Effects of curcumin and $B_{1-6-12}$ on alterations in cell viability, oxidative stress, expression of tight junction proteins and barrier property of endothelial cell and pericyte induced by cisplatin and oxaliplatin in vitro

จุฬาลงกรณ์มหาวิทยาลัย
CHULALONGKORN UNIVERSITY

Miss Amornrat Tothonglor

A Dissertation Submitted in Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy in Medical Sciences
Common Course
FACULTY OF MEDICINE
Chulalongkorn University
Academic Year 2021
Copyright of Chulalongkorn University

ผลของ curcumin และวิตามิน $B_{1-6-12}$ ต่อการเปลี่ยนแปลงในด้านการอยู่รอดของเซลล์ oxidative stress การแสดงออกของโปรตีนใน tight junction และคุณสมบัติทาง barrier ของเซลล์ endothelial และ pericyte ที่เกิดจากยา cisplatin และ oxaliplatin ในสภาวะเพาะเลี้ยง

น.ส.อมรรัตน์ โตทองหล่อ

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
สาขาวิชาวิทยาศาสตร์การแพทย์ ไม่สังกัดภาควิชา/เทียบเท่า
คณะแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
ปีการศึกษา 2564

ลิขสิทธิ์ของจุฬาลงกรณ์มหาวิทยาลัย

| | |
|---|---|
| Thesis Title | Effects of curcumin and $B_{1\text{-}6\text{-}12}$ on alterations in cell viability, oxidative stress, expression of tight junction proteins and barrier property of endothelial cell and pericyte induced by cisplatin and oxaliplatin in vitro |
| By | Miss Amornrat Tothonglor |
| Field of Study | Medical Sciences |
| Thesis Advisor | Professor SITHIPORN AGTHONG, M.D., Ph.D. |

Accepted by the FACULTY OF MEDICINE, Chulalongkorn University in Partial Fulfillment of the Requirement for the Doctor of Philosophy

.................................................. Dean of the FACULTY OF MEDICINE

(Associate Professor CHANCHAI SITTIPUNT, M.D.)

DISSERTATION COMMITTEE

.................................................. Chairman

(Professor VILAI CHENTANEZ, M.D., Ph.D.)

.................................................. Thesis Advisor

(Professor SITHIPORN AGTHONG, M.D., Ph.D.)

.................................................. Examiner

(Assistant Professor DEPICHA JINDATIP, Ph.D.)

.................................................. Examiner

(Assistant Professor SEKH THANPRASERTSUK, M.D.)

.................................................. External Examiner

(Assistant Professor AREE WANASUNTRONWONG, Ph.D.)

จุฬาลงกรณ์มหาวิทยาลัย
CHULALONGKORN UNIVERSITY

อมรรัตน์ โตทองหล่อ : ผลของ curcumin และวิตามิน $B_{1-6-12}$ ต่อการเปลี่ยนแปลงในด้านการอยู่รอดของเซลล์ oxidative stress การแสดงออกของโปรตีนใน tight junction และคุณสมบัติทาง barrier ของเซลล์ endothelial และ pericyte ที่เกิดจากยา cisplatin และ oxaliplatin ในสภาวะเพาะเลี้ยง. ( Effects of curcumin and $B_{1-6-12}$ on alterations in cell viability, oxidative stress, expression of tight junction proteins and barrier property of endothelial cell and pericyte induced by cisplatin and oxaliplatin in vitro) อ.ที่ปรึกษาหลัก : ศ. ดร.นพ.สิทธิพร แอกทอง

Cisplatin และ oxaliplatin เป็นยาเคมีบำบัดที่ทำให้เกิดผลข้างเคียงคือการเกิดพยาธิสภาพต่อระบบประสาทส่วนปลาย (peripheral neuropathy) ได้ แต่ปัจจุบันยังไม่ทราบพยาธิกำเนิดที่แน่ชัด นอกจากนี้ยาเคมีบำบัดทั้งสองตัวยังทำให้เกิดอันตรายต่อหลอดเลือดซึ่งอาจเป็นกลไกที่ทำให้เกิดภาวะ peripheral neuropathy ได้ วัตถุประสงค์ของงานวิจัยเพื่อศึกษาผลของ curcumin และวิตามินบี 1-6-12 ต่อการเปลี่ยนแปลงในด้านการอยู่รอดของเซลล์ การตายแบบ apoptosis การเกิด oxidative stress การแสดงออกของโปรตีนใน tight junction และคุณสมบัติทาง barrier ของเซลล์ endothelial และ pericyte ที่เกิดจากยา cisplatin และ oxaliplatin ในสภาวะเพาะเลี้ยง โดยใช้ human umbilical vein endothelial cell (HUVEC) และ human brain vascular pericyte (HBVP) เป็นตัวแทนของเซลล์ endothelial และ pericyte ผลการศึกษาพบว่ายา cisplatin จะทำให้การอยู่รอดของเซลล์ลดลง เซลล์เกิด oxidative stress และ apoptosis ซึ่งทำให้เกิดการแสดงออกของโปรตีน tight junction ลดลง และนำไปสู่การทำหน้าที่เป็น barrier ของเซลล์ลดลงทั้งในเซลล์ endothelial และ pericyte การให้ curcumin ร่วมกับ cisplatin ทำให้ภาวะดังกล่าวดีขึ้นในทั้งสองเซลล์ นอกจากนี้การให้วิตามินบี 1-6-12 ร่วมกับเคมีบำบัดดังกล่าวก็ช่วยทำให้ภาวะที่เกิดจากการให้ cisplatin ดีขึ้น ยกเว้นไม่สามารถลด oxidative stress ได้ในทั้งสองเซลล์ ยา oxaliplatin ก็ทำให้เกิดอันตรายต่อ HUVEC และ HBVP เช่นเดียวกับ cisplatin การให้ curcumin ร่วมกับ oxaliplatin ทำให้ภาวะดังกล่าวดีขึ้นในทั้งสองเซลล์ แต่การให้วิตามินบี 1-6-12 ไม่สามารถบรรเทาภาวะที่เกิดจากยา oxaliplatin ได้ ผลจากการให้ทั้ง curcumin และ วิตามินบี 1-6-12 ร่วมกับยา cisplatin หรือยา oxaliplatin ก็สามารถบรรเทาภาวะที่เซลล์ทั้งสองถูกทำลายจากยาเคมีบำบัดทั้งสองชนิดได้ แต่ไม่ได้มีประสิทธิภาพดีกว่าการให้ curcumin หรือ $B_{1-6-12}$ เพียงอย่างเดียว ทั้ง curcumin และวิตามินบี 1-6-12 มีประสิทธิภาพที่จะเป็นตัวเลือกในการรักษาอันตรายต่อหลอดเลือดที่เกิดจากการให้ยา cisplatin ส่วนการรักษาภาวะดังกล่าวที่เกิดจากยา oxaliplatin มีเพียง curcumin เท่านั้นที่มีประสิทธิภาพในการบรรเทาภาวะดังกล่าว นอกจากนี้ยังไม่คุ้มค่าที่จะใช้ทั้ง curcumin และวิตามินบี 1-6-12 ในการรักษาภาวะดังกล่าวที่เกิดจากยา cisplatin และ oxaliplatin

| | | |
|---|---|---|
| สาขาวิชา | วิทยาศาสตร์การแพทย์ | ลายมือชื่อนิสิต ............................................... |
| ปีการศึกษา | 2564 | ลายมือชื่อ อ.ที่ปรึกษาหลัก ............................. |

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

# # 5974761330 : MAJOR MEDICAL SCIENCES
KEYWORD: Endothelial cell Cisplatin Curcumin Oxaliplatin Pericyte Peripheral neuropathy Vitamin B1-6-12

Amornrat Tothonglor : Effects of curcumin and $B_{1-6-12}$ on alterations in cell viability, oxidative stress, expression of tight junction proteins and barrier property of endothelial cell and pericyte induced by cisplatin and oxaliplatin in vitro. Advisor: Prof. SITHIPORN AGTHONG, M.D., Ph.D.

Cisplatin and oxaliplatin are platinum chemotherapeutic drugs that can cause peripheral neuropathy. Nowadays, the pathogenesis of cisplatin/oxaliplatin-induced neuropathy has not been completely elucidated. Emerging evidence revealed that both drugs cause vascular complications. Both drugs might damage nerve capillaries leading to cisplatin/oxaliplatin-induced neuropathy. The objective of this study is to examine the effect of curcumin and $B_{1-6-12}$ on cisplatin and oxaliplatin-induced alterations in cell viability, apoptosis (caspase-3), oxidative stress (ROS and GSH/GSSG ratio), expression of tight junction proteins (claudin-5, occludin, ZO-1, and ZO-2), and barrier property in endothelial cell and pericyte in vitro. Human umbilical vein endothelial cell (HUVEC) and human brain vascular pericyte (HBVP) were utilized as a model of endothelial cells and pericytes, respectively. Cisplatin caused the reduction of cell viability, induction of caspase-3, overproduction of oxidative stress, depletion of tight junction protein expression, and barrier dysfunction in both HUVEC and HBVP. Co-treatment with curcumin improved these toxic effects in both cells. Moreover, co-treatment with $B_{1-6-12}$ improved all damage except oxidative stress in both HUVEC and HBVP. Oxaliplatin also caused alterations in HUVEC and HBVP similar to cisplatin and curcumin relieved the alterations in both cell types. In contrast, $B_{1-6-12}$ was not effective against oxaliplatin-induced cytotoxicity in both HUVEC and HBVP. The combination of curcumin and $B_{1-6-12}$ was not significantly more effective than curcumin or $B_{1-6-12}$ alone in most parameters. Both curcumin and $B_{1-6-12}$ are effective against cisplatin-induced endothelial cell and pericyte cytotoxicity: however, only curcumin is the potential treatment against oxaliplatin-induced microvascular damage. In addition, it is not worth combining these two agents for the management of platinum chemotherapy-induced microvascular toxicity.

| Field of Study: | Medical Sciences | Student's Signature ............................... |
|---|---|---|
| Academic Year: | 2021 | Advisor's Signature ............................. |

# ACKNOWLEDGEMENTS

This successful thesis is assisted and supported by my advisor Prof. Dr. Sithiporn Agthong. I would like to greatly thank my advisor for valuable advice and guidance in this thesis. Moreover, I would like to thank my all committees for their suggestion and helpfulness.

I am grateful to all lecturers in the neuroscience program, anatomy department staff and medical science staff, faculty of medicine, Chulalongkorn University for their assistance and kindness.

Additionally, I would like to thank Mr. Vitavat Aksornkitti who teaches me about cell culture techniques, and the staff of the peripheral nerve research unit for providing the equipment or materials used in this thesis.

I deeply thank my friends in our lab, pericyte lab, and cell culture MRC members for encouragement and supporting me in resolving problems and sharing protocol in this thesis. In addition, I also deeply grateful to my parent and my sister for encouragement, counseling, and love.

Finally, I would like to thank Huachiew Chalermprakiet University for the opportunity and financial support. This research was supported by the the 90th Anniversary of Chulalongkorn University Fund (Ratchadaphiseksomphot Endowment Fund) and Ratchadaphiseksomphot fund (faculty of medicine).

Amornrat Tothonglor

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

# TABLE OF CONTENTS

Page

ABSTRACT (THAI) ..... iii

ABSTRACT (ENGLISH) ..... iv

ACKNOWLEDGEMENTS ..... v

TABLE OF CONTENTS ..... vi

LIST OF TABLES ..... xvi

LIST OF FIGURES ..... xvii

LIST OF ABBREVIATIONS ..... xxii

CHAPTER I INTRODUCTION ..... 1

1.1 Introduction ..... 1

1.2 Keywords ..... 3

1.3 Research questions ..... 3

1.4 Objectives ..... 3

1.5 Conceptual Framework ..... 4

1.6 Expected benefits and applications ..... 4

CHAPTER II REVIEW OF THE RELATED LITERATURES ..... 5

2.1 Platinum-based anticancer drugs ..... 5

2.2 Anticancer mechanisms of platinum drugs ..... 6

2.3 Side effects of platinum chemotherapy ..... 7

2.4 Peripheral neuropathy induced by cisplatin and oxaliplatin ..... 7

2.5 Mechanisms of cisplatin and oxaliplatin induced-neurotoxicity ..... 8

2.6 Sensory neuropathy induced by cisplatin and oxaliplatin ..... 12

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

2.7 Cisplatin and oxaliplatin-induced morphologic alterations in the peripheral nervous system ........ 13
2.8 Effects of cisplatin and oxaliplatin on blood vessels ........ 13
2.9 Treatments of cisplatin/oxaliplatin induced-peripheral neuropathy ........ 16
2.9.1 Neurotrophic factors ........ 16
2.9.2 Antioxidants ........ 16
2.9.2.1 Curcumin ........ 17
2.9.3 Vitamin B ........ 18
2.9.4 Other compounds ........ 19
2.10 Blood-nerve barrier (BNB) ........ 20
2.10.1 Endothelial cell ........ 21
2.10.2 Pericyte ........ 23
2.10.2.1 Pericyte identification ........ 23
2.10.2.2 Properties of pericyte ........ 24
2.10.2.3 Pericyte and endothelial cell interaction ........ 25
2.10.2.4 Molecular signaling between pericyte and endothelial cell ........ 25
2.10.2.5 Functions of pericyte ........ 27
2.10.2.6 Pericyte culture ........ 27
2.11 BNB alterations in peripheral neuropathy ........ 28
CHAPTER III MATERIAL AND METHOD ........ 29
3.1 Research framework ........ 29
3.2 Pilot study of HUVEC ........ 30
3.3 Pilot study of HBVP ........ 33
3.4 Research methodology ........ 36

จุฬาลงกรณ์มหาวิทยาลัย
CHULALONGKORN UNIVERSITY

3.4.1 Materials and methods ........ 36
3.4.1.1 Reagents ........ 36
3.4.2 Cell culture ........ 36
3.4.3 Experimental groups ........ 37
3.5 MTT assay (Micro-culture tetrazolium assay) ........ 38
3.6 Caspase-3 activity assay ........ 39
3.7 ROS assay ........ 40
3.8 GSH/GSSG assay ........ 42
3.9 Western blot analysis ........ 43
3.10 Transendothelial electrical resistance (TEER) study ........ 44
3.11 Data analysis ........ 45
CHAPTER IV RESULTS ........ 46
Effects of curcumin on cisplatin- and oxaliplatin-induced alterations in HUVEC ........ 46
1. Cell viability ........ 46
1.1 Effects of co-treatment with cisplatin and curcumin on HUVEC viability ........ 46
1.2 Effects of co-treatment with oxaliplatin and curcumin on HUVEC viability ........ 47
2. Caspase-3 activity ........ 48
2.1 Effects of co-treatment with cisplatin and curcumin on caspase-3 activity ........ 48
2.2 Effects of co-treatment with oxaliplatin and curcumin on caspase-3 activity ........ 48
3. ROS level ........ 49

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

3.1 Effects of co-treatment with cisplatin and curcumin on ROS production ........ 49

3.2 Effects of co-treatment with oxaliplatin and curcumin on ROS production ........ 49

4. Total glutathione level and GSH/GSSG ratio ........ 50

4.1 Effects of co-treatment with cisplatin and curcumin on total glutathione level and GSH/GSSG ratio ........ 50

4.2 Effects of co-treatment with oxaliplatin and curcumin on total glutathione level and GSH/GSSG ratio ........ 51

5. Expression of endothelial tight junction proteins ........ 51

5.1 Effects of co-treatment with cisplatin and curcumin on expression of tight junction proteins ........ 51

5.2 Effects of co-treatment with oxaliplatin and curcumin on expression of tight junction proteins ........ 53

6. TEER value ........ 55

6.1 Effects of co-treatment with cisplatin and curcumin on TEER value . 55

6.2 Effects of co-treatment with oxaliplatin and curcumin on TEER value ........ 56

Effects of curcumin on cisplatin-and oxaliplatin-induced alterations in HBVP ........ 57

1. MTT assay ........ 57

1.1 Effects of co-treatment with cisplatin and curcumin on HBVP viability ........ 57

1.2 Effects of co-treatment with oxaliplatin and curcumin on HBVP viability ........ 58

2. Caspase-3 activity ........ 58

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

2.1 Effects of co-treatment with cisplatin and curcumin on caspase-3 activity ... 58

2.2 Effects of co-treatment with oxaliplatin and curcumin on caspase-3 activity ... 59

3. ROS assay ... 60

3.1 Effects of co-treatment with cisplatin and curcumin on ROS production ... 60

3.2 Effects of co-treatment with oxaliplatin and curcumin on ROS production ... 60

4. Total glutathione level and GSH/GSSG ratio ... 61

4.1 Effects of co-treatment with cisplatin and curcumin on total glutathione level and GSH/GSSG ratio ... 61

4.2 Effects of co-treatment with oxaliplatin and curcumin on total glutathione level and GSH/GSSG ratio ... 62

5. TEER value ... 62

5.1 Effects of co-treatment with cisplatin and curcumin on TEER value ... 62

5.2 Effects of co-treatment with oxaliplatin and curcumin on TEER value ... 63

Effects of $B_{1-6-12}$ on cisplatin- and oxaliplatin-induced alterations in HUVEC ... 64

1. MTT assay ... 64

1.1 Effects of co-treatment with cisplatin and $B_{1-6-12}$ on HUVEC viability ... 64

1.2 Effects of co-treatment with oxaliplatin and $B_{1-6-12}$ on HUVEC viability ... 65

2. Caspase-3 activity ... 65

2.1 Effects of co-treatment with cisplatin and $B_{1-6-12}$ on caspase-3 activity ... 65

2.2 Effects of co-treatment with oxaliplatin and $B_{1-6-12}$ on caspase-3 activity ........ 66

3. ROS assay ........ 67

3.1 Effects of co-treatment with cisplatin and $B_{1-6-12}$ on ROS production 67

3.2 Effects of co-treatment with oxaliplatin and $B_{1-6-12}$ on ROS production ........ 67

4. Total glutathione level and GSH/GSSG ratio ........ 68

4.1 Effects of co-treatment with cisplatin and $B_{1-6-12}$ on total glutathione level and GSH/GSSG ratio ........ 68

4.2 Effects of co-treatment with oxaliplatin and $B_{1-6-12}$ on total glutathione level and GSH/GSSG ratio ........ 68

5. Expression of endothelial tight junction proteins ........ 69

5.1 Effects of co-treatment with cisplatin and $B_{1-6-12}$ on expression of tight junction proteins ........ 69

5.2 Effects of co-treatment with oxaliplatin and $B_{1-6-12}$ on expression of tight junction proteins ........ 70

6. TEER value ........ 72

6.1 Effects of co-treatment with cisplatin and $B_{1-6-12}$ on TEER value ........ 72

6.2 Effects of co-treatment with oxaliplatin and $B_{1-6-12}$ on TEER value ..... 73

Effects of $B_{1-6-12}$ on cisplatin- and oxaliplatin-induced alterations in HBVP ........ 74

1. MTT assay ........ 74

1.1 Effects of co-treatment with cisplatin and $B_{1-6-12}$ on HBVP viability .... 74

1.2 Effects of co-treatment with oxaliplatin and $B_{1-6-12}$ on HBVP viability 75

2. Caspase-3 activity ........ 75

2.1 Effects of co-treatment with cisplatin and $B_{1-6-12}$ on caspase-3 activity ........ 75

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

2.2 Effects of co-treatment with oxaliplatin and $B_{1-6-12}$ on caspase-3 activity ........ 76

3. ROS assay ........ 77

3.1 Effects of co-treatment with cisplatin and $B_{1-6-12}$ on ROS production 77

3.2 Effects of co-treatment with oxaliplatin and $B_{1-6-12}$ on ROS production ........ 77

4. Total glutathione level and GSH/GSSG ratio ........ 78

4.1 Effects of co-treatment with cisplatin and $B_{1-6-12}$ on total glutathione level and GSH/GSSG ratio ........ 78

4.2 Effects of co-treatment with oxaliplatin and $B_{1-6-12}$ on total glutathione level and GSH/GSSG ratio ........ 78

5. TEER value ........ 79

5.1 Effects of co-treatment with cisplatin and $B_{1-6-12}$ on TEER value ........ 79

5.2 Effects of co-treatment with oxaliplatin and $B_{1-6-12}$ on TEER value ..... 79

Effects of combination between curcumin and $B_{1-6-12}$ on cisplatin- and oxaliplatin-induced alterations in HUVEC ........ 81

1. MTT assay ........ 81

1.1 Effects of co-treatment with cisplatin, curcumin, and $B_{1-6-12}$ on HUVEC viability ........ 81

1.2 Effects of co-treatment with oxaliplatin, curcumin, and $B_{1-6-12}$ on HUVEC viability ........ 82

2. Caspase-3 activity ........ 83

2.1 Effects of co-treatment with cisplatin, curcumin, and $B_{1-6-12}$ on caspase-3 activity ........ 83

2.2 Effects of co-treatment with oxaliplatin, curcumin, and $B_{1-6-12}$ on caspase-3 activity ........ 84

จุฬาลงกรณ์มหาวิทยาลัย
CHULALONGKORN UNIVERSITY

3. ROS assay ........................................ 84

3.1 Effects of co-treatment with cisplatin, curcumin, and $B_{1-6-12}$ on ROS production ........................................ 84

3.2 Effects of co-treatment with cisplatin, curcumin, and $B_{1-6-12}$ on ROS production ........................................ 85

4. Total glutathione level and GSH/GSSG ratio ........................................ 86

4.1 Effects of co-treatment with cisplatin, curcumin, and $B_{1-6-12}$ on total glutathione level and GSH/GSSG ratio ........................................ 86

4.2 Effects of co-treatment with oxaliplatin, curcumin, and $B_{1-6-12}$ on total glutathione level and GSH/GSSG ratio ........................................ 86

5. Expression of endothelial tight junction proteins ........................................ 87

5.1 Effects of co-treatment with cisplatin, curcumin, and $B_{1-6-12}$ on expression of tight junction proteins ........................................ 87

5.2 Effects of co-treatment with oxaliplatin, curcumin, and $B_{1-6-12}$ on expression of tight junction proteins ........................................ 89

6. TEER value ........................................ 91

6.1 Effects of co-treatment with cisplatin, curcumin, and $B_{1-6-12}$ on TEER value ........................................ 91

6.2 Effects of co-treatment with oxaliplatin, curcumin, and $B_{1-6-12}$ on TEER value ........................................ 92

Effects of combination between curcumin and $B_{1-6-12}$ on cisplatin- and oxaliplatin-induced alterations in HBVP ........................................ 94

1. MTT assay ........................................ 94

1.1 Effects of co-treatment with cisplatin, curcumin, and $B_{1-6-12}$ on HBVP viability ........................................ 94

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

1.2 Effects of co-treatment with oxaliplatin, curcumin, and $B_{1-6-12}$ on HBVP viability ... 95

2. Caspase-3 activity ... 96

2.1 Effects of co-treatment with cisplatin, curcumin, and $B_{1-6-12}$ on caspase-3 activity ... 96

2.2 Effects of co-treatment with oxaliplatin, curcumin, and $B_{1-6-12}$ on caspase-3 activity ... 96

3. ROS assay ... 97

3.1 Effects of co-treatment with cisplatin, curcumin, and $B_{1-6-12}$ on ROS production ... 97

3.2 Effects of co-treatment with oxaliplatin, curcumin, and $B_{1-6-12}$ on ROS production ... 98

4. Total glutathione level and GSH/GSSG ratio ... 98

4.1 Effects of co-treatment with cisplatin, curcumin, and $B_{1-6-12}$ on total glutathione level and GSH/GSSG ratio ... 98

4.2 Effects of co-treatment with oxaliplatin, curcumin, and B1-6-12 total glutathione level and GSH/GSSG ratio ... 99

5. TEER value ... 100

5.1 Effects of co-treatment with cisplatin, curcumin, and $B_{1-6-12}$ on TEER value ... 100

5.2 Effects of co-treatment with cisplatin, curcumin, and $B_{1-6-12}$ on TEER value ... 100

CHAPTER V DISCUSSION ... 104

5.1 Effects of curcumin on cisplatin-induced alterations in HUVEC and HBVP ... 104

5.2 Effects of curcumin on oxaliplatin-induced alterations in HUVEC and HBVP ... 106

5.3 Effects of $B_{1-6-12}$ on cisplatin-induced alterations in HUVEC and HBVP ... 108

5.4 Effects of $B_{1-6-12}$ on oxaliplatin-induced alterations in HUVEC and HBVP .......... 110

5.5 Effects of combination between curcumin and $B_{1-6-12}$ on cisplatin-induced alterations in HUVEC and HBVP .......... 111

5.6 Effects of combination between curcumin and $B_{1-6-12}$ on oxaliplatin-induced alterations in HUVEC and HBVP .......... 112

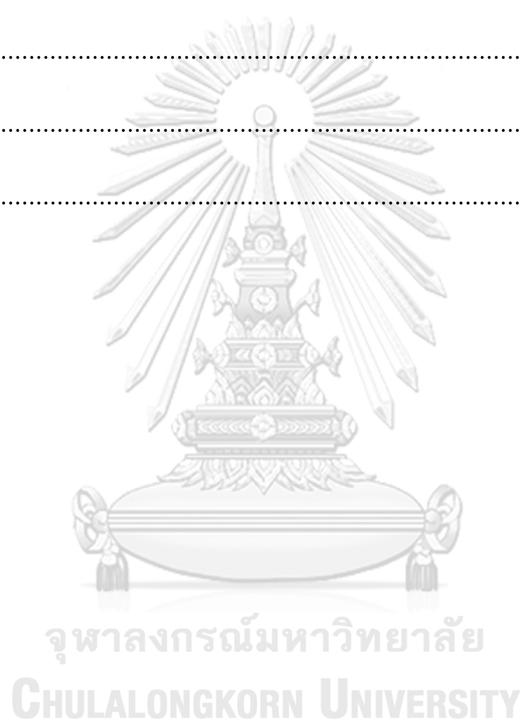
5.7 Further study .......... 112

5.8 Limitation of this study .......... 112

REFERENCES .......... 115

APPENDIX .......... 127

VITA .......... 139



จุฬาลงกรณ์มหาวิทยาลัย
CHULALONGKORN UNIVERSITY

# LIST OF TABLES

**Page**

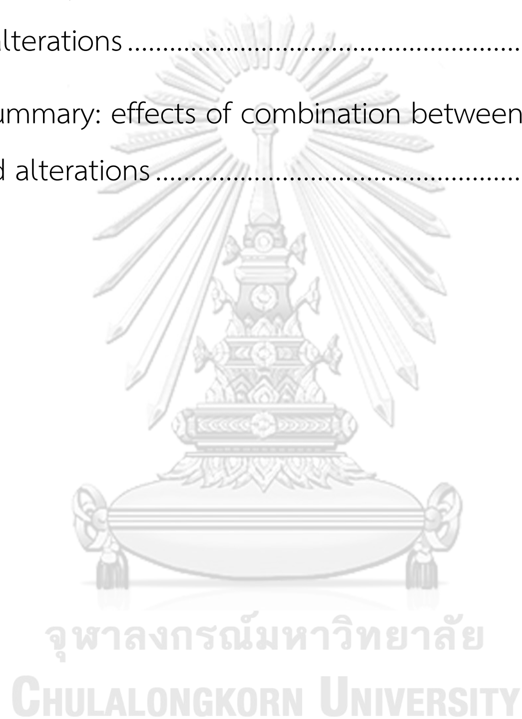
Table 1 Studies of cisplatin in endothelial cells in vitro ..... 15

Table 2 Study of oxaliplatin in endothelial cells in vitro ..... 16

Table 3 Pericyte markers ..... 24

Table 4 Result summary: effects of combination between curcumin and $B_{1-6-12}$ on cisplatin-induced alterations ..... 102

Table 5 Results summary: effects of combination between curcumin and $B_{1-6-12}$ on oxaliplatin-induced alterations ..... 103


จุฬาลงกรณ์มหาวิทยาลัย
CHULALONGKORN UNIVERSITY

# LIST OF FIGURES

Page

Figure 1 chemical structures of cisplatin, carboplatin and oxaliplatin ............................ 5

Figure 2 Anticancer mechanisms of cisplatin ........................................................................ 6

Figure 3 The possible mechanisms of cisplatin and oxaliplatin-induced neurotoxicity ........................................................................................................................................ 11

Figure 4 Components of blood-nerve barrier........................................................................ 20

Figure 5 A) Normal blood-nerve barrier (BNB), B) disrupted BNB ................................... 21

Figure 6 Inter-endothelial cell junction .................................................................................. 23

Figure 7 Junctional complex between endothelial cell and pericyte ............................ 25

Figure 8 Pericyte-endothelial cell molecular interaction .................................................. 26

Figure 9 Cell viability of HUVEC treated with various concentrations of cisplatin ...... 30

Figure 10 Cell viability of HUVEC treated with curcumin................................................... 31

Figure 11 Cell viability of HUVEC treated with $B_{1-6-12}$............................................................ 31

Figure 12 Cell viability of HUVEC treated with oxaliplatin................................................. 32

Figure 13 Cell viability of HBVP treated with various concentration of cisplatin......... 33

Figure 14 Cell viability of HBVP treated with curcumin ..................................................... 34

Figure 15 Cell viability of HBVP treated with $B_{1-6-12}$.............................................................. 34

Figure 16 Cell viability of HBVP treated with oxaliplatin ................................................... 35

Figure 17 Principle of reactions of the GSH/GSSG assay ................................................... 42

Figure 18 Schematic of TEER value measurement.............................................................. 45

Figure 19 Cell viability of HUVEC after treatments. ............................................................ 46

Figure 20 Morphology of HUVEC under phase contrast microscope ............................. 46

Figure 21 Cell viability of HUVEC after treatments. .......... 47
Figure 22 Morphology of HUVEC under phase contrast microscope .......... 47
Figure 23 Caspase-3 activity in HUVEC after treatments. .......... 48
Figure 24 Caspase-3 activity in HUVEC after treatments. .......... 49
Figure 25 ROS production in HUVEC after treatments. .......... 49
Figure 26 ROS production in HUVEC after treatments. .......... 50
Figure 27 Total glutathione level and GSH/GSSG ratio in HUVEC after treatments. .......... 50
Figure 28 Total glutathione level and GSH/GSSG ratio in HUVEC after treatments. .......... 51
Figure 29 Claudin-5 and occludin protein expression in HUVEC after treatments. .......... 52
Figure 30 ZO-1 and ZO-2 protein expression HUVEC after treatments. .......... 53
Figure 31 Claudin-5 and occludin protein expression in HUVEC after treatments. .......... 54
Figure 32 ZO-1 and ZO-2 protein expression in HUVEC after treatments. .......... 55
Figure 33 TEER value of HUVEC after treatments. .......... 56
Figure 34 TEER value of HUVEC after treatments. .......... 56
Figure 35 Cell viability of HBVP after treatments. .......... 57
Figure 36 Morphology of HBVP under phase contrast microscope .......... 57
Figure 37 Cell viability of HBVP after treatments. .......... 58
Figure 38 Morphology of HBVP under phase contrast microscope .......... 58
Figure 39 Caspase-3 activity in HBVP after treatments. .......... 59
Figure 40 Caspase-3 activity in HBVP after treatments. .......... 59
Figure 41 ROS production in HBVP after treatments. .......... 60
Figure 42 ROS production in HBVP after treatments. .......... 61
Figure 43 Total glutathione level and GSH/GSSG ratio in HBVP after treatments. .......... 61
Figure 44 Total glutathione level and GSH/GSSG ratio in HBVP after treatments. .......... 62

จุฬาลงกรณ์มหาวิทยาลัย
CHULALONGKORN UNIVERSITY

Figure 45 The TEER value of HBVP after treatments. .... 63
Figure 46 The TEER value of HBVP after treatments. .... 63
Figure 47 Cell viability of HUVEC after treatments. .... 64
Figure 48 Morphology of HUVEC under phase contrast microscope .... 64
Figure 49 Cell viability of HUVEC after treatments. .... 65
Figure 50 Morphology of HUVEC under phase contrast microscope .... 65
Figure 51 Caspase-3 activity in HUVEC after treatments. .... 66
Figure 52 Caspase-3 activity in HUVEC after treatments. .... 66
Figure 53 ROS production in HUVEC after treatments. .... 67
Figure 54 ROS production in HUVEC after treatments. .... 67
Figure 55 Total glutathione level and GSH/GSSG ratio in HUVEC after treatments. .... 68
Figure 56 Total glutathione level and GSH/GSSG ratio in HUVEC after treatments. .... 68
Figure 57 Claudin-5 and occludin protein expression in HUVEC after treatments. .... 69
Figure 58 ZO-1 and ZO-2 protein expression in HUVEC after treatments. .... 70
Figure 59 Claudin-5 and occludin protein expression in HUVEC after treatments. .... 71
Figure 60 ZO-1 and ZO-2 protein expression in HUVEC after treatments. .... 72
Figure 61 TEER value of HUVEC after treatments. .... 73
Figure 62 TEER value of HUVEC after treatments. .... 73
Figure 63 Cell viability of HBVP after treatments. .... 74
Figure 64 Morphology of HBVP under phase contrast microscope .... 74
Figure 65 Cell viability of HBVP after treatments. .... 75
Figure 66 Morphology of HBVP under phase contrast microscope .... 75
Figure 67 Caspase-3 activity in HBVP after treatments. .... 76
Figure 68 Caspase-3 activity in HBVP after treatments. .... 76

จุฬาลงกรณ์มหาวิทยาลัย
CHULALONGKORN UNIVERSITY

Figure 69 ROS production in HBVP after treatments. ........................................................ 77

Figure 70 ROS production in HBVP after treatments. ........................................................ 77

Figure 71 Total glutathione level and GSH/GSSG ratio in HBVP after treatments. ...... 78

Figure 72 Total glutathione level and GSH/GSSG ratio in HBVP after treatments. ...... 79

Figure 73 TEER value of HBVP after treatments. ................................................................ 79

Figure 74 TEER value of HBVP after treatments. ................................................................ 80

Figure 75 Cell viability of HUVEC after treatments. .......................................................... 81

Figure 76 Morphology of HUVEC under phase contrast microscope ............................ 82

Figure 77 Cell viability of co-treatment with oxaliplatin, curcumin, and $B_{1\text{-}6\text{-}12}$ on HUVEC. ........................................................................................................................... 83

Figure 78 Morphology of HUVEC under phase contrast microscope ............................ 83

Figure 79 Caspase-3 activity in HUVEC after treatments. ................................................ 83

Figure 80 Caspase-3 activity in HUVEC after treatments. ................................................ 84

Figure 81 ROS production in HUVEC after treatments. .................................................... 85

Figure 82 ROS production in HUVEC after treatments. .................................................... 85

Figure 83 Total glutathione level and GSH/GSSG ratio in HUVEC after treatments. ... 86

Figure 84 Total glutathione level and GSH/GSSG ratio in HUVEC after treatments. ... 87

Figure 85 Claudin-5 and occludin protein expression in HUVEC after treatments. ...... 88

Figure 86 ZO-1 and ZO-2 protein expression in HUVEC after treatments. .................... 89

Figure 87 Claudin-5 and occludin protein expression in HUVEC after treatments. ...... 90

Figure 88 ZO-1 and ZO-2 protein expression in HUVEC after treatments. .................... 91

Figure 89 TEER value of HUVEC after treatments. ............................................................ 92

Figure 90 TEER value of HUVEC after treatments. ............................................................ 93

Figure 91 Cell viability of HBVP after treatments. ............................................................ 94

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

Figure 92 Morphology of HBVP under phase contrast microscope ..... 94

Figure 93 Cell viability of HBVP after treatments. ..... 95

Figure 94 Morphology of HBVP under phase contrast microscope ..... 95

Figure 95 Caspase-3 activity in HBVP after treatments. ..... 96

Figure 96 Caspase-3 activity in HBVP after treatments. ..... 97

Figure 97 ROS production in HBVP after treatments. ..... 97

Figure 98 ROS production of co-treatment with oxaliplatin, curcumin, and $B_{1-6-12}$ on HBVP. ..... 98

Figure 99 Total glutathione level and GSH/GSSG ratio in HBVP after treatments. ..... 99

Figure 100 Total glutathione level and GSH/GSSG ratio in HBVP after treatments. ..... 99

Figure 101 TEER value of HBVP after treatments. ..... 100

Figure 102 TEER value of HBVP after treatments. ..... 101

Figure 103 Result summary: effects of curcumin on cisplatin/oxaliplatin-induced endothelial and pericyte cytotoxicity. ..... 113

Figure 104 Result summary: effects of $B_{1-6-12}$ on cisplatin/oxaliplatin-induced endothelial and pericyte cytotoxicity. ..... 114

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

# LIST OF ABBREVIATIONS

| | |
|---|---|
| AGEs | Advanced glycation end-products |
| Ang1 | Angiopoietin-1 |
| BBB | Blood-brain barrier |
| BDNF | Brain-derived neurotrophic factor |
| bFGF | Basic fibroblastic growth factor |
| BNB | Blood-nerve barrier |
| CIDP | Chronic inflammatory demyelinating polyradiculoneuropathy |
| Cis | Cisplatin |
| CTR1 | Copper transporter-1 |
| Cur | Curcumin |
| DCF | Fluorescence form |
| DCFH-DA | 2’, 7’-dichlorofluorescein diacetate |
| DPBS | Dulbecco's phosphate-buffered saline |
| DRG | Dorsal root ganglion |
| FBS | Fetal bovine serum |
| GLUT1 | Glucose transporter 1 |
| GPx | Glutathione peroxidase |
| GSH | Glutathione |
| GSSG | Oxidized glutathione |
| GST | Glutathione-S-transferase |
| $H_2O_2$ | Hydrogen peroxide |
| HBVP | Human brain vascular pericytes |
| HUMEC-1 | Human dermal microvascular endothelial cell |
| HUVEC | Human umbilical vein endothelial cell |
| ICAM-1 | Intercellular adhesion molecule 1 |
| IENF | Intra-epidermal nerve fibers |
| LPO | Lipid peroxidation |
| MMP-2 | Matrix metalloproteinase-2 |
| MMR | Mismatch repair |

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

| | |
|---|---|
| mPTP | Mitochondrial permeability transition pore |
| MTT | Micro-culture tetrazolium assay |
| NCV | Nerve conduction velocity |
| NER | Nuclear excision repair |
| NF-kB | Nuclear factor kappa-B |
| NG2 | Nerve-glial antigen-2 |
| NGF | Nerve growth factor |
| NO | Nitric oxide |
| OCT | Organic cation transporters |
| OXP | Oxaliplatin |
| PBS | Phosphate buffered saline |
| PDGFR-β | Platelet-derived growth factor receptor-beta |
| ROS | Reactive oxygen species |
| SOD | Superoxide dismutase |
| TEER | Trans-endothelial electrical resistance |
| TGF-β | Transforming growth factor beta |
| TRP | Transient receptor potential |
| ZO | Zonula occludens |
| α-SMA | alpha-smooth muscle actin |

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

# CHAPTER I

# INTRODUCTION

## 1.1 Introduction

Cancer is one leading cause of death worldwide and the incidence is accelerating. This phenomenon leads to the increased use of chemotherapeutic drugs. Cisplatin and oxaliplatin are in the platinum derivative class of chemotherapy drugs.[1] Cisplatin is used to treat several solid tumors such as testicular and ovarian cancers, whereas oxaliplatin is mainly used in metastatic colorectal cancer.[1] However, both drugs have a common side effect: peripheral neuropathy with sensory predominance.[2] Oxaliplatin can induce two types of neuropathy: acute and chronic forms which differ from cisplatin.[3] Acute neurotoxicity occurs during or immediately after drug administration, while chronic neuropathy occurs later.[4] Neuropathy can develop with cisplatin and oxaliplatin treatments in approximately 30% and 20-30%, respectively.[5, 6] Cisplatin and oxaliplatin-induced neuropathy can produce severe pain leading to the reduction of efficacious therapeutic dose or treatment withdrawal.[6] Moreover, after the end of treatment, the neuropathic symptoms can continue for several months.[7] Even if this abnormality recovers, it takes a long time and restoration is frequently incomplete.[8] The neuropathic symptoms may also cause disability and reduced quality of life.[8]

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

The proposed mechanisms of neuropathy induced by platinum anticancer drugs are nuclear DNA damage, altered calcium homeostasis, oxidative stress, and mitochondrial dysfunction in DRG neurons and/or nerve fibers.[9] However, some studies have shown the effects of both drugs on endothelial cells and blood vessels.[10-12] In addition, endothelial cells co-operate with pericytes in the control of blood flow and permeability.[13] If the endothelial cells are damaged, the pericytes are likely affected.[14] Therefore, the vascular disruption may be another potential mechanism of cisplatin/oxaliplatin-induced peripheral neuropathy. The recent study in our lab has demonstrated significant pericyte loss and detachment from endothelial cells in the nerves from cisplatin-treated rats compared with controls.[15]

However, whether oxaliplatin has the same effects as cisplatin and the specific molecular mechanisms remain unclear.

Oxidative stress or increased levels of reactive oxygen species (ROS) is one potential mechanism of cisplatin neuropathy. Accordingly, the antioxidant agents can ameliorate this neuropathy. Several antioxidants such as N-acetylcysteine, glutathione, alpha-lipoic acid, and amifostine were effective in experimental models.(16) However, they had side-effects or caused minimal improvement in clinical trials.(16)

Curcumin, a polyphenol antioxidant, is isolated from turmeric plant.(17) It has demonstrated various properties such as antioxidant, anti-inflammation, and neuroprotection.(18) It also enhances the anticancer activity of cisplatin and oxaliplatin.(18, 19) Curcumin can ameliorate chemotherapy-induced demyelination via alleviation of oxidative stress.(20) Besides, it reduces the free radicals generated by cisplatin and oxaliplatin as well as restores the antioxidant enzymes.(21, 22) Curcumin exhibits the neuroprotective effect in cisplatin-treated PC12 cell.(19) It also corrects structural and functional abnormalities in the rats receiving cisplatin and oxaliplatin.(17, 23) Nevertheless, the effects of curcumin on cisplatin/oxaliplatin induced-endothelial and pericyte alterations have not been examined.

Another potential therapeutic agent are B vitamins. They are commonly utilized in peripheral neuropathic treatment. The advantages of B vitamins are low cost, low side effects, and availability.(24) The main functions of B vitamins especially $B_1$, $B_6$, and $B_{12}$ in the nervous system are the coenzymes. They are important for the transmission of nerve impulse, myelin formation and neurotransmitter synthesis.(24-26) Consequently, the deficiencies of these vitamins can cause peripheral neuropathy.(27) The reduction of vitamin $B_{12}$ in patients who received chemotherapeutic drug was observed.(27) Because $B_1$, $B_6$, and $B_{12}$ play different important roles in the nervous system, the combination of these vitamins may have synergistic effects.(25) The combination of these three vitamins demonstrated the effectiveness in diabetic and alcoholic neuropathies.(25, 28, 29) Unpublished data from our lab indicated that $B_{1\text{-}6\text{-}12}$ could ameliorate cisplatin-induced neuropathy in rats. Nonetheless, whether this

beneficial effect of B vitamins is related to vascular damage induced by cisplatin remains to be investigated.

### 1.2 Keywords

Endothelial cell, Cisplatin, Curcumin, Oxaliplatin, Pericyte, Peripheral neuropathy, Vitamin $B_{1-6-12}$

### 1.3 Research questions

1. What are the alterations in cell viability, oxidative stress, expression of tight junction proteins, and barrier property in endothelial cell and pericyte induced by cisplatin and oxaliplatin in vitro?
2. What are the effects of curcumin on cisplatin and oxaliplatin-induced alterations in cell viability, oxidative stress, expression of tight junction proteins, and barrier property in endothelial cell and pericyte in vitro?
3. What are the effects of vitamin $B_{1-6-12}$ on cisplatin and oxaliplatin-induced alterations in cell viability, oxidative stress, expression of tight junction proteins, and barrier property in endothelial cell and pericyte in vitro?
4. What is the combination effect of curcumin and vitamin $B_{1-6-12}$ on cisplatin and oxaliplatin-induced alterations in cell viability, oxidative stress, expression of tight junction proteins, and barrier property in endothelial cell and pericyte in vitro?

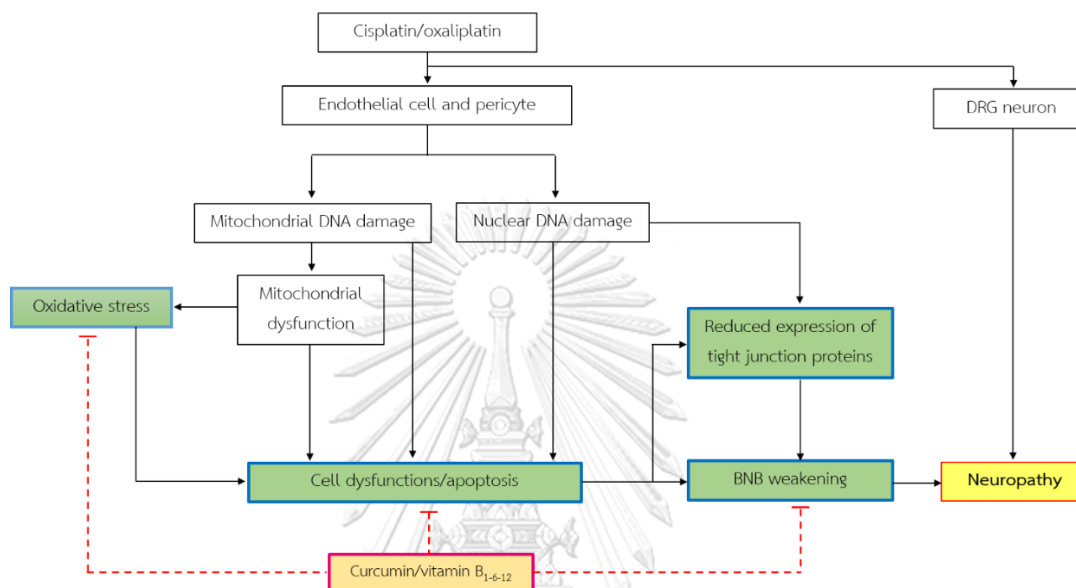
จุฬาลงกรณ์มหาวิทยาลัย
CHULALONGKORN UNIVERSITY

### 1.4 Objectives

1. To study the alterations in cell viability, oxidative stress, expression of tight junction proteins, and barrier property in endothelial cell and pericyte induced by cisplatin and oxaliplatin in vitro.
2. To evaluate the effects of curcumin on cisplatin and oxaliplatin-induced alterations in cell viability, oxidative stress, expression of tight junction proteins, and barrier property in endothelial cell and pericyte in vitro.
3. To examine the effects of vitamin $B_{1-6-12}$ on cisplatin and oxaliplatin-induced alterations in cell viability, oxidative stress, expression of tight junction proteins, and barrier property in endothelial cell and pericyte in vitro.

4. To explore the combination effect of curcumin and vitamin $B_{1-6-12}$ on cisplatin and oxaliplatin-induced alterations in cell viability, oxidative stress, expression of tight junction proteins, and barrier property in endothelial cell and pericyte in vitro.

## 1.5 Conceptual Framework




## 1.6 Expected benefits and applications

This study will clarify whether cisplatin (3 µg/ml in HUVEC and 1.5 µg/ml in HBVP) and oxaliplatin (5 µg/ml and 8 µg/ml) induce endothelial and pericyte cytotoxicity and through which mechanisms. Additionally, if curcumin and $B_{1-6-12}$ have beneficial effects on the cisplatin/oxaliplatin-induced alterations in endothelial cells and pericytes, they can be effective treatments for cisplatin/oxaliplatin-induced neuropathy.

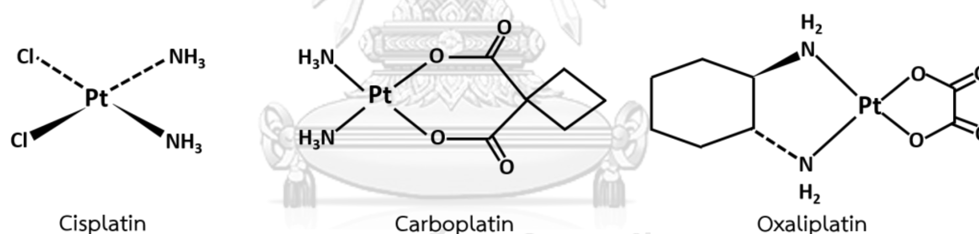
# CHAPTER II

# REVIEW OF THE RELATED LITERATURES

## 2.1 Platinum-based anticancer drugs

The platinum based-anticancer agents currently in use are cisplatin, carboplatin, and oxaliplatin.

