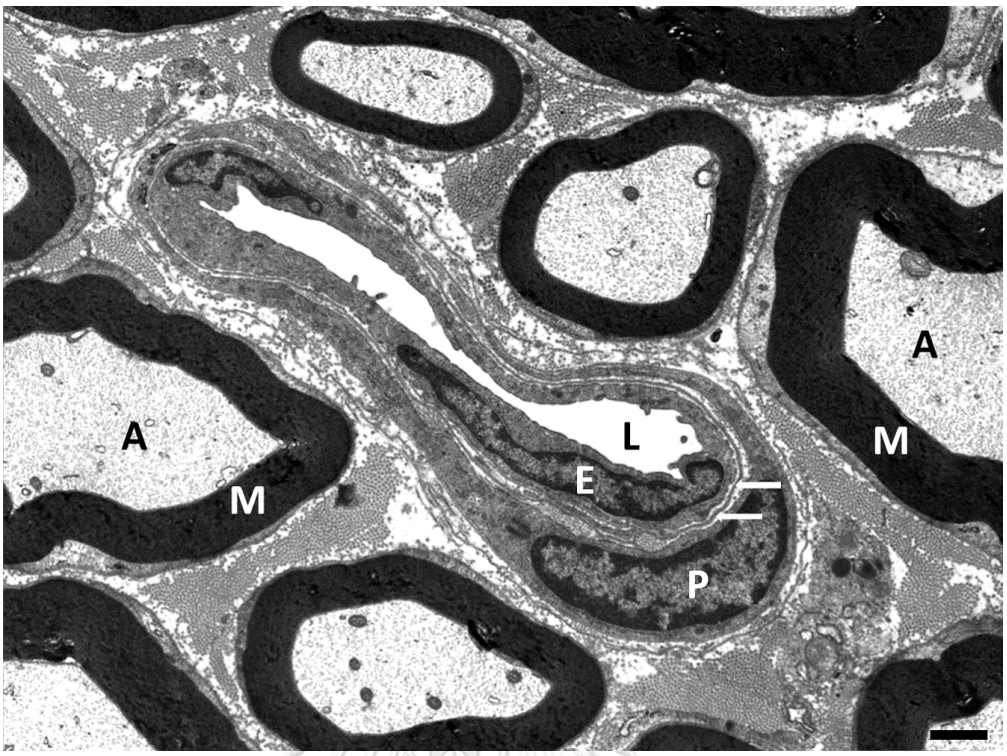


Figure 7 Components of blood-nerve barrier (TEM, rat sciatic nerve) (P = Pericyte, E = Endothelial cell, L = Capillary lumen, Arrows = Vascular basement membrane, A = axon, M = myelin sheath) (scale bar = 1 μm)

2.3.2.1 Endothelial cells in the BNB

The markers of endothelial cells are von Willebrand factor (vWF) and platelet endothelial cell adhesion molecule (PECAM). The endothelial cells also express components of the BNB including occludin, zonula occluden-1 & 2 (ZO-1 and ZO-2), junctional adhesion protein (JAM) and claudin-5 & 12 (43). Kanda et al., (2004) showed the decreased expression of claudin-5 and ZO-1 in the nerve endothelial cells from chronic inflammatory demyelinating polyradiculoneuropathy (CIDP) (10).

2.3.2.2 Vascular basement membrane in BNB

The vascular basement membrane is an essential component of the BNB that is located between endothelial cell and pericyte. The vascular basement membrane is composed mainly of collagen type IV, elastin, proteoglycan, laminin and fibronectin (51).

2.3.2.3 Pericytes in the BNB

The pericytes have been shown to express mRNAs for occludin, ZO-1&2, JAM and claudin-12 but not claudin-5 (43, 52). In addition, pericytes secret growth factors including basic fibroblast growth factor (bFGF), vascular endothelial growth factor (VEGF), glial cell line-derived neurotrophic factor (GDNF), Angiopoietin 1 (Ang1), and transforming growth factor beta (TGF-β) (14, 52). These growth factors regulate the claudin-5 expression in endothelial cells affecting the BNB functions (43).

The BNB maintains the endoneurial microenvironment by controlling exchange of materials between the endoneurial and intravascular spaces (53). Moreover, it also blocks the entry of toxic mediators, e.g. chemokines, cytokines, and immunoglobulin from the circulatory blood (53, 54). The endoneurial homeostasis is likely important for normal functions of axons and associated Schwann cells (53).

As described above, the BNB breakdown might play pathophysiological roles in the onset of various immune-mediated neuropathies, including chronic inflammatory demyelinating polyradiculoneuropathy (CIDP), multifocal motor neuropathy (MMN), and Guillain-Barre syndrome (55). Pathological breakdown of BNB can be divided into two steps (55). Firstly, paracellular leakage of soluble mediators occurs through the disruption of tight junctions. Then, transcellular entrance of inflammatory T cells across nerve microvascular endothelial cells (PnMEC) due to the increase of adhesion molecules on these cells. Moreover, in other causes of neuropathy, Giannini et al., (1995) showed that pericyte loss and degeneration with basement membrane thickening are found in diabetic neuropathy (9).

2.4 Cisplatin

2.4.1 What is cisplatin?

Cisplatin (*cis*-diamminedichloroplatinum or *cis*-DDP) is an antineoplastic agent for the treatment of cancer of various organs including testis, ovary, lung, head and neck (56). After intravenous administration, cisplatin undergoes diffusion to nearly all organs with the particularly high levels in kidney, bone, skin, uterus, ovary, and liver. In the nervous system, cisplatin shows the highest concentration in dorsal root ganglion (DRG) (5).

2.4.2 Mechanism of action

Cisplatin is a derivative of platinum, which comprises NH_3 and Cl groups (Figure 8).

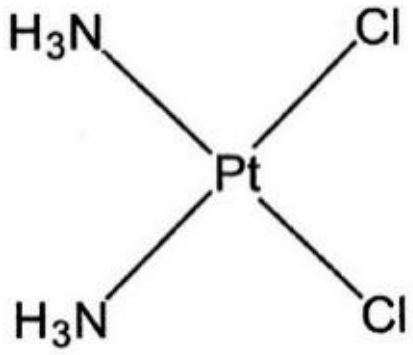


Figure 8 Structure of cisplatin

Cisplatin enters the cell by passive diffusion because the concentration of chloride ions outside is higher than inside the cell (57). In another way, the previous study presented a direct link between the uptakes of platinum and copper. These two metals have the same transporter, copper transporter 1 (58). Copper transporter 1 is a transmembrane protein participated in copper homeostasis and plays a key role in the cisplatin uptake as well (56). However, the efflux of cisplatin needs the copper-transporting P-type adenosine triphosphate (ATP7B), it contributes to cisplatin resistance (58, 59).

Inside the cell, cisplatin is converted to the aqueous form, due to the lower intracellular concentration of chloride ions which facilitates ligand exchange of chloride for water (59). In addition, the aqueous form of cisplatin is very reactive toward DNA in the nucleus (60). Therefore, the primary target of cisplatin is DNA.

Inside the nucleus, the platinum atom of cisplatin forms covalent adduct to the N7 positions of guanines or adenine of DNA to form intrastrand and interstrand crosslinks (61). The formation of cisplatin DNA-crosslink distorts the structure of DNA and normal transcription and replication (60). Previous study identified proteins which specifically bind to cisplatin-damaged DNA, as high mobility group (HMG)-domain

proteins (62). HMG-domain proteins are a class of non-histone chromosomal proteins which bind cisplatin-DNA adducts both in vivo and in vitro. This binding plays an important role in the cisplatin-induced cell death (61). The main role of HMG-domain proteins is disruption of transcription machinery leading to cell death (61). Furthermore, the cisplatin-DNA adducts become unrecognisable by the repair machinery when the HMG-domain proteins attach to them. Therefore, these DNA damage and dysfunction are likely the mechanisms responsible for cancer cell apoptosis induced by cisplatin (Figure 9).

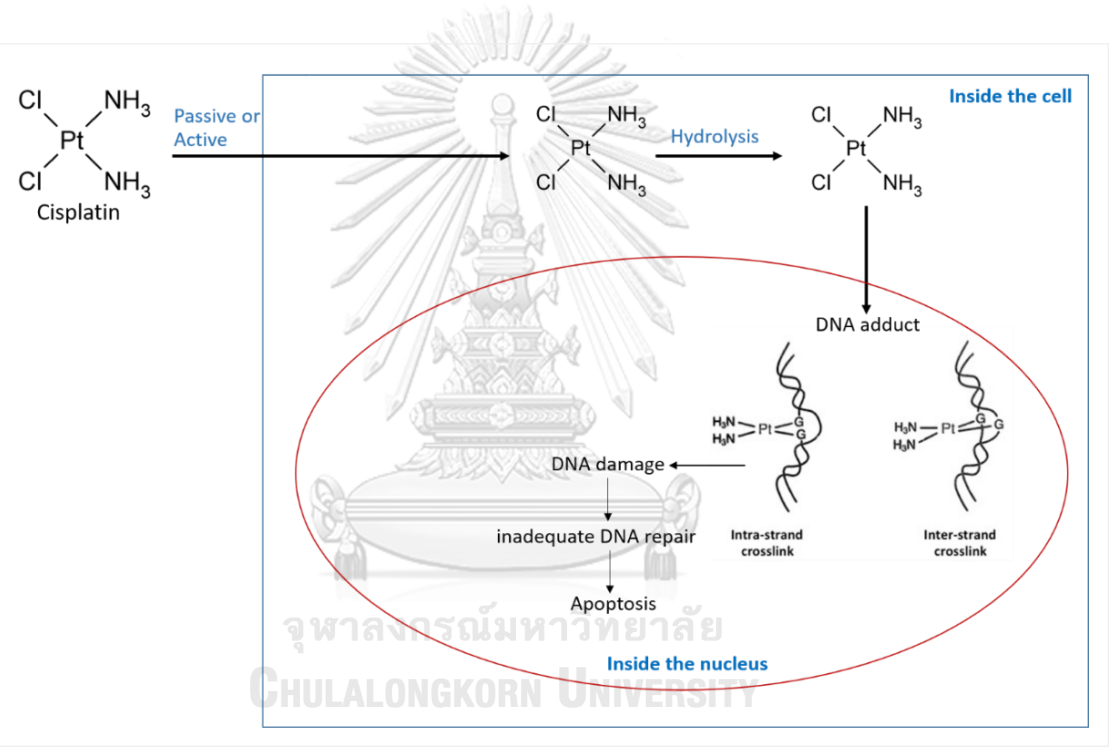


Figure 9 Mechanism of cisplatin (57)

2.4.3 Side effects of cisplatin

Several side effects of cisplatin are observed e.g. nephrotoxicity, ototoxicity due to damage of hair cells in organ of Corti, nausea and vomiting and neurotoxicity. Accordingly, the treatment can be limited by these side effects. Neurotoxicity is one of important dose-limiting toxic effects of cisplatin, resulting in peripheral neuropathy (63).

2.4.4 Cisplatin-induced neuropathy

Abnormalities of peripheral neuropathy induced by cisplatin include motor, sensory and autonomic dysfunctions (3). However, the sensory symptoms, such as loss of proprioception, loss of pain/temperature perception, paresthesia, dysesthesia, hyperesthesia, predominate. This might be explained by the fact that cisplatin highly accumulates in the DRG (5).

This preference of cisplatin towards primary sensory neurons compared with motor neurons may be because they are located outside the BBB of CNS. Furthermore, the presence of capillary fenestrations in the DRG might allow the platinum agents to penetrate into the DRG (64). In contrast, the ventral horn of the spinal cord contains the cell bodies of motor neurons protected by the BBB.

The potential mechanisms of cisplatin-induced neuropathy include:

2.4.4.1 Nuclear DNA damage in DRG neurons

DNA damage-induced neuronal apoptosis of DRG neurons is probably the major cause of neurologic symptoms. The global transcriptional inhibition of DRG neurons is the main consequence of the formation of DNA adducts, resulting in DNA damage (Figure 9) (64). However, the process for removing platinum DNA adducts and for repairing DNA damage is nucleotide excision repair (NER) (56). Thus, the ability of DNA repair mainly by NER is a main factor determining the severity of neurotoxicity (64).

In addition, the studies in DRG neurons showed that apoptosis of neurons was preceded by abnormal re-entry into G0 phase of the cell cycle (65, 66). Cisplatin might induce apoptosis via activation of p53 leading to induction of Bax. The Bax binds to the mitochondrial membrane stimulating cytochrome *c* release and induces caspase-3 and caspase-9 activation, resulting in apoptosis of DRG neurons (67).

2.4.4.2 Mitochondrial DNA damage

Furthermore, cisplatin may also directly bind to mitochondrial DNA, and inhibits transcription and replication of mitochondrial genes. This might result in reduction of proteins essential for mitochondrial functions. Therefore, cisplatin may induce mitochondrial DNA damage causing decreased energy generation (68). This

abnormality might cause the impairment of the axonal transport of organelles and synaptic function. Russell et al., (1995) showed that the cisplatin impaired axonal transport which resulted in the reduction of both number and velocity of the organelles moving in the anterograde and retrograde directions (69). Apart from energy production failure, oxidative stress likely occurs after mitochondrial damage (64). As a result, oxidative stress has been shown to play role in cisplatin neuropathy (70) and antioxidants have demonstrated beneficial effects (this will be discussed later in the treatment section).

In the peripheral nerves, according to recent findings, cisplatin can alter the expression of mitochondrial fusion and fission proteins. These proteins regulate number, shape, and size of mitochondria. Bobylev et al., (2018) presented that mitochondrial fusion protein in tibial nerves and DRG neurons significantly decreased in the cisplatin-treated mice, resulting in vacuolization and swelling of mitochondria (71).

2.4.4.3 Enhanced reactivity of thermosensitive transient receptor potential (TRP) channels

The enhanced exhibition and response of transient receptor potential ion channels: transient receptor potential melastatin 8 (TRPM8), transient receptor potential vanilloid 1 (TRPV1), and transient receptor potential ankyrin-1 (TRPA1) play a main role in the development of platinum-induced neuropathy (64). These transient receptor potential channels all play an important part in the sensation, especially generation of inflammatory and neuropathic pain.

The previous studies demonstrated that the DRG neurons in mice treated with cisplatin showed an increase in TRPA1, TRPV1, and TRMP8 mRNA expression and this was related with enhanced mechanical and heat hypersensitivity (72).

2.4.4.4 Cisplatin-induced neuropathy in human

Cisplatin chemotherapy usually develops asymptomatic and clinically detectable sensory neuropathy (73). Pain and/or paresthesia as signs of small sensory fiber dysfunction are symmetric and usually worse distally (70).

Signs of large fiber loss: the reduction of vibration, proprioception and diminish or absence of muscle stretch reflexes, are also found (74). Sensory ataxia may be observed in those patients with severe neuropathy (73). In sural nerve biopsies from patients receiving high dose cisplatin, the pathologic changes showed decreased number of large myelinated nerve fibers (75).

Moreover, the entire platinum compounds produce "coasting effect" in which the neuropathy can progress for a week or several months after the cessation of treatment (3, 76). Thus, neurotoxicity is a major reason that cisplatin is discontinued or its dose is limited, resulting in reduced chemotherapeutic efficacy. For this reason, it is important to study the molecular mechanisms of cisplatin-induced neuropathy**.**

2.4.4.5 Cisplatin-induced neuropathy in rats

In the experimental animal, the cisplatin-induced neuropathy is characterized by involvement of sensory neurons in the DRG.

Neurophysiologic studies presented cisplatin decreased the amplitude of nerve action potentials and the conduction velocities of sensory nerves with little motor involvement (77).

After repeated cisplatin injections, behavioral assessments showed sensory (thermal hypoalgesia) which the latency of the response to a fixed pain stimulus was increased (7, 78).

Furthermore, the motor and proprioceptive functions were evaluated by using rota-rod test, the result showed impairment in coordination (78-80).

As for structural changes, several previous studies presented that administration of cisplatin 2 mg/kg twice a week for five weeks in the rats resulted in the decrease of somatic, nuclear and nucleolar areas of DRG neurons (6, 8, 80-82). Moreover, Tredici et al., (1999) and Wongtawatchai et al., (2009, 2012) showed that the number of DRG neurons in rats treat with cisplatin tended to decrease compared with that of untreated rats (7, 8, 83). Nevertheless, decreased number of DRG may not be caused by apoptosis because the marker of apoptosis was not increased after cisplatin treatment (84). This issue needs more studies.

In nerve, loss of large myelinated fibers, decrease in myelinated fiber density, decrease in axonal diameter, and axonal degeneration were observed (7, 8, 74, 79, 84). Moreover, Wongtawatchai et al., (2009, 2012) showed significantly decreased myelin thickness (8, 83).

2.4.5 Effect of cisplatin on cell culture

In vitro, the previous study showed cisplatin-induced apoptosis in neuroblastoma cells (SH-SY5Y) (85). In the same way, Rathinam et al., (2015) presented cisplatin-induced cytotoxicity leading to apoptosis in HK2 (epithelial cell line from human kidney), SH-SY5Y and UBOC1 (organ of Corti) cells (86).

In the embryonic sensory neuronal cell line (50B11s), there was increased expression of caspase 3 as well as a reduction in vascular endothelial growth factor-A (VEGF-A) expression following cisplatin treatment at the dose of 5 μg/ml (87). Moreover, cultured rat DRG neurons were exposed to 2μg/ml cisplatin which induced degradation of mitochondria and extensive mitochondrial vacuolization (68). Santin et al., (2012) presented the organelle changes including endoplasmic reticulum dilation, Golgi apparatus with abnormal cisternae, reduced number of lysosomes and dense perinuclear masses of mitochondria after cisplatin treatment in neuroblastoma cells (88).

2.4.6 Treatment of the cisplatin-induced neuropathy

To date, many potential agents have been tested in animals and humans treated with cisplatin.

2.4.6.1 Growth factors

Exogenous administration of nerve growth factor (NGF) is able to recover functional, structural, and biochemical changes induced by cisplatin (89). Brain-derived neurotrophic factor (BDNF) has been demonstrated to enhance survival of differentiated neuroblastoma cells after cisplatin treatment (90).

Leukemia inhibitory factor (LIF) is effective in correcting some functional (prolonged heat latency of tail flick test) and morphological deteriorations (small nuclear and somatic sizes of neurons) (84).

These results suggest that decrease of growth factors may play a role in cisplatin-induced neuropathy and exogenous administration of growth factors may prevent or diminish cisplatin neurotoxicity. However, although growth factors have been successful in animal models, they are less effective in human showing side effects and are of concern for their potential to promote cancer. Therefore, further human study has not been conducted.

2.4.6.2 Neuroprotective agents

Acetyl-L-Carnitine (ALC) has a neuroprotective property including the stabilization of intracellular membrane. In the animal model, following cisplatin treatment, ALC was able to reduce the mechanical nociception (91). Another study showed that the ALC co-treatment was able to significantly decrease the neurotoxicity of paclitaxel and cisplatin (82). Similarly, nerve conduction velocity was recovered and the mechanical nociceptive threshold was supported by ALC injection (92). Moreover, in the human study, ALC prevented progression and reversed symptoms during cisplatin treatment (93).

Amifostine is a cytoprotective drug that can diminish the toxicity of platinum compounds. In head and neck cancer patients, the combination of cisplatin and amifostine administration reduced subclinical neurotoxicity (94).

Erythropoietin has a wide range of neuroprotective effects. In the previous studies, erythropoietin was shown effective against cisplatin-induced peripheral neuropathy (95, 96).

The above agents need further human studies to confirm their clinical effectiveness.

2.4.6.3 Antioxidants

Glutathione (GSH) is also effective for the prevention of cisplatin-induced neuropathy. Based on the assessment of the sural, ulnar, and median sensory nerve conduction, GSH treatment was associated with a statistically significant improvement in these values (97). The neurophysiological and pathological changes induced by cisplatin administration were less severe in rats co-treated with GSH (97,

98). Beneficial effects of GSH on cisplatin neuropathy were also demonstrated in patients (99).

N-acetylcysteine (NAC) is a precursor of glutathione which can block cisplatin-induced apoptosis. It has an inhibitory effect on cisplatin-induced p53 accumulation but not Fas/Fas-L accumulation (100).

Vitamin A or retinoic acid which is the active metabolite of vitamin A has neurotrophic effects via upregulation of nerve growth factor (NGF) and antioxidant properties. However, Tredici et al., (1998) demonstrated retinoic acid had a mild effect on electrophysiological abnormalities and morphometric changes in DRG neurons (78).

As for vitamin E, Bove et al., (2001) demonstrated that decrease in plasma vitamin E level was found in patients with severe cisplatin neuropathy (101). However, Albers et al., (2014) showed that vitamin E could not limit or prevent the neurotoxicity of cisplatin in patients (73). Therefore, data regarding the effect of vitamin E on cisplatin neuropathy are still inconclusive.

Polyphenols are micronutrients with antioxidant activity. Curcumin is a polyphenol in the root of *Curcuma longa* L. (Zingiberaceae family), commonly known as turmeric. It is commonly used as a spice. Moreover, use in medicine, with the anti-inflammatory, anti-oxidant, anti-aging, antiproliferative properties including neuroprotection is also documented (15). Therefore, it is effective against several human diseases e.g. neurological diseases, arthritis, diabetes, cardiovascular diseases, cancer, and Crohn's disease (16). Recently, in models of Alzheimer's disease, the curcumin has also been found to bind to Beta-amyloid proteins (102). Several human and animal studies suggest that the curcumin is very safe even at high doses (16).

Particularly, curcumin exhibits strong antioxidant activity. The protective effect of curcumin was attributed to its antioxidant properties by blocking the formation of reactive oxygen species (ROS) including superoxide anion radicals, hydroxyl radicals, and nitrogen dioxide radicals (15). Moreover, it was also reported to inhibit lipid peroxidation (103, 104).

