ผลของเควอซิตินต่อความผิดปกติของไทท์จังชันที่ถูกเหนี่ยวนำด้วยไฮโดรเจนเปอร์ออกไซด์

นางสาว สมฤดี ชื่นกิติญานนท์

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
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EFFECT OF QUERCETIN ON TIGHT JUNCTION DISRUPTION INDUCED BY HYDROGEN PEROXIDE

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A Dissertation Submitted in Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy Program in Pharmacology
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Thesis Title	EFFECT OF QUERCETIN ON TIGHT JUNCTION DISRUPTION INDUCED BY HYDROGEN PEROXIDE
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สมฤดี ชื่นกิติญานนท์ : ผลของเควอซิตินต่อความผิดปกติของไทท์จังชันที่ถูกเหนี่ยวนำด้วยไฮโดรเจนเปอร์ออกไซด์. (EFFECT OF QUERCETIN ON TIGHT JUNCTION DISRUPTION INDUCED BY HYDROGEN PEROXIDE) อ. ที่ปรึกษาวิทยานิพนธ์หลัก: ผศ. ดร. สุรีย์ เจียรณ์มงคล, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม : รศ. ดร. ธิติมา เพ็งสุภาพ, 99 หน้า.

ภาวะเครียดออกซิเดชันเป็นสาเหตุหนึ่งที่ทำให้เอนโดทีเลียมสูญเสียหน้าที่ในการเป็นเยื่อเลือกผ่านของสารจากภายนอกเข้าสู่ระบบอวัยวะ ทั้งนี้ได้มีรายงานการศึกษาที่แสดงให้เห็นว่าภาวะเครียดออกซิเดชันมีผลกระทบต่อการแสดงออกและการกระจายตัวของไทท์จังชันโปรตีน ทำให้การซึมผ่านเยื่อดังกล่าวเพิ่มขึ้น ในงานวิจัยนี้ได้ศึกษาผลของเควอซิตินซึ่งเป็นสารในกลุ่มฟลาโวนอยด์ที่มีฤทธิ์ต้านอนุมูลอิสระต่อความเสียหายของไทท์จังชันที่ถูกทำลายด้วยไฮโดรเจนเปอร์ออกไซด์ในแบบจำลองเยื่อเลือกผ่านจากเซลล์ ECV304 ในการศึกษานี้เซลล์ ECV304 ได้รับเควอซิตินที่ความเข้มข้น 10 ไมโครโมลาร์เป็นเวลา 30 นาทีก่อนที่จะได้รับไฮโดรเจนเปอร์ออกไซด์ที่ความเข้มข้น 100 ไมโครโมลาร์ในเวลา 4 ชั่วโมงต่อมาทำการตรวจวัดความแน่นของไทท์จังชันโดยวัดการเปลี่ยนแปลงของค่าความต้านทานไฟฟ้าของเยื่อเลือกผ่าน (TEER) และการเคลื่อนที่ของ phenol red ผ่านช่องว่างระหว่างเซลล์ตลอดจนวัดการแสดงออกและการกระจายตัวของไทท์จังชันโปรตีนด้วยวิธี western blot และ immunofluorescence ซึ่งผลการศึกษาพบว่าไฮโดรเจนเปอร์ออกไซด์ (100 ไมโครโมลาร์, 4 ชั่วโมง) ไม่ก่อให้เกิดความเป็นพิษต่อเซลล์ แต่ทำให้การเคลื่อนที่ของ phenol red ผ่านช่องว่างระหว่างเซลล์เพิ่มขึ้นในขณะที่ความต้านทานไฟฟ้าของเยื่อเลือกผ่านมีค่าลดลง ทั้งนี้เควอซิติน (10 μM) ที่เซลล์ได้รับก่อนได้รับไฮโดรเจนเปอร์ออกไซด์ 30 นาทีนั้น สามารถป้องกันผลของไฮโดรเจนเปอร์ออกไซด์ดังกล่าวได้ นอกจากนี้เควอซิตินสามารถป้องกันการลดลงของโปรตีน occludin และ ZO-1 บริเวณขอบเซลล์จากการได้รับไฮโดรเจนเปอร์ออกไซด์ได้อย่างมีนัยสำคัญ นอกจากนั้นการออกฤทธิ์ของเควอซิตินในการป้องกันความเสียหายของไทท์จังชันยังอาจเกี่ยวข้องกับการทำงานของเอนไซม์ MAP kinase โดยเฉพาะอย่างยิ่ง phosphorylated p38 และการสังเคราะห์ไนตริกออกไซด์ การศึกษานี้จึงสรุปได้ว่าเควอซิตินสามารถป้องกันผลของไฮโดรเจนเปอร์ออกไซด์ ต่อความแน่นของเยื่อเลือกผ่านเซลล์ ECV304 ได้โดยป้องกันการลดการแสดงออกของโปรตีนที่เป็นองค์ประกอบของไทท์จังชันผ่านทางกลไกที่เกี่ยวข้องกับ MAPK pathway

สาขาวิชา เภสัชวิทยา ลายมือชื่อนิสิต .....

ปีการศึกษา 2551 ลายมือชื่ออ.ที่ปรึกษาวิทยานิพนธ์หลัก .....

ลายมือชื่ออ.ที่ปรึกษาวิทยานิพนธ์ร่วม .....

4789685420 : MAJOR PHARMACOLOGY

KEYWORDS : QUERCETIN / TIGHT JUNCTION DISRUPTION/H_2O_2

SOMRUDEE CHUENKITIYANON : EFFECT OF QUERCETIN ON TIGHT JUNCTION DISRUPTION INDUCED BY HYDROGEN PEROXIDE. ADVISOR : ASST. PROF. SUREE JIANMONGKOL, Ph.D., CO-ADVISOR : ASSOC. PROF. THITIMA PENGSUPARP, Ph.D., 99 pp.

Oxidative stress has been identify as a major cause of endothelium dysfunction and loss in vascular protective barrier against xenobiotics. Several studies showed that oxidative stress affected the expression and distribution of tight junction proteins, leading to tight junction disruption and increase in permeability. The proposed of this study were to investigate the effect of quercetin, a known antioxidant flavonoid, on the H_2O_2-mediated disruption of tight junction. ECV304 cells were pretreated with 10 μM quercetin for 30 min prior to the addition of 100 μM H_2O_2. After the incubation period of 4 hr, the integrity of tight junction was evaluated through an alteration of TEER values and paracellular permeability of phenol red. In addition, the expression and localization of tight junction proteins were determined with western blot and immunofluorescent techniques. In this study, exposure to 100 μM H_2O_2 for 4 hr markedly increased the paracellular permeability and decreased TEER value without an observable effect on cell viability. Pretreatment the cells with quercetin prevented H_2O_2-induced hyperpermeability as well as a loss of TEER value. The disruption of occludin and ZO-1 at the cell border was not observed in the quercetin pretreatment group. In addition, pretreatment with quercetin could significantly prevent the H_2O_2-induced reduction of expression of these two tight junction proteins, Furthermore, experiments with anti-MAP kinases activities revealed that the protective effect of quercetin against H_2O_2-induced tight junction disruption was involved with inhibition of phosphorylated p38 MAP activity along with NO production. In conclusion, quercetin could preserve the barrier integrity of ECV304 upon challenging with H_2O_2. The results suggested that quercetin was able to prevent the loss of tight junction protein expression as well as its assembly through the mechanisms involved with MAPK pathways.

Field of Study : Pharmacology Student's Signature : .....

Academic Year : 2008 Advisor's Signature : .....

Co-Advisor's Signature : .....

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LIST OF ABBREVIATIONS

%	Percentage
°C	Degree Celsius
µg	Microgram (s)
µl	Microlitre (s)
$\bullet O^{2-}$	Superoxide
BBMEC	Bovine brain microvessel endothelial cells
BPAEC	Bovine pulmonary artery endothelial cells
BSA	Bovine serum albumin
Ca^{2+}	Calcium ion
Caco-2 cell	Colorectal adenocarcinoma cell
Cd	Cadmium
CEC	Choroidal endothelial cells
cGMP	Cyclic guanosine 3',5'-cyclic monophosphate
DCF	2',7'-dichlorofluorescein
DCFH-DA	2',7'-dichlorofluorescein diacetate
DMSO	Dimethyl sulfoxide
ECV304	Human bladder carcinoma cell
EDTA	Ethylenediamine tetraacetic acid
EGF	Epidermal growth factor
ERK	Extracellular signal-regulated kinase
ERS®	Electrical resistance system
et al.	*et alii*, and others
FBS	Fetal bovine serum
g	Gram
GMP	Guanosine 3',5'-cyclic monophosphate
Grb2	Growth factor receptor-bound protein 2
GSH	Glutathion
GTP	Guanosine 5'-triphosphate

H_2O_2	Hydrogen peroxide
HCE	Human corneal epithelial cells
HCl	Hydrochloric acid
hr	Hour
HRP	Horseradish peroxidase
HSP27	Heat shock protein 27
HUVEC	Human vascular endothelium cell
IFN-α	Interferon gramma
IgG	Immunoglobulin
IL	Interleukin
iNOS	Inducible nitric oxide synthase
JAM	Junctional adhesion molecule
JNK	c-Jun NH_2-terminal kinase
kDa	Kilodalton
L	Liter
LDH	Lactase dehydrogenase
LLC-PK1	Porcine kidney cell line
MAGUK	Membrane-associated guanylate kinase-like protein
MAPK	Mitogen activated protein kinase
MDCK	Madin-Darby canine kidney
mg	Milligram (s)
min	Minute (s)
ml	Mililitre (s)
mM	Milimolar
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
MyD88	Myeloid differentiation primary response gene 88
N	Normality

NAC	N-acetylcysteine
NaCl	Sodium chloride
NaF	Sodium fluoride
NaOH	Sodium hydroxide
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cell
nm	Nanometer
nM	Nanomolar
NO	Nitric oxide
PBS	Phosphate-buffer saline
PC12 cells	Transplatable rat pheochromocytoma
pH	The negative logarithm of hydrogen ion concentration
PKA	Protein kinase A
PKC	Protein kinase C
PLC	Phospholipase C
PMA	Phorbol 12-myristate 13-acetate
pmol	Picomole
PVDF	Polyvinylidine fluoride
Rho	Rhomboid protein
ROS	Reactive oxygen species
rpm	Revolutions per minute
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
sec	Second
SH3	The Src homology 3 domain
smGP	Small G-protein
SOD	Superoxide dismutase
TBS	Tris-buffered saline
TBST	Tris-buffered saline, 0.1%Tween 20

TEER	Transcellular endothelial electrical resistance
VEGF	Vascular endothelial growth factor
VEGFR	Vascular endothelial growth factor receptor
ZO-1	Zonular occludens
ZONAB	ZO-1-associated nucleic acid binding protein

CHAPTER I

INTRODUCTION

Hydrogen peroxide (H_2O_2) was the most stable and long lasting member of the reactive oxygen species (ROS) (Channon, 2004). It could cause cellular damage, leading to cell death via mechanism of apoptosis and necrosis (Ueda and Shah, 1992; Takeda *et al*., 1999). H_2O_2 had been reported to decrease the activity of membrane transporters (Andréoli *et al*., 1994) and to disrupt the tight junction barrier (Ying and Harold, 1998; Vepa *et al*., 1999). Consequently, treatment of H_2O_2 resulted in a reduction of transendothelial electrical resistance (TEER) value and an increase of transcellular permeability.

Tight junction was the crucial element of endothelium and epithelium barrier in preventing the invasion of xenobiotics and blood-borne pathogens into an organ system. Tight junction was constructed from four major types of proteins including occludin, claudin, junctional adhesion molecule (JAM), and zonular occudens (ZO) (Figure 1). These proteins interacted with each other to form and maintain the structural integrity of tight junction. Occludin and claudin were structural proteins, which connected to actin cytoskeleton through ZO proteins to form tight junction. In addition, the interaction between JAM, ZO-1, and another protein such as cingulin provided the tightness of the junction. The formation of tight junction sealed the gap between cells, and consequently restricted the transport of any ions and particles through paracellular pathway.

Oxidative stress could cause the disruption of tight junction via several mechanisms. For example, it had been reported that ROS caused an increase of intracellular Ca^{2+} and activated its consequential signaling cascade such as nitric oxide (NO) and protein kinase C (PKC) (Tiruppathi *et al*., 2003; Siflinger-Birnboim *et al*., 1996). An increase of NO production activated NF-κB and vascular endothelial growth factor (VEGF) expression. Consequently, an increase in cell proliferation and the disintegration of tight junction were observed (Chua *et al*., 1998). In addition, H_2O_2 induced an alteration of endothelial cell morphology from cobblestone to elongation, suggesting its interference on actin cytoskeleton.

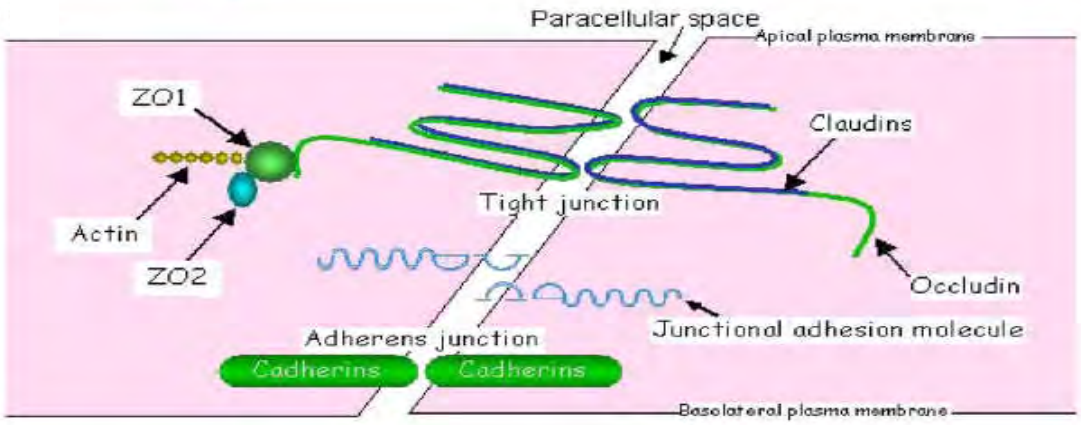


Figure 1: Tight junction proteins and their arrangement

Disruption of tight junction integrity could occur from a loss of expression of tight junction proteins or disorganization of the structure. For example, it was demonstrated that a decrease of occludin protein was responsible for a barrier leakage and loss of TEER values in BPAEC (bovine pulmonary artery endothelial cells) treated with H_2O_2 (Kevil *et al.,* 1998). The disruption of tight junction had been related to a number of signaling molecules as well as protein kinase family such as mitogen-activated protein kinase (MAPK), extracellular signal-regulated kinase (ERK or p44/42 MAPK), c-Jun NH_2-terminal kinase (JNK) and p38 kinases (Fialkow *et al.*,1994; Guyton *et al.*, 1996). An alteration of kinase family in H_2O_2-induced tight junction disruption was still inconclusive. Some studies suggested that p38 MAPK, but not ERK or JNK, played a regulatory role in barrier dysfunction of microvascular endothelial cells upon H_2O_2 treatment (Usatyuk and Natarajan, 2004). However, it has been reported that the phosphorylation of ERK1/2 led to an increase in endothelial permeability in response to H_2O_2 (Kevil *et al.*, 2000).

The disintegration of tight junction structure was a major loss of the control over paracellular transport, leading to a leakage of endothelium or epithelium barrier. Consequently, it compromised the defense mechanism of the organ. Moreover, the disruption of tight junction had been well-correlated to several pathologic states such as edema, jaundice, diarrhea, and blood-borne metastasis. Hence, a compound with ability to protect tight junction integrity would be beneficial to reduce the potential threat to the organ system and preserve a normal physiological state of blood-organ barrier.

Flavonoids are a large group of natural polyphenolic compounds found in many fruits, vegetables, and beverages (Hertog *et al*., 1992). Several experiments had demonstrated the preventive effects of flavonoids against coronary heart disease (Knekt *et al*., 1996), stroke (Keli *et al.,* 1996) and cancers (Weber *et al*., 1996). Moreover, several studies had shown that various flavonoids could inhibit MAP kinase pathway, reduce cell proliferation, and inactivate NF-κB (Formica and Regelson, 1995). Quercetin is one of the most widely distributed flavonoids in the plant kingdom. Quercetin was able to inhibit MAP kinases via suppression of tyrosine phosphorylation and intervention in downstream signaling processes (Formica and Regelson, 1995; Miura *et al*., 1998).

Taken together, it could be hypothesized that quercetin was able to prevent the disintegration of tight junction from an exposure to H_2O_2. In this study, H_2O_2 at non-cytotoxic concentration could induce the disintegration of tight junction structure through activation of MAPK cascade. The potential inhibitory effect of quercetin on these kinase pathways may enable its protective capability against H_2O_2-induced disruption of tight junction. It was possible that quercetin enhanced the expression and localization of tight junction proteins, which had been affected and disorganized by H_2O_2 exposure.

Objectives

1. To study the effect of quercetin on tight junction disruption induced by H_2O_2
2. To elucidate the potential mechanisms of quercetin on tight junction protection under H_2O_2-induced stress condition.

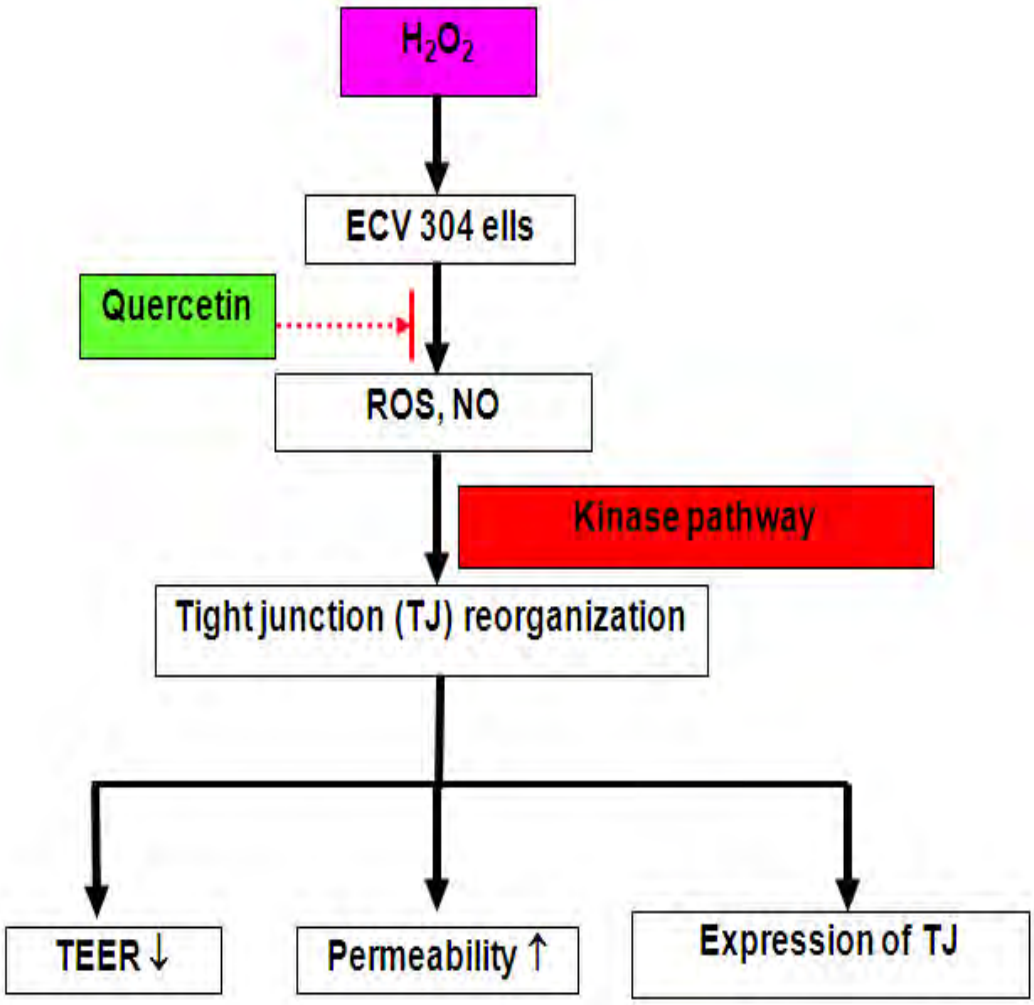
Hypothesis

Quercetin had intrinsic activity to prevent and/or protect the disruption of tight junction barrier which occurred in oxidative stress condition. It was possible that quercetin exerted its action through an alteration of MAP kinase signaling pathway.