Cisplatin (cis-diamminedichloroplatinum) is the first of platinum chemotherapy drugs.[1] Its anticancer properties were discovered in 1965, and it was approved by Food and Drug Administration (FDA) for testicular and ovarian cancer treatment in 1978.[30] Nowadays, cisplatin has been used in solid tumors such as testicular, ovarian, bladder, head and neck, and lung cancers.[9] Chemical structure of cisplatin is composed of a central atom of platinum surrounded by two chloride and two ammonia molecules (Figure 1).[31] Although cisplatin has been an effective anti-cancer agent for decades, it can cause several side effects which lead to dose reduction or discontinuation.




จุฬาลงกรณ์มหาวิทยาลัย
CHULALONGKORN UNIVERSITY

**Figure 1** chemical structures of cisplatin, carboplatin and oxaliplatin

Carboplatin (cis-diammine-1, 1-cyclobutane dicarboxylate platinum II) is the second platinum derivative drug.[1] It was developed to reduce the toxicity of cisplatin.[1] Chemical structure of this drug was similar to that of cisplatin except the chloride ligand is replaced by bidentate dicarboxylate (Figure 1).[31] Carboplatin is used to treat ovarian, lung, and head-neck cancers.[31] Although carboplatin can be administered to patients in higher dose than cisplatin due to the minimized toxicity, its therapeutic efficacy is restricted.[1, 31]

Oxaliplatin (trans-R,R-cyclohexane-1,2-diamineoxalatoplatinum II) is the third platinum chemotherapy drug.[1] It is used to treat metastatic colorectal cancer with

5-fluorouracil (5-FU) and folinate.[1] The chemical structure of oxaliplatin is composed of platinum, (1R,2R)-1,2-diaminocyclohexane (DACH) and oxalate (Figure 1).[1] This modified structure is helpful to minimize side effects and enhances drug uptake into the cell.[1]

## 2.2 Anticancer mechanisms of platinum drugs

When the patient receives intravenous cisplatin, it quickly spreads into tissues.[32] Inside the cell, cisplatin is hydrolyzed resulting in generation of reactive charged platinum complex $[Pt(NH_3)_2ClH_2O]^+$. This complex preferably interacts with nitrogen on position 7 (N7) of guanine or adenine bases by forming covalent bond on the same strand of DNA (intrastrand crosslink) or between two DNA strands (interstrand crosslink) which is defined as DNA adducts.[9, 33] DNA adducts mostly form in the S phase of the cell cycle and destroy double helix structure of DNA which result in impaired DNA replication and final induction of cell cycle arrest at G2/M phase.[9] In addition, DNA adducts also alter multiple signaling pathways such as induction of p53, down-regulation of proto-oncogenes and anti-apoptotic proteins, and activation of both intrinsic and extrinsic pathways of apoptosis.[31] Thus, cisplatin kills cancer cells by damaging DNA that finally results in cell cycle arrest and apoptotic cell death.

จุฬาลงกรณ์มหาวิทยาลัย
CHULALONGKORN UNIVERSITY

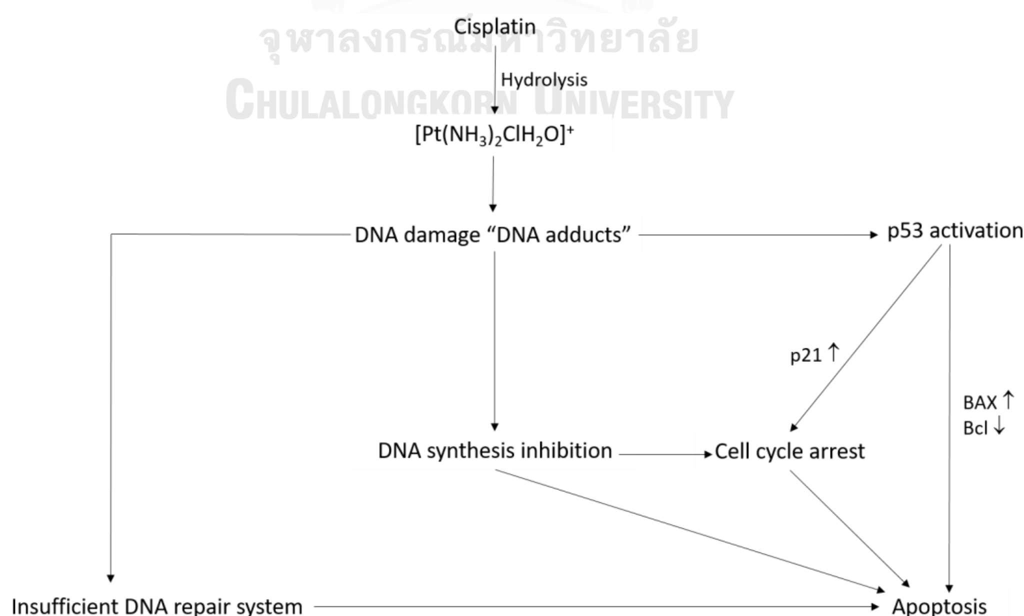



**Figure 2** Anticancer mechanisms of cisplatin

Anticancer mechanism of carboplatin is similar to that of cisplatin, but the DNA reaction rate of carboplatin is slower.[1, 31]

As for oxaliplatin, after administration, it is converted to the reactive compound, dichloro (DACH) platinum complexes (DACH-$Cl_2$-platin or $PtCl_2(DACH)$), followed by hydrolysis ($[Pt(H_2O)(Cl)(DACH)]^+$, $[Pt(H_2O)_2(DACH)]^{2+}$). Then it binds to the DNA to form DNA adducts.[34-36] Like cisplatin, these adducts block the replication and transcription of DNA which promote cell cycle arrest and apoptosis.[34] However, the DNA binding rate is slower than cisplatin but DNA adducts from oxaliplatin have more capability to inhibit DNA synthesis owing to the large size and hydrophobic property [1, 35] Additionally, oxaliplatin-DNA adducts are less detected by repair mechanism especially mismatch repair (MMR) protein.[35, 36]

## 2.3 Side effects of platinum chemotherapy

Although these platinum compounds have been effective anticancer drugs, they can cause several side effects which lead to dose reduction or discontinuation.[2]

The dose-limiting side effects of cisplatin are nephrotoxicity, ototoxicity, neurotoxicity, severe nausea and vomiting, and mild hematologic toxicity.[9, 37] The neurotoxicity is characterized by peripheral neuropathy with sensory predominance.[2]

Regarding carboplatin and oxaliplatin, their toxicity are lower than that of cisplatin especially nephrotoxicity.[4] Nevertheless, oxaliplatin can still induce neurotoxicity similar to cisplatin while high doses of carboplatin are required to induce this side effect.[2]

## 2.4 Peripheral neuropathy induced by cisplatin and oxaliplatin

In the nervous system, dorsal root ganglion (DRG) is the main target of platinum-based drugs because the DRG is not protected by blood brain barrier. Moreover, large-sized DRG neurons express the copper transporter-1 (CTR1) and organic cation transporters (OCT), which also transports cisplatin into the cytoplasm.[38] Therefore, cisplatin largely affects the large sensory nerve fibers.[9, 39] Consequently, cisplatin induces sensory neuropathy rather than motor neuropathy.[9]

จุฬาลงกรณ์มหาวิทยาลัย
CHULALONGKORN UNIVERSITY

This event is supported by the higher accumulation of cisplatin in the DRG and nerve than other nervous tissues.[40] Oxaliplatin is uptake into the cell via passive transport due to its lipophilic structure and organic cation transporters (OCT).[1]

The risk to develop neuropathy increases with higher cumulative doses and longer durations of treatment.[41] In general, cisplatin and oxaliplatin induced-peripheral neurotoxicity occurs after the cumulative doses higher than 250-350 and 540 mg/m$^2$, respectively.[9, 38] According to the clinical presentation, oxaliplatin generates two types of neuropathy— acute and chronic neuropathies which differ from cisplatin.[3] Acute neurotoxicity occurs during or immediately after drug administration, whereas chronic neuropathy occurs after prolonged drug administration and related to cumulative dose (dose limiting toxicity).[4] The incidence of cisplatin-induced neuropathy has been reported in 30-50% of patients[38], while the incidence of acute and chronic oxaliplatin-induced neuropathy is up to 90% of cases.[35]

Taken together, toxic neuropathy induced by cisplatin and oxaliplatin is the important clinical complication due to its high incidence, lack of efficacious treatment and it may restrict the chemotherapy treatment.[11]

### 2.5 Mechanisms of cisplatin and oxaliplatin induced-neurotoxicity

The proposed mechanisms of both cisplatin and oxaliplatin induced-peripheral neurotoxicity are nuclear DNA damage, altered calcium homeostasis, oxidative stress, and mitochondrial dysfunction in DRG neuron and/or axon.[9]

After cisplatin and oxaliplatin infiltrate the DRG neuron, they create DNA adducts similar to cancer cell.[9] However, cisplatin can generate more adducts than oxaliplatin likely explaining more severity of cisplatin-induced neuropathy.[42] Evidence in DRG culture showed the levels of cell death and DNA damage were increased with higher doses of cisplatin.[43] The possible mechanism to explain how DNA adducts cause peripheral neuropathy is the effort of postmitotic DRG neuron to return into cell cycle leading to apoptosis.[40] This idea is supported by the increase in the amount of cell cycle regulatory elements and apoptotic genes such as cyclin D1, CDK 4, p21, Bid 3 in DRG neuron treated with cisplatin.[40, 44] Nonetheless, the DRG

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

neuron has low capacity of nuclear excision repair (NER) system, the main repair mechanism of DNA adducts, resulting in impaired DNA replication, transcription and protein synthesis.[9] Subsequently, these different kinds of DNA lesions accumulated in DRG neuron trigger a specific apoptotic signaling cascade.[9]

The disturbance of intracellular calcium is one possible mechanism of cisplatin and oxaliplatin induced-neuropathy.[45, 46] Intracellular calcium, a secondary messenger, plays a role in several cellular processes such as apoptotic regulation, initiation of intracellular signaling, and controlling neuronal functions— cell survival/death, synaptic plasticity, and neurotransmitter release.[45, 46] This mechanism is supported by emerged evidences in vitro and in vivo treatments with cisplatin or oxaliplatin which revealed the elevation of intracellular calcium.[45, 47, 48]

Moreover, both cisplatin and oxaliplatin also induce overproduction of reactive oxygen species (ROS) that bring about apoptosis via intrinsic and extrinsic pathways in cancer and normal cells.[9, 12, 43] Oxidative stress are generated when ROS production exceeds defensive mechanisms.[22] The DNA, protein, and lipid are the main targets of ROS. Cisplatin as well as oxaliplatin promote oxidative stress with a subsequent acceleration of lipid peroxidation, protein and DNA oxidation, depletion of glutathione peroxidase (GPx) and catalase (ROS defensive enzymes).[49-51] Furthermore, frataxin, an antioxidant protein, is downregulated following cisplatin treatment.[41] The frataxin deficiency is associated with mitochondrial dysfunctions.[52] The chloride ions of cisplatin and oxaliplatin metabolites ($PtCl_2$(DACH)) are replaced by water inside the cell and this complex can elevate the ROS levels.[9, 53] Besides, mammalian nerves are susceptible to oxidative stress because it has more phospholipid content in myelin sheath and less free radical scavengers.[54] ROS can promote neuronal apoptotic cell death via the accumulation of p53 and Fas/Fas-L.[55] Thus, accumulation of ROS in peripheral neurons can progress to neuropathy.[56]

Finally, cisplatin and oxaliplatin also interact with mitochondrial DNA to form DNA adduct which inhibits mitochondrial DNA replication and proper transcription and brings about interruption of electron transport chain, and ATP depletion.[5, 57] Although the binding of cisplatin to nuclear DNA and mitochondrial DNA is likely

equivalent, mitochondria do not have NER for repair mechanisms.[5] Accordingly, the mitochondrial DNA adduct cannot be eliminated.[5]

Since mitochondria are the source of ROS generation and target for ROS attack, the increased ROS alter mitochondrial DNA, protein, and lipid that result in mitochondrial dysfunctions e.g. electron transport chain impairment, prolonged mitochondrial permeability transition pore (mPTP) opening.[22, 58] The prolonged mPTP opening can lead to several events: mitochondrial membrane potential disruption, ATP depletion, ROS production, intracellular calcium elevation, and finally cell death.[52] Vice versa, the mitochondrial dysfunction also provokes oxidative stress.[52] The evidence supports the relationship between mitochondria and ROS following oxaliplatin treatment.[43]

The mitochondrial damage is also evident by morphological abnormalities: swelling, vacuolization, and loss of cristae. These morphological changes are present in peripheral nerve axons of cisplatin and oxaliplatin-treated animals.[57, 59] Mitochondrial swelling and vacuole formation affect the mitochondrial inner membrane which maintains the proton gradient for energy synthesis. Hence, the morphological alterations are correlated with the functional impairments.[60] In addition, the number of functional mitochondria decreased whereas autophagic vacuoles containing degenerative mitochondria increased in DRG neurons after exposing to cisplatin.[41] Accordingly, loss of mitochondrial number and functions likely play a role in cisplatin neurotoxicity.[50] In addition, the number of functional mitochondria decreased whereas autophagic vacuoles containing degenerative mitochondria increased in DRG neurons after exposing to cisplatin.[41] Accordingly, loss of mitochondrial functions or BAX-mitochondrion-cytochrome c pathways can lead to cellular apoptosis.[50]

The mitochondrial alterations may influence axonal transport which requires ATP.[5, 46] The axonal transport defect has been shown to play a role in inherited and acquired peripheral neuropathies.[61] Emerging evidence in animal models showed the disturbance of axonal transport after cisplatin exposure.[62, 63] Thus mitochondrial alterations in neurons might induce impaired axonal transport due to energy failure and finally leading to neuropathy.

Nonetheless, the potential mechanisms mentioned above cannot explain the cold-aggravated burning pain that appears after oxaliplatin administration.[2] The voltage-gated ion channel dysfunction and/or enhanced thermosensitive transient receptor potential (TRP) channel of sensory neurons are possible underlying mechanisms.[2] The first mechanism is defined as “channelopathy”. The channel that involves in oxaliplatin-induced acute neurological side effects is the voltage-gated sodium channels.[34] Oxalate, one of the main metabolites of oxaliplatin, changes the function of voltage-gated sodium channels via calcium ion participation.[46, 64] In detail, oxalate chelates intracellular calcium that brings about the functional disruption of calcium-dependent voltage-gated sodium channel.[65] Oxaliplatin opens $Na^+$ channel, accelerates $Na^+$ influx, prolongs action potential resulting in hyperexcitability of peripheral nerve.[4, 66] Another mechanism involves the stimulation of cold-induced TRP channels such as TRP melastatin 8 (TRPM8), TRP ankyrin 1 (TRPA1).[2, 48] Additionally, oxidative stress also triggers the TRPA1; therefore, the oxidative stress generated from oxaliplatin probably causes cold hypersensitivity via activation of this channel.[67]

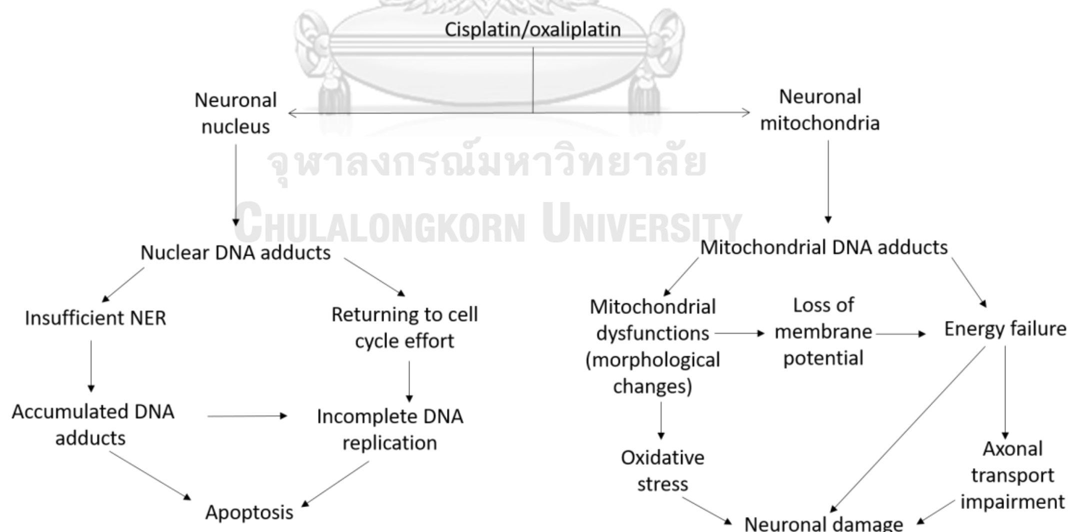



**Figure 3** The possible mechanisms of cisplatin and oxaliplatin-induced neurotoxicity

In addition, chemotherapy drugs also induce axonal degeneration from distal to proximal nerve which is defined as “dying back”. The possible causes of axon degeneration are ROS provoked-demyelination and neuronal cytoskeleton

alteration.[68] The neuronal damage, impaired axonal transport, mitochondrial dysfunctions, or alteration of calcium homeostasis are probable to initiate the degeneration of axon.[9, 69]

### 2.6 Sensory neuropathy induced by cisplatin and oxaliplatin

Characteristic of cisplatin and oxaliplatin neuropathy in human are pain, tingling or numbness in distal extremities which is defined as glove and stocking distribution, as well as slow nerve conduction.[9, 70] The continuous exposure may progress to absence or decrease of proprioception (joint position sense) and deep tendon reflexes which result in severe neurologic symptoms—sensory ataxia and gait disturbance.[6, 9] Gait abnormality may occur which possibly results from the loss of proprioception rather than muscle weakness.[71] The abnormal sensory nerve conduction velocity (NCV) and axonal degeneration are also described.[71] These neuropathic symptoms especially pain may cause dose reduction or early cessation of chemotherapy potentially impacting patient survival.[6] In addition, after discontinuation of these drugs, symptoms may continue and this effect is called "coasting phenomenon".[7] Hence, peripheral neuropathy generates considerable morbidity which leads to the decrease in quality of life and less effective cancer treatment.[72]

The signs and symptoms of cisplatin and chronic oxaliplatin induced-neuropathy are identical; however, acute oxaliplatin-induced neuropathy differs. The symptoms in acute state include cold hypersensitivity, paresthesia and dysesthesia.[3] Besides, acute oxaliplatin neurotoxicity also shows motor symptoms: cramp or jaw tightness.[4] These symptoms are temporary and disappear within hours or days.[7] In acute oxaliplatin-induced neuropathy, there was no or mild degree of axonal degeneration. On the other hands, the axonal degeneration is likely more severe in the chronic neuropathy.[73]

In animal models, behavioral examinations show pain-related behaviors: heat hypoalgesia/hyperalgesia, mechanical allodynia, and mechanical hyperalgesia.[69, 74] However, both drugs produce different types of hyperalgesia: heat hyperalgesia for

cisplatin and cold hyperalgesia for oxaliplatin.[75] The slowing of NCV following cisplatin or oxaliplatin administration is also reported.[76, 77]

**2.7 Cisplatin and oxaliplatin-induced morphologic alterations in the peripheral nervous system**

Size of cell body, nucleus, and nucleolus of DRG neurons decreased after treatments with cisplatin or oxaliplatin.[65, 66, 76, 78] The apoptotic morphological features of DRG neurons: cell shrinkage, nuclear condensation and fragmentation are observed in vitro and in vivo after exposure to cisplatin.[40, 41] Moreover, the segregation of nucleolus is also observed in DRG neurons treated with cisplatin and oxaliplatin.[65, 79] These alterations are likely evidence of neuronal damage due to these antineoplastic agents.

As for the nerve fiber, the electron microscopic study in sural nerve necropsy of cisplatin-treated patients demonstrates the damage of large and medium-sized nerve fibers including demyelination, whereas the non-myelinated nerve fibers are relatively normal.[80] However, several studies in animal models demonstrate the axon degeneration and demyelination including loss of intra-epidermal nerve fibers (IENF), the unmyelinated dermal nociceptors, after long-term administration of cisplatin or oxaliplatin.[17, 65, 76]

The sensory nerve fiber degeneration has also been reported in cisplatin/oxaliplatin-treated animals that can be detected by PGP9.5 staining, a pan-neuronal marker to detect IENF density.[75, 81] These findings imply the development of small-fiber neuropathy.[73]

จุฬาลงกรณ์มหาวิทยาลัย
CHULALONGKORN UNIVERSITY

**2.8 Effects of cisplatin and oxaliplatin on blood vessels**

The neovascularization in tumor growth and metastasis require endothelial cell migration.[82] The study by Montiel and co-workers demonstrated that cisplatin decreased the migration of endothelial cells through suppression the activity of matrix metalloproteinase-2 (MMP-2) which plays a role in collagen type IV degradation.[82] Thus, cisplatin may have an indirect antitumor effect through the inhibition of angiogenesis required for tumor growth.[82, 83]

Vascular complications such as thromboembolism, phlebitis, and Raynaud's phenomenon after treatment with cisplatin and oxaliplatin has been reported. The venous thromboembolism (pulmonary embolism or deep vein thrombosis), and Raynaud's phenomenon appear in 8.5% and 50% of cisplatin-treated patients, respectively.(84, 85) The phlebitis and pulmonary embolism, and sinusoidal injury occur in 3.5%, and 51% of oxaliplatin-treated patients, respectively.(86, 87) Moreover, cisplatin has been associated with the microvascular damage in the stria vascularis, glomerular capillaries, and the vasa nervorum (blood vessel of nerve).(11, 88, 89) Accordingly, cisplatin and oxaliplatin possibly damage the blood vessels outside the tumor.

Kirchmair and colleagues hypothesized that the antiangiogenic chemotherapeutic agents caused neuropathy, and this hypothesis is encouraged by lower nerve perfusion in cisplatin-induced neuropathy.(11) The results of their study showed that cisplatin triggered apoptosis of the endothelial cells, damage of vasa nervorum and nerve organization, resulting in disrupted nerve function.(11) Therefore, it is possible that cisplatin-induced neuropathy is caused by nerve ischemia as a result of endothelial cell apoptosis in the vasa nervorum with subsequent pathological alterations.(11)

Mechanisms of cisplatin/oxaliplatin induced-vascular toxicity are mainly studied using in vitro models. Human umbilical vein endothelial cell (HUVEC), human dermal microvascular endothelial cell (HMEC-1), and rat brain endothelial cell are commonly used. The dose range of cisplatin was 3.3-80 μM (0.79-24.24 μg/ml), while the dose of oxaliplatin in one study was 10 μM (3.97 μg/ml) (Table 1, 2). The evidence shows that cisplatin decreases migration, induces apoptosis, and alters functions of endothelial cells.(10, 82, 90) Besides, cisplatin promotes the toxicity on endothelial cells through upregulation of ICAM-1 (intercellular adhesion molecule 1) which mediates leukocyte-endothelial cell interactions and the inflammatory response via a nuclear factor kappa-B (NF-kB)-dependent pathway or cytokine production such as IL-1 and IL-6.(10, 91, 92)

The main effect of oxaliplatin on endothelial cell culture is the reduced expression of—endothelial tight junction protein (zonula occludens-1) and

cytoskeleton (F-actin). Thus, this finding suggests that oxaliplatin might loosen the blood-brain barrier.[12]

The studies of effects of cisplatin and oxaliplatin on cultured endothelial cells are summarized in Table 1 and Table 2, respectively.

**Table 1** Studies of cisplatin in endothelial cells in vitro

| Model | Cisplatin dose (μg/ml) | Treatment duration (hr.) | Results | Reference |
|---|---|---|---|---|
| HUVEC | 6.06 | 18, 48 | Cisplatin induces apoptosis in endothelial cell via Akt pathway | Kirchmair et al., 2005[11] |
| Pancreatic microvascular endothelial (MS1) cells | 3.03, 15.15 | 24 | Cisplatin induces apoptosis and necrosis in endothelial cell via caspases and calpain mediator | Dursun et al., 2006[90] |
| HUVEC | 1, 2, 4, 6, 8, 10 | 24 | Cisplatin induces endothelial cell toxicity via ICAM-1 up-regulation | Yu et al., 2008[91] |
| HUVEC | 6.06, 24.24 | 1-6 | Cisplatin decreases endothelial cell migration | Montiel et al., 2009[82] |
| HUVEC | 3.79, 7.58, 15.15 | 24, 48 | Cisplatin induces apoptosis of endothelial cell via caspase mediator | Eguchi et al., 2010[93] |
| HMEC-1 | 0.79, 3.91 | 24, 48, 72 | Cisplatin alters the endothelial function (proliferation, fibrinolysis, inflammation) | Nuver et al.,2010[10] |
| HUVEC | 1 | 24 | Cisplatin induces endothelial cell injury via up-regulation of peroxynitrite and ICAM-1 | Zhu et al., 2014[94] |

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

**Table 2** Study of oxaliplatin in endothelial cells in vitro

| Model | Oxaliplatin dose (µg/ml) | Treatment duration (hr.) | Results | Reference |
|---|---|---|---|---|
| Rat brain endothelial cell line (RBE4) | 3.97 | 8, 16 | Oxaliplatin loosen endothelial tight junction | Branca et al., 2018[12] |

The potential mechanisms of endothelial toxicity caused by the platinum drugs are–apoptosis, activated endothelial inflammation, inhibited endothelial cell proliferation, and decreased nitric oxide (NO) level.[95] Additionally, in the cisplatin–treated rats, vitamin E can ameliorate the endothelial damage in superior mesenteric artery. Consequently, cisplatin might generate oxidative stress which causes endothelial damage.[96]

### 2.9 Treatments of cisplatin/oxaliplatin induced-peripheral neuropathy

#### 2.9.1 Neurotrophic factors

Since the neurotrophic factors are involved in the maintenance and development of neuron, several neurotrophic factors such as nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), basic fibroblastic growth factor (bFGF), and neurotrophins are examined in their efficacy against platinum drug neurotoxicity.[97] Even though these agents showed the effectiveness in experimental studies, there were no significant benefits in the clinical trials.[98] However, ORG 2766, which stimulates the NGF synthesis, showed neuroprotection in the cisplatin-induced neuropathic patients.[97] Nevertheless, other clinical trials found no such effect.[16]

#### 2.9.2 Antioxidants

Since cisplatin and oxaliplatin can induce oxidative stress, antioxidants may be helpful against the drug-induced toxicity.[9] A number of antioxidants e.g. N-acetylcysteine, glutathione, alpha-lipoic acid, amifostine, acetyl-L-carnitine, vitamin E ($\alpha$-tocopherol) and curcumin have been tested for neuropathy therapy.[56, 99] Although N-acetylcysteine, glutathione, alpha-lipoic acid, vitamin E, and amifostine acid showed the beneficial effects on cisplatin/oxaliplatin-induced neurotoxicity in

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

the experimental models, they failed to show significant efficacy in clinical trials.[16, 100, 101]

#### 2.9.2.1 Curcumin

Curcumin is a natural phenolic compound isolated from turmeric. The turmeric is prepared from the root of *Curcuma longa*.[102] Diferuloylmethane is active hydrophobic polyphenol compound that provides a yellow color.[103] It is used as food addition and traditional medicine in Southeast Asia, China, and India.[104] It has shown several beneficial effects such as anti-inflammation, anti-cancer, analgesic, and antioxidant.[102] The bioavailability of curcumin is low due to its hydrophobic property, poor intestinal absorption and rapid systemic elimination.[102]

Regarding its antioxidant properties, curcumin can directly eliminate the free radicals and elevate the levels of antioxidant enzymes such as superoxide dismutase (SOD), glutathione-S-transferase (GST) and glutathione peroxidase (GPx).[21, 102] Moreover, curcumin scavenges several free radicals: superoxide anion ($O_2^{\bullet -}$), hydroxyl radical ($OH^{\bullet}$), peroxyl radical ($ROO^{\bullet}$), singlet oxygen ($^1O_2$), peroxynitrite anion ($ONOO^-$), hypochlorous acid (HOCl) and hydrogen peroxide ($H_2O_2$).[104] Besides, it also reduces lipid peroxidation (LPO) and enhances glutathione (GSH) level.[102]

Regarding the antitumor effect, curcumin can kill the cancer cell by several mechanisms such as cell cycle interruption, apoptotic induction, and NF-kB suppression.[102] Curcumin has a synergist effect to chemotherapeutic drugs. It enhances the therapeutic efficacy of cisplatin and oxaliplatin.[105, 106] Furthermore, it also reduces the chemotherapeutic drug resistance.[102] In addition, curcumin affects the proliferation, migration and tube formation of endothelial cell which represents its anti-angiogenic property.[107, 108]

The studies of curcumin on neuron-like cell (e.g. PC12 cell) culture demonstrates the amelioration of cisplatin toxicity on neurite outgrowth.[19] In animals, curcumin can reduce cisplatin induced-sensory deficits in rats.[23] Furthermore, improvement in pathological changes in the DRG and nerve after cisplatin administration were observed with curcumin treatment.[17, 23] Regarding oxaliplatin, curcumin reduces lipid peroxidation and protein oxidation, restores GST, GPx, and electron transport chain enzymes in the mitochondria of rat brain.[22]

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

Moreover, curcumin ameliorates the cisplatin-induced decrease of tight junction proteins (claudin-2 and occludin) and adherens junction protein (E-cadherin) in rats.[104]

**2.9.3 Vitamin B**

Vitamin B family is composed of eight B vitamins: $B_1$ (thiamine), $B_2$ (riboflavin), $B_3$ (nicotinic acid), $B_5$ (pantothenic acid), $B_6$ (pyridoxine), $B_7$ (biotin), $B_9$ (folic acid), and $B_{12}$ (cobalamin).[24] They are water-soluble; hence, the overdose toxicity is unlikely.[24] B vitamins act as the coenzymes in the energy metabolism pathways which are important in the nervous tissue due to high-energy demand.[24] From the reasons mentioned above including its availability, B vitamins are commonly used as supportive treatment in peripheral neuropathies.[24] Since the deficiencies of vitamin $B_1$, $B_6$, $B_{12}$ cause the impairment of nervous system, the combination of these three B vitamins is frequently used.

Vitamin $B_1$ (thiamine) acts as a coenzyme in carbohydrate metabolism in thiamine pyrophosphate form (an active form). $B_1$ is important for a transmission of nerve impulse.[25] Additionally, thiamine pyrophosphate also plays a role in ribose synthesis which is crucial for cell proliferation.[109] The effect of thiamine on cancer cell depends on dose used. It promotes cancer cell growth at the low dose, whereas it suppresses the growth at the high dose. The mechanism that might explain how high dose inhibits the proliferation of cancer cell is caspase-3 induction leading to apoptosis.[110]

Vitamin $B_6$ (pyridoxine) has three forms: pyridoxine, pyridoxal, pyridoxamine.[111] Pyridoxal phosphate is an active form of this vitamin.[24] The functions of vitamin $B_6$ in the nervous system is synthesis of sphingolipid and amino acid (transaminases and L-amino acid decarboxylases) which is essential for myelin formation and synthesis of neurotransmitters (dopamine, serotonin and $\gamma$-aminobutyric-acid or GABA), respectively.[24, 26] Besides, it also shows anti-inflammatory activity through suppression of NF-k$\beta$.[111] Vitamin $B_6$ has anti-tumor activity in several types of cancer e.g. colorectal, lung, and breast cancers via several mechanisms such as disruption of DNA, decreased inducible nitric oxide synthase.[26]

CHULALONGKORN UNIVERSITY

This vitamin diminishes cisplatin induced-neurotoxicity in patients without interruption of anticancer effect. Similarly, vitamin $B_6$ at concentration 1-25 μM does not inhibit the antitumor effect of oxaliplatin in vitro.(112) However, the toxicity of this vitamin, peripheral neuropathy, can still occur in very high dose intake (more than 500 mg/day).(26)

Vitamin $B_{12}$ (cobalamin or cyanocobalamin) possesses two active forms: methylcobalamin, and adenosylcobalamin.(113) This vitamin participates in fatty acid metabolism which is crucial for maintenance of myelin sheath.(25) Moreover, it may encourage nerve regeneration as well as improve nerve function by neurotropic activity, and neurotoxic cytokine reduction.(111) Emerging evidence shows that $B_{12}$ can ameliorate neuropathic symptoms via GSH restoration and oxidative stress neutralization.(28) Furthermore, $B_{12}$ also functions in cell growth and replication.(24) In the proliferation of cancer cell in vitro and in vivo, $B_{12}$ (methylcobalamin, 100-500 μg/ml) suppresses proliferation of tumor cell.(114)

As mentioned above, $B_1$, $B_6$, and $B_{12}$ play different vital roles in the nervous system. Accordingly, the combination of these vitamins may have synergistic effects on peripheral neuropathy.(25) There are several studies that supports this hypothesis. One study demonstrated the alleviation of neuropathic pain and improvement of NCV in diabetic rats after daily treatment with the cocktail of B vitamins, but not with each vitamin alone.(29) In diabetic and alcoholic patients, this combination of B vitamins also relieves neuropathic symptoms.(25, 28) In addition, B vitamin cocktail ($B_1$:$B_6$:$B_{12}$=100:100:1) promotes axonal regeneration after nerve injury.(115) Although those previous studies used the different ratio of this combination, all studies showed the effectiveness of the combination in the treatment of neuropathy. However, the effectiveness of this combination in clinical trials remains unclear due to insufficient data.(116) Moreover, its efficacy in cisplatin/oxaliplatin neuropathy has not been studied.

### 2.9.4 **Other compounds**

Calcium and magnesium, electrolytes, may play a role against channelopathy in acute oxaliplatin-induced peripheral neuropathy via oxalate chelation. However,

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

these electrolytes fail to improve oxaliplatin neuropathy and disrupts the antitumor activity.[8, 16]

Carbamazepine and oxcarbazepine, antiseizure drugs, are the candidate agents in acute oxaliplatin neuropathy through blocking the voltage-gated sodium channel.[16] Nonetheless, the efficacy of these drugs remains to be proved by further clinical trials.

Although many experimental studies have explored the potential agents to relieve or prevent platinum provoked-neuropathy, currently there are no effective agents recommended for clinical cisplatin/oxaliplatin-induced neuropathy. Most effective drugs in preclinical studies had severe side-effects or were unsuccessful to reverse neuropathy in clinical trials.[74, 99, 117]

**2.10 Blood-nerve barrier (BNB)**

Peripheral nerve or nerve is a group of nerve fibers that connects the central nervous system (CNS, brain and spinal cord) with other body regions.[118] The crucial function of the nerve is transmission of impulse to and from the CNS. The environment within the nerve is strictly regulated to maintain the normal functions.[118]

The nerve is enveloped with three layers of connective tissue: epineurium, perineurium, and endoneurium from outermost to innermost, respectively.[118] Deep to epineurium, nerve fibers are grouped as fascicles which are enveloped by perineurium. Within each fascicle, nerve fibers are surrounded by endoneurium (Figure 4).

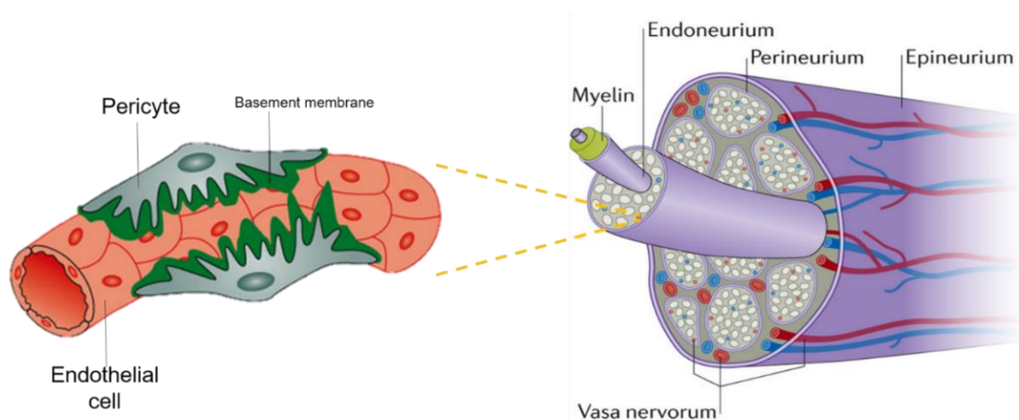



**Figure 4** Components of blood-nerve barrier (from Lange et al., 2016)[119]

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

The blood supply of peripheral nerve (vasa nervorum) comes from neighboring arteries that travel along the epineurium and give the penetrating branches until becoming the capillaries in the endoneurium.[118] Blood-nerve exchange occurs at endoneurial capillary and transperineurium. Nevertheless, the endoneurial capillary is the major site of exchange.[120]

Blood-nerve barrier (BNB) is located at the endoneurial microvessels within the fascicle of peripheral nerve. The structure of BNB is composed of endothelial cell, pericyte and vascular basement membrane in between (Figure 4). The function of BNB is the prevention of toxic substances from circulating blood to contact the nerve fibers and maintaining the endoneurial homeostasis.[14]

In comparison with the blood-brain barrier (BBB) which is located around the small blood vessels in the CNS and composed of endothelial cell, pericyte, and astrocyte; the BNB is less strong than the BBB.[14]

The disruption of BNB can cause neuropathy.[14] Several neuropathies e.g. tellurium neuropathy, immune-mediated neuropathy, diabetic neuropathy including chemotherapy induced-neuropathy have the BNB breakdown (Figure 5).[14, 119, 121]

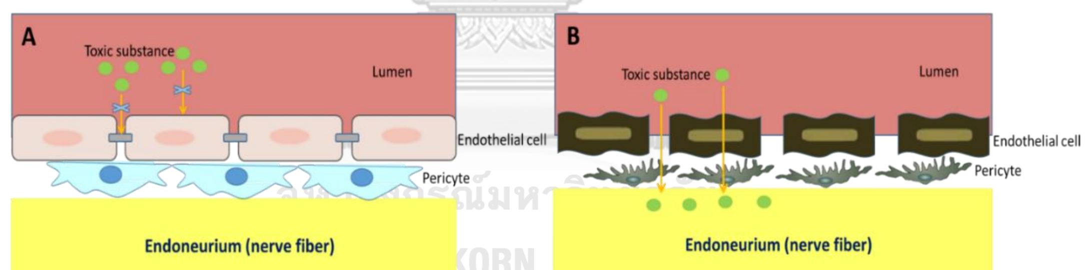



**Figure 5** A) Normal blood-nerve barrier (BNB), B) disrupted BNB

**2.10.1 Endothelial cell**

Endothelial cell is located at the luminal surface of blood vessels including capillaries. The capillary can be classified into 3 types according to porosity of its wall: continuous, fenestrated and sinusoidal. The capillary in peripheral nerve is continuous type in which endothelial cells are tightly attached by junctional complexes.[122]

The endoneurial endothelial cells have several transporters and receptors in order to eliminate the toxic metabolites or the harmful substances to contact the

nerve fibers while allowing the necessary substances to pass.[14, 123] Influx and efflux transporters are such as glucose transporter 1 (GLUT1), L-type amino acid transporter 1 (LAT1), and ATP-binding cassette (ABC) transporter similar to the brain endothelial cell.[123]

The endothelial component of BNB is formed between endothelial cells by tight junction and adherens junction.[124]

Tight junction is composed of two main groups of proteins: claudins, and zonula occludens (ZO).[125] The ZO, an intracellular protein, links the transmembrane proteins (occludin and claudins) with the cellular cytoskeleton (Figure 6).[126] The most important tight junction proteins that serve the barrier integrity is claudin-5 and ZO-1.[125] Moreover, another tight junction protein— occludin probably play a crucial role in the barrier function.[125]

These junctional proteins control the paracellular transport and maintain the microenvironment in the nerve.[124] In the study of Yosef and co-worker, the microvascular endothelial cells from human sciatic nerve expressed occludin, ZO-1, and claudin-5.[127] Apart from claudin-5, human endoneurial endothelial cell also expresses claudin-1, claudin-4, claudin-11, claudin-12, and claudin-19.[124] The depletion of tight junction proteins is demonstrated in several causes of neuropathy e.g. immune-mediated neuropathy, diabetic neuropathy, chronic inflammatory demyelinating polyradiculoneuropathy (CIDP), as well as chemotherapy-induced neuropathy.[12, 14, 104, 122, 128] In inflammatory neuropathy, the decrease of claudin-5, alteration of ZO-1 location were shown in sural nerve biopsy from CIDP patients.[128] The reduction of claudin-5 also occurs in the cultured endoneurial endothelial cells treated with advanced glycation end-products (AGEs) or sera from CIDP patients.[129, 130] Additionally, the depletion of claudin-2, occludin, and E-cadherin was found in cisplatin treated-rats.[104]

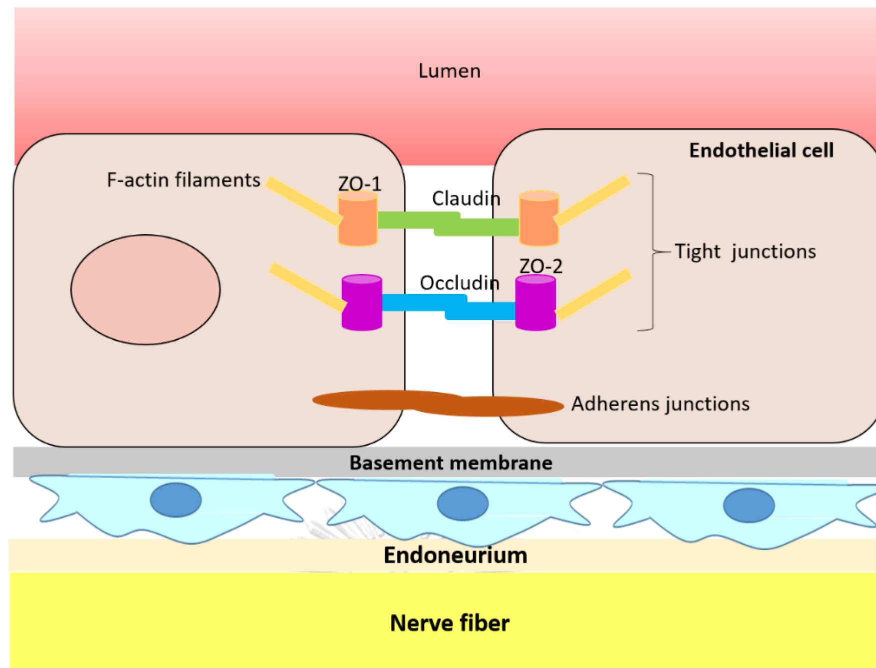



**Figure 6** Inter-endothelial cell junction

Trans-endothelial electrical resistance (TEER) and solute permeability are the in vitro measurements of BNB functions in endothelial cell culture.[118] The solute permeability evaluates the barrier permission of solute e.g. sodium fluorescein, radioactive inulin, and fluoresceinated dextran.[118]

### 2.10.2 Pericyte

Pericyte is the perivascular cell that wraps around the endothelial cell of pre-capillary arteriole, capillary and post-capillary venule. It is embedded within the same basement membrane as the endothelial cell (Figure 7).[13]

The embryonic origin of pericyte is from mesoderm except in the CNS and thymus that comes from the neural crest (ectoderm).[131] The density of pericyte around capillary varies in each organ.[132, 133] The highest density is found in the brain and retina where pericyte is a part of blood-nerve and blood-retina barriers.[131]

#### 2.10.2.1 Pericyte identification

The identification of pericyte should utilize the location, morphology, and pericyte markers due to absence of specific marker.[131]

In general, the pericyte cell body possesses a rounded shape with a prominent nucleus and scant cytoplasm.[131] It has several elongated processes around the vessel wall.[13] However, pericytes have different morphology depending on their locations: elongated and spindle-shaped for capillary pericytes, short and stellate-shaped for pre/post-capillary pericytes.[131]

As for pericyte marker, the common markers to identify pericyte are platelet-derived growth factor receptor-beta (PDGFR-β), alpha-smooth muscle actin (α-SMA), nerve-glial antigen-2 (NG2), the regulator of G-protein signaling-5 (RGS5), CD 13, CD 146, and desmin (Table 3).[132, 133] Nevertheless, there is no single specific marker since the expression varies in different organs. Moreover, these markers are also expressed by other cell types (Table 3). Accordingly, identification of pericyte should use two or more markers.[132]

**Table 3** Pericyte markers

| Pericyte marker | Cells that also express the marker |
|---|---|
| PDGFR-β | fibroblast, astrocyte |
| α-SMA | smooth muscle cell |
| NG2 | vascular smooth muscle cell |
| CD13 | smooth muscle cell, endothelial cell |
| CD146 | endothelial cell, smooth muscle cell |
| Desmin | smooth muscle cell |

**2.10.2.2 Properties of pericyte**

Pericyte is the contractile cell as it expresses microfilament as well as α-SMA, and myosin.[133] Hence, the contraction of pericyte can control the constriction of capillaries in order to regulate blood flow.[132]

It is believed that pericyte may give rise to the vascular smooth muscles cell and vice versa.[13] Moreover, pericyte may also differentiate into multiple types of mesenchymal cell such as fibroblast, adipocyte, and chondrocyte.[13]

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

### 2.10.2.3 Pericyte and endothelial cell interaction

Pericyte is attached to the endothelial cell via junctional complex in the basement membrane which allows them to communicate through various paracrine signals.[131] There are two different types of direct contact between pericyte and endothelial cell: peg–socket junctional complex, and adhesion plaque (Figure 7).[13]

Peg-socket junctional complex attaches the hole of pericyte cytoplasm with the extended cytoplasm of endothelial cell through the absent basement membrane.[13] This contact contains tight junction, gap junction, and adherens junction.[13] Accordingly, pericyte can regulate the proliferation and differentiation of endothelium and transfer the contractile force to the endothelial cell.[133]

The adhesion plaque links between the pericyte plasma membrane and endothelial cytoplasm by fibronectin (microfilament).[132] Since there are direct contacts between one pericyte and several endothelial cells, pericyte may play a role in the integration and co-ordination of adjacent endothelial cells.[13]

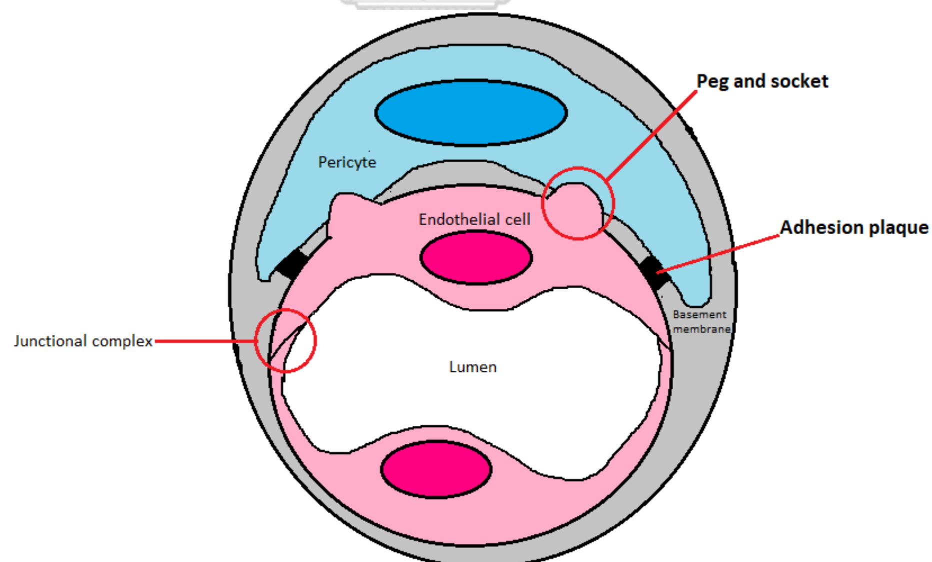



Figure 7 Junctional complex between endothelial cell and pericyte

### 2.10.2.4 Molecular signaling between pericyte and endothelial cell

Multiple molecular signaling molecules between pericyte and endothelial cell has been identified such as platelet-derived growth factor beta/

platelet-derived growth factor receptor beta (PDGF-β/PDGFR-β), Angiopoietin-1 (Ang1)/Tie2, and transforming growth factor beta (TGF-β) (Figure 8).[13]