In an animal model of focal cerebral ischemia, curcumin caused significant reduction of lipid peroxidation and increase in endogenous antioxidant defense enzymes after ischemia (105).

In spinal cord injury, the study on effects of curcumin in rats showed that curcumin efficiently protects tissues of spinal cord against oxidative damage by significantly increased serum SOD levels after spinal cord injury (106). Curcumin inhibited neuronal loss and apoptosis, and significant improvement in neurologic deficit after spinal cord hemisection in rats (107).

In peripheral nerve injury, Al Moundhri et al., (2012) reported the protective effect of curcumin with improved motor recovery and histology of sciatic nerve (108). Agthong et al., (2015) showed that the curcumin could improve the structural changes in sciatic nerve and DRG neurons in rats with cisplatin-induced neuropathy (6). In addition, the curcumin also ameliorated the reduction of myelin thickness in the sciatic nerve in cisplatin-induced neuropathy (6).

In the cisplatin-induced neurotoxicity, the treatment of PC12 (pheochromocytoma) cells with 10 μg/mL cisplatin showed significant reduction in neurite outgrowth and curcumin could partially reverse this effect (109). These results indicate that curcumin can protect against cisplatin toxicity to neurites of PC12 cells which might explain its beneficial effects on cisplatin neuropathy.

In conclusion, curcumin shows a diversity of beneficial effects and possesses significant antioxidant property. It is effective against several human diseases including neurological diseases. In particular, it has shown effectiveness against experimental cisplatin neuropathy. Nevertheless, the effects of curcumin on endothelial cell, pericyte, and vascular basement membrane in the peripheral nervous system and effects on the expression of tight junction proteins synthesized by pericytes after cisplatin administration have not been studied.

2.4.6.4 B vitamins

The B vitamins are a group of water-soluble compounds. They function as coenzymes, or as part of coenzymes, thereby implicating in the metabolic pathways such as energy metabolism (17, 18). Their collective effects on various aspects of

the functions of the nervous system include energy production, synthesis of neurotransmitters and signaling molecules, genomic and non-genomic methylation, neuronal membrane synthesis and DNA/RNA synthesis/repair (17). Due to their essential roles in the nervous system, they have usually been prescribed to patients with neurological diseases.

The B vitamins have been applied for peripheral neuropathy, especially thiamine (B1), pyridoxine (B6) and cyanocobalamin (B12). Deficiency in B1, B6, and B12 is associated with nerve damage and dysfunction that can cause peripheral neuropathy (110).

1. Thiamine (B1)

B1 plays a role as coenzyme precursor in metabolic functions and a structural component in the membranes of central and peripheral nervous system. B1 is converted to thiamine pyrophosphate in the brain that is a coenzyme in glucose metabolism (111). Especially, thiamine pyrophosphate plays an important role in the nerve impulse transmission (18). It acts as a maintenance of cell membrane stability and the deficiency causes nerve conduction impairment (112).

2. Pyridoxine (B6)

Pyridoxine acts as a co-factor in many metabolic processes and also as an antioxidant.

Pyridoxine is converted to pyridoxal phosphate that functions in the metabolism of glucose, fatty acids, amino-acids, and synthesis of neurotransmitters including serotonin, epinephrine, norepinephrine, dopamine, and gamma-aminobutyric acid (GABA) (113). In addition, pyridoxal phosphate is required for the sphingolipid synthesis for the formation of myelin as well (18, 114).

The studies reported the antioxidant properties of pyridoxine, including prevention of hydrogen peroxide (H_2O_2)-induced formation of oxygen radical and lipid peroxidation in U937 monocytes (115). Moreover, reduction of vitamin B6 has been correlated with an increased susceptibility to oxidative stress (116).

Pyridoxine deficiency produces a length-dependent neuropathy. The early signs of neuropathy include numbness or burning pain and neurological examination shows reduced distal sensation and deep tendon reflexes (117).

Moreover, deficiency of B6 can cause neurological symptoms: depression, headaches, convulsions, and confusion (118).

3. Cobalamin (B12)

Cobalamin is converted to methylcobalamin that is essential in cell replication and cell growth (18). Methylcobalamin plays a role in neurotransmitter metabolism (119). In addition, it also participates in the synthesis of nucleoproteins and myelin. Methylcobalamin has been shown to promote the synthesis of lecithin, one of the main components of myelin sheath (120).

B12 deficiency induces neurological impairments due to the disruption of myelin sheath (121). The recent study has presented that B12 was rapidly decreased during chemotherapy treatment (122).

Furthermore, the level of B12 was lower in the injured nerve compared with the intact nerve suggesting that B12 supplementation might augment nerve repair (123).

The B vitamins have no side effects when given in appropriate doses, the dose of B vitamins is under the Recommended Daily Allowance (RDA) in adults, thiamine 1.2 mg/day, pyridoxine 1.3 mg/day, and cobalamin 2.4 μg/day (17). It is unclear whether the appropriate dose for treatment is similar or should be higher than the RDA recommendation for specific vitamin B types. In addition, the appropriate treatment duration and the effects of combined different B vitamins for treatment of peripheral neuropathy are also not known.

Excessive intake of B vitamin is normally harmless as they are excreted in the urine. There are no known toxic effects in a high dose of thiamine and cobalamin, but pyridoxine overdose can cause neurotoxicity by presenting as ataxia and peripheral neuropathy (124, 125).

Cochrane review reported that vitamin B was usually used to treat the peripheral neuropathy in human but its effectiveness was not clear (18). However, recent evidence seems to support the efficacy of B vitamins, Brito et al., (2016) reported that the B12 treatment of deficient, asymptomatic, elderly Chileans improved the myelinated nerve conduction (126). Rispoli et al., (2017) presented that when 30 patients with Parkinson's disease using Levodopa-Carbidopa intestinal

gel (LCIG) infusion received early and continuous B vitamins integration, after a long follow-up, they reported a low rate (19%) of newly developed peripheral neuropathy that remained stable. (127).

In animal studies, a mixture of B1, B6, and B12 increased the antinociceptive effect of nonsteroidal anti-inflammatory drug in rats with carrageenan-induced hyperalgesia (128). B1, B6, and B12 vitamins had antinociceptive effect via inhibition of the action and/or synthesis of the inflammatory mediators in the formaldehyde-induced pain (129). Moreover, B1, B6, and B12 vitamins are shown to alleviate neuropathic pain and improved sensory nerve conduction in diabetic rats (130).

In the neuropathic pain, B1, B6 and B12 vitamins significantly reduced thermal hyperalgesia caused by loose ligation of the sciatic nerve (131). In diabetic peripheral neuropathy, treatment with vitamin B complex prevented the damaging effects of hyperglycemia on the sciatic nerve and preserved the normal structural characteristics of the perineurium, Schwann cells, myelin sheath, nerve fibers, and blood capillaries (132).

In vitro, vitamin B6 promoted the antineoplastic activity of cisplatin in non-small cell lung carcinoma (NSCLC) cells (133) and metabolism of vitamin B6 influenced the response of NSCLC to the cisplatin DNA-damage (134). Nevertheless, the effects of B vitamins on cisplatin-treated normal cells including pericytes remain unstudied.

Our pilot data indicated that vitamin B1-6-12 were able to ameliorate hind-paw thermal perception and reduced a number of DRG neuronal loss on cisplatin-induced neuropathy in rats. However, the effects of vitamin B1-6-12 on endothelial cell, pericyte, and vascular basement membrane in the peripheral nerve and effects on the expression of tight junction proteins synthesized by pericytes after cisplatin administration have not been studied.

2.4.7 Effect of cisplatin on endothelial cell, pericyte and blood-nerve barrier

Currently, there are few studies on the effect of cisplatin on pericytes in the peripheral nervous system. The previous study showed that patients treated with cisplatin chemotherapy have increased incidence of arterial occlusion (135). Furthermore, in patients treated with cisplatin-based chemotherapy with germ cell tumor had damaged endothelial cells (12).

In the rat model, administration of cisplatin 2 mg/kg/week for 9 weeks decreased nerve blood flow, number of vasa nervorum associated with marked endothelial apoptosis (13). Moreover, in the albino guinea pigs treated with cisplatin there were changed in the vascular endothelium with swelling or shrinkage of mitochondria and presence of lipid bodies in the cytoplasm of endothelial cells (136).

In vitro, cisplatin 1-10 µg/mL can up-regulate the expression of intercellular adhesion molecule (ICAM-1) in human umbilical vein endothelial cells (HUVECs) and induce vascular endothelial injury (137, 138). These results indicate that ICAM-1 involves in the pathophysiologic process of cisplatin-induced vascular toxicity. Furthermore, cisplatin induced dysfunctions of microvascular endothelial cell (HMEC-1) concerning fibrinolysis, inflammation and proliferation (139).

Results from recent work in our lab showed that density of pericyte around capillaries in the sciatic nerve was reduced in the cisplatin-treated compared with that of the control rats, especially in the distal part. EM analysis demonstrated the separation of pericytes from endothelial cells including the disruption of basement membrane in the sciatic nerve from the cisplatin group. These data indicate the loss of pericyte and structural abnormalities of the BNB in cisplatin neuropathy. Moreover, BNB protein components were determined using Western blot analysis. Zonula occludens-2 (ZO-2) expression tended to decrease in the nerves of the cisplatin group. This data indicate that cisplatin might adversely affect pericytes and BNB.

Taken together, curcumin and B vitamins have shown beneficial effects on functional and structural changes of nerve fibers in experimental cisplatin neuropathy. However, their possible effects on the endothelial cell, pericyte and vascular

basement membrane have not been investigated. Therefore, this study aims to examine the ultrastructural changes in the PNS of rats receiving vehicle, cisplatin only and co-treatment of cisplatin with either curcumin or vitamin B1-6-12 using TEM.

In addition, it is unknown whether curcumin and vitamin B1-6-12 have any effects on the expression of tight junction proteins synthesized by pericytes treated with cisplatin. Thus, this study also aims to evaluate changes in the expression of tight junction proteins in the cultured pericytes receiving cisplatin only and co-treatment of cisplatin with either curcumin or B1-6-12 using Western blot analysis.

CHAPTER III

MATERIAL AND METHOD

3.1 Research Methodology

3.1.1 Animal specimens

The specimens from two previous experiments were used in this study.

3.1.1.1 Experiment 1 Effect of curcumin on cisplatin-induced neuropathy in rats.

This experiment was done by Mr. Thanthawat Charoensap. The results have been published (6).

- **Materials and methods**

1. Animal experiment

Twenty female Wistar rats weighing 200 to 250 g each were used.

2. Drug administration

The rats were divided into 3 groups which were control (C, n=6), cisplatin (P, n=7) and cisplatin + curcumin (S, n=7) groups. The cisplatin and cisplatin + curcumin groups received cisplatin (Pfizer, 50 mg in 50 ml) at a dose of 2 mg/kg. Cisplatin was diluted in normal saline to the 0.5 mg/ml concentration. The rats received the intraperitoneal injection (IP) of cisplatin twice a week for five consecutive weeks to induce neuropathy (20 mg/kg accumulative dose). This dosage regimen of cisplatin has been shown to induce both functional and structural abnormalities of peripheral nerve (140).

In the co-treatment group, curcumin administration (Cayman Chemical, Cat. No. 81025) 200 mg/kg dissolved in 1% sodium carboxy methyl cellulose (SCMC) (Sigma, Cat. No. 419273) was given by gavage during the cisplatin treatment to the cisplatin + curcumin group once daily for five weeks. The cisplatin group was received the vehicle (1% SCMC) and the control group received the vehicles for curcumin and cisplatin. After five weeks, all rats were left untreated until the eighth weeks, and then sacrificed.

3. Assessment of hind-paw thermal perception using hot plate test

The sensory function was determined by using the hot plate test. Each animal was placed on the hot plate analgesia meter where a constant temperature of 55^{o}C was maintained. Time from the start when the hind paws of the rats touched the hot plate until lifted to lick was recorded. This time was defined as the withdrawal latency.

4. Sciatic motor nerve conduction velocity

Motor nerve conduction velocity (MNCV) test was examined in the sciatic nerve. The compound muscle action potential (CMAP) is recorded from foot muscles and analyzed for MNCV.

5. Tissue collection

At the 8th week, the rats were sacrificed by overdose anesthesia with isoflurane and underwent intracardiac perfusion with normal saline 200 ml followed by 4% paraformaldehyde (PFA) 400 ml. The DRG (L4-6) and sciatic nerve were removed and post-fixed in 3% glutaraldehyde.

6. Nerve morphometry

The nerve sections were cut to a thickness of 1 mm, mounted on slides, and stained with 1% para-phenylenediamine. A light microscope was used to examine the sections. Images of the fascicles in the section were imported to the computer and the total fascicular area (mm^2) was calculated under a 4x objective len. For the three-window sampling procedure, only large fascicles enough to contain three windows of 0.012 mm^2 were further analyzed. Three window areas were positioned at random, one in the center and the other two on the periphery of fascicle. Under a 40x objective lens, the windows were imported to the computer. Only nerve fibers completely located in the window was examined. Using the Image Pro-Plus software, each window was analyzed for myelin thickness, the number of myelinated fibers, axon diameter, and g ratio.

7. DRG morphometry

Toluidine blue was used to stain the DRG sections, which were cut at 2 μm thickness. To prevent counting the same neuron, one out of every twenty sections was chosen. The selected sections were included for analysis**.** All neurons in the section with a prominent nucleus and nucleolus were counted to estimate the total number of neurons per DRG, and this was done for all selected sections. Then, the total number of neurons in the whole DRG was then estimated using the number of counted neurons. In addition, using the Image-Pro Plus software, at least 300 neurons from each DRG were randomly analyzed for nucleus and nucleolus areas.

- **Results**

The mean body weight (BW) of the control group was continuously increased over the study period. Conversely, at the 3rd week after the start of cisplatin administration, BW of the cisplatin and curcumin-treated groups was significantly reduced compared with that of the control group and remained lower until the end of the experiment.

Hind-paw thermal threshold

In the fifth week, the cisplatin group had the significantly longer latency than the other groups, indicating thermal hypoalgesia.

Motor nerve conduction velocity

In the fifth week, the cisplatin group had a significantly lower sciatic MNCV than the control. Moreover, in the eighth week, the cisplatin group still had significantly lower MNCV than the control group. In addition, the MNCV of the control and curcumin-treated groups were not significantly different.

Nerve morphometric study

In the cisplatin group, there was slightly decreased myelin thickness compared with the control group with no significant difference. In the curcumin-treated group, the myelin thickness was similar to the control group.

DRG morphometric study

The nuclear and nucleolar areas were significantly decreased in the cisplatin group when compared with those of the controls. The nucleolar area was significantly higher in the curcumin-treated group when compared with that of the cisplatin group. The total number of L4 DRG neurons in the cisplatin group was significantly lower than that of the control group. The number of neuron in the curcumin-treated group was between the values of the control and cisplatin groups.

Taken together, the results above indicate that cisplatin-treated rats had neuropathy which was alleviated by curcumin treatment.

3.1.1.2 Experiment 2 Effects of B1-6-12 on cisplatin-induced neuropathy in rats. The results have been published.

- **Materials and methods**

1. Animal experiment

Thirty female Wistar rats weighing approximately 250 g each were used.

2. Drug administration

The rats were divided into 5 groups which were control (C, n=6), cisplatin (P, n=6), cisplatin + low-dose B1-6-12 (n=6), cisplatin + medium-dose B1-6-12 (n=6) and cisplatin + high-dose B1-6-12 (n=6) groups.

The cisplatin (P) and cisplatin + B1-6-12 groups received cisplatin (Pfizer) at a dose of 2 mg/kg. Cisplatin was diluted in normal saline to the 0.5 mg/ml concentration. The rats received the intraperitoneal injection (IP) of cisplatin twice a week for five consecutive weeks to induce neuropathy (20 mg/kg accumulative dose).

In the co-treatment groups, B1-6-12 included B1 (Sigma, Cat. No. 67038), B6 (Sigma, Cat. No. 58560) and B12 (Sigma, Cat. No. 68199), B1-B6-B12 (100:100:1) dissolved in normal saline and were given by gavage during the cisplatin treatment once daily for five weeks. Low-dose, medium-dose and high-dose B1-6-12 groups received 100, 300 and 600 mg/kg/day, respectively. The cisplatin and the control groups received the vehicles for B1-6-12. This proportion of B1-6-12 were used according to the previous studies (128, 130).

3. Assessment of hind-paw thermal perception using hotplate test

The procedures were the same as in the experiment 1.

4. Tissue collection

At the 5th week, the rats were sacrificed. The procedures were the same as in the experiment 1.

5. Nerve morphometric study

The procedures were the same as in the experiment1.

6. DRG morphometric study

The procedures were the same as in the experiment1.

- **Results**

The mean body weight (BW) of the control group was continuously increased over the study period. Conversely, at the 3rd and 5th weeks after the start of cisplatin administration, BW of all the groups receiving cisplatin was significantly reduced compared with that of the control.

Hind-paw thermal threshold

In the fifth week, the cisplatin group had a significantly longer latency than the other groups, indicating thermal hypoalgesia. Furthermore, the latency of the low-dose B1-6-12 group was close to that of the cisplatin group and significantly longer than that of the control groups. However, the medium-dose and high-dose B1-6-12 groups had significantly shorter latencies than the cisplatin group and not statistically different from the control group.

Nerve morphometric study

In the fifth week, the cisplatin group had significantly decreased fiber diameter compared with the control and medium-dose B1-6-12 groups. Moreover, the cisplatin group had significantly increased fiber density compared with the control and medium-dose B1-6-12 groups. Therefore, in the medium-dose B1-6-12 group, the fiber diameter and fiber density were similar to the control group. In the cisplatin, low-dose, medium-dose, and high-dose B1-6-12 groups, the myelin thickness tended to decrease with no significant difference from the control group.

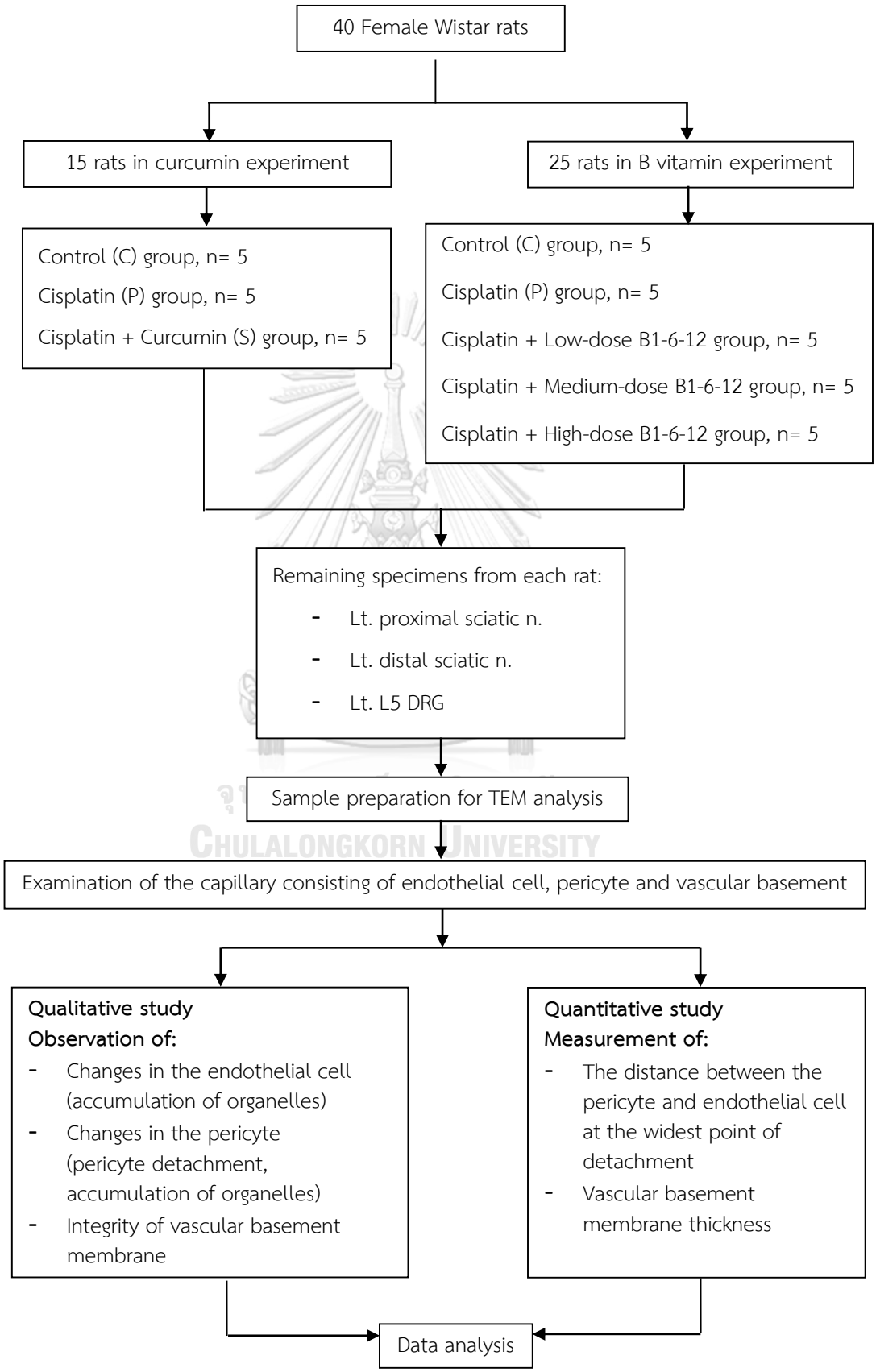
DRG morphometric study

The total number of L4 DRG neurons in the cisplatin group was significant lower than that of the control, medium-dose and high-dose B1-6-12 groups. The number of neurons in the B1-6-12-treated groups was between the values of the control and cisplatin groups.