Contributions of the study

This study will clarify the mechanisms of quercetin on the protection of tight junction and barrier integrity which may be disrupted in the oxidative stress condition. This information will be helpful in considering the possibility of using quercetin as well as other flavonoids to protect the disruptions of blood brain barrier from oxidative insults.

Conceptual framework



CHAPTER II

LITERATURE REVIEWS

Epithelium was a tissue composed of cells that line up the cavities and surfaces of organ and structures throughout the body. Epithelial tissue on the surfaces of the interior of the body was known as endothelium. Generally, epithelium or endothelium cells were closely packed and arranged in one or more layers. In addition, the adjacent cells also formed special structures including the tight junction to provide the tightness of their bonding (Figure 2). Epithelial tissue, regardless of the type, was usually separated from the underlying tissue by a thin sheet of connective tissue, known as basement membrane. The basement membrane provided structure support for the epithelium and also bound them to neighboring structures. Both, epithelium and endothelium, functioned as protective barriers and metabolic base (Welker *et al*., 2007). These tissues acted as restrictive layers throughout the body involving several physiological processes such as absorption, excretion, secretion. They also prevented an invasion of bacteria and other foreign materials into the body or organ system (Gobe and David, 2007).

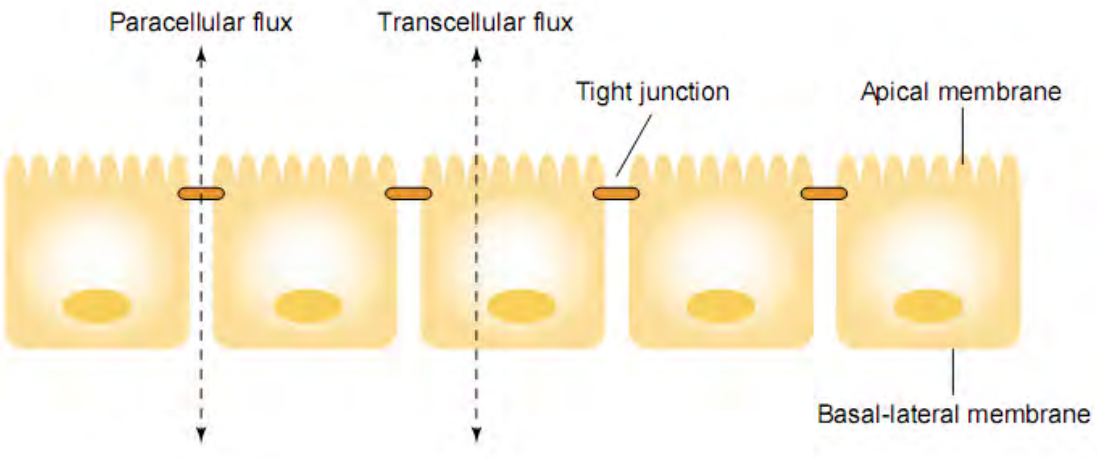


Figure 2: Basic epithelial architecture in simple epithelial monolayer (Jame *et al*., 2005)

The structure of tight junction

The formation of tight junction established the continuous circumferential intercellular contacts between epithelial cells. This structure played a central role in the regulation of permeability and leakage of solutes, water, and immune cells across the epithelial barrier (Tsukita *et al*., 2001). Tight junction was localized toward the apical face of the cell. Its structure formed a branching network of several sealing stands, each of which functions independently from the others. The structural proteins of tight junctions include: (1) integral membrane proteins such as occludin, claudin, and junctional adhesion molecules (JAM); (2) the peripheral proteins of zonular occludens (ZO) family; and (3) other proteins associated with tight junctions (Figure 3) (Brown and Stow, 1996; Ye *et al*., 2003). As shown in Figure 3, tight junction bridged the two adjacent cells together through the connection of its proteins (claudin and occludin) and the actin cytoskeleton. Tight junction performed three vital functions, including held the cells together, blocked the movement of integral membrane proteins between the apical and basolateral surfaces of the cell, and prevented the flux of molecules and ions through the intracellular space between cells (Karen and Thomas, 2002)

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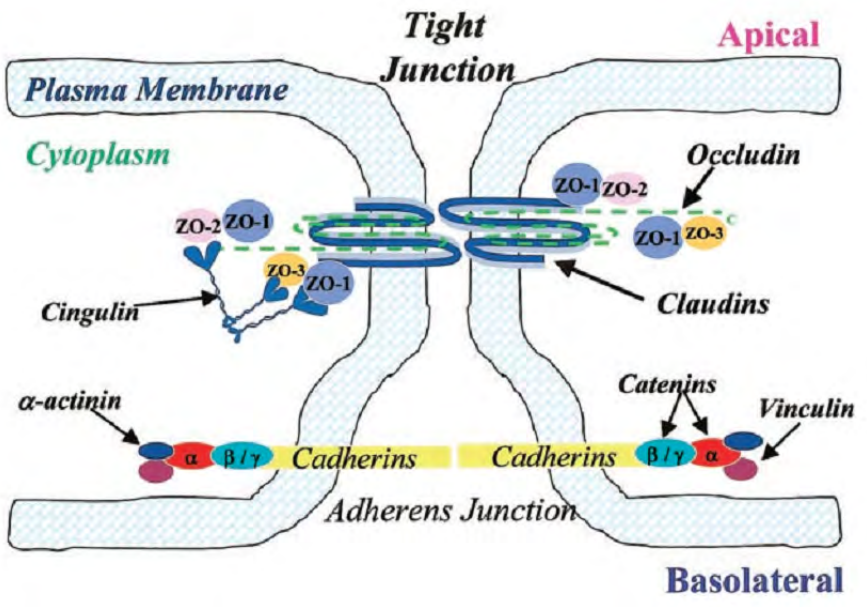


Figure 3: The basic structure of tight junction complex showing its junction proteins as well as its bridging to the cytoskeleton proteins (Karen and Thomas, 2002)

ZO family was a group of cytoplasmic proteins which localize close to the tight junction membrane, apparently forming a continuously scaffolding seal at the intracellular cleft. Z0-1 was the first protein to be identified and cloned within tight junction (Willott *et al*., 1993). Currently, the ZO family contained three members, including ZO-1, ZO-2, and ZO-3. These proteins belonged to a large family of multidomain proteins known as the membrane-associated guanylate kinase homologs (MAGUKs). Their molecular structure contained a core cassette of protein-binding domains including PDZ domains, an SH3 domain, and a region of homology to guanylate kinase which were involved in organizing the structural and functional linkage between transmembrane proteins and the cytoskeleton and signaling pathway (Fanning *et al*., 1996; Lapierre, 2000). ZO-1 associated with protein involved in the regulation of gene transcription and cell proliferation such as the transcription factors, ZONAB, Jun, and Fos. Furthermore, there were indications that phosphorylation of ZO-1 was important for junction regulation (Tsukamoto and Nigam, 1999).

Occludin (Figure 4), contained four membrane-spanding regions and had two extracellular loops, which a short amino-terminal domain and a long carboxyl-terminal domain oriented into the cytoplasm. Occludin linked claudins together with tight junction accessory proteins including ZO-1 and ZO-2, as well as the plasma membranes of adjacent cells. This 60-65 kDa phosphoprotein was important for the regulation of paracellular permeability (Balda *et al*., 2000; Huber *et al*., 2001). It also regulated transepithelial migration of neutrophils, which process was independent of transepithelial resistance (Huber *et al*., 2001). The expression of occludin correlated well with the barrier properties in various tissues. For example, it was highly expressed in brain endothelium, allowing the formation of a very tight blood brain barrier. High expression of occludin was associated with an increase of electrical resistance (tightness) and a decrease of paracellular transport (McCarthy *et al*., 1996; Balda *et al*., 2000). Form *in vivo* study, it was found that the transmigration of neutrophils across brain endothelium induced by interleukin-1β was associated with the loss of occludin and ZO-1 at cell borders (Bolton *et al.,* 1998). Recent data demonstrated that the phosphorylation of occludin regulated the permeability of tight junction in both G-protein-dependent or –

independent manner, depending on the types of activated receptor. However, this phosphorylation process was independent of cytoskeleton changes (Hirase *et al*., 1997).

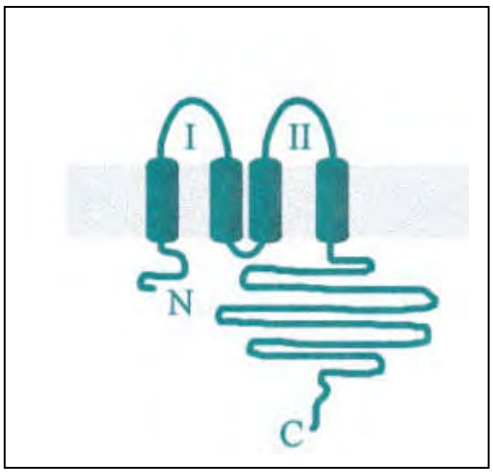


Figure 4: The transmembrane structure of occludin showing four membrane-spanding regions and two extracellular loops (Alan *et al*., 1999).

Occludin and ZO-1 were two well-studied tight junction proteins. Their alterations could reflect the integrity and function of tight junction as well as the barrier functional status. It had been suggested that protein phosphorylation played an important role in tight junction assembly and function (Balda *et al*., 1991; Citi, 1992; Balda *et al*., 1993; Stuart and Nigam, 1995). Furthermore, the consequences of the phosphorylation and dephosphorylation of tight junction proteins could be influenced by the type of stimulus (inflammatory cytokines, oxidative stress, temperature, and growth factors) and by which amino acid residues where phosphorylation occurred (serine, threonine, and tyrosine). The studied of Lechner *et al*. (1999) shown that exposure of LLC-PK1 monolayer to IFN-α resulted in a dose- and time-dependent decrease in TEER and increase of tyrosine phosphorylation and overexpression of occludin. Oxidative stress-induced increase in permeability was associated with Tyrosine-phosphorylation, redistribution from the intercellular junctions of occludin and ZO-1 (Rao *et al.,* 2002). The TEER dropped to about 70% of the initial value after the temperature shift from the non permissive to the permissive. This temperature shift facilitated the tyrosine phosphorylation of the ZO-1 and ZO-2 (Takeda and Tsukita, 1995). In addition, epidermal growth factor (EGF) treatment of A431 human epidermal carcinoma cells also

resulted in the redistribution and tyrosine phosphorylation of ZO-1 (Van Itallie *et al.,* 1995).

Assembly and modification of the tight junction complexes

Upon changing the physiological and pathological states of epithelial barrier, the structure and function of the tight junction complexes may be affected due to the alteration of intracellular signaling event. There was a number of evidence showing that tight junction proteins underwent rapid changes in expression, subcellular redistribution, and posttranslational modifications, which in turn affected protein-protein interaction (Huber *et al*., 2001; Feldman *et al*., 2005). Consequently, the assembly of the tight junction complexes was disturbed.

The formation of the structure of tight junction complexes involved with several signaling pathways, especially enzymes in the MAPK family. In addition, it had been reported that a number of molecules such as nitric oxide render their influences over the assembly and stability of the structure of tight junction complexes.

The MAPK signaling pathway

Mitogen-activated protein kinases (MAPK) were serine/threonine protein kinases that respond to extracellular stimuli such as growth factors and stress. The MAPK signaling pathways were essential in several cellular responses including signal amplification, specificity determination, and tight regulation of the transmitted signal. The members of MAPK family included extracellular regulated protein kinase 1 and 2 (ERK1/2), c-Jun amino-terminal kinases (JNK1/2/3), p38 kinases (p38α/β/γ/δ), and big MAPK (BMK), also known as ERK5 (Chang and Karin, 2001). The activation of MAPK signaling pathway could be triggered either by a small guanosine 5'-triphosphate (GTP)-binding protein (smGP; Ras family protein) or by an adapter protein such as MyD88, Grb2, and Sch. MAPK were organized in a three-kinase module, which transmits the signal either directly or through a mediator kinase (MAP4K) to the MAPK kinase kinase (MAP3K) level of the cascades (Figure 5).

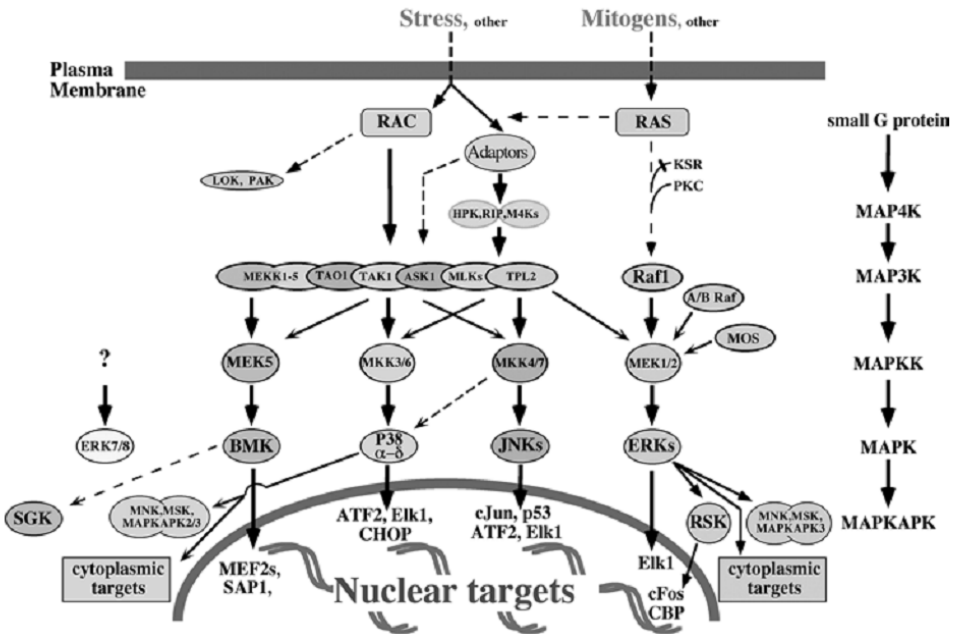


Figure 5: Schematic representation of MAPK signaling pathways.

There were a growing evidence supporting roles of MAPK proteins in cellular function and survival. As known, ERKs were ubiquitous serine/threonine kinases that involved in transduction of externally derived signals regulating cell proliferation, cell growth and differentiation (Schaeffer and Weber, 1999; Pearson *et al*., 2001). ERK1 had been found in epithelial cells to interact with occludin. Basal ERK activity was required for proper organization of the actin cytoskeleton and adherents junctions in PC12 cells. In addition, ERK/MAPK had been shown to involve in the formation and maintain of tight junction structure of epithelial barrier (Chen *et al.,* 2000). Activation of ERK1/2 could lead to the phosphorylation of a wide array of potential targets including cytosolic and cell-surface proteins which may translocalize into the nucleus (Lewis, 1998). JNK and p38-MAPK were other MAPK proteins which ubiquitously expressed and could be activated by stress (Yosimichi *et al*., 2001). The members of MAPK proteins controlled a spectrum of cellular processes including, cell growth, differentiation, transformation, or apoptosis (Waskiewicz and Cooper, 1995). Moreover, inhibition of MEK-1/2 could prevent barrier formation and upregulation of the tight junction protein claudin-2 in epithelial cells (Kinugasa *et al*., 2000).

MAPK signaling pathway had crucial roles in modulation the paracellular transport through tight junction structures by affecting the expression of several tight junction proteins. Consequently, the molecular composition within tight junction complexes could be disturbed. It had been reported that a number of conditions could result in the activation of the MAPK pathway and loss in tight junction assembly. Those conditions included cellular transfection with Ras or Raf, treatment with alcohol, oxidative stress, cytokine, and VEGF (Yuan *et al*., 2004). For example, oxidative assaults caused an increase of paracellular permeability of endothelium barrier, which correlated to an activation of MAPK pathways. In addition, an activation of ERK1/2 in the transfected MDCK with an activated Ras mutant resulted in an increase of the transepithelial permeability of manitol by six-fold, along with a disappearance of occludin from cell-cell contact sites (Chen *et al*., 2000). Application of the specific ERK1/2 inhibitor (PD98059) could also inhibit the increase of permeability and affected the redistribution of occludin (Kevil *et al*., 2000). Treatment of the p38 inhibitor (SB202190) attenuated an increase of solute permeability in HUVEC monolayer treated with H_2O_2 (Kevil *et al.,* 2001). Hence, these data suggested that ERK1/2 and p38 were responsible for the junctional damage induced by oxidative stress.

In addition, the PKC-ε and -δ caused phosphorylation of Raf-1, leading to activation of ERK pathway (Kolch *et al*., 1993; Cai *et al*., 1997). In human corneal epithelial cells (HCE), the activation of PKC isozyme by phorbol 12-myristate 13-acetate (PMA) resulted in ERK phosphorylation and activation which was independent of EGF stimulation (Xu *et al*., 2001). The results of Yuan *et al.* (2004) demonstrated that activation of PKC caused the disruption of tight junction through activation of MAK kinase, and that the MAP kinase signaling pathway played a key role in the regulation of epithelial cell morphology and barrier function in cornea.

Taken together, the MAPK signaling pathway related to the assembly and integrity of tight junction complexes in two major ways. First, the activation of MAPK pathway affected the expression of several tight junction proteins. Secondly, the interaction of tight junction integral protein with other membrane protein required the activation of ERK signaling pathway.

Signaling molecules

Assembly and function of tight junction could be modulated by a number of signaling molecules including cyclic GMP (cGMP), calcium, NF-κB, nitric oxide (NO), and VEGF (Balda *et al*., 1993; Mullin *et al*., 1998; Saha *et al*., 1998). The relationship between those signaling molecules and the modulation of tight junction complexes had been briefly described as follows:

1. **cGMP (guanosine 3',5'-cyclic monophosphate)**

Guanylyl cyclase was an enzyme found in vascular smooth muscle cells and others. This enzyme catalyzes the dephosphorylation of GTP to cGMP. As known, cGMP served as a second messenger for several important cellular functions such as smooth muscle relaxation, regulation of the cardiovascular system, renal fluid retention, bone marrow progenitor cell proliferation (Ignarro, 2002). Its molecular targets include cGMP-gated cation channel and cGMP-regulated phosphodiesterase. In addition, cGMP may involve with the integrity of tight junction structure. It had been shown that an administration of cGMP analogues resulted in an elevation of microvascular permeability, causing a leakage of aminoisobutyric acid in brain cortex cerebral (Chi *et al*., 1999) as well as albumin in rat cerebral microvessels (Joó *et al*., 1983). Furthermore, the reduction of cGMP levels resulted in the tightness of the tight junction barrier (Lee and Cheng, 2003).

2. **Calcium**

Extracellular calcium was first shown to be a critical component of tight junction regulation in models of Ca^{2+} addition/depletion (Palant *et al*., 1983; Gonzalez-Marisca *et al*., 1985). The exposure of epithelial cells to low Ca^{2+} levels compromised the assembly of tight junction, leading to an increased permeability and decreased transepithelial resistance of MDCK and Caco-2 cell monolayers (Balda *et al*., 1991; Said and Ma, 1994). It had been known that alteration of intracellular Ca^{2+} played a pivotal role in regulation of several cellular events such as reorganization of cytoskeleton structure, disassembly of tight junction, increased nitric oxide production. Hence, it is very likely that intracellular Ca^{2+} influenced the assembly and integrity of the tight junction.

Elevation of intracellular Ca^{2+} may activate the signaling cascades that directly altered the transcription and translation processes of protein expression. In addition, Ca^{2+} could affect the posttranslational modification of the tight junction proteins which render the alteration of protein distribution, localization and assembly of the tight junction complexes (Brown and Davis 2002). As known, a rise of cytosolic Ca^{2+} either from extracellular space or from intracellular stores following exposure to stressful stimuli could activate the Ca^{2+}/calmudulin kinases. Subsequently, all three MAPK in the MAPK signaling cascade were activated, and their effects on modification of tight junction complexes could be observed. Fischer *et al*. (2005) demonstrated that increase paracellular permeability related to activation of phospholipase C (PLC) and increase of intracellular release of Ca^{2+} which, consequently, activated the ERK activity. Moreover, removal of extracellular calcium could deprive intracellular calcium which eventually led to barrier dysfunction. This event could be due to an alteration of the conformation of tight junction complexes (Alexander *et al*., 1998). It was also possible that under the calcium-free condition, certain signaling cascades were activated. As a result, the structure of tight junction disrupted and underwent a process of disassembly. Under this circumstance, ZO-1 and occludin were redistributed away from apical-lateral borders (Ma *et al*., 2000).