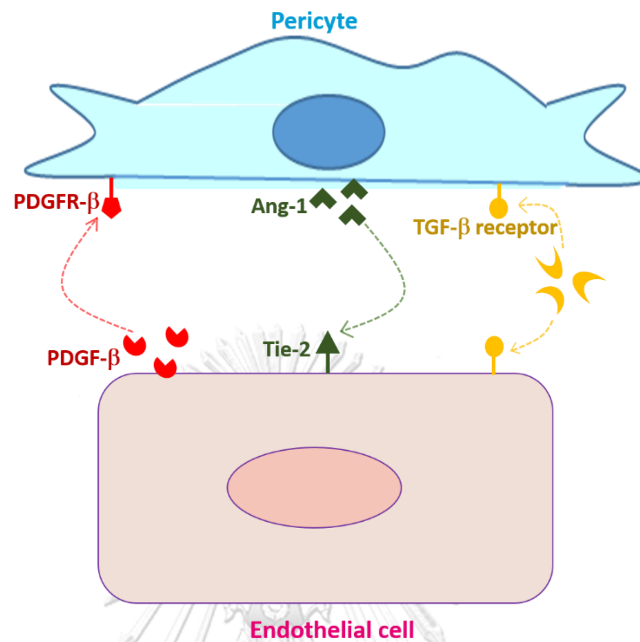



**Figure 8** Pericyte-endothelial cell molecular interaction

PDGF-β is secreted from endothelial cell and binds to PDGFR-β expressed on the surface of pericyte.[131] The PDGF-β/PDGFR-β signaling has an important role in angiogenesis through recruitment of pericyte to the endothelial cell.[13]

Ang1 is the ligand released from pericyte which binds to Tie2, the receptor on the surface of endothelial cell.[13] The Ang1/Tie2 signaling plays an essential role in vessel maturation and stabilization (endothelial integrity).[131] Besides, endothelial cell can produce Ang2, a Tie2 antagonist ligand. The Ang2/Tie2 pathway is involved in the degeneration of pericyte.[133]

TGF-β is synthesized by endothelial cell and is activated by the contact between both cells.[133] TGF-β receptor is present in both pericyte and endothelial cell. The TGF-β signaling regulates the proliferation and differentiation of pericyte and endothelial cell during angiogenesis.[131]

#### 2.10.2.5 Functions of pericyte

Pericyte plays a role in the formation of BBB/BNB, vascular permeability, vascular homeostasis, and blood flow as well as angiogenesis.[131] From the anatomical contact and paracrine signaling between pericyte and endothelial cell, pericyte can regulate proliferation and maturation of endothelial cell.[131, 134] Pericyte promotes these effects by secreting the paracrine growth factors e.g. Ang-1, basic fibroblast growth factor (bFGF). For instance, pericytes secrete the bFGF to enhance the expression of claudin-5, a component of basement membrane, in endothelial cell.[135]

In wound healing, it is believed that pericyte can differentiate into the fibroblast-like cell that produces collagen to heal the wound.[13] Additionally, pericyte can promote the tissue repair via inhibition of immune response and inflammation.[131]

Moreover, pericyte is also involved in the immune system. It can excrete cytokines and chemokines which attract the immune cells to the inflammatory site and upregulate the adhesion molecules such as ICAM-1 which results in trans-endothelial migration.[131]

#### 2.10.2.6 Pericyte culture

Primary pericyte is isolated from brain, retina, and fetal tissue such as placenta.[133] In 2008, Kanda and colleagues developed the first successful isolation of pericyte from the rat sciatic nerve.[136]

The rat nerve pericyte express the markers: PDGFR-β, α-SMA, NG2, osteopontin, desmin, tight junction proteins (ZO-1, ZO-2, claudin, occludin), transporters, and growth factors (Ang-I, VEGF, TGF-β, bFGF) similar to rat brain pericyte.[136] This study demonstrated that pericytes also express barrier proteins similar to endothelial cells.[136]

Subsequently, the pericytes derived from human sciatic nerve are successfully generated.[135] These human nerve pericytes express the markers and paracrine growth factors seen in the human brain pericytes and rat nerve pericytes. However, the expression levels of Ang-I, bFGF, and TGF-β in this type of pericyte are

higher than the human brain pericyte. Both human nerve and brain pericytes also express the neurotrophic factors (NGF, BDNF, and GDNF); nonetheless, the levels of these factors are higher in the nerve pericytes.[135]

## 2.11 BNB alterations in peripheral neuropathy

The abnormal alterations of wall and basement membrane of endoneurial microvessels including the disruption of tight junction are exhibited in several neuropathies such as diabetic neuropathy, immune-mediated neuropathy.[14, 122]

The number of nerve pericytes in diabetic neuropathy is also reduced. This event leads to the disorganization of endothelium, the reduction of oxygen diffusion into nerve fibers (hypoxia), and finally ischemia.[131] Nerve pericytes exposed to AGEs increased the expression of basement membrane components (collagen type IV, fibronectin) via the upregulation of VEGF and TGF-$\beta$ signaling.[129] This result implies that basement membrane of BNB can be regulated by pericytes.[129]

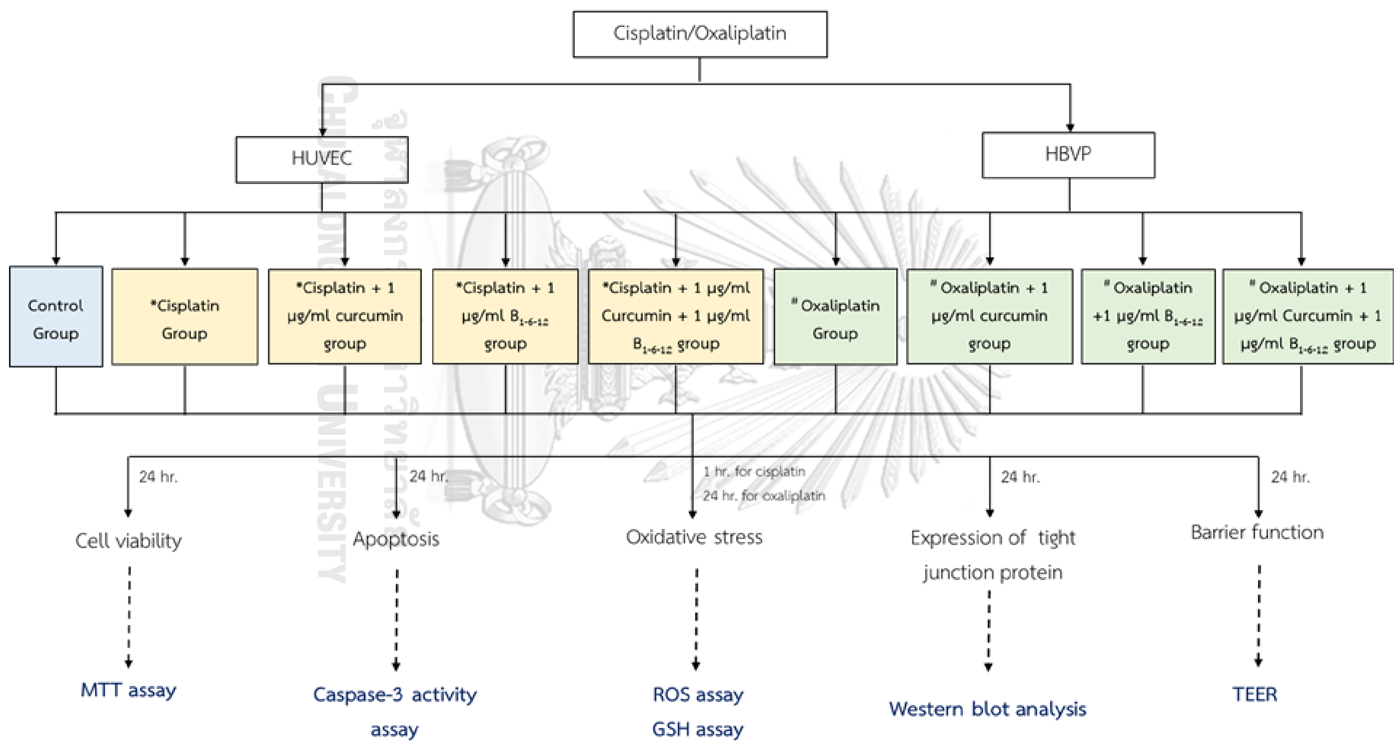
The recent work from our laboratory found the alteration of BNB in peripheral nerve from cisplatin-induced neuropathic rats.[15] Reduction of the density of pericytes in the neuropathic group compared with control group was found. The separation of pericyte from the endothelial cell was also demonstrated in ultrastructural study. Therefore, preliminary evidence suggests that cisplatin adversely affects pericytes and results in BNB pathology and finally neuropathy.

จุฬาลงกรณ์มหาวิทยาลัย
CHULALONGKORN UNIVERSITY

# CHAPTER III

# MATERIAL AND METHOD

## 3.1 Research framework




* 3 µg/ml in HUVEC and 1.5 µg/ml in HBVP

\# 5 µg/ml in HUVEC and 8 µg/ml in HBVP

**3.2 Pilot study of HUVEC**

3.2.1. To optimize cisplatin (Cis) concentrations for further investigation using MTT assay

When HUVEC were incubated with different concentrations of cisplatin (0-60 µg/ml), their viability decreased dose-dependently (Figure 9). HUVEC viability at 3, 6, 15, 30, and 60 µg/ml was significantly lower than the control group ($p<0.001$). We selected the lowest significant dose ($p<0.001$), 3 µg/ml, to conduct the subsequent experiments.

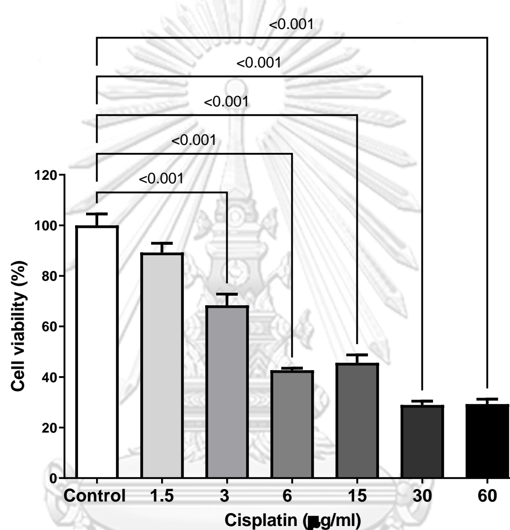



**Figure 9** Cell viability of HUVEC treated with various concentrations of cisplatin (0-60 µg/ml) for 24 hr. The graph shows mean and SEM.

จุฬาลงกรณ์มหาวิทยาลัย
CHULALONGKORN UNIVERSITY

3.2.2. To optimize curcumin (Cur) concentrations for further investigation using MTT assay

HUVEC were treated with various concentrations of curcumin (0-60 µg/ml). The treatment concentration higher than 1 µg/ml were cytotoxic to the cells when compared with the control group (Figure 10). Accordingly, we selected the concentration of 0.1 and 1 µg/ml to find out the appropriate treatment concentration when combined with cisplatin and oxaliplatin. The 1 µg/ml improved the viability of the cell more than the 0.1 µg/ml (appendix table 1.9-1.10). Hence, curcumin 1 µg/ml was used for future experiments.

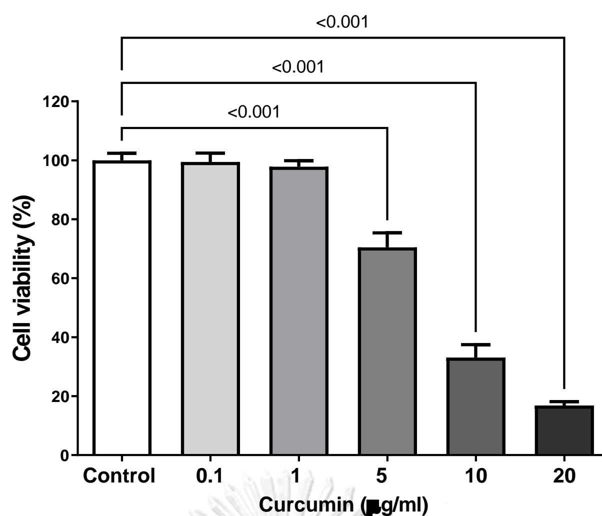



**Figure 10** Cell viability of HUVEC treated with curcumin (0-20 μg/ml). The graph represents the mean and SEM.

3.2.3. To optimize $B_{1-6-12}$ concentrations for further investigation using MTT assay

HUVEC received $B_{1-6-12}$ treatment at concentration 0, 1, 5, 10, 20, 40, 80, and 160 μg/ml. All treatment doses of $B_{1-6-12}$ increased cell viability without any statistical significance when compared with the control group (Figure 11). Subsequently, the lowest concentration of 1 μg/ml was selected to perform the experiments with cisplatin and oxaliplatin.

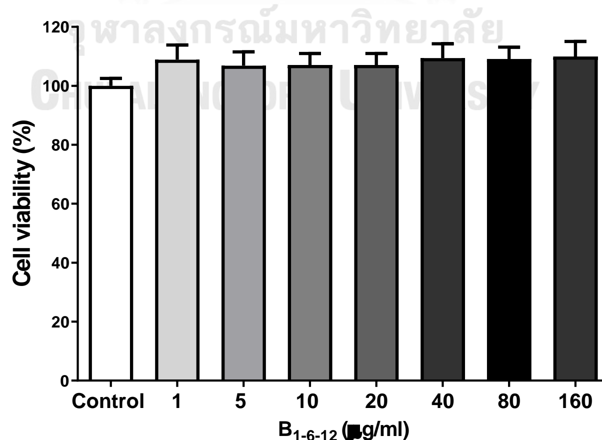



**Figure 11** Cell viability of HUVEC treated with $B_{1-6-12}$ (0-160 μg/ml). Values represent Mean ± SEM.

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

3.2.4. To optimize oxaliplatin (OXP) concentrations for further investigation using MTT assay

HUVEC treated with 0-150 µg/ml oxaliplatin had decreased cell viability in a dose-dependent manner. A significant reduction in viability was first observed in the HUVEC exposed to 5 µg/ml oxaliplatin (*p*<0.001). Hence, the 5 µg/ml oxaliplatin was selected to use for the subsequent experiments (Figure 12).

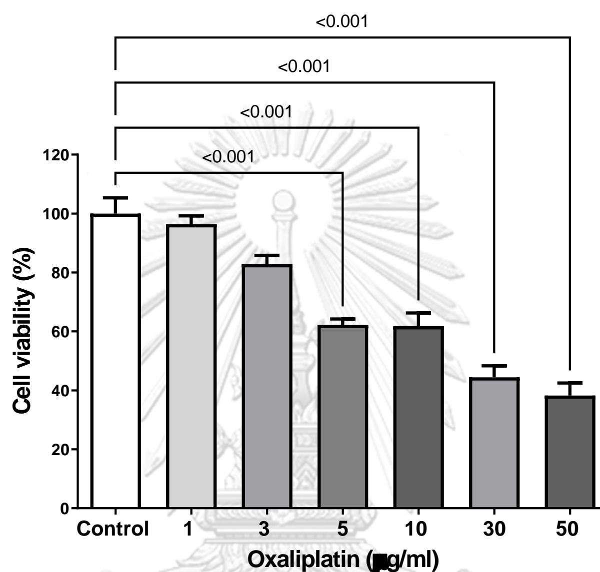



**Figure 12** Cell viability of HUVEC treated with oxaliplatin (0-150 µg/ml). The graph shows the mean ± SEM.

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

3.2.5. To optimize the ROS measurement time for further investigation

The measurement was examined at 1-24 hours and the time with peak generation of ROS was selected. The peaked ROS level in HUVEC was at 1 hour after treatment with cisplatin and 24 hours after treatment with oxaliplatin. Hence, the ROS results in HUVEC at these times were shown.

### 3.3 Pilot study of HBVP

3.3.1. To optimize cisplatin concentrations for further investigation using MTT assay

The HBVP were incubated with cisplatin at 0, 1.5, 3, 4, 5, 6 and 15 µg/ml for 24 hours. The viability was inhibited in a dose-dependent manner. The first significant dose of cisplatin at 1.5 µg/ml was optimal for further study (Figure 13).

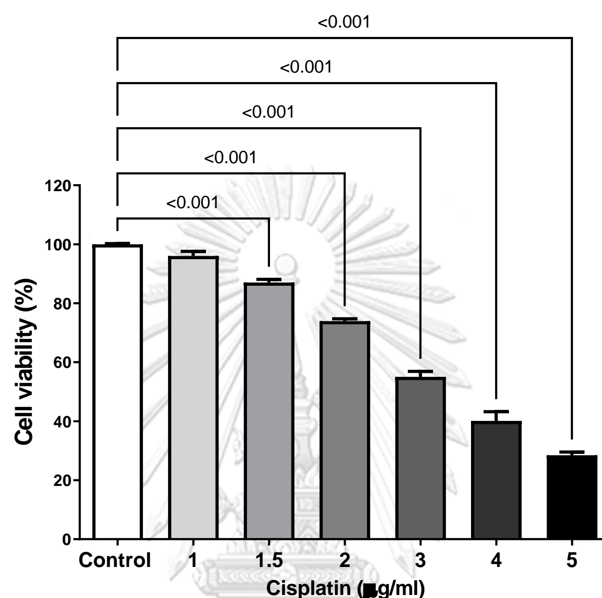



**Figure 13** Cell viability of HBVP treated with various concentration of cisplatin (0-15 µg/ml) for 24 hr. The graph presents mean and SEM.

จุฬาลงกรณ์มหาวิทยาลัย
CHULALONGKORN UNIVERSITY

3.3.2. To optimize curcumin concentrations for further investigation using MTT assay

Treatment with curcumin at 0.1 and 1 µg/ml exhibited no cytotoxic effect on the viability of HBVP (Figure 14), indicating these doses did not induce cellular damage and were suitable for the next experiments. However, when combined these doses with cisplatin and oxaliplatin, the 1 µg/ml could improve more cell viability than the dose 0.1 µg/ml (appendix table 1.11-1.12). Hence, the 1 µg/ml was chosen for the next experiment.

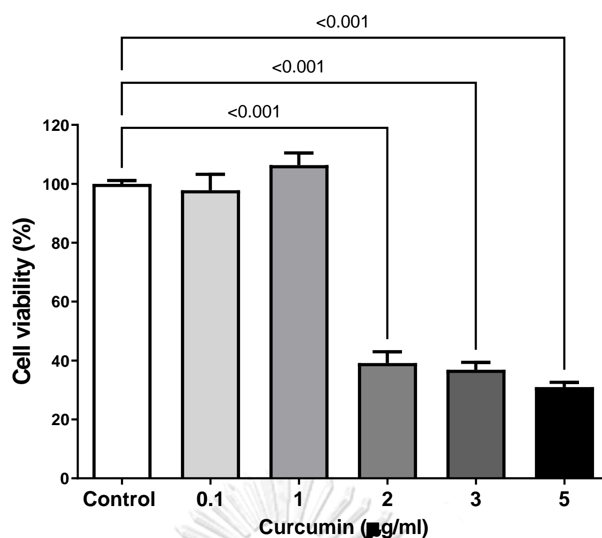



**Figure 14** Cell viability of HBVP treated with curcumin (0-5 μg/ml). The graph represents the mean and SEM.

3.3.3. To optimize $B_{1-6-12}$ concentrations for further investigation using MTT assay

After incubation with $B_{1-6-12}$ μg/ml, all doses (1-80 μg/ml) caused the increase of HBVP viability (Figure 15). Nonetheless, 1, 5, and 10 μg/ml were not statistically significant. Accordingly, the lowest dose, the 1 μg/ml, was selected for the future experiment.

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

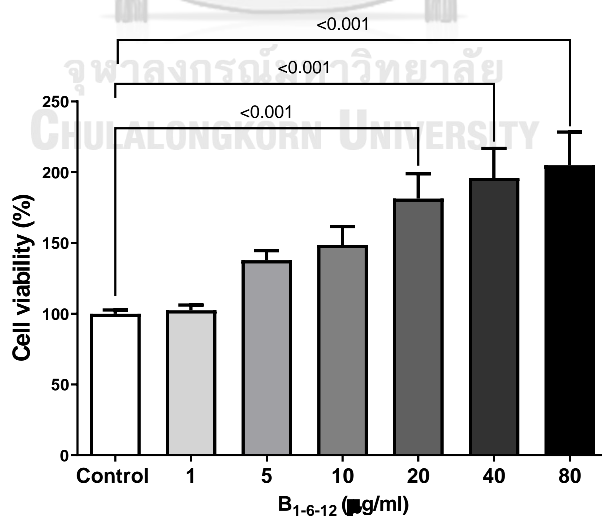



**Figure 15** Cell viability of HBVP treated with $B_{1-6-12}$ (0-80 μg/ml). Values represent Mean ± SEM.

3.3.4. To optimize oxaliplatin concentrations for further investigation using MTT assay

After exposure to oxaliplatin for 24 hours, the first significant change in HBVP viability was 8 µg/ml when compared with the control cells (Figure 16). Hence, the 8 µg/ml oxaliplatin was chosen for further investigation.

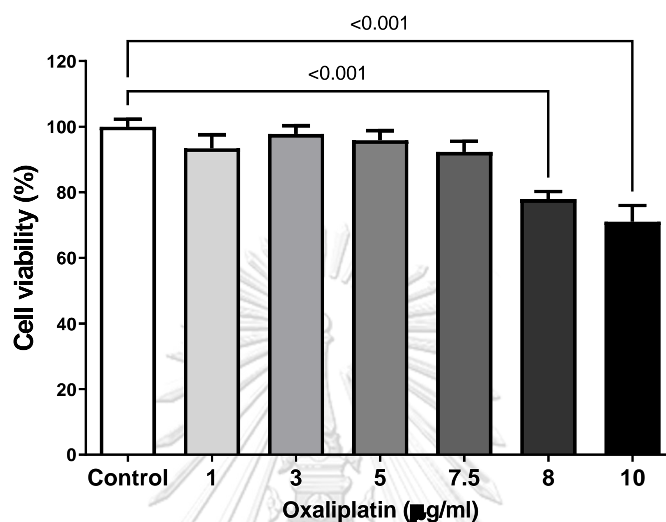



**Figure 16** Cell viability of HBVP treated with oxaliplatin (0-10 µg/ml). The graph shows the mean ± SEM.

3.3.5. To optimize the ROS measurement time for further investigation

The measurement was examined at 1-24 hours and the time with peak generation of ROS was selected. The peak ROS levels in HBVP treated with cisplatin and oxaliplatin were at 1 and 24 hours, respectively. Thus, the ROS results in HBVP at these times were shown.

**3.4 Research methodology**

3.4.1 Materials and methods

3.4.1.1 Reagents

Cisplatin (Unistin, Korea United Pharm, South Korea, Reg. No. 1C 257/51; Lot E687F804) and oxaliplatin (Pharmachemie BV, Netherlands, Reg. No. 1C 46/53(NG)) were purchased from King Chulalongkorn Memorial Hospital. Then, the stock concentration of cisplatin and oxaliplatin were 1 mg/ml and 5 mg/ml, respectively. Cisplatin and oxaliplatin were further diluted to concentration 3 μg/ml (from the pilot study) and 5 μg/ml (from the pilot study) with the culture medium just before use, respectively.

Curcumin (Cayman Chemical, USA, Cat. No. 81025) was dissolved in dimethylsulfoxide (DMSO) (Sigma-Aldrich, USA, Cat. No. D4540). The stock solution of curcumin was prepared with concentration of 1 mg/ml and 5 mg/ml, diluted with medium to concentration 1 μg/ml (from the pilot study) and the final concentration of DMSO is 0.1%.

Vitamin $B_1$ (Sigma-Aldrich, USA, Cat. No. T4625; Lot # SLBT0878), $B_6$ (Sigma-Aldrich, USA, Cat. No. P9755; Lot # SLBW4952), and $B_{12}$ (Sigma-Aldrich, USA, Cat. No. V2876; Lot # MKCF8778) were dissolved in sterile water to prepare the stock solution with concentration of 2.01 mg/ml. This stock solution was prepared from the 100 mg vitamin $B_1$, 100 mg vitamin $B_6$, and 1 mg vitamin $B_{12}$ or ratio of $B_1$:$B_6$:$B_{12}$ is 100:100:1[29], and then dissolved in 50 ml sterile water. The used Vitamin $B_{1-6-12}$ was diluted with the medium to concentration 1 μg/ml (from the pilot study).

Fetal bovine serum (FBS), antibiotic-antimycotic, trypan blue, Dulbecco's phosphate-buffered saline (DPBS), phosphate buffered saline (PBS), 0.25% trypsin-EDTA, and trypsin neutralizer solution were purchased from Invitrogen, Thermo Fisher Scientific, USA. Poly-L-lysine and T/E neutralization solution were purchased from ScienCell research laboratory, USA.

3.4.2 Cell culture

Human umbilical vascular endothelial cell (HUVEC) (Cat. No. #C-015-5C; Lot #2029025) and human brain vascular pericyte (HBVP) (Cat. No. 1200; Lot 27288) were

purchased from Invitrogen and ScienceCell, respectively. The cell culture protocol followed the data sheet of each company. Briefly, the cryopreserved cell was thawed and plated on the T-25 flask. The HUVEC and pericyte were grown in HUVEC medium (medium 200) and pericyte growth medium respectively with 1% antibiotic-antimycotic added. The medium was changed every 3–4 days. When the cells reach 80-90% confluency, they were transferred to the new containers using 0.25% trypsin-EDTA. The cells were maintained within humidified incubator in an atmosphere of 95% air and 5% $CO_2$ at 37 °C. In all experiments, the cells were used between passage 2 and 15.

3.4.3 Experimental groups

*Effect of combination between curcumin/$B_{1-6-12}$ on cisplatin- and oxaliplatin-induced alterations in HUVEC.*

HUVEC were divided into 9 groups:

1.) Control group: the cells were incubated in culture medium.
2.) Cisplatin group: the cells were treated with cisplatin 3 µg/ml for 24 hours.
3.) Cisplatin + curcumin group: the cells were treated with 3 µg/ml cisplatin and 1 µg/ml curcumin.
4.) Cisplatin + $B_{1-6-12}$ group: the cells were treated with 3 µg/ml cisplatin and 1 µg/ml $B_{1-6-12}$.
5.) Cisplatin + curcumin + $B_{1-6-12}$ group: the cells were treated with 3 µg/ml cisplatin, 1 µg/ml curcumin, and 1 µg/ml $B_{1-6-12}$.
6.) Oxaliplatin group: the cells were treated with 5 µg/ml oxaliplatin for 24 hours.
7.) Oxaliplatin + curcumin group: the cell was treated with 5 µg/ml oxaliplatin and 1 µg/ml curcumin.
8.) Oxaliplatin + $B_{1-6-12}$ group: the cell was treated with 5 µg/ml oxaliplatin and 1 µg/ml $B_{1-6-12}$.
9.) Oxaliplatin + curcumin + $B_{1-6-12}$ group: the cells were treated with 5 µg/ml oxaliplatin, 1 µg/ml curcumin, and 1 µg/ml $B_{1-6-12}$.

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

*Effect of combination between curcumin and $B_{1-6-12}$ on cisplatin- and oxaliplatin-induced alterations in HBVP.*

HBVP were divided into 9 groups:

1.) Control group: the cells were incubated in culture medium.
2.) Cisplatin group: the cells were treated with cisplatin 1.5 μg/ml for 24 hours.
3.) Cisplatin + curcumin group: the cells were treated with 1.5 μg/ml cisplatin and 1 μg/ml curcumin.
4.) Cisplatin + $B_{1-6-12}$ group: the cells were treated with 1.5 μg/ml cisplatin and 1 μg/ml $B_{1-6-12}$.
5.) Cisplatin + curcumin + $B_{1-6-12}$ group: the cells were treated with 1.5 μg/ml cisplatin, 1 μg/ml curcumin, and 1 μg/ml $B_{1-6-12}$.
6.) Oxaliplatin group: the cells were treated with 8 μg/ml oxaliplatin for 24 hours.
7.) Oxaliplatin + curcumin group: the cell was treated with 8 μg/ml oxaliplatin and 1 μg/ml curcumin.
8.) Oxaliplatin + $B_{1-6-12}$ group: the cell was treated with 8 μg/ml oxaliplatin and 1 μg/ml $B_{1-6-12}$.
9.) Oxaliplatin + curcumin + $B_{1-6-12}$ group: the cells were treated with 8 μg/ml oxaliplatin, 1 μg/ml curcumin, and 1 μg/ml $B_{1-6-12}$.

จุฬาลงกรณ์มหาวิทยาลัย
CHULALONGKORN UNIVERSITY

**3.5 MTT assay (Micro-culture tetrazolium assay)**

The MTT assay was conducted to evaluate the cytotoxicity of cisplatin, oxaliplatin, curcumin, and vitamin $B_{1-6-12}$ on HUVEC and pericytes. MTT is a yellow tetrazolium dye which is converted to purple formazan crystals by mitochondrial enzyme in living cells. Hence, the intensity of purple color indicates the number of living cells. [137]

*MTT Solution preparation*[137]

1. Prepare the MTT stock solution by dissolving the MTT powder (Life technologies, Molecular Probes, USA, Cat. No. M6494; Lot 1392119) 50 mg in 10 ml PBS and protect it from light in the -20°C.

2. Prepare the working MTT solution by 10x dilution of stock solution in the medium to establish the final concentration 0.5 mg/ml.

*Method*

1. Plate HUVEC and HBVP at $1\times10^4$ and $5\times10^3$ cells/well, respectively in 96-well plate with 100 µl of medium and then allow cells to attach for 24 hours.
2. Treat cells according to the experimental conditions for 24 hours.
3. Discard the culture medium.
4. Incubate with the 100 µl MTT solution (0.5 mg/mL) for 2 hours.
5. Discard the MTT solution.
6. Dissolve the purple formazan crystals in 100 µl DMSO.
7. Measure the absorbance of each well at 570 nm by a microplate reader (Thermo Fisher Scientific, Multiscan GO 1510-02675).
8. Calculate the percentage of cell viability in each group from the mean absorbance of test samples divided by the mean of negative control.

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

**3.6 Caspase-3 activity assay**

The caspase-3 activity was determined since the activation of caspase-3 enzyme is involved in both intrinsic and extrinsic apoptotic pathways.[49] The caspase-3/cpp32 colorimetric assay kit (BioVision, USA, Cat. No. #K106--200, Lot 6E30K01060) was used following the manufacturer's protocol. The principle of this assay is based on the reaction between caspases that recognize the sequence DEVD and the labeled substrate DEVD-*p*NA. This assay detects the *p*NA after it cleaved from the substrate.

*Method*

1. Seed 1-5x$10^6$ cells in 60 mm cell culture dish for HUVEC or T-25 flask for HBVP.
2. Treat cells according to the experimental conditions for 24 hours.
3. Harvest the cell pellet and wash in PBS.
4. Resuspend the cells in 50 µl of chilled cell lysis buffer and incubate on ice for 10 minutes.
5. Centrifuge the lysed cells 10,000 x g for 1 min and transfer supernatant (cytosolic extract) into a flesh tube.
6. Measure the protein concentration using PierceTM BCA protein assay (Thermo Scientific, Cat. No. 23227, Lot UK292602).
7. Dilute protein sample into cell lysis buffer to obtain 1 µg/ul (50 µg protein to 50 µl cell lysis buffer)
8. Add 50 µl of diluted protein sample in each well of 96-well plate.
9. Prepare working reaction buffer immediately before use by add DTT to 2x reaction buffer to obtain 10 mM final concentration.
10. Add 50 µl working reaction buffer to each sample.
11. Add 5 µl of DEVD-*p*NA substrate and incubate at 37°C for 2 hours.
12. Measure the cleaved substrate (*p*NA) of each sample using a microplate reader (Thermo Fisher Scientific, Multiscan GO 1510-02675) at 405 nm.
13. Calculate the caspase-3 activity (fold over control) from induced sample divided by uninduced (control) sample.

**3.7 ROS assay**

The intracellular ROS was measured using 2’, 7’-dichlorofluorescein diacetate (DCFH-DA) (Sigma-Aldrich, Cat. No. D6883, Lot #059M4133V). When the DCFH-DA diffuses into the cell, it is metabolized to DCFH, a non-fluorescent compound, by intracellular esterase. After that, DCFH rapidly reacts with ROS especially peroxide and changes to fluorescence form (DCF).

*DCFH-DA Solution preparation*

1. Prepare the DCFH-DA (molecular weight 487.29) stock solution by dissolving the DCFH-DA powder 121.82 mg in 5 ml DMSO and protect it from light in the -20°C.

2. Prepare the working solution by 100x dilution of stock solution in the medium to establish the final concentration 0.5 mM.

*Method*

1. Seed HUVEC $1x10^4$ cells/well or HBVP $1x10^4$ in 96-well black plate.
2. Remove the culture medium from each well.
3. Incubate cells with 100 µl of working DCFH-DA at 37°C for 30 minutes.
4. Remove DCFH-DA.
5. Wash with PBS to remove the excessive DCFH-DA, and then resuspend in 100 µl of PBS.
6. Treat cells according to the experimental conditions.
7. Measure the level of fluorescence ($T_0$) (DCF) by fluorescence microplate reader (Thermo Fisher Scientific, Varioskan Flash Multimode reader) at 480, and 535 nm for excitation and emission, respectively.
8. Incubate cells that treated with cisplatin and oxaliplatin for 1 and 24 hours, respectively.
9. Measure the level of fluorescence ($T_1$ and $T_{24}$) again by fluorescence microplate reader at 480, and 535 nm for excitation and emission, respectively.
10. Calculate the relative ROS production following the formula: $T_{24}(T_1)/T_0$, and then set the value of the control group as 1. The value of the treatment group was presented as fold over the control group which calculates for the following:

$$\text{Relative ROS production} = \frac{\frac{T24(T1)}{T0}\ of\ tretment\ group}{\frac{T24(T1)}{T0}\ of\ control\ group}$$

Chulalongkorn University

### 3.8 GSH/GSSG assay

This assay was conducted to quantify total glutathione and oxidized glutathione (GSSG) by glutathione colorimetric detection kit (Promega, USA, Cat. No. #9135, Lot #21F17). The total glutathione composes of the reduced form (GSH) and the oxidized form (GSSG). Regarding the total glutathione detection, the free thiol group of GSH reacts with the detection substrate to produce the colored product (Figure 17A). As the GSSG detection, sample will be treated with 2-vinylpyridine (2VP) to block GSH before the steps of the detection of total glutathione (Figure 17B).

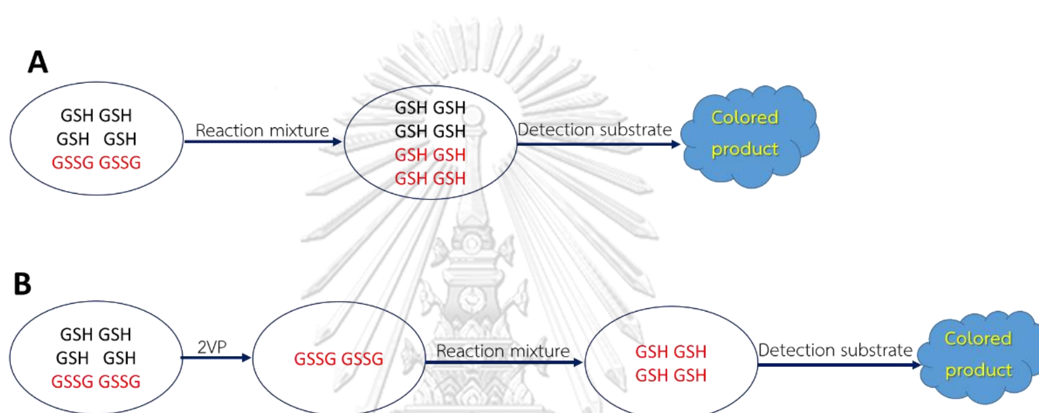



**Figure 17** Principle of reactions of the GSH/GSSG assay

The protocol of this assay was done following the manufacturer's protocol which is briefly described below.

1. Plate HUVEC or HBVP $1x10^6$ cells/ml into T-25 flask.
2. Treat cells according to the experimental conditions for 24 hours.
3. Remove medium from the plate.
4. Collect the pellet of cells.
5. Resuspend in cold 5% 5-Sulfosalicylic acid (Sigma, Cat. No. S2130), and then vigorously vortex.
6. Incubate at 4°C for 10 minutes.
7. Centrifuge at 14,000 RPM at 4°C for 10 minutes, after that collect supernatant.
8. Treat standard, blank, and sample with 2 VP (Sigma, Cat. No. 132292) (for GSSG detection).

จุฬาลงกรณ์มหาวิทยาลัย
CHULALONGKORN UNIVERSITY

9. Dilute sample or 2VP-treated sample with glutathione assay buffer in the ratio 1:4.
10. Future dilute sample or 2VP-treated sample with sample diluent in the ratio 1:10 and 1:20 for HUVEC and HBVP, respectively.
11. Add 50 µl of either 2VP-treated or untreated standard, sample, and blank into each well of 96-well plate.
12. Add 25 µl of detection substrate to each well.
13. Add 25 µl of reaction mixture to each well.
14. Incubate at room temperature for 20 minutes.
15. Measure the optical density at 405 nm using microplate reader (Thermo Fisher Scientific, Multiscan GO 1510-02675) and calculate the GSH/GSSG ratio following the formula below.

$$\text{GSH/GSSG ratio} = \frac{\textbf{Total glutathione} - \textbf{GSSG}}{\textbf{GSSG}}$$

### 3.9 Western blot analysis

Western blot analysis was performed in order to determine the expression of endothelial tight junction proteins (ZO-1, ZO-2, claudin-5, occludin).

*Method*

1. Seed HUVEC 2 x $10^6$ cells in 90 mm cell culture dish.
2. Treat cells according to experimental protocols for 24 hours.
3. Wash with cold PBS.
4. Add 50 µl of 1x ice-cold RIPA lysis buffer (Cell Signaling, Cat. No. #9806, Lot 20) containing 1x protease inhibitor cocktail (Cell Signaling, Cat. No. #5871, Lot 14).
5. Incubate on ice for 5 minutes and then scrape cells using cell scraper.
6. Sonicate briefly.
7. Centrifuge at 14,000 x g for 10 minutes at 4°C and then collect the supernatant.
8. Determine the protein concentration by Pierce™ BCA protein assay (Thermo Scientific, Cat. No. 23227, Lot UK292602).

Chulalongkorn University

9. Dilute sample to 6 μg protein in 4 ul of 1x sample buffer (ProteinSimple, California; Lot 15134) and further mix sample with fluorescent dye with the ratio of 4:1.
10. Heat the sample that containing dye at 95°C for 5 minutes.
11. Dilute primary antibodies: 1:200 **β**-actin (Cell Signaling, Cat. No. #4970), 1:200 ZO-1 (Invitrogen, Cat. No. #PA5-28858), 1:200 ZO-2 (Invitrogen, Cat. No. # PA5-17155), 1:200 claudin-5 (Invitrogen, Cat. No. # 34-1600), and 1:200 occludin (Invitrogen, Cat. No. #PA5-20755with antibody diluent (ProteinSimple, California).
12. Load the ladder, samples, antibody diluent, primary antibody, rabbit secondary conjugate, streptavidin-HRP, and Luminol-peroxide following the manufacturer's plate map.
13. Centrifuge plate at 2500 rpm for 10 minutes at 25°C.
14. automate protein separation and immunodetection using WES automated Protein Simple system (ProteinSimple, California).
15. Analyze the density of digital image (the area under the curve) with Compass software (ProteinSimple).
16. Calculate the relative protein expression from the density of each band divided by the density of **β**-actin.

จุฬาลงกรณ์มหาวิทยาลัย

**3.10 Transendothelial electrical resistance (TEER) study** [135]

CHULALONGKORN UNIVERSITY

The aim of TEER is to determine the integrity of tight junction between endothelial cells in vitro and the barrier function of pericyte. The integrity is represented in electrical resistance (ohm).

*Method*

1. Seed $1x10^4$ cell of HUVEC or HBVP on the upper chamber of transwell insert (pore size 0.4 μM, 0.3 $cm^2$, Merck, USA, Cat. No. MCHT24H48) that is placed in the 24-well plate (Figure 18).
2. Culture for 24 hours or until cells grow to confluency.
3. Treat cells according to the experimental conditions for 24 hours.

4. Measure the cell resistance(R) using Millicell electrical resistance apparatus (Millicell® ERS-2, Merck, USA) following the manufacturer's user guide.
5. Calculate the TEER value following this formula:

   TEER value($\Omega cm^2$) = ($R_{sample}$ - $R_{blank}$) x effective membrane area (0.6 $cm^2$)

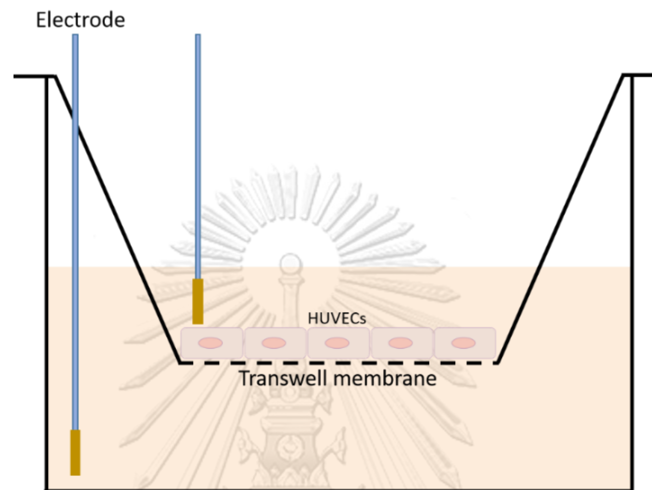



**Figure 18** Schematic of TEER value measurement

### 3.11 Data analysis

Each experiment was performed in triplicate and repeated three times. The numerical data were expressed as mean ± SEM and analyzed for statistically significant differences between groups using one-way ANOVA followed by Tukey's post hoc test. GraphPad Prism 9 program was used for statistical analysis. Statistical significance was considered when *p-value* < 0.05.

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

# CHAPTER IV

# RESULTS

**Effects of curcumin on cisplatin- and oxaliplatin-induced alterations in HUVEC**

**1. Cell viability**

1.1 Effects of co-treatment with cisplatin and curcumin on HUVEC viability

The treatment of HUVEC with 3 µg/ml cisplatin significantly reduced cell viability when compared with the control group (p<0.001). The combination of cisplatin and 1 µg/ml curcumin significantly enhanced HUVEC viability (p<0.01) (Figure 19).

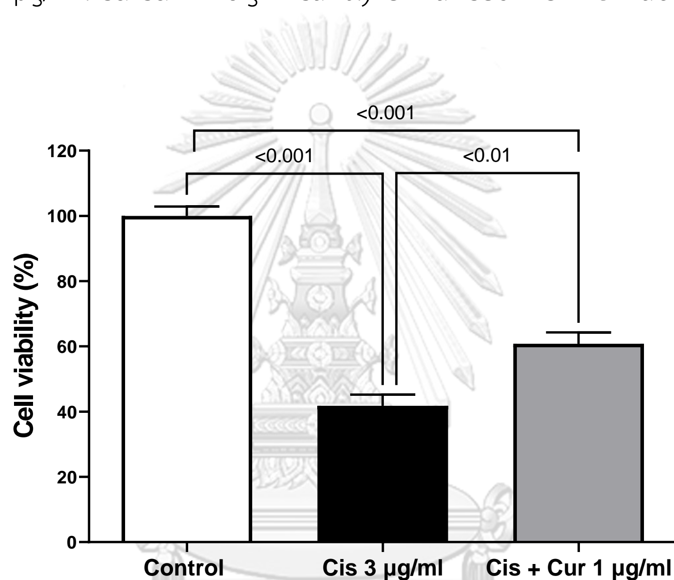



**Figure 19** Cell viability of HUVEC after treatments. The graph shows the average percent of cell viability with SEM. Cis=cisplatin, Cur=curcumin.

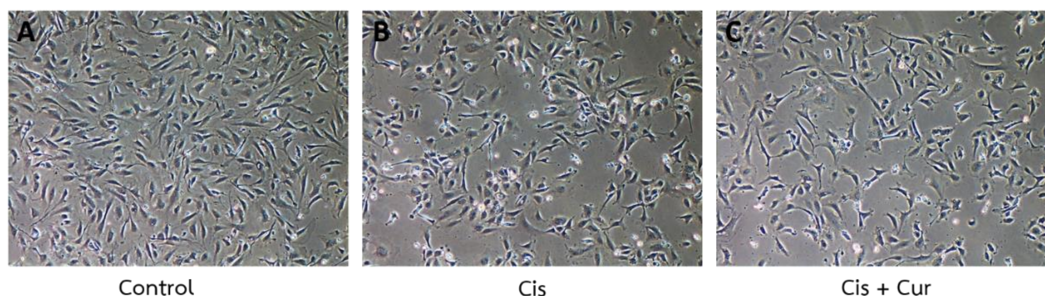



**Figure 20** Morphology of HUVEC under phase contrast microscope (magnification: 10x). A. Control, B. Cisplatin (Cis), C. Co-treatment with cisplatin and curcumin (Cis + Cur)

1.2 Effects of co-treatment with oxaliplatin and curcumin on HUVEC viability

The dose 5 µg/ml of oxaliplatin significantly minimized the HUVEC viability when compared with their control group ($p$<0.001). A combination of oxaliplatin and 1 µg/ml curcumin significantly enhanced the growth inhibitory activity of HUVEC ($p$<0.01) (Figure 21).

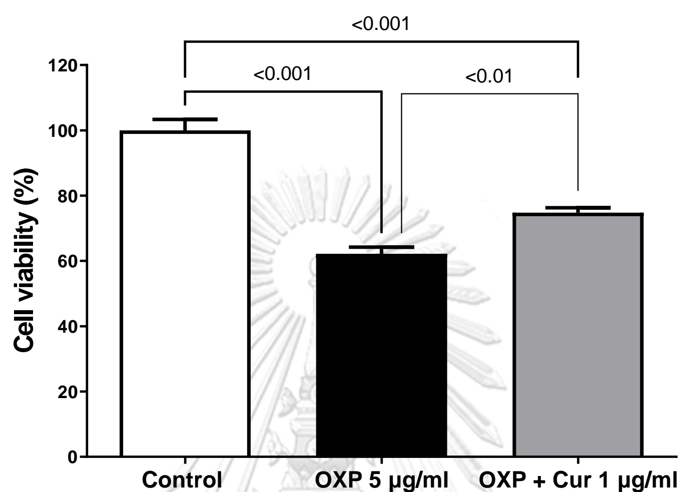



**Figure 21** Cell viability of HUVEC after treatments. The graph shows the average percent of cell viability with SEM. OXP=oxaliplatin, Cur=curcumin.

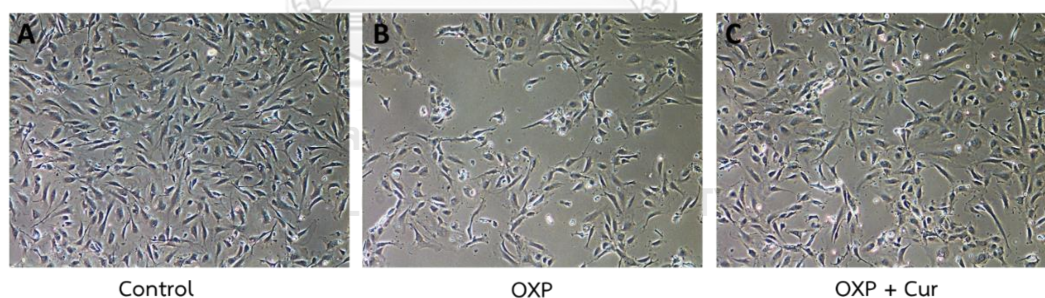



**Figure 22** Morphology of HUVEC under phase contrast microscope (magnification: 10x). A. Control, B. Oxaliplatin (OXP), C. Co-treatment with oxaliplatin and curcumin (OXP + Cur).

**2. Caspase-3 activity**

2.1 Effects of co-treatment with cisplatin and curcumin on caspase-3 activity

The incubation of HUVEC with 3 μg/ml of cisplatin induced an increase in caspase-3 activity (*p*<0.001). Nonetheless, the caspase-3 activity did not significantly diminish after co-treatment with curcumin (Figure 23).