Taken together, the results above indicate that cisplatin-treated rats had neuropathy which was alleviated by B1-6-12 treatment. The best effects were seen in the medium-dose B1-6-12 group.

3.2 Experimental procedures

3.2.1 Research framework: *In vivo* (animal model)



3.2.1.1 Tissue collection and sample preparation

The left sciatic nerve was divided into proximal and distal nerve stumps and suspended in 3% glutaraldehyde for 24 hours. The first 5-mm segment was used as the representative of the proximal stump. The last 5-mm segment before the bifurcation of sciatic nerve was used as the representative of the distal stump (Figure 10).

Furthermore, the 5-mm nerve segment was divided into 3 shorter (1.6 mm) segments or blocks for better penetration of fixative and sampling.

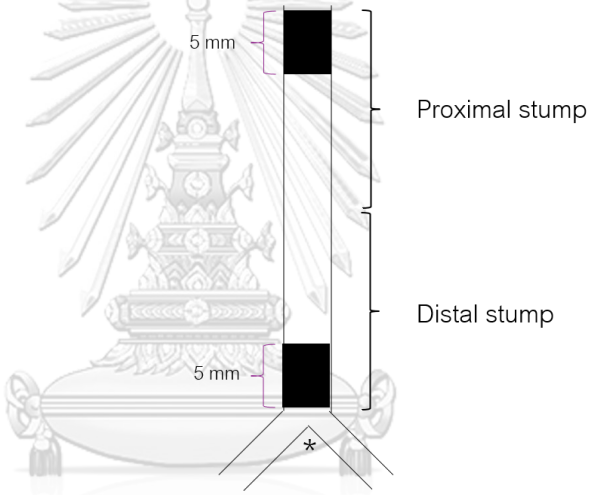


Figure 10 Segments of left sciatic nerve for capillary analysis.

3.2.1.2 Transmission electron microscope study of endothelial cell, pericyte and vascular basement membrane

The DRG and sciatic nerve segments were further fixed in 3% glutaraldehyde for 24 hr at 4 ºC and were incubated with 0.1 M PBS pH 7.4 overnight at 4 ºC. For next step, the specimens were washed with PBS pH 7.4 for 3 times 10 min each. Fixation was done with 1% osmium tetroxide for 2 hr. The samples were 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. Next, they were embedded in resin with 100% Epon

in capsule block and left at 60 ºC for 3 days. After that, the specimen blocks were taken out of the mold for sectioning.

The sections were cut at 1 µm thickness for sciatic nerve and 2 µm thickness for DRG using ultramicrotome (RMC Powertome X, USA). Then, the sciatic nerve and DRG sections were stained with toluidine blue for screening of artifacts under light microscope (Motic BA310, USA). After that, the ultrathin sections of 75 nm thickness were cut from selected areas of the blocks using the ultramicrotome (Leica EM UC6, German), and placed on the copper grid (G150-Cu). The ultrathin sections on the grid were stained with 2% uranyl acetate for 15 min and washed with distilled water for 3 times. Next step, the ultrathin sections were stained for 15 minutes with lead citrate before being rinsed three times with distilled water. Finally, the sections were examined under transmission electron microscope (JEM-1400 plus, JEOL USA) for studying the endothelial cell, pericyte, and vascular basement membrane.

3.2.1.3 Ultrastructural study of sciatic nerve and DRG

The ultrastructural study was performed to evaluate the endothelial cell, pericyte, and vascular basement membrane. The analysis was made over ultrathin section photographs (TEM images).

3.2.1.4 Sample size determination

From the pilot study of 160 capillaries from 8 female rats, the standard deviation of the basement membrane thickness was 6.22. The sample size was calculated from the following formula (141):

$$n = \frac{z^2 \sigma^2}{d^2}$$

n = sample size

Z = 1.96 (two tail), Alpha (α) = 0.05

σ = standard deviation = 6.22

d = acceptable error = 3 nm

Therefore $$n = \frac{(1.96)^2 \times (6.22)^2}{(3)^2}$$

$$n = 16.51$$

The calculated sample size was at least 16.51 capillaries per each part of the specimens (proximal nerve, distal nerve and DRG) of each rat. Consequently, this study included 20 capillaries for each part of each rat.

The capillaries with endothelial cells, vascular basement membrane and pericytes (Figure 11) were chosen randomly from each specimen. The nuclei of endothelial cells and pericyte must appear in the chosen capillaries.

- Lt. proximal sciatic nerve: 20 capillaries
- Lt. distal sciatic nerve: 20 capillaries
- Lt. L5 DRG: 20 capillaries (L4 had already been used)

The random 20 capillaries were collected from 3 blocks of proximal or distal nerves.

Each block was sectioned (75 nm thickness). The best one in 5 serial ultrathin sections were chosen.

The total number of capillaries for TEM analysis in this study is shown in Table1.

Table 1 The total number of capillaries for EM analysis

Groups / Specimens	Experiment 1 (15 rats)			Experiment 2 (25 rats)				
	C (5)	P (5)	S (5)	C (5)	P (5)	LB (5)	MB (5)	HB (5)
Lt. proximal sciatic n. (20 capillaries/each rat)	100	100	100	100	100	100	100	100
Lt. distal sciatic n. (20 capillaries/each rat)	100	100	100	100	100	100	100	100
L5 DRG (20 capillaries/each rat)	100	100	100	100	100	100	100	100
Total	300	300	300	300	300	300	300	300
	900 capillaries			**1,500 capillaries**				
	2,400 capillaries							

C = Control, P = Cisplatin, S = Curcumin, LB = low-dose B1-6-12, MB = medium-dose B1-6-12, HB = high-dose B1-6-12

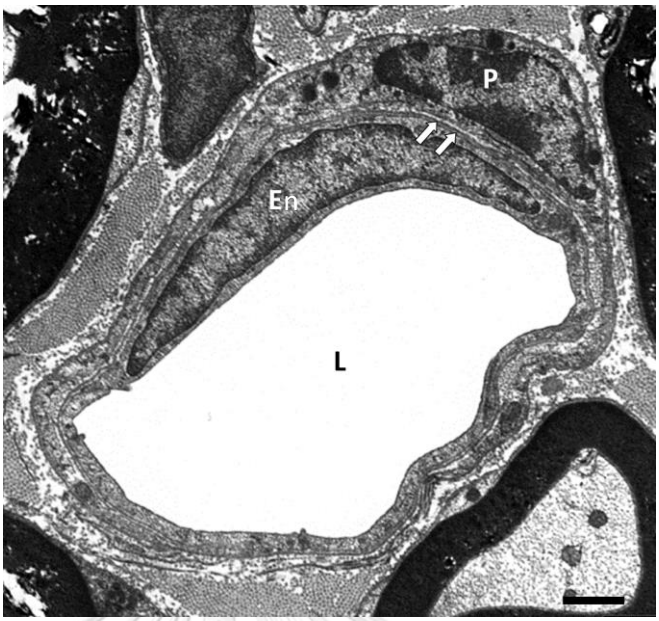


Figure 11 The capillary in the rat sciatic nerve, consisting of pericyte (P) and shared vascular basement membrane (arrows) with endothelial cell (En). L = Capillary lumen; scale bar = 1 μm

Qualitative analysis

The endothelial cell, pericyte and vascular basement membrane were examined on varying magnification of TEM images.

- The endothelial cell was studied for changes in the accumulation of organelles e.g. mitochondria, vacuole, lysosome and cytoplasmic inclusion body.
- The pericyte was studied for changes in accumulation of organelles and detachment from the endothelial cell.
- The vascular basement membrane was studied for the structural integrity.

For next step, each capillary was examined for the presence of pericyte detachment from endothelial cell which was classified into 2 categories:

Category 1: Pericyte completely attached to the endothelial cell.

Category 2: Pericyte detached from the endothelial cell at some points.

Quantitative analysis

Measurement of:

- **The distance between the pericyte and endothelial cell at the widest point of detachment**

In category 2, at the widest point of pericyte migration from the endothelial cell where nuclei of endothelial cell and pericyte were present, the distance between the pericyte and vascular basement membrane was measured at 10000X magnification (Figure 12).

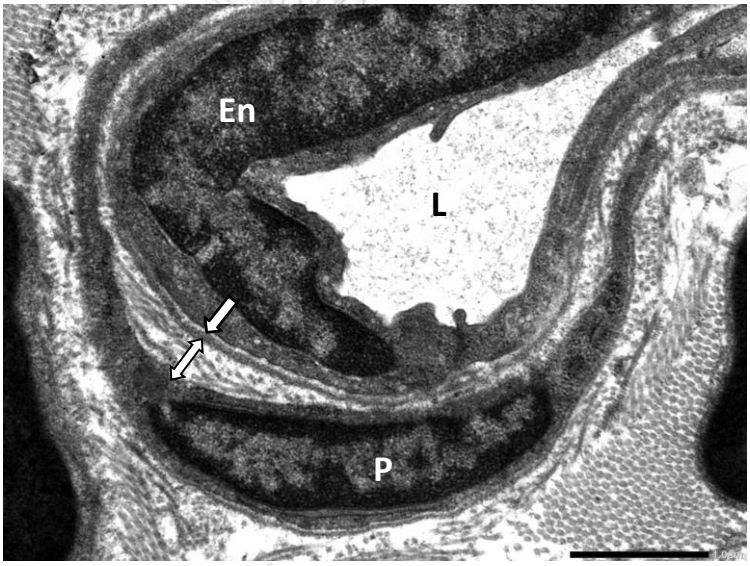


Figure 12 Distance between the pericyte (P) and vascular basement membrane (one-headed arrow) at the widest point of detachment (two-headed arrow). En = Endothelial cell; L = Capillary lumen; scale bar = 1 μm

- **The thickness of vascular basement membrane**

Thickness of vascular basement membrane in the capillary of category 1 was measured 3 points randomly at the site where nuclei of endothelial cell and pericyte were present. Then, 3 values were used to calculate the average of the basement membrane thickness for each capillary (Figure 13). The thickness was measured at 10,000x magnification.

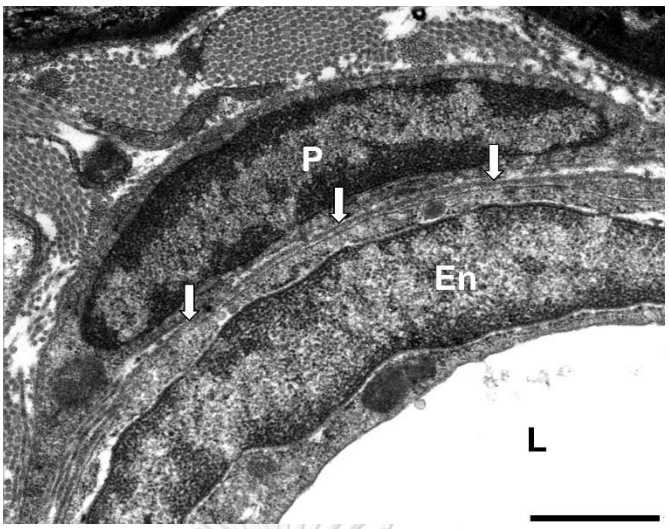


Figure 13 Vascular basement membrane (one-headed arrows) at the site where nuclei of endothelial cell and pericyte are present. En = Endothelial cell; P = Pericyte; L = Capillary lumen; scale bar = 1 µm

In the category 2, the thickness was measured at the widest point of detachment between the pericyte and endothelial cell (Figure 12).

3.2.2 In vitro experiment

Each experiment was done in triplicate.

3.2.2.1 Pericyte culture

The experiments were performed using the rat brain vascular pericyte (RBVP) from ScienCell Research Laboratories (California, USA, Cat. No. R1200). RBVP was grown in pericyte medium (ScienCell, USA) containing 500 ml of basal medium, 5 ml of pericyte growth supplement (PGS, Cat. No.1252), 10 ml of fetal bovine serum (FBS, Cat. No. 0010), and 5 ml of penicillin/streptomycin solution (P/S Solution, Cat. No.0503). The cells were subcultured when the culture reached 90-95% confluency and used between 2^{nd} - 5^{th} passages. The medium was changed every three days.

RBVP cells were plated in 6-well tissue culture plates with 3 ml of medium, cisplatin (Unistin, Korea United Pharm, South Korea, Reg. No. 1C 257/51) 3 µg/mL, curcumin (Cayman Chemical, USA, Cat. No. 81025) 1 µg/mL and B1-6-12 vitamins 1 µg/mL. Vitamin B1 (Sigma-Aldrich, USA, Cat. No. T4625), B6 (Sigma-Aldrich, USA, Cat. No. P9755), and B12 (Sigma-Aldrich, USA, Cat. No.

V2876) were prepared in a ratio of 100: 100: 1 (B1: B6: B12), similar to the animal experiment, and then dissolved in 50 ml sterile water. The culture was incubated for 24 h at 37 °C in a humidified atmosphere containing 5% CO_2.

The cells were divided into 4 groups:

- Group 1: Control pericyte (C)
- Group 2: Pericyte treated with cisplatin (P)
- Group 3: Pericyte treated with cisplatin + curcumin (PS)
- Group 4: Pericyte treated with cisplatin + B1-6-12 (PB)

3.2.2.2 MTT assay

The micro-culture tetrazolium (MTT) assay was conducted to evaluate the cytotoxicity of cisplatin, curcumin, and vitamin B1-6-12 on RBVP. The cells were seeded at $5x10^3$ cells/well in 6-well plates and allowed to attach for 24 hours. The cells were then treated for 24 hours under the experimental conditions. Finally, the cells were incubated with MTT solution 0.5 mg/mL (Life technologies, Molecular Probes, USA, Cat. No.M6494) for 2 hours. A 100 µl of DMSO were used to dissolve the purple formazan crystals. The absorbance was measured at 570 nm using a microplate reader (Thermo Fisher Scientific, Multiskan GO 1510-02675). The mean absorbance of the test samples divided by the mean of the negative control is used to calculate the percentage of cell viability in each group.

3.2.2.3 Western blot analysis

Western blot analysis was performed in order to determine the expression of endothelial tight junction proteins including ZO-1 and ZO-2.

3.2.2.4 Sample preparation

RBVP 5 x 10^3 cells were seeded in cell culture dish. Cells were treated for 24 hours in accordance with the experimental protocols. Cell pellets were lysed in 50 µl of 1x ice-cold lysis buffer (RIPA buffer from Cell signaling, USA, Cat. No. #9806) with the 1x protease inhibitor (Cell signaling, USA, Cat. No.5871) in the proportion of 100:1. The cell culture plate was placed on ice and the cells were washed twice with cold PBS. Then, the PBS was discarded and lysis

buffer was added to the cells and kept on ice for 5 min. The cells were collected and transferred to a 1.5 ml microcentrifuge tube. The samples were cooled for 30 min at 4 ℃. Then, the samples were centrifuged at 14,000 rpm at 4 ℃ for 10 min and the supernatant was transferred to a new microcentrifuge tube kept on ice. The BCA protein assay was then used to calculate the protein concentration for each cell lysate. (Thermo Scientific, USA, Cat. No. 23227). This technique is used for quantitation of total protein in a sample 3 µg of each sample was diluted with 0.1x sample buffer (ProteinSimple California) and mixed with the fluorescent dry (4:1 ratio) and denatured at 95℃ for 5 min.

3.2.2.5 Automated protein detection

The automated Western blot WESTM Simple Western™ assay system (ProteinSimple, USA) was used for the protein investigation. Primary antibodies were 1:200 anti-zonula occuden1 (ZO-1, Invitrogen, Cat. No. #PA5-28858), 1:200 anti-zonula occluden2 (ZO-2, Invitrogen, Cat. No. #PA5-17155) and 1:200 Beta-actin (Cell signaling, Cat. No. #4970). Beta-actin was used for loading control. The samples, antibody diluent, primary antibodies, rabbit secondary conjugate HRP, and luminol-peroxide chemiluminescence were pipetted into the plate following the manufacturer's plate map. The plate were centrifuged at 2500 rpm for 10 minutes at 25℃. Finally, the protein separation and immunodetection were processed and density of digital image was analyzed using Compass software (ProteinSimple, California). The expression of each protein was normalized to that of beta-actin.

3.3 Data Analysis

The statistical analysis was performed using SPSS software version 23.0. The numerical data were expressed as mean ± SEM and tested for significant differences between groups using one-way ANOVA with Tukey post-hoc test. Statistically significant differences were considered when $p < 0.05$.

3.4 Ethical Consideration

The rat specimens were taken from the previous studies which were already approved by the institutional ethics committee of the Faculty of Medicine, Chulalongkorn University (Reference No. 13/52 for curcumin experiment and No.19/58 for B1-6-12 experiment).

CHAPTER IV

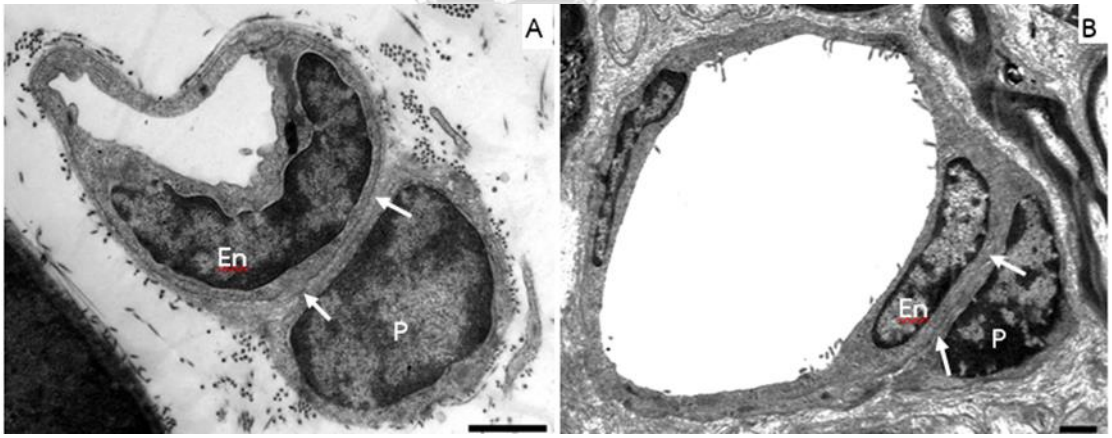
RESULTS

4.1 Effects of curcumin on cisplatin-induced alterations in the blood-nerve barrier in rats.

In the capillaries of proximal and distal sciatic nerves from the control (C) group, the pericytes wrapped around the endothelial cells with shared vascular basement membrane (VBM) (Figure 14A, 14B). However, the detachment of the pericytes from the endothelial cells was observed in the cisplatin (P) group (Figure 14C, 14D).

Furthermore, treatment with curcumin seemed to prevent the detachment since the pericyte remained attached to the endothelial cell with shared VBM (Figure 14E, 14F).

Accumulation of lysosomes or vacuoles were not observed in the pericytes and endothelial cells in any group.



Control-proximal n. **Control-distal n.**

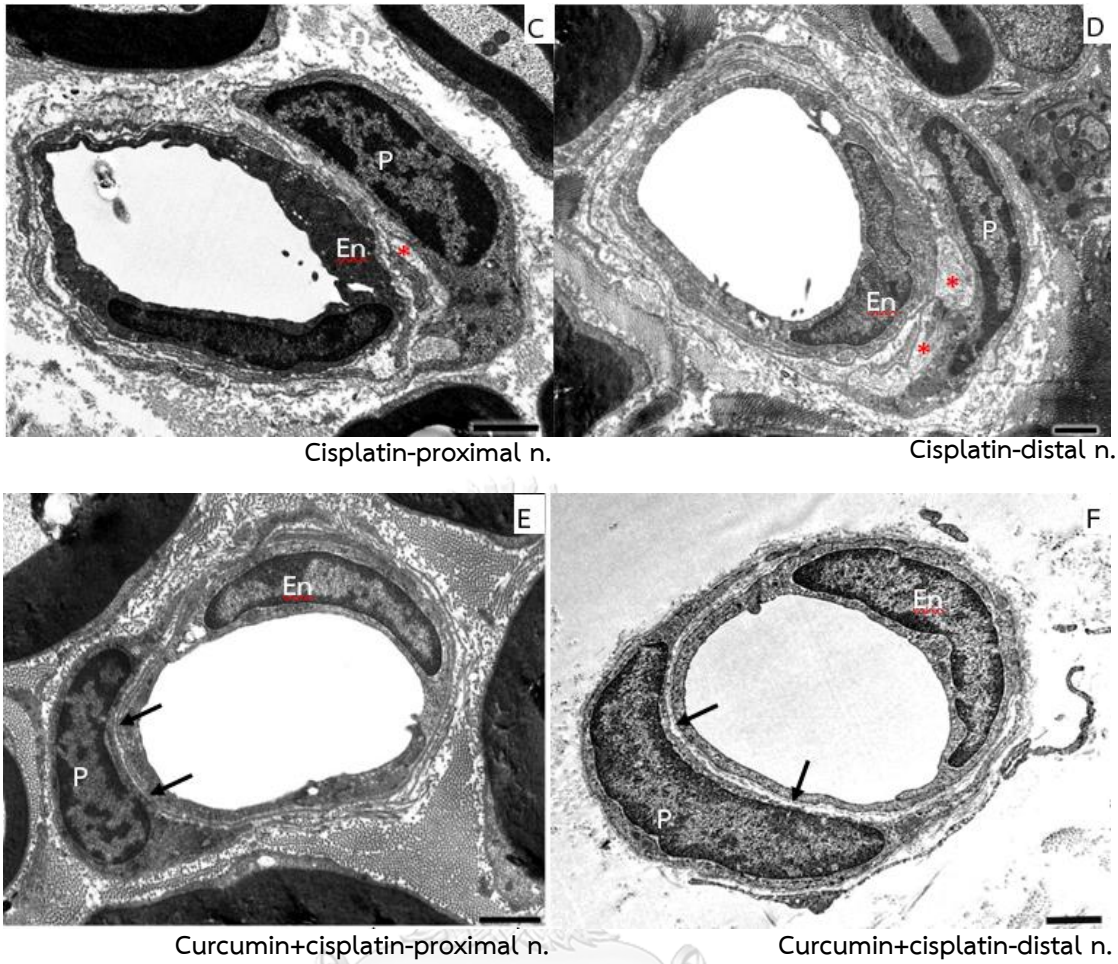
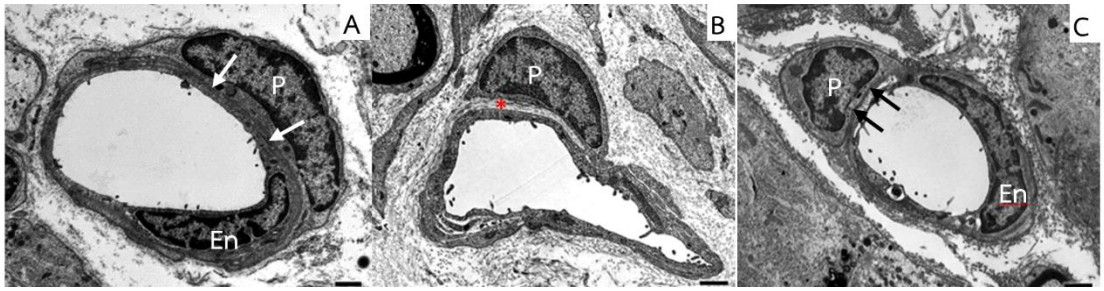


Figure 14 Transmission electron microscope images of capillaries from the sciatic nerves of rats including control-proximal (A), control-distal (B), cisplatin-proximal (C), cisplatin-distal (D), curcumin + cisplatin-proximal (E), and curcumin + cisplatin-distal (F). Arrows = vascular basement membrane shared between the endothelial cell (En) and pericyte (P); * pericyte detachment or separation between the endothelial cell and pericyte; Scale bars = 1 μm.