3. NF-κB

NF-κB was known as a transcription factor presented in most cell types. Several genes that were involved in cellular transformation, proliferation, invasion and angiogenesis were regulated by NF-κB. Targets of NF-κB include the genes corresponding to the production of cytokines (including IL-1, IL-6, IL-8, and TNF-α), iNOS, and adhesion molecules. In addition, NF-κB could activate iNOS, increased NO and VEGF released, activated PKC and induced phosphorylation of MAP kinase. It had been reported that an increase in permeability of Caco-2 was associated with NF-κB-dependent downregulation of ZO-1 protein expression as well as alteration of junctional localization (Thomas *et al*., 2004).

4. Vascular endothelial grown factor (VEGF)

Vascular endothelial grown factor (VEGF) was a vascular permeability factor which could be induced by certain stress and assaults such as hypoxia, inflammation. An increased of VEGF augments paracellular permeability. For example, the state of hypoxia induced hyperpermeability through the released of VEGF in human choroidal endothelial cells (CECs). This particular event correlated to the dislocalization, decreased expression, and enhanced phosphorylation of ZO-1 (Fischer *et al*., 2002). It had been proposed that the effects of VEGF on tight junction complexes involved with the released of nitric oxide and subsequent activation of guanylate cyclase *in vivo* (Mayhan, 1999). Consequently, VEGF could be seen as a survival and maintenance factor that preserves endothelial integrity. VEGF acted specifically on endothelial cells via its receptors VEGFR1, and VEGFR2 to induce vascular permeability, cellular proliferation, cellular migration and tube formation of human microvessels (Ferrara *et al*., 2003). The study of Antonetti *et al.* (1999) had shown that an increase of permeability triggered by VEGF was associated with phosphorylation of occludin and ZO-1 protein in the retinal endothelial cells. In aortic endothelial cells, the effects of VEGF on hyperpermeability involved with activation of ERK, JNK, and Scr. Consequently, these led to Serine/Threonine phosphorylation and redistribution of ZO-1 and occludin.

5. Nitric oxide (NO)

NO was a free radical gas with well characterized signaling roles in mammalian systems, acting as a second messenger molecule in a number of organ systems including endothelium, nervous system and cardiovascular system (Furchgott, 1995). NO had a dual role as a physiological messenger and a mediator of pathological processes in a variety of degenerative disorders.

In blood vessels, NO from endothelial cells mediates several physiological functions including control of vasodilatation, inhibitions of platelet aggregation, inhibition of neutrophil/platelet adhesion to endothelial cells, inhibition of smooth muscle cell proliferation and migration, and regulation of programmed cell death. In addition to its major roles in vasodilatation, NO may influence the barrier function of endothelium cells. It had been demonstrated that treatment of NO increased the paracellular permeability of

brain endothelial cells. This effect was apparently concentration dependent. Low levels of NO (30 μM) had no influence on the monolayer permeability, but high concentration of NO (150 μM) caused an increase of paracellular permeability (Utepbergenov *et al*., 1998). An increase of NO production led to an activation of NF-κB and VEGF expression, which resulted in cell proliferation and tight junction disruption (Chua *et al*., 1998). In addition, an increased expression of iNOS and total NO productions in hypoxic condition were responsible for the increased paracellular permeability in cerebral microvascular (Karen and Thomas, 2002). Inhibition of NOS activity attenuated this barrier disruption.

Oxidative stress and its influence on the integrity of tight junction

Oxidative stress was a state of imbalance between free radical production and the ability of the organism to defend against free radicals that could lead to progressive oxidative damage. Oxidative stress could disrupt several cellular homeostasis and functions, leading to the pathological stress such as increase in intracellular Ca^{2+} and activation of protein kinase family (Pariente *et al*., 2001; Tiruppathi *et al*., 2003). Hence, oxidative stress could attribute to the aging process and to the pathogenesis of several degenerative diseases such as Alzheimer's disease, Parkinson's disease, cerebral ischemia/reperfusion injuries, seizures, stroke, and trauma. In epithelial cell, oxidative stress caused an impairment of tight junction complexes which related to phosphorylation of occludin and ZO-1 at tyrosine residues. Consequently, the dissociation of these junctional proteins from the actin cytoskeleton along with redistribution from junctional area occurred (Rao *et al*., 2002; Kale *et al*., 2003).

In this study, H_2O_2 had been employed to induce the oxidative stress and the disruption of tight junction. In general, H_2O_2 was an important by product of normal cellular oxidative metabolism and could be found in human plasma at micromolar concentrations (Lacy *et al*., 1998; Raimondi *et al*., 2000; Liu and Zweier, 2001). H_2O_2 was a small molecule (MW=34.01) with a melting point of -0.43°C and boiling point of 153°C, making it liquid at room temperature. H_2O_2 was a fairly unstable molecule in water, particularly in the presence of metal contaminants. H_2O_2 had been implicated in the pathophysiology of a variety of cardiovascular disorders including hypertension, myocardial ischemia and atherosclerosis (Hearse *et al*., 1993; Ross,1993; Lacy *et al*.,

1998). However, there was a growing evidence to support the function of H_2O_2 as an inter- and intra-cellular signaling molecule. For example, at site of inflammation, H_2O_2 which was generated by activated adhesion molecules had a role in controlling cell proliferation or apoptosis and in modulating platelet aggregation.

H_2O_2 was freely miscible with water and was apparently able to cross cell membranes readily. H_2O_2 may undergo further transformations into highly reactive hydroxyl radicals such as superoxide in the presence of transition metals (particularly iron and copper) (Halliwell and Gutteridege, 1990) (Table 1). Hence, H_2O_2 could produce oxidative damage to macromolecules at the plasma membrane as well as those resided in cytoplasm as well as nucleus of the cell (Cochrane, 1991; Halliwell and Gutteridge, 1987).

Table 1.

Formation of ROS by reduction of molecular oxygen in the electron transport chain

(i) $O_2 + e + H^+ \longrightarrow HO_2$ (hydroperoxyl radical)

(ii) $HO_2 \longrightarrow H^+ + O_{2^-}$ (superoxide radical)

(iii) $O_2 + 2H^+ + e \longrightarrow H_2O_2$ (hydrogen peroxide)

(iv) $H_2O_2 + e \longrightarrow OH^- + OH$ (hydroxyl radical)

(v) $OH + e + H^+ \longrightarrow H_2O$

(vi) $O_2 + H_2O_2 \longrightarrow OH^- + OH + O_2$ (Haber–Weiss reaction)

(vii) $Fe^{2+} + H_2O_2 \longrightarrow OH^- + OH + Fe^{3+}$ (Fenton reaction)

H_2O_2 and Tight junction

It had been reported that H_2O_2 generated by xanthine oxidase or glucose oxidase stimulated endothelial cell growth (Ruiz-Gines *et al*., 2000). Consistently, scavenging H_2O_2 by catalase or cytosolic glutathione peroxidase inhibited endothelial cell proliferation (Zanetti *et al*., 2002, Faucher *et al*., 2003). In addition to cellular oxidative injury, H_2O_2 could cause a corrupt of tight junction complexes, leading to an increase of paracellular permeability of endothelium/ epithelium barrier. In the hypoxic state of lung endothelial cells, it had been demonstrated that H_2O_2 was a key responsible player for the injury through an activation of ERK1/2 and p38 (Parinandi *et al*., 1999).

High concentrations of H_2O_2 increased endothelial cell permeability which had been related to several signaling pathways including activations of PKC, phosphodiesterase, small G protein Rho, p38 MAPK, and an increase of intracellular calcium (Siflinger-Birnboim *et al*.,1992; Lum *et al*., 1993; Suttorp *et al*.,1993; Siflinger-Birnboim *et al.,*1996; Usatyuk *et al*., 2003; Hippenstiel *et al*., 2003). Activity of MAPK was also involved with the disruption of tight junction complexes in the endothelial cell monolayer exposed to H_2O_2 at 1 mM for 30 min (Kevil *et al*., 2000) and by phorbol ester in corneal epithelium (Wang *et al*., 2004). Furthermore, H_2O_2 triggered intracellular events, leading to the phosphorylation of occludin and loss of its interaction with actin cytoskeleton. These processes could be protected with VEGF through the inhibition of ERK and ERK1/2 activity (Shyamali *et al*., 2006).

The effects of H_2O_2 on barrier function had been linked to the modification of endothelial actin cytoskeleton. It had been shown that H_2O_2 mediated the reorganization of actin cytoskeleton through the phosphorylation of HSP27, which was an actin binding protein. In addition, this phosphorylation process could result from the activation of p38-MAPK activity (Huot *et al*., 1998). Moreover, treatment of H_2O_2 caused an increase of endothelial solute permeability which related to an activation of PKC along with an increase of intracellular Ca^{2+} (Shasby *et al*., 1985; Siflinger-Birnboim *et al*.1992; 1996). The increase of intracellular Ca^{2+} activated Ca^{2+}/calmudulin kinases, which subsequently activated all three MAPKs (ERK, JNK, and p38) (Nimnual *et al*., 1998).

The integrity of tight junction complexes was very crucial component of epithelium barrier function. The disintegration of tight junction structure was a major cause of barrier leakage that led to weaken the defense mechanism of the organ system. Therefore, any compounds with ability to protect tight junction integrity would be beneficial to maintain organ function. In this study, oxidative stress could results in tight junction disruption. It was possible that flavonoid compounds, which contain broad spectrum of pharmacological action including antioxidant, will protect tight junction integrity and function.

Flavonoids

The term “flavonoid” was generally used to describe a broad collection of natural products. All flavonoids had the same basic chemical structure, a three-ringed molecule with hydroxyl (OH) groups attached. Flavonoids (Figure 6) were polyphenolic compounds that occurred ubiquitously in fruits and vegetables as well as in food products and beverages derived from plants such as olive oil, tea, and red wine (Croft, 1998). The polyphenolic flavonoids, containing diphenylpropane skeleton were subclassed as monomeric favonols, flavones, flavanones, and flavonols. A number of flavonoids had been found to possess various clinically relevant properties such as antitumor (Adlecreutz, 2002; Frei and Higdon, 2003), anti-inflammatory (Middelton, 1998), antioxidant *in vitro* (Rice-Evans *et al*., 1996; Pannala *et al*., 1997; Oldreive *et al*., 1998; Kerry and Rice-Evans, 1999), antiviral (Jassim and Naji, 2003), antiplatelet, and anti-ischemia (Avila *et al*., 1994; Gerritsen *et al*., 1995).

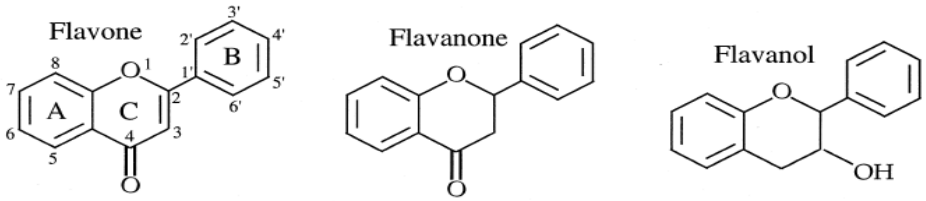


Figure 6: Chemical structure of flavonoid

Quercetin

Quercetin (Figure 7) was one of the most widely distributed flavonoids in the plant kingdom. It was found in many often-consumed foods including apple, onion, berries (blackcurrants, lingonberries and bilberries) beans, black tea, green tea, red wine, and brassica vegetables. It was also found as a major component in several herbal medicines, including *Ginkgo biloba, Hypericum perforatum* (St. John's Wort), *Sambucus canadensis* (Elder), *Vaccinium macrocarpon* (cranberry) and *Oenothera biennis* (evening primrose). Quercetin, also known as meletin and sopheretin, was known chemically as 2-(3,4-dihydroxyphenyl)-3,5,7-trihydroxyl-4H-1-benzopyran-4-one and 3,3’,4’,5,7-penthydroxyflavone. The edible portion of some foodstuffs had an unusually high concentration of quercetin. Hertog *et al*. (1992) examined hydrolysed

samples from numerous fruits and vegetables for total aglycone content and found the highest concentration of quercetin in onions (284-486 mg/kg), kale (110 mg/kg), French beans (32-45 mg/kg), broccoli (30 mg/kg), lettuce (14 mg/kg) and tomatoes (8 mg/kg). Among the fruit examined, the quercetin concentration averaged 15 mg/kg, with apples having the highest concentration (21-72 mg/kg). In beverages such as beer, coffee, chocolate milk and white wine were below 1mg/litre. By contrast, the quercetin concentration in red wine ranged from 4-16 mg/liter, while grape juice contained 7-9 mg/liter, fruit juices other than lemon (7mg/liter) and tomato (13 mg/liter) contained below 5 mg/liter quercetin. Tea infusions were the highest, ranging in quercetin from 10-25 mg/liter.

After absorption, quercetin was transported to the liver via the portal circulation, where it underwent significant first pass metabolism. Peak plasma levels of quercetin occurred from 0.7-9 hrs following ingestion, and the elimination half-life of quercetin was approximately 23-28 hrs (Hollman *et al*., 1997; PDRHealth, 2008). Due to its long half-life, repeated consumption of quercetin-containing foods should cause accumulation of quercetin in the body. Excretion was likely to be via the biliary system (Erlund, 2004). Quercetin had carcinostatic and antiviral activities. It could suppress cell proliferation, modified eicosanoid synthesis, protected low-density lipoprotein from oxidation, prevented platelet aggregation, stabilized immune cells, and promoted relaxation of cardiovascular smooth muscle (Formica and Regelson, 1995).

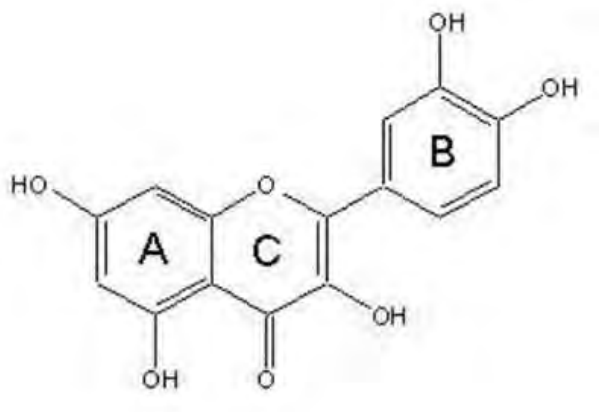


Figure 7: Chemical structure of quercetin

Effects of quercetin

There were several reports of the biological activities of quercetin especially as an antioxidant which may be the results of its ability to neutralize free radical, to chelate the metal ions, to inhibit certain enzymes, or to induce the expression of the protective enzymes (Erlund 2004). Effects of short-period treatment of quercetin related to its antioxidant effect whereas long term treatment involved with a reduction of GSH levels and increased in cellular oxidative status (Ferraresi *et al*., 2005). In the absence of GSH, potentially harmful oxidation product such as orthoquinone may be produced when quercetin exerts its antioxidant activity. Therefore, adequate GSH levels should be maintained when quercetin was supplemented (Boots *et al*., 2003).

Quercetin had also been shown to inhibit the enzyme involved in proliferation and signal transduction pathway including protein kinase C, tyrosine kinase, PI-3 kinase, DNA topoisomerase II, proline-directed protein kinase fatty acid in human prostate carcinoma cells, Na^+ K^+ ATPase, NF-κB, and MAPK family (Gamet-Payrastre *et al*., 1999; Constantinou *et al*., 1995; Lee *et al*., 1998; Yoshizumi *et al*., 2001; Yoshihisa *et al.,* 1999). *In vivo* and *in vitro* studies, quercetin could inhibit inflammatory process by modulating neutrophil function, prostanoid synthesis, cytokine production, and iNOS expression. The action of quercetin had been linked to the inhibition of the NF-κB pathway (Morikawa *et al*., 2003; Comalada *et al*., 2005).

It had been shown that quercetin contains the protective effect against oxidative stress. Quercetin could protect neuronal cells from oxidative stress-induced neurotoxicity (Dok-Go *et al*., 2003; Heo and Lee, 2004) and inflammatory-related neuronal injury (Chen *et al*., 2005). Quercetin could increase the *in vitro* survival rate of neuron under specific condition (Ternaux and Portalier 2002). Furthermore, the neuroprotective effect of quercetin was demonstrated in certain pathological conditions such as ischemia (Cho *et al*., 2006; Pu *et al*., 2007).

Quercetin had also possessed antiproliferative activity against ovarian, breast, and stomach cancer cells (Scambia *et al*., 1990; Yanagihara *et al*., 1993). Its actions include cell cycle regulation, interaction with several proteins such as type II estrogen binding sites, multidrug-resistant protein, and induction apoptosis of tumor cells. Moreover, quercetin had been shown to inhibit several important steps of angiogenesis

including proliferation, migration, and tube formation of human microvascular dermal endothelial cell (Wen-fu, *et al.,* 2003).

Quercetin and its potential to maintain the integrity of tight junction

Quercetin had been considered as an antioxidant and inhibited some biological processes triggered by H_2O_2. It had been shown that quercetin was able to suppress the activity of MAP kinase including ERK and JNK (Formica and Regelson, 1995; Miura *et al.,*1998). The inhibitory effects of quercetin against MAP kinases may be through direct suppression of tyrosine phosphorylation of these enzymes (Whitmarsh and Davis, 1996). As known, the assembly and integrity of tight junction structure had been linked to these kinases activity and the sequential signaling pathways. Considering that quercetin was able to inhibit several kinases including cAMP-dependent kinase, PKC, and calmodulin-dependent kinase as well as NF-κB activity (Formica and Regelson, 1995), which related to the signaling associated the assembly and function of tight junction proteins, it was possible that quercetin could prevent or restore the disruption of tight junction structure. The study of Takuya and Hiroshi (2009) demonstrated that quercetin enhanced the intestinal barrier function through the assembly of ZO-1, claudin, and occludin. This effect was related to its inhibitory action on PKC. Recently, quercetin had been shown to rapidly increase the TEER values across the monolayer of Caco2 cells. Moreover, the effect of quercetin on the NO level may influence the barrier function of endothelium cells. It had been demonstrated that administration of NO resulted in an increased of the paracellular permeability of brain endothelial cells (Karen *et al*., 2002).

Taken together, it could be hypothesized that quercetin was able to prevent the disintegration of tight junction from an exposure to H_2O_2. In the present study, H_2O_2 at non-lethal concentration induced the disintegration of tight junction structure through activation of MAPK cascade. The potential inhibitory effect of quercetin on these kinase pathways may enable its protective capability against H_2O_2-induced disruption of tight junction. It was possible that quercetin enhances the expression and localization of tight junction proteins, which have been affected and disorganized by H_2O_2 exposure.

CHAPTER III

MATERIALS AND METHODS

Materials

Chemicals

The following chemicals were purchased from Sigma Chemical Company (St. Louis, USA); Acrylamide, Albumin from bovine serum (BSA), Ammonium persulfate, 2',7'-dichlorofluorescein diacetate (DCFH-DA), Dimetyl sulphoxide (DMSO), EDTA ([Ethylenedinitrilo]tetraacetic acid), Glycine, Glycerol, Penicillin G sodium, MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenltetrazolium bromide), N',N'-Methylendiacrylamide, Quercetin, Potassium chloride, Sodium dodecyl sulfate, Sodium phosphate dibasic anhydrous, Streptomycin sulfate, Secondary anti-rabbit Cy-3 conjugated, 0.04%Trypan blue, Trypsin, Tween 20, and Tritron x-100.

The following chemicals were purchased from Merck (Darmstadt, Germany); Calcium chloride, Magnesium Chloride, Methanol, Pefabloc, Potassium dihydrogen phosphate, Potassium hydroxide, Skimmed milk powder, Sodium bicarbonate, Sodium chloride, Sodium hydroxide, Sodium nitrite, Tris-(hydroxymethyl) aminomethane.

The rabbit monoclonal anti-ZO-1 and anti-occludin were from Zymed (South San Francisco, CA, USA).

The rabbit polyclonal anti-ERK, anti-phosphorylated ERK, anti-p38, and anti phosphorylated p38 were purchased from Calbiochem (San Diego, CA, USA).

Antibody against β-actin was purchased from Cell Signaling Technology (Beverly, MA, USA).

Fetal bovine serum (FBS) was purchased from Hyclon (USA).