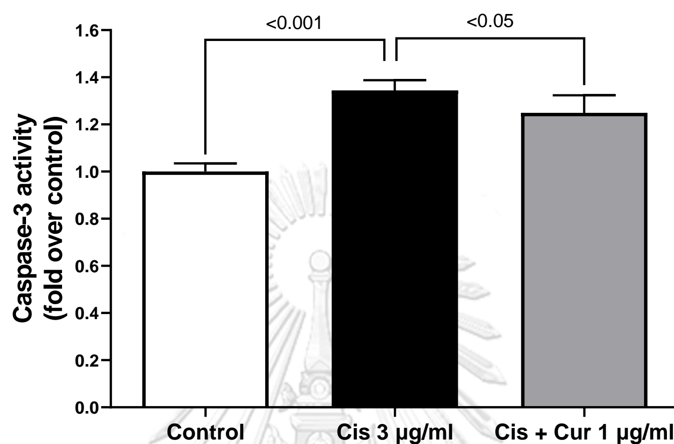



**Figure 23** Caspase-3 activity in HUVEC after treatments. Data in graph are mean ± SEM. Cis=cisplatin, Cur=curcumin.

2.2 Effects of co-treatment with oxaliplatin and curcumin on caspase-3 activity

Comparable to the control group, oxaliplatin caused the significant enhancement of caspase-3 level (*p*<0.001). However, the co-treatment with curcumin did not significantly decrease this event (Figure 24).

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

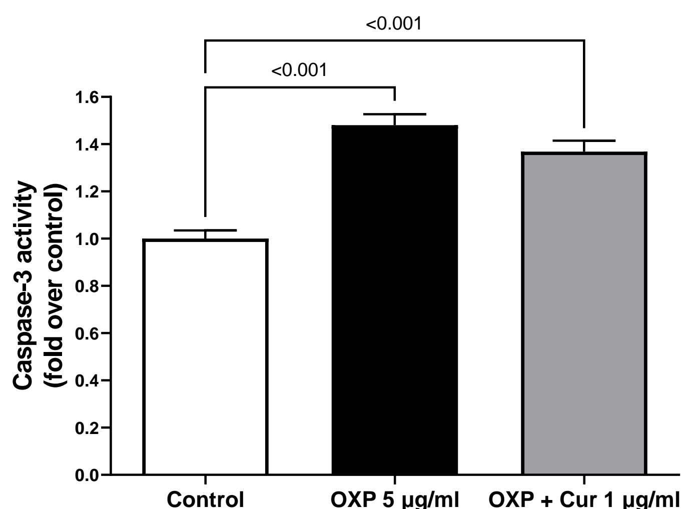


**Figure 24** Caspase-3 activity in HUVEC after treatments. Data in graph are mean ± SEM. OXP=oxaliplatin, Cur=curcumin.

**3. ROS level**

3.1 Effects of co-treatment with cisplatin and curcumin on ROS production

After treating HUVEC with 3 μg/ml cisplatin, ROS level was significantly increased in comparison to the control group ($p<0.01$). Combined treatment with curcumin significantly reduced this effect of cisplatin ($p<0.05$) (Figure 25).

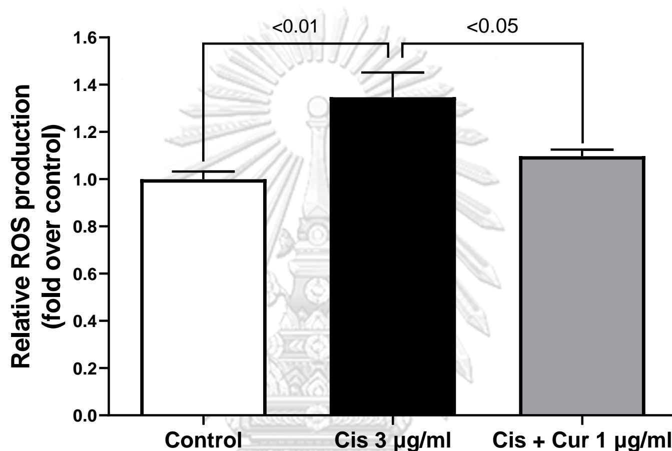



**Figure 25** ROS production in HUVEC after treatments. Average of relative ROS production (fold over control) and SEM are shown. Cis=cisplatin, Cur=curcumin.

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

3.2 Effects of co-treatment with oxaliplatin and curcumin on ROS production

ROS assay for HUVEC incubated with 5 μg/ml of oxaliplatin revealed a significant production of ROS ($p<0.05$). In the presence of curcumin, the ROS level was significantly diminished as compared with the oxaliplatin group ($p<0.01$) (Figure 26).

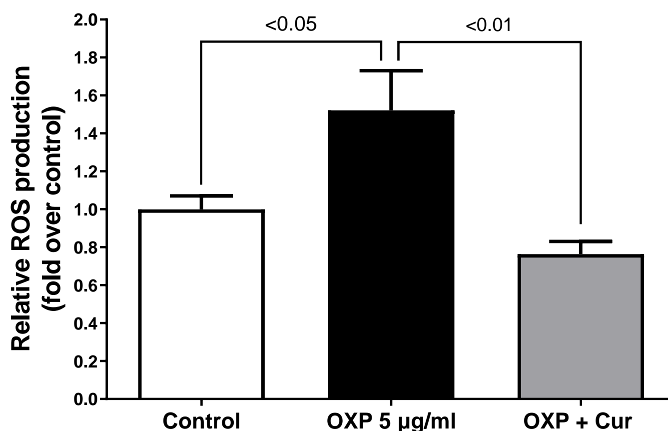



**Figure 26** ROS production in HUVEC after treatments. Average of relative ROS production (fold over control) and SEM are shown. OXP=oxaliplatin, Cur=curcumin.

**4. Total glutathione level and GSH/GSSG ratio**

4.1 Effects of co-treatment with cisplatin and curcumin on total glutathione level and GSH/GSSG ratio

After exposure to cisplatin, the concentration of total glutathione and GSH/GSSG ratio were significantly decreased (*p*<0.001). The co-treatment with curcumin significantly reversed only GSH/GSSG ratio (*p*<0.01) but not the total glutathione level (Figure 27).

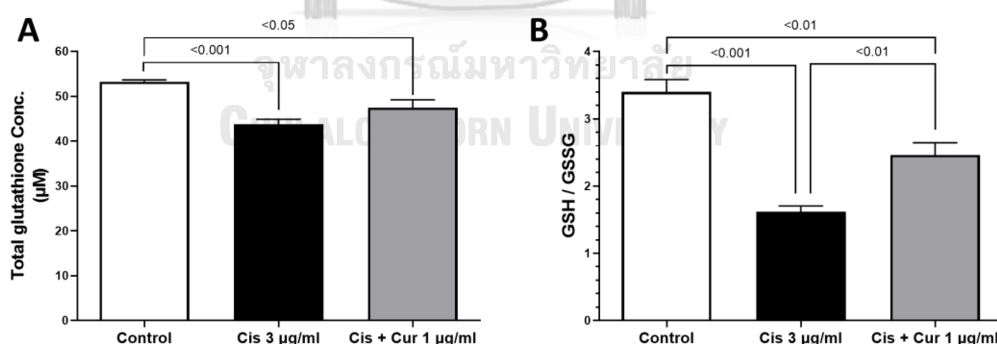



**Figure 27** Total glutathione level and GSH/GSSG ratio in HUVEC after treatments. (A) total glutathione concentration, (B) the ratio of reduced glutathione (GSH) to oxidized glutathione (GSSG). Graphs represent Mean ± SEM. Cis=cisplatin, Cur=curcumin.

4.2 Effects of co-treatment with oxaliplatin and curcumin on total glutathione level and GSH/GSSG ratio

Oxaliplatin caused a significant decrease in total glutathione level ($p$<0.001) and GSH/GSSG ratio ($p$<0.001). Concurrent incubation with curcumin significantly restored the GSH/GSSG ratio ($p$<0.001). However, the total glutathione level was unchanged after curcumin treatment (Figure 28).

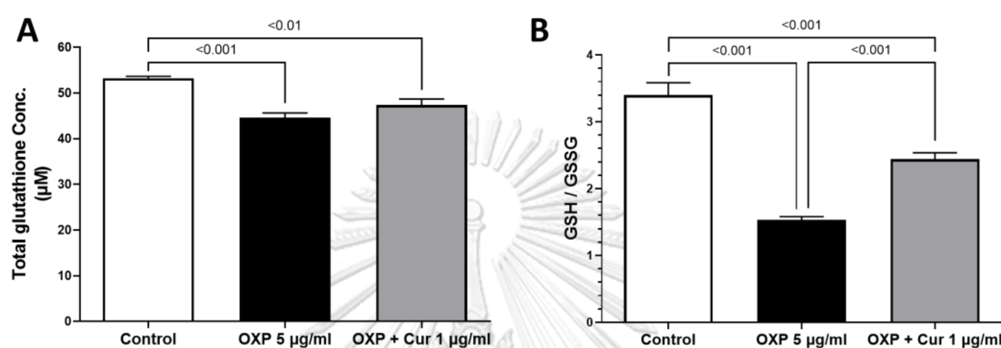



**Figure 28** Total glutathione level and GSH/GSSG ratio in HUVEC after treatments. (A) total glutathione concentration, (B) the ratio of reduced glutathione (GSH) to oxidized glutathione (GSSG). Graphs represent Mean ± SEM. OXP=oxaliplatin, Cur=curcumin.

**5. Expression of endothelial tight junction proteins**

5.1 Effects of co-treatment with cisplatin and curcumin on expression of tight junction proteins

จุฬาลงกรณ์มหาวิทยาลัย
CHULALONGKORN UNIVERSITY

The exposure of HUVEC to cisplatin caused the downregulation of occludin without statistical significance. However, cisplatin did not change the expression of claudin-5. After co-treatment with curcumin, the expression of claudin-5 was upregulated but not statistically significant. The expression of occludin did not change after curcumin treatment (Figure 29).

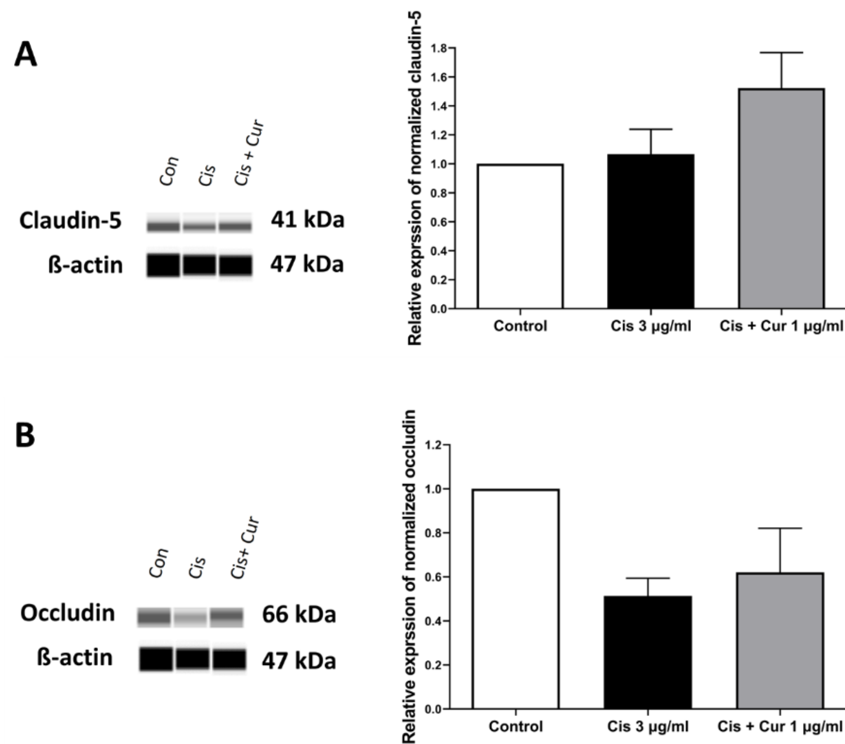



**Figure 29** Claudin-5 and occludin protein expression in HUVEC after treatments. (A) Claudin-5, (B) Occludin. The graphs show the average of relative protein expression normalized to beta-actin (fold over control) with SEM. Cis=cisplatin, Cur=curcumin.

Treatment with cisplatin caused a trend to suppress the ZO-1 and ZO-2. However, the combination treatment between cisplatin and curcumin significantly enhanced the expression of ZO-1 ($p$<0.05) and ZO-2 ($p$<0.05) (Figure 30).

จุฬาลงกรณ์มหาวิทยาลัย
CHULALONGKORN UNIVERSITY

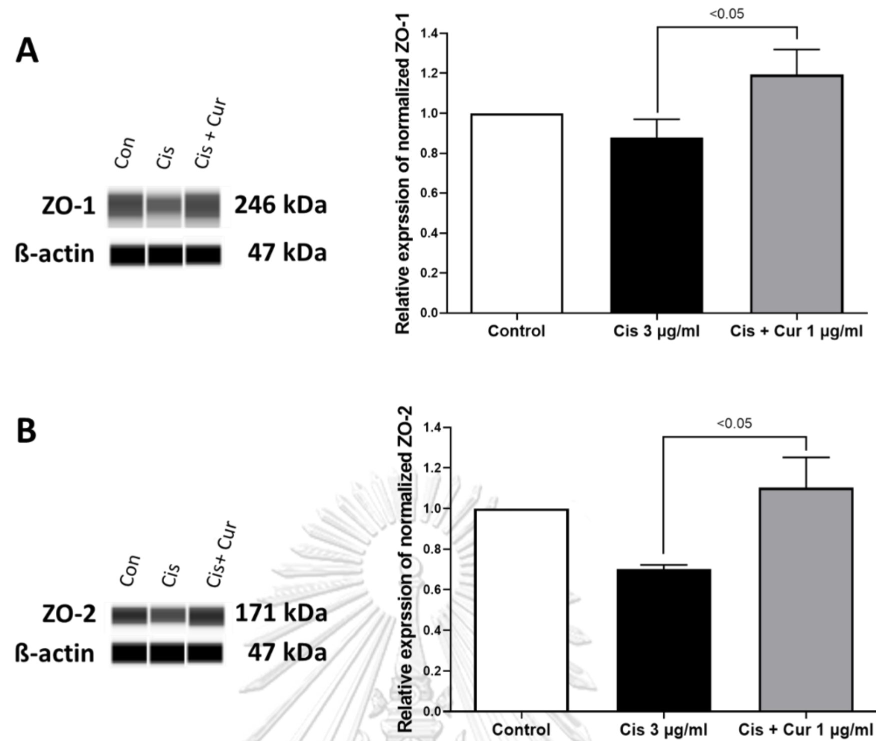



**Figure 30** ZO-1 and ZO-2 protein expression HUVEC after treatments. (A) ZO-1, (B) ZO-2. The graphs show the average of relative protein expression normalized to beta-actin (fold over control) with SEM. Cis=cisplatin, Cur=curcumin, *p<0.05 vs. cisplatin group.

5.2 Effects of co-treatment with oxaliplatin and curcumin on expression of tight junction proteins

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

Oxaliplatin treatment induced the depletion of claudin-5 which was not statistically significant. Nonetheless, it did not change the expression of occludin protein. Co-treatment with curcumin tended to elevate the expression of these proteins despite no statistical significance (Figure 31).

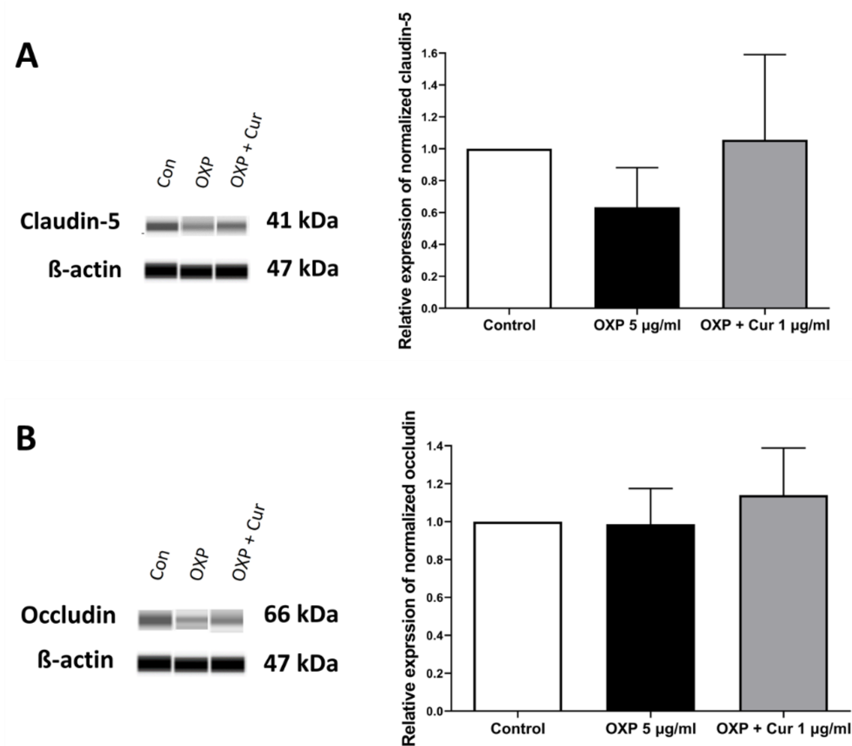



**Figure 31** Claudin-5 and occludin protein expression in HUVEC after treatments. (A) Claudin-5, (B) Occludin. The graphs show the average of relative protein expression normalized to beta-actin (fold over control) with SEM. OXP=oxaliplatin, Cur=curcumin.

Oxaliplatin significantly decreased the expression of ZO-1 ($p<0.05$), whereas it did not change the expression of ZO-2. Co-treatment with curcumin tended to improve the expression of both proteins despite no statistical significance (Figure 32).

จุฬาลงกรณ์มหาวิทยาลัย
CHULALONGKORN UNIVERSITY

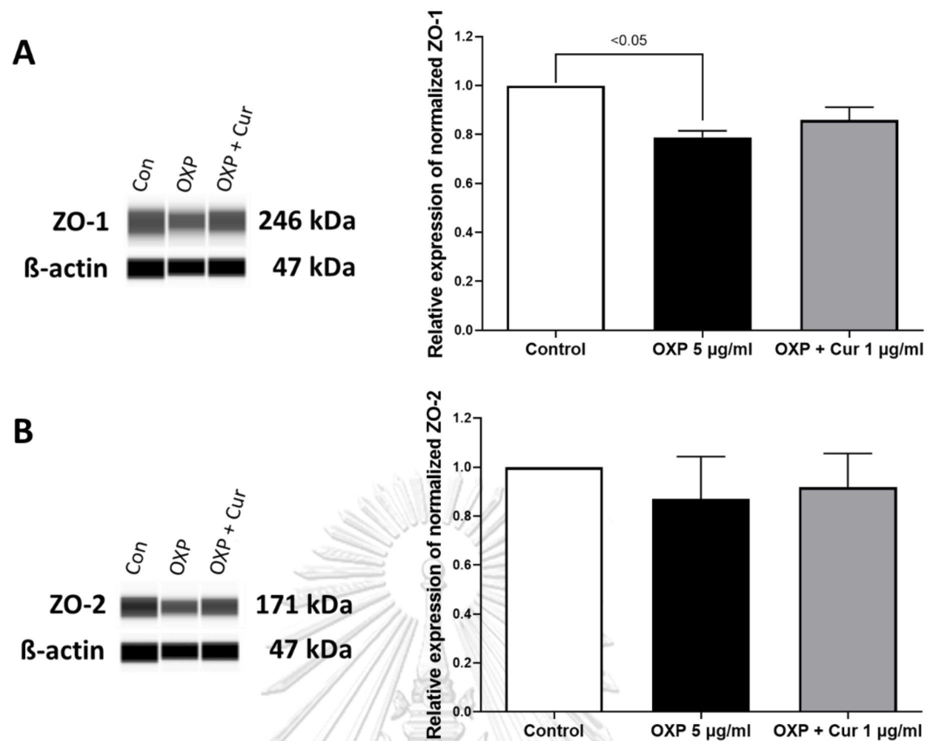



**Figure 32** ZO-1 and ZO-2 protein expression in HUVEC after treatments. (A) ZO-1, (B) ZO-2. The graphs show the average of relative protein expression normalized to beta-actin (fold over control) with SEM. OXP=oxaliplatin, Cur=curcumin.

**6. TEER value**

6.1 Effects of co-treatment with cisplatin and curcumin on TEER value

After 24 hours of incubation with 3 µg/ml of cisplatin, the TEER value of HUVEC significantly decreased when compared with the control group ($p<0.001$). Co-treatment with curcumin led to a significant increase in the TEER value ($p<0.01$) (Figure 33). Hence, curcumin enhanced the barrier property of HUVEC after cisplatin treatment.

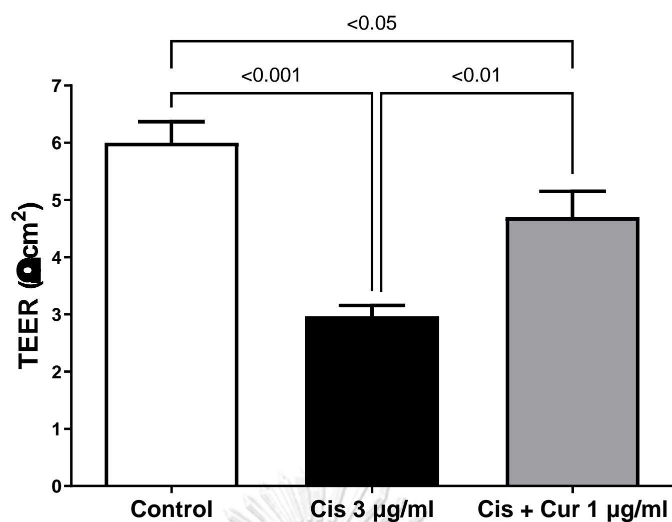



**Figure 33** TEER value of HUVEC after treatments. Data are mean ± SEM. Cis=cisplatin, Cur=curcumin.

6.2 Effects of co-treatment with oxaliplatin and curcumin on TEER value

The decreased TEER value of HUVEC was also observed following exposure to 5 μg/ml of oxaliplatin ($p<0.001$). After co-treatment with curcumin, the TEER value was significantly enhanced when compared with the oxaliplatin group ($p<0.001$) (Figure 34).

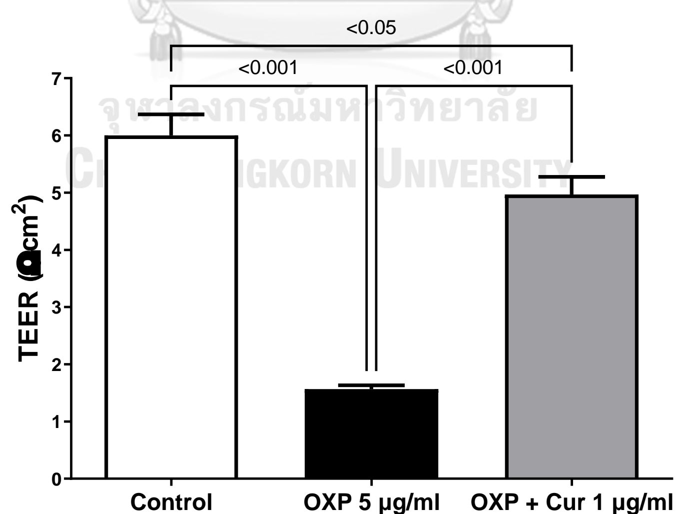



**Figure 34** TEER value of HUVEC after treatments. Data are mean ± SEM. OXP=oxaliplatin, Cur=curcumin

จุฬาลงกรณ์มหาวิทยาลัย
CHULALONGKORN UNIVERSITY

**Effects of curcumin on cisplatin-and oxaliplatin-induced alterations in HBVP**

**1. MTT assay**

1.1 Effects of co-treatment with cisplatin and curcumin on HBVP viability

HBVP were co-treated with 1.5 μg/ml cisplatin and 1 μg/ml curcumin for 24 hours. The cell viability in the cisplatin-treated group significantly decreased when compared with the control group ($p$<0.001), whereas the cell viability in the curcumin-treated group significantly increased when compared with the cisplatin-treated group ($p$<0.01) (Figure 35).

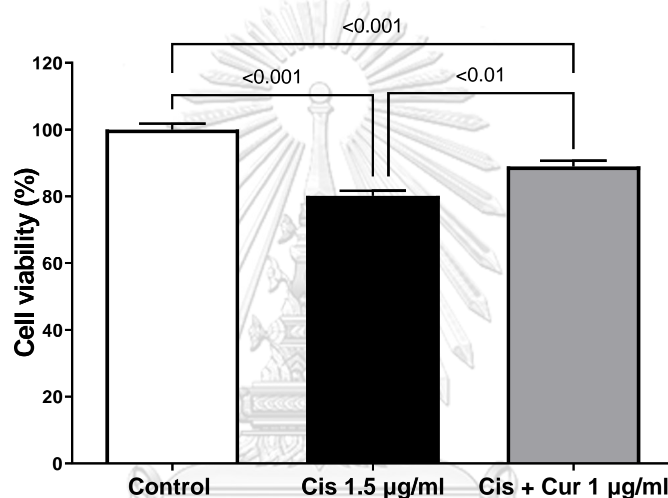



**Figure 35** Cell viability of HBVP after treatments. The graph shows the average percent of cell viability with SEM. Cis=cisplatin, Cur=curcumin.

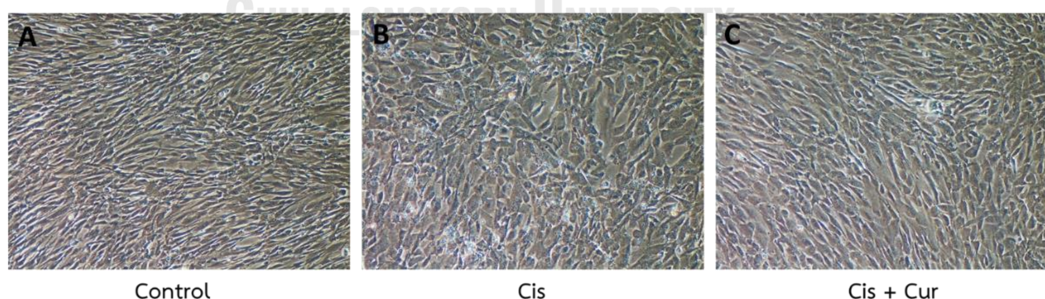



**Figure 36** Morphology of HBVP under phase contrast microscope (magnification: 10x). A. Control, B. Cisplatin (Cis), C. Co-treatment with cisplatin and curcumin (Cis + Cur).

1.2 Effects of co-treatment with oxaliplatin and curcumin on HBVP viability

After HBVP were exposed to 8 µg/ml of oxaliplatin for 24 hours, cell viability was significantly reduced ($p<0.01$). Curcumin 1 µg/ml exhibited a protective effect against the oxaliplatin-induced decrease in viability ($p<0.05$) (Figure 37).

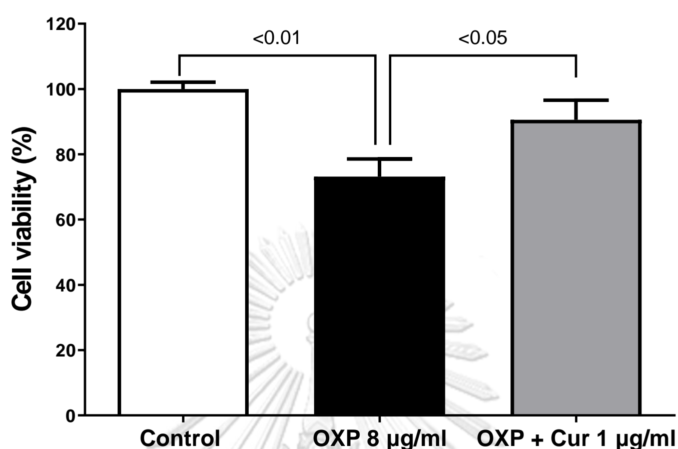
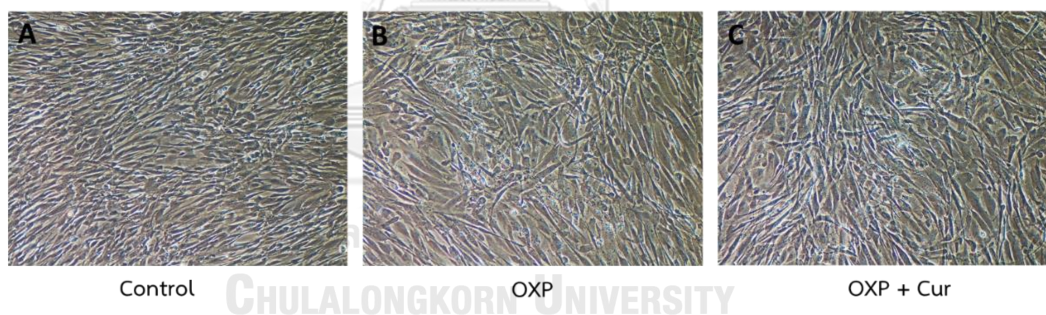



**Figure 37** Cell viability of HBVP after treatments. The graph shows the average percent of cell viability with SEM. OXP=oxaliplatin, Cur=curcumin.




CHULALONGKORN UNIVERSITY

**Figure 38** Morphology of HBVP under phase contrast microscope (magnification: 10x). A. Control, B. Oxaliplatin (OXP), C. Co-treatment with oxaliplatin and curcumin (OXP + Cur).

**2. Caspase-3 activity**

2.1 Effects of co-treatment with cisplatin and curcumin on caspase-3 activity

Cisplatin significantly increased the caspase-3 activity in comparison with the control group ($p<0.05$). However, co-treatment with curcumin significantly attenuated the activity of caspase-3 ($p<0.05$) (Figure 39).

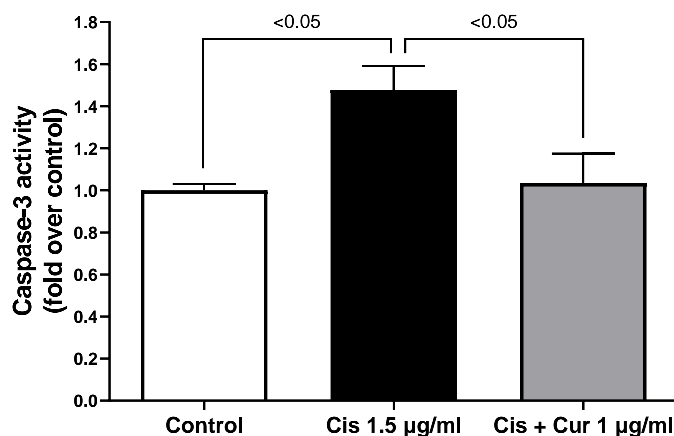



**Figure 39** Caspase-3 activity in HBVP after treatments. Data in graph are mean ± SEM. Cis=cisplatin, Cur=curcumin.

2.2 Effects of co-treatment with oxaliplatin and curcumin on caspase-3 activity

After exposure to oxaliplatin, the activity of caspase-3 was significantly elevated when compared with the control group ($p<0.05$). However, the caspase-3 activity in the co-treatment group was between those of control and oxaliplatin groups (Figure 40).

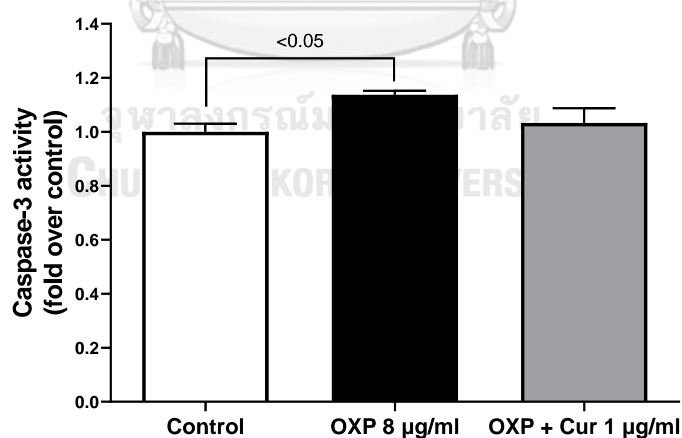



**Figure 40** Caspase-3 activity in HBVP after treatments. Data in graph are mean ± SEM. OXP=oxaliplatin, Cur=curcumin.

**3. ROS assay**

3.1 Effects of co-treatment with cisplatin and curcumin on ROS production

When HBVP were incubated with cisplatin alone, ROS level significantly increased from the control ($p$<0.001). However, when the cells were incubated with curcumin and cisplatin, the production of ROS significantly decreased when compared with the cisplatin treatment ($p$<0.001) (Figure 41).

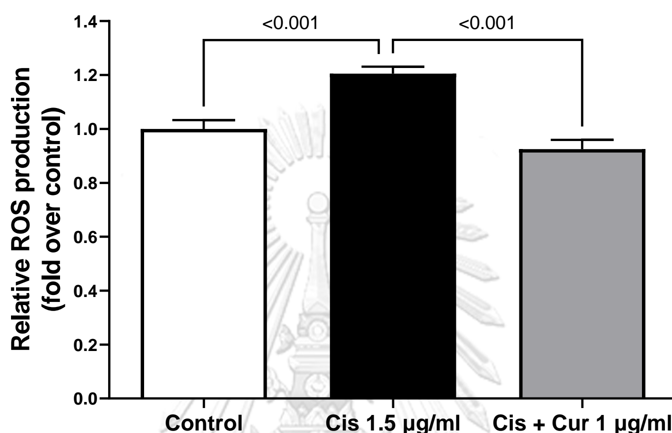



**Figure 41** ROS production in HBVP after treatments. Results show the relative ROS production (fold over control). Cis=cisplatin, Cur=curcumin.

3.2 Effects of co-treatment with oxaliplatin and curcumin on ROS production

ROS level was significantly increased in the oxaliplatin-treated group compared with the control group ($p$<0.001). Nevertheless, the curcumin-treated group had significantly reduced ROS production under oxaliplatin cytotoxicity ($p$<0.001) (Figure 42).

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

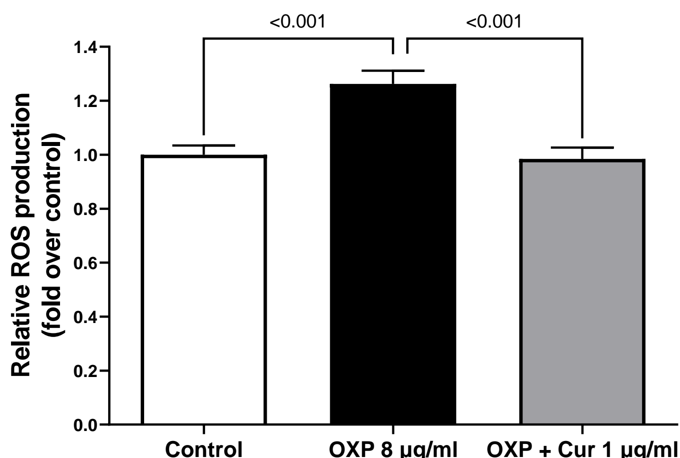



**Figure 42** ROS production in HBVP after treatments. Results show the relative ROS production (fold over control). OXP=oxaliplatin, Cur=curcumin.

**4. Total glutathione level and GSH/GSSG ratio**

4.1 Effects of co-treatment with cisplatin and curcumin on total glutathione level and GSH/GSSG ratio

Cisplatin exposure significantly minimized the level of total glutathione and GSH/GSSG ratio ($p$<0.001). Curcumin treatment significantly elevated both total glutathione level ($p$<0.001) and GSH/GSSG ratio ($p$<0.01) (Figure 43).

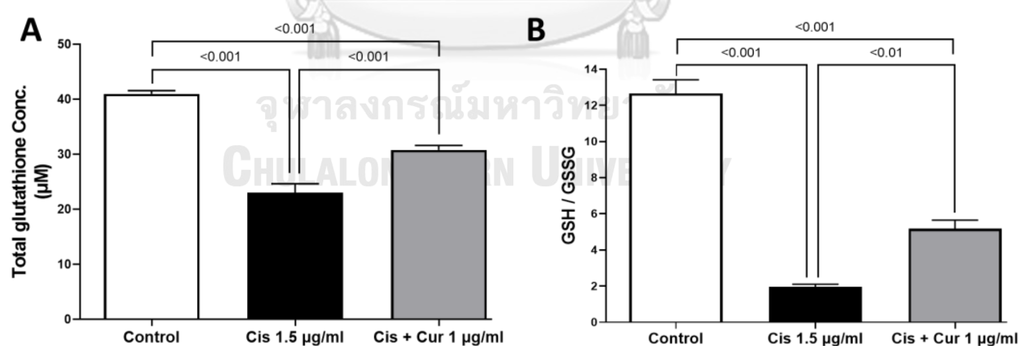



**Figure 43** Total glutathione level and GSH/GSSG ratio in HBVP after treatments. (A) total glutathione concentration, (B) the ratio of reduced glutathione (GSH) to oxidized glutathione (GSSG). Graphs represent Mean ± SEM. Cis=cisplatin, Cur=curcumin.

4.2 Effects of co-treatment with oxaliplatin and curcumin on total glutathione level and GSH/GSSG ratio

Oxaliplatin exposure also significantly attenuated both total glutathione level and the GSH/GSSG ratio (p<0.001). Curcumin treatment enhanced the total glutathione level (p<0.05) and GSH/GSSG ratio (*p*<0.01) (Figure 44).

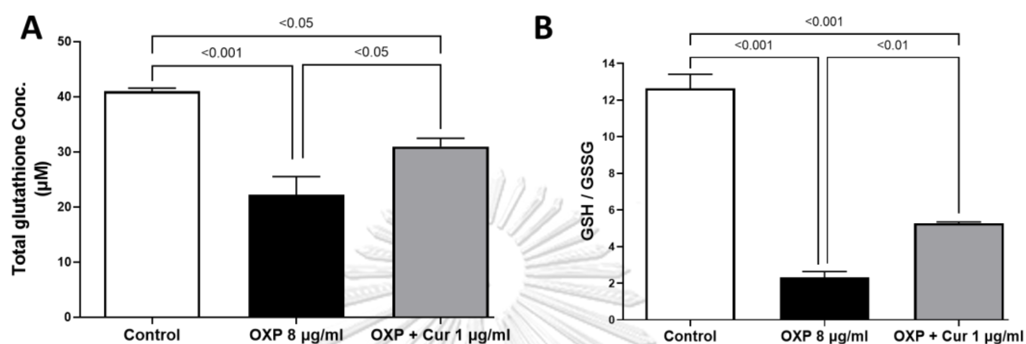



**Figure 44** Total glutathione level and GSH/GSSG ratio in HBVP after treatments. (A) total glutathione concentration. (B) the ratio of reduced glutathione (GSH) to oxidized glutathione (GSSG). Graphs represent Mean ± SEM. OXP=oxaliplatin, Cur=curcumin.

**5. TEER value**

5.1 Effects of co-treatment with cisplatin and curcumin on TEER value

The TEER value in HBVP significantly dropped after exposed to the 1.5 μg/ml of cisplatin (*p*<0.001). The TEER value was improved by co-treatment with 1.5 μg/ml of curcumin (*p*<0.001) (Figure 45).

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

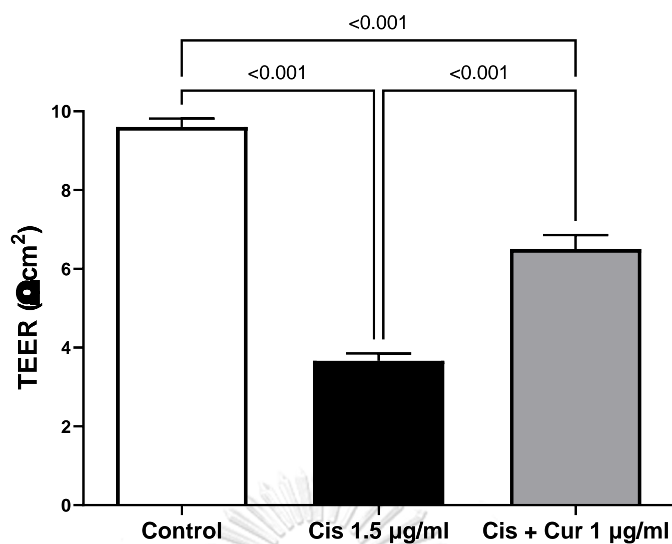



**Figure 45** The TEER value of HBVP after treatments. Data are mean ± SEM. Cis=cisplatin, Cur=curcumin.

5.2 Effects of co-treatment with oxaliplatin and curcumin on TEER value

Oxaliplatin caused remarkable TEER value reduction ($p<0.001$). However, this reduction was ameliorated by treatment with 1 μg/ml of curcumin ($p<0.001$) (Figure 46).

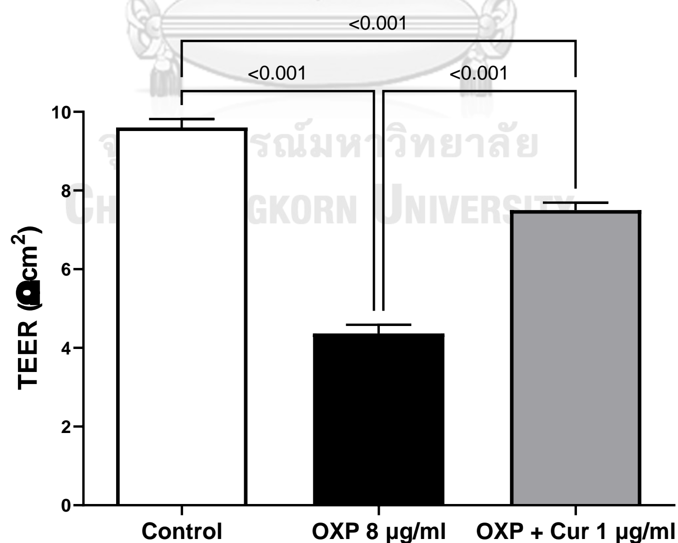



**Figure 46** The TEER value of HBVP after treatments. Data are mean ± SEM. OXP=oxaliplatin, Cur=curcumin.

**Effects of $B_{1-6-12}$ on cisplatin- and oxaliplatin-induced alterations in HUVEC**

**1. MTT assay**

1.1 Effects of co-treatment with cisplatin and $B_{1-6-12}$ on HUVEC viability

A significant reduction was observed in cisplatin-treated group ($p<0.001$). Combination with 1 μg/ml of $B_{1-6-12}$ could protect these cells from cisplatin cytotoxicity ($p<0.001$) (Figure 47).

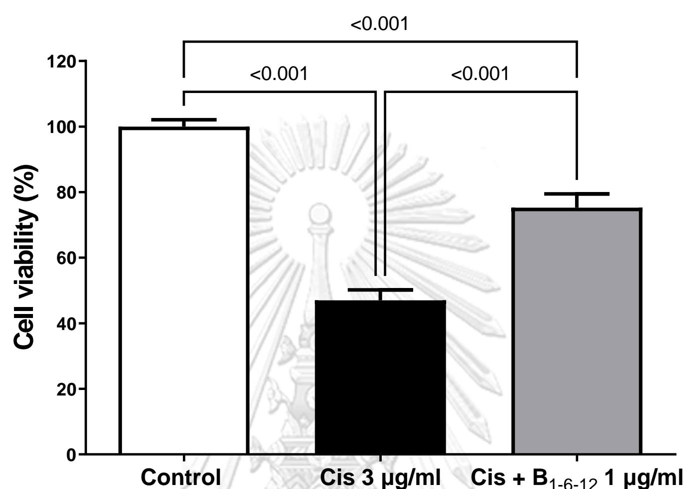



**Figure 47** Cell viability of HUVEC after treatments. The graph shows the average percent of cell viability with SEM. Cis=cisplatin.

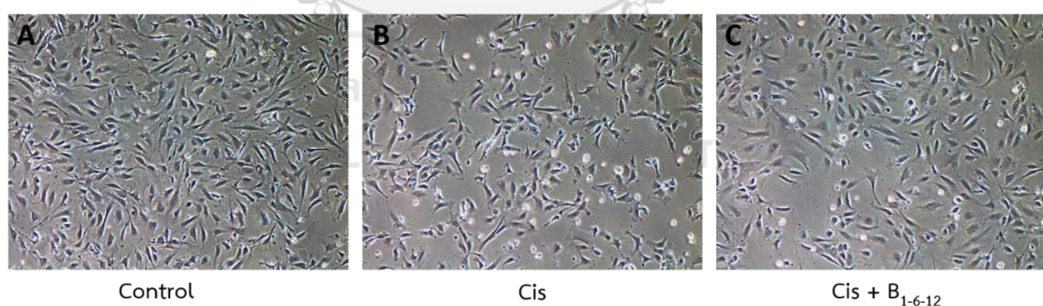



**Figure 48** Morphology of HUVEC under phase contrast microscope (magnification: 10x). A. Control, B. Cisplatin (Cis), C. Co-treatment with cisplatin and B1-6-12 (Cis + $B_{1-6-12}$).

1.2 Effects of co-treatment with oxaliplatin and $B_{1-6-12}$ on HUVEC viability

The treatment with 5 µg/ml oxaliplatin significantly decreased cell viability ($p$<0.001). Combined treatment with 1 µg/ml $B_{1-6-12}$ did not alleviate reduced cell viability caused by oxaliplatin (Figure 49).

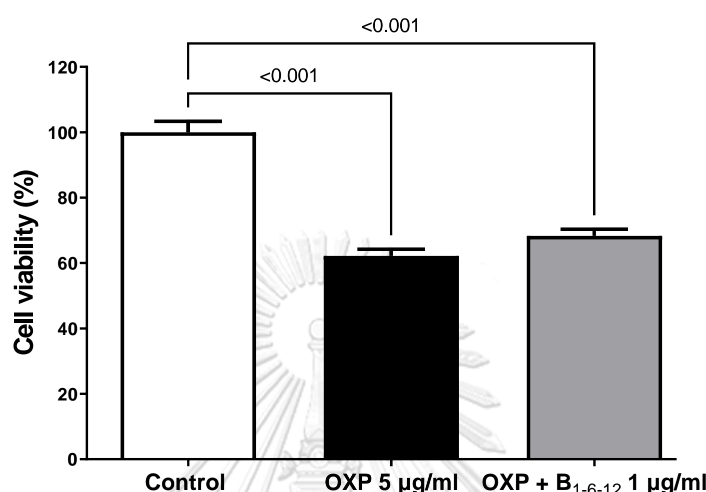



**Figure 49** Cell viability of HUVEC after treatments. The graph shows the average percent of cell viability with SEM. OXP=oxaliplatin.

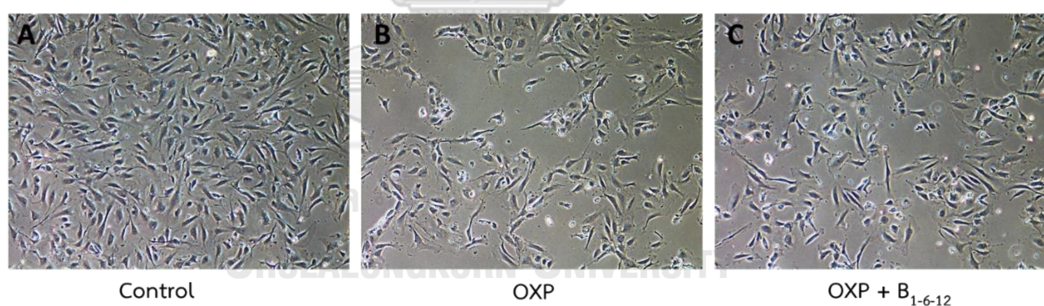



**Figure 50** Morphology of HUVEC under phase contrast microscope (magnification: 10x). A. Control, B. Oxaliplatin (OXP), C. Co-treatment with oxaliplatin and B1-6-12 (OXP + $B_{1-6-12}$).

**2. Caspase-3 activity**

2.1 Effects of co-treatment with cisplatin and $B_{1-6-12}$ on caspase-3 activity

After cisplatin treatment, the caspase-3 activity of HUVEC was induced ($p$<0.01 vs. control group). However, co-treatment with $B_{1-6-12}$ did not significantly reduce the caspase-3 activity (Figure 51).