In the capillaries of DRG from the control group, the pericyte attached to the endothelial cell with shared VBM (Figure 15A). However, separation between the endothelial cell and pericyte was observed in the cisplatin group (Figure 15B). Moreover, the curcumin treatment corrected these abnormalities (Figure 15C).

Abnormal accumulation of lysosomes or vacuoles in the pericytes and endothelial cells were not observed in any group.



Control-DRG **Cisplatin-DRG** **Curcumin + Cisplatin-DRG**

Figure 15 Representative ultrastructural images of capillaries in the DRG including control-DRG (A), cisplatin-DRG (B), and curcumin + cisplatin-DRG (C). Arrows = vascular basement membrane shared between the endothelial cell and pericyte; * pericyte detachment or separation between the endothelial cell and pericyte; En = Endothelial cell; P = Pericyte; Scale bars = 1 μm.

Quantitative analysis showed that the ratio of capillaries with pericyte detachment was increased in the cisplatin vs. control groups with significant reduction in the curcumin treatment group (Figure 16).

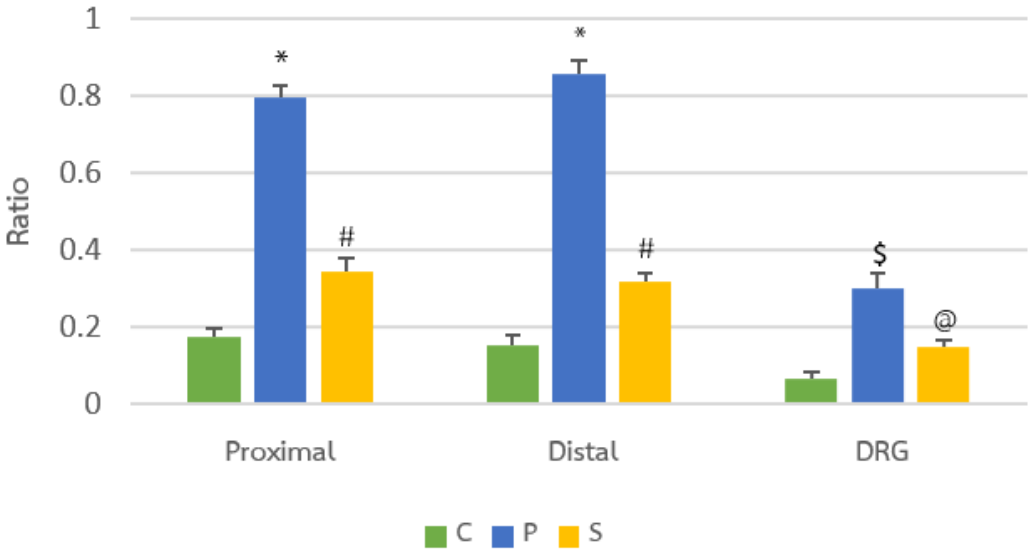


Figure 16 Ratio of the number of capillaries with pericyte detachment from endothelial cells to the total number of capillaries investigated in the proximal and distal parts of sciatic nerves including DRG from the control (C), cisplatin (P), cisplatin + curcumin (S) groups. The graph shows means and SEM. * $p < 0.001$ C vs. P, # $p < 0.001$ S vs. P & $p < 0.01$ S vs. C, $ $p < 0.01$ P vs. C, @ $p < 0.05$ S vs. P

The separation distance between the endothelial cell and pericyte was increased in the cisplatin vs. control groups. However, the curcumin treatment group had significantly decreased separation distance compared with the cisplatin group (Figure 17).

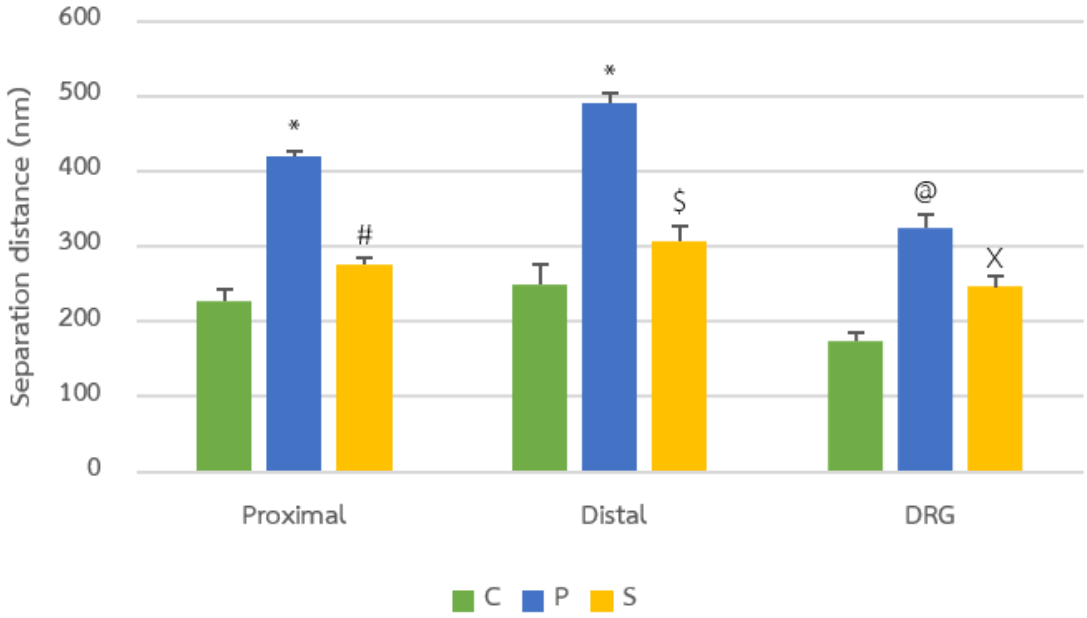


Figure 17 Distance at the widest separation between the endothelial cells and pericytes of capillaries in the proximal and distal parts of sciatic nerves including DRG from the control (C), cisplatin (P), cisplatin + curcumin (S) groups. The graph shows means and SEM. * $p < 0.001$ P vs. C, # $p < 0.001$ S vs. P & $p < 0.05$ S vs. C; $ $p < 0.001$ S vs. P; @ $p < 0.05$ P vs. C; x $p < 0.05$ S vs. P

Moreover, the thickness of vascular basement membrane of category 2 was not significantly different between groups (Figure 18).

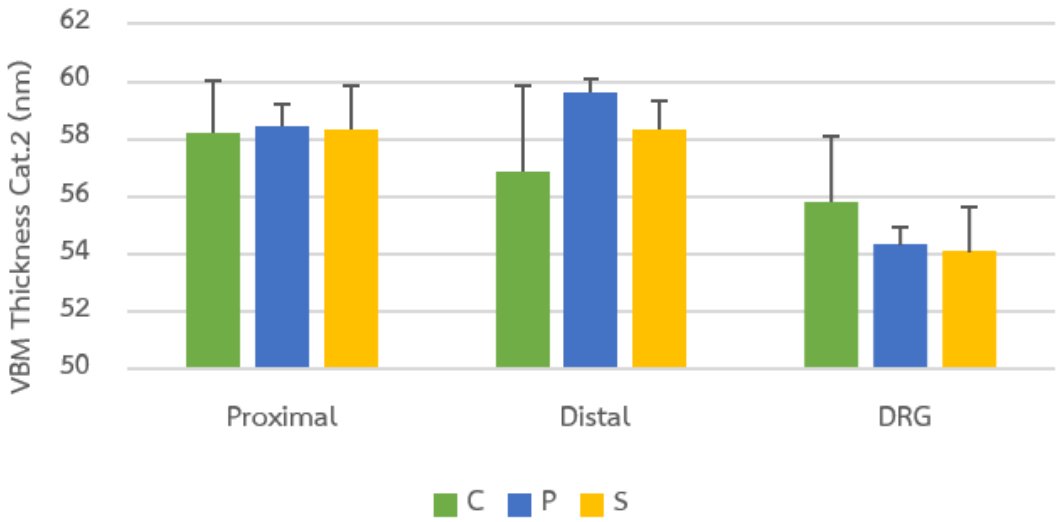


Figure 18 Vascular basement membrane (VBM) thickness at the widest point of detachment between the endothelial cell and pericyte of the capillaries in the proximal and distal parts of sciatic nerves including DRG from the control (C), cisplatin (P), cisplatin + curcumin (S) groups. The graph shows means and SEM.

In the same way, the thickness of vascular basement membrane of category 1 was not significantly different between groups (Figure 19).

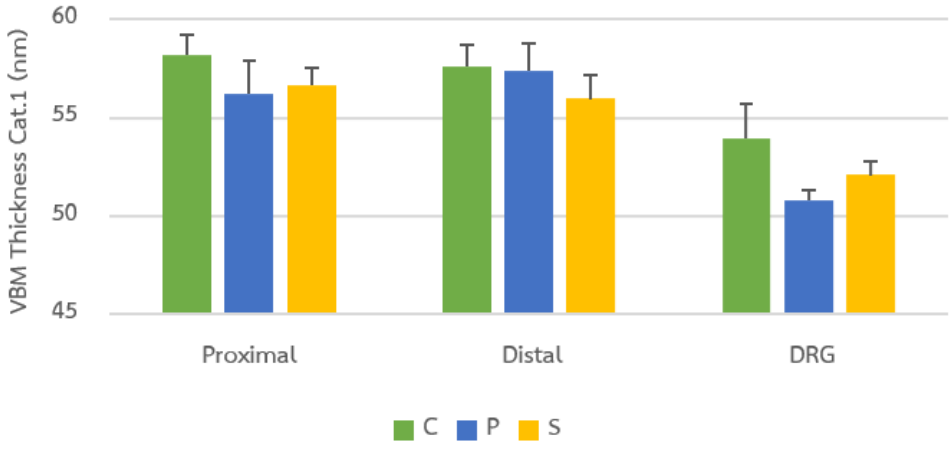


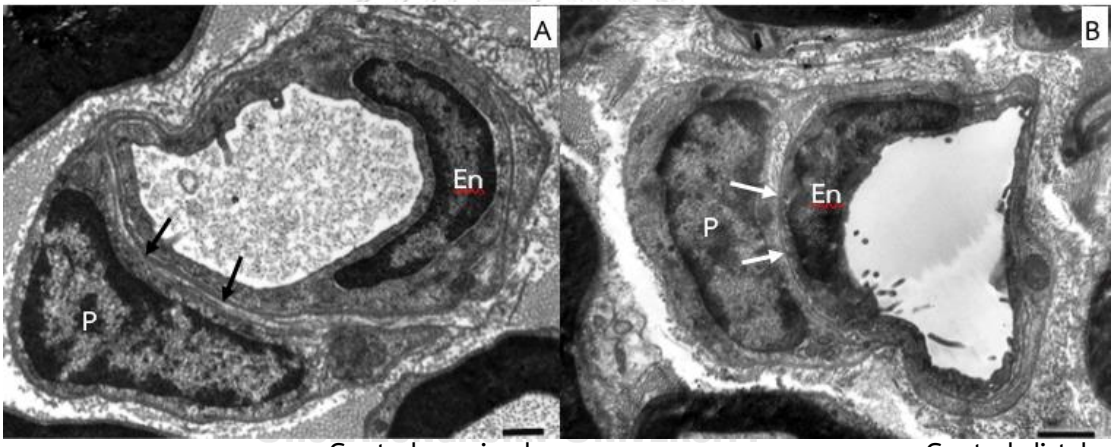
Figure 19 Thickness of vascular basement membrane at the site between the endothelial cell and pericyte of capillaries in the proximal and distal parts of sciatic nerves including DRG from the control (C), cisplatin (P), cisplatin + curcumin (S) groups. The graph shows means and SEM.

4.2 Effects of B Vitamins on cisplatin-induced alterations in the blood-nerve barrier in rats.

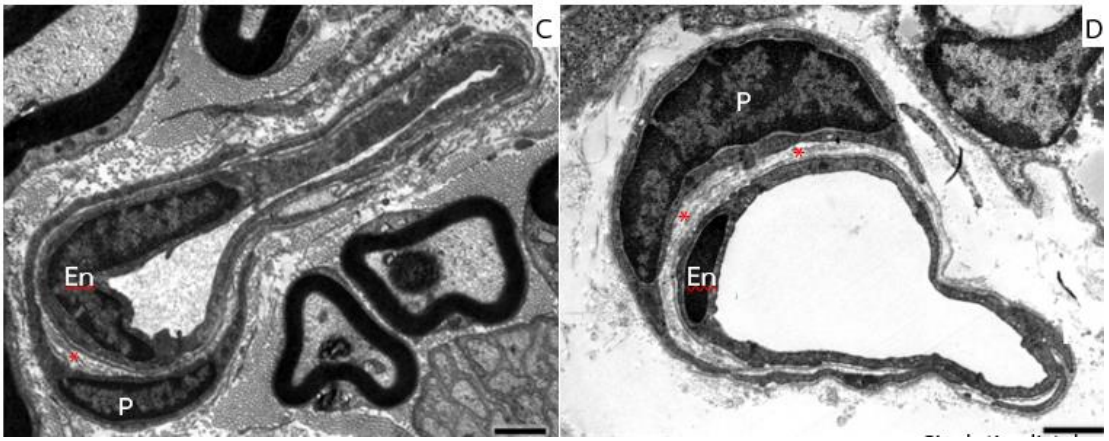
In the capillaries of proximal and distal sciatic nerves from the control (C) group, the pericytes attached to the endothelial cells with shared VBM in between (Figure 20A, 20B). The pericyte detachment/ migration from the endothelial cells was observed in the cisplatin (P) group (Figure 20C, 20D).

However, the detachment was less prominent in the cisplatin + B1-6-12 groups. Treatments with low-dose, medium-dose and high-dose of B1-6-12 seemed to prevent the detachment (Figure 20E, 20F, 20G, 20H, 20I, 20J).

In all groups, accumulation of mitochondria, vacuole, lysosome and cytoplasmic inclusion body were not observed in the pericytes and endothelial cells.



Control-proximal n. **Control-distal n.**



Cisplatin-proximal n. **Cisplatin-distal n.**

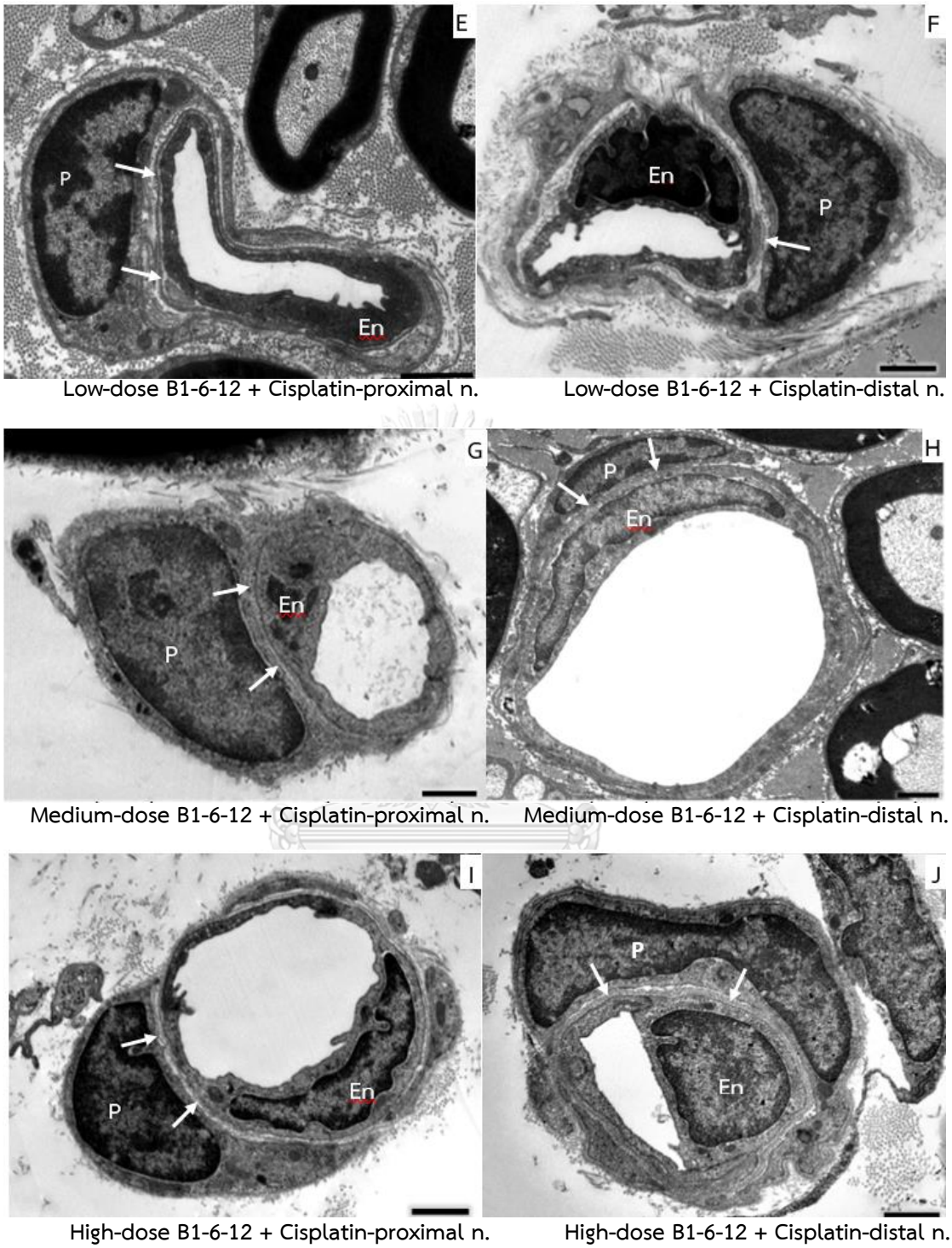


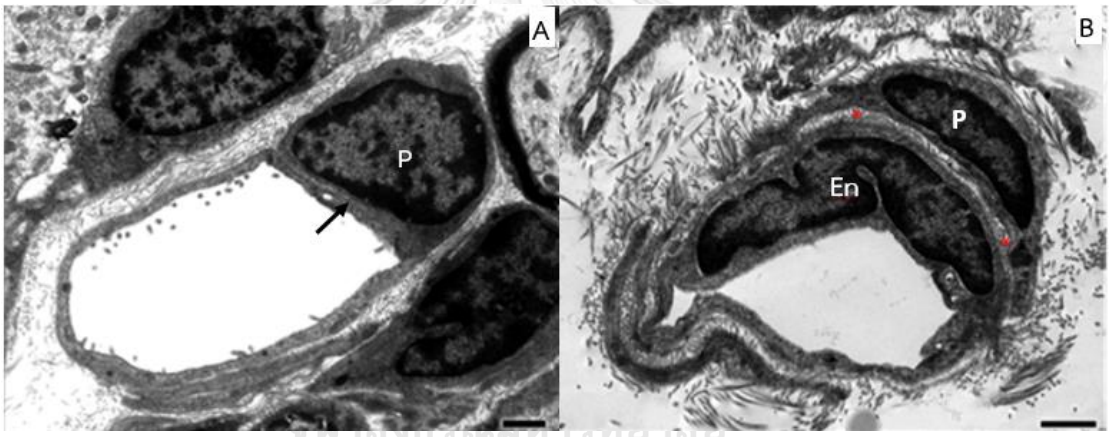
Figure 20 TEM images of capillaries from the sciatic nerves of B1-6-12 experiment including control-proximal (A), control-distal (B), cisplatin- proximal (C), cisplatin-distal (D), low-dose B1-6-12 + cisplatin-proximal (E), low-dose B1-6-12 + cisplatin-distal (F), medium-dose B1-6-12 + cisplatin-proximal (G), medium-dose B1-6-12 + cisplatin-distal

(H), high-dose B1-6-12 + cisplatin-proximal (I), and high-dose B1-6-12 + cisplatin-distal (J). Arrows = vascular basement membrane shared between the endothelial cell and pericyte; * pericyte detachment or separation between the endothelial cell and pericyte; En = Endothelial cell; P = Pericyte; Scale bars = 1 μm.