Film X-ray blue, developer replenishes, and fixer replenishes were purchased from Eastman Kodax Co. (Rochester, NY, USA).

M199 medium and M199 medium free phenol red were purchased from Gibco Life Technologies, Inc. (Grand Island, NY, USA).

Page Ruler™ Prestained Protein Ladder was purchased from Fermentus Life Science (Hanover, MD, USA).

Polyvinylidine difluoride (PVDF) transfer membranes were purchased from Pall Gelman Laboratory (Pensacola, FL, USA).

Super signal West Pico chemilluminescent substrate and secondary goat anti-rabbit IgG (H&L) horseradish peroxidase (HRP) were purchase from Pierce Biotechnology, Inc. (Rockford, IL, USA).

All other chemicals and solvents used throughout this study were commercially available reagents or analytical grade reagent.

Equipments

The following instruments were major equipment used in this study:

1. 12-well, 24-well, 96-well cell culture plate, Transwell® insert, Tissue culture flasks, and Sterilization filtration membranes 0.22 μm (cellulose acetate membrane) were purchase from Corning (USA.).
2. Inverted microscope; Axiovert 135 (Zeiss, Germany)
3. Microplate reader (Anthos Labtec HT2 version 1.21E, Australia)
4. Multilabel microplate reader (Perkin Elmer VICTOR^3 Wallac 1420, Germany)
5. Milicell®-ERS (Millipore, USA.)
6. Mini-PROTEAN® 3 Electrophoresis Cell and Mini Trans-Blot electrophoretic Transfer cell (Bio-Rad, USA.)
7. Fluorescence microscope (BX-FLA, Olympus, Japan)

Preparation of ECV304 cell culture

The ECV304 cell line (Figure 8) was purchased from Cell Line Service (Germany, Lot No. 600560-2). The cells were grown in M199 medium containing 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin in a humidified incubator maintaining at 37°C and containing 5%CO_2, 95% air atmosphere. The medium was replaced every 48 hr. When the cultures reached 80-90% confluence, the culture medium was aspirated and the cells were trypsinized in 0.25% trypsin-EDTA solution for 4 min at 37°C. Then, the trypsinization reaction was stopped by adding of culture medium. The cell suspension was centrifuged at 1,200 rpm for 4 min at 4 °C and collected the pellets. The pellets were resuspended in fresh M199 containing 10%FBS for further experiment (Figure 9). For the viability assay, the cells were seed at $2x10^5$cells/ml in a well (96 well-plates). For the permeability assay, the cells were seed at $2x10^5$cells/transwell. For the immunofluorescent assay, the cells were seed at $2x10^5$cells/well (24 well-plates).

The morphology of cultured cells was monitored by an inverted microscope with phase contrast optics in combination with microcomputer-assisted image capture system (Pinnacle 8, Pinnacle system, Germany).

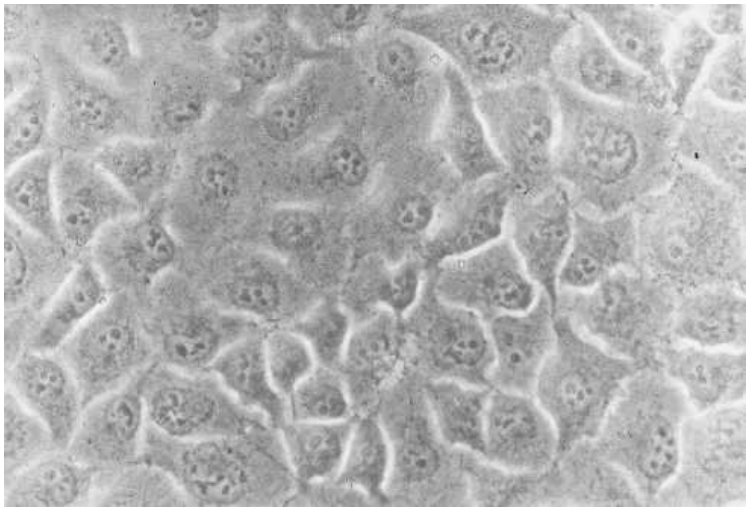


Figure 8: The morphology of ECV 304 cells which were photographed under normal culture condition.

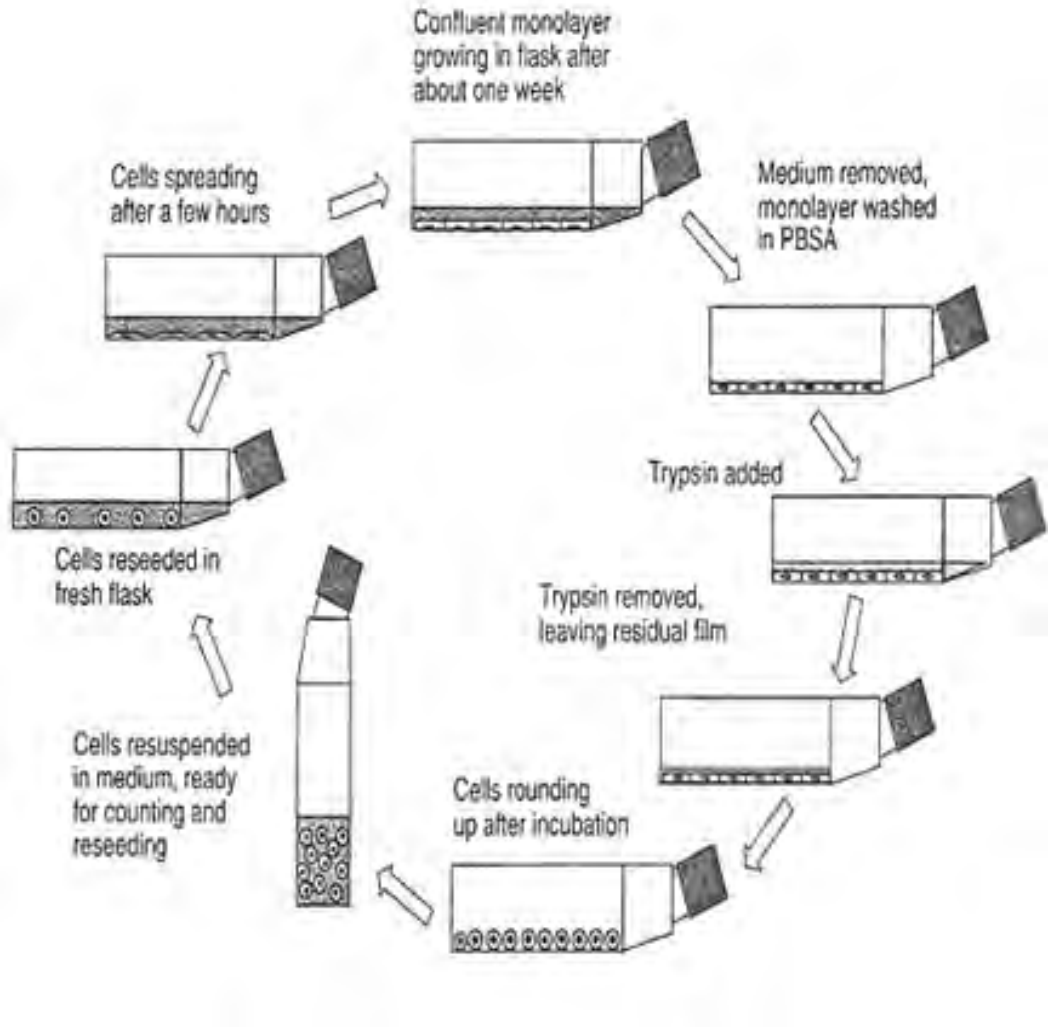


Figure 9: Subculture method (Freshney, 2000)

Preparation of test compounds

Quercetin and H_2O_2 were freshly prepared on the day of experiment. Quercetin was dissolved in 100%DMSO and further diluted with culture medium. The final concentration of DMSO in each experiment was less than 0.5%. In addition, the solvent control was also conducted in each experiment. H_2O_2 was diluted with double distilled water to the desired concentration at the beginning of experiment.

Experimental procedures

1. The cytotoxicity studies

In order to investigate the cytotoxic effects of quercetin and H_2O_2, ECV304 cells were plated in 96-well plates at density of 2 x 10^5 cell/ml. After 24 hr, either quercetin or H_2O_2 at various concentrations were added to the cultures. The concentrations of H_2O_2 were in the range of 0-1000 μM whereas those of quercetin were at 0-100 μM. In addition, the effects of exposed times to H_2O_2 and quercetin were also determined. In this study, the durations of treatment were at 4 and 24 hr for H_2O_2, and 30 min and 24 hr for quercetin. After treatment, cell viability was determined by an MTT reduction assay. ECV304 cells which were received the equivalent amount of solvent vehicle were used as the control group.

In separated experiments, the protective effects of quercetin against H_2O_2-induced toxicity were also determined. In brief, after plating the cells for 24 hr, the cells were pretreated with quercetin at the concentration of 10 μM for 30 min, and then the medium was replaced with fresh medium containing H_2O_2 at various concentrations (0-1000 μM). After 4 hr of incubation, the cell viability was determined by an MTT and LDH assay.

MTT assay

The MTT assay was a well-established method for studying cell proliferation and viability. This method was based on the reduction of yellow colored 3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenl tetrazolium bromide into a purple insoluble formazan product by active mitochondrial dehydrogenase (Mosmann, 1983). Hence, only living cells, but not dead cells or their lytic debris, contained this dehydrogenase activity to generate intracellular formazan (Figure 10). A decrease in cellular MTT reduction could be an index of cell damage. The resulting purple chromophore could be solubilized with DMSO, and quantitatively measured by spectrophotometry.

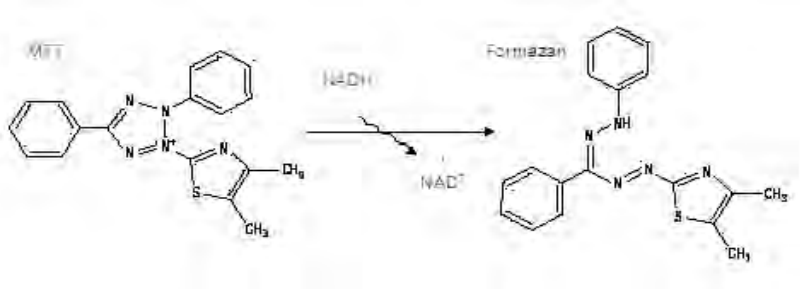


Figure 10: Molecular structure of MTT and their corresponding reaction products.

The colorimetric method to determine MTT reduction was initiated by addition of 100 µl of MTT solution (0.4mg/ml) in each well. Then, cultures were incubated in a 5% CO_2 incubator at 37 $^{\circ}$C for 4 hr. At the end of incubation, the culture medium was aspirated off carefully, without disturbing the formazan precipitate. The next step was adding of 100 µl of DMSO to solubilize the formazan crystals. After formazan solubilization, the absorbance was measured spectrophotometrically, using an Anthos Labtec HT2 microplate reader at a measuring wavelength of 570 nm and a reference wavelength of 620 nm. The cellular reduction of MTT which represents metabolic activity and viability was expressed as the percentage of the values obtained from control group.

LDH release assay

The lactase dehydrogenase assay was a mean of measuring cell viability as well as membrane integrity. This assay measured the presence of cytoplasmic LDH in the medium. LDH was a stable cytoplasmic enzyme found in all cell type. It could be rapidly released into extracellular environment when the plasma membrane was damaged. In this regard, the existence of LDH in cell culture medium could be used as an indicator of the integrity of plasma membrane and cell viability. The principle of LDH release assay was to determine LDH activity via measuring the reduction of NAD^+ to NADH and H^+ (Figure 11) (Petrik *et al*., 2005). Subsequently, the reduced NAD (NADH) was utilized in the stoichiometric conversion of a tetrazolium dye which could be measured with spectrophotometer.

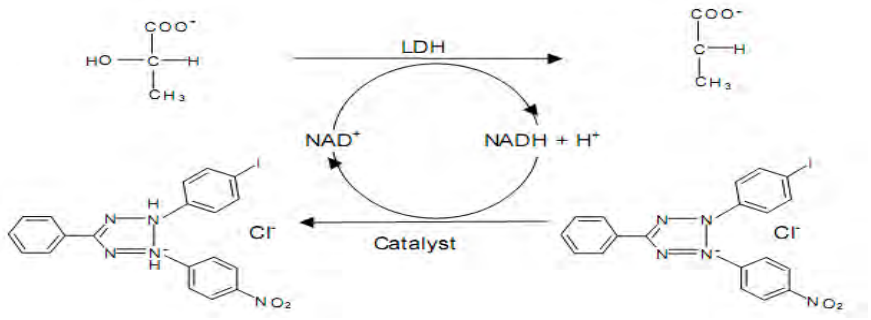


Figure 11: The reactions in lactate dehydrogenase (LDH) assay. (Cytotoxicity detection kit (LDH): instruction manual version 5, 1999).

In this study, LDH activity was determined by using an *In vitro* Toxicology Assay Kit Lactate dehydrogenase based (Sigma, USA) with a procedure according to the manufacturer's instruction. In brief, ECV 304 cells were plated at $4x10^5$ cells per well in 24 wells-plates and cultured for 24 hr. At the time of measurement, medium LDH (100µl) was collected and transferred into a 96-well micro plate. Cellular LDH was also collected by lysis cells with 500 µl of 0.5% Triton X-100 in PBS. Then, 100 µl of cell lysed were transferred for LDH measurement. The reaction was initiated by mixing 50 µl of assay mixture with samples, and incubating in the dark for 30 min at room temperature. Then, the reaction was terminated by addition of 50 µl of 0.5 N HCl into each sample. The spectrophotometric absorbance was determined with a measuring wavelength of 490 nm with reference wavelength 690 nm, using a microplate reader (Anthos LabtecHT2 version 1.21E, Australia). The LDH release was expressed as the percentage of total LDH activity, calculated as the following equation.

$$\% \text{ LDH release} = \frac{\text{LDH activity in medium} \times 100}{\text{LDH activity in medium} + \text{LDH activity in cells}}$$

2. The studies of localization and expression of the tight junction complexes

2.1 Immunofluoresent analysis

The localization of major tight junction proteins was visualized by an immunofluorescent staining method (Chen *et al*., 2000; Lee *et al.,* 2004), with the use of Fluorescence microscope (BX-FLA, Olympus, Japan). ECV304 cells were plated at the density of 2 x 10^5 cell/well for 48 hr. On the day of the experiment, cells were treated with quercetin 10 μM for 30 min prior to H_2O_2 (100, 125 μM) exposure for 4 hr.

The immunofluorescent analysis started with washing the cell monolayer twice with iced-cold PBS, then fixed the cells with the ethanolic solution containing 5% acetic acid at -20 °C for 10 min. The next step was washing the cells with iced-cold PBS, followed by addition of 0.05% Triton X-100 to permeabilize the cells for 10 min at room temperature, and washed again with iced-cold PBS three times. Cell samples were treated with 3% BSA for 15 min at room temperature, followed by incubated with primary antibodies against occludin (dilutions: 1:200), or ZO-1 (dilutions: 1:200) for 1 hr at room temperature. After washing the cells with PBS, the cell samples were incubated with secondary antibody conjugated with Cy-3 for 1 hr in the dark at room temperature. Finally, the cells were washed and visualized under fluorescence microscopy to determine the localization of tight junction proteins.

2.2 Western blot analysis

In this experiment, the cells were plated at the density of 2 x 10^5 cell/well for 48 hr. On the day of the experiment, cells were treated with quercetin (10 μM) for 30 min prior to H_2O_2 (100, 125 μM) exposure. After 4 hr incubation with H_2O_2, cells were washed twice with ice-cold PBS on ice, and lysed with the lysis buffer containing 20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% Triton X-100, 1mM Pefabloc, 1% Na deoxyclolate, 10 mM NaF, 1 mM Na orthovanadate, 5μg/ml aprotinin. Then, cells were harvested using a cell scraper. The cell lysate were collected and pooled from three culture wells into a 1.5 ml-microcentrifuge tube, kept on ice for 30 min prior to the centrifugation at 10,000 g for 10 min at 4 °C. After centrifugation, the amount of protein was quantified by Bradford method, (Bradford, 1976), using bovine serum albumin as a protein standard. The expression of protein was determined by western blot analysis. The samples were

aliquot, and mixed with loading dye (60 mM Tris-HCl pH 6.8, 25% glycerol, 2%SDS, 14.4 mM 2-mercaptoerhanol and 0.1% bromophenol blue) (5:1), boiled at 95 °C for 4 min and stored at -20 °C until use.

In order to perform the western blot analysis of occludin and ZO-1, 30 µg of protein samples were loaded onto a 12% or 8% polyacrylamide gel. The electrophoresis was run under constant voltage of 180 V for 45 min. After gel electrophoresis, the proteins were electrotransferred to polyvinylidene difluoride (PVDF) membrane under constant voltage of 60 V for 90 min. The membranes were blocked with 5% skimmed milk in Tris-buffered saline-Tween 20 (TBST) for 60 min at room temperature. The membranes were probed with specific primary antibodies at the dilution of 1:2000 for anti-occludin, 1:1000 for anti-ZO-1, and 1:500 for anti-Actin, for 2 hr at room temperature. After washing the membrane with TBST three times, the membranes were incubated with the corresponding horseradish peroxidase (HRP)-conjugated secondary antibody, goat anti-rabbit immunoglobulin (IgG) for 1 hr at room temperature. Subsequently, the membranes were incubated with an enhanced chemiluminescence detection reagent, Supersignal® chemiluminescent substrate (West Pico, Pierce) for 5 min. The immunoblots were exposed to X-ray films (Eastman Kodak). Actin bands were monitored on the same blot to verify the consistency of protein loading. Films were scanned and the band intensity of each treatment was compared to that of control.

The effect of quercetin on MAPK expression was also determined by western blot analysis. Protein samples (30 µg) were loaded onto 12% polyacrylamide gel. The electrophoresis was run under constant voltage of 180 V for 45 min. Then, proteins were blotted onto PVDF membrane, using 3%BSA in TBST as blocking solution. The membranes were probed with specific primary antibodies including anti-ERK1/2, anti-phosphorylation ERK1/2, anti-p38, and anti-phosphorylation p38 at a dilution of 1:1000, and incubated overnight at 4 °C. Subsequently, the membranes were incubated with secondary antibodies and the blots were developed using the Supersignal® chemiluminescent (West Pico, Pierce) as described above. The samples were collected from four separate experiments (n=4).

3. Measurement of reactive oxygen species and nitric oxide

3.1 Measurement of reactive oxygen species (ROS)

The accumulation of intracellular ROS in ECV 304 cells was determined with the use of 2', 7'-dichlorofluorescein (DCF) fluorescence (Bastianetto *et al*., 2000). The principle of the assay was the conversion of 2', 7'-Dichlorofluorescein diacetate (DCFH-DA) which was a peroxide/redox-sensitive fluorescent probe into DCFH by intracellular esterases. DCFH-DA was non polar and freely diffusible whereas DCFH was polar and could be trapped within the cell. Then, DCFH interacted with intracellular free radicals and peroxide and generated the highly fluorescent compound DCF.

In this study, a stock solution of DCFH-DA at the concentration of 20 mM was prepared in DMSO and stored at -20 °C in the dark. ECV304 cells were seeded at a density of 2 x10^5 cells/well in 24-well plates. After treatment with quercetin and H_2O_2 as described in other experiments, cells were washed twice with cooled PBS on ice and loaded with 50 μM of DCFH-DA at 37 °C in the dark for 45 min incubation. ECV304 cells that were not treated with DCFH-DA were used as a blank. After loading the dye, cells were washed three times with iced-cold PBS, and collected for fluorescent determination. The fluorescent intensity was quantified with a Perkin-Elmer VICTOR3 Wallac 1420 microplate reader using excitation and emission wavelengths of 483 and 535 nm, respectively. The fluorescence of the cell population reflected the level of intracellular ROS.