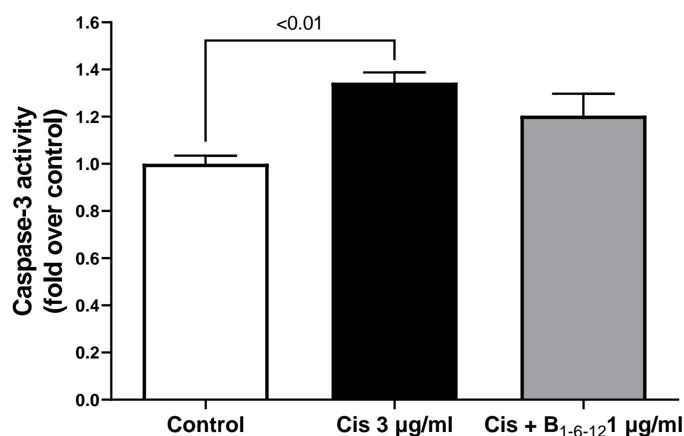



**Figure 51** Caspase-3 activity in HUVEC after treatments. Data in graph are mean ± SEM. Cis=cisplatin.

2.2 Effects of co-treatment with oxaliplatin and $B_{1\text{-}6\text{-}12}$ on caspase-3 activity

Oxaliplatin also induced the caspase-3 activity (*p*<0.001 vs. control group). Nevertheless, the caspase-3 activity did not significantly decrease when $B_{1\text{-}6\text{-}12}$ was co-treated (Figure 52).

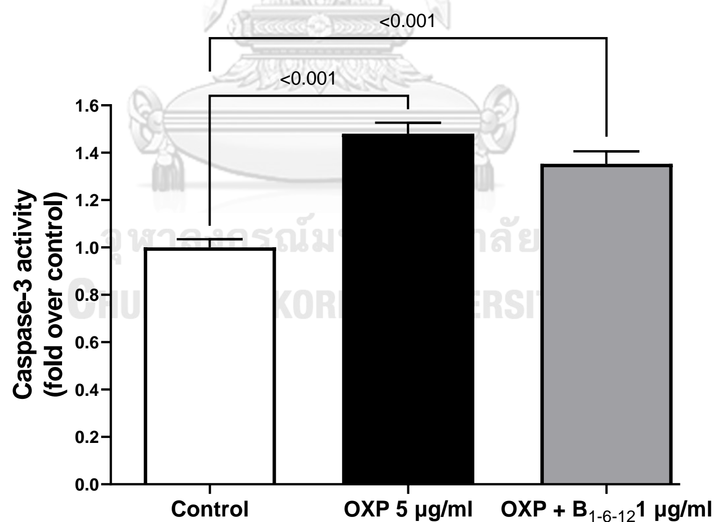



**Figure 52** Caspase-3 activity in HUVEC after treatments. Data in graph are mean ± SEM. OXP=oxaliplatin.

**3. ROS assay**

3.1 Effects of co-treatment with cisplatin and $B_{1-6-12}$ on ROS production

Cisplatin caused an increase in ROS level ($p$<0.01). However, the co-treatment with $B_{1-6-12}$ slightly decrease ROS level compared with cisplatin alone (Figure 53).

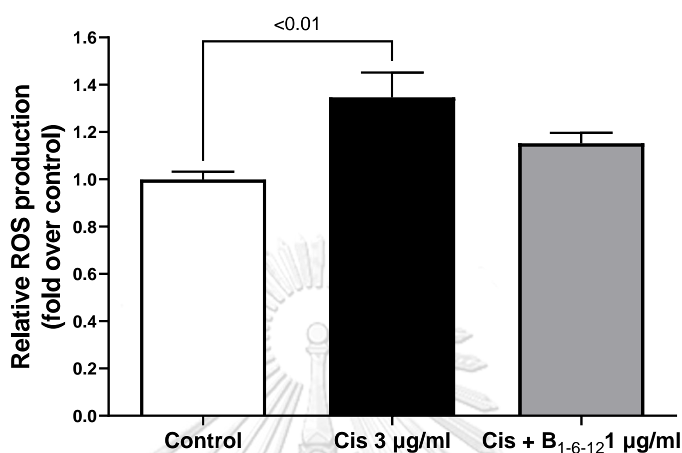



**Figure 53** ROS production in HUVEC after treatments. Average of relative ROS production (fold over control) and SEM are shown. Cis=cisplatin.

3.2 Effects of co-treatment with oxaliplatin and $B_{1-6-12}$ on ROS production

Oxaliplatin significantly elevated ROS production ($p$<0.05 vs. control group). Combined treatment with $B_{1-6-12}$ partially lower the ROS level (Figure 54).

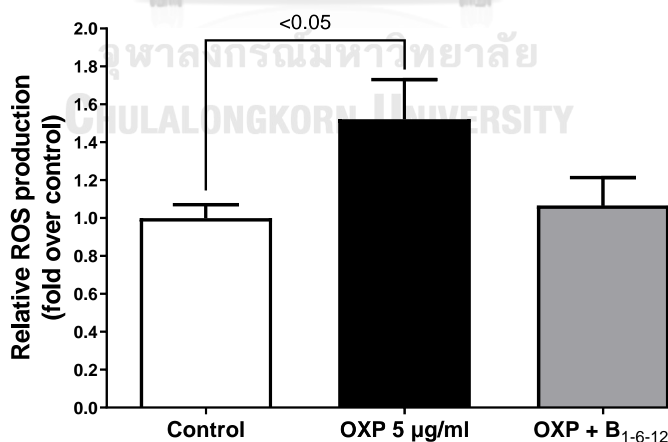



**Figure 54** ROS production in HUVEC after treatments. Average of relative ROS production (fold over control) and SEM are shown. OXP=oxaliplatin.

จุฬาลงกรณ์มหาวิทยาลัย
CHULALONGKORN UNIVERSITY

**4. Total glutathione level and GSH/GSSG ratio**

4.1 Effects of co-treatment with cisplatin and $B_{1-6-12}$ on total glutathione level and GSH/GSSG ratio

Cisplatin treatment alone significantly minimized total glutathione ($p$<0.001) and the GSH/GSSG ratio ($p$<0.001). $B_{1-6-12}$ treatment did not affect both total glutathione concentration and the GSH/GSSG ratio (Figure 55).

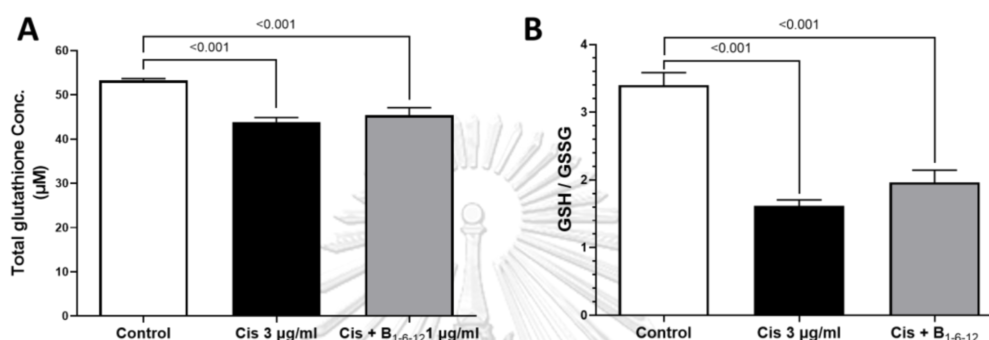



**Figure 55** Total glutathione level and GSH/GSSG ratio in HUVEC after treatments. (A) total glutathione concentration, (B) the ratio of reduced glutathione (GSH) to oxidized glutathione (GSSG). Graphs represent Mean ± SEM. Cis=cisplatin.

4.2 Effects of co-treatment with oxaliplatin and $B_{1-6-12}$ on total glutathione level and GSH/GSSG ratio

Oxaliplatin treatment alone reduced both total glutathione level ($p$<0.001) and GSH/GSSG ratio ($p$<0.001) (Figure 56). $B_{1-6-12}$ treatment did not affect the total glutathione level and GSH/GSSG ratio.

จุฬาลงกรณ์มหาวิทยาลัย
CHULALONGKORN UNIVERSITY

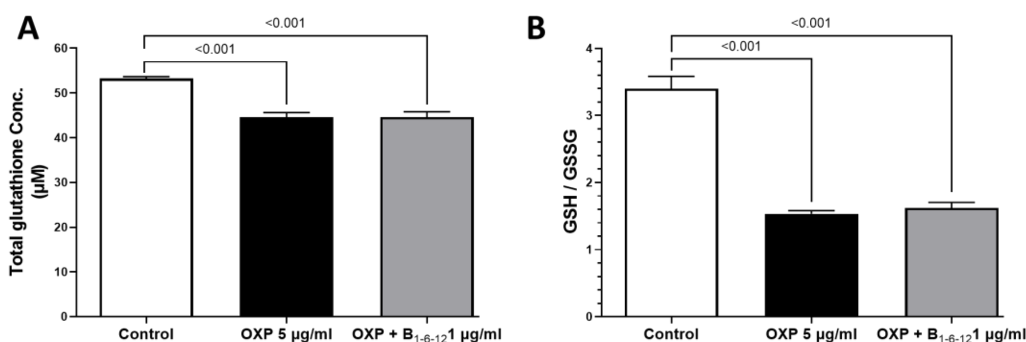



**Figure 56** Total glutathione level and GSH/GSSG ratio in HUVEC after treatments. (A) total glutathione concentration, (B) the ratio of reduced glutathione (GSH) to oxidized glutathione (GSSG). Graphs represent Mean ± SEM. OXP=oxaliplatin.

**5. Expression of endothelial tight junction proteins**

5.1 Effects of co-treatment with cisplatin and $B_{1-6-12}$ on expression of tight junction proteins

The expression of claudin-5 protein did not alter after exposure to cisplatin, whereas the expression of occludin significantly reduced ($p$<0.05). Co-treatment with $B_{1-6-12}$ did not improve the expression of claudin-5 but it significantly ameliorated the reduced expression of occludin ($p$<0.01) (Figure 57).

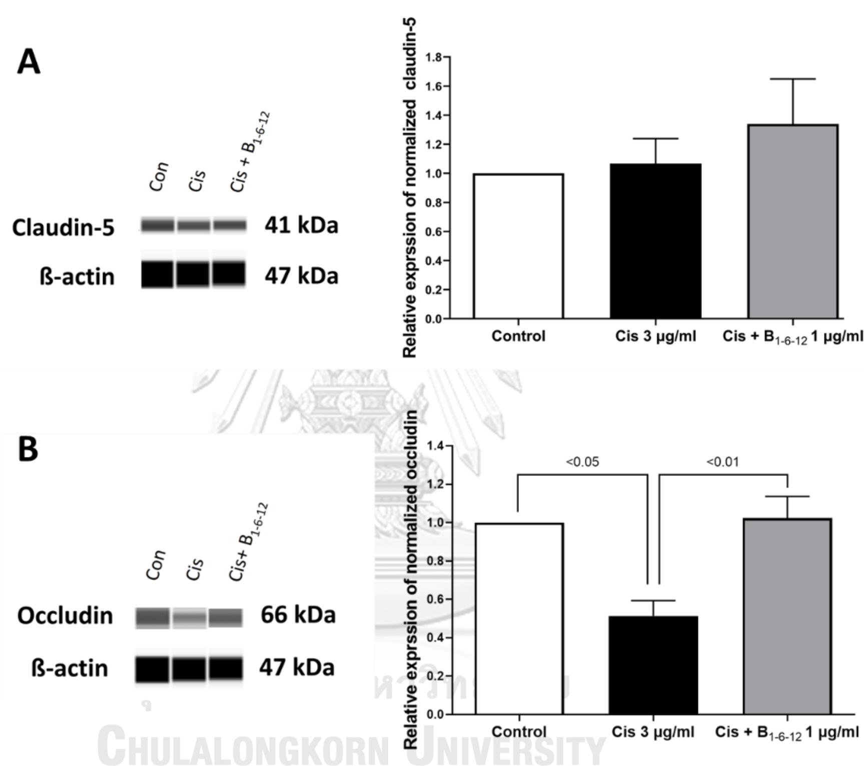



**Figure 57** Claudin-5 and occludin protein expression in HUVEC after treatments. (A) Claudin-5, (B) Occludin. The graphs show the average of relative protein expression normalized to beta-actin (fold over control) with SEM. Cis=cisplatin.

Expression of ZO-1 was slightly reduced in HUVEC treated with cisplatin compared with the the control group, whereas expression of ZO-2 was significantly decreased ($p$<0.01). $B_{1-6-12}$ treatment significantly improved the expression level of ZO-1 ($p$<0.05) and ZO-2 ($p$<0.01) as compared with the cisplatin-treated group (Figure 58).

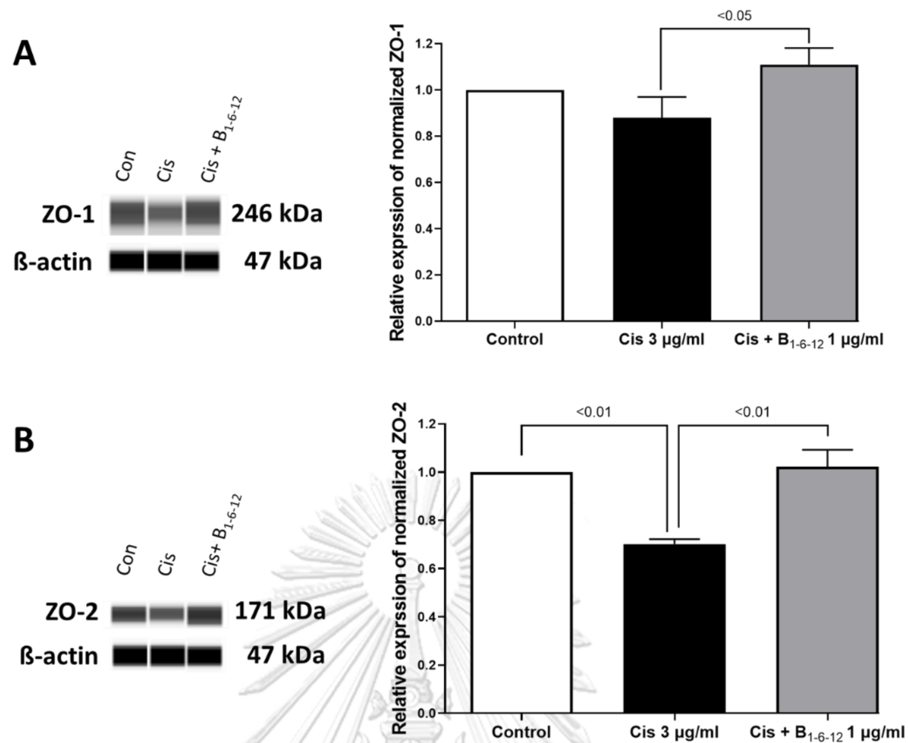



**Figure 58** ZO-1 and ZO-2 protein expression in HUVEC after treatments. (A) ZO-1, (B) ZO-2. The graphs show the average of relative protein expression normalized to beta-actin (fold over control) with SEM. Cis=cisplatin, Cur=curcumin.

5.2 Effects of co-treatment with oxaliplatin and $B_{1-6-12}$ on expression of tight junction proteins

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

Oxaliplatin did not significantly change the expression of both claudin-5 and occludin proteins. Co-treatment with $B_{1-6-12}$ also did not alter the expression of these proteins (Figure 59).

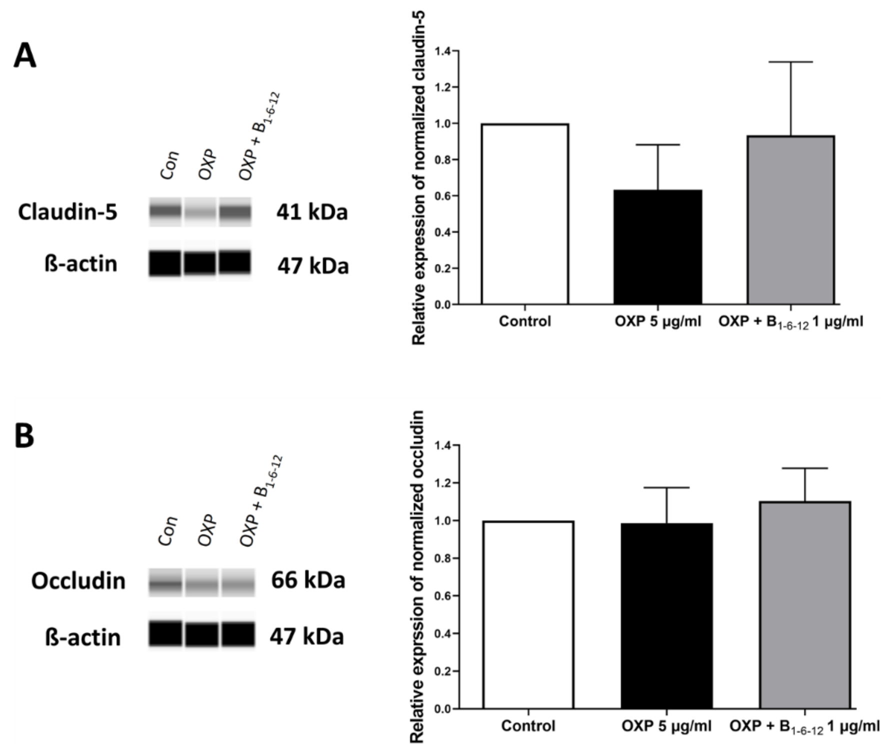



**Figure 59** Claudin-5 and occludin protein expression in HUVEC after treatments. (A) Claudin-5, (B) Occludin. The graphs show the average of relative protein expression normalized to beta-actin (fold over control) with SEM. OXP=oxaliplatin.

In addition, oxaliplatin did not significantly lower the expression of ZO-1 and ZO-2. Co-treatment with $B_{1\text{-}6\text{-}12}$ did not upregulate the expression of both proteins (Figure 60).

จุฬาลงกรณ์มหาวิทยาลัย
CHULALONGKORN UNIVERSITY

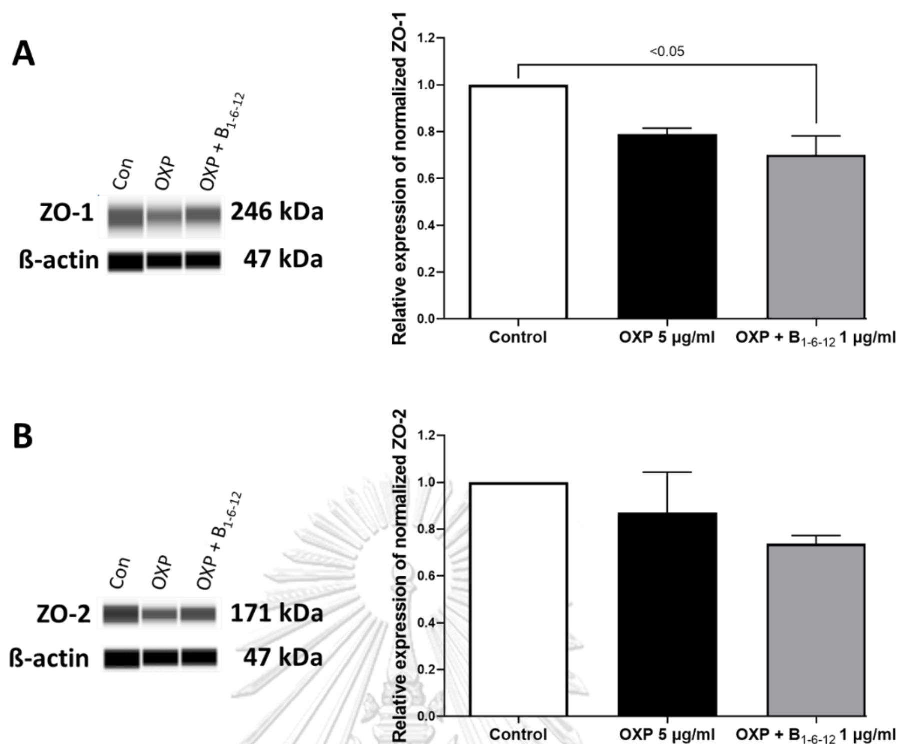



**Figure 60** ZO-1 and ZO-2 protein expression in HUVEC after treatments. (A) ZO-1, (B) ZO-2. The graphs show the average of relative protein expression normalized to beta-actin (fold over control) with SEM. OXP=oxaliplatin.

**6. TEER value**

6.1 Effects of co-treatment with cisplatin and $B_{1-6-12}$ on TEER value

Exposure to the cisplatin resulted in decreased TEER value in comparison with the control group (*p*<0.001). However, the TEER value was ameliorated by treatment with $B_{1-6-12}$ (*p*<0.001) (Figure 61).

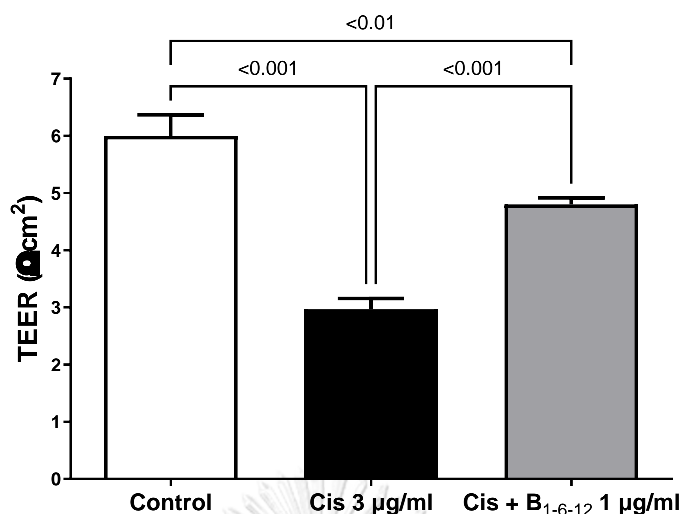



**Figure 61** TEER value of HUVEC after treatments. Data are mean ± SEM. Cis=cisplatin.

6.2 Effects of co-treatment with oxaliplatin and $B_{1-6-12}$ on TEER value

The 5 µg/ml of oxaliplatin caused the reduction of TEER value (*p*<0.001). Nonetheless, the TEER value of HUVEC did not significantly improve after incubation with $B_{1-6-12}$ (Figure 62).

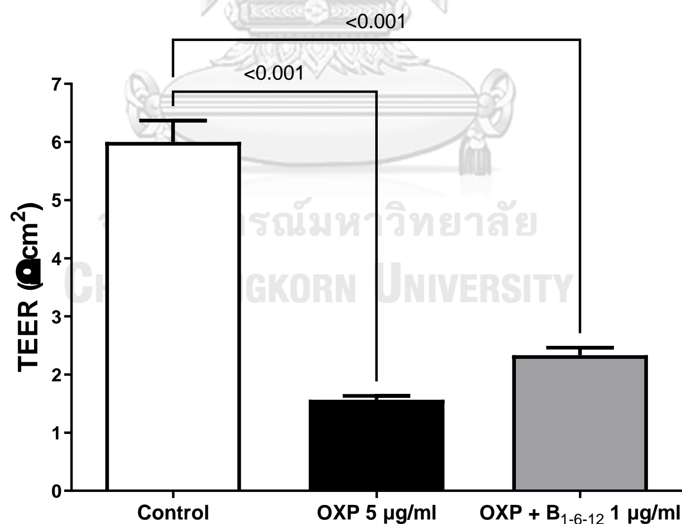



**Figure 62** TEER value of HUVEC after treatments. Data are mean ± SEM. OXP=oxaliplatin.

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

**Effects of $B_{1-6-12}$ on cisplatin- and oxaliplatin-induced alterations in HBVP**

**1. MTT assay**

1.1 Effects of co-treatment with cisplatin and $B_{1-6-12}$ on HBVP viability

The HBVP viability decreased after exposure to the 1.5 µg/ml of cisplatin for 24 hours ($p$<0.001). Nonetheless, the 1 µg/ml of $B_{1-6-12}$ ameliorated this event ($p$<0.001) (Figure 63).

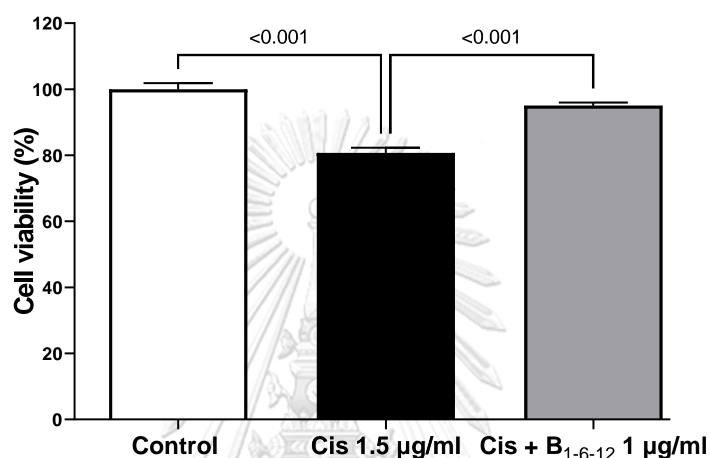



**Figure 63** Cell viability of HBVP after treatments. The graph shows the average percent of cell viability with SEM. Cis=cisplatin.

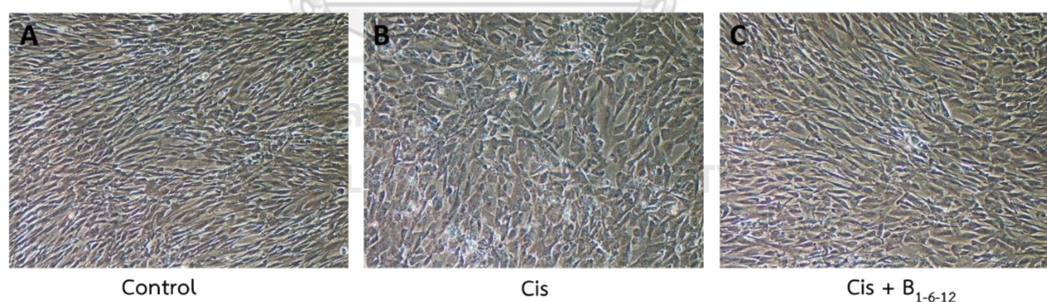



**Figure 64** Morphology of HBVP under phase contrast microscope (magnification: 10x). A. Control, B. Cisplatin (Cis), C. Co-treatment with cisplatin and $B_{1-6-12}$ (Cis + $B_{1-6-12}$).

1.2 Effects of co-treatment with oxaliplatin and $B_{1-6-12}$ on HBVP viability

The HBVP viability significantly diminished after treatment with 8 μg/ml of oxaliplatin ($p$<0.001). Co-treatment with $B_{1-6-12}$ significantly increased cell viability in comparison with oxaliplatin alone ($p$<0.05) (Figure 65).

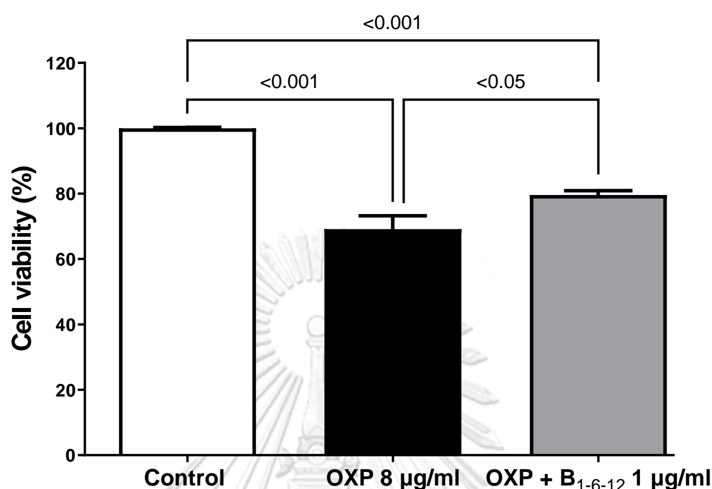



**Figure 65** Cell viability of HBVP after treatments. The graph shows the average percent of cell viability with SEM. OXP=oxaliplatin.

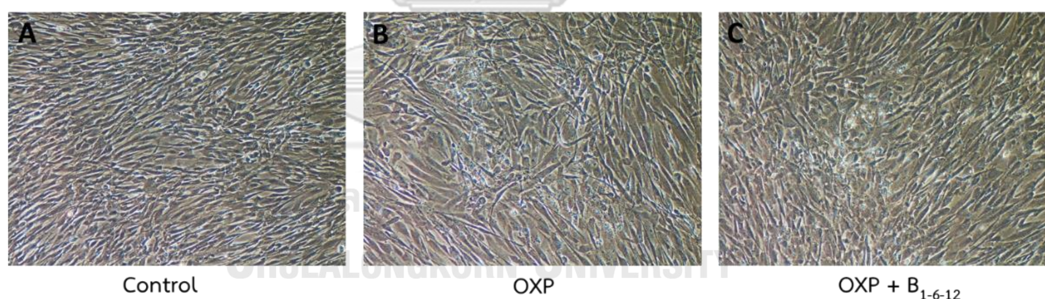



**Figure 66** Morphology of HBVP under phase contrast microscope (magnification: 10x). A. Control, B. Oxaliplatin (OXP), C. Co-treatment with oxaliplatin and $B_{1-6-12}$ (OXP+ $B_{1-6-12}$).

**2. Caspase-3 activity**

2.1 Effects of co-treatment with cisplatin and $B_{1-6-12}$ on caspase-3 activity

Caspase-3 activity of HBVP was significantly elevated after incubation with cisplatin in comparison with the control group ($p$<0.01). Nevertheless, the co-

incubation between cisplatin and $B_{1\text{-}6\text{-}12}$ caused the slight lowering of caspase-3 activity when compared with the cisplatin-treated group (Figure 67).

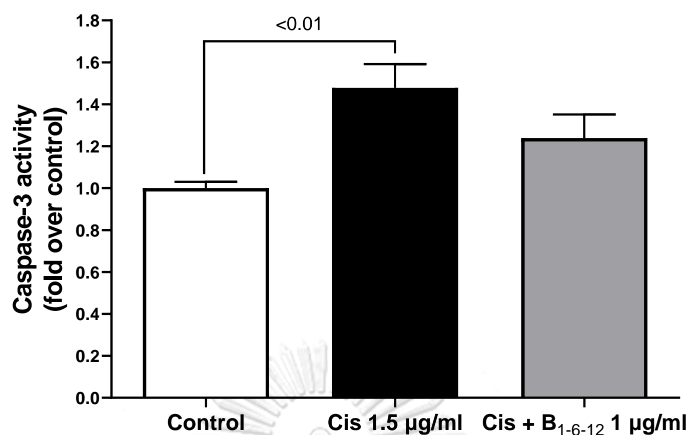



**Figure 67** Caspase-3 activity in HBVP after treatments. Data in graph are mean ± SEM. Cis=cisplatin.

2.2 Effects of co-treatment with oxaliplatin and $B_{1\text{-}6\text{-}12}$ on caspase-3 activity

Treatment with oxaliplatin caused the higher activation of caspase-3 than the control group ($p<0.05$). The co-treatment with $B_{1\text{-}6\text{-}12}$ did not reduce the caspase-3 activity (Figure 68).

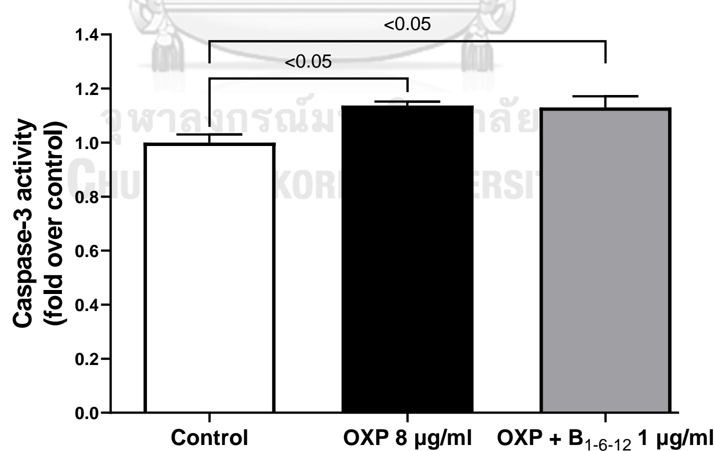



**Figure 68** Caspase-3 activity in HBVP after treatments. Data in graph are mean ± SEM. OXP=oxaliplatin.

**3. ROS assay**

3.1 Effects of co-treatment with cisplatin and $B_{1-6-12}$ on ROS production

The ROS production of HBVP significantly increased after exposure to cisplatin in comparison to the control group ($p<0.01$). The co-treatment with $B_{1-6-12}$ did not significantly diminish the ROS elevation caused by cisplatin (Figure 69).

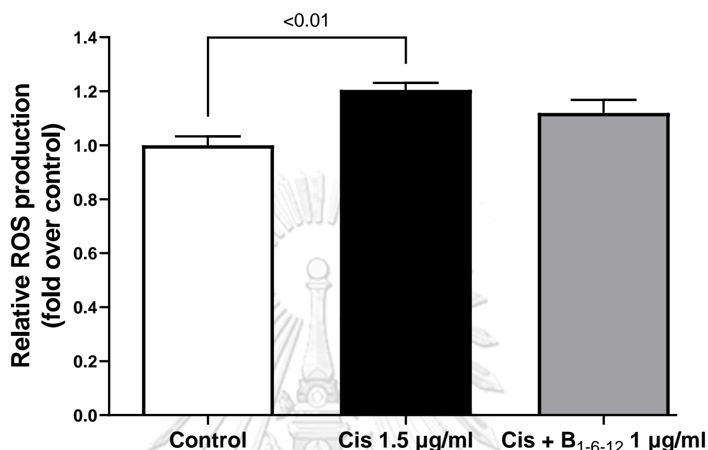



**Figure 69** ROS production in HBVP after treatments. Average of relative ROS production (fold over control) and SEM are shown. Cis=cisplatin.

3.2 Effects of co-treatment with oxaliplatin and $B_{1-6-12}$ on ROS production

Oxaliplatin also induced an increase in the ROS level ($p<0.001$). However, $B_{1-6-12}$ did not significantly reduce the oxaliplatin-induced ROS production (Figure 70).

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

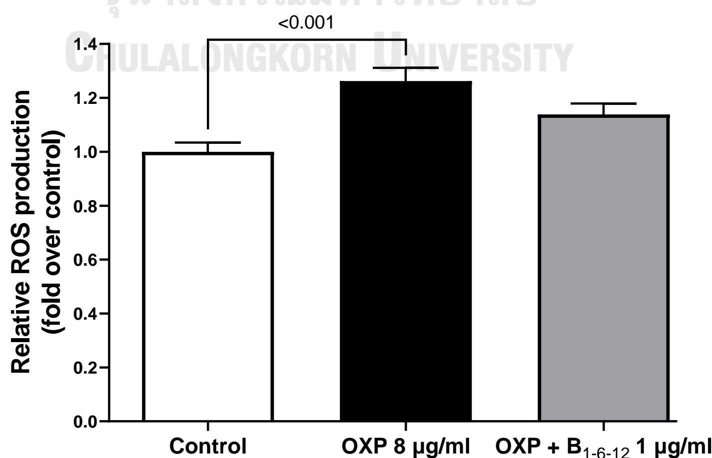



**Figure 70** ROS production in HBVP after treatments. Average of relative ROS production (fold over control) and SEM are shown. OXP=oxaliplatin.

**4. Total glutathione level and GSH/GSSG ratio**

4.1 Effects of co-treatment with cisplatin and $B_{1-6-12}$ on total glutathione level and GSH/GSSG ratio

Cisplatin induced the depletion of total glutathione level in comparison to the control group ($p$<0.001) and caused the reduction of GSH/GSSG ratio ($p$<0.001). Nevertheless, co-treatment with $B_{1-6-12}$ did not significantly alter these parameters (Figure 71).

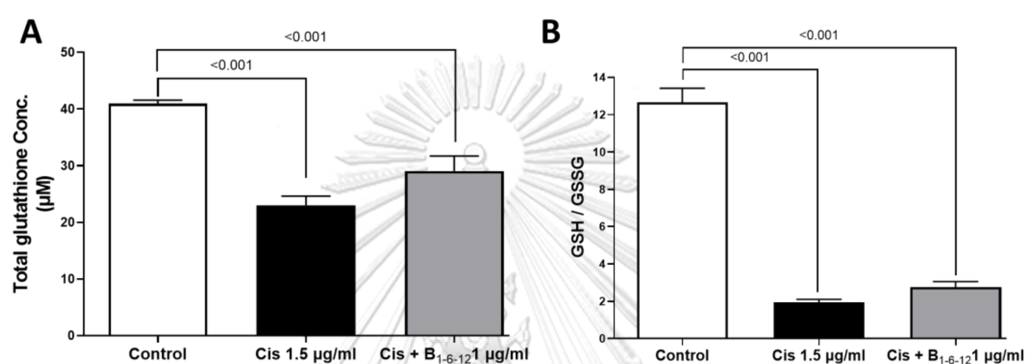



**Figure 71** Total glutathione level and GSH/GSSG ratio in HBVP after treatments. (A) total glutathione concentration, (B) the ratio of reduced glutathione (GSH) to oxidized glutathione (GSSG). Graphs represent Mean ± SEM. Cis=cisplatin.

4.2 Effects of co-treatment with oxaliplatin and $B_{1-6-12}$ on total glutathione level and GSH/GSSG ratio

จุฬาลงกรณ์มหาวิทยาลัย
CHULALONGKORN UNIVERSITY

Exposure to oxaliplatin also produced the lowering of total glutathione level ($p$<0.001) and the GSH/GSSG ratio ($p$<0.001) when compared with the control group. The co-incubation with $B_{1-6-12}$ did not ameliorate these changes (Figure 72).

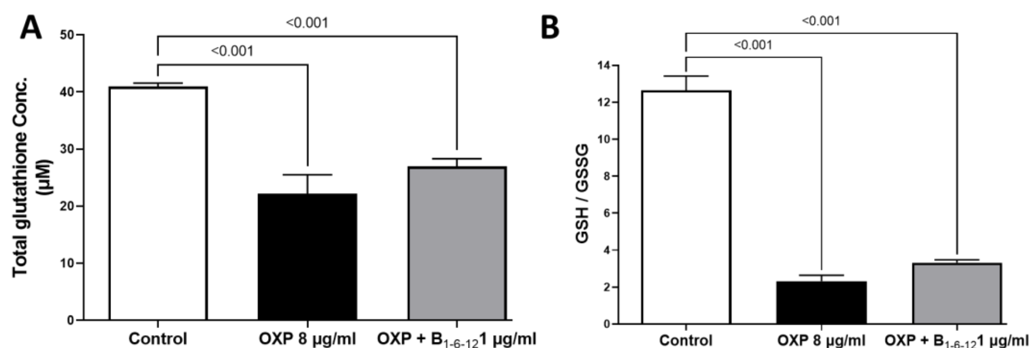



**Figure 72** Total glutathione level and GSH/GSSG ratio in HBVP after treatments. (A) total glutathione concentration, (B) the ratio of reduced glutathione (GSH) to oxidized glutathione (GSSG). Graphs represent Mean ± SEM. OXP=oxaliplatin.

**5. TEER value**

5.1 Effects of co-treatment with cisplatin and $B_{1-6-12}$ on TEER value

The TEER value of HBVP decreased after treatment with cisplatin ($p$<0.001). After incubation with $B_{1-6-12}$, the TEER value significantly increased compared with the only cisplatin group ($p$<0.001) (Figure 73).

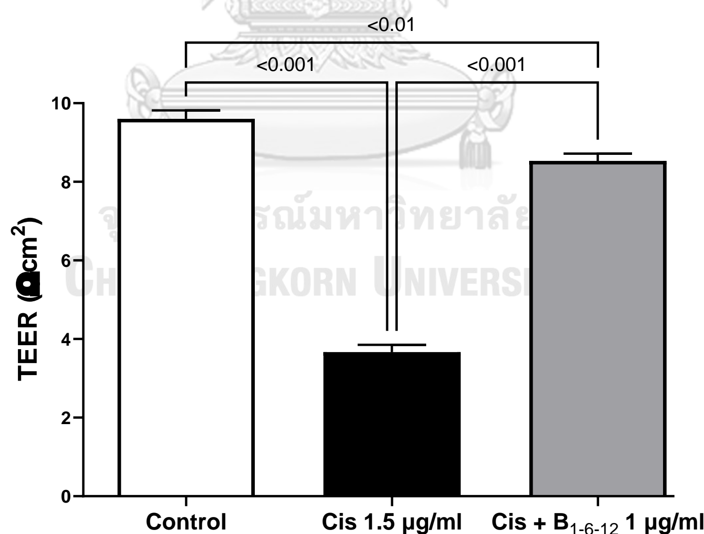



**Figure 73** TEER value of HBVP after treatments. Data are mean ± SEM. Cis=cisplatin.

5.2 Effects of co-treatment with oxaliplatin and $B_{1-6-12}$ on TEER value

The TEER value of HBVP diminished after incubated with oxaliplatin ($p$<0.001). Nonetheless, $B_{1-6-12}$ slightly increased the TEER value (Figure 74).

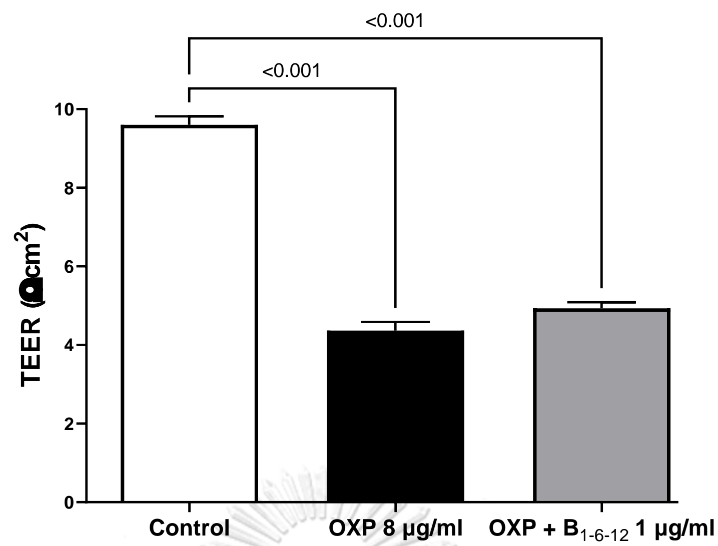



**Figure 74** TEER value of HBVP after treatments. Data are mean ± SEM. OXP=oxaliplatin.

จุฬาลงกรณ์มหาวิทยาลัย
CHULALONGKORN UNIVERSITY

**Effects of combination between curcumin and $B_{1\text{-}6\text{-}12}$ on cisplatin- and oxaliplatin-induced alterations in HUVEC**

**1. MTT assay**

1.1 Effects of co-treatment with cisplatin, curcumin, and $B_{1\text{-}6\text{-}12}$ on HUVEC viability

Cisplatin 3 µg/ml significantly reduced the viability of HUVEC ($p<0.001$). The treatment of 1 µg/ml curcumin and 1 µg/ml $B_{1\text{-}6\text{-}12}$ with cisplatin significantly increased cell viability compared with the cisplatin group ($p<0.001$). Moreover, combined treatment of curcumin and $B_{1\text{-}6\text{-}12}$ significantly improved HUVEC viability more than curcumin ($p<0.001$) and $B_{1\text{-}6\text{-}12}$ ($p<0.05$) treatment alone (Figure 75).

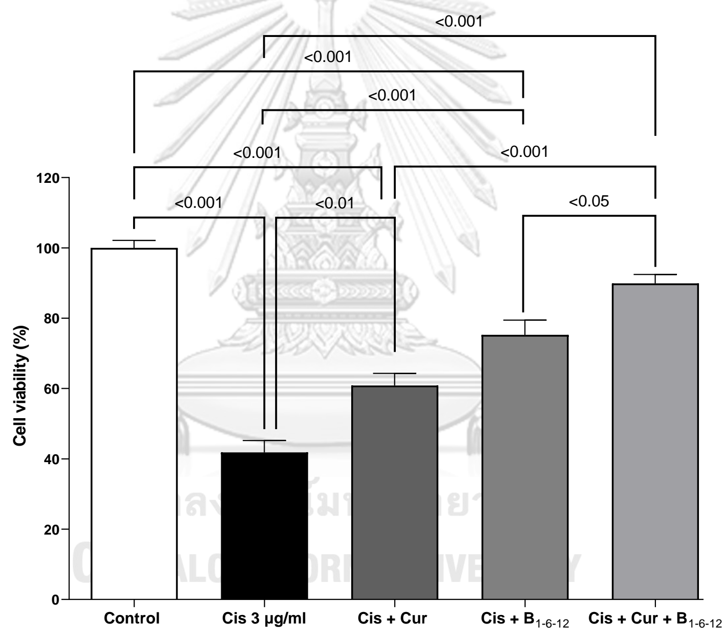



**Figure 75** Cell viability of HUVEC after treatments. The graph shows the average percent of cell viability with SEM. Cis=cisplatin, Cur=curcumin.

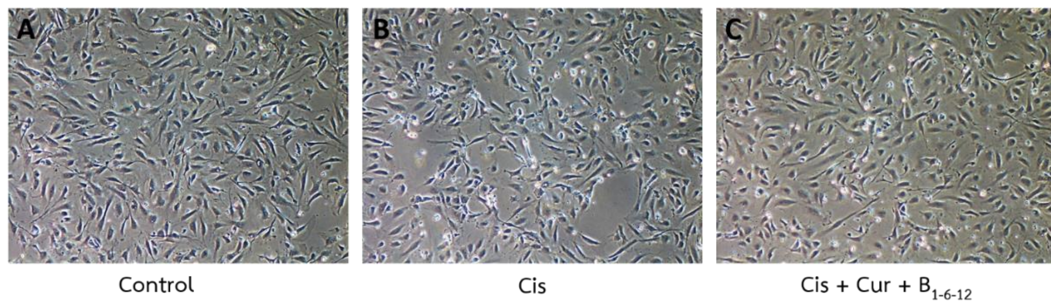



**Figure 76** Morphology of HUVEC under phase contrast microscope (magnification: 10x). A. Control, B. Cisplatin (Cis), C. Co-treatment with cisplatin, curcumin, and $B_{1\text{-}6\text{-}12}$ (Cis + Cur + $B_{1\text{-}6\text{-}12}$).

1.2 Effects of co-treatment with oxaliplatin, curcumin, and $B_{1\text{-}6\text{-}12}$ on HUVEC viability

After HUVEC were incubated with oxaliplatin for 24 hours, a significant reduction of cell viability occurred ($p<0.001$). This effect of oxaliplatin could be significantly improved by co-treatment with 1 µg/ml curcumin and 1 µg/ml $B_{1\text{-}6\text{-}12}$ ($p<0.001$). This co-treatment significantly enhanced the viability more than $B_{1\text{-}6\text{-}12}$ treatment alone ($p<0.05$) but not significantly more than curcumin treatment alone (Figure 77).

จุฬาลงกรณ์มหาวิทยาลัย
CHULALONGKORN UNIVERSITY

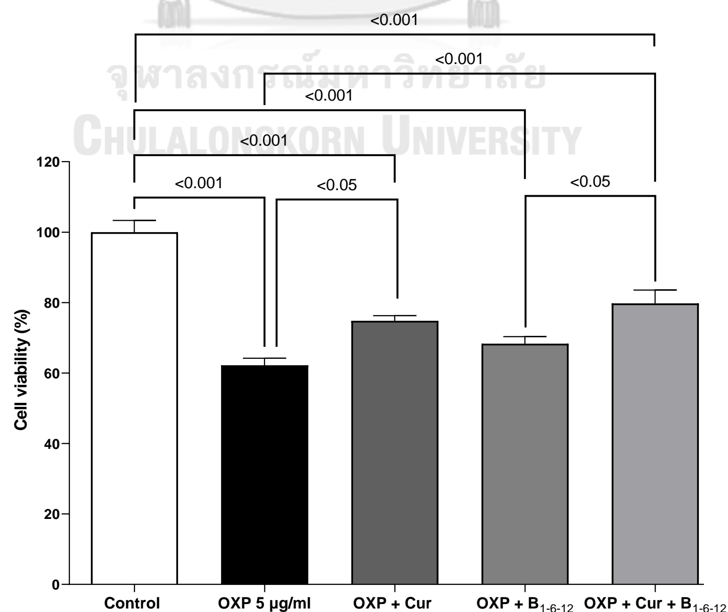


**Figure 77** Cell viability of co-treatment with oxaliplatin, curcumin, and $B_{1-6-12}$ on HUVEC. The graph shows the average percent of cell viability with SEM. OXP=oxaliplatin, Cur=curcumin.