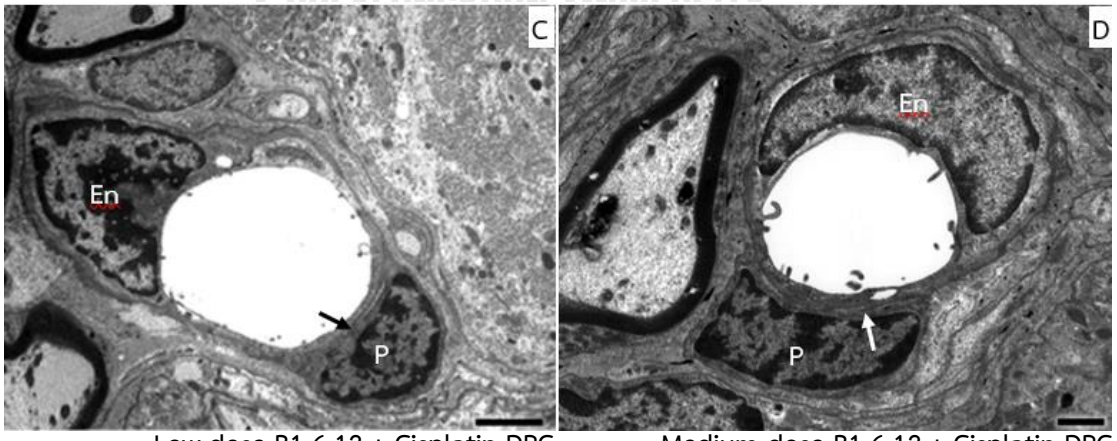
In the capillaries of DRG from the control group, the pericyte attachment to endothelial cell with shared VBM was observed (Figure 21A).

The separation of the pericytes from the endothelial cells appeared to be wider in the capillaries from the cisplatin group (Figure 21B).

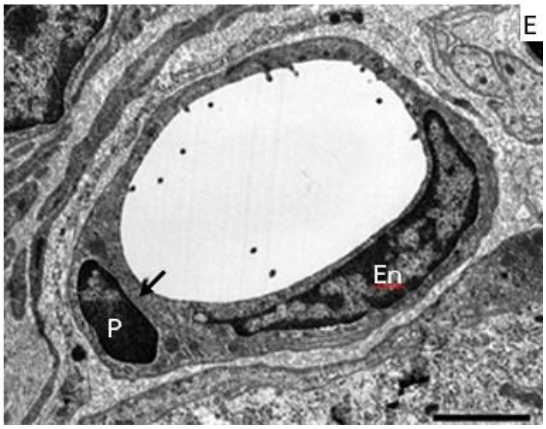
Treatments with all doses of B1-6-12 appeared to preserve the pericyte attachment to the endothelial cell (Figure 21C, 21D, 21E).



Control-DRG Cisplatin-DRG



Low-dose B1-6-12 + Cisplatin-DRG Medium-dose B1-6-12 + Cisplatin-DRG



High-dose B1-6-12 + Cisplatin-DRG

Figure 21 TEM images of capillaries from the DRG of B vitamins experiment including control-DRG (A), cisplatin-DRG (B), low-dose B1-6-12 + cisplatin-DRG (C), medium-dose B1-6-12 + cisplatin-DRG (D), and high-dose B1-6-12 + cisplatin-DRG (E) groups. Arrows = vascular basement membrane shared between the endothelial cell (En) and pericyte (P); * pericyte detachment or separation between the endothelial cells and pericyte; Scale bars = 1 μm.

When the number of capillaries with detachment was compared with that of total capillaries included, the ratio of capillaries with detachment was significantly higher in the cisplatin than the control groups. All doses of B1-6-12 had the significantly lower ratio than the cisplatin group but still higher than that of the control group. (Figure 22). However, after comparing between three doses, the ratio of the cisplatin + medium-dose B1-6-12 group was the least different from that of the control group. Moreover, similar alterations occurred in the proximal and distal parts of the nerve.

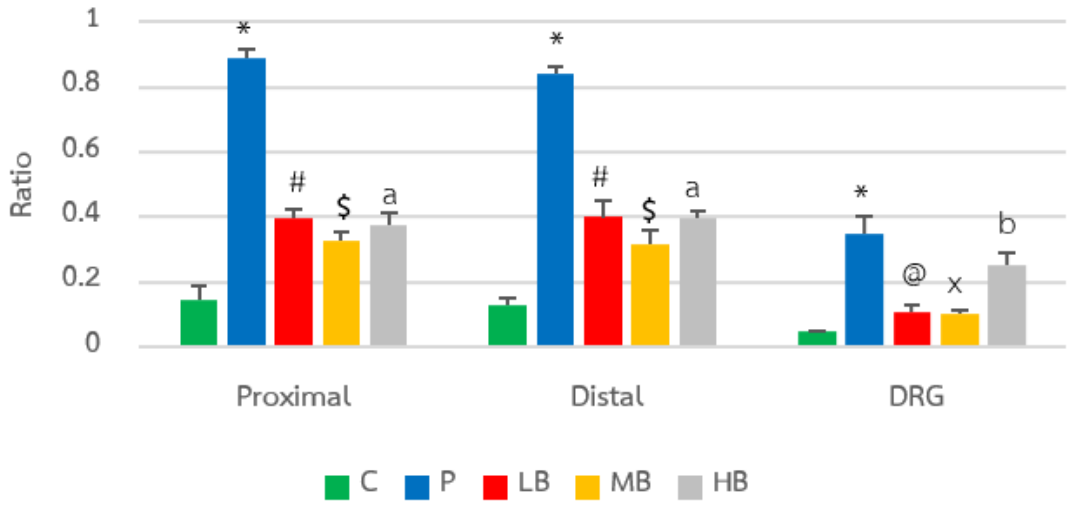


Figure 22 Ratio of the number of capillaries with pericyte detachment from endothelial cells to the total number of capillaries examined in the proximal and distal parts of sciatic nerves including DRG from the control (C), cisplatin (P), low-dose B1-6-12 + cisplatin (LB), medium-dose B1-6-12 + cisplatin (MB), High-dose B1-6-12 + cisplatin (HB) groups. The graph shows means and SEM. * $p < 0.001$ P vs. C; # $p < 0.001$ LB vs. P & $p < 0.001$ LB vs. C; $ $p < 0.001$ MB vs. P & $p < 0.01$ MB vs. C; a $p < 0.001$ HB vs. P & $p < 0.01$ HB vs. C; @ $p < 0.001$ LB vs. P; x $p < 0.001$ MB vs. P; b $p < 0.01$ HB vs. C

In the cisplatin group, the separation distance was significantly wider than that of the control group in the sciatic nerves but not the DRG (Figure 23). All cisplatin + B1-6-12 groups had shorter distances compared with the cisplatin group. The values of the cisplatin + medium-dose B1-6-12 group were the closest to those of the control group. It is worth mentioning that only the cisplatin + high-dose B1-6-12 group had significantly wider distance than the control group in the DRG.

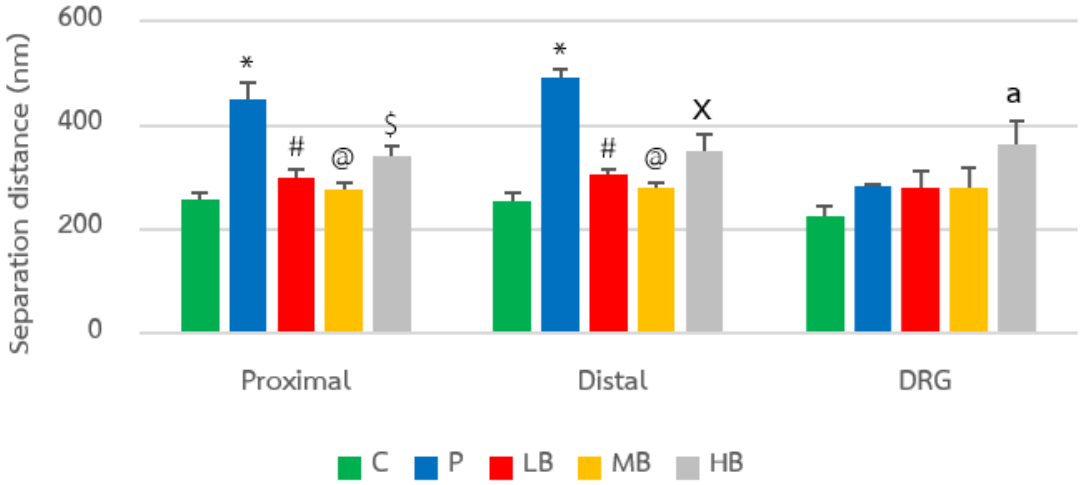


Figure 23 Separation distance between the endothelial cells and pericytes of capillaries in the proximal and distal parts of sciatic nerves including DRG from the control (C), cisplatin (P), low-dose B1-6-12 + cisplatin (LB), medium-dose B1-6-12 + cisplatin (MB), High-dose B1-6-12 + cisplatin (HB) groups. The graph shows means and SEM. * $p < 0.001$ P vs. C, # $p < 0.001$ LB vs. P, @ $p < 0.001$ MB vs. P, $ $p < 0.001$ HB vs. P, x $p < 0.001$ HB vs. P & $p < 0.01$ HB vs. C, a $p < 0.05$ HB vs. C

The thickness of vascular basement membrane at the separation of category 2 was not significantly different between groups (Figure 24).

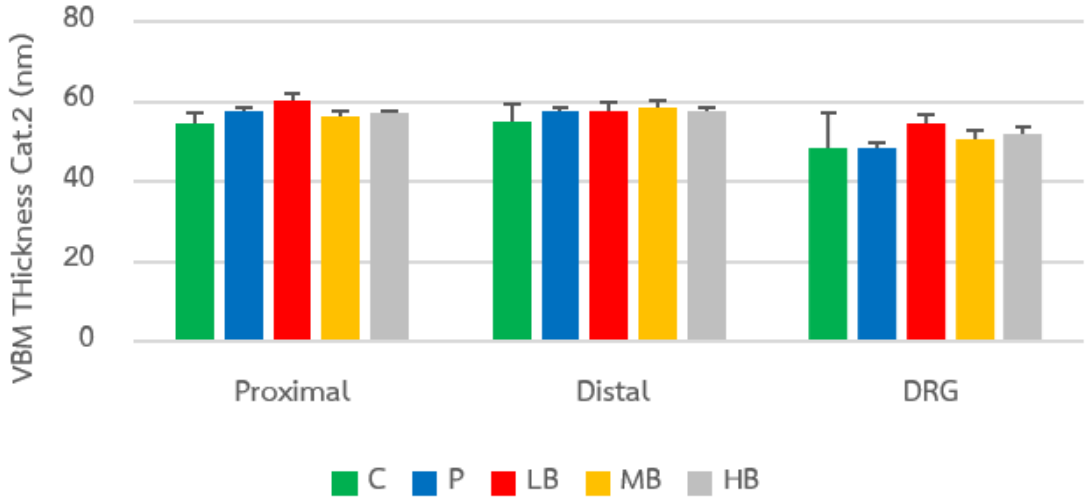


Figure 24 Vascular basement membrane (VBM) thickness at the widest point of detachment between endothelial cell and pericyte of the capillaries in the proximal and distal parts of sciatic nerves including DRG from the control (C), cisplatin (P), low-dose B1-6-12 + cisplatin (LB), medium-dose B1-6-12 + cisplatin (MB), High-dose B1-6-12 + cisplatin (HB) groups. The graph shows means and SEM.

Moreover, the thickness of vascular basement membrane of category 1 was not significantly different between groups (Figure 25).

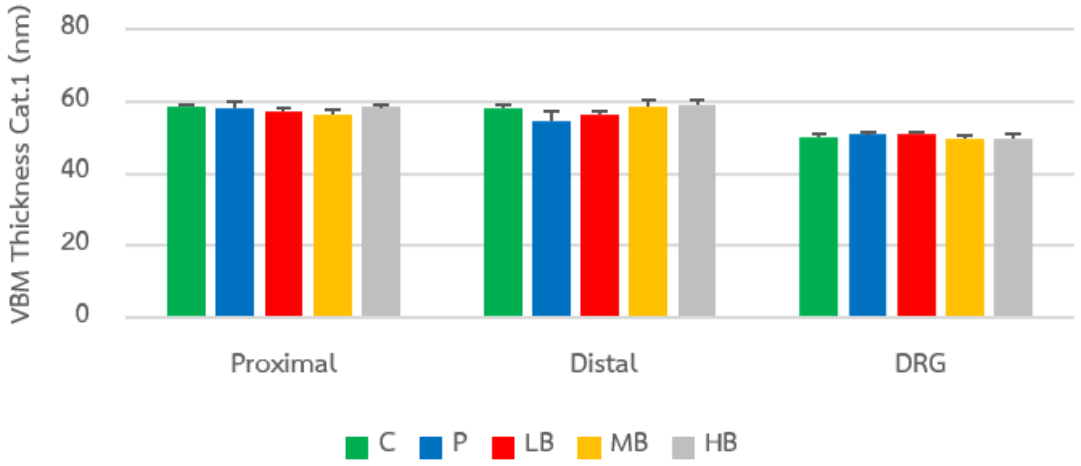


Figure 25 Thickness of vascular basement membrane at the site between endothelial cell and pericyte of capillaries in the proximal and distal parts of sciatic nerves including DRG from the control (C), cisplatin (P), low-dose B1-6-12 + cisplatin (LB), medium-dose B1-6-12 + cisplatin (MB), High-dose B1-6-12 + cisplatin (HB) groups. The graph shows means and SEM.

4.3 Effects of curcumin and B1-6-12 on cisplatin-induced alterations in rat brain vascular pericyte (RBVP)

4.3.1 Cell viability

4.3.1.1 To optimize cisplatin concentrations for further investigation using MTT assay

The RBVP were incubated with cisplatin at 0, 1, 3, 5, 10, 20, 40 and 60 µg/ml for 24 hours. The viability was significantly reduced starting from 3 µg/ml. Hence, the dose of cisplatin at 3 µg/ml was selected for further study (Figure 26).

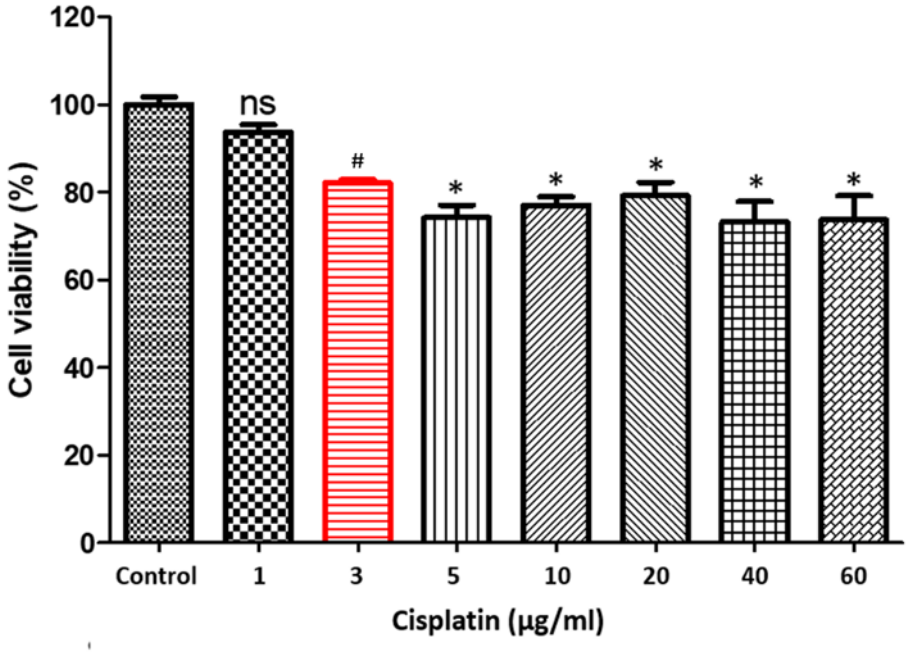


Figure 26 Cell viability of RBVP treated with various concentrations of cisplatin (0-60 µg/ml). The graph presents mean and SEM. #p < 0.01 vs. control group; *p < 0.001 vs. control group.

4.3.1.2 To optimize curcumin concentrations for further investigation using MTT assay

Reduced viability of RBVP was observed starting from 5 ug/ml (Figure 27). Therefore, the 1 µg/ml was chosen for the future experiments.

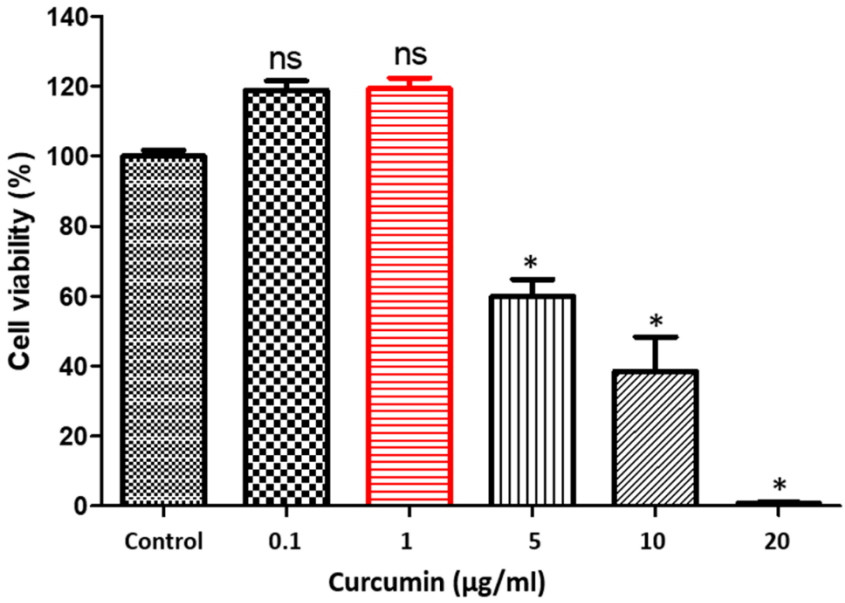


Figure 27 Cell viability of RBVP treated with curcumin (0-20 µg/ml). The graph represents the mean and SEM. * $p < 0.001$ vs. control group.

4.3.1.3 To optimize B1-6-12 concentrations for further investigation using MTT assay

RBVP were treated with B1-6-12, all doses (1 - 80 µg/ml) caused significant increase of RBVP viability (Figure 28). Accordingly, the lowest dose, 1 µg/ml, was selected for the next experiments.

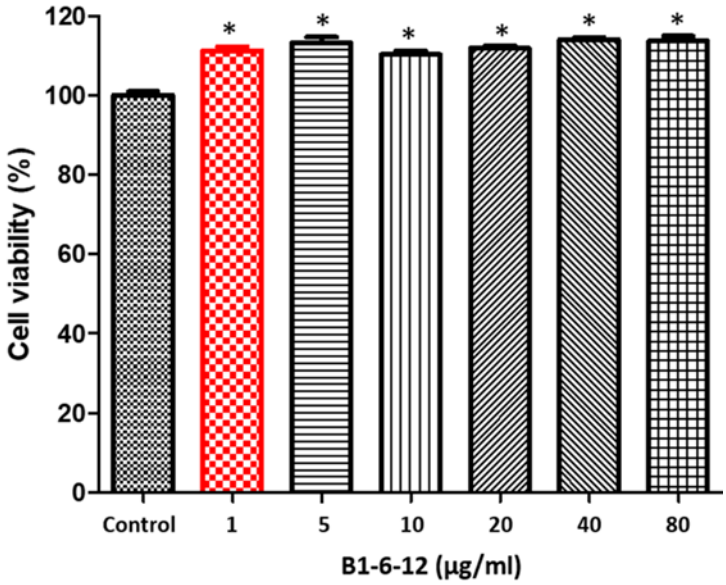


Figure 28 Cell viability of RBVP treated with B1-6-12 (1 - 80 µg/ml). The graph represents the mean and SEM. * $p < 0.001$ vs. control group.

4.3.2 Effects of curcumin and B1-6-12 on cisplatin-induced alterations in RBVP

- Cell viability

4.3.2.1 Effect of co-treatment with cisplatin and curcumin on RBVP viability

The treatment of RBVP with 3 µg/ml cisplatin significantly reduced cell viability when compared with the control group ($p < 0.001$). However, the combination of cisplatin and 1 µg/ml curcumin significantly increased RBVP viability when compared with the cisplatin group ($p < 0.001$) (Figure 29).

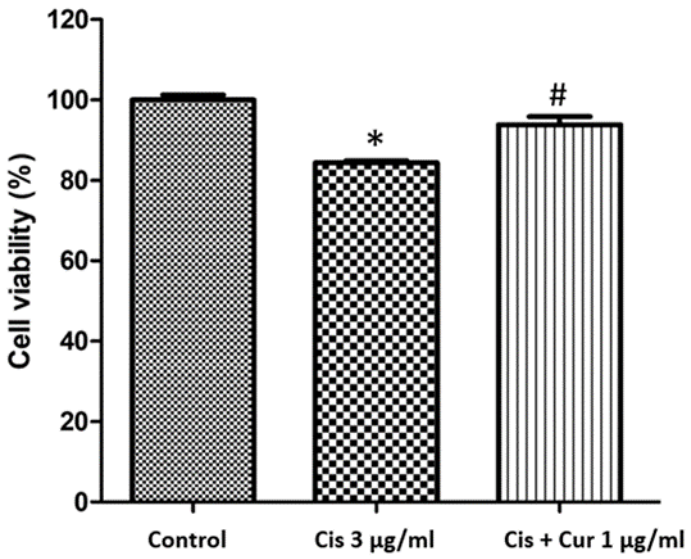


Figure 29 Cell viability of RBVP after treatments. The graph shows the average percent of cell viability with SEM. Cis = cisplatin, Cur = Curcumin, * $p < 0.001$ vs. control; # $p < 0.001$ vs. cisplatin

The control RBVP were polygonal with several processes (Figure 30A, 30D). However, partial retraction of cell processes was found in the cisplatin group (Figure 30B, 30E) with normalization in the combination (cis +cur) group (Figure 30C, 30F).