3.2 Measurement of nitric oxide

Total production of nitrite and nitrate, which were the end products of nitric oxide reactions, in the cultured medium was determined by colorimetric non-enzymatic assay. The procedures included addition of 30% $ZnZO_4$ to deproteinate the medium. Then, the samples were incubated at room temperature for 15 min, followed by centrifugation at 4000 rpm for 5 min. The supernatants were collected and incubated with 0.5 g dry granulated cadmium (Cd) at room temperature overnight. Then, the samples were mixed with color reagent #1 (1% Sulhanilamide (p-Aminobenzenesulfonamide) in 3 N HCl) 50 μL and shaken briefly prior to addition of color reagent #2 (0.1% N-(1-Naphthyl) ethylenediamine dihydrochloride in deionized water) 50 μL. After shaking for 5 min at

room temperature, the absorbance values at 540 nm were determined with microtiter plate reader. Concentration of nitric oxide was calculated from a standard curve derived from the $NaNO_2$ reaction in the assay. The concentration of nitrite standard at 500 pmol/µL is equivalent to 500 µM of NO. Example of a standard curve was shown in Figure 12.

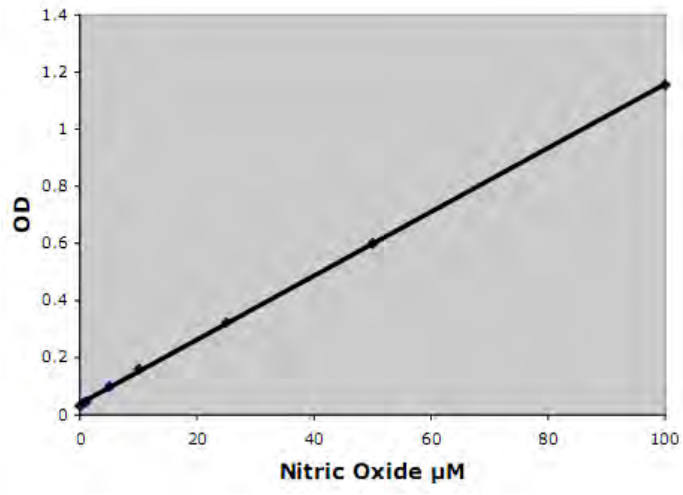


Figure 12: A typical standard curve of nitric oxide

4. The functional studies of tight junction complexes

4.1 Measurment of transepithelial electrical resistance (TEER)

The restrictions of paracellular transport of small ions could be determined by determination of TEER values. ECV304 cells were plated on polycarbonate filter with a pore size of 0.4 µm and a diameter 12mm. (Transwell®, Coning, USA.) at the confluent density of $2x10^5$ cell/well. Then, the filter was immersed in the culture medium, of which the volume was adjusted to 0.5 ml in the upper side and to 1.6 ml in the lower side. The electrical resistance across the membrane was measured, using an electrical resistance system (ERS®) with voltage-measuring (Milicell-ERS®, Millipore, USA) (Figure 13). The instrument had a chopstick-like electrode with the short electrode being inserted in the apical side whereas the longer electrode was placed in the basolateral side. The apparent TEER value was obtained by multiplying the measured electrical resistance (Ω) with the surface area of the monolayer (cm^2). Generally, the basal TEER values of ECV304 cells varied from 80-120 $\Omega \cdot cm^2$ (Kuchler-Bopp *et al.*, 1999). Changes of TEER

values were calculated as a percentage of the basal values. In this study, duplicate cell monolayers were used for each group, and the experiment was conducted at least three times, repetitively.

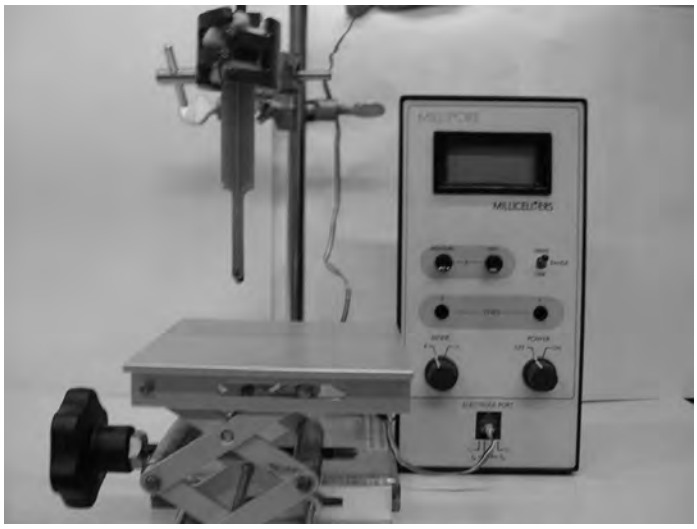


Figure 13: Photo of Millicell-ERS® (Millipore, USA.)

4.2 Measurment of paracellular permeability

It has been established that movement of phenol red across the monolayer of cultured cells were achieved by paracellular passage. This was due to the unique property of phenol red that hardly permeates through plasma membrane. Apparently, the crossing of phenol red through monolayer of cells in normal condition was at rate of less than 5% per hr (Martel *et al*., 2003). Therefore, determination of phenol red transport could be a measure of the integrity and tightness of tight junction complexes.

In this study, ECV304 cells were seeded at the confluent density of 2 x 10^5 cell/well onto Transwell® insert, and culture in phenol red-free completed medium for 12 days prior to the experiment. On the day of an experiment, quercetin at the concentration of 1 and 10 μM (if needed) was added into the Transwell® insert for 30 min, then the medium were replaced with 0.5 ml of phenol red-free medium which was supplemented with phenol red at the concentration 50 μM in the apical side. In this step, H_2O_2 (if needed) might be mixed with the apical medium at the final concentration of 100 μM. The phenol red-free medium at the volume of 1.6 ml was added to the basolateral side.

Then, a 100 μl-aliquots of medium in the basolateral side was taken hourly to measure the amount of phenol red spectrophotometrically at the wavelength of 430 nm. The apparent permeability coefficient (P_{app}) was calculated according to the following formula.

$$P_{app} = \frac{dQ}{dt} \times \frac{1}{A \times C_0}$$

Where P_{app} = apparent permeability coefficient (cm/sec)

dQ/dt = the amount of drug appearing in the receiver compartment in function of time (nmol/sec)

A = the surface area across which the transport occurred (cm^2)

C_0 = the initial concentration in the donor compartment (μM)

Statistical analysis

The results were expressed as the mean ± SEM (standard error of means), calculated from at least 3-4 separated experiment (n=3-4). The significant differences between groups were evaluated by analysis of variance (ANOVA), followed by Scheffe's post hoc analysis, where appropriated. Differences were considered significant at *p*-values less than 0.05.

CHAPTER IV

RESULTS

1. The cytotoxicity studies

1.1 Effects of H_2O_2 and quercetin on cell viability

As shown in Figure 14, H_2O_2 caused the cytotoxic effect to ECV304 cells in concentration- and time-dependent manners, as measuring by an MTT assay. At 4-hr exposure, H_2O_2 at the concentrations of 100 and 125 μM had no significant effects on the viability of ECV304 cells. The survival rate reduced significantly when the concentration of H_2O_2 was at 250 μM or higher. At 24-hr treatment, H_2O_2 markedly increased its cytotoxic activity. The cell viability significantly reduced to under 80% at the concentration of H_2O_2 greater than 10 μM. In addition, none of the ECV304 cells could survive the 24-hr exposure to H_2O_2 at the concentration greater than 50 μM.

As shown in Figure 15, quercetin at the concentration of less than 10 μM had no effect on cell viability after 30 min and 24 hr of incubation. However, when the concentration of quercetin increased to 100 μM, cell survival significantly decreased to less than 50%. Moreover, there was no significant difference in ECV304 viability between 30-min and 24-hr exposure to quercetin, as seen in Figure 15. These finding suggested that the exposure time of quercetin had no significant effects on the viability of ECV304 in this study.

1.2 The protective effect of quercetin on H_2O_2-mediated cytotoxicity

Based upon the previous results, the noncytotoxic concentration of quercetin at 10 μM was selected for further studies on its protective effects against H_2O_2-mediated cell injury and disruption of tight junction structure. The cytoprotective effect of quercetin was evaluated at the exposure time of 30 min prior to the treatment with H_2O_2 for 4 hr. As shown in Figure 16, pretreated ECV304 cells with quercetin (10 μM) was able to

significantly attenuate the cytotoxicity caused by H_2O_2 even at the high concentration of 1000 μM.

1.3 Assessment of cell morphology

It has been well established that cell morphology reflected cell function to some degree. In this study, the morphological alteration of the ECV304 upon exposure to H_2O_2 was observed with inverted microscope. As shown in Figure 17 A, the morphology of ECV 304 was normally seen in spindle-shaped. Quercetin at the concentration of 10 μM had no effect on the cell morphology (Figure 17 B). In addition, at the concentration of 100 μM, H_2O_2 had no observable effect on the morphology of ECV 304 (Figure17 C). By contrast, a markedly change in the cell morphology was observed when the H_2O_2 was at the concentration of 125 μM (Figure17 D).

1.4 Effect of quercetin on LDH releases

As shown in Figure 18, there was an increase in LDH release by approximately 20% in the cells treated with H_2O_2. This result suggested that H_2O_2 might damage the integrity of plasma membrane. However, the cells viability was also evaluated with trypan blue exclusion test and Hoechst 33342 staining. It was found that at the concentration of 100 μM, H_2O_2 had no observable cytotoxic effects in the trypan blue exclusion test. In addition, the fluorescence images of Hoechst 33342 staining cells treated with H_2O_2 100 μM were comparable to those of the control group. Furthermore, treatment of quercetin for 30 min prior to H_2O_2 could prevent the increase in H_2O_2-mediated LDH release.

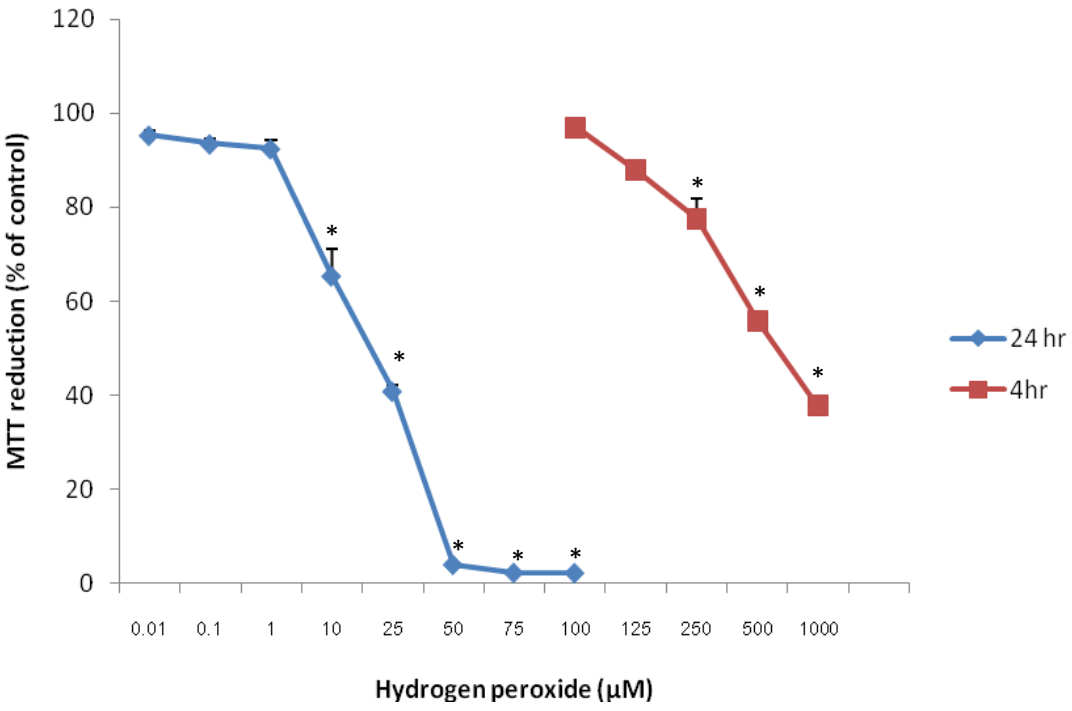


Figure 14: Concentration and time courses of the viability of ECV304 cells upon exposure to H_2O_2. The viability of the cells was determined by an MTT reduction assay. Data were expressed as mean ± SEM of six separated experiments, each of which was performed in triplicate samples.

*p <0.001; significant differences in comparison with the control cultures.

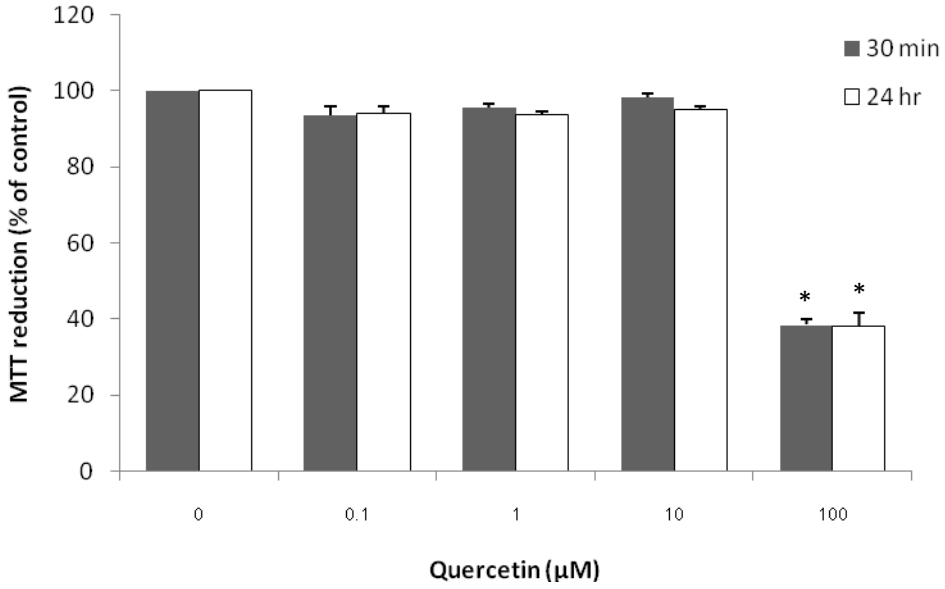


Figure 15: Effects of quercetin on the viability of ECV304 cells. ECV304 cells were grown and treated with quercetin for 30 min and 24 hr. Cell viability was determined by an MTT reduction assay. Data were expressed as mean ± SEM of six separated experiments, each of which was performed in triplicate samples.

*p <0.001; significant differences in comparison with the control cultures.

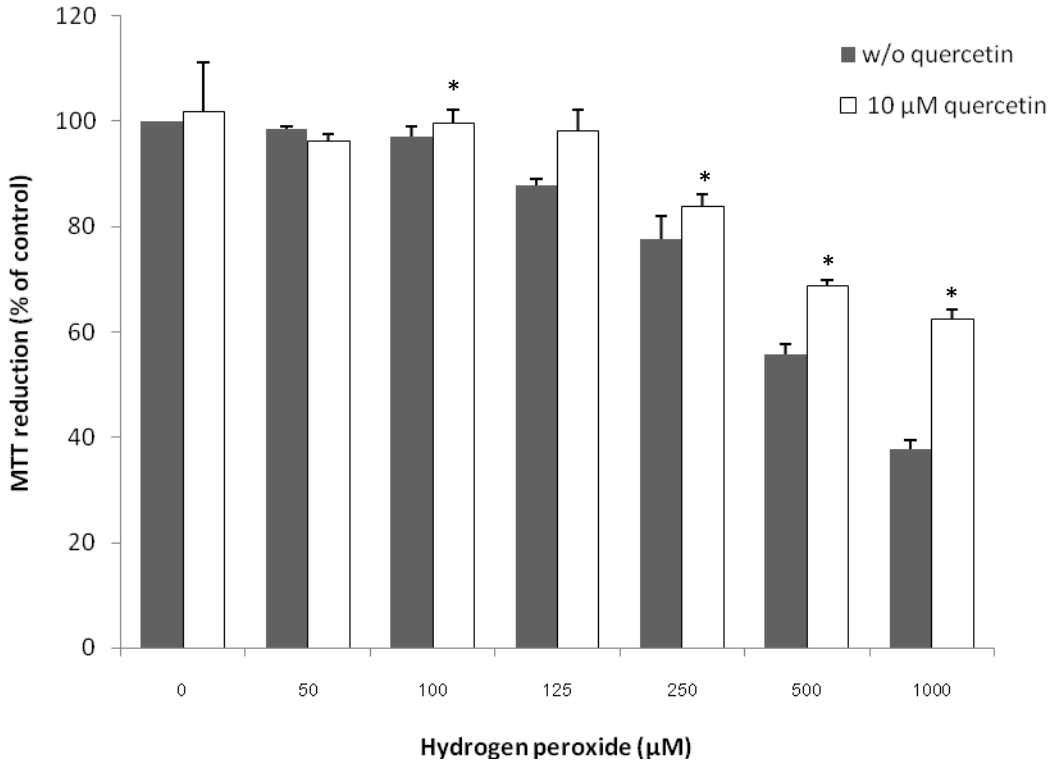
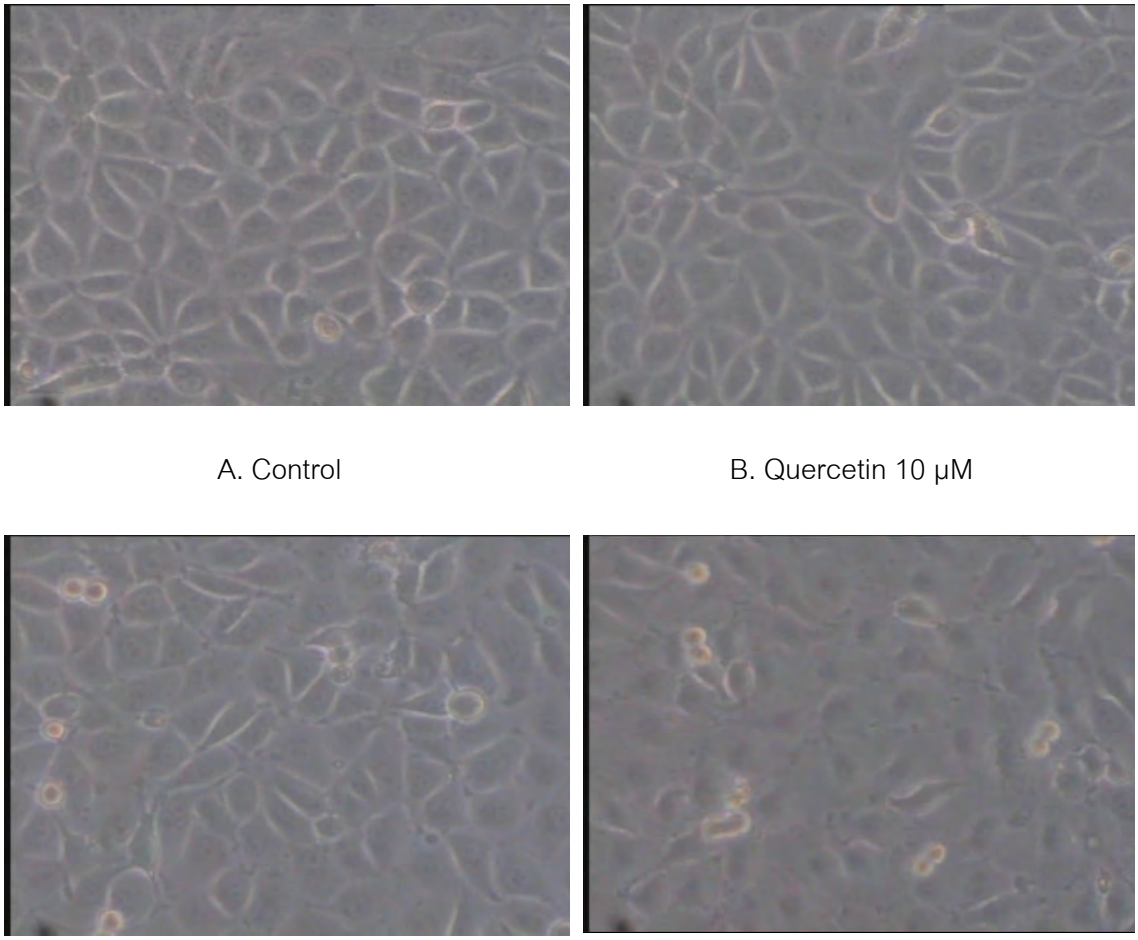


Figure 16: The protective effect of quercetin on the viability of ECV304 cells which exposed to H_2O_2. ECV304 cells were treated with quercetin 10 µM for 30 min prior to 4-hr exposure of H_2O_2 at various concentrations (0-1000 µM). The cell viability was measured by an MTT assay. The cell viability in the absence of H_2O_2 was set to 100%. Data were expressed as the mean±SEM of six separated experiments, each of which was performed in triplicate samples.