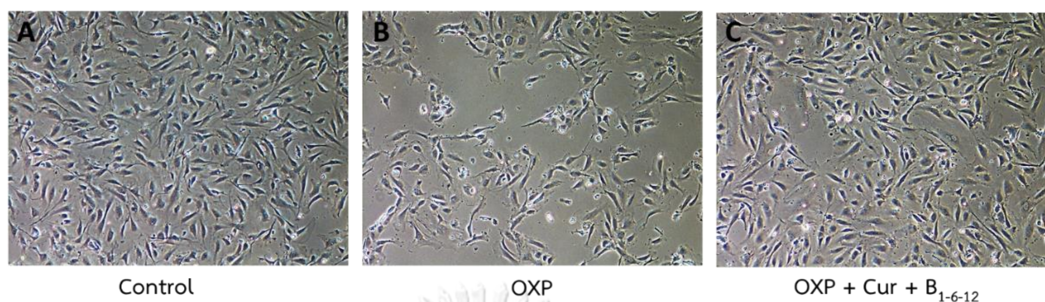



**Figure 78** Morphology of HUVEC under phase contrast microscope (magnification: 10x). A. Control, B. Oxaliplatin (OXP), C. Co-treatment with oxaliplatin, curcumin, and $B_{1-6-12}$ (OXP + Cur + $B_{1-6-12}$).

## 2. Caspase-3 activity

2.1 Effects of co-treatment with cisplatin, curcumin, and $B_{1-6-12}$ on caspase-3 activity

Cisplatin treatment activated the caspase-3 level in HUVEC ($p<0.05$). The co-treatment with curcumin and $B_{1-6-12}$ did not significantly inhibit the caspase-3 caused by cisplatin, similar to curcumin and $B_{1-6-12}$ alone (Figure 79).

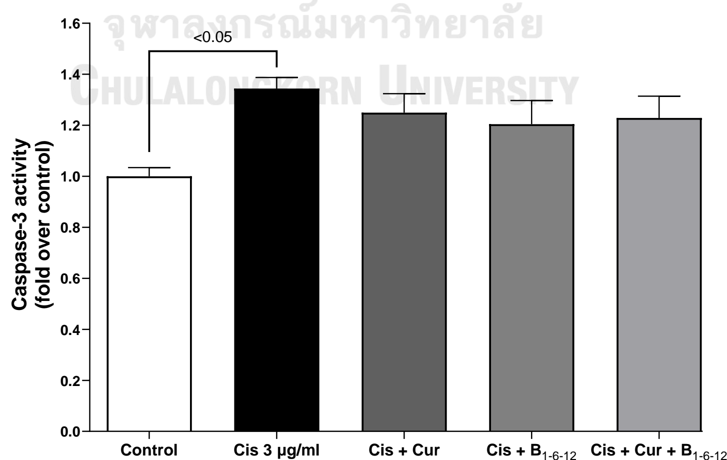



**Figure 79** Caspase-3 activity in HUVEC after treatments. Data in graph are mean ± SEM. Cis=cisplatin, Cur=curcumin.

2.2 Effects of co-treatment with oxaliplatin, curcumin, and $B_{1-6-12}$ on caspase-3 activity

Oxaliplatin also significantly activated the caspase-3 level compared with the control group ($p$<0.001). Co-treatment with curcumin and $B_{1-6-12}$ significantly decreased the caspase-3 level in comparison with the oxaliplatin alone ($p$<0.01). This reduction was lower than curcumin or $B_{1-6-12}$ treatment alone (Figure 80).

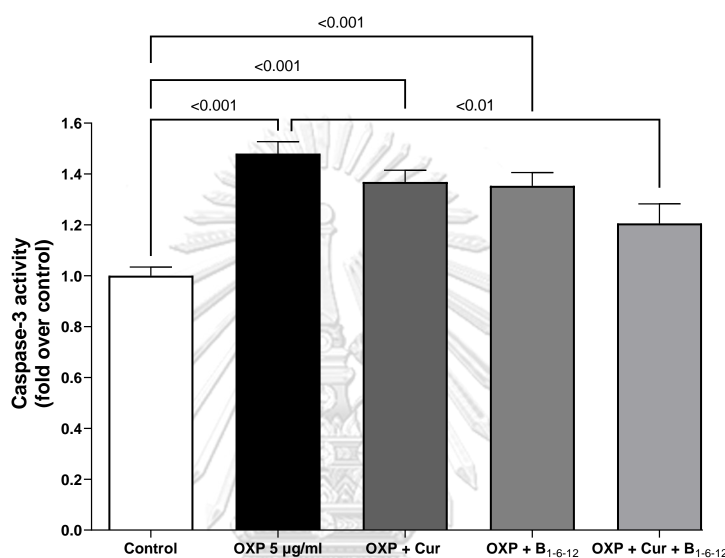



**Figure 80** Caspase-3 activity in HUVEC after treatments. Data in graph are mean ± SEM. OXP=oxaliplatin, Cur=curcumin.

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

**3. ROS assay**

3.1 Effects of co-treatment with cisplatin, curcumin, and $B_{1-6-12}$ on ROS production

When HUVEC were exposed to cisplatin, the ROS level increased in comparison with the control ($p$<0.01). The combined treatment with curcumin and $B_{1-6-12}$ decreased the ROS level ($p$<0.05) comparable to that of curcumin alone (Figure 81).

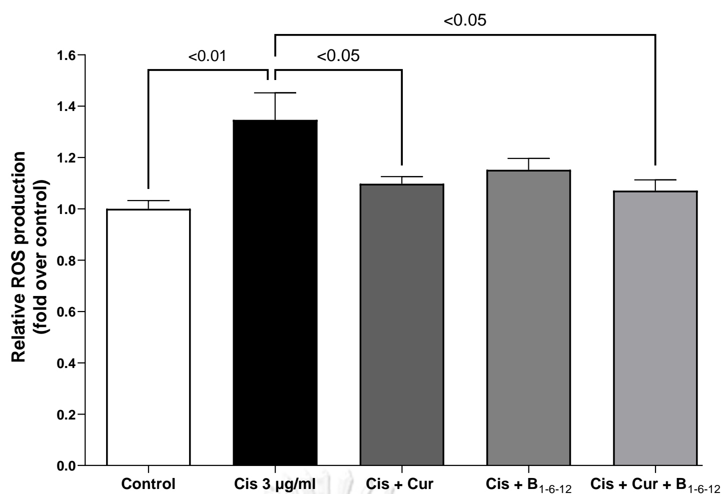



**Figure 81** ROS production in HUVEC after treatments. Average of relative ROS production (fold over control) and SEM are shown. Cis=cisplatin, Cur=curcumin.

3.2 Effects of co-treatment with cisplatin, curcumin, and $B_{1-6-12}$ on ROS production

With oxaliplatin treatment, there was a statistically significant elevation in ROS production ($p$<0.05). Moreover, the combined treatment with curcumin and $B_{1-6-12}$ also minimized the oxaliplatin-induced ROS augmentation ($p$<0.01). This combined treatment reduced the ROS level comparable to the treatment with curcumin alone (Figure 82).

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

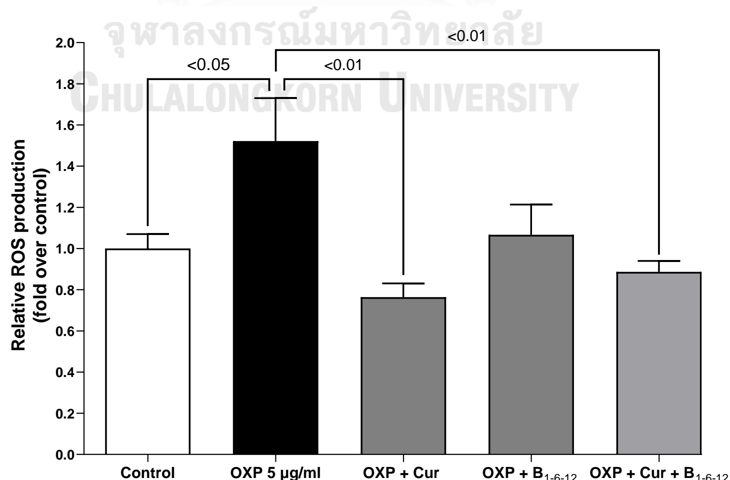



**Figure 82** ROS production in HUVEC after treatments. Average of relative ROS production (fold over control) and SEM are shown. OXP=oxaliplatin, Cur=curcumin.

**4. Total glutathione level and GSH/GSSG ratio**

4.1 Effects of co-treatment with cisplatin, curcumin, and $B_{1-6-12}$ on total glutathione level and GSH/GSSG ratio

Significant reduction of total glutathione concentration ($p$<0.001) and GSH/GSSG ratio ($p$<0.001) were observed after exposure to cisplatin. However, co-treatment with curcumin and $B_{1-6-12}$ slightly improve both reduction in total glutathione and GSH/GSSG level compared with cisplatin alone, similar to $B_{1-6-12}$ alone. These results were less favorable than curcumin treatment alone (Figure 83).

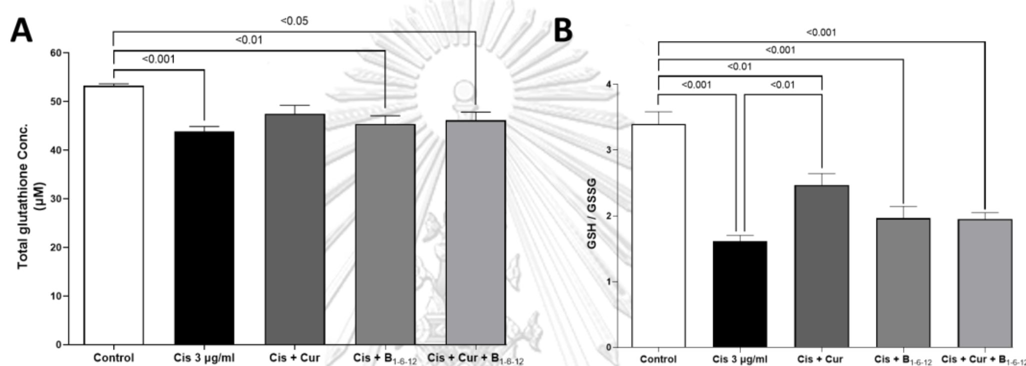



**Figure 83** Total glutathione level and GSH/GSSG ratio in HUVEC after treatments. (A) total glutathione concentration, (B) the ratio of reduced glutathione (GSH) to oxidized glutathione (GSSG). Graphs represent Mean ± SEM. Cis=cisplatin, Cur=curcumin.

4.2 Effects of co-treatment with oxaliplatin, curcumin, and $B_{1-6-12}$ on total glutathione level and GSH/GSSG ratio

CHULALONGKORN UNIVERSITY

Total glutathione level ($p$<0.001) and GSH/GSSG ratio ($p$<0.001) significantly lowered after incubation with oxaliplatin. Co-treatment with curcumin and $B_{1-6-12}$ enhanced only the GSH/GSSG ratio ($p$<0.01). This combined treatment enhanced the GSSH/GSSG ratio more than $B_{1-6-12}$ treatment alone ($p$<0.05) but was non-different from curcumin treatment alone (Figure 84).

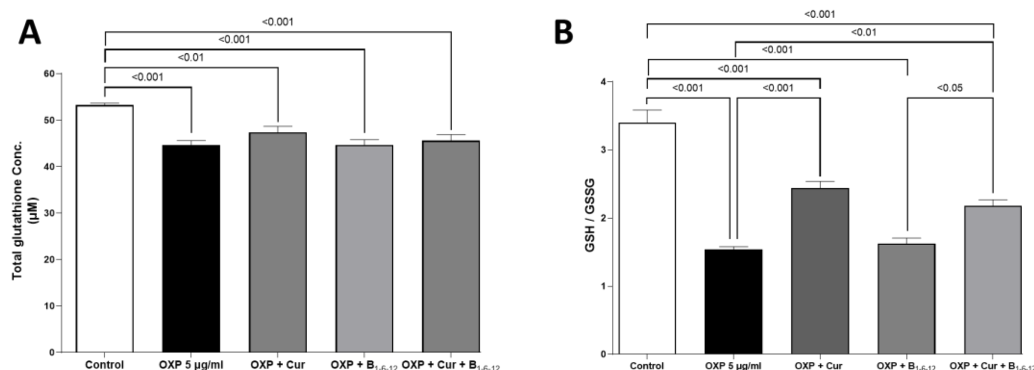



**Figure 84** Total glutathione level and GSH/GSSG ratio in HUVEC after treatments. (A) total glutathione concentration, (B) the ratio of reduced glutathione (GSH) to oxidized glutathione (GSSG). Graphs represent Mean ± SEM. OXP=oxaliplatin, Cur=curcumin.

**5. Expression of endothelial tight junction proteins**

5.1 Effects of co-treatment with cisplatin, curcumin, and $B_{1-6-12}$ on expression of tight junction proteins

The expression of claudin-5 did not alter after incubation with cisplatin. Co-treatment with curcumin and $B_{1-6-12}$ slightly increased an expression of this protein similar to curcumin or $B_{1-6-12}$ treatment alone (Figure 85).

The occludin protein level tended to decrease after treatment with cisplatin. The expression was modestly up-regulated after co-treatment with curcumin. The expression was higher after the treatments with combined curcumin and $B_{1-6-12}$ or $B_{1-6-12}$ alone (Figure 85). However, there were no significant differences between any groups.

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

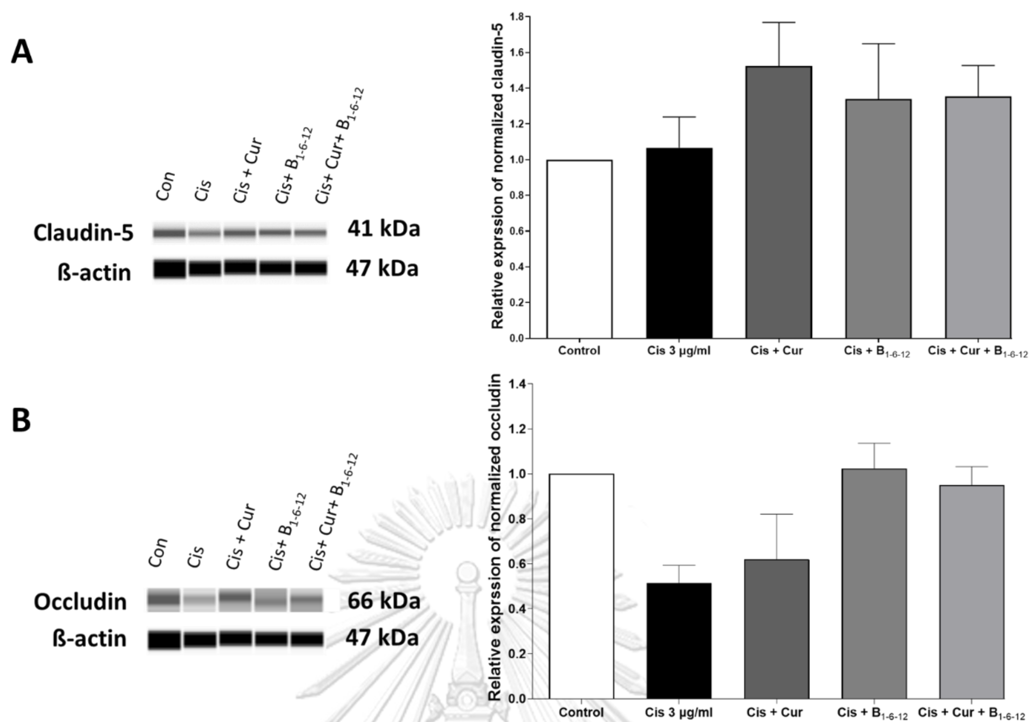



**Figure 85** Claudin-5 and occludin protein expression in HUVEC after treatments. (A) Claudin-5, (B) Occludin. The graphs show the average of relative protein expression normalized to beta-actin (fold over control) with SEM. Cis=cisplatin, Cur=curcumin.

Treatment with cisplatin did not significantly change the expression of ZO-1 and ZO-2. Co-treatments with either combined curcumin and $B_{1\text{-}6\text{-}12}$ or each drug alone did not significantly alter the expression compared with cisplatin alone (Figure 86).

Chulalongkorn University

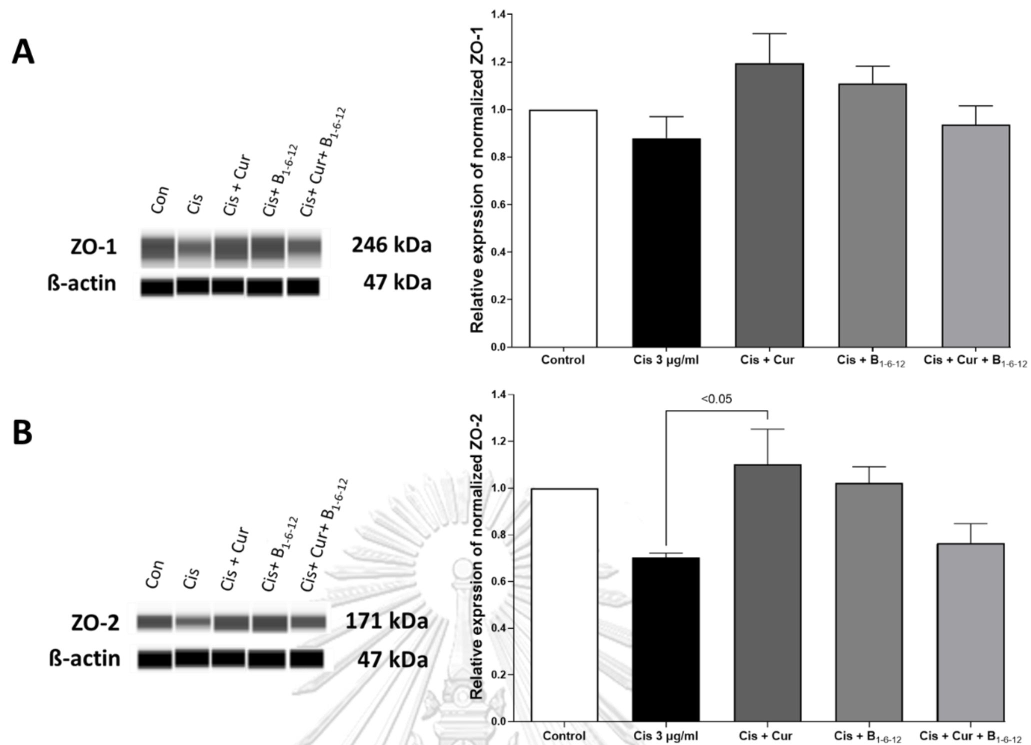



**Figure 86** ZO-1 and ZO-2 protein expression in HUVEC after treatments. (A) ZO-1, (B) ZO-2. The graphs show the average of relative protein expression normalized to beta-actin (fold over control) with SEM. Cis=cisplatin, Cur=curcumin

5.2 Effects of co-treatment with oxaliplatin, curcumin, and $B_{1-6-12}$ on expression of tight junction proteins

จุฬาลงกรณ์มหาวิทยาลัย
CHULALONGKORN UNIVERSITY

Oxaliplatin did not significantly change the expression of claudin-5 and occludin proteins. In addition, co-treatment with curcumin and $B_{1-6-12}$ did not significantly enhance both proteins similar to curcumin or $B_{1-6-12}$ treatment alone (Figure 87).

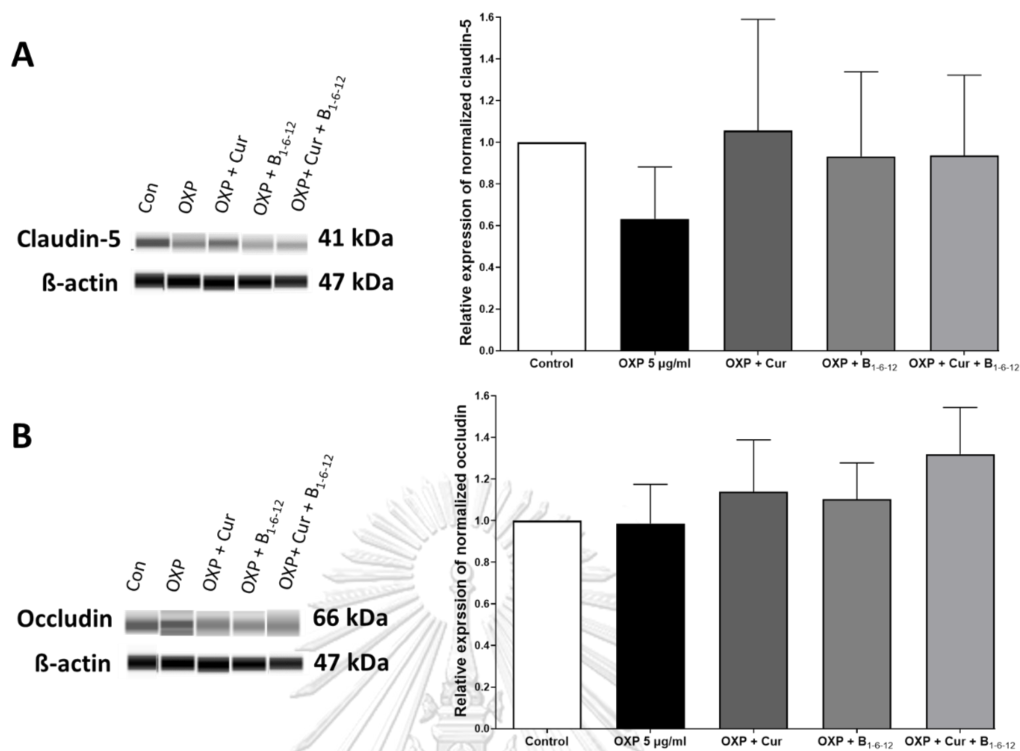



**Figure 87** Claudin-5 and occludin protein expression in HUVEC after treatments. (A) Claudin-5, (B) Occludin. The graphs show the average of relative protein expression normalized to beta-actin (fold over control) with SEM. OXP=oxaliplatin, Cur=curcumin.

The expression of ZO-1 and ZO-2 did not significantly alter after treatment with oxaliplatin. Treatment with curcumin and $B_{1-6-12}$ did not improve the expression of these proteins alike curcumin or $B_{1-6-12}$ treatment alone (Figure 88).

จุฬาลงกรณ์มหาวิทยาลัย
CHULALONGKORN UNIVERSITY

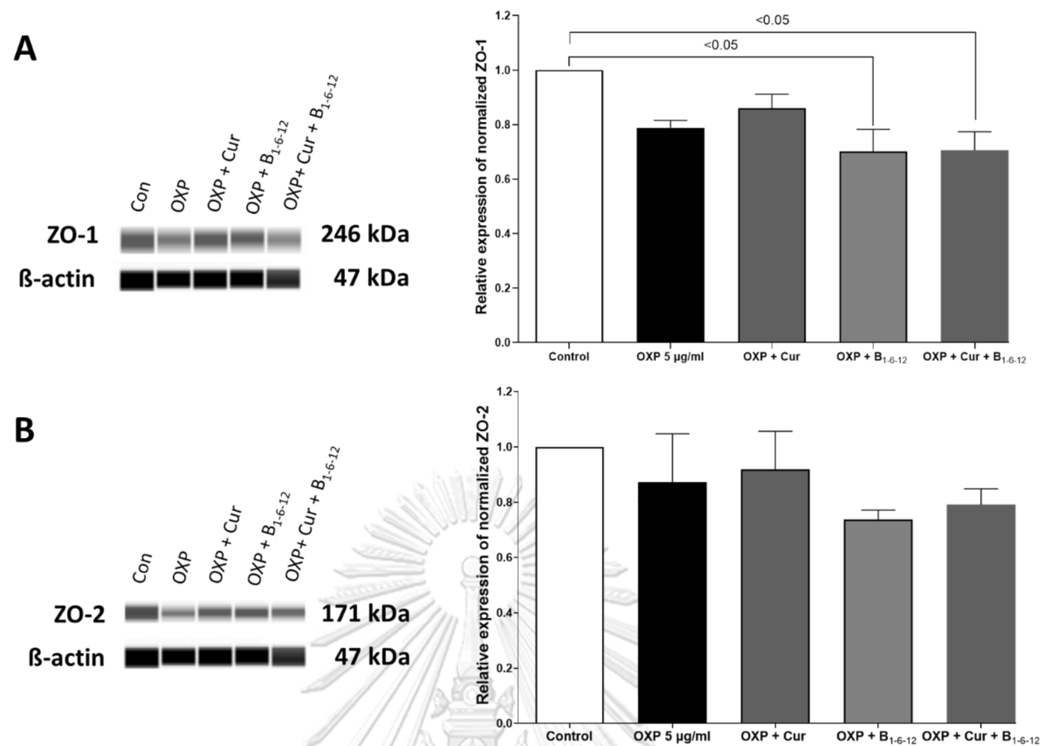



**Figure 88** ZO-1 and ZO-2 protein expression in HUVEC after treatments. (A) ZO-1, (B) ZO-2. The graphs show the average of relative protein expression normalized to beta-actin (fold over control) with SEM. OXP=oxaliplatin, Cur=curcumin, # $p<0.05$ vs. control group.

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

**6. TEER value**

6.1 Effects of co-treatment with cisplatin, curcumin, and $B_{1-6-12}$ on TEER value

The treatment with cisplatin decreased the TEER value ($p<0.001$). This effect was partially blocked by the co-treatment with curcumin and $B_{1-6-12}$ ($p<0.01$) (Figure 89) which was comparable to the treatment with only curcumin or $B_{1-6-12}$ alone.

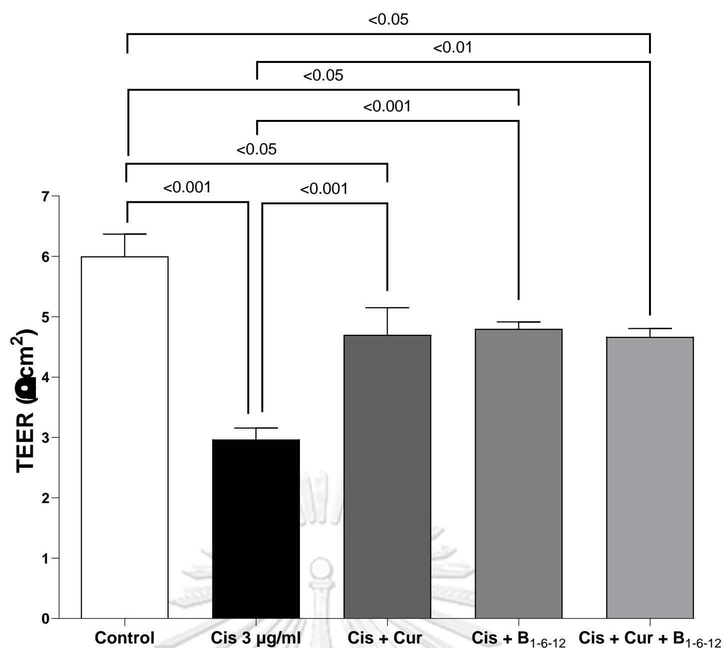



**Figure 89** TEER value of HUVEC after treatments. Data are mean ± SEM. Cis=cisplatin, Cur=curcumin.

6.2 Effects of co-treatment with oxaliplatin, curcumin, and $B_{1-6-12}$ on TEER value

The TEER value was significantly reduced after incubation with oxaliplatin ($p$<0.001). This was improved by incubation with curcumin and $B_{1-6-12}$ ($p$<0.001) or curcumin alone. the TEER value of $B_{1-6-12}$ treatment alone was slightly higher than the cisplatin group (Figure 90).

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

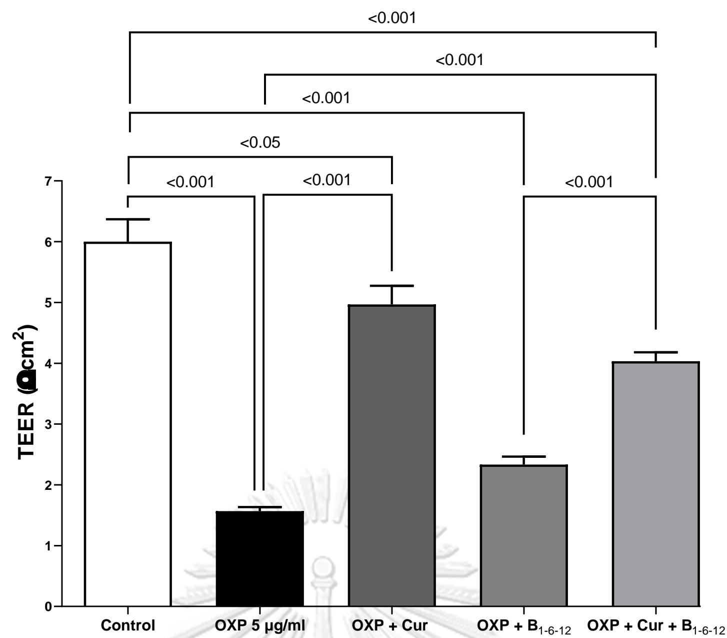



**Figure 90** TEER value of HUVEC after treatments. Data are mean ± SEM. OXP=oxaliplatin, Cur=curcumin.

จุฬาลงกรณ์มหาวิทยาลัย
CHULALONGKORN UNIVERSITY

**Effects of combination between curcumin and $B_{1\text{-}6\text{-}12}$ on cisplatin- and oxaliplatin-induced alterations in HBVP**

**1. MTT assay**

1.1 Effects of co-treatment with cisplatin, curcumin, and $B_{1\text{-}6\text{-}12}$ on HBVP viability

The cell viability significantly decreased in cisplatin-treated group compared with the control group ($p$<0.001). However, the co-treatment with 1 µg/ml curcumin and 1 µg/ml $B_{1\text{-}6\text{-}12}$ significantly increased against cisplatin-induced reduction of cell viability ($p$<0.001) which was similar to either curcumin or $B_{1\text{-}6\text{-}12}$ alone (Figure 91).

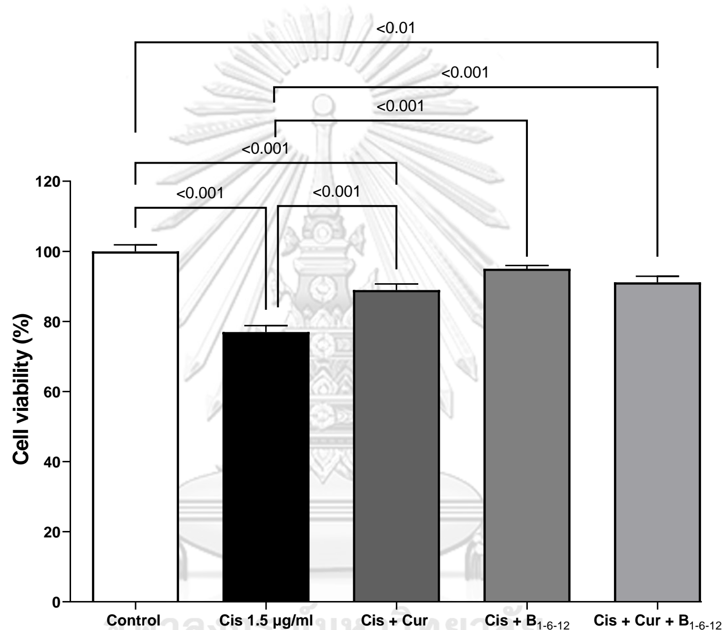



**Figure 91** Cell viability of HBVP after treatments. The graph shows the average percent of cell viability with SEM. Cis=cisplatin, Cur=curcumin.

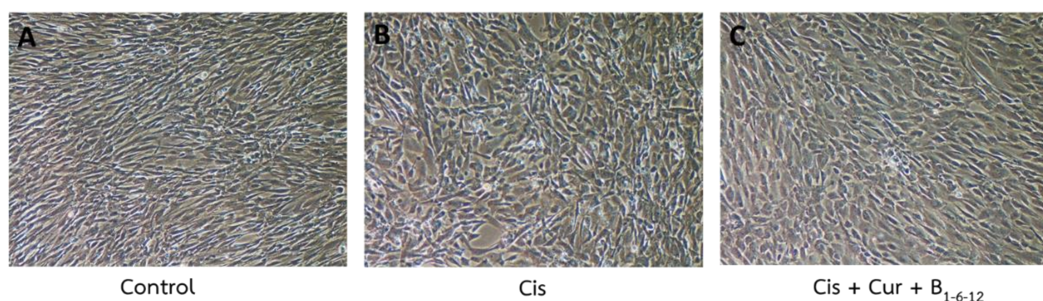



**Figure 92** Morphology of HBVP under phase contrast microscope (magnification: 10x). A. Control, B. Cisplatin (Cis), C. Co-treatment with cisplatin, curcumin, and $B_{1\text{-}6\text{-}12}$ (Cis + Cur + $B_{1\text{-}6\text{-}12}$).

จุฬาลงกรณ์มหาวิทยาลัย
CHULALONGKORN UNIVERSITY

1.2 Effects of co-treatment with oxaliplatin, curcumin, and $B_{1-6-12}$ on HBVP viability

Oxaliplatin also reduced the HBVP viability when compared with the control group (*p*<0.001). The combination treatment between curcumin and $B_{1-6-12}$ enhanced the reduced viability caused by oxaliplatin (*p*<0.01). The enhancement from this combined treatment did not differ from curcumin treatment alone (Figure 93). The viability of B1-6-12 alone was similar to that of oxaliplatin only.

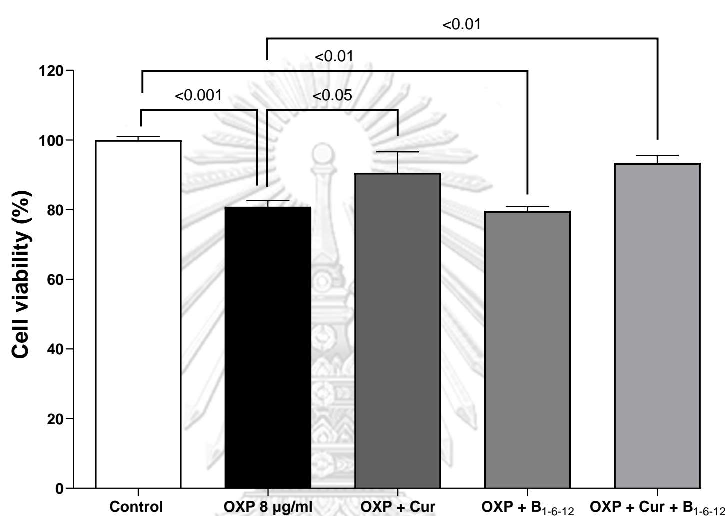



**Figure 93** Cell viability of HBVP after treatments. The graph shows the average percent of cell viability with SEM. OXP=oxaliplatin, Cur=curcumin.

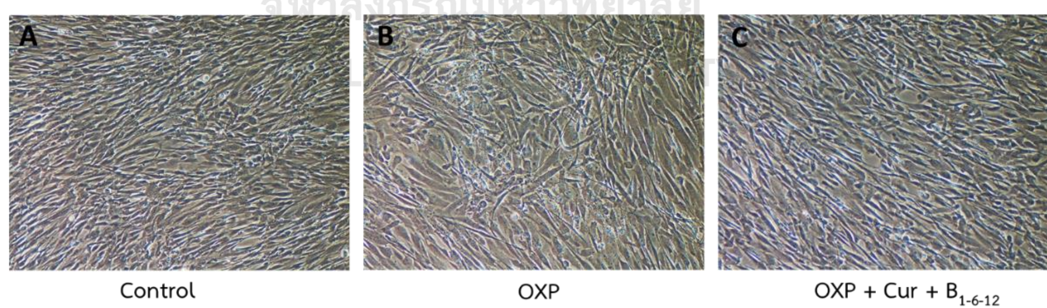



**Figure 94** Morphology of HBVP under phase contrast microscope (magnification: 10x). A. Control, B. Oxaliplatin (OXP), C. Co-treatment with oxaliplatin, curcumin, and $B_{1-6-12}$ (OXP + Cur + $B_{1-6-12}$).

**2. Caspase-3 activity**

2.1 Effects of co-treatment with cisplatin, curcumin, and $B_{1-6-12}$ on caspase-3 activity

When treated HBVP with cisplatin, the caspase-3 level elevated in comparison to the control group ($p$<0.05). Co-treatment with curcumin and $B_{1-6-12}$ significantly decreased the caspase-3 activity ($p$<0.05). The reduction observed in curcumin treatment alone did not differ from that of $B_{1-6-12}$ alone (Figure 95).

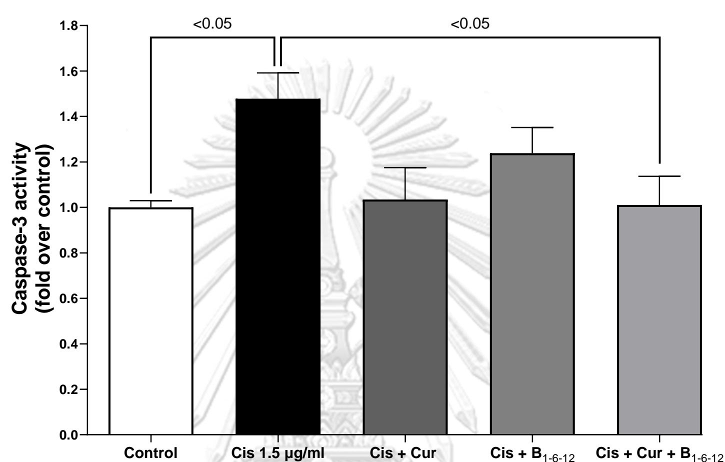



**Figure 95** Caspase-3 activity in HBVP after treatments. Data in graph are mean ± SEM. Cis=cisplatin, Cur=curcumin.

2.2 Effects of co-treatment with oxaliplatin, curcumin, and $B_{1-6-12}$ on caspase-3 activity

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

The caspase-3 activity significantly increased after incubation with oxaliplatin ($p$<0.05). Moreover, co-treatment with curcumin and $B_{1-6-12}$ did not significantly decrease the caspase-3 level compared with the oxaliplatin group similar to curcumin or $B_{1-6-12}$ alone (Figure 96).

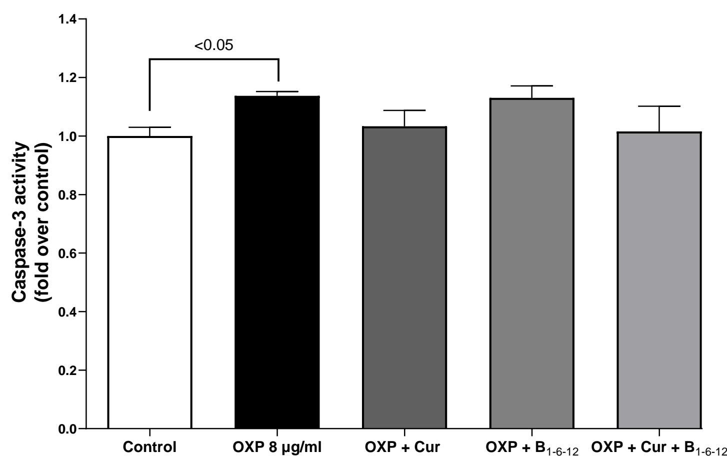



**Figure 96** Caspase-3 activity in HBVP after treatments. Data in graph are mean ± SEM. OXP=oxaliplatin, Cur=curcumin.

**3. ROS assay**

3.1 Effects of co-treatment with cisplatin, curcumin, and $B_{1-6-12}$ on ROS production

Cisplatin induced a higher production of ROS than the control group ($p$< 0.01). However, this augmentation was significantly ameliorated by the co-treatment with curcumin and $B_{1-6-12}$ ($p$<0.001) comparable to the treatment with curcumin alone. This combined treatment suppressed the ROS level more than the $B_{1-6-12}$ treatment alone ($p$<0.001) (Figure 97).

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

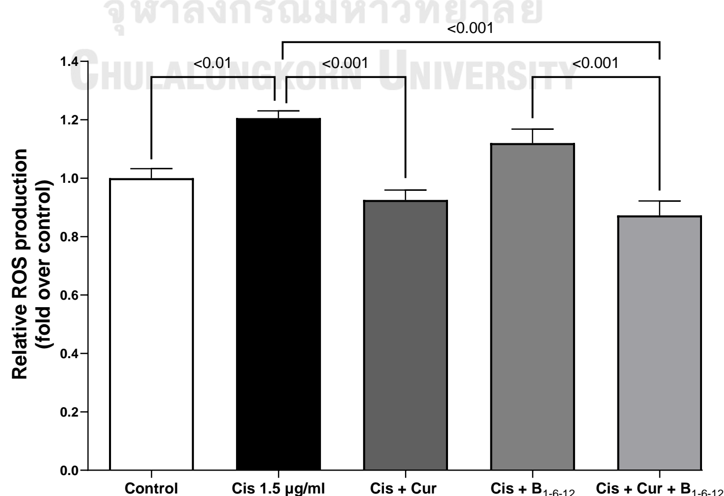



**Figure 97** ROS production in HBVP after treatments. Average of relative ROS production (fold over control) and SEM are shown. Cis=cisplatin, Cur=curcumin.

3.2 Effects of co-treatment with oxaliplatin, curcumin, and $B_{1-6-12}$ on ROS production

HBVP treated with oxaliplatin for 24 hours had a significant rising in ROS level ($p$<0.01). Combination treatment with curcumin and $B_{1-6-12}$ reduced the ROS level ($p$<0.001) comparable to the treatment with curcumin alone (Figure 98).

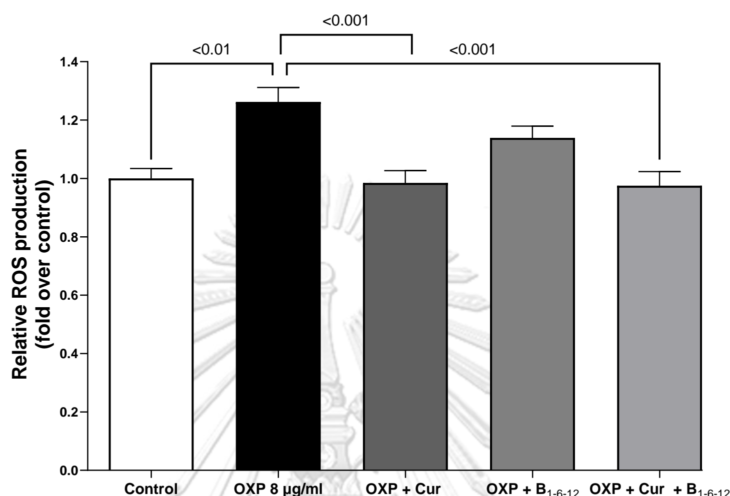



**Figure 98** ROS production of co-treatment with oxaliplatin, curcumin, and $B_{1-6-12}$ on HBVP. Result shows the relative ROS production (fold over control). OXP=oxaliplatin, Cur=curcumin.

## 4. Total glutathione level and GSH/GSSG ratio

4.1 Effects of co-treatment with cisplatin, curcumin, and $B_{1-6-12}$ on total glutathione level and GSH/GSSG ratio

Total glutathione level ($p$<0.001) and GSH/GSSG ratio ($p$<0.001) significantly depleted after exposure to cisplatin. The combined treatment with curcumin and $B_{1-6-12}$ did not change the level of total glutathione; nonetheless, it significantly elevated the GSH/GSSG ratio ($p$<0.05) similar to curcumin treatment alone (Figure 99).

CHULALONGKORN UNIVERSITY

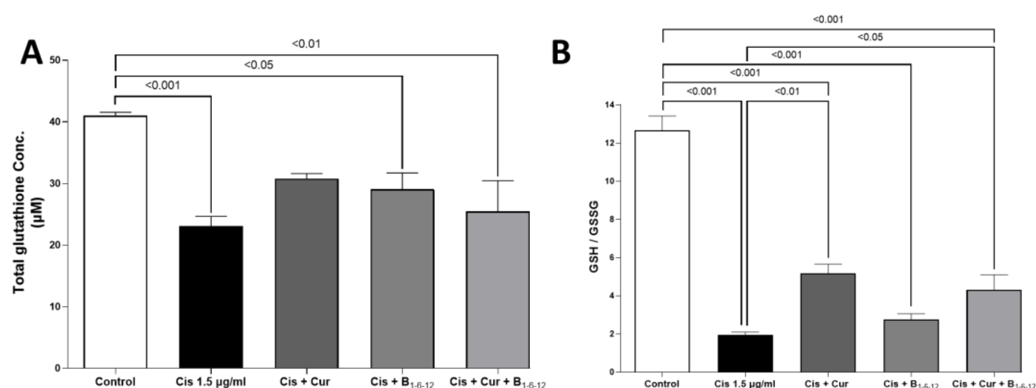



**Figure 99** Total glutathione level and GSH/GSSG ratio in HBVP after treatments. (A) total glutathione concentration, (B) the ratio of reduced glutathione (GSH) to oxidized glutathione (GSSG). Graphs represent Mean ± SEM. Cis=cisplatin, Cur=curcumin.

4.2 Effects of co-treatment with oxaliplatin, curcumin, and B1-6-12 total glutathione level and GSH/GSSG ratio

Oxaliplatin also significantly decreased the total glutathione level ($p<0.001$) and a more significant reduction in GSH/GSSG ratio ($p<0.001$) (Figure 100). The co-treatment with curcumin and $B_{1\text{-}6\text{-}12}$ did not increase the level of total glutathione but increased the ratio of GSH/GSSG ($p<0.001$). This co-treatment enhanced the GSSH/GSSG ratio more than $B_{1\text{-}6\text{-}12}$ treatment alone ($p<0.05$) but was not different compared with curcumin treatment alone (Figure 100).

จุฬาลงกรณ์มหาวิทยาลัย
CHULALONGKORN UNIVERSITY

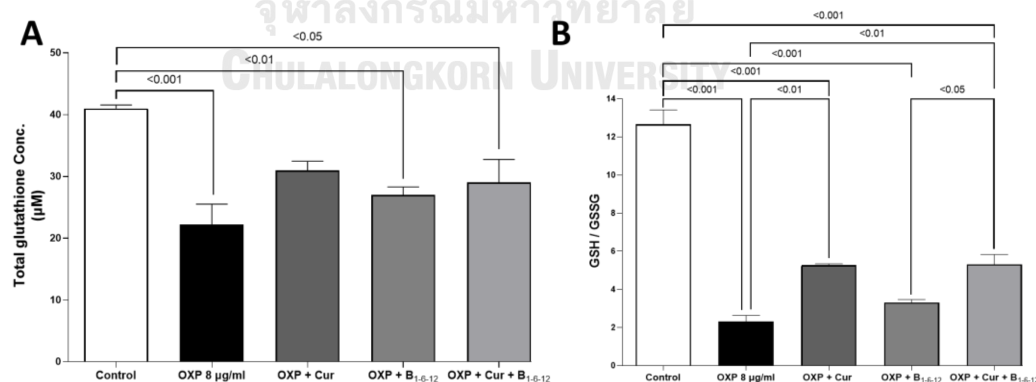
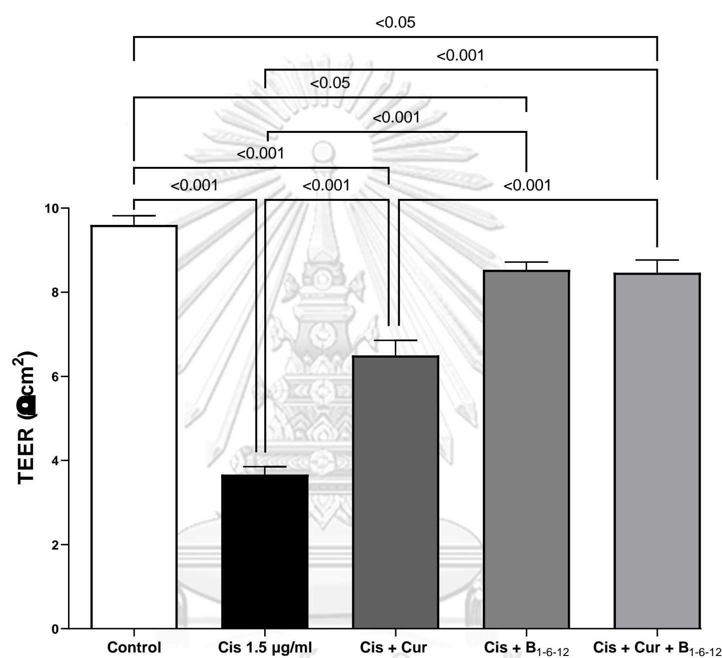



**Figure 100** Total glutathione level and GSH/GSSG ratio in HBVP after treatments. (A) total glutathione concentration, (B) the ratio of reduced glutathione (GSH) to oxidized glutathione (GSSG). Graphs represent Mean ± SEM. OXP=oxaliplatin, Cur=curcumin.