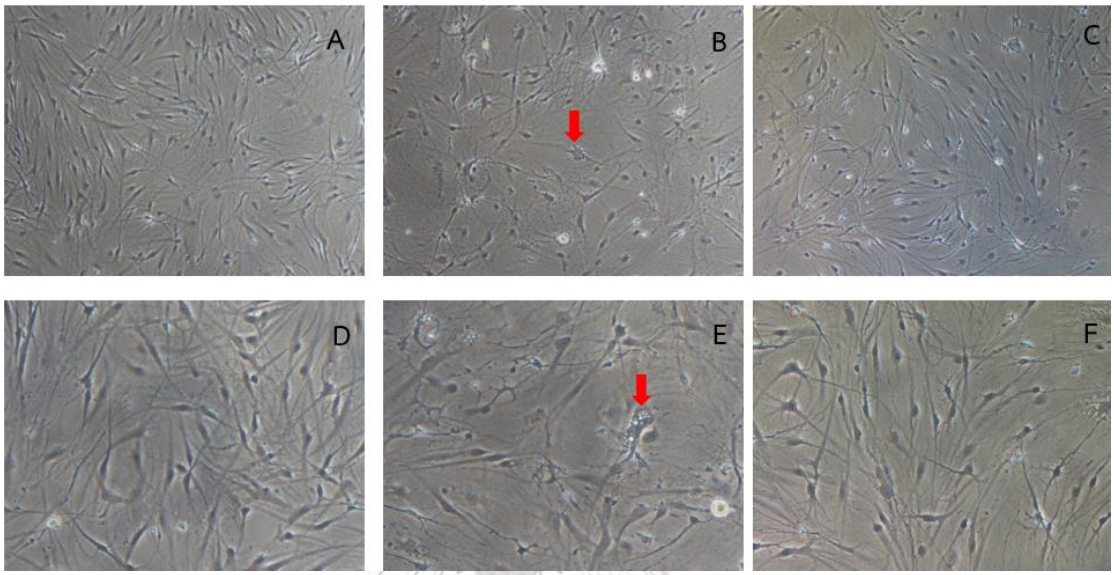


Figure 30 Morphology of rat brain vascular pericyte (RBVP) culture under phase contrast microscope (A, B, C = magnification 10X and D, E, F = 20X). A, D = control; B, E = cisplatin; C, F = Co-treatment with cisplatin and curcumin; Arrow = retraction of cell process

4.3.2.2 Effect of co-treatment with cisplatin and B1-6-12 on RBVP viability

A significant reduction was observed in the cisplatin-treated group ($p<0.001$). Combination with 1 µg/ml of B1-6-12 exhibited a protective effect against the cisplatin-induced decrease in viability ($p<0.001$) (Figure 31).

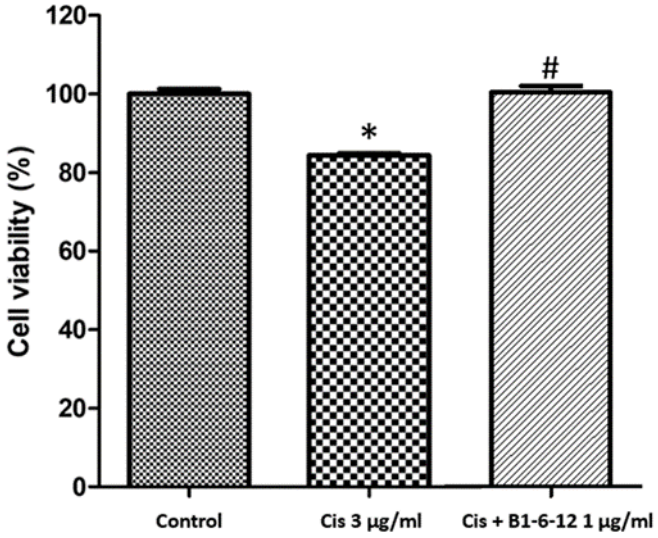


Figure 31 Cell viability of RBVP after treatments. The graph shows the average percent of cell viability with SEM. Cis=cisplatin, * $p < 0.001$ vs. control; # $p < 0.001$ vs. cisplatin

In the control RBVP had polygonal body with long processes (Figure 32A, 32D). However, RBVP shrinkage of cell processes was observed after treatment with cisplatin (Figure 32B, 32E). Treatment combination (cis + B1-6-12) seemed to prevent the abnormalities (Figure 32C, 32F).

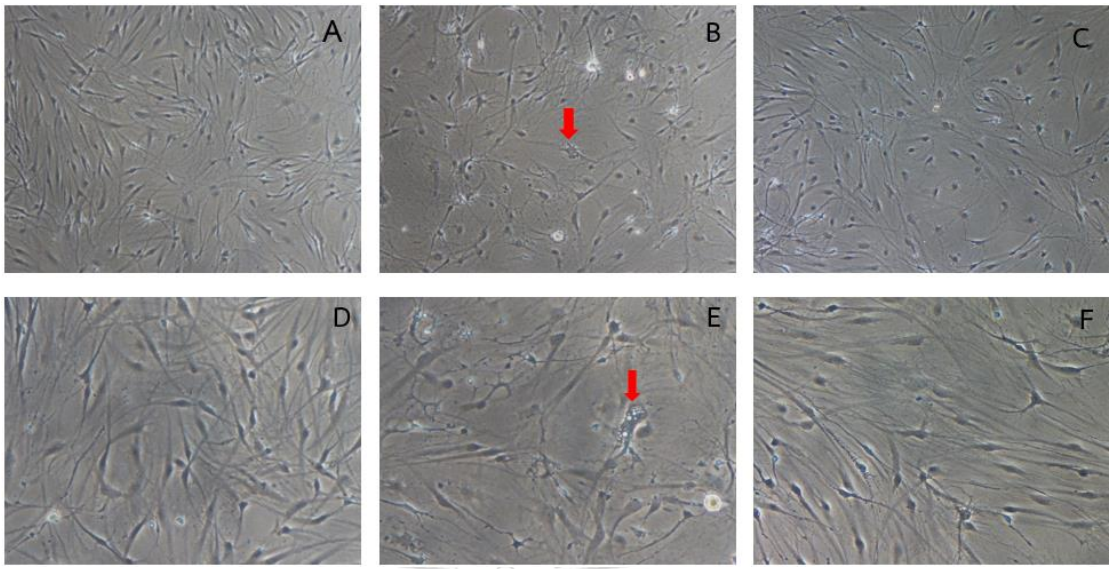


Figure 32 Morphology of rat brain vascular pericyte (RBVP) culture under phase contrast microscope (A, B, C = magnification 10X and D, E, F = 20X). A, D = control; B, E = cisplatin; C, F = Co-treatment with cisplatin and B1-6-12; Arrow = retraction of cell process

4.3.2.3 Expression of tight junction proteins in RBVP

- Zonula occludens 1 (ZO-1)

The ZO-1 expression was not significantly different between groups. However, the cisplatin (P) group had trend toward down-regulation of ZO-1 compared with the control (C), cisplatin + curcumin (PS) and cisplatin + B1-6-12 (PB) groups (Figure 33, 34).

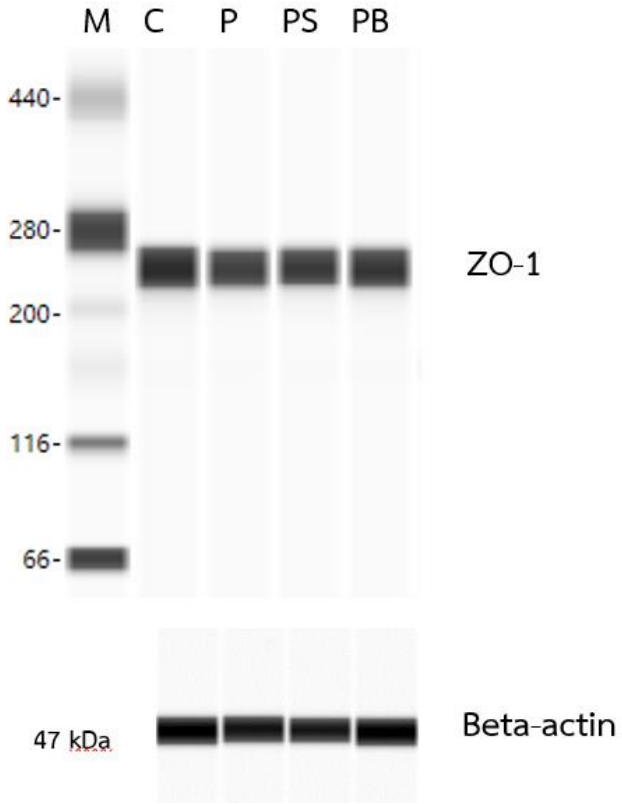


Figure 33 The immunoblots show the bands of ZO-1 and beta-actin in rat brain vascular pericyte in the control (C), cisplatin (P), curcumin + cisplatin (PS), and B vitamins + cisplatin (PB) groups. M = marker

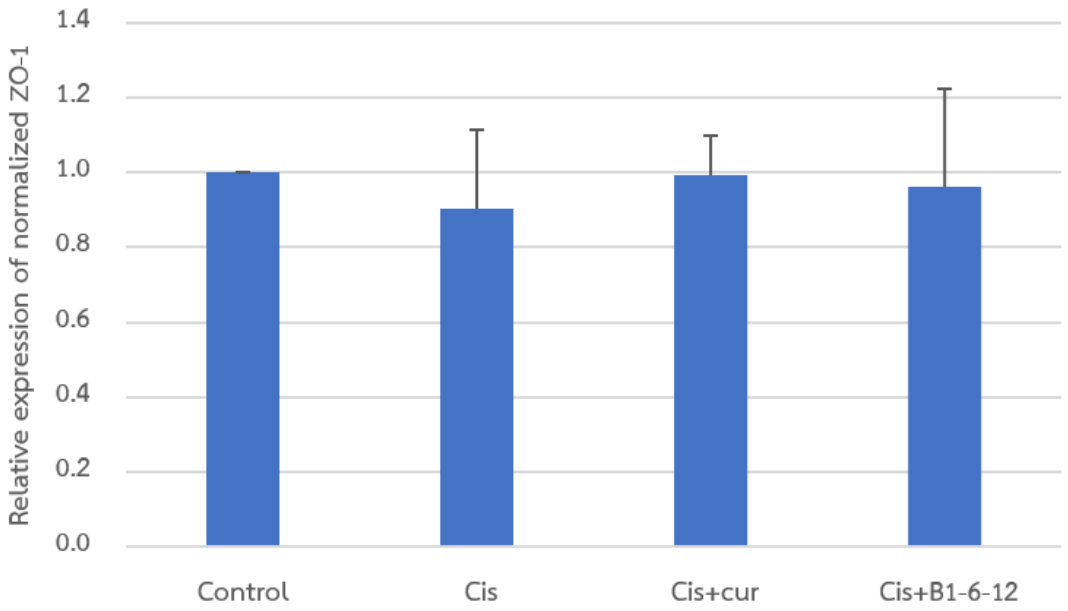


Figure 34 ZO-1 protein expression in RBVP after treatments. The graph shows the average ratio of ZO-1 normalized to beta-actin with SEM. Cis = cisplatin; Cur = curcumin

- **Zonula occludens 2 (ZO-2)**

Similar to ZO-1, the ZO-2 expression was not significantly different between groups (Figure 35, 36).

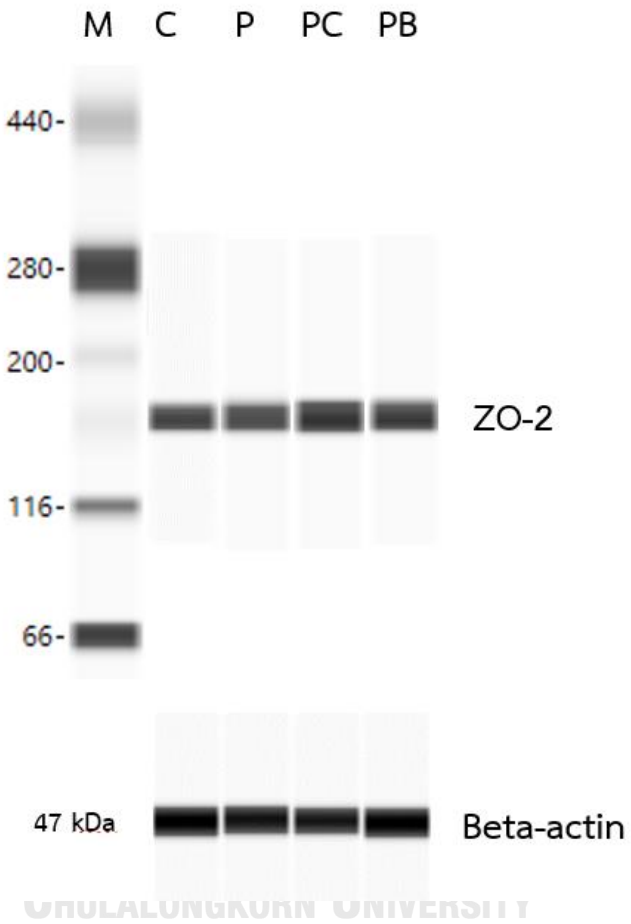


Figure 35 ZO-2 in RBVP in the control (C), cisplatin (P), cisplatin + curcumin (PS), and cisplatin + B1-6-12 (PB) groups. The western blots show the bands of ZO-2. M = marker

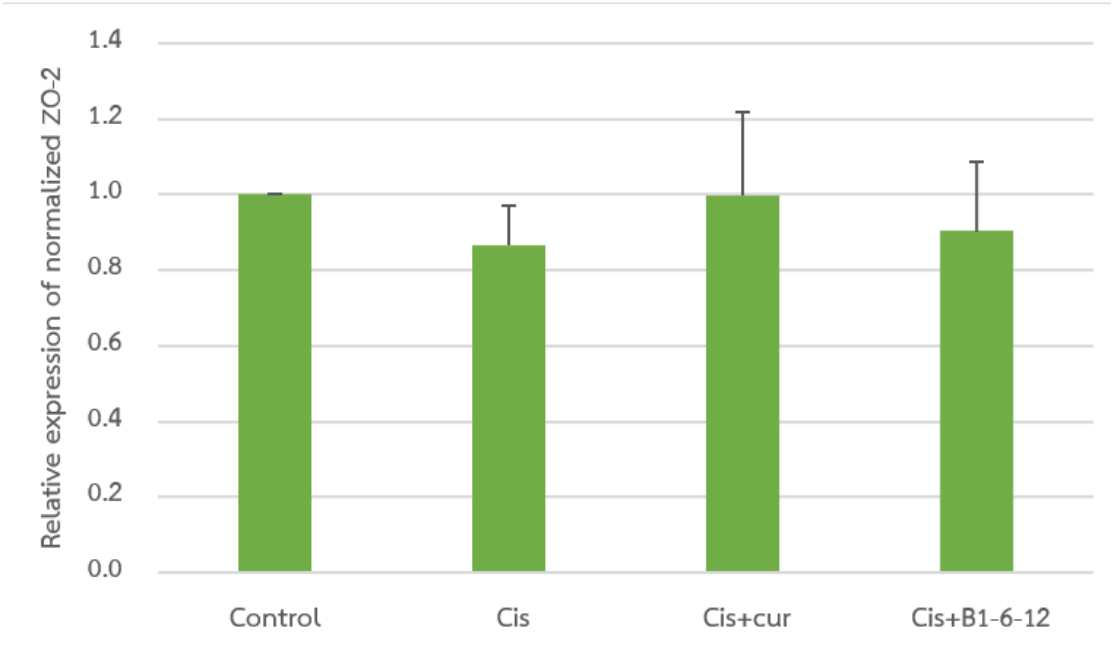


Figure 36 ZO-2 protein expression in RBVP after treatments. The graph shows the average ratio of ZO-2 normalized to beta-actin with SEM. Cis = cisplatin; Cur = curcumin

In addition, the expression of beta-actin was not significantly different between groups (Figure 37).

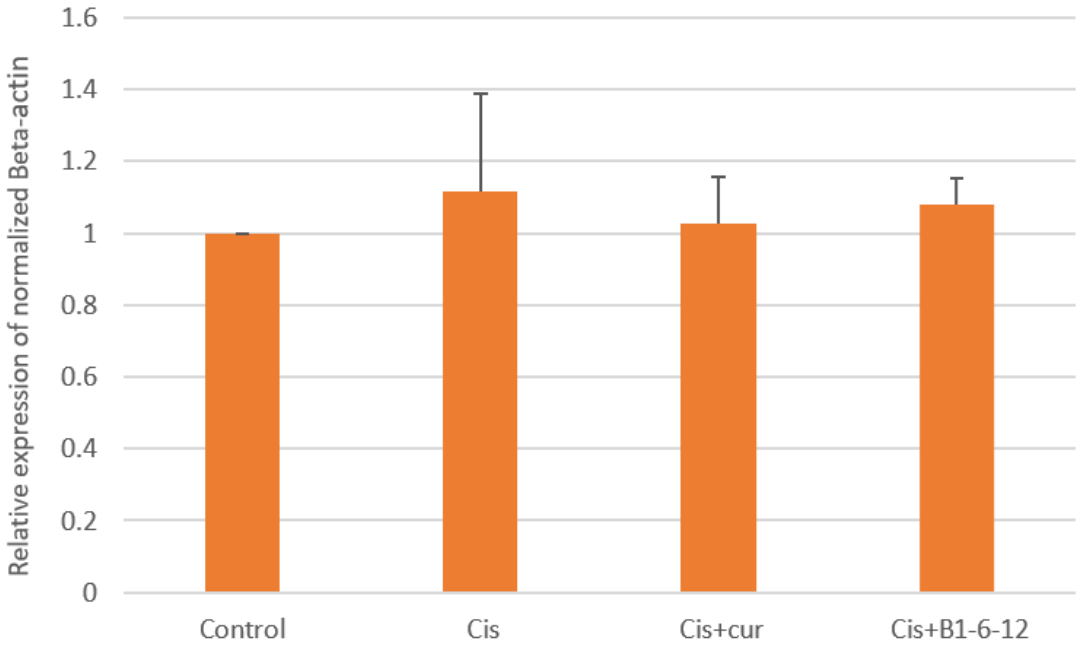


Figure 37 Expression of beta-actin in RBVP in the control, cisplatin, cisplatin + curcumin, and cisplatin + B1-6-12 groups. The graph shows the average ratio of beta-actin with SEM. Cis = cisplatin; Cur = curcumin

Table 2 Result summary: effect of curcumin on cisplatin-induced alterations in the blood nerve barrier in rats

Specimens	Groups	Ratio of capillaries with pericyte detachment		Separation distance between the endothelial cell and pericyte	
		Alterations	Sig.	Alterations	Sig.
Proximal nerve	Cis vs. Control	↑	Yes ($p < 0.001$)	↑	Yes ($p < 0.001$)
	Cis + Cur vs. Cis	↓	Yes ($p < 0.001$)	↓	Yes ($p < 0.001$)
Distal nerve	Cis vs. Control	↑	Yes ($p < 0.001$)	↑	Yes ($p < 0.001$)
	Cis + Cur vs. Cis	↓	Yes ($p < 0.001$)	↓	Yes ($p < 0.001$)
DRG	Cis vs. Control	↑	Yes ($p < 0.01$)	↑	Yes ($p < 0.05$)
	Cis + Cur vs. Cis	↓	Yes ($p < 0.05$)	↓	Yes ($p < 0.05$)

Cis = Cisplatin group; Cur = Curcumin group; Sig. = significance; ↓ = decrease; ↑ = increase

Table 3 Result summary: effect of B1-6-12 on cisplatin-induced alterations in the blood nerve barrier in rats

Specimens	Groups	Ratio of capillaries with pericyte detachment		Separation distance between the endothelial cell and pericyte	
		Alterations	Sig.	Alterations	Sig.
Proximal nerve	Cis vs. Control	↑	Yes ($p < 0.001$)	↑	Yes ($p < 0.001$)
	Cis + LB vs. Cis	↓	Yes ($p < 0.001$)	↓	Yes ($p < 0.001$)
	Cis + MB vs. Cis	↓	Yes ($p < 0.001$)	↓	Yes ($p < 0.001$)
	Cis + HB vs. Cis	↓	Yes ($p < 0.001$)	↓	Yes ($p < 0.001$)
Distal nerve	Cis vs. Control	↑	Yes ($p < 0.001$)	↑	Yes ($p < 0.001$)
	Cis + LB vs. Cis	↓	Yes ($p < 0.001$)	↓	Yes ($p < 0.001$)
	Cis + MB vs. Cis	↓	Yes ($p < 0.001$)	↓	Yes ($p < 0.001$)
	Cis + HB vs. Cis	↓	Yes ($p < 0.001$)	↓	Yes ($p < 0.001$)
DRG	Cis vs. Control	↑	Yes ($p < 0.001$)	↑	No
	Cis + LB vs. Cis	↓	Yes ($p < 0.001$)	↓	No
	Cis + MB vs. Cis	↓	Yes ($p < 0.001$)	↓	No
	Cis + HB vs. Cis	↓	No	↑	No

Cis = Cisplatin group; LB = Low dose of B1-6-12 group; MB = Medium dose of B1-6-12 group; HB = High dose of B1-6-12 group; Sig. = significance; ↓ = decrease; ↑ = increase

Table 4 Result summary: effect of curcumin on cisplatin-induced alterations in rat brain vascular pericyte (RBVP)

Specimens	Groups	Cell viability		ZO-1		ZO-2	
		Alterations	Sig.	Alterations	Sig.	Alterations	Sig.
RBVP	Cis vs. Control	↓	Yes (p<0.001)	↓	No	↓	No
	Cis + Cur vs. Cis	↑	Yes (p<0.001)	↑	No	↑	No

Cis = Cisplatin group; Cur = Curcumin group; Sig. = significance; ↓ = decrease; ↑ = increase

Table 5 Result summary: effect of B1-6-12 on cisplatin-induced alterations in rat brain vascular pericyte (RBVP)

Specimens	Groups	Cell viability		ZO-1		ZO-2	
		Alterations	Sig.	Alterations	Sig.	Alterations	Sig.
RBVP	Cis vs. Control	↓	Yes (p<0.001)	↓	No	↓	No
	Cis + B1-6-12 vs. Cis	↑	Yes (p<0.001)	↑	No	↑	No

Cis = Cisplatin group; Sig. = significance; ↓ = decrease; ↑ = increase

CHAPTER V

DISCUSSION

5.1 Effect on cisplatin-induced alterations in BNB and RBVP

This study confirmed the findings of a previous study by our lab that cisplatin was likely to induce pericyte detachment from endothelial cells in the nerves (142). The quantitative analysis of this study showed that the cisplatin group had a significantly higher frequency of separation and wider separation distance between endothelial cells and pericytes of capillaries in the proximal and distal parts of the sciatic nerve including DRG than the control rats. These results were similar between the proximal and the distal nerve segments.