*p <0.05; significant differences in comparison with the without quercetin cultures.



A. Control B. Quercetin 10 μM

C. H_2O_2 100 μM D. H_2O_2 125 μM

Figure 17: The morphology of ECV304 cells as observed by inverted microscopy at 100x magnification. Control ECV 304 cells (A), ECV304 cells pretreated with quercetin 10 μM for 30 min (B) and ECV304 cells treated with H_2O_2 100 and 125 μM for 4 hr, respectively (C and D).

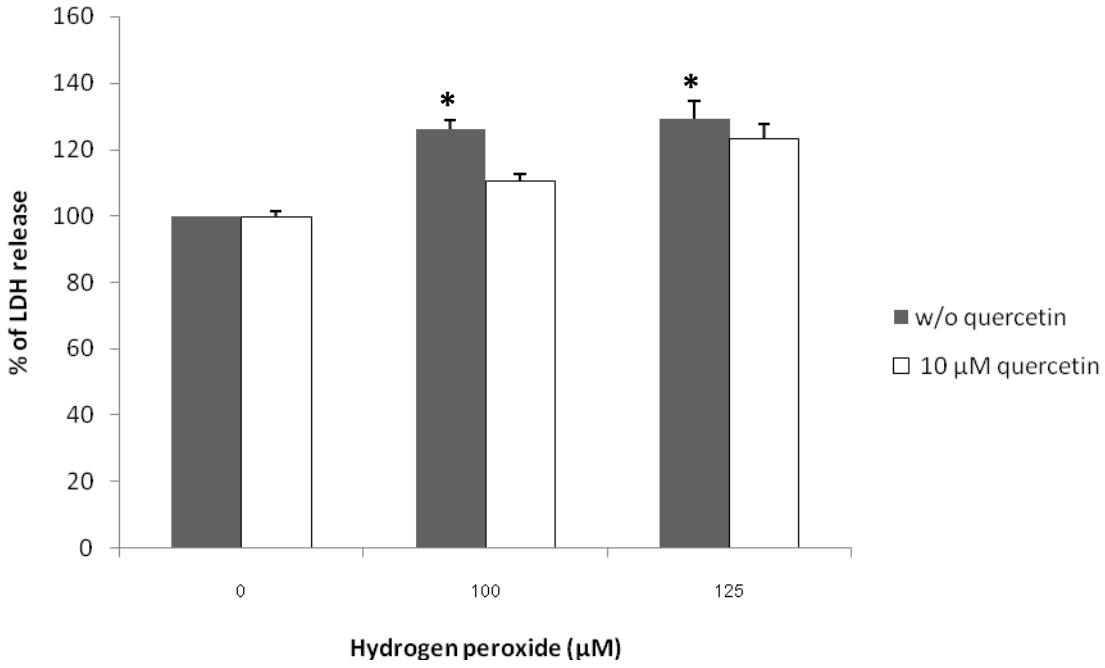


Figure 18: LDH release from quercetin-treated ECV304 cells after exposure with H_2O_2. ECV304 were incubated with H_2O_2 (100 and 125 µM) in the absence and presence of quercetin (10 µM). LDH release was expressed as percent of control.

*p <0.05; significant differences in comparison with the control cultures.

2. The studies of localization and expression of tight junction complexes

2.1 Immunofluorescent analysis

In order to evaluate the involvement of ZO-1 and occludin in H_2O_2-mediated the loss of barrier function. The cellular distribution of these tight junction proteins was determined with the immunofluoresence microscopy. Under the normal circumstance, ECV304 monolayer expressed the continuous distribution pattern of ZO-1 and occludin proteins at the cell boundaries, as shown in Figures 19 A and 20 A. In addition, quercetin had no influence on the localization of these junction proteins (Figures 19 B and 20 B). In contrast, H_2O_2 at the concentrations of 100 and 125 μM apparently changed the distribution pattern as seen by a discontinuous circumference of the cell border at in Figures 19 C, E, Figures 20 C, E, respectively (arrow point). These findings suggested the abnormality of localization, and the possible loss of expression of tight junction proteins. Pretreatment the monolayer with quercetin at the concentration of 10 μM for 30 min prior to addition of H_2O_2 was able to restore the complete circumference of the cell border, as shown in Figures 19 D, F, Figures 20 D, F, respectively. These results suggested that H_2O_2 even at the low concentrations of 100 and 125 μM could induced the disturbance of tight junction structure and loss of the organization of its major protein components, specifically ZO-1 and occludin proteins. In addition, these disruptions of tight junction proteins at cell-to-cell contact sites could be prevented by treating the monolayer with quercetin even at the concentration of 10 μM.

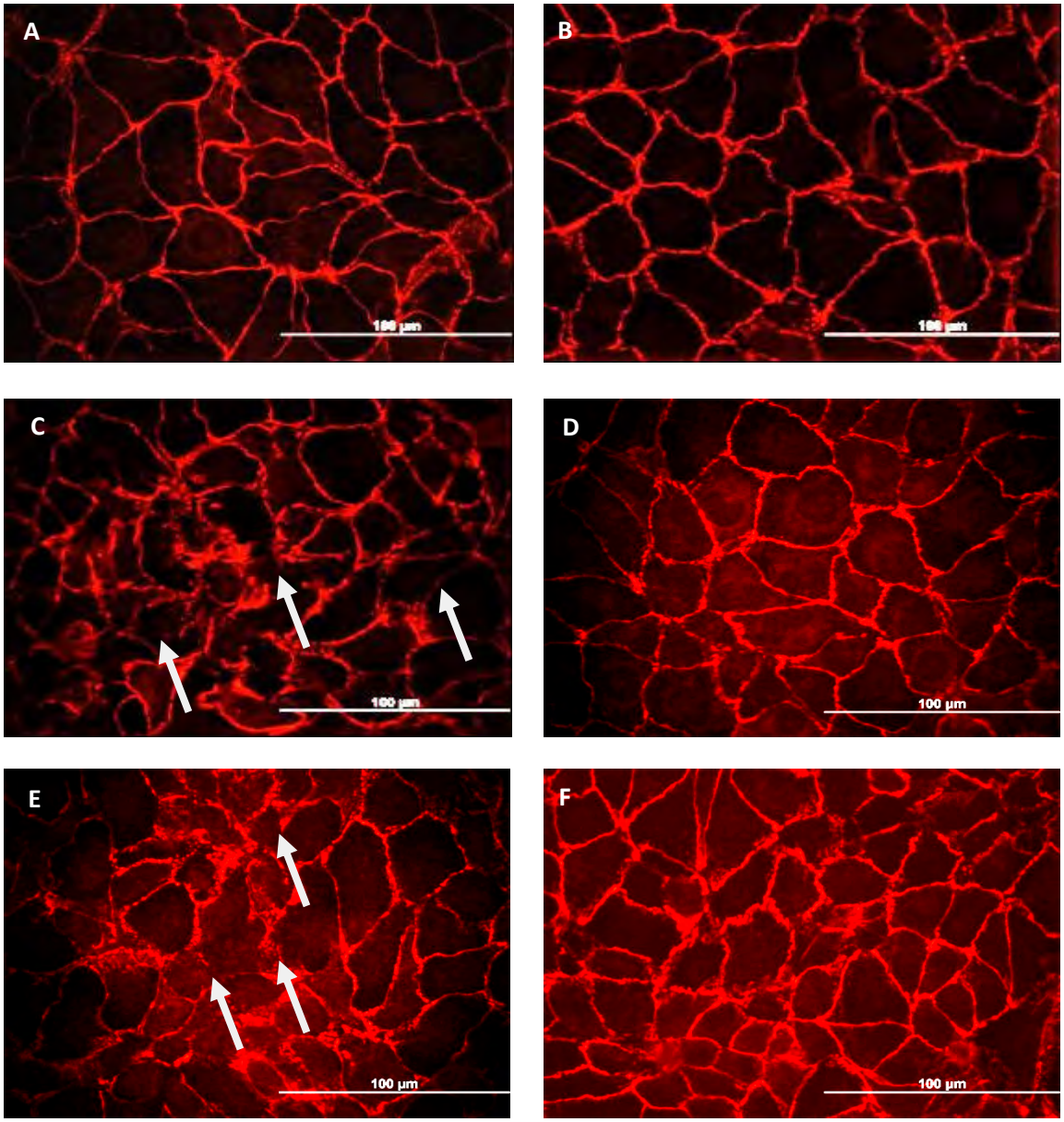


Figure 19: Immunofluorescent staing showing localization of ZO-1 protein in ECV304 cells. (A) control, (B) quercetin 10 µM for 30 min, (C) H_2O_2 100 for 4 hr, (D) quercetin 10 µM for 30 min followed by H_2O_2 100 for 4 hr, (E) H_2O_2 125 for 4 hr, and (F) quercetin 10 µM for 30 min followed by H_2O_2 125 for 4 hr. The arrow showed the ZO-1 disruption. All fields were shot at x40. Bar, 100 µm.

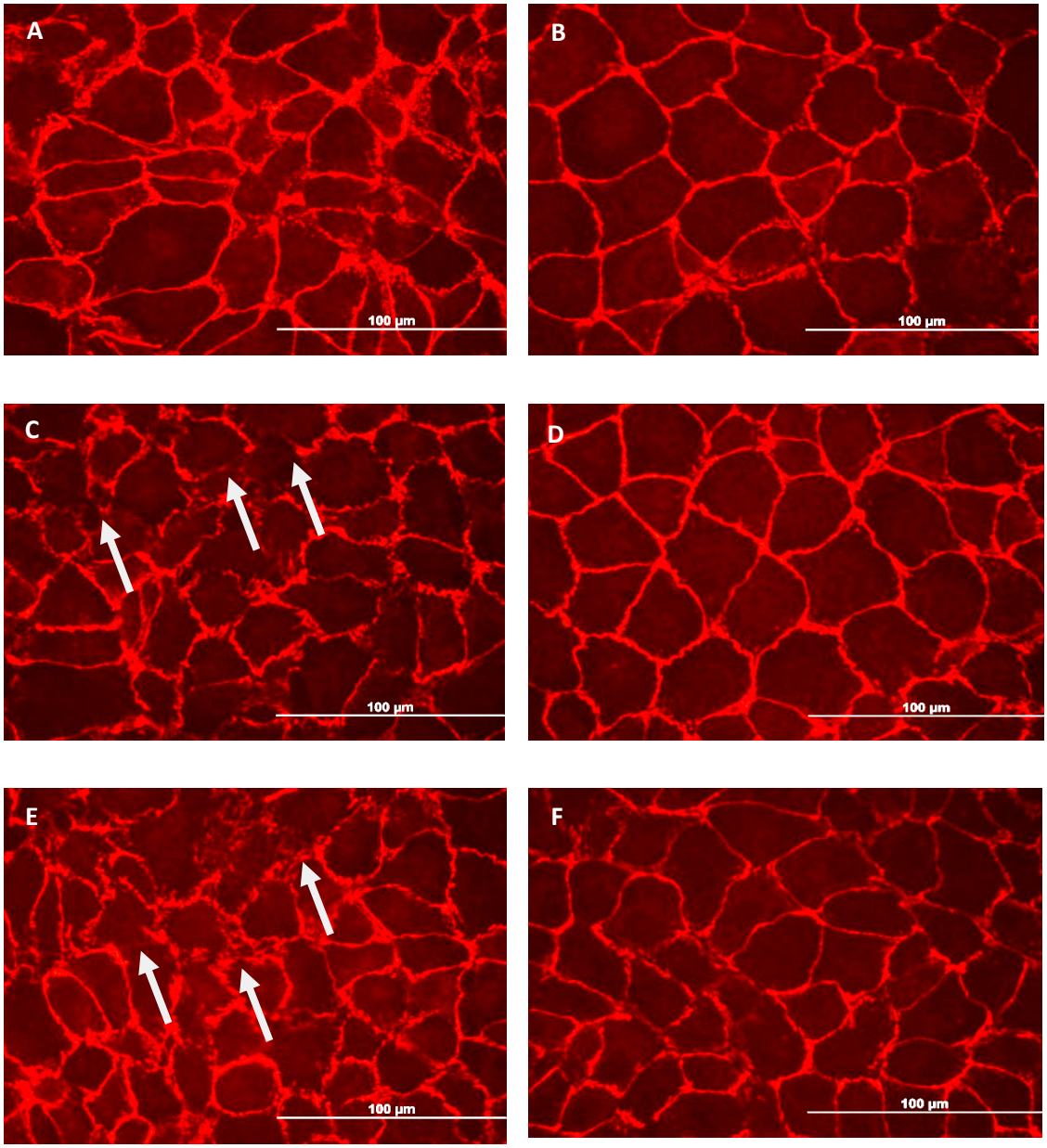


Figure 20: Immunofluorescent staing showing localization of occludin protein in ECV304 cells. (A)control , (B) quercetin 10 µM for 30 min, (C) H_2O_2 100 for 4 hr, (D) quercetin 10 µM for 30 min followed by H_2O_2 100 for 4 hr, (E) H_2O_2 125 for 4 hr, and (F) quercetin 10 µM for 30 min followed by H_2O_2 125 for 4 hr. The arrow showed the occludin disruption. All fields were shot at x40. Bar, 100 µm.

2.2 Western blot analysis

In order to determine the expression of tight junction-associated proteins including occludin and ZO-1, the immunoblots was applied in this study. The specific antibodies for occludin, ZO-1, and actin proteins recognized the proteins at the expected molecular weight of target proteins in homogenate of ECV304 cells. The occludin, ZO-1, and actin bands were detected at 65, 210, and 42 kDa, respectively. In addition, the intensity of the protein bands was quantitatively assessed by image capture and further analysis with Image J program from The National Institute of Health (NIH).

As shown in Figures 21 A and B, H_2O_2 at the concentrations of 100 and 125 μM were able to significantly suppress the expression of the occludins and ZO-1 proteins. Exposure to H_2O_2 caused approximately 30% reduction in expression of occludins and ZO-1 proteins. On the contrary, quercetin at the concentration of 10 μM had no influence on the expression of these proteins (Figures 22 A and B). Pretreatment of the cells with quercetin for 30 min prior to H_2O_2 was able to significantly preserved at least 90% of the occludin and ZO-1 expression. These findings indicated that quercetin was able to prevent the H_2O_2 mediated loss of the expression of occludin and ZO-1.

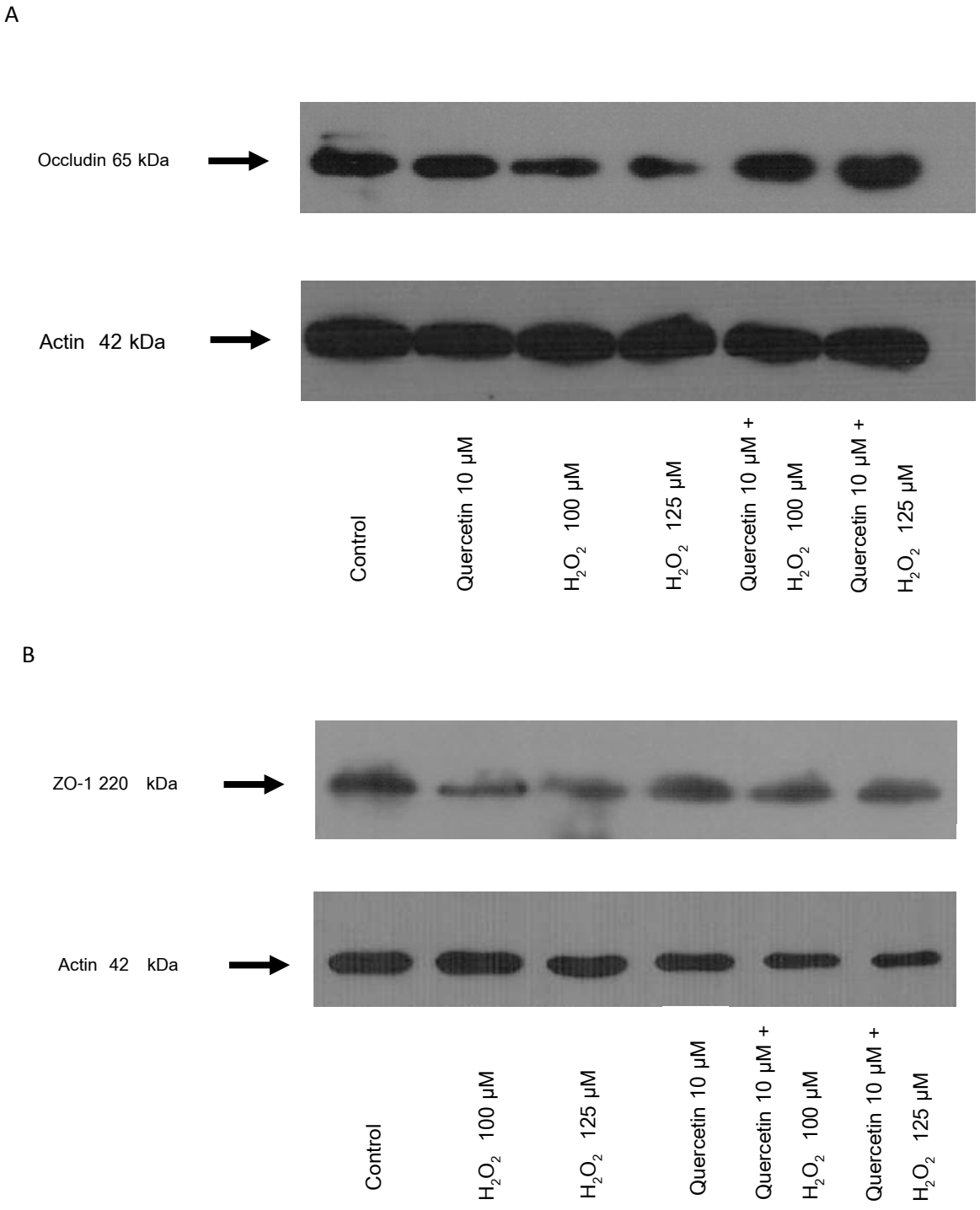


Figure 21: Effects of quercetin on the expression of occludins (A) and ZO-1 (B) proteins in ECV304 cells. Cell lysated were run on 12% (for occludin and β-actin), 8% (for ZO-1) SDS-PAGE. Data are representative of four separated experiments.

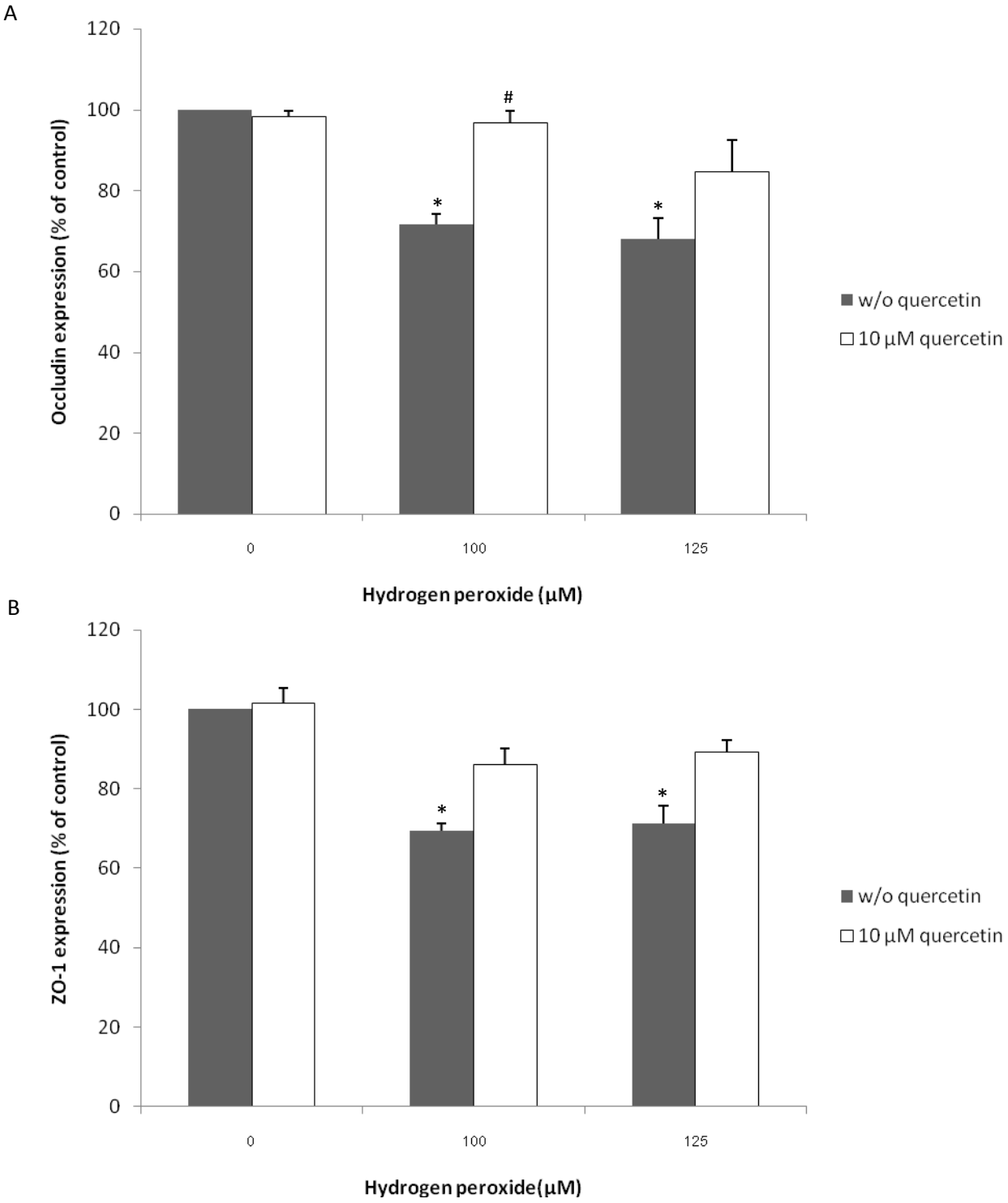


Figure 22: Densitometrical analysis of immunoblots of occludin (A) and ZO-1 (B). Data represent the mean±SEM obtained from four separated experiments.