## 5. TEER value

5.1 Effects of co-treatment with cisplatin, curcumin, and $B_{1-6-12}$ on TEER value

Cisplatin caused a significantly decreased TEER value compared with the control group ($p$<0.001). The combination of curcumin and $B_{1-6-12}$ enhanced the TEER value compared with the cisplatin-treated group ($p$<0.001). This combined treatment improved the TEER value more than curcumin treatment alone ($p$<0.001) and similar to $B_{1-6-12}$ treatment alone (Figure 101).




จุฬาลงกรณ์มหาวิทยาลัย
CHULALONGKORN UNIVERSITY

**Figure 101** TEER value of HBVP after treatments. Data are mean ± SEM. Cis=cisplatin, Cur=curcumin.

5.2 Effects of co-treatment with cisplatin, curcumin, and $B_{1-6-12}$ on TEER value

Oxaliplatin also caused the reduction of TEER value compared with the control group ($p$<0.001). The co-treatment with curcumin and $B_{1-6-12}$ improved this reduction ($p$<0.001). Additionally, this combined treatment elevated the TEER value more than $B_{1-6-12}$ treatment alone ($p$<0.001) but did not significantly differ from curcumin treatment alone (Figure 102).

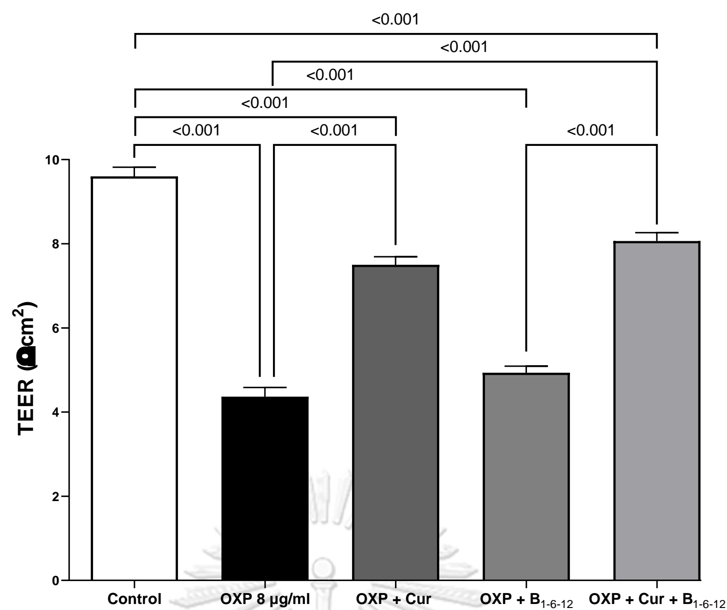



**Figure 102** TEER value of HBVP after treatments. Data are mean ± SEM. OXP= oxaliplatin, Cur=curcumin.

จุฬาลงกรณ์มหาวิทยาลัย
CHULALONGKORN UNIVERSITY

**Table 4** Result summary: effects of combination between curcumin and $B_{1-6-12}$ on cisplatin-induced alterations

| | HUVEC | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Cis | | Cis + Cur | | Cis + $B_{1-6-12}$ | | Cis + Cur + $B_{1-6-12}$ | |
| | Alterations | Significance# | Alterations | Significance* | Alterations | Significance# | Alterations | Significance* |
| Cell viability | ↓ | Yes (p<0.001) | ↑ | Yes (p<0.01) | ↑ | Yes (p<0.001) | ↑ | Yes (p<0.001) |
| Caspase-3 | ↑ | Yes (p<0.05) | ↓ | No | ↓ | No | ↓ | No |
| ROS | ↑ | Yes (p<0.01) | ↓ | Yes (p<0.05) | ↓ | No | ↓ | Yes (p<0.05) |
| Total GSH | ↓ | Yes (p<0.001) | ↑ | No | ↔ | No | ↑ | No |
| GSH/GSSG | ↓ | Yes (p<0.001) | ↑ | Yes (p<0.01) | ↑ | No | ↑ | No |
| Claudin-5 | ↔ | No | ↑ | No | ↑ | No | ↑ | No |
| Occludin | ↓ | No | ↑ | No | ↑ | No | ↑ | No |
| ZO-1 | ↓ | No | ↑ | No | ↑ | No | ↑ | No |
| ZO-2 | ↓ | No | ↑ | Yes (p<0.05) | ↑ | No | ↑ | No |
| TEER | ↓ | Yes (p<0.001) | ↑ | Yes (p<0.001) | ↑ | Yes (p<0.001) | ↑ | Yes (p<0.01) |

# vs. control group, * vs. Cis group, ↓= decrease, ↑= increase, ↔= unchanged

| | HBVP | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Cis | | Cis + Cur | | Cis + $B_{1-6-12}$ | | Cis + Cur + $B_{1-6-12}$ | |
| | Alterations | Significance# | Alterations | Significance* | Alterations | Significance# | Alterations | Significance* |
| Cell viability | ↓ | Yes (p<0.001) | ↑ | Yes (p<0.01) | ↑ | Yes (p<0.001) | ↑ | Yes (p<0.001) |
| Caspase-3 | ↑ | Yes (p<0.05) | ↓ | Yes (p<0.05) | ↓ | No | ↓ | Yes (p<0.05) |
| ROS | ↑ | Yes (p<0.01) | ↓ | Yes (p<0.001) | ↓ | No | ↓ | Yes (p<0.001) |
| Total GSH | ↓ | Yes (p<0.001) | ↑ | Yes (p<0.001) | ↑ | No | ↑ | Yes (p<0.001) |
| GSH/GSSG | ↓ | Yes (p<0.001) | ↑ | Yes (p<0.01) | ↑ | No | ↑ | Yes (p<0.05) |
| TEER | ↓ | Yes (p<0.001) | ↑ | Yes (p<0.001) | ↑ | Yes (p<0.001) | ↑ | Yes (p<0.001) |

# vs. control group, * vs. Cis group, ↓= decrease, ↑= increase, ↔= unchanged

จุฬาลงกรณ์มหาวิทยาลัย
CHULALONGKORN UNIVERSITY

**Table 5** Results summary: effects of combination between curcumin and $B_{1\text{-}6\text{-}12}$ on oxaliplatin-induced alterations

| | HUVEC | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | OXP | | OXP + Cur | | OXP + $B_{1\text{-}6\text{-}12}$ | | OXP + Cur + $B_{1\text{-}6\text{-}12}$ | |
| | Alterations | Significance[#] | Alterations | Significance[*] | Alterations | Significance[#] | Alterations | Significance[*] |
| Cell viability | ↓ | Yes ($p<0.001$) | ↑ | Yes ($p<0.01$) | ↔ | No | ↑ | Yes ($p<0.001$) |
| Caspase-3 | ↑ | Yes ($p<0.001$) | ↓ | No | ↓ | No | ↓ | Yes ($p<0.01$) |
| ROS | ↑ | Yes ($p<0.05$) | ↓ | Yes ($p<0.001$) | ↓ | No | ↓ | Yes ($p<0.01$) |
| Total GSH | ↓ | Yes ($p<0.001$) | ↑ | No | ↔ | No | ↔ | No |
| GSH/GSSG | ↓ | Yes ($p<0.001$) | ↑ | Yes ($p<0.001$) | ↔ | No | ↑ | Yes ($p<0.01$) |
| Claudin-5 | ↓ | No | ↑ | No | ↑ | No | ↑ | No |
| Occludin | ↔ | No | ↑ | No | ↑ | No | ↑ | No |
| ZO-1 | ↓ | Yes ($p<0.05$) | ↑ | No | ↔ | No | ↔ | No |
| ZO-2 | ↓ | No | ↑ | No | ↔ | No | ↔ | No |
| TEER | ↓ | Yes ($p<0.001$) | ↑ | Yes ($p<0.001$) | ↑ | No | ↑ | Yes ($p<0.001$) |

[#] vs. control group, [*] vs. OXP group, ↓= decrease, ↑= increase, ↔= unchanged

| | HBVP | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | OXP | | OXP + Cur | | OXP + $B_{1\text{-}6\text{-}12}$ | | OXP + Cur + $B_{1\text{-}6\text{-}12}$ | |
| | Alterations | Significance[#] | Alterations | Significance[*] | Alterations | Significance[#] | Alterations | Significance[*] |
| Cell viability | ↓ | Yes ($p<0.001$) | ↑ | Yes ($p<0.05$) | ↔ | No | ↑ | Yes ($p<0.01$) |
| Caspase-3 | ↑ | Yes ($p<0.001$) | ↓ | No | ↔ | No | ↓ | No |
| ROS | ↑ | Yes ($p<0.05$) | ↓ | Yes ($p<0.001$) | ↓ | No | ↓ | Yes ($p<0.001$) |
| Total GSH | ↓ | Yes ($p<0.001$) | ↑ | Yes ($p<0.05$) | ↑ | No | ↑ | No |
| GSH/GSSG | ↓ | Yes ($p<0.001$) | ↑ | Yes ($p<0.001$) | ↑ | No | ↑ | Yes ($p<0.001$) |
| TEER | ↓ | Yes ($p<0.001$) | ↑ | Yes ($p<0.001$) | ↑ | No | ↑ | Yes ($p<0.001$) |

[#] vs. control group, [*] vs. OXP group, ↓= decrease, ↑= increase, ↔= unchanged

จุฬาลงกรณ์มหาวิทยาลัย
CHULALONGKORN UNIVERSITY

# CHAPTER V
# DISCUSSION

## 5.1 Effects of curcumin on cisplatin-induced alterations in HUVEC and HBVP

Cisplatin and oxaliplatin caused the reduction of viability in both HUVEC and HBVP. The dose of cisplatin (3 μg/ml) that utilized in HUVEC was lower than oxaliplatin (5 μg/ml) due to the less toxicity of oxaliplatin. This result is consistent with the study of Donzelli and co-workers.[138] Similarly, the dose of cisplatin (1.5 μg/ml) was less than the dose of oxaliplatin (8 μg/ml) in HBVP. This reduction in viability was possibly related to the activation of caspase-3. This was in line with the previous studies that reported cisplatin-induced endothelial apoptosis via enhancement of caspase-3.[10, 11, 90, 93]

One mechanism of cisplatin-induced toxicity is via oxidative stress due to overproduction of ROS and depletion of antioxidant.[50] This was in accordance with our results. Cisplatin elevated ROS, reduced total glutathione and GSH/GSSG ratio in both HUVEC and HBVP. Oxidative stress can cause apoptosis. Therefore, it is possible that cisplatin-induced cytotoxicity in both cell types was mediated by oxidative stress leading to activation of caspase-3 and cell death.

In addition, we found that cisplatin reduced TEER value in endothelial cells and pericytes. Furthermore, we explored the expression of endothelial tight junction proteins. The expression of occludin, ZO-1, and ZO-2 had a tendency to decrease. Oxidative stress is known to induce disruption of endothelial tight junctions.[139] Previous study also demonstrated that ROS altered the localization of ZO-1 and ZO-2 between endothelial cells correlating with impaired barrier function.[140] This alteration in tight junction proteins along with the reduced TEER values suggests that cisplatin might damage the BNB. These results agree with a previous study of our lab that demonstrated pericyte loss and detachment from endothelial cells in the nerves of cisplatin-treated rats.[15] Taken together, cisplatin could damage endothelial cells and pericytes via generation of oxidative stress leading to cell dysfunction and apoptosis. Since endothelial cells and pericytes play important roles in the normal

functions of BNB[141], cytotoxicity of cisplatin to both cell types finally results in the BNB dysfunction.

This study showed that curcumin alleviated endothelial and pericyte viability. Furthermore, it reduced the ROS level and restored GSH/GSSG ratio in both cell types. These results indicate that curcumin has an antioxidant property against cisplatin in endothelial cells and pericytes. The antioxidant property of curcumin is well recognized.[106] Although, curcumin diminished ROS overproduction, it did not significantly ameliorate higher caspase-3 activity in endothelial cells. Oxidative stress is not the only cause of cisplatin-induced apoptosis. Cisplatin also causes DNA damage leading to caspase-3 activation and apoptosis.[9] Co-treatment with curcumin probably attenuates mainly the oxidative stress but not DNA damage that needs further study to clarify. On the other hand, curcumin reduced both ROS and caspase-3 levels in pericytes. Hence, the inhibition of apoptosis in pericytes likely resulted from the reduction of ROS.

As for the tight junction protein expression, claudin-5 and occludin are the transmembrane tight junction proteins, whereas ZO-1 and ZO-2 are the intracellular tight junction proteins.[126] Results of this study showed that curcumin could significantly upregulate the protein expression of ZO-1 and ZO-2 in endothelial cells. Expression of claudin-5 was also increased despite no significant difference. This improvement of ZO-1 and ZO-2 levels was consistent with the TEER result. Consequently, the enhancement of endothelial barrier function probably resulted from the reduction of oxidative stress leading to improvement in the expression of ZO-1 and ZO-2.

In summary, cisplatin damages both endothelial cells and pericytes. Co-treatment with curcumin effectively alleviated cisplatin-induced toxicity in both cell types via its antioxidant property. This was correlated with improved barrier function. Consequently, curcumin is the potential treatment against cisplatin-induced microvascular damage.

## 5.2 Effects of curcumin on oxaliplatin-induced alterations in HUVEC and HBVP

In the present study, oxaliplatin induced the reduction of HUVEC and HBVP viability which corresponded with the enhancement of caspase-3 activity. This was in line with the previous finding that demonstrated the elevation of caspase-3 in endothelial cell apoptosis following oxaliplatin treatment.[12]

Oxidative stress caused by excessive generation of ROS and antioxidant reduction is one mechanism of oxaliplatin-induced toxicity.[9] This was in line with our findings. Oxaliplatin increased ROS, decreased total glutathione, and decreased the GSH/GSSG ratio in both HUVEC and HBVP. Apoptosis can be triggered by oxidative stress. Consequently, it is possible that oxidative stress plays a major role in oxaliplatin-induced cytotoxicity in both cell types, resulting in caspase-3 activation and cell death.

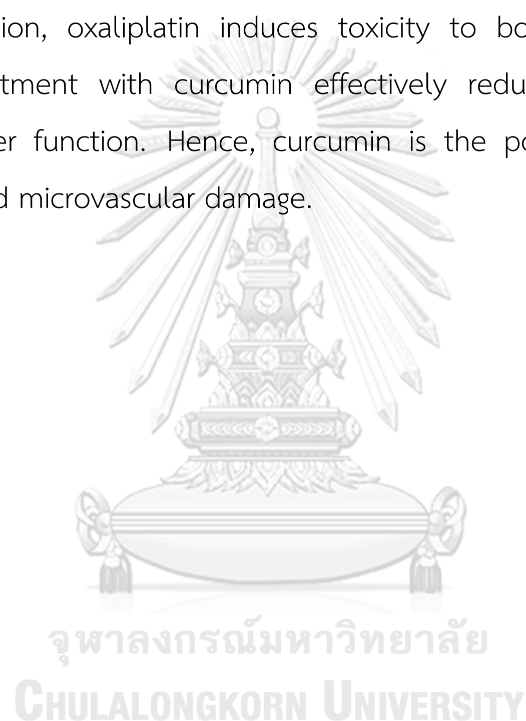
Furthermore, we discovered that oxaliplatin decreased the TEER value in both endothelial cells and pericytes. After that, we assessed the expression of endothelial tight junction proteins. We found the downregulation of ZO-1. Moreover, the expression of claudin-5 and ZO-2 tended to reduce. Endothelial tight junctions are known to be disrupted by oxidative stress.[139] Excessive production of ROS causes tight junction dislocation.[12] The study of Fischer and co-workers also demonstrated that ROS affected the localization of ZO-1.[140] Our findings are accordant with those of Branca and co-workers that oxaliplatin generates oxidative stress, produces apoptosis, suppresses tight junction protein expression, and finally brings about barrier dysfunction.[12] Taken together, oxaliplatin enhances the oxidative stress likely resulting in lower expression of some tight junction proteins and impaired barrier function.

This study demonstrated that curcumin enhanced both HUVEC and HBVP viability. In addition, curcumin eliminated the overproduction of ROS and reversed the GSH/GSSG ratio caused by oxaliplatin in both cell types. Nonetheless, curcumin did not inhibit oxaliplatin-induced apoptosis in both endothelial cells and pericytes similar to the results of cisplatin. It also tended to upregulate all endothelial tight junction proteins (claudin-5, occludin, ZO-1, ZO-2), although there was no significant

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

difference. However, the barrier function was enhanced despite no significant change in tight junction protein expression. Tight junction is composed of intracellular proteins that link actin cytoskeleton with transmembrane proteins.[126] The highly dynamic property of actin cytoskeleton affects the endothelial barrier function.[142] Since ROS overproduction induces intercellular gap formation and the actin filament rearrangement, it influences endothelial integrity.[142] Accordingly, curcumin-mediated barrier improvement might be via the ROS depletion. Further study is needed to clarify this hypothesis.

In conclusion, oxaliplatin induces toxicity to both endothelial cells and pericytes. Co-treatment with curcumin effectively reduced oxidative stress and ameliorated barrier function. Hence, curcumin is the potential treatment against oxaliplatin-induced microvascular damage.



จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

### 5.3 Effects of $B_{1-6-12}$ on cisplatin-induced alterations in HUVEC and HBVP

As mentioned in the previous section, cisplatin induced lower viability, caspase-3 activation, oxidative stress, downregulate the expression of tight junction proteins (occludin and ZO-2), and barrier dysfunction in both endothelial cells and pericytes.

$B_{1-6-12}$ ameliorated cell viability but did not diminish caspase-3 activation in both HUVEC and HBVP. The main causes of apoptosis induced by cisplatin are oxidative stress and DNA damage.[9] In addition, $B_{1-6-12}$ did not inhibit an increase in ROS level. Furthermore, it also did not correct a decrease in the total glutathione and GSH/GSSG ratio in both cell types. Although $B_{12}$ possesses the antioxidant property,[28] the combination of $B_{1-6-12}$ did not have significant effects on oxidative stress. Accordingly, $B_{1-6-12}$ did not prevent apoptosis caused by oxidative stress. The question remains to be answered is how $B_{1-6-12}$ improved the cell viability. Since B vitamins are vital to various cellular metabolic pathways[24], their supplement might be associated with higher resistance to cell death. More research is needed to clarify this issue.

In addition, $B_{1-6-12}$ restored the endothelial barrier function. This result agrees with the expression of tight junction protein. $B_{1-6-12}$ treatment upregulated the expression of occludin, ZO-1, and ZO-2. These results suggest that $B_{1-6-12}$ improved both intracellular and transmembrane tight junction proteins via oxidative stress-independent mechanism. Apart from ROS, inflammation also causes tight junction disruption.[143] Cisplatin exposure can generate inflammation in endothelial cells.[10] B vitamin complex ($B_1$, $B_2$, $B_3$, $B_5$, $B_6$, and $B_{12}$) also express an anti-inflammatory effect.[144] Several studies have found that p53 improves endothelial barrier function via an anti-inflammatory mechanism such as reducing pro-inflammatory and inflammatory cytokines, suppressing NF-kB, or inhibiting the Rho pathway.[145-147] Additionally, $B_6$ can elevate the p53 level.[148] Consequently, $B_{1-6-12}$ probably increased the amount of p53 leading to reduction of inflammation, and subsequently up-regulation of tight junction proteins. However, further exploration is required to prove this hypothesis. However, the improvement of barrier function in the pericyte could also possibly result from improvement in cell dysfunction.

In conclusion, $B_{1-6-12}$ improved the endothelial barrier function and upregulated–tight junction protein expression. Nonetheless, the precise mechanism that $B_{1-6-12}$ increased the expression is unknown. Moreover, $B_{1-6-12}$ also enhanced pericyte barrier integrity. Accordingly, $B_{1-6-12}$ is an effective candidate for treatment against cisplatin-induced endothelial cell and pericyte cytotoxicity.

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

### 5.4 Effects of $B_{1-6-12}$ on oxaliplatin-induced alterations in HUVEC and HBVP

As mentioned in the previous section, oxaliplatin induced lower viability, caspase-3 activation, oxidative stress, and barrier dysfunction in both endothelial cells and pericytes. Nevertheless, it did not significantly downregulate the expression of tight junction proteins.

$B_{1-6-12}$ did not ameliorate cell viability, caspase-3 activity, ROS level, total glutathione, GSH/GSSG ratio, expression of tight junction proteins as well as barrier integrity in both cell types. These results implied that $B_{1-6-12}$ was ineffective against oxaliplatin-induced cytotoxicity.

Cisplatin-induced endothelial and pericyte damage may occur from several causes, such as oxidative stress and inflammation. Hence, curcumin and $B_{1-6-12}$ are efficacious against cisplatin-induced cytotoxicity. In contrast, oxaliplatin-induced endothelial and pericyte toxicity might be mainly due to oxidative stress since only curcumin was effective. $B_{1-6-12}$ which has weak antioxidant property was therefore ineffective against oxaliplatin-induced endothelial and pericyte toxicity. This hypothesis remains to be proved by applying other antioxidants in oxaliplatin-induced cytotoxicity.

In summary, $B_{1-6-12}$ did not improve any toxicity parameter by oxaliplatin. It implies that $B_{1-6-12}$ might be an ineffective treatment for oxaliplatin-induced endothelial and pericyte damage.

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

**5.5 Effects of combination between curcumin and $B_{1-6-12}$ on cisplatin-induced alterations in HUVEC and HBVP**

Combined treatment with curcumin and $B_{1-6-12}$ restored cell viability, inhibited ROS production, and improved barrier function in both cell types. Additionally, this combined treatment suppressed the activation of caspase-3, recovered GSH/GSSG level in pericytes, but not in endothelial cells. Moreover, it did not upregulate the tight junction proteins in endothelial cells.

Combined treatment with curcumin and $B_{1-6-12}$ was not significantly better than curcumin or $B_{1-6-12}$ treatment alone in all parameters except the HUVEC viability. This may be due to the synergistic effect from both curcumin and $B_{1-6-12}$. In addition, the combined treatment elevated barrier function similar to $B_{1-6-12}$ treatment alone, whereas it lessened the caspase-3 level and oxidative stress comparable to curcumin treatment alone in both cell types.

In conclusion, the combination treatment between curcumin and $B_{1-6-12}$ against cisplatin-induced endothelial cells and pericytes toxicity was not superior to the curcumin or $B_{1-6-12}$ treatment alone. Thus, it is unnecessary to utilize this combination for management of cisplatin-induced microvascular damage.

จุฬาลงกรณ์มหาวิทยาลัย
CHULALONGKORN UNIVERSITY

### 5.6 Effects of combination between curcumin and $B_{1-6-12}$ on oxaliplatin-induced alterations in HUVEC and HBVP

The combination treatment between curcumin and $B_{1-6-12}$ reduced apoptosis (not in HBVP), inhibited ROS production, recovered GSH/GSSG level, and improved barrier function in both endothelial cells and pericytes following oxaliplatin exposure. Nonetheless, it could not significantly upregulate endothelial tight junction proteins.

$B_{1-6-12}$ treatment alone did not improve any events caused by oxaliplatin. This was in agreement with the results from the combined treatment. The combination between curcumin and $B_{1-6-12}$ significantly ameliorated all parameters better than $B_{1-6-12}$ alone but not curcumin alone in both cells. Thus, the favorable effects of this co-treatment against oxaliplatin toxicity are likely due to the action of curcumin.

In conclusion, the combination treatment between curcumin and $B_{1-6-12}$ against oxaliplatin-induced endothelial cell and pericyte damage can improve each experiment parameter close to the curcumin treatment alone. Hence, it is not worth combining these two agents in the treatment for oxaliplatin-induced microvascular damage.

### 5.7 Further study

Curcumin/$B_{1-6-12}$, should be investigated further in endothelial co-culture with pericytes, animal studies, and clinical trials, respectively.

CHULALONGKORN UNIVERSITY

### 5.8 Limitation of this study

In some Western blot results, the differences were not statistically significant due to wide SEM. These experiments should be repeated in the future with higher number of samples to prove if there are any significant changes.

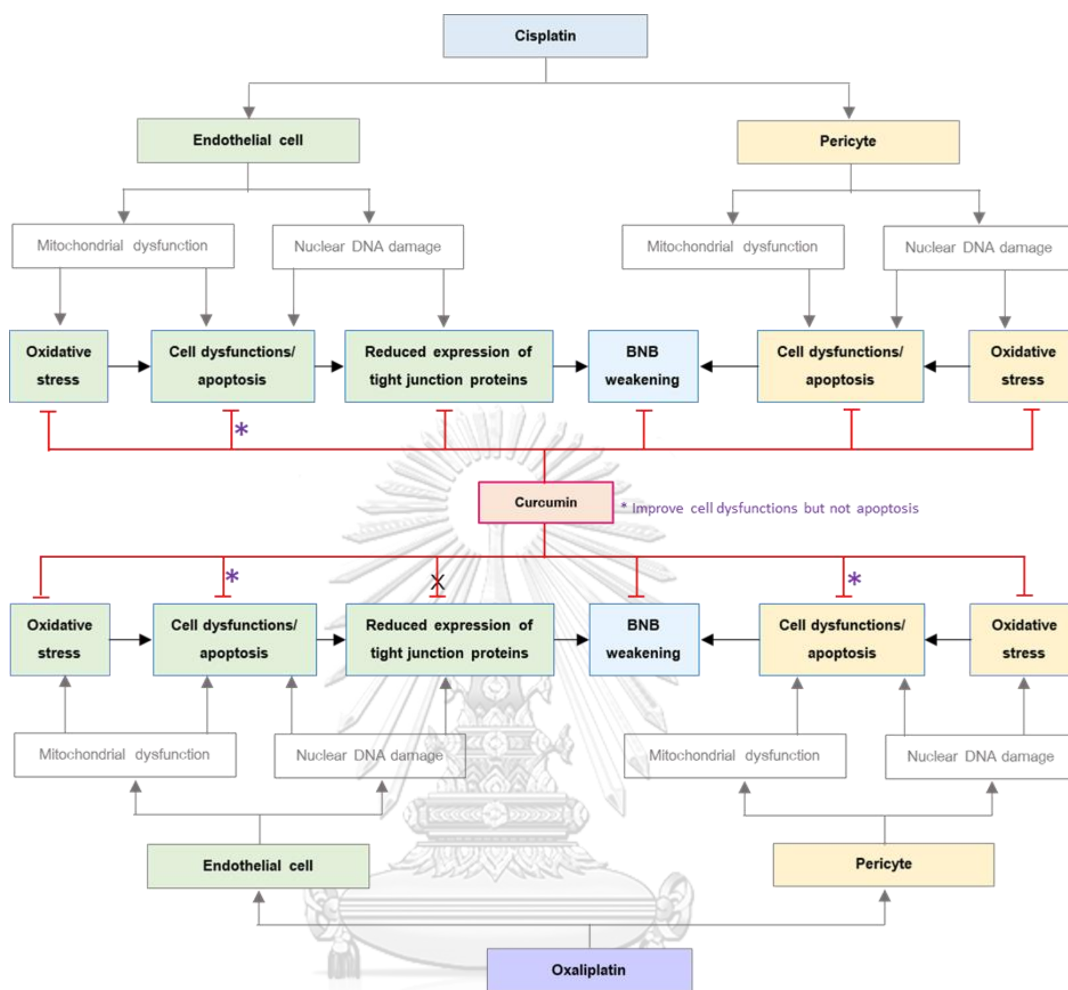



**Figure 103** Result summary: effects of curcumin on cisplatin/oxaliplatin-induced endothelial and pericyte cytotoxicity.

จุฬาลงกรณ์มหาวิทยาลัย
CHULALONGKORN UNIVERSITY

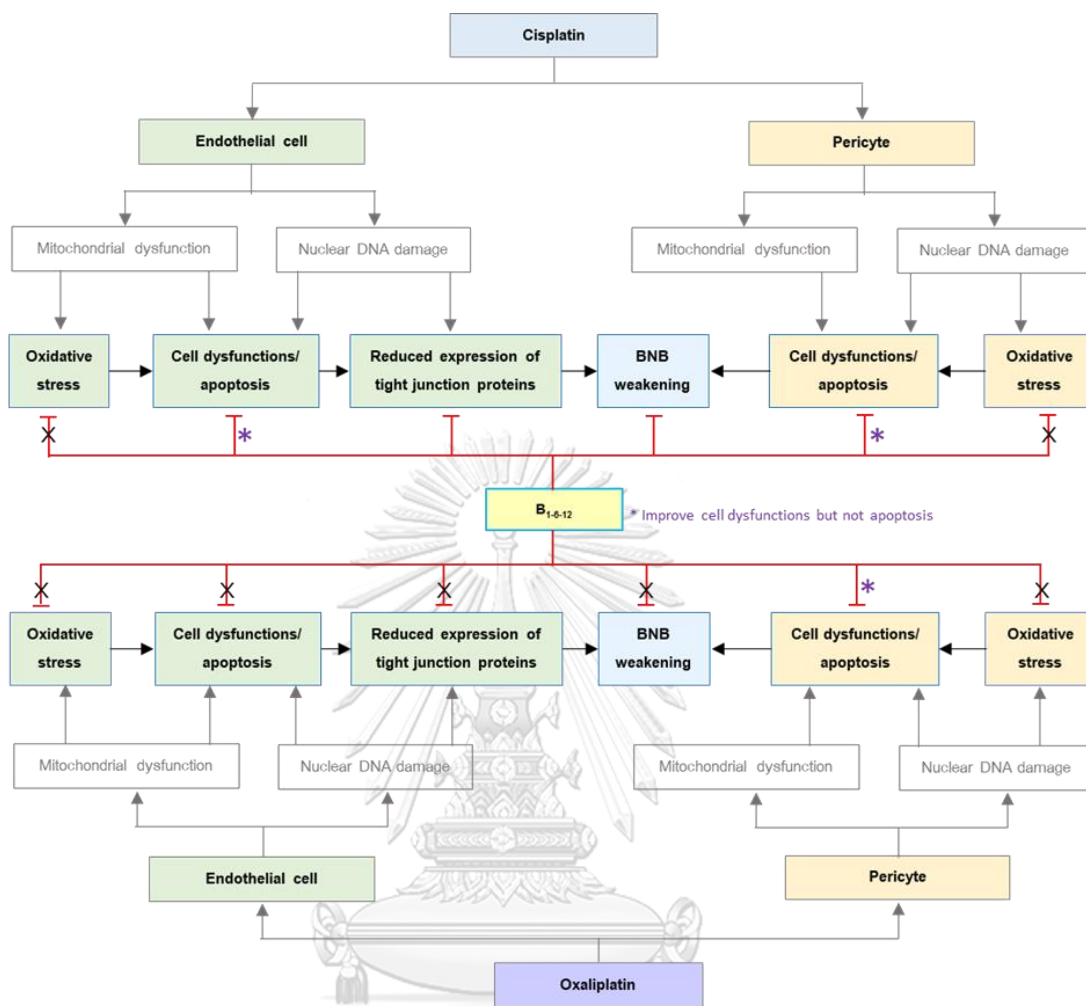



**Figure 104** Result summary: effects of $B_{1\text{-}6\text{-}12}$ on cisplatin/oxaliplatin-induced endothelial and pericyte cytotoxicity.

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

# REFERENCES


1. Dilruba S, Kalayda GV. Platinum-based drugs: past, present and future. Cancer Chemother Pharmacol 2016;77:1103-24.

2. Kanat O, Ertas H, Caner B. Platinum-induced neurotoxicity: A review of possible mechanisms. World J Clin Oncol. 2017;8:329.

3. Verstappen CC, Heimans JJ, Hoekman K, Postma TJ. Neurotoxic complications of chemotherapy in patients with cancer. Drugs. 2003;63:1549-63.

4. Pasetto LM, D'Andrea MR, Rossi E, Monfardini S. Oxaliplatin-related neurotoxicity: how and why? Crit Rev Oncol Hematol. 2006;59:159-68.

5. Podratz JL, Knight AM, Ta LE, Staff NP, Gass JM, Genelin K, et al. Cisplatin induced mitochondrial DNA damage in dorsal root ganglion neurons. Neurobiol Dis. 2011;41:661-8.

6. Park SB, Goldstein D, Krishnan AV, Lin CSY, Friedlander ML, Cassidy J, et al. Chemotherapy-induced peripheral neurotoxicity: A critical analysis. CA Cancer J Clin. 2013;63:419-37.

7. Argyriou AA, Bruna J, Marmiroli P, Cavaletti G. Chemotherapy-induced peripheral neurotoxicity (CIPN): an update. Crit Rev Oncol Hematol 2012;82:51-77.

8. Miltenburg N, Boogerd W. Chemotherapy-induced neuropathy: a comprehensive survey. Cancer Treat Rev. 2014;40:872-82.

9. Chiorazzi A, Semperboni S, Marmiroli P. Current view in platinum drug mechanisms of peripheral neurotoxicity. Toxics. 2015;3:304-21.


จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University


10. Nuver J, De Haas EC, Van Zweeden M, Gietema JA, Meijer C. Vascular damage in testicular cancer patients: a study on endothelial activation by bleomycin and cisplatin in vitro. Oncol Rep. 2010;23:247-53.

11. Kirchmair R, Walter DH, Ii M, Rittig K, Tietz AB, Murayama T, et al. Antiangiogenesis mediates cisplatin-induced peripheral neuropathy: attenuation or reversal by local vascular endothelial growth factor gene therapy without augmenting tumor growth. Circulation. 2005;111:2662-70.

12. Branca JJV, Maresca M, Morucci G, Becatti M, Paternostro F, Gulisano M, et al. Oxaliplatin-induced blood brain barrier loosening: a new point of view on chemotherapy-induced neurotoxicity. Oncotarget. 2018;9:23426.

13. Armulik A, Abramsson A, Betsholtz C. Endothelial/pericyte interactions. Circ Res.

2005;97:512-23.

14. Kanda T. Biology of the blood–nerve barrier and its alteration in immune mediated neuropathies. J Neurol Neurosurg Psychiatry. 2013;84:208-12.

15. Jindatip D, Nopparat W, Kobutree P, Roumwong A, Agthong S. Pericyte loss and detachment in experimental cisplatin-induced neuropathy. Int J Morphol. 2019;37:509-14.

16. Avan A, Postma TJ, Ceresa C, Avan A, Cavaletti G, Giovannetti E, et al. Platinum-induced neurotoxicity and preventive strategies: past, present, and future. oncologist. 2015:theoncologist. 2014-0044.

17. Al Moundhri MS, Al-Salam S, Al Mahrouqee A, Beegam S, Ali BH. The effect of curcumin on oxaliplatin and cisplatin neurotoxicity in rats: some behavioral, biochemical, and histopathological studies. J Med Toxicol. 2013;9:25-33.

18. Zangui M, Atkin SL, Majeed M, Sahebkar A. Current evidence and future perspectives for curcumin and its analogues as promising adjuncts to oxaliplatin: state-of-the-art. Pharmacol Res. 2019.

19. Mendonça LM, da Silva Machado C, Teixeira CCC, de Freitas LAP, Bianchi MdLP, Antunes LMG. Curcumin reduces cisplatin-induced neurotoxicity in NGF-differentiated PC12 cells. Neurotoxicology. 2013;34:205-11.

20. Oveissi V, Ram M, Bahramsoltani R, Ebrahimi F, Rahimi R, Naseri R, et al. Medicinal plants and their isolated phytochemicals for the management of chemotherapy-induced neuropathy: therapeutic targets and clinical perspective. DARU J Pharm Sci. 2019.

21. Waly MI, Al Moundhri MS, Ali BH. Effect of curcumin on cisplatin-and oxaliplatin-induced oxidative stress in human embryonic kidney (HEK) 293 cells. Renal Failure. 2011;33:518-23.

22. Waseem M, Parvez S. Neuroprotective activities of curcumin and quercetin with potential relevance to mitochondrial dysfunction induced by oxaliplatin. Protoplasma. 2016;253:417-30.

23. Agthong S, Kaewsema A, Charoensub T. Curcumin ameliorates functional and structural abnormalities in cisplatin-induced neuropathy. Exp Neurobiol. 2015;24:139-45.

24. Ang CD, Alviar MJM, Dans AL, Bautista‐Velez GGP, Villaruz‐Sulit MVC, Tan JJ, et al. Vitamin B for treating peripheral neuropathy. Cochrane Database Syst Rev. 2008.

25. RIZVI A, AHMAD A, RIZVI Z. Efficacy of Combination of Vitamin B1, B6, and B12 in Management of Diabetic Peripheral Neuropathy. PJMHS. 2013;7:801-4.

26. Spinneker A, Sola R, Lemmen V, Castillo M, Pietrzik K, Gonzalez-Gross M. Vitamin B6

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

status, deficiency and its consequences-an overview. Nutr Hosp. 2007;22.

27. Schloss J, Colosimo M. B vitamin complex and chemotherapy-induced peripheral neuropathy. Curr Oncol Rep. 2017;19:76.

28. Hakim M, Kurniani N, Pinzon RT, Tugasworo D, Basuki M, Haddani H, et al. Management of peripheral neuropathy symptoms with a fixed dose combination of high-dose vitamin B1, B6 and B12: A 12-week prospective non-interventional study in Indonesia. Asian j med sci (Pokhara).9:32-40.

29. Jolivalt CG, Mizisin LM, Nelson A, Cunha JM, Ramos KM, Bonke D, et al. B vitamins alleviate indices of neuropathic pain in diabetic rats. Eur J Pharmacol. 2009;612:41-7.

30. Kelland L. The resurgence of platinum-based cancer chemotherapy. Nat Rev Cancer. 2007;7:573.

31. Dasari S, Tchounwou PB. Cisplatin in cancer therapy: molecular mechanisms of action. Eur J Pharmacol. 2014;740:364-78.

32. O'dwyer PJ, Stevenson JP, Johnson SW. Clinical pharmacokinetics and administration of established platinum drugs. Drugs. 2000;59:19-27.

33. Mehmood RK. Review of cisplatin and oxaliplatin in current immunogenic and monoclonal antibody treatments. Oncol Rev. 2014;8.

34. Alcindor T, Beauger N. Oxaliplatin: a review in the era of molecularly targeted therapy. Curr Oncol. 2011;18:18.

35. Raymond E, Chaney S, Taamma A, Cvitkovic E. Oxaliplatin: a review of preclinical and clinical studies. Ann Oncol 1998;9:1053-71.

36. Di Francesco A, Ruggiero A, Riccardi R. Cellular and molecular aspects of drugs of the future: oxaliplatin. Cell Mol Life Sci. 2002;59:1914-27.

37. Fuertes M, Castilla J, Alonso C, Prez J. Cisplatin biochemical mechanism of action: from cytotoxicity to induction of cell death through interconnections between apoptotic and necrotic pathways. Curr Med Chem. 2003;10:257-66.

38. Dos Santos NAG, Ferreira RS, Dos Santos AC. Overview of cisplatin-induced neurotoxicity and ototoxicity, and the protective agents. Food Chem Toxicol 2020;136:111079.

39. Ip V, Liu JJ, Mercer JF, McKeage MJ. Differential expression of ATP7A, ATP7B and CTR1 in adult rat dorsal root ganglion tissue. Mol Pain. 2010;6:53.

40. Gill JS, Windebank AJ. Cisplatin-induced apoptosis in rat dorsal root ganglion neurons is associated with attempted entry into the cell cycle. J Clin Invest 1998;101:2842-50.

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

41. Melli G, Taiana M, Camozzi F, Triolo D, Podini P, Quattrini A, et al. Alpha-lipoic acid prevents mitochondrial damage and neurotoxicity in experimental chemotherapy neuropathy. Exp Neurol. 2008;214:276-84.

42. Ta LE, Espeset L, Podratz J, Windebank AJ. Neurotoxicity of oxaliplatin and cisplatin for dorsal root ganglion neurons correlates with platinum–DNA binding. Neurotoxicology. 2006;27:992-1002.

43. Kelley MR, Jiang Y, Guo C, Reed A, Meng H, Vasko MR. Role of the DNA base excision repair protein, APE1 in cisplatin, oxaliplatin, or carboplatin induced sensory neuropathy. PloS one. 2014;9:e106485.

44. Alaedini A, Xiang Z, Kim H, Sung Y-J, Latov N. Up-regulation of apoptosis and regeneration genes in the dorsal root ganglia during cisplatin treatment. Exp Neurol. 2008;210:368-74.

45. Leo M, Schmitt L-I, Jastrow H, Thomale J, Kleinschnitz C, Hagenacker T. Cisplatin alters the function and expression of N-type voltage-gated calcium channels in the absence of morphological damage of sensory neurons. Mol Pain. 2017;13:1744806917746565.

46. Carozzi V, Canta A, Chiorazzi A. Chemotherapy-induced peripheral neuropathy: what do we know about mechanisms? Neurosci Lett 2015;596:90-107.

47. Günes DA, Florea A-M, Splettstoesser F, Büsselberg D. Co-application of arsenic trioxide (As2O3) and cisplatin (CDDP) on human SY-5Y neuroblastoma cells has differential effects on the intracellular calcium concentration ([Ca2+] i) and cytotoxicity. Neurotoxicology. 2009;30:194-202.

48. Schulze C, McGowan M, Jordt S-E, Ehrlich BE. Prolonged oxaliplatin exposure alters intracellular calcium signaling: a new mechanism to explain oxaliplatin-associated peripheral neuropathy. Clin Colorectal Cancer. 2011;10:126-33.

49. Mannelli LDC, Zanardelli M, Failli P, Ghelardini C. Oxaliplatin-induced oxidative stress in nervous system-derived cellular models: Could it correlate with in vivo neuropathy? Free Radic Biol Med. 2013;61:143-50.

50. Carozzi V, Marmiroli P, Cavaletti G. The role of oxidative stress and anti-oxidant treatment in platinum-induced peripheral neurotoxicity. Curr Cancer Drug Targets. 2010;10:670-82.

51. Mannelli LDC, Zanardelli M, Failli P, Ghelardini C. Oxaliplatin-induced neuropathy: oxidative stress as pathological mechanism. Protective effect of silibinin. J Pain. 2012;13:276-84.

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

52. Canta A, Pozzi E, Carozzi VA. Mitochondrial dysfunction in chemotherapy-induced peripheral neuropathy (CIPN). Toxics. 2015;3:198-223.

53. Gheorghe-Cetean S, Cainap C, Oprean L, Hangan A, Virag P, Fischer-Fodor E, et al. Platinum derivatives: A multidisciplinary approach. J BUON. 2017;22:568-77.

54. Low PA, Nickander KK, Tritschler HJ. The roles of oxidative stress and antioxidant treatment in experimental diabetic neuropathy. Diabetes. 1997;46:S38-S42.

55. Park SA, Choi KS, Bang JH, Huh K, Kim SU. Cisplatin-induced apoptotic cell death in mouse hybrid neurons is blocked by antioxidants through suppression of cisplatin-mediated accumulation of p53 but not of Fas/Fas ligand. J Neurochem. 2000;75:946-53.

56. Areti A, Yerra VG, Naidu V, Kumar A. Oxidative stress and nerve damage: role in chemotherapy induced peripheral neuropathy. Redox Biol. 2014;2:289-95.

57. Zheng H, Xiao WH, Bennett GJ. Functional deficits in peripheral nerve mitochondria in rats with paclitaxel-and oxaliplatin-evoked painful peripheral neuropathy. Exp Neurol. 2011;232:154-61.

58. Masuda H, Tanaka T, Takahama U. Cisplatin generates superoxide anion by interaction with DNA in a cell-free system. Biochem Biophys Res Commun. 1994;203:1175-80.

59. Bobylev I, Joshi AR, Barham M, Neiss WF, Lehmann HC. Depletion of mitofusin-2 causes mitochondrial damage in cisplatin-induced neuropathy. Mol Neurobiol. 2018;55:1227-35.

60. Bennett GJ, Doyle T, Salvemini D. Mitotoxicity in distal symmetrical sensory peripheral neuropathies. Nat Rev Neurol. 2014;10:326.

61. Baloh RH. Mitochondrial dynamics and peripheral neuropathy. Neuroscientist. 2008;14:12-8.

62. Russell JW, Windebank AJ, McNiven MA, Brat DJ, Brimijoin WS. Effect of cisplatin and ACTH4–9 on neural transport in cisplatin induced neurotoxicity. Brain Res. 1995;676:258-67.

63. Goshima Y, Usui H, Shiozawa T, Hida T, Kuraoka S, Takeshita S, et al. Computational analysis of the effects of antineoplastic agents on axonal transport. J Pharmacol Sci. 2010;114:168-79.

64. Grolleau F, Gamelin L, Boisdron-Celle M, Lapied B, Pelhate M, Gamelin E. A possible explanation for a neurotoxic effect of the anticancer agent oxaliplatin on neuronal voltage-gated sodium channels. J Neurophysiol. 2001;85:2293-7.

65. Renn CL, Carozzi VA, Rhee P, Gallop D, Dorsey SG, Cavaletti G. Multimodal

assessment of painful peripheral neuropathy induced by chronic oxaliplatin-based chemotherapy in mice. Mol Pain. 2011;7:29.

66. Lersch C, Schmelz R, Eckel F, Erdmann J, Mayr M, Schulte-Frohlinde E, et al. Prevention of oxaliplatin-induced peripheral sensory neuropathy by carbamazepine in patients with advanced colorectal cancer. Clin Colorectal Cancer 2002;2:54-8.

67. Nassini R, Gees M, Harrison S, De Siena G, Materazzi S, Moretto N, et al. Oxaliplatin elicits mechanical and cold allodynia in rodents via TRPA1 receptor stimulation. pain. 2011;152:1621-31.

68. Starobova H, Vetter I. Pathophysiology of chemotherapy-induced peripheral neuropathy. Front Mol Neurosci. 2017;10:174.

69. Fukuda Y, Li Y, Segal RA. A mechanistic understanding of axon degeneration in chemotherapy-induced peripheral neuropathy. Front Neurosci. 2017;11:481.

70. Head KA. Peripheral neuropathy: pathogenic mechanisms and alternative therapies. Altern Med Rev. 2006;11:294.

71. Cersosimo RJ. Cisplatin neurotoxicity. Cancer Treat Rev. 1989;16:195-211.

72. Addington J, Freimer M. Chemotherapy-induced peripheral neuropathy: an update on the current understanding. F1000Res. 2016.

73. Zedan AH, Hansen TF, Svenningsen ÅF, Vilholm OJ. Oxaliplatin-induced neuropathy in colorectal cancer: many questions with few answers. Clin Colorectal Cancer. 2014;13:73-80.

74. Flatters S, Dougherty P, Colvin L. Clinical and preclinical perspectives on Chemotherapy-Induced Peripheral Neuropathy (CIPN): a narrative review. Br J Anaesth. 2017;119:737-49.

CHULALONGKORN UNIVERSITY

75. Vencappa S, Donaldson LF, Hulse RP. Cisplatin induced sensory neuropathy is prevented by vascular endothelial growth factor-A. Am J Transl Res. 2015;7:1032.

76. Wongtawatchai T, Agthong S, Kaewsema A, Chentanez V. Altered phosphorylation of mitogen-activated protein kinases in dorsal root ganglia and sciatic nerve of rats with cisplatin-induced neuropathy. Asian Biomed (Res Rev News). 2012;6:397-411.

77. Cavaletti G, Tredici G, Petruccioli M, Donde E, Tredici P, Marmiroli P, et al. Effects of different schedules of oxaliplatin treatment on the peripheral nervous system of the rat. Eur J Cancer. 2001;37:2457-63.

78. Donertas B, Cengelli Unel C, Aydin S, Ulupinar E, Ozatik O, Kaygisiz B, et al. Agmatine co-treatment attenuates allodynia and structural abnormalities in cisplatin-induced neuropathy in rats. Fundam Clin Pharmacol. 2018;32:288-96.