Pericyte loss and separation from endothelial cells in the nerves of cisplatin-treated rats were demonstrated in a prior investigation by our team (142). Pericyte detachment or migration has been reported in different conditions and organs. Increased migration of pericytes was found in the retinal capillary of diabetic rats (143). Pericyte migrates from the vascular wall in response to traumatic brain injury in the rat (49). The pericytes migrate from the capillary walls in the anterior pituitary gland of rats with prolactinoma (144). In this study, pericytes in the nerves migrate from the capillary walls in response to cisplatin treatment. Implications of the pericyte detachment are still unclear. However, pericytes and endothelial cells, share vascular basement membrane and collaborate in BNB to regulate the microvascular functions (43). Detachment of the pericyte from the endothelial cell likely impairs BNB function and may be associated with cisplatin-induced neuropathy.

There is evidence that cisplatin causes vascular abnormalities such as ischemic stroke (135). It was also found that chemotherapy containing cisplatin caused endothelial cell damage (12). In addition, cisplatin-treated rats showed reductions in the number of blood vessels and nerve blood flow, as well as endothelial cell apoptosis (13). Ultrastructural alterations in endothelial cells and pericyte migration from blood vessels in the stria vascularis of the cochlea were partially related to

auditory impairment in the cisplatin-induced ototoxicity model (145). The findings of this study also imply that cisplatin has a deleterious effect on pericytes, causing BNB to become dysfunctional. These data indicate that changes in the BNB are correlated with severity of cisplatin neuropathy and might play a role in this condition. In diabetic neuropathy, BNB changes also play an important pathophysiological role (9, 146).

In this study, cisplatin caused the reduction of cell viability in cultured pericytes confirming the cytotoxic effect of cisplatin to pericytes. Oxidative stress caused by excessive ROS generation and antioxidant depletion is one mechanism of cisplatin-induced cytotoxicity (99), which can lead to cell dysfunction and apoptosis. Previous research has suggested that tight junction proteins are important for BNB integrity and nerve function (43). Moreover, endothelial tight junctions are known to be disrupted by oxidative stress (147). This cisplatin-induced cytotoxicity in the pericytes might also explain the detachment from endothelial cells in our EM results.

Pericyte plays an important role in BNB integrity by secreting growth factors that affect claudin-5 production of endothelial cells (52). Claudin-5 is a major component of BNB (43), and reductions in this protein have been found to be associated with impaired BNB function in chronic inflammatory demyelinating polyradiculoneuropathy (CIDP) (10). In addition, the expression of tight junction proteins in the RBVP was examined in this study. Expression of ZO-1 and ZO-2 was not significant altered in the cisplatin-treated cells compared with the controls. However, the cisplatin group had a tendency toward lower levels of ZO-1 and ZO-2. In cisplatin-treated mice, tight junction protein expression was shown to be reduced in the stria vascularis of the cochlea (145).

Furthermore, abnormalities of organelles or the accumulation of organelles such as lysosomes or vacuoles in pericytes and endothelial cells in cisplatin-induced neuropathy were not seen in any groups in this study. However, in cisplatin-induced stria vascularis damage, swollen endoplasmic reticulum was observed in the endothelial cells (145). The effects of cisplatin in neuroblastoma cells include

structural damage to organelles such as mitochondria, golgi apparatus and endoplasmic reticulum as well as decreased number of the lysosome (88). The disparity between the previous and this studies might be due to differences in the cell types and concentrations of cisplatin.

In this study, no changes were found in the vascular basement membrane thickness. In contrast, in a rat model of diabetic retinopathy, the basement membrane thickening and pericyte loss were found (148). Therefore, vascular basement membrane may be differentially altered in different models of BNB dysfunction.

5.2 Effect of curcumin on cisplatin-induced alterations in BNB and RBVP

The potential of curcumin for the treatment of peripheral neuropathies is well recognized (149, 150). Moreover, combined curcumin and cisplatin therapy is a novel approach for improving chemotherapeutic efficacy while minimizing side effects (151). Our previous study found that curcumin improved both functional and structural abnormalities of the nerves and DRG in cisplatin-induced neuropathy (6).

In this study, curcumin given in combination with cisplatin, caused a significant reduction in the ratio of capillaries with pericyte detachment and separation distance between the endothelial cell and pericyte compared with the cisplatin alone. In addition, curcumin significantly improved pericyte viability. This suggests that the reduced pericyte detachment by curcumin is partially due to its direct effect on the pericyte viability.

As mentioned above, one mechanism of cisplatin-induced cytotoxicity, which can lead to cell malfunction and apoptosis, is oxidative stress (99). Curcumin has antioxidant properties (151, 152), by activating antioxidant enzymes and accelerating the elimination of ROS (152). Furthermore, cisplatin also induces DNA damage, which activates caspase-3 and causes apoptosis (153). This was consistent with previous studies which found that cisplatin increased caspase-3 expression, causing endothelial apoptosis (13, 139, 154). Curcumin inhibits cellular apoptosis and attenuates cerebral ischemia-induced injury by upregulating the expression of anti-apoptotic proteins

including Bcl-2 and downregulating the expression of apoptosis-related proteins including caspase-3 (155). Therefore, curcumin might alleviate the oxidative stress and apoptotic processes induced by cisplatin resulting in improved viability of pericytes in this study.

In this study, no changes were found in the levels of ZO-1 and ZO-2 proteins in the pericytes. However, the expression of ZO-1 and ZO-2 had a tendency to decrease in the cisplatin group, while the curcumin-treated tended to increase the protein expression. Previous study also reported a reduction in the levels of these proteins in the cochlea vascular wall in the cisplatin-treated mice (145). Reduction in the protein expression might be due to cisplatin-induced cytotoxicity. Moreover, oxidative stress caused by cisplatin might also impair the expression since oxidative stress can disrupt the tight junction (147). Previously, curcumin was found to restore the expression of ZO-1 protein after oxygen glucose deprivation in BBB in vitro (156). Curcumin increases the expression of tight junction proteins such as (ZO-1), occludin, and claudin-5, which protect the integrity of the BBB (157, 158). The findings of this study that curcumin-treated tended to increase the ZO-1 expression was consistent with those of the previous studies.

Therefore, curcumin co-treatment possibly reduces oxidative stress and cytotoxicity induced by cisplatin leading to improved expression of tight junction proteins in pericytes.

5.3 Effect of B1-6-12 on cisplatin-induced alterations in BNB and RBVP

In the cisplatin-treated rats, EM analysis revealed higher frequencies and wider separation distances between the pericytes and endothelial cells than in the control groups. All doses of B1-6-12 were effective in reducing cisplatin-induced pericyte detachment in both sciatic nerves and DRG, with the medium-dose group showing the best results. However, a high-dose of B1-6-12, especially in the DRG, may be less favorable or even harmful. The detachment distance was significantly longer in the

high-dose group than in the other groups. The dosage-dependent effects of B1-6-12 will be discussed later.

This study showed that cisplatin lower viability and tended to decrease the levels of tight junction proteins (ZO-1 and ZO-2), while B1-6-12 improved cell viability. Regarding to tight junction proteins, the B1-6-12-treated cells tend to have higher expression of ZO-1 and ZO-2 than the cisplatin group. Since B vitamins are important for cellular metabolic pathways (18, 159), their supplement might be associated with higher resistance to cell death and improved expression of tight junction proteins. In addition, B1 and B12 have antioxidant properties (159-162). Therefore, it is possible that B vitamins may improve cell viability by preventing oxidative damage. Furthermore, cisplatin also directly binds to mitochondrial DNA and inhibits the transcription and replication of mitochondrial genes (68). This might result in mitochondrial DNA damage or mitochondrial toxicity, causing apoptosis. In the previous studies, B6 and B12 have been shown to protect mitochondria from damage or toxicity (163, 164). B vitamins might improve pericyte viability through this process.

Aside from ROS, inflammation also causes disruption of tight junctions (165). Moreover, cisplatin can cause endothelial cells to upregulate expression of inflammatory mediators (139). Treatment with a vitamin B complex reduces local inflammation after peripheral nerve injury (166). P53 improves endothelial barrier function through an anti-inflammatory mechanism (167-169). Furthermore, B6 has the ability to elevate the amount of p53 (170). Therefore, B1-6-12 might also cause up-regulation of tight junction proteins via reducing inflammation. More research is necessary to clarify this issue.

These findings imply that the beneficial effects of B1-6-12 on cisplatin neurotoxicity described earlier could be mediated at least in part by improved BNB functions. It is possible that this is attributable to reduced toxicity and increased expression of tight junction proteins in the pericytes. Although numerous agents targeting various mechanisms were effective in experimental cisplatin neuropathy,

clinical trials failed to show significant improvements (171). B1-6-12 are widely used for the treatment of peripheral neuropathy (18). However, current evidence on the clinical effectiveness of B vitamins in chemotherapy-induced neuropathy is still inconclusive (172). The results of this study support continued efforts to develop B1-6-12 as a potential treatment for cisplatin-induced neuropathy. It is worth noting that high-dose B1-6-12 had less or unfavorable effects in the ultrastructural analysis of DRG and sciatic nerves in this study. This might be caused by toxicity of all or specific B vitamins. Excessive intake of pyridoxine (B6) can result in neuropathy (125). Therefore, the optimal dose of these B vitamins must be determined to avoid the side effects of overdose. According to our findings, the medium-dose of B1-6-12 (300 mg/kg/day per oral) might be the most suitable.

The findings of this study also suggest that BNB impairment is a potential additional mechanism for cisplatin-induced neuropathy. However, whether these changes in the BNB occur in patients with cisplatin neuropathy is uncertain and need to be confirmed in clinical specimens. In addition, in neuropathies caused by other chemotherapeutic agents or other etiologies, the BNB integrity should be evaluated. In the future, drugs with beneficial effects on endothelial cells or pericytes could be evaluated for potential treatments for peripheral neuropathy with impaired BNB.

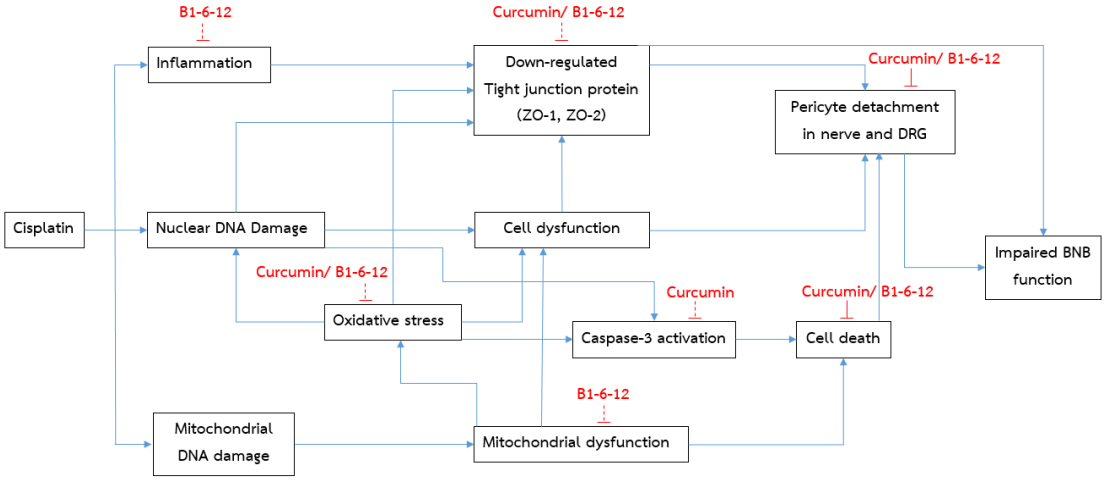


Figure 38 Summary of effects of curcumin and B1-6-12 on cisplatin-induced alterations in pericytes

CHAPTER VI

CONCLUSIONS

This study has demonstrated the beneficial effects of curcumin and B1-6-12 on ultrastructural changes in the sciatic nerves and DRG induced by cisplatin. Ultrastructural studies showed that cisplatin caused higher frequency and wider distance of pericyte detachment in the capillaries of those tissues. Detachment of pericytes was significantly improved by curcumin and B1-6-12.

Furthermore, cell culture investigations revealed reduced pericyte viability caused by cisplatin. Curcumin and B1-6-12 also improved pericyte viability. However, no significant changes were found in the levels of ZO-1 and ZO-2 tight junction proteins. These findings explain the effects of cisplatin, curcumin and B vitamins on pericyte detachment in the EM study.

These data suggest that additional pathological mechanism underlying cisplatin-induced neuropathy is BNB disruption and curcumin and B1-6-12 could be used to treat the condition. The precise mechanisms underlying the beneficial effects of curcumin and B1-6-12 remain unclear but may be partly due to the antioxidant and metabolism-enhancing properties, respectively. Further studies are needed to determine the levels of tight junction proteins in cisplatin-treated endothelial cells. In addition, the BNB integrity and effects of curcumin and B1-6-12 should be further investigated in cisplatin-induced neuropathy in the clinical trials.

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APPENDIX

Effects of curcumin on cisplatin-induced alterations in the blood-nerve barrier in rats.

Table 6 The data of percentage of category 1 and 2, separation distance of category 2 and vascular basement membrane thickness of category 1 and 2 in the control group.

No. of rat	Group	Part			Separation distance (mean±SE), nm	VBM thickness (mean±SE), nm
CC1	**Control 1**	Proximal n=20				
	(1st rat)	Category 1	n=15	75.00 %		61.89 ± 1.31
		Category 2	n=5	25.00 %	272.04 ± 29.37	61.13 ± 2.20
		Distal n=20				
		Category 1	n=16	80.00 %		60.49 ± 1.33
		Category 2	n=4	20.00 %	339.16 ± 67.30	63.25 ± 1.62
		L5 DRG n=20				
		Category 1	n=18	90.00 %		59.19 ± 1.49
		Category 2	n=2	10.00 %	195.42 ± 14.33	51.38 ± 5.91
CC2	**Control 2**	Proximal n=18				
	(2nd rat)	Category 1	n=15	83.33 %		58.24 ± 1.84
		Category 2	n=3	16.67 %	248.33 ± 13.96	54.36 ± 1.10
		Distal n=18				
		Category 1	n=15	83.33 %		59.59 ± 1.23
		Category 2	n=3	16.67 %	234.87 ± 50.90	64.71 ± 3.06
		L5 DRG n=20				
		Category 1	n=20	100.00%		48.09 ± 1.25
		Category 2	n=0			
CC3	**Control 3**	Proximal n=18				
	(3rd rat)	Category 1	n=16	88.89 %		55.72 ± 1.71
		Category 2	n=2	11.11 %	173.61 ± 37.20	54.25 ± 3.04
		Distal n=18				
		Category 1	n=17	94.44 %		54.42 ± 1.66
		Category 2	n=1	5.56 %	171.85	50.21
		L5 DRG n=20				

		Category 1	n=19	95.00 %		53.70 ± 1.45
		Category 2	n=1	5.00 %	274.52	55.99
CC4	**Control 4**	Proximal n=17				
	(4^{th} rat)	Category 1	n=14	82.35 %		58.30 ± 1.67
		Category 2	n=3	17.65 %	205.33 ± 31.29	63.20 ± 3.22
		Distal n=18				
		Category 1	n=15	83.33 %		57.49 ± 1.68
		Category 2	n=3	16.67 %	268.40 ± 44.80	53.07 ± 2.14
		L5 DRG n=20				
		Category 1	n=20	100.00%		55.02 ± 1.32
		Category 2	n=0			
CC5	**Control 5**	Proximal n=18				
	(5^{th} rat)	Category 1	n=15	83.33 %		56.79 ± 1.44
		Category 2	n=3	16.67 %	233.80 ± 33.76	58.29 ± 3.83
		Distal n=17				
		Category 1	n=14	82.35 %		56.07 ± 0.88
		Category 2	n=3	17.65 %	232.13 ± 28.01	53.25 ± 1.59
		L5 DRG n=20				
		Category 1	n=19	95.00 %		53.64 ± 1.32
		Category 2	n=1	5.00 %	209.93	58.57

Table 7 The data of percentage of category 1 and 2, separation distance of category 2 and vascular basement membrane thickness of category 1 and 2 in the cisplatin group.

No. of rat	**Group**	**Part**			**Separation distance (mean±SE), nm**	**VBM thickness (mean±SE), nm**
PC1	**Cisplatin 1**	Proximal n=20				
	(1^{st} rat)	Category 1	n=3	15.00 %		61.40 ± 1.54
		Category 2	n=17	85.00 %	433.31 ± 44.17	59.95 ± 1.12
		Distal n=20				
		Category 1	n=2	10.00 %		61.76 ± 1.08
		Category 2	n=18	90.00 %	511.48 ± 27.75	58.93 ± 1.47
		L5 DRG n=20				

		Category 1	n=11	55.00 %		52.99 ± 1.66
		Category 2	n=9	45.00 %	327.93 ± 26.49	56.52 ± 1.68
PC2	**Cisplatin 2**	Proximal n=18				
	(2^{nd} rat)	Category 1	n=3	16.67 %		52.97 ± 5.64
		Category 2	n=15	83.33 %	390.45 ± 34.46	55.98 ± 1.12
		Distal n=18				
		Category 1	n=2	11.11 %		53.45 ± 0.93
		Category 2	n=16	88.89 %	444.22 ± 42.77	61.33 ± 1.82
		L5 DRG n=20				
		Category 1	n=14	70.00 %		50.41 ± 1.15
		Category 2	n=6	30.00 %	373.61 ± 42.00	53.32 ± 3.07
PC3	**Cisplatin 3**	Proximal n=18				
	(3^{rd} rat)	Category 1	n=3	16.67 %		56.89 ± 2.89
		Category 2	n=15	83.33 %	423.28 ± 41.76	57.96 ± 1.70
		Distal n=17				
		Category 1	n=1	5.88 %		58.5
		Category 2	n=16	94.12 %	504.37 ± 59.88	59.15 ± 1.49
		L5 DRG n=20				
		Category 1	n=15	75.00 %		50.27 ± 1.19
		Category 2	n=5	25.00 %	325.02 ± 56.36	54.77 ± 2.33
PC4	**Cisplatin 4**	Proximal n=18				
	(4^{th} rat)	Category 1	n=4	22.22 %		52.06 ± 4.65
		Category 2	n=14	77.78 %	424.92 ± 31.35	58.32 ± 1.48
		Distal n=19				
		Category 1	n=4	21.05 %		56.37 ± 2.07
		Category 2	n=15	78.95 %	492.50 ± 68.31	58.84 ± 2.33
		L5 DRG n=20				
		Category 1	n=16	80.00 %		50.31 ± 1.93
		Category 2	n=4	20.00 %	256.77 ± 47.46	54.07 ± 4.00
PC5	**Cisplatin 5**	Proximal n=20				
	(5^{th} rat)	Category 1	n=6	30.00 %		57.75 ± 1.34
		Category 2	n=14	70.00 %	426.82 ± 44.41	60.20 ± 1.66
		Distal n=18				
		Category 1	n=4	22.22 %		56.93 ± 2.02

		Category 2	n=14	77.78 %	504.10 ± 57.64	59.99 ± 1.88
		L5 DRG n=20				
		Category 1	n=14	70.00 %		50.01 ± 1.47
		Category 2	n=6	30.00 %	336.3 ± 48.91	52.97 ± 3.33

Table 8 The data of percentage of category 1 and 2, separation distance of category 2 and vascular basement membrane thickness of category 1 and 2 in the curcumin treated group.