*$p < 0.05$; significant difference in comparison with the control cultures

#$p < 0.05$; significant difference in comparison with H_2O_2 treatment.

3. **The possible involvement of reactive oxygen species, nitric oxide, and MAP kinase activity in H_2O_2-mediated disruption of tight junction integrity**

3.1 Measurement of reactive oxygen species

In this study, 4-hr exposure of H_2O_2 affected the amount of ROS in the ECV304 cells. At the concentration of 100 μM, H_2O_2 caused a slight but not significant increase of the ROS level. Its effects was more prominent at the concentration of 125 μM, which it significantly increased the ROS level by approximately 20% ($p< 0.05$). The ROS level in the group pretreated with quercetin 10 μM for 30 min prior to H_2O_2 exposure was statistically significant less than that of treatment with H_2O_2 alone. In addition, quercetin was able to equally prevent the increase of ROS levels in the groups exposed to H_2O_2 at the concentration of 100 and 125 μM ($p<0.01$) (Figure 23). These results suggested that the protective effect of quercetin on H_2O_2-induced tight junction disruption was partly due to its ability to preserve the oxidative status of the cells.

3.2 Measurement of nitric oxide level

The measurement of nitric oxide in the culture medium elicited that H_2O_2 significantly induced the production of nitric oxide in ECV304. In addition, the effect of H_2O_2 on nitric oxide production was concentration-dependent. However, pretreatment of ECV 304 cells with quercetin 10 μM for 30 min prior to addition of H_2O_2 could prevent the increased nitric oxide in the culture medium. The results suggested that quercetin was able to hinder the production of nitric oxide in ECV304 cells (Figure 24).

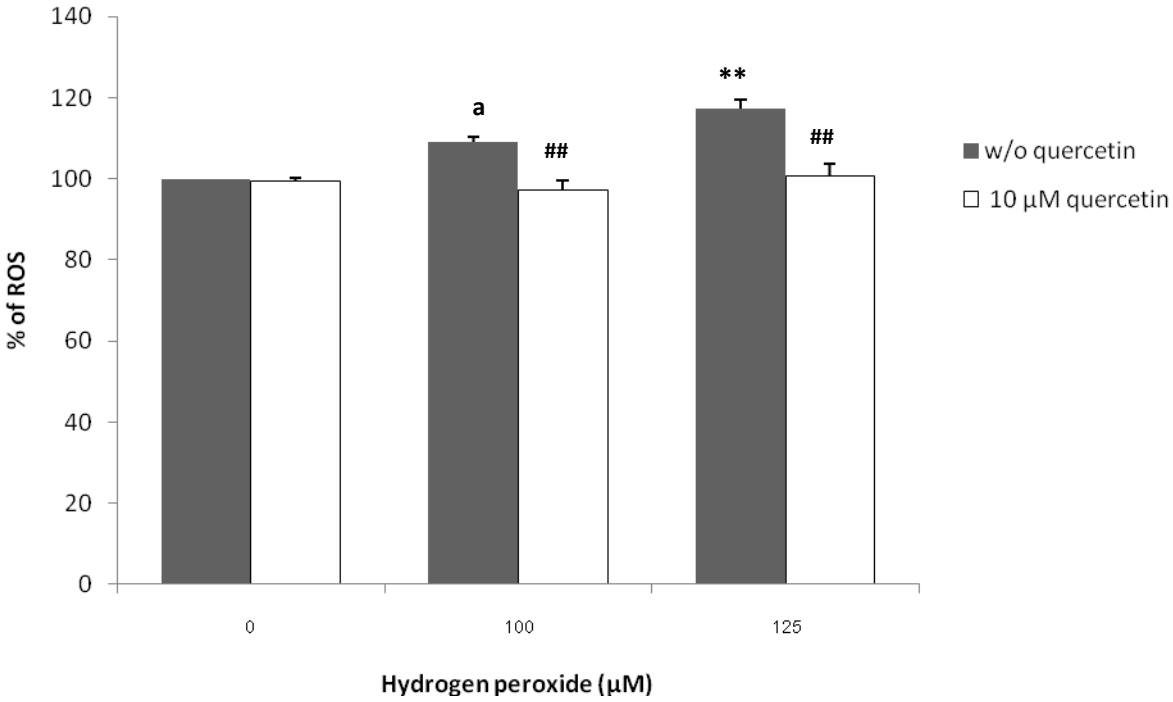


Figure 23: Effects of quercetin on the amount of ROS in ECV304 cells. ECV304 were pretreated with quercetin 10 µM for 30 min prior to addition of H_2O_2. After 4-hr exposure, the level of ROS was measured, and the value obtained in the absence of H_2O_2 was set to 100%. Data are expressed as mean ±SEM of four separated experiments, each of which was performed in triplicate samples.

$^{**}p$ <0.001; the significant difference in comparison with the control cultures,

$^{\#\#}$ p <0.001; the significant difference in comparison with the group treated with H_2O_2 alone,

^{a}p <0.05; the significant difference in comparison with the group treated with quercetin alone.

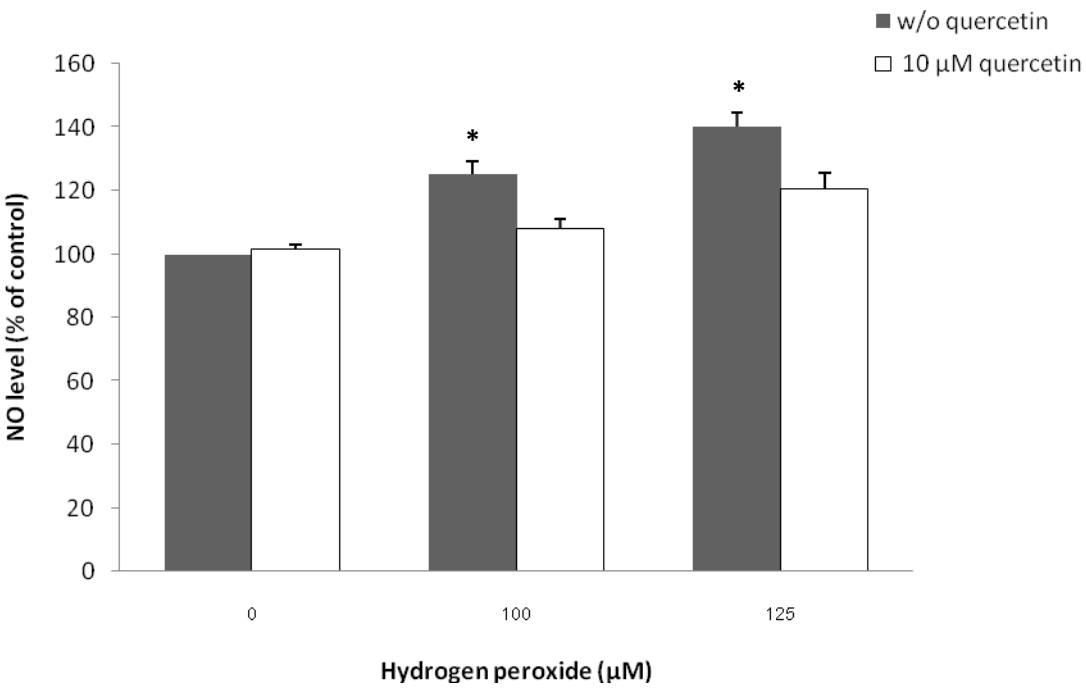


Figure 24: Effects of quercetin and H_2O_2 on the release of nitric oxide from ECV304 cells. ECV304 were pretreatment with quercetin 10 µM for 30 min prior addition of H_2O_2. After 4-hr incubation, the culture medium was taken for measurement of nitric oxide. The value obtained in the absence of H_2O_2 was set to 100%. Data were expressed as mean ±SEM of six separated experiment, each of which performed in triplicate samples.

*p <0.05; the significant difference in comparison with control cultures.

3.3 Determination of MAPK expression

The involvement of two members of the MAPK family was examined for their expression in this study. As seen in Figures 25 A and Figures 26 B, the expression of ERK1/2 was shifted into the phosphorylated form upon treatment of H_2O_2. Although quercetin alone had no effect on the balance of ERK1/2 and its phosphorylated form, it could potentiate the effects of H_2O_2 on the phosphorylation of ERK1/2. As seen in Figures 26 B, pretreatment of ECV304 cells with quercetin at the concentration of 10 μM prior to addition of H_2O_2 resulted in the increase in phosphorylated ERK1/2 at the greater level than those expressed in the group treated with H_2O_2 alone.

In contrast, quercetin pretreatment caused the reduction of the phosphorylated p38, which increased from exposure to H_2O_2. As seen in Figures 25 B and Figures 27 B, H_2O_2 caused an almost two-fold increase in the expression of phosphorylated p38 after 4 hr-exposure. Quercetin at the concentration of 10 μM had no effect on an alteration of phosphorylated and non-phosphorylated of p38. However, the expression of phosphorylated p38 in the ECV304 pretreated with quercetin for 30 min prior to addition of H_2O_2 was at the lesser degree than those expressed in the group treated with H_2O_2 alone (Figure 27 B). These results suggested that quercetin was able to suppress the phosphorylation process of p38 which was activated during cells exposure to H_2O_2.

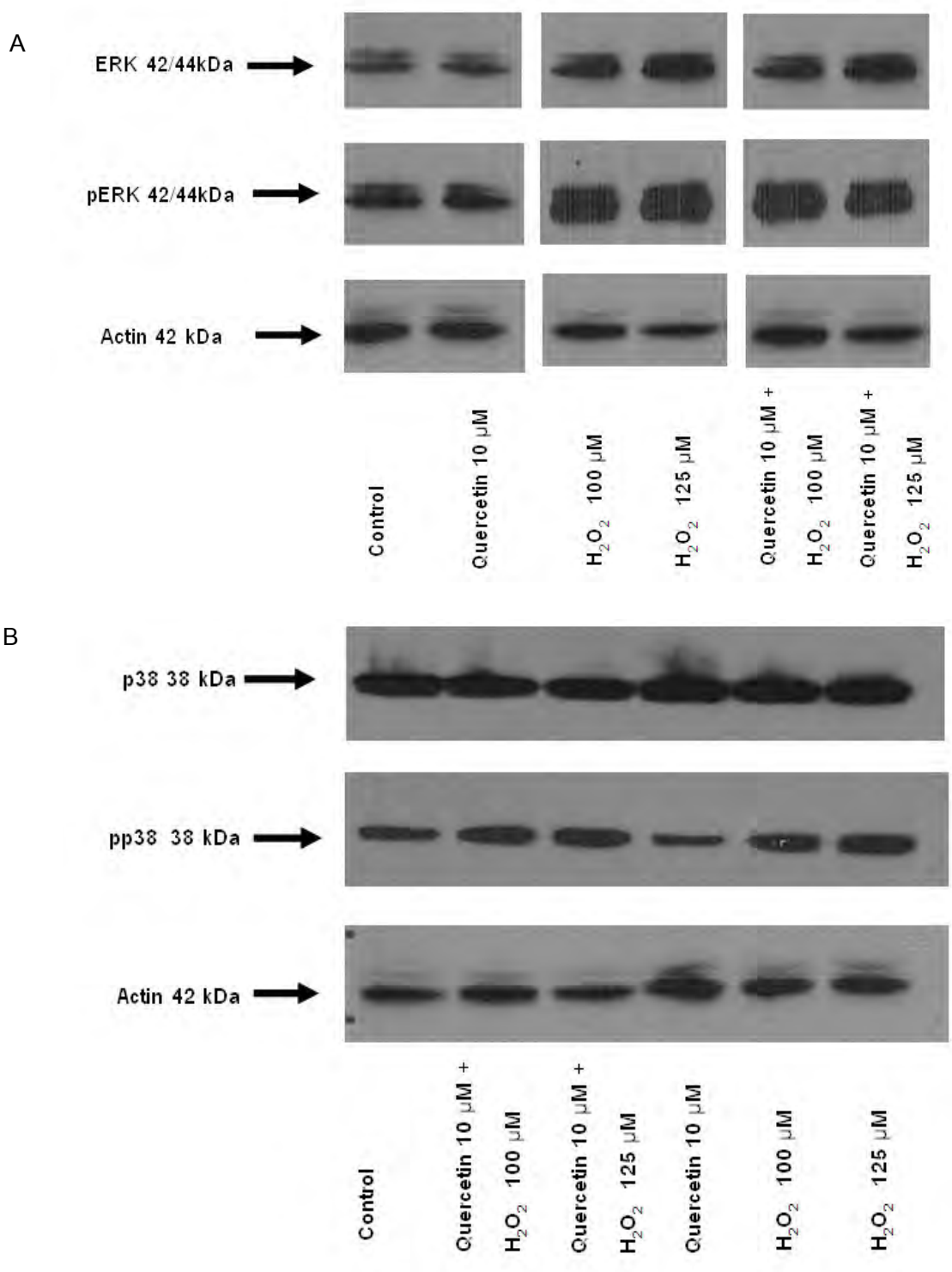
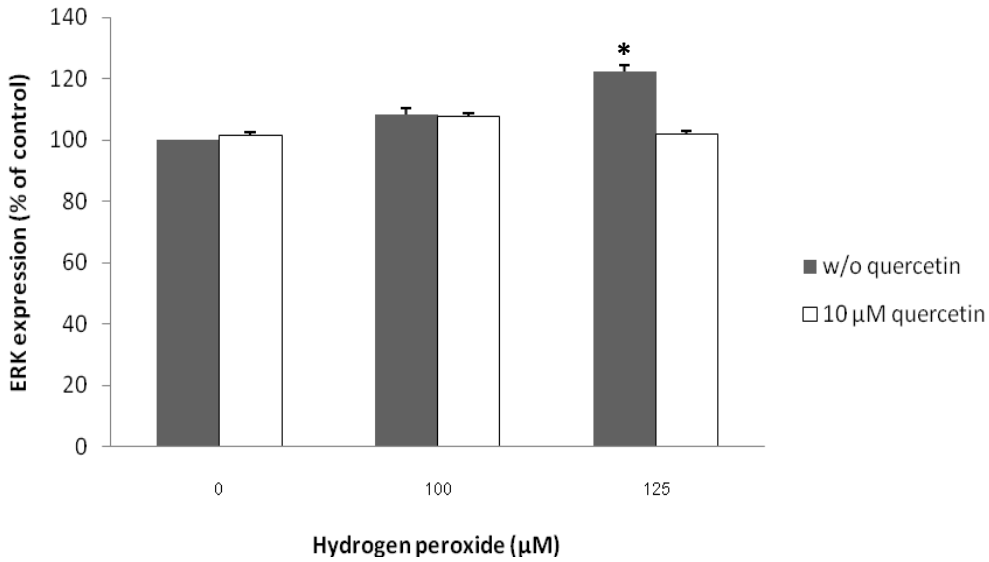


Figure 25: Effects of quercetin on expression of ERK1/2, p38 and its phosphorylation form in ECV304 cells. (A) Immunoblot of ERK1/2 and phosphorylation ERK1/2. (B) Immunoblot of p38 and phosphorylation p38.

A



B

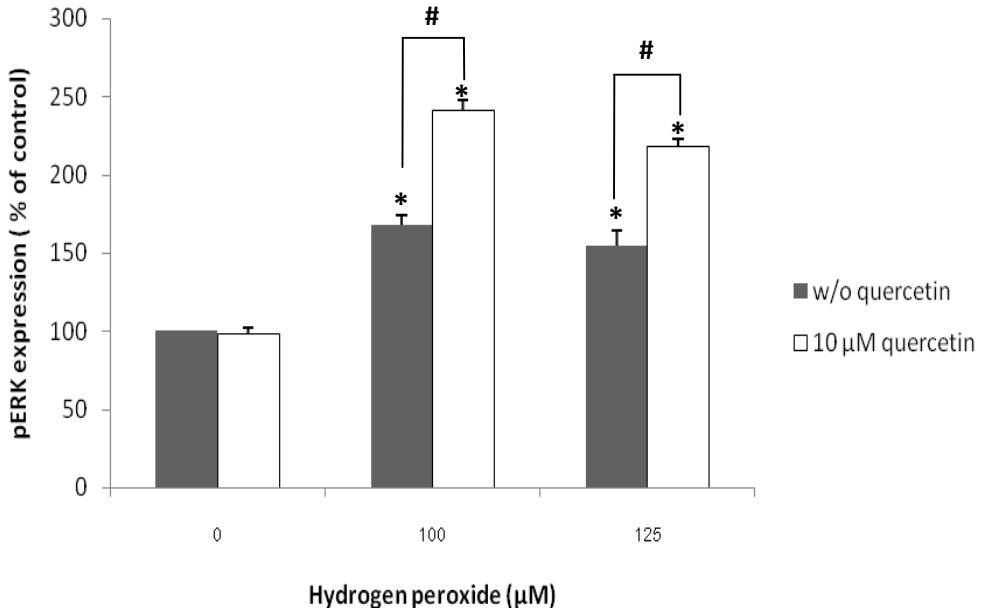


Figure 26: Effects of quercetin on expression of ERK1/2 and its phosphorylation form in ECV304 cells. (A) Densitometrical analysis of immunoblots of ERK1/2 and, (B) Densitometrical analysis of immunoblots of phosphorylation of ERK1/2. Data were presented as mean±SEM obtained from four separated experiments.

*$p<0.05$; significant difference in comparison with the control group,

$^{\#}p<0.05$; significant difference in comparison with the group treated with H_2O_2 alone.