79. Cavaletti G, Tredici G, Marmiroli P, Petruccioli M, Barajon I, Fabbrica D. Morphometric study of the sensory neuron and peripheral nerve changes induced by chronic cisplatin (DDP) administration in rats. Acta Neuropathol. 1992;84:364-71.

80. Thompson SW, Davis LE, Kornfeld M, Hilgers RD, Standefer JC. Cisplatin neuropathy. Clinical, electrophysiologic, morphologic, and toxicologic studies. Cancer. 1984;54:1269-75.

81. Boyette-Davis J, Dougherty P. Protection against oxaliplatin-induced mechanical hyperalgesia and intraepidermal nerve fiber loss by minocycline. Eep Neurol. 2011;229:353-7.

82. Montiel M, Urso L, de la Blanca EP, Marsigliante S, Jimenez E. Cisplatin reduces endothelial cell migration via regulation of type 2-matrix metalloproteinase activity. Cell Physiol Biochem. 2009;23:441-8.

83. Yoshikawa A, Saura R, Matsubara T, Mizuno K. A mechanism of cisplatin action: antineoplastic effect through inhibition of neovascularization. Kobe J Med Sci. 1997;43:109-20.

84. Yeh ET, Bickford CL. Cardiovascular complications of cancer therapy: incidence, pathogenesis, diagnosis, and management. J Am Coll Cardiol. 2009;53:2231-47.

85. Hansen S, Olsen N, Rossing N, RΦrth M. Vascular toxicity and the mechanism underlying Raynaud's phenomenon in patients treated with cisplatin, vinbiastine and bleomycin. Ann Oncol. 1990;1:289-92.

86. Tournigand C, Cervantes A, Figer A, Lledo G, Flesch M, Buyse M, et al. OPTIMOX1: a randomized study of FOLFOX4 or FOLFOX7 with oxaliplatin in a stop-and-go fashion in advanced colorectal cancer—a GERCOR study. J Clin Oncol. 2006;24:394-400.

87. Rubbia-Brandt L, Audard V, Sartoretti P, Roth A, Brezault C, Le Charpentier M, et al. Severe hepatic sinusoidal obstruction associated with oxaliplatin-based chemotherapy in patients with metastatic colorectal cancer. Ann Oncol. 2004;15:460-6.

88. Kohn S, Fradis M, Podoshin L, Ben-David J, Zidan J, Robinson E. Endothelial injury of capillaries in the stria vascularis of guinea pigs treated with cisplatin and gentamicin. Ultrastruct Pathol 1997;21:289-99.

89. Kohn S, Fradis M, Ben-David J, Zidan J, Robinson E. Nephrotoxicity of combined treatment with cisplatin and gentamicin in the guinea pig: glomerular injury findings. Ultrastruct Pathol. 2002;26:371-82.

90. Dursun B, He Z, Somerset H, Oh D-J, Faubel S, Edelstein CL. Caspases and calpain are independent mediators of cisplatin-induced endothelial cell necrosis. Am J Physiol Renal Physiol 2006;291:F578-F87.

91. Yu M, Han J, Cui P, Dai M, Li H, Zhang J, et al. Cisplatin up-regulates ICAM-1 expression in endothelial cell via a NF-κB dependent pathway. Cancer Sci 2008;99:391-7.

92. Shi Y, Inoue S, Shinozaki R, Fukue K, Kougo T. Release of cytokines from human umbilical vein endothelial cells treated with platinum compounds in vitro. Cancer Sci 1998;89:757-67.

93. Eguchi R, Fujimori Y, Ohta T, Kunimasa K, Nakano T. Calpain is involved in cisplatin-induced endothelial injury in an in vitro three-dimensional blood vessel model. Int J Oncol 2010;37:1289-96.

94. Zhu M, Chen J, Yin H, Jiang H, Wen M, Miao C. Propofol protects human umbilical vein endothelial cells from cisplatin-induced injury. Vasc Pharmacol. 2014;61:72-9.

95. Soultati A, Mountzios G, Avgerinou C, Papaxoinis G, Pectasides D, Dimopoulos M-A, et al. Endothelial vascular toxicity from chemotherapeutic agents: preclinical evidence and clinical implications. Cancer Treat Rev. 2012;38:473-83.

96. Ito H, Okafuji T, Suzuki T. Vitamin E prevents endothelial injury associated with cisplatin injection into the superior mesenteric artery of rats. Heart Vessels. 1995;10:178-84.

97. Screnci D, McKeage MJ. Platinum neurotoxicity: clinical profiles, experimental models and neuroprotective approaches. J Inorg Biochem. 1999;77:105-10.

98. Forman AD. Peripheral neuropathy and cancer. Curr Oncol Rep. 2004;6:20-5.

99. Albers JW, Chaudhry V, Cavaletti G, Donehower RC. Interventions for preventing neuropathy caused by cisplatin and related compounds. Cochrane Database Syst Rev. 2014.

100. Amptoulach S, Tsavaris N. Neurotoxicity caused by the treatment with platinum analogues. Chemother Res Pract. 2011;2011.

101. Han Y, Smith MT. Pathobiology of cancer chemotherapy-induced peripheral neuropathy (CIPN). Front Pharmacol. 2013;4:156.

102. Rezaee R, Momtazi AA, Monemi A, Sahebkar A. Curcumin: a potentially powerful tool to reverse cisplatin-induced toxicity. Pharmacol Res. 2017;117:218-27.

103. Epstein J, Sanderson IR, MacDonald TT. Curcumin as a therapeutic agent: the evidence from in vitro, animal and human studies. Br J Nutr. 2010;103:1545-57.

104. Trujillo J, Molina-Jijón E, Medina-Campos ON, Rodríguez-Muñoz R, Reyes JL, Loredo ML, et al. Curcumin prevents cisplatin-induced decrease in the tight and adherens junctions: relation to oxidative stress. Food funct. 2016;7:279-93.

105. Howells LM, Mitra A, Manson MM. Comparison of oxaliplatin-and curcumin-mediated

antiproliferative effects in colorectal cell lines. Int J Cancer 2007;121:175-83.

106. Hussain Y, Islam L, Khan H, Filosa R, Aschner M, Javed S. Curcumin–cisplatin chemotherapy: A novel strategy in promoting chemotherapy efficacy and reducing side effects. Phytother Res. 2021;35:6514-29.

107. Arbiser JL, Klauber N, Rohan R, Van Leeuwen R, Huang M-T, Fisher C, et al. Curcumin is an in vivo inhibitor of angiogenesis. Mol Med. 1998;4:376.

108. Binion DG, Otterson MF, Rafiee P. Curcumin inhibits VEGF mediated angiogenesis in human intestinal microvascular endothelial cells through COX-2 and MAPK inhibition. Gut. 2008.

109. Comín-Anduix B, Boren J, Martinez S, Moro C, Centelles JJ, Trebukhina R, et al. The effect of thiamine supplementation on tumour proliferation: a metabolic control analysis study. Eur J Biochem. 2001;268:4177-82.

110. Hanberry BS, Berger R, Zastre JA. High-dose vitamin B1 reduces proliferation in cancer cell lines analogous to dichloroacetate. Cancer Chemother. Pharmacol. 2014;73:585-94.

111. Tjokroprawiro A. Review Article and Clinical Experience: EMERGING MULTIPLE PROPERTIES OF HIGH DOSE THIAMINE AND B6-B12 VITAMINS: Therapeutic Possibilities for Diabetic Vascular Complications. Folia Med (Plovdiv). 2009;45:165.

112. Garg MB, Ackland SP. Pyridoxine to protect from oxaliplatin-induced neurotoxicity without compromising antitumour effect. Cancer Chemother Pharmacol. 2011;67:963-6.

113. Thakkar K, Billa G. Treatment of vitamin B12 deficiency–methylcobalamine? cyancobalamine? hydroxocobalamin?—clearing the confusion. Eur J Clin Nutr. 2015;69:1.

114. Volkov I. The master key effect of vitamin B12 in treatment of malignancy–A potential therapy? Med Hypotheses. 2008;70:324-8.

115. Becker K, Kienecker E-W, Dick P. A contribution to the scientific assessment of degenerative and regenerative processes in peripheral nerve fibers following axonotmesis under the systemic administration of vitamins B1, B6 and B12-light and electron microscopy findings in the saphenous nerve of the rabbit. Neurochirurgia. 1990;33:113-21.

116. Liu Y-W, Liu C-T, Su Y-L, Tsai M-Y. A Narrative Review of Complementary Nutritional Supplements for Chemotherapy-induced Peripheral Neuropathy. Altern Ther Health Med. 2020;26.

117. Hershman DL, Lacchetti C, Loprinzi CL. Prevention and management of chemotherapy-induced peripheral neuropathy in survivors of adult cancers: American Society of Clinical Oncology clinical practice guideline summary. J Oncol Pract. 2014;10:e421-e4.

118. Ubogu EE. The molecular and biophysical characterization of the human blood-nerve barrier: current concepts. J Vasc Res. 2013;50:289-303.

119. Lange C, Storkebaum E, De Almodóvar CR, Dewerchin M, Carmeliet P. Vascular endothelial growth factor: a neurovascular target in neurological diseases. Nat Rev Neurol. 2016;12:439.

120. Weerasuriya A, Mizisin AP. The blood-nerve barrier: structure and functional significance. The Blood-Brain and Other Neural Barriers: Springer; 2011. p. 149-73.

121. Bouldin T, Earnhardt T, Goines N, Goodrum J. Temporal relationship of blood-nerve barrier breakdown to the metabolic and morphologic alterations of tellurium neuropathy. Neurotoxicology. 1989;10:79-89.

122. Yasuda H, Dyck PJ. Abnormalities of endoneurial microvessels and sural nerve pathology in diabetic neuropathy. Neurology. 1987;37:20-.

123. Sano Y, Shimizu F, Nakayama H, Abe M, Maeda T, Ohtsuki S, et al. Endothelial cells constituting blood-nerve barrier have highly specialized characteristics as barrier-forming cells. Cell Struct Funct. 2007;32:139-47.

124. Liu Q, Wang X, Yi S. Pathophysiological changes of physical barriers of peripheral nerves after injury. Front Neurosci. 2018;12:597.

125. Ma X, Zhang H, Pan Q, Zhao Y, Chen J, Zhao B, et al. Hypoxia/Aglycemia-induced endothelial barrier dysfunction and tight junction protein downregulation can be ameliorated by citicoline. PloS one. 2013;8:e82604.

126. Chen C-J, Ou Y-C, Li J-R, Chang C-Y, Pan H-C, Lai C-Y, et al. Infection of pericytes in vitro by Japanese encephalitis virus disrupts the integrity of the endothelial barrier. J Virol. 2014;88:1150-61.

127. Yosef N, Xia RH, Ubogu EE. Development and characterization of a novel human in vitro blood-nerve barrier model using primary endoneurial endothelial cells. J Neuropathol Exp Neurol. 2010;69:82-97.

128. Kanda T, Numata Y, Mizusawa H. Chronic inflammatory demyelinating polyneuropathy: decreased claudin-5 and relocated ZO-1. J Neurol Neurosurg Psychiatry. 2004;75:765-9.

129. Shimizu F, Sano Y, Haruki H, Kanda T. Advanced glycation end-products induce basement membrane hypertrophy in endoneurial microvessels and disrupt the blood–nerve barrier by stimulating the release of TGF-β and vascular endothelial growth factor (VEGF) by pericytes. Diabetologia. 2011;54:1517-26.

จุฬาลงกรณ์มหาวิทยาลัย
CHULALONGKORN UNIVERSITY

130. Shimizu F, Sawai S, Sano Y, Beppu M, Misawa S, Nishihara H, et al. Severity and patterns of blood-nerve barrier breakdown in patients with chronic inflammatory demyelinating polyradiculoneuropathy: correlations with clinical subtypes. PloS one. 2014;9:e104205.

131. Harrell CR, Markovic BS, Fellabaum C, Arsenijevic A, Djonov V, Volarevic V. Molecular mechanisms underlying therapeutic potential of pericytes. J Biomed Sci. 2018;25:21.

132. Armulik A, Genové G, Betsholtz C. Pericytes: developmental, physiological, and pathological perspectives, problems, and promises. Dev Cell. 2011;21:193-215.

133. Gökçinar-Yagci B, Uçkan-Çetinkaya D, Çelebi-Saltik B. Pericytes: properties, functions and applications in tissue engineering. Stem Cell Rev. 2015;11:549-59.

134. Stratman AN, Malotte KM, Mahan RD, Davis MJ, Davis GE. Pericyte recruitment during vasculogenic tube assembly stimulates endothelial basement membrane matrix formation. Blood. 2009;114:5091-101.

135. Shimizu F, Sano Y, Abe Ma, Maeda T, Ohtsuki S, Terasaki T, et al. Peripheral nerve pericytes modify the blood–nerve barrier function and tight junctional molecules through the secretion of various soluble factors. J Cell Physiol. 2011;226:255-66.

136. Shimizu F, Sano Y, Maeda T, Abe Ma, Nakayama H, Takahashi Ri, et al. Peripheral Nerve pericytes originating from the blood–nerve barrier expresses tight junctional molecules and transporters as barrier-forming cells. J Cell Physiol. 2008;217:388-99.

137. van Meerloo J, Kaspers GJL, Cloos J. Cell Sensitivity Assays: The MTT Assay. In: Cree IA, editor. Cancer Cell Culture: Methods and Protocols. Totowa, NJ: Humana Press; 2011. p. 237-45.

Chulalongkorn University

138. Donzelli E, Carfì M, Miloso M, Strada A, Galbiati S, Bayssas M, et al. Neurotoxicity of platinum compounds: comparison of the effects of cisplatin and oxaliplatin on the human neuroblastoma cell line SH-SY5Y. J Neurooncol. 2004;67:65-73.

139. Rao R. Oxidative stress-induced disruption of epithelial and endothelial tight junctions. Front Biosci. 2008;13:7210.

140. Fischer S, Wiesnet M, Renz D, Schaper W. H2O2 induces paracellular permeability of porcine brain-derived microvascular endothelial cells by activation of the p44/42 MAP kinase pathway. Eur J Cell Biol. 2005;84:687-97.

141. Peltonen S, Alanne M, Peltonen J. Barriers of the peripheral nerve. Tissue barriers. 2013;1:e24956.

142. Lum H, Roebuck KA. Oxidant stress and endothelial cell dysfunction. Am J Physiol

Cell Physiol. 2001;280:C719-C41.

143. Rahimi N. Defenders and challengers of endothelial barrier function. Front immunol. 2017;8:1847.

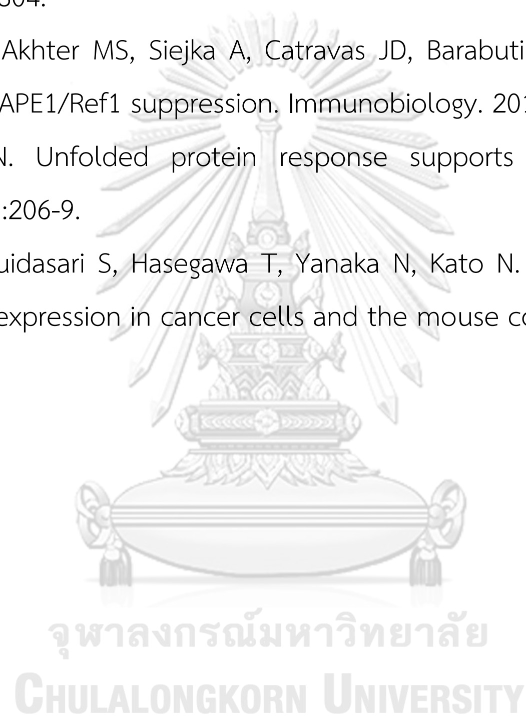
144. Ehmedah A, Nedeljkovic P, Dacic S, Repac J, Draskovic Pavlovic B, Vucevic D, et al. Vitamin B complex treatment attenuates local inflammation after peripheral nerve injury. Molecules. 2019;24:4615.

145. Barabutis N, Dimitropoulou C, Gregory B, Catravas JD. Wild-type p53 enhances endothelial barrier function by mediating RAC 1 signalling and RhoA inhibition. J Cell Mol Med. 2018;22:1792-804.

146. Uddin MA, Akhter MS, Siejka A, Catravas JD, Barabutis N. P53 supports endothelial barrier function via APE1/Ref1 suppression. Immunobiology. 2019;224:532-8.

147. Barabutis N. Unfolded protein response supports endothelial barrier function. Biochimie. 2019;165:206-9.

148. Zhang P, Suidasari S, Hasegawa T, Yanaka N, Kato N. Vitamin B6 activates p53 and elevates p21 gene expression in cancer cells and the mouse colon. Oncol Rep. 2014;31:2371-6.


จุฬาลงกรณ์มหาวิทยาลัย
CHULALONGKORN UNIVERSITY

# APPENDIX

## 1. Cell viability

### 1.1 Effect of cisplatin on HUVEC viability

| Group | % Cell viability (Mean ± SEM) |
|---|---|
| Control | 100.0 ± 4.467 |
| Cisplatin 1.5 μg/ml | 89.26 ± 3.650 |
| Cisplatin 3 μg/ml | 68.39 ± 4.411 |
| Cisplatin 6 μg/ml | 42.68 ± 0.7823 |
| Cisplatin 15 μg/ml | 45.60 ± 3.121 |
| Cisplatin 30 μg/ml | 28.98 ± 1.458 |
| Cisplatin 60 μg/ml | 29.32 ± 1.935 |

### 1.2 Effect of curcumin on HUVEC viability

| Group | % Cell viability (Mean ± SEM) |
|---|---|
| Control | 100.0 ± 2.382 |
| Curcumin 0.1 μg/ml | 99.46 ± 2.986 |
| Curcumin 1 μg/ml | 97.86 ± 2.023 |
| Curcumin 5 μg/ml | 70.46 ± 4.947 |
| Curcumin 10 μg/ml | 33.08 ± 4.424 |
| Curcumin 20 μg/ml | 16.86 ± 1.291 |

### 1.3 Effect of $B_{1-6-12}$ on HUVEC viability

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

| Group | % Cell viability (Mean ± SEM) |
|---|---|
| Control | 100.0 ± 2.535 |
| $B_{1-6-12}$ 1 μg/ml | 108.9 ± 5.016 |
| $B_{1-6-12}$ 5 μg/ml | 106.8 ± 4.694 |
| $B_{1-6-12}$ 10 μg/ml | 107.1 ± 3.858 |
| $B_{1-6-12}$ 20 μg/ml | 107.1 ± 3.894 |
| $B_{1-6-12}$ 40 μg/ml | 109.4 ± 4.862 |
| $B_{1-6-12}$ 80 μg/ml | 109.1 ± 4.023 |
| $B_{1-6-12}$ 160 μg/ml | 109.9 ± 5.103 |

1.4 Effect of oxaliplatin on HUVEC viability

| Group | % Cell viability (Mean ± SEM) |
|---|---|
| Control | 100.0 ± 5.335 |
| Oxaliplatin 1 µg/ml | 96.42 ± 2.799 |
| Oxaliplatin 3 µg/ml | 82.84 ± 3.013 |
| Oxaliplatin 5 µg/ml | 62.17 ± 2.05 |
| Oxaliplatin 10 µg/ml | 61.78 ± 4.513 |
| Oxaliplatin 30 µg/ml | 44.41 ± 3.919 |
| Oxaliplatin 50 µg/ml | 38.23 ± 4.261 |
| Oxaliplatin 80 µg/ml | 33.19 ± 4.518 |
| Oxaliplatin 100 µg/ml | 34.86 ± 5.939 |
| Oxaliplatin 160 µg/ml | 22.90 ± 4.789 |

1.5 Effect of cisplatin on HBVP viability

| Group | % Cell viability (Mean ± SEM) |
|---|---|
| Control | 100.0 ± 0.228 |
| Cisplatin 1 µg/ml | 96.04 ± 1.556 |
| Cisplatin 1.5 µg/ml | 87.01 ± 1.087 |
| Cisplatin 2 µg/ml | 73.93 ± 0.799 |
| Cisplatin 3 µg/ml | 55.08 ± 1.828 |
| Cisplatin 4 µg/ml | 40.10 ± 3.115 |
| Cisplatin 5 µg/ml | 28.49 ± 1.081 |

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

1.6 Effect of curcumin on HBVP viability

| Group | % Cell viability (Mean ± SEM) |
|---|---|
| Control | 100.0 ± 1.146 |
| Curcumin 0.1 µg/ml | 97.81 ± 5.394 |
| Curcumin 1 µg/ml | 106.4 ± 4.103 |
| Curcumin 2 µg/ml | 39.12 ± 3.897 |
| Curcumin 3 µg/ml | 36.88 ± 2.500 |
| Curcumin 5 µg/ml | 31.01 ± 1.620 |

**1.7 Effect of $B_{1-6-12}$ on HBVP viability**

| Group | % Cell viability (Mean ± SEM) |
|---|---|
| Control | 100.0 ± 2.749 |
| $B_{1-6-12}$ 1 µg/ml | 102.4 ± 3.757 |
| $B_{1-6-12}$ 5 µg/ml | 137.9 ± 6.755 |
| $B_{1-6-12}$ 10 µg/ml | 148.7 ± 12.93 |
| $B_{1-6-12}$ 20 µg/ml | 181.4 ± 17.51 |
| $B_{1-6-12}$ 40 µg/ml | 196.0 ± 20.93 |
| $B_{1-6-12}$ 80 µg/ml | 205.0 ± 23.46 |

**1.8 Effect of oxaliplatin on HBVP viability**

| Group | % Cell viability (Mean ± SEM) |
|---|---|
| Control | 100.0 ± 2.251 |
| Oxaliplatin 1 µg/ml | 93.37 ± 4.147 |
| Oxaliplatin 3 µg/ml | 97.75 ± 2.559 |
| Oxaliplatin 5 µg/ml | 95.83 ± 2.963 |
| Oxaliplatin 7.5 µg/ml | 92.31 ± 3.225 |
| Oxaliplatin 8 µg/ml | 77.93 ± 2.305 |
| Oxaliplatin 10 µg/ml | 71.09 ± 4.870 |

**1.9 Effect of co-treatment between cisplatin and curcumin on HUVEC viability**

| Group | % Cell viability (Mean ± SEM) |
|---|---|
| Control | 100.0 ± 2.870 |
| Cisplatin 3 µg/ml | 41.80 ± 3.426 |
| Cisplatin 3 µg/ml + Curcumin 0.1 µg/ml | 51.24 ± 4.917 |
| Cisplatin 3 µg/ml + Curcumin 1 µg/ml | 60.83 ± 3.477 |

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

**1.10 Effect of co-treatment between oxaliplatin and curcumin on HUVEC viability**

| Group | % Cell viability (Mean ± SEM) |
|---|---|
| Control | 100.0 ± 3.34 |
| Oxaliplatin 5 µg/ml | 62.17 ± 2.05 |
| Oxaliplatin + Curcumin 0.1 µg/ml | 71.53 ± 1.61 |
| Oxaliplatin + Curcumin 1 µg/ml | 74.80 ± 1.50 |

**1.11 Effect of co-treatment between cisplatin and curcumin on HBVP viability**

| Group | % Cell viability (Mean ± SEM) |
|---|---|
| Control | 100.0 ± 1.819 |
| Cisplatin 1.5 μg/ml | 80.20 ± 1.505 |
| Cisplatin + Curcumin 0.1 μg/ml | 90.09 ± 2.203 |
| Cisplatin + Curcumin 1 μg/ml | 88.97 ± 1.703 |

**1.12 Effect of co-treatment between oxaliplatin and curcumin on HBVP viability**

| Group | % Cell viability (Mean ± SEM) |
|---|---|
| Control | 100.0 ± 2.085 |
| Oxaliplatin 8 μg/ml | 73.18 ± 5.418 |
| Oxaliplatin + Curcumin 0.1 μg/ml | 82.29 ± 2.436 |
| Oxaliplatin + Curcumin 1 μg/ml | 90.56 ± 6.019 |

**1.13 Effect of co-treatment between cisplatin and $B_{1-6-12}$ on HUVEC viability**

| Group | % Cell viability (Mean ± SEM) |
|---|---|
| Control | 100.00 ± 2.096 |
| Cisplatin 3 μg/ml | 47.03 ± 3.189 |
| Cisplatin + $B_{1-6-12}$ 1 μg/ml | 75.27 ± 4.181 |

**1.14 Effect of co-treatment between oxaliplatin and $B_{1-6-12}$ on HUVEC viability**

| Group | % Cell viability (Mean ± SEM) |
|---|---|
| Control | 100.00 ± 3.34 |
| Oxaliplatin 5 μg/ml | 62.17 ± 2.05 |
| Oxaliplatin + $B_{1-6-12}$ 1 μg/ml | 68.32 ± 2.04 |

จุฬาลงกรณ์มหาวิทยาลัย
CHULALONGKORN UNIVERSITY

**1.15 Effect of co-treatment between cisplatin and $B_{1-6-12}$ on HBVP viability**

| Group | % Cell viability (Mean ± SEM) |
|---|---|
| Control | 100.00 ± 1.821 |
| Cisplatin 1.5 μg/ml | 80.76 ± 1.503 |
| Cisplatin + $B_{1-6-12}$ 1 μg/ml | 95.05 ± 0.915 |
| Cisplatin + $B_{1-6-12}$ 5 μg/ml | 90.89 ± 2.029 |
| Cisplatin + $B_{1-6-12}$ 10 μg/ml | 92.10 ± 4.038 |

1.16 Effect of co-treatment between oxaliplatin and $B_{1-6-12}$ on HBVP viability

| Group | % Cell viability (Mean ± SEM) |
|---|---|
| Control | 100.0 ± 0.2557 |
| Oxaliplatin 8 μg/ml | 69.16 ± 4.092 |
| Oxaliplatin 8 μg/ml + $B_{1-6-12}$ 1 μg/ml | 79.57 ± 1.346 |
| Oxaliplatin 8 μg/ml + $B_{1-6-12}$ 5 μg/ml | 74.88 ± 2.809 |
| Oxaliplatin 8 μg/ml + $B_{1-6-12}$ 10 μg/ml | 74.91 ± 4.774 |

1.17 Effect of co-treatment of cisplatin, curcumin, and $B_{1-6-12}$ on HUVEC viability

| Group | % Cell viability (Mean ± SEM) |
|---|---|
| Control | 100.0 ± 2.122 |
| Cisplatin 3 μg/ml | 41.80 ± 3.426 |
| Cisplatin + Curcumin 1 μg/ml | 60.83 ± 3.477 |
| Cisplatin + $B_{1-6-12}$ 1 μg/ml | 75.27 ± 4.181 |
| Cisplatin + Curcumin +$B_{1-6-12}$ | 89.87 ± 2.572 |

1.18 Effect of co-treatment of oxaliplatin, curcumin, and $B_{1-6-12}$ on HUVEC viability

| Group | % Cell viability (Mean ± SEM) |
|---|---|
| Control | 100.0 ± 3.344 |
| Oxaliplatin 5 μg/ml | 62.17 ± 2.052 |
| Oxaliplatin + Curcumin 1 μg/ml | 74.80 ± 1.499 |
| Oxaliplatin + $B_{1-6-12}$ 1 μg/ml | 68.32 ± 2.043 |
| Oxaliplatin + Curcumin +$B_{1-6-12}$ | 79.77 ± 3.796 |

จุฬาลงกรณ์มหาวิทยาลัย
CHULALONGKORN UNIVERSITY

1.19 Effect of co-treatment of cisplatin, curcumin, and $B_{1-6-12}$ on HBVP viability

| Group | % Cell viability (Mean ± SEM) |
|---|---|
| Control | 100.0 ± 1.849 |
| Cisplatin 1.5 μg/ml | 76.99 ± 1.792 |
| Cisplatin + Curcumin 1 μg/ml | 88.97 ± 1.703 |
| Cisplatin + $B_{1-6-12}$ 1 μg/ml | 95.05 ± 0.915 |
| Cisplatin + Curcumin +$B_{1-6-12}$ | 91.16 ± 1.714 |

1.20 Effect of co-treatment of oxaliplatin, curcumin, and $B_{1\text{-}6\text{-}12}$ on HBVP viability

| Group | % Cell viability (Mean ± SEM) |
| --- | --- |
| Control | 100.0 ± 1.025 |
| Oxaliplatin 8 µg/ml | 80.80 ± 1.795 |
| Oxaliplatin + Curcumin 1 µg/ml | 90.56 ± 6.019 |
| Oxaliplatin + $B_{1\text{-}6\text{-}12}$ 1 µg/ml | 79.57 ± 1.346 |
| Oxaliplatin + Curcumin + $B_{1\text{-}6\text{-}12}$ | 93.34 ± 2.167 |

2. Caspase-3 level

2.1 Effect of cisplatin treatment induced caspase-3 on HUVEC

| Group | Caspase-3 level (Mean ± SEM) |
| --- | --- |
| Control | 1.000 ± 0.0344 |
| Cisplatin 3 µg/ml | 1.344 ± 0.0433 |
| Cisplatin + Curcumin 1 µg/ml | 1.249 ± 0.0745 |
| Cisplatin +$B_{1\text{-}6\text{-}12}$ 1 µg/ml | 1.205 ± 0.0927 |
| Cisplatin + Curcumin +$B_{1\text{-}6\text{-}12}$ | 1.229 ± 0.0851 |

2.2 Effect of oxaliplatin treatment induced caspase-3 on HUVEC

| Group | Caspase-3 level (Mean ± SEM) |
| --- | --- |
| Control | 1.000 ± 0.0344 |
| Oxaliplatin 5 µg/ml | 1.480 ± 0.0468 |
| Oxaliplatin + Curcumin 1 µg/ml | 1.368 ± 0.0467 |
| Oxaliplatin +$B_{1\text{-}6\text{-}12}$ 1 µg/ml | 1.353 ± 0.0524 |
| Oxaliplatin + Curcumin +$B_{1\text{-}6\text{-}12}$ | 1.206 ± 0.0766 |

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

2.3 Effect of cisplatin treatment induced caspase-3 on HBVP

| Group | Caspase-3 level (Mean ± SEM) |
| --- | --- |
| Control | 1.000 ± 0.0298 |
| Cisplatin 1.5 µg/ml | 1.478 ± 0.1139 |
| Cisplatin + Curcumin 1 µg/ml | 1.034 ± 0.1408 |
| Cisplatin + $B_{1\text{-}6\text{-}12}$ 1 µg/ml | 1.239 ± 0.1129 |
| Cisplatin + Curcumin +$B_{1\text{-}6\text{-}12}$ | 1.010 ± 0.1260 |

2.4 Effect of oxaliplatin treatment induced caspase-3 on HBVP

| Group | Caspase-3 level (Mean ± SEM) |
|---|---|
| Control | 1.000 ± 0.0298 |
| Oxaliplatin 8 μg/ml | 1.137 ± 0.0145 |
| Oxaliplatin + Curcumin 1 μg/ml | 1.033 ± 0.0545 |
| Oxaliplatin + $B_{1-6-12}$ 1 μg/ml | 1.130 ± 0.0413 |
| Oxaliplatin + Curcumin +$B_{1-6-12}$ | 1.016 ± 0.0861 |

3. ROS level

3.1 ROS level on HUVEC after treated with cisplatin and curcumin/$B_{1-6-12.}$

| Group | Relative ROS production (Mean ± SEM) |
|---|---|
| Control | 1.000 ± 0.0321 |
| Cisplatin 3 μg/ml | 1.347 ± 0.1051 |
| Cisplatin + Curcumin 1 μg/ml | 1.097 ± 0.0281 |
| Cisplatin + $B_{1-6-12}$ 1 μg/ml | 1.152 ± 0.0446 |
| Cisplatin + Curcumin +$B_{1-6-12}$ | 1.071 ± 0.0422 |

3.2 ROS level on HUVEC after treated with oxaliplatin and curcumin/$B_{1-6-12}$.

| Group | Relative ROS production (Mean ± SEM) |
|---|---|
| Control | 1.000 ± 0.0703 |
| Oxaliplatin 5 μg/ml | 1.521 ± 0.2090 |
| Oxaliplatin + Curcumin 1 μg/ml | 0.764 ± 0.0673 |
| Oxaliplatin + $B_{1-6-12}$ 1 μg/ml | 1.066 ± 0.1476 |
| Oxaliplatin + Curcumin +$B_{1-6-12}$ | 0.886 ± 0.0537 |

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

3.3 ROS level on HBVP after treated with cisplatin and curcumin/$B_{1-6-12}$.

| Group | Relative ROS production (Mean ± SEM) |
|---|---|
| Control | 1.000 ± 0.03274 |
| Cisplatin 1.5 μg/ml | 1.205 ± 0.02543 |
| Cisplatin + Curcumin 1 μg/ml | 0.9250 ± 0.03454 |
| Cisplatin + $B_{1-6-12}$ 1 μg/ml | 1.120 ± 0.04815 |
| Cisplatin + Curcumin +$B_{1-6-12}$ | 0.8726 ± 0.04937 |

3.4 ROS level on HBVP after treated with oxaliplatin and curcumin/$B_{1\text{-}6\text{-}12}$

| Group | Relative ROS production (Mean ± SEM) |
|---|---|
| Control | 1.000 ± 0.03438 |
| Oxaliplatin 8 μg/ml | 1.262 ± 0.04949 |
| Oxaliplatin + Curcumin 1 μg/ml | 0.985 ± 0.04201 |
| Oxaliplatin + $B_{1\text{-}6\text{-}12}$ 1 μg/ml | 1.139 ± 0.04044 |
| Oxaliplatin + Curcumin +$B_{1\text{-}6\text{-}12}$ | 0.9750 ± 0.04864 |

4. GSH level

4.1 GSH level on HUVEC after treated with cisplatin and curcumin/$B_{1\text{-}6\text{-}12}$

| Group | Total GSH (μM) (Mean ± SEM) | GSH (μM) (Mean ± SEM) | GSSG (μM) (Mean ± SEM) | GSH/GSSG |
|---|---|---|---|---|
| Control | 53.24 ± 0.404 | 41.05 ± 0.774 | 12.20 ± 0.465 | 3.400 ± 0.1847 |
| Cisplatin 3 μg/ml | 43.82 ± 1.053 | 27.02 ± 1.174 | 16.80 ± 0.327 | 1.615 ± 0.0899 |
| Cisplatin + Curcumin 1 μg/ml | 47.45 ± 1.777 | 33.66 ±1.870 | 13.79 ± 0.440 | 2.463 ± 0.1808 |
| Cisplatin + $B_{1\text{-}6\text{-}12}$ 1 μg/ml | 45.38 ± 1.716 | 29.94 ± 1.986 | 15.44 ± 0.518 | 1.963 ± 0.1819 |
| Cisplatin + Curcumin +$B_{1\text{-}6\text{-}12}$ | 46.14 ± 1.697 | 30.52 ± 1.618 | 15.62 ± 0.170 | 1.952 ± 0.0958 |

4.2 GSH level on HUVEC after treated with oxaliplatin and curcumin/$B_{1\text{-}6\text{-}12}$

| Group | Total GSH (μM) (Mean ± SEM) | GSH (μM) (Mean ± SEM) | GSSG (μM) (Mean ± SEM) | GSH/GSSG |
|---|---|---|---|---|
| Control | 53.24 ± 0.4036 | 41.05 ± 0.7741 | 12.20 ± 0.465 | 3.400 ± 0.1847 |
| Oxaliplatin 5 μg/ml | 44.59 ± 1.035 | 26.97 ± 0.8656 | 17.62 ± 0.318 | 1.535 ± 0.0452 |
| Oxaliplatin + Curcumin 1 μg/ml | 47.36 ± 1.302 | 33.57 ± 1.284 | 13.79 ± 0.185 | 2.440 ± 0.0975 |
| Oxaliplatin + $B_{1\text{-}6\text{-}12}$ 1 μg/ml | 44.64 ± 1.171 | 27.55 ± 1.224 | 17.08 ± 0.352 | 1.620 ± 0.0860 |
| Oxaliplatin + Curcumin + $B_{1\text{-}6\text{-}12}$ | 45.58 ± 1.324 | 31.24 ± 1.281 | 14.34 ± 0.197 | 2.177 ±0.0901 |

4.3 GSH level on HBVP after treated with cisplatin and curcumin/$B_{1\text{-}6\text{-}12}$

| Group | Total GSH (μM) (Mean ± SEM) | GSH (μM) (Mean ± SEM) | GSSG (μM) (Mean ± SEM) | GSH/GSSG |
|---|---|---|---|---|
| Control | 40.99 ± 0.5703 | 37.94 ± 0.571 | 3.048 ± 0.1702 | 12.66 ± 0.7612 |
| Cisplatin 1.5 μg/ml | 23.03 ± 1.617 | 15.22 ± 1.386 | 7.813 ± 0.3944 | 1.948 ± 0.1539 |
| Cisplatin + Curcumin 1 μg/ml | 30.78 ± 0.829 | 25.66 ± 0.903 | 5.122 ± 0.3702 | 5.170 ± 0.4834 |
| Cisplatin + $B_{1\text{-}6\text{-}12}$ 1 μg/ml | 29.07 ± 2.642 | 21.36 ± 2.522 | 7.710 ± 0.2280 | 2.758 ± 0.2997 |
| Cisplatin + Curcumin + $B_{1\text{-}6\text{-}12}$ | 25.47 ± 4.993 | 20.83 ± 4.704 | 4.638 ± 0.3631 | 4.305 ± 0.7993 |

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

4.4 GSH level on HBVP after treated with oxaliplatin and curcumin/$B_{1-6-12}$

| Group | Total GSH (μM) (Mean ± SEM) | GSH (μM) (Mean ± SEM) | GSSG (μM) (Mean ± SEM) | GSH/GSSG |
|---|---|---|---|---|
| Control | 40.99 ± 0.5703 | 37.94 ± 0.571 | 3.048 ± 0.1702 | 12.66 ± 0.7612 |
| Oxaliplatin 8 μg/ml | 22.20 ± 3.317 | 15.65 ± 2.900 | 6.545 ± 0.4869 | 2.318 ± 0.3141 |
| Oxaliplatin + Curcumin 1 μg/ml | 30.96 ± 1.503 | 26.01 ± 1.261 | 4.947 ± 0.2537 | 5.265 ± 0.0772 |
| Oxaliplatin + $B_{1-6-12}$ 1 μg/ml | 26.97 ± 1.330 | 20.66 ± 0.9792 | 6.322 ± 0.4523 | 3.313 ± 0.1523 |
| Oxaliplatin + Curcumin +$B_{1-6-12}$ | 29.00 ± 3.732 | 24.49 ± 3.486 | 4.513 ± 0.2776 | 5.313 ± 0.5170 |

5. Tight junction protein expression

5.1 Cladin-5 level on HUVEC after treated with cisplatin and curcumin/$B_{1-6-12}$.

| Group | Claudin-5 (Mean ± SEM) |
|---|---|
| Control | 1.0000 ± 0.0000 |
| Cisplatin 3 μg/ml | 1.067 ± 0.1723 |
| Cisplatin + Curcumin 1 μg/ml | 1.523 ± 0.2451 |
| Cisplatin +$B_{1-6-12}$ 1 μg/ml | 1.340 ± 0.3092 |
| Cisplatin + Curcumin +$B_{1-6-12}$ | 1.353 ± 0.1733 |

5.2 Occludin level on HUVEC after treated with cisplatin and curcumin/$B_{1-6-12}$.

| Group | Occludin (Mean ± SEM) |
|---|---|
| Control | 1.0000 ± 0.0000 |
| Cisplatin 3 μg/ml | 0.5133 ± 0.0801 |
| Cisplatin + Curcumin 1 μg/ml | 0.6200 ± 0.2011 |
| Cisplatin +$B_{1-6-12}$ 1 μg/ml | 1.0230 ± 0.1129 |
| Cisplatin + Curcumin +$B_{1-6-12}$ | 0.9500 ± 0.0819 |

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

5.3 ZO-1 level on HUVEC after treated with cisplatin and curcumin/$B_{1-6-12}$.

| Group | ZO-1 (Mean ± SEM) |
|---|---|
| Control | 1.0000 ± 0.0000 |
| Cisplatin 3 μg/ml | 0.8791 ± 0.0906 |
| Cisplatin + Curcumin 1 μg/ml | 1.1940 ± 0.1253 |
| Cisplatin +$B_{1-6-12}$ 1 μg/ml | 1.1100 ± 0.0721 |
| Cisplatin + Curcumin +$B_{1-6-12}$ | 0.9360 ± 0.0787 |

**5.4 ZO-2 level on HUVEC after treated with cisplatin and curcumin/$B_{1-6-12}$.**

| Group | ZO-2 (Mean ± SEM) |
|---|---|
| Control | 1.0000 ± 0.0000 |
| Cisplatin 3 μg/ml | 0.7020 ± 0.0199 |
| Cisplatin + Curcumin 1 μg/ml | 1.1020 ± 0.1499 |
| Cisplatin +$B_{1-6-12}$ 1 μg/ml | 1.0220 ± 0.0697 |
| Cisplatin + Curcumin +$B_{1-6-12}$ | 0.7639 ±0.0838 |

**5.5 Cladin-5 level on HUVEC after treated with oxaliplatin and curcumin/$B_{1-6-12}$.**

| Group | Claudin-5 (Mean ± SEM) |
|---|---|
| Control | 1.0000 ± 0.0000 |
| Oxaliplatin 5 μg/ml | 0.6333 ± 0.2485 |
| Oxaliplatin + Curcumin 1 μg/ml | 1.0570 ± 0.5339 |
| Oxaliplatin +$B_{1-6-12}$ 1 μg/ml | 0.9333 ± 0.4054 |
| Oxaliplatin + Curcumin +$B_{1-6-12}$ | 0.9367 ± 0.3856 |

**5.6 Occludin level on HUVEC after treated with oxaliplatin and curcumin/$B_{1-6-12}$.**

| Group | Occludin (Mean ± SEM) |
|---|---|
| Control | 1.0000 ± 0.0000 |
| Oxaliplatin 5 μg/ml | 0.9867 ± 0.1882 |
| Oxaliplatin + Curcumin 1 μg/ml | 1.1400 ± 0.2488 |
| Oxaliplatin + $B_{1-6-12}$ 1 μg/ml | 1.1030 ± 0.1748 |
| Oxaliplatin + Curcumin +$B_{1-6-12}$ | 1.3200 ± 0.2248 |

จุฬาลงกรณ์มหาวิทยาลัย
CHULALONGKORN UNIVERSITY

**5.7 ZO-1 level on HUVEC after treated with oxaliplatin and curcumin/$B_{1-6-12}$.**

| Group | ZO-1 (Mean ± SEM) |
|---|---|
| Control | 1.0000 ± 0.0000 |
| Oxaliplatin 5 μg/ml | 0.7884 ± 0.0266 |
| Oxaliplatin + Curcumin 1 μg/ml | 0.8593 ± 0.0523 |
| Oxaliplatin +$B_{1-6-12}$ 1 μg/ml | 0.7014 ± 0.0809 |
| Oxaliplatin + Curcumin +$B_{1-6-12}$ | 0.7055 ± 0.0685 |

5.8 ZO-2 level on HUVEC after treated with oxaliplatin and curcumin/$B_{1-6-12}$.

| Group | ZO-2 (Mean ± SEM) |
|---|---|
| Control | 1.0000 ± 0.0000 |
| Oxaliplatin 8 µg/ml | 0.8728 ± 0.1749 |
| Oxaliplatin + Curcumin 1 µg/ml | 0.9193 ± 0.1376 |
| Oxaliplatin +$B_{1-6-12}$ 1 µg/ml | 0.7380 ± 0.0341 |
| Oxaliplatin + Curcumin +$B_{1-6-12}$ | 0.7908 ± 0.0581 |

6. TEER value

6.1 TEER value on HUVEC after treated with cisplatin and curcumin/$B_{1-6-12}$.

| Group | TEER value ($\Omega cm^3$) (Mean ± SEM) |
|---|---|
| Control | 6.000 ± 0.3697 |
| Cisplatin 3 µg/ml | 2.967 ± 0.1900 |
| Cisplatin + Curcumin 1 µg/ml | 4.700 ± 0.4491 |
| Cisplatin +$B_{1-6-12}$ 1 µg/ml | 4.800 ± 0.1155 |
| Cisplatin + Curcumin +$B_{1-6-12}$ | 4.667 ± 0.1394 |

6.2 TEER value on HUVEC after treated with oxaliplatin and curcumin/$B_{1-6-12}$.

| Group | TEER value ($\Omega cm^3$) (Mean ± SEM) |
|---|---|
| Control | 6.000 ± 0.3697 |
| Oxaliplatin 5 µg/ml | 1.567 ± 0.0667 |
| Oxaliplatin + Curcumin 1 µg/ml | 4.967 ± 0.3087 |
| Oxaliplatin + $B_{1-6-12}$ 1 µg/ml | 2.333 ± 0.1302 |
| Oxaliplatin + Curcumin +$B_{1-6-12}$ | 4.033 ± 0.1481 |

6.3 TEER value on HBVP after treated with cisplatin and curcumin/$B_{1-6-12}$.

| Group | TEER value ($\Omega cm^3$) (Mean ± SEM) |
|---|---|
| Control | 9.600 ± 0.2198 |
| Cisplatin 1.5 µg/ml | 3.667 ± 0.1856 |
| Cisplatin + Curcumin 1 µg/ml | 6.500 ± 0.3559 |
| Cisplatin + $B_{1-6-12}$ 1 µg/ml | 8.533 ± 0.1833 |
| Cisplatin + Curcumin +$B_{1-6-12}$ | 8.467 ± 0.2991 |

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

**6.4 TEER value on HBVP after treated with oxaliplatin and curcumin/$B_{1-6-12}$.**

| Group | TEER value ($\Omega cm^3$) (Mean ± SEM) |
|---|---|
| Control | 9.600 ± 0.2198 |
| Oxaliplatin 8 µg/ml | 4.367 ± 0.2186 |
| Oxaliplatin + Curcumin 1 µg/ml | 7.500 ± 0.1915 |
| Oxaliplatin + $B_{1-6-12}$ 1 µg/ml | 4.933 ± 0.1563 |
| Oxaliplatin + Curcumin +$B_{1-6-12}$ | 8.067 ± 0.1986 |

จุฬาลงกรณ์มหาวิทยาลัย
CHULALONGKORN UNIVERSITY

# VITA

| | |
|---|---|
| **NAME** | Amornrat Tothonglor |
| **DATE OF BIRTH** | 16 December 1986 |
| **PLACE OF BIRTH** | Chainat |
| **INSTITUTIONS ATTENDED** | - Bachelor of Science (Physical therapy), Faculty of Allied Health Sciences, Chulalongkorn University, Bangkok, Thailand, 2008.<br>- Masters of Science in Medical Science: anatomy, Faculty of Medicine, Chulalongkorn University, Thailand, 2010. |
| **HOME ADDRESS** | 78 Moo 9 Tambon Chainat, Amphoe Muang, Chainat 17000 |
| **PUBLICATION** | 1. Tothonglor, A., Agthong, S., Huanmanop, T., Chentanez, V., TOTHONGLOR, A., AGTHONG, S., CHENTANEZ, V. (2013). Sartorial branch of saphenous nerve: anatomical relationship with bony landmarks and great saphenous vein. Int. J. Morphol, 31(2), 432-437.<br>2. Phoglin, M., Bangsumruj, J., Choombuathong, A., Sanguansit, P., & Tothonglor, A. (2019). On the Relationship among Age, Sex, Body Mass Index and Audiovisual Reaction Time in Normal Thai Subjects-ความ สัมพันธ์ ระหว่าง อายุ เพศ ค่า ดัชนี มวล กาย และ ค่า เวลา ปฏิกิริยา การ ตอบ สนอง ต่อ แสง และ เสียง ของ อาสา สมัคร คน ไทย ปกติ. Journal of Health Science-วารสาร วิชาการ สาธารณสุข, 28(1), 56-67.<br>3. Choombuathong, A., Chaitiamwong, R., Sanguansit, P., & Tothonglor, A. (2017). Effect of Aerobic Exercise on Reaction Time and Memory in the Elderly-ผล ของ การ ออกกำลัง กาย แบบ แอโรบิค ต่อ ปฏิกิริยา ตอบ สนอง และ ความ จำ ใน ผู้ สูงอายุ. Journal of Health Science-วารสาร วิชาการ สาธารณสุข, 24(2), 283-295. |

จุฬาลงกรณ์มหาวิทยาลัย
CHULALONGKORN UNIVERSITY