No. of rat	Group	Part			Separation distance (mean±SE), nm	VBM thickness (mean±SE), nm
S1	**Curcumin + Cisplatin1**	Proximal n=18				
	(1^{st} rat)	Category 1	n=10	55.56 %		59.14 ± 1.91
		Category 2	n=8	44.44 %	268.40 ± 26.17	63.16 ± 3.00
		Distal n=18				
		Category 1	n=11	61.11 %		57.19 ± 1.39
		Category 2	n=7	38.89 %	300.52 ± 28.25	60.06 ± 2.1
		L5 DRG n=20				
		Category 1	n=16	80.00 %		54.80 ± 1.67
		Category 2	n=4	20.00 %	240.73 ± 19.20	57.30 ± 3.6
S2	**Curcumin + Cisplatin 2**	Proximal n=17				
	(2^{nd} rat)	Category 1	n=10	58.82 %		56.14 ± 1.64
		Category 2	n=7	41.18 %	250.85 ± 22.77	59.67 ± 2.76
		Distal n=18				
		Category 1	n=13	72.22 %		58.97 ± 1.7
		Category 2	n=5	27.78 %	261.15 ± 17.51	59.68 ± 2.18
		L5 DRG n=20				
		Category 1	n=17	85.00 %		50.94 ± 1.08
		Category 2	n=3	15.00 %	299.08 ± 41.49	50.23 ± 2.74
S3	**Curcumin + Cisplatin 3**	Proximal n=18				

	(3rd rat)	Category 1	n=12	66.67 %		56.77 ± 1.52
		Category 2	n=6	33.33 %	275.69 ± 35.08	55.35 ± 1.46
		Distal n=17				
		Category 1	n=12	70.59 %		54.10 ± 1.44
		Category 2	n=5	29.41 %	275.86 ± 17.29	59.92 ± 5.26
		L5 DRG n=20				
		Category 1	n=17	85.00 %		51.22 ± 1.32
		Category 2	n=3	15.00 %	248.36 ± 51.12	55.29 ± 3.40
S4	**Curcumin + Cisplatin 4**	Proximal n=20				
	(4th rat)	Category 1	n=15	75.00 %		57.32 ± 0.93
		Category 2	n=5	25.00 %	282.66 ± 24.2	58.62 ± 0.92
		Distal n=18				
		Category 1	n=12	66.67 %		52.43 ± 2.37
		Category 2	n=6	33.33 %	383.20 ± 75.81	55.34 ± 3.38
		L5 DRG n=20				
		Category 1	n=17	85.00 %		51.02 ± 1.53
		Category 2	n=3	15.00 %	235.46 ± 42.66	50.57 ± 4.07
S5	**Curcumin + Cisplatin 5**	Proximal n=18				
	(5th rat)	Category 1	n=13	72.22 %		54.07 ± 1.48
		Category 2	n=5	27.78 %	300.83 ± 36.41	54.88 ± 1.72
		Distal n=20				
		Category 1	n=14	70.00 %		57.16 ± 1.18
		Category 2	n=6	30.00 %	308.94 ± 62.30	56.82 ± 3.87
		L5 DRG n=20				
		Category 1	n=18	90.00 %		52.49 ± 1.49
		Category 2	n=2	10.00 %	206.23 ± 4.16	57.09 ± 6.85

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Table 9 The data of percentage of category 1 and 2, separation distance of category 2 and vascular basement membrane thickness of category 1 and 2 in the control group.

No. of rat	Group	Part			Separation distance (mean±SE), nm	VBM thickness (mean±SE), nm
CB1	**Control 1**	Proximal n=18				
	(1st rat)	Category 1	n=12	70.59 %		59.46 ± 1.79
		Category 2	n=5	29.41 %	278.14 ± 19.48	56.56 ± 2.94
		Distal n=17				
		Category 1	n=15	88.24 %		58.19 ± 1.62
		Category 2	n=2	11.76 %	306.75 ± 34.46	57.25 ± 0.04
		L5 DRG n=20				
		Category 1	n=19	95.00 %		47.58 ± 1.13
		Category 2	n=1	5.00 %	245.2	57.25
CB2	**Control 2**	Proximal n=17				
	(2nd rat)	Category 1	n=16	94.12 %		56.72 ± 0.99
		Category 2	n=1	5.88 %	252.51	44.3
		Distal n=18				
		Category 1	n=17	94.44 %		59.79 ± 1.27
		Category 2	n=1	5.56 %	222.36	40.16
		L5 DRG n=20				
		Category 1	n=19	95.00 %		53.06 ± 0.75
		Category 2	n=1	5.00 %	205.9	40.16
CB3	**Control 3**	Proximal n=17				
	(3rd rat)	Category 1	n=15	88.24 %		60.0 2± 0.99
		Category 2	n=2	11.76 %	209.52 ± 51.67	55.45 ± 7.80
		Distal n= 17				
		Category 1	n=15	88.24 %		54.98 ± 1.85
		Category 2	n=2	11.76 %	245.90 ± 36.83	62.30 ± 2.05
		L5 DRG n=20				
		Category 1	n=20	100.00%		51.36 ± 1.05
		Category 2	n=0	0.00 %		

CB4	**Control 4**	Proximal n=18				
	(4^{th} rat)	Category 1	n=16	88.89 %		58.22 ± 2.32
		Category 2	n=2	11.11 %	261.01 ± 24.31	57.76 ± 5.78
		Distal n= 17				
		Category 1	n=14	82.35 %		60.43 ± 1.68
		Category 2	n=3	17.65 %	218.94 ± 33.59	61.89 ± 5.09
		L5 DRG n=20				
		Category 1	n=20	100.00%		49.75 ± 1.21
		Category 2	n=0	0.00 %		
CB5	**Control 5**	Proximal n=20				
	(5^{th} rat)	Category 1	n=17	85.00 %		57.92 ± 1.16
		Category 2	n=3	15.00 %	284.15 ± 57.14	58.88 ± 2.39
		Distal n= 17				
		Category 1	n=14	82.35 %		58.11 ± 2.25
		Category 2	n=3	17.65 %	280.59 ± 10.43	54.90 ± 6.29
		L5 DRG n=20				
		Category 1	n=20	100.00%		49.86 ± 1.08
		Category 2	n=0	0.00 %		

Table 10 The data of percentage of category 1 and 2, separation distance of category 2 and vascular basement membrane thickness of category 1 and 2 in the cisplatin group.

No. of rat	**Group**	**Part**			**Separation distance (mean±SE), nm**	**VBM thickness (mean±SE), nm**
PB1	**Cisplatin 1**	Proximal n=20				
	(1^{st} rat)	Category 1	n=1	5.00 %		62.93
		Category 2	n=19	95.00 %	370.42 ± 31.79	59.15 ± 1.24
		Distal n=18				
		Category 1	n=2	11.11 %		62.30 ± 1.63
		Category 2	n=16	88.89 %	471.44 ± 66.79	57.63 ± 1.27
		L5 DRG n=20				
		Category 1	n=11	55.00 %		52.63 ± 1.95

		Category 2	n=9	45.00 %	279.06 ± 27.32	47.99 ± 1.07
PB2	**Cisplatin 2**	Proximal n=18				
	(2^{nd} rat)	Category 1	n=3	16.67 %		59.29 ± 1.31
		Category 2	n=15	83.33 %	397.39 ± 47.86	54.22 ± 1.17
		Distal n=18				
		Category 1	n=2	11.11 %		57.38 ± 0.14
		Category 2	n=16	88.89 %	500.99 ± 51.26	56.69 ± 2.28
		L5 DRG n=20				
		Category 1	n=14	70.00 %		52.38 ± 1.35
		Category 2	n=6	30.00 %	265.57 ± 47.37	48.98 ± 3.49
PB3	**Cisplatin 3**	Proximal n= 18				
	(3^{rd} rat)	Category 1	n=1	5.56 %		51.53
		Category 2	n=17	94.44 %	443.58 ± 42.58	61.32 ± 1.29
		Distal n= 18				
		Category 1	n=4	22.22 %		56.35 ± 2.57
		Category 2	n=14	77.78 %	458.77 ± 54.60	58.66 ± 1.86
		L5 DRG n=20				
		Category 1	n=16	80.00 %		50.63 ± 1.40
		Category 2	n=4	20.00 %	295.93 ± 15.78	44.71 ± 3.18
PB4	**Cisplatin 4**	Proximal n= 18				
	(4^{th} rat)	Category 1	n=3	16.67 %		57.70 ± 7.52
		Category 2	n=15	83.33 %	502.05 ± 35.55	56.41 ± 1.18
		Distal n= 19				
		Category 1	n=4	21.05 %		45.48 ± 1.99
		Category 2	n=15	78.95 %	537.33 ± 56.87	59.85 ± 1.73
		L5 DRG n=20				
		Category 1	n=10	50.00 %		49.32 ± 2.31
		Category 2	n=10	50.00 %	293.20 ± 43.28	52.14 ± 1.89
PB5	**Cisplatin 5**	Proximal n= 18				
	(5^{th} rat)	Category 1	n=2	11.11 %		58.68 ± 7.45
		Category 2	n=16	88.89 %	541.79 ± 41.73	56.82 ± 1.65
		Distal n= 20				
		Category 1	n=3	15.00 %		52.11 ± 0.96
		Category 2	n=17	85.00 %	498.30 ± 37.72	56.15 ± 1.07

		L5 DRG n=20				
		Category 1	n=14	70.00 %		49.13 ± 1.63
		Category 2	n=6	30.00 %	275.55 ± 54.09	48.32 ± 1.49

Table 11 The data of percentage of category 1 and 2, separation distance of category 2 and vascular basement membrane thickness of category 1 and 2 in the low-dose of B1-6-12 (LB) treated group.

No. of rat	Group	Part			Separation distance (mean±SE), nm	VBM thickness (mean±SE), nm
LB1	**Low-dose B1-6-12**	Proximal n=17				
	(1^{st} rat)	Category 1	n=10	58.82 %		54.42 ± 1.93
		Category 2	n=7	41.18 %	281.09 ± 24.41	62.27 ± 1.81
		Distal n=18				
		Category 1	n=10	55.56 %		59.28 ± 1.99
		Category 2	n=8	44.44 %	321.72 ± 61.47	60.89 ± 1.79
		L5 DRG n=20				
		Category 1	n=17	85.00 %		50.59 ± 1.26
		Category 2	n=3	15.00 %	328.04 ± 48.83	57.33 ± 3.12
LB2	**Low-dose B1-6-12**	Proximal n=17				
	(2^{nd} rat)	Category 1	n=9	52.94 %		57.40 ± 2.58
		Category 2	n=8	47.06 %	354.22 ± 34.74	66.68 ± 1.80
		Distal n=17				
		Category 1	n=10	58.82 %		57.38 ± 1.71
		Category 2	n=7	41.18 %	295.23 ± 48.96	55.43 ± 3.11
		L5 DRG n=20				
		Category 1	n=19	95.00 %		50.42 ± 1.33
		Category 2	n=1	5.00 %	161.51	49.47
LB3	**Low-dose B1-6-12**	Proximal n=18				
	(3^{rd} rat)	Category 1	n=12	66.67 %		57.53 ± 1.32

		Category 2	n=6	33.33 %	260.56 ± 20.86	58.78 ± 1.78
		Distal n= 20				
		Category 1	n=16	80.00 %		54.04 ± 1.35
		Category 2	n=4	20.00 %	326.86 ± 41.72	51.76 ± 3.23
		L5 DRG n=20				
		Category 1	n=17	85.00 %		53.14 ± 0.96
		Category 2	n=3	15.00 %	269.93 ± 47.97	60.37 ± 3.32
LB4	**Low-dose B1-6-12**	Proximal n=19				
	(4^{th} rat)	Category 1	n=12	63.16 %		57.61 ± 1.34
		Category 2	n=7	36.84 %	298.68 ± 37.54	56.12 ± 1.97
		Distal n= 17				
		Category 1	n=9	52.94 %		56.01 ± 2.77
		Category 2	n=8	47.06 %	302.52 ± 41.34	62.30 ± 1.52
		L5 DRG n=20				
		Category 1	n=18	90.00 %		49.52 ± 1.46
		Category 2	n=2	10.00 %	312.27 ± 106.37	50.19 ± 0.02
LB5	**Low-dose B1-6-12**	Proximal n=17				
	(5^{th} rat)	Category 1	n=10	58.82 %		60.18 ± 1.79
		Category 2	n=7	41.18 %	306.30 ± 38.98	58.50 ± 2.30
		Distal n= 17				
		Category 1	n=9	52.94 %		56.10 ± 1.80
		Category 2	n=8	47.06 %	280.34 ± 54.22	59.22 ± 1.72
		L5 DRG n=20				
		Category 1	n=18	90.00 %		51.57 ± 1.24
		Category 2	n=2	10.00 %	333.07 ± 107.08	55.95 ± 8.30

Table 12 The data of percentage of category 1 and 2, separation distance of category 2 and vascular basement membrane thickness of category 1 and 2 in the medium-dose of B1-6-12 (MB) treated group.

No. of rat	Group	Part			Separation distance (mean±SE), nm	VBM thickness (mean±SE), nm
MB1	**Medium-dose B1-6-12**	Proximal n=18				
	(1st rat)	Category 1	n=12	66.67 %		60.42 ± 2.09
		Category 2	n=6	33.33 %	283.08 ± 34	57.40 ± 1.60
		Distal n=17				
		Category 1	n=10	58.82 %		56.24 ± 0.82
		Category 2	n=7	41.18 %	297.61 ± 65.04	54.58 ± 1.73
		L5 DRG n=20				
		Category 1	n=18	90.00 %		52.34 ± 1.26
		Category 2	n=2	10.00 %	193.75 ± 58.11	50.70 ± 0.49
MB2	**Medium-dose B1-6-12**	Proximal n=18				
	(2nd rat)	Category 1	n=13	72.22 %		56.73 ± 2.02
		Category 2	n=5	27.78 %	291.27 ± 41.03	59.84 ± 1.75
		Distal n=18				
		Category 1	n=12	66.67 %		65.91 ± 1.71
		Category 2	n=6	33.33 %	287.45 ± 32.63	63.24 ± 2.40
		L5 DRG n=20				
		Category 1	n=17	85.00 %		48.08 ± 1.08
		Category 2	n=3	15.00 %	266.94 ± 33.97	50.75 ± 3.14
MB3	**Medium-dose B1-6-12**	Proximal n=20				
	(3rd rat)	Category 1	n=15	75.00 %		55.54 ± 1.49
		Category 2	n=5	25.00 %	225.78 ± 19.66	53.88 ± 2.72
		Distal n= 20				
		Category 1	n=16	80.00 %		56.62 ± 1.78
		Category 2	n=4	20.00 %	244.49 ± 30.35	60.73 ± 1.42
		L5 DRG n=20				

		Category 1	n=18	90.00 %		51.68 ± 1.51
		Category 2	n=2	10.00 %	274.95 ± 96.96	54.47 ± 2.81
MB4	**Medium-dose B1-6-12**	Proximal n=17				
	(4^{th} rat)	Category 1	n=11	64.71 %		57.26 ± 1.49
		Category 2	n=6	35.29 %	285.38 ± 34.48	54.97 ± 2.70
		Distal n= 20				
		Category 1	n=12	60.00 %		59.93 ± 1.67
		Category 2	n=8	40.00 %	303.83 ± 26.1	57.71 ± 1.80
		L5 DRG n=20				
		Category 1	n=19	95.00 %		48.89 ± 1.24
		Category 2	n=1	5.00 %	413.48	55.45
MB5	**Medium-dose B1-6-12**	Proximal n=17				
	(5^{th} rat)	Category 1	n=10	58.82 %		52.96 ± 1.16
		Category 2	n=7	41.18 %	292.28 ± 35.98	56.77 ± 1.40
		Distal n= 20				
		Category 1	n=15	75.00 %		54.15 ± 0.86
		Category 2	n=5	25.00 %	259.60 ± 33.57	57.63 ± 2.57
		L5 DRG n=20				
		Category 1	n=18	90.00 %		47.05 ± 1.73
		Category 2	n=2	10.00 %	255.67 ± 106.80	42.22 ± 5.43

Table 13 The data of percentage of category 1 and 2, separation distance of category 2 and vascular basement membrane thickness of category 1 and 2 in the high-dose of B1-6-12 (HB) treated group.

No. of rat	Group	Part			Separation distance (mean±SE), nm	VBM thickness (mean±SE), nm
HB1	**High-dose B1-6-12**	Proximal n=17				
	(1^{st} rat)	Category 1	n=9	52.94 %		60.10 ± 1.49
		Category 2	n=8	47.06 %	312.89 ± 38.70	57.35 ± 1.19
		Distal n=17				

		Category 1	n=9	52.94 %		59.01 ± 2.31
		Category 2	n=8	47.06 %	391.55 ± 76.21	57.70 ± 1.92
		L5 DRG n=20				
		Category 1	n=13	65.00 %		50.96 ± 1.33
		Category 2	n=7	35.00 %	282.13 ± 32.21	53.13 ± 3.47
HB2	**High-dose B1-6-12**	Proximal n=18				
	(2^{nd} rat)	Category 1	n=10	55.56 %		56.66 ± 1.39
		Category 2	n=8	44.44 %	344.73 ± 44.97	56.63 ± 2.04
		Distal n=17				
		Category 1	n=10	58.82 %		62.27 ± 1.57
		Category 2	n=7	41.18 %	322.82 ± 31.74	59.33 ± 3.67
		L5 DRG n=20				
		Category 1	n=16	80.00 %		50.01 ± 1.09
		Category 2	n=4	20.00 %	318.88 ± 36.28	54.87 ± 2.41
HB3	**High-dose B1-6-12**	Proximal n=17				
	(3^{rd} rat)	Category 1	n=11	64.71 %		59.53 ± 1.42
		Category 2	n=6	35.29 %	384.39 ± 60.29	56.70 ± 0.77
		Distal n= 18				
		Category 1	n=12	66.67 %		55.61 ± 1.24
		Category 2	n=6	33.33 %	265.74 ± 33.46	59.41 ± 2.04
		L5 DRG n=20				
		Category 1	n=16	80.00 %		49.67 ± 1.97
		Category 2	n=4	20.00 %	419.04 ± 28.55	56.25 ± 2.05
HB4	**High-dose B1-6-12**	Proximal n=19				
	(4^{th} rat)	Category 1	n=14	73.68 %		56.31 ± 1.47
		Category 2	n=5	26.32 %	273.14 ± 43.24	59.97 ± 1.96
		Distal n= 20				
		Category 1	n=13	65.00 %		57.34 ± 1.49
		Category 2	n=7	35.00 %	332.31 ± 49.82	57.33 ± 1.89
		L5 DRG n=20				
		Category 1	n=13	65.00 %		45.32 ± 1.98

		Category 2	n=7	35.00 %	514.85 ± 108.78	50.32 ± 1.75
HB5	**High-dose B1-6-12**	Proximal n=17				
	(5^{th} rat)	Category 1	n=11	64.71 %		59.68 ± 2.41
		Category 2	n=6	35.29 %	386.03 ± 37.08	54.79 ± 3.43
		Distal n= 17				
		Category 1	n=10	58.82 %		61.44 ± 2.67
		Category 2	n=7	41.18 %	448.28 ± 77.55	54.44 ± 2.05
		L5 DRG n=20				
		Category 1	n=17	85.00 %		53.50 ± 1.15
		Category 2	n=3	15.00 %	285.58 ± 111	45.61 ± 3.19

Cell viability

1. **Effect of cisplatin on RBVP viability**

Group	% Cell viability (Mean ± SEM)
Control	100.0 ± 0
Cisplatin 1 µg/ml	93.77 ± 3.56
Cisplatin 3 µg/ml	82.18 ± 0.87
Cisplatin 5 µg/ml	74.25 ± 6.78
Cisplatin 10 µg/ml	77.07 ± 4.51
Cisplatin 20 µg/ml	79.21 ± 7.43
Cisplatin 40 µg/ml	73.26 ± 11.38
Cisplatin 60 µg/ml	73.71 ± 13.21

2. **Effect of curcumin on RBVP viability**

Group	% Cell viability (Mean ± SEM)
Control	100.0 ± 0
Curcumin 0.1 µg/ml	118.88 ± 6.32
Curcumin 1 µg/ml	119.28 ± 6.6
Curcumin 5 µg/ml	60.00 ± 11.47
Curcumin 10 µg/ml	38.55 ± 24.12
Curcumin 20 µg/ml	0.89 ± 0.46

3. **Effect of B1-6-12 on RBVP viability**

Group	% Cell viability (Mean ± SEM)
Control	100.0 ± 0
B1-6-12 1 μg/ml	111.19 ± 0.68
B1-6-12 5 μg/ml	113.27 ± 0.39
B1-6-12 10 μg/ml	110.24 ± 1.87
B1-6-12 20 μg/ml	111.85 ± 0.14
B1-6-12 40 μg/ml	114.02 ± 0.92
B1-6-12 80 μg/ml	113.65 ± 1.73
B1-6-12 160 μg/ml	101.86 ± 4.58

4. **Effect of co-treatment between cisplatin and curcumin on RBVP viability**

Group	% Cell viability (Mean ± SEM)
Control	100.0 ± 0
Cisplatin 3 μg/ml	83.69 ± 0.87
Cisplatin 3 μg/ml + Curcumin 0.1 μg/ml	87.98 ± 0.74
Cisplatin 3 μg/ml + Curcumin 1 μg/ml	89.85 ± 4.23

5. **Effect of co-treatment between cisplatin and B1-6-12 on RBVP viability**

Group	% Cell viability (Mean ± SEM)
Control	100.0 ± 0
Cisplatin 3 μg/ml	83.69 ± 0.87
Cisplatin + B1-6-12 1 μg/ml	97.21 ± 4.78
Cisplatin + B1-6-12 40 μg/ml	96.65 ± 3.42
Cisplatin + B1-6-12 160 μg/ml	95.60 ± 4.85

Tight junction protein expression

- **ZO-1 level on RBVP after treated with cisplatin and curcumin/B1-6-12.**

Group	ZO-1 (Mean ± SEM)
Control	1.0000 ± 0
Cisplatin 3 µg/ml	0.9018 ± 0.2138
Cisplatin + Curcumin 1 µg/ml	0.9946 ± 0.1026
Cisplatin + B1-6-12 1 µg/ml	0.9629 ± 0.2594

- **ZO-2 level on RBVP after treated with cisplatin and curcumin/B1-6-12.**

Group	ZO-1 (Mean ± SEM)
Control	1.0000 ± 0
Cisplatin 3 µg/ml	0.8670 ± 0.1026
Cisplatin + Curcumin 1 µg/ml	0.9978 ± 0.2214
Cisplatin + B1-6-12 1 µg/ml	0.9027 ± 0.1842

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