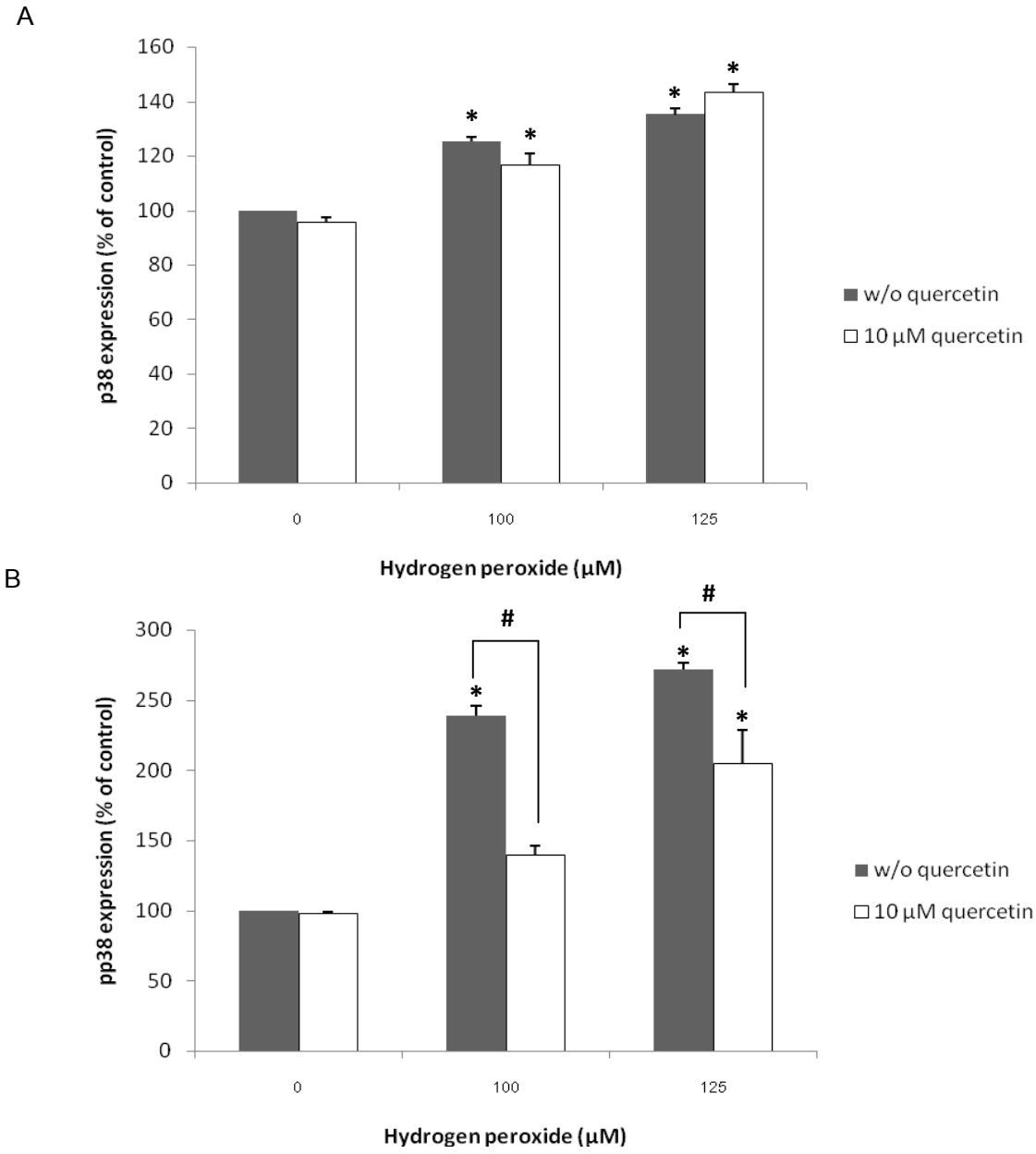


Figure 27: Effects of quercetin on expression of p38 and its phosphorylation form in ECV304 cells. (A) Densitometrical analysis of immunoblots of p38 and, (B) Densitometrical analysis of immunoblots of phosphorylation of p38. Data were presented as mean±SEM obtained from four separated experiments.

*$p<0.05$; significant difference in comparison with the control group,

#$p<0.05$; significant difference in comparison with the group treated with H_2O_2 alone.

4. The functional studies of tight junction complexes

4.1 Measurement of the TEER values

The measurement of TEER values which reflected membrane conductance could be applied to evaluate the formation of the tight junction complexes of the ECV304 cell monolayers. Hence, after seeding the cells, the TEER values were followed with Milicell-ERS® everyday in order to evaluate the development of tight junction structure. As shown in Figure 28, the TEER values increased gradually over time, and reached plateau on day 10. The maximal TEER values was 95.3 ± 8.36 $\Omega \cdot cm^2$, as measured on day 12. When the TEER values reached maximal and plateau state, it suggested that the ECV304 monolayer developed the structure of tight junction and was ready for the study of the paracellular transport with the permeability assay.

As shown in Figure 29, H_2O_2 at the concentration of 100 μM significantly decreased the TEER values approximately by 20% after 4-hr exposure. By contrast, under the similar 4-hr exposure time, the TEER values of the control group were stable. Quercetin at the concentration of 10 μM had no significant effect on the TEER values. Meanwhile, a protective effect on the H_2O_2-induced reduction in TEER was observed when 10 μM of quercetin was administered 30 min prior to H_2O_2. These results indicated that quercetin was able to protect the loss of barrier integrity mediated by H_2O_2.

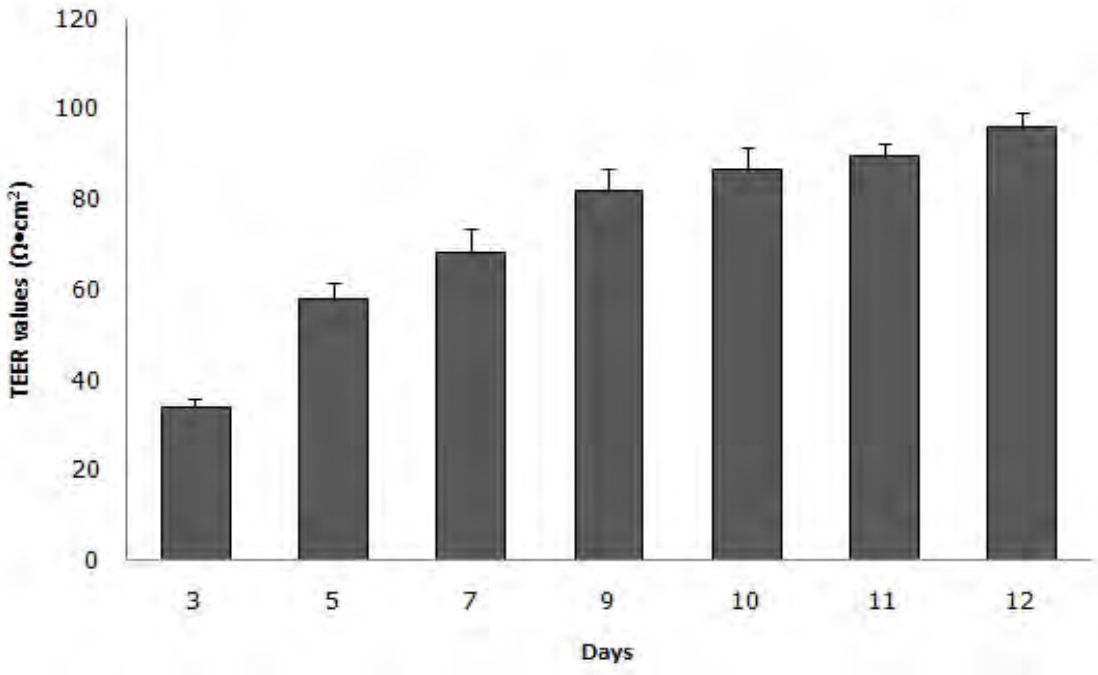


Figure 28: TEER values of ECV304 cells during 3 to 12 day-culture. The TEER increased almost fourfold after 12 day, indicated the readiness of barrier function for a phenol red permeability assay. The TEER values were obtained with background subtraction and normalized for area of the filter. The data were expressed as the mean ± SEM of four separated experiments, each of which was performed three times, repetitively.

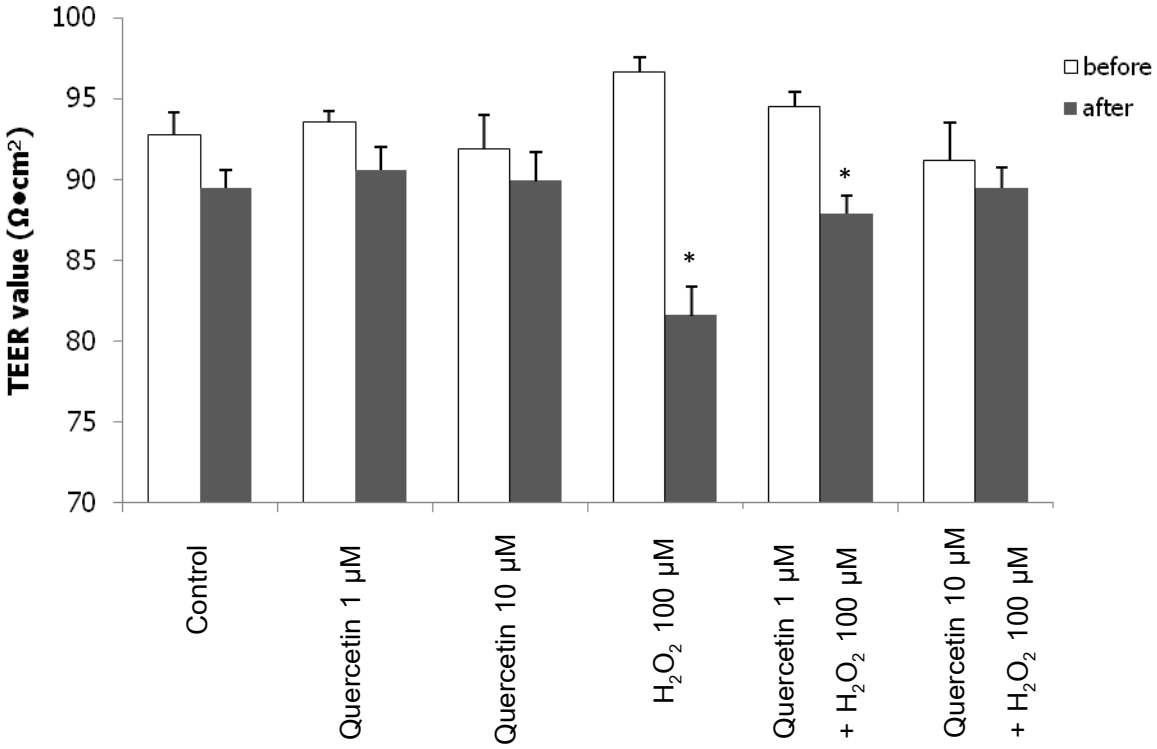


Figure 29: Effects of quercetin and H_2O_2 on the TEER values. After seeding the ECV304 for 12 days, the monolayer was ready for an experiment. The TEER values of ECV304 cell monolayer were measured before and after 4-hr period of treatment. Data were presented as mean±SEM obtained from four separated experiments.

*$p<0.05$; significant differences in comparison with the values before treatment.

4.2 Measurement of paracellular permeability

In addition to measurement of the TEER values, the paracellular transport of phenol red could be applied to measure the integrity of tight junction barrier. The healthy tight junction structure would tightly restrict the movement of phenol red through the paracellular route of the monolayer. As shown in Figure 30, H_2O_2 at the concentration of 100 μM significantly increased the paracellular permeability of phenol red by two-fold, whereas quercetin at concentration of 1 and 10 μM had no effects on the permeability. Pretreatment with quercetin for 30 min prior to the addition of H_2O_2 resulted in the decrease of the H_2O_2-induced hyperpermeability. In addition, the preventive effect of quercetin increased when its concentration increased from 1 μM to 10 μM. these observations were in agreement with those observed in the previous analysis of the TEER values. Hence, the results strongly indicated the intrinsic activity of quercetin in protection the loss of tight junction integrity upon H_2O_2 exposure.

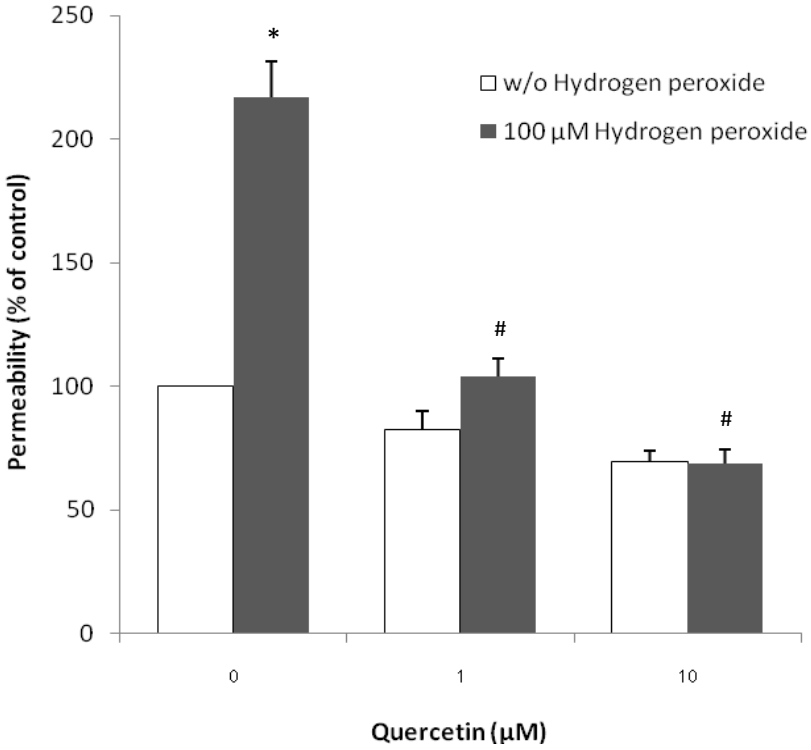


Figure 30: Effect of quercetin on H_2O_2–induced hyperpermeability of ECV304 monolayer. The ECV304 cells were pre-treated for 30 min with or without quercetin at the concentration of 1 and 10 µM. Subsequently, 100 µM of H_2O_2 was added for another 4 hr. At the end of incubation, the phenol red permeability assay was performed. Data were obtained from four separated experiments, and expressed as the mean±SEM of the percentage of the control group.

*p <0.05; significant differences in comparison with the control cultures.

#p <0.05; significant differences in comparison with H_2O_2 100 µM.

CHAPTER V

DISCUSSION AND CONCLUSION

The main objectives of the present study were (1) to study the effect of quercetin on tight junction disruption induced by H_2O_2; (2) to elucidate the potential mechanisms of quercetin on tight junction protection under H_2O_2-induced stress condition in ECV304 cells.

The formation of tight junction structure at cell-to-cell contact created a strong barrier to limit paracellular permeability of water, solutes, and immune cells. The dysfunction of tight junction complexes could be reflected by reduction of electrical resistance (TEER values) and increase of paracellular transport (Gonzales-Marisca *et al.,* 1985). In this study, ECV304 cells were employed as a model of endothelial barrier to investigate the function and integrity of tight junction complexes upon oxidative assault. Based on the TEER values, it had been established that the cell-to-cell junctional complexes of ECV304 cells provided the significant tightness at the greater degree than those of other endothelial cell lines such as RBE4, b.End3, b.End5, and RBEC (Winfried *et al*., 2008).

In this study, ECV304 cells grown as monolayer exhibited increasing TEER values over time with the maximal values of 95.3±8.36 $\Omega \cdot cm^2$. These electrical resistant values were higher than the numbers measured in primary culture of BBMECs which were used as endothelial solute barriers model of blood brain barrier (Lee *et al*., 2004). Moreover, the immunofluorescent staining demonstrated the expression and localization of occludin and ZO-1, which are tight junction associated proteins at the cell border. Hence, the monolayer of ECV304 cells grown in this study could be considered as an appropriated model for further study of tight junction.

Oxidative stress had been linked to several pathological conditions such as inflammation and ischemia-reperfusion injury. The reactive oxygen species readily react

with macromolecules in the cell, consequently they could damage cellular function, membrane integrity, and ultimately lead to cell death (Waskiewicz and Cooper, 1995; Pariente *et al*., 2001; Tiruppathi *et al*., 2003). In addition, it had been well established that H_2O_2 increased the permeability of several endothelial barrier models through disruption the tight junction structure (Kevil *et al.,* 2000; Meyer *et al.,* 2001; Jepson, 2003). Although the mechanism of H_2O_2-induced hyperpermeability was incompletely understood, a number of evidence indicated that H_2O_2 activated the MAP kinase pathway, leading to a loss of normal expression and localization of junctional regulatory proteins, especially occludin and ZO-1 (Rao *et al*., 2002; Kale *et al*., 2003).

In this study, exposure of ECV304 cells to H_2O_2 resulted in an increased paracellular permeability along with a decreased electrical resistance across barrier. These finding were in agreement with the effects of H_2O_2 on barrier function in other reports in the literature (Rao *et al*., 1997; Chapman *et al*., 2002; Fischer *et al.,* 2005). The tight junction structure was very important for control paracellular flux of solute between endothelial cells. The restrictiveness of tight junction barrier could be assessed by transport of phenol red, a marker for paracellular pathway across the monolayer. It had been demonstrated that the low level transport of phenol red across the monolayer occurred in concurrent with the high TEER values, which gave an estimate of the level of tight junctions expressed in the *in vitro* cell culture models of endothelial barrier (Martel *et al*., 2003).

Under the culture condition of this study, tight junction complexes could be the primary lesion by treated with H_2O_2. Considering that its localization at the cholesterol-enriched region on plasma membrane, the junctional associated proteins and signaling molecule were readily to interact with exogenous permeated H_2O_2. Although exposure to H_2O_2 could lead to cell injury and cell death, the exposure effects were concentration- and time-dependent. At the high concentration of greater than 1 mM, H_2O_2 caused

apoptosis of several cells type including endothelium (Huot *et al*., 1998; Wang *et al*., 2004). In addition, Fischer *et al.* (2005) treated endothelium cells with H_2O_2 at the concentration of 500 μM for 3 hr to create their hyperpermeability models. In this study, H_2O_2 at the concentration of 100 μM had no effect on cell viability. Hence, the H_2O_2-induced hyperpermeability were not resulted from the loss of ECV304 cells.

The findings in this study clearly demonstrated that an increase in paracellular permeability of ECV304 monolayer after 4-hr exposure to H_2O_2 (100 μM) resulted from a decrease in expression and localization of occludin and ZO-1 proteins. Occludin and ZO-1 were key tight junction proteins, whose expression level could determine the barrier properties. Occludin was an integral membrane protein which regulated and maintained endothelial barrier. Reduction in expression and/or disorganization of occludin lead to permeability changes of the barrier (Hirase *et al*., 1997; Denker and Nigam, 1998; Itoh *et al*., 1999; Tsukita and Fususe, 1999; Wachtel *et al*., 1999). ZO-1 was also an important component of the tight junction complexes as a linkage to the cytoskeleton. Like occludin, abnormality with ZO-1 could be resulted in the junctional disruption. The western blotting analysis of occludin and ZO-1 in this study revealed a reduction of their amount. These results were in correlation with the discontinuous pattern of immunofluorescent staining for these two junctional proteins which suggested the loss in their localization. These findings were similar to those seen in other reports (István *et al*., 2005). Hence, it was likely that disruption of tight junction integrity in response to oxidative stress occurred from downregulation of junctional protein expression as well as from their disorganization. However, these were studies reported that H_2O_2 caused an increase of occludin expression. For example, Lee *et al*. (2004) showed that exposure the bovine brain microvessel endothelial cells (BBMECs) with H_2O_2 resulted in the reduction of TEER values and rearrangement of occludin and ZO-1 with an increased of occludin protein expression.

The most interesting findings in this study were the protective effect of quercetin against H_2O_2-induced hyperpermeability. Quercetin had been one of the most studied flavonoids with variety of pharmacological actions including vasodilatation, antiinflammation, and antioxidant. This study was the first to demonstrate that pretreatment the cells with quercetin could preserve the integrity and function of tight junction barrier against H_2O_2 assaults.

The regulation and maintenance of microvascular endothelial permeability involved with several processes including receptor activation, second messenger mobilization, cytoskeleton and junction associated protein assembly, and junction reorganization (Malik and Fenton,1992; Siflinger-Birnboim *et al*., 1996; Haselton and Heimarh, 1997; Menger *et al.,*1997). Multiple signaling transduction pathways and secondary messengers had been implicated in tight junction biogenesis including Src kinase (Kevil *et al*., 2001), MAP kinase (Huot *et al*., 1998; Chen *et al.,* 2000; Kevil *et al.,* 2001), Ca^{2+} (Ueda and Shah, 1992; Stuart *et al*., 1996), G-protein (Denger *et al*., 1996), PKC (Shasby *et al*., 1988), PLA_2 (Goligorsky *et al*., 1993), and c-AMP (Ishizaki *et al*., 2003). It had been well established that the effects of H_2O_2 on hyperpermeability were mediated through the activation of MAP kinase signaling pathway, in particular through ERK1/ERK2 (Chen *et al*., 2000; Kevil *et al.,* 2000).

In this study, H_2O_2 (100 μM) led to an activation of MAPK pathways without significant effect on cell viability. It was likely that the increases of phosphorylated ERK1/2 and p38 were well corroborated with an increase of paracellular permeability and the disruption of tight junction assembly. These finding were similar to those reports in other endothelial cells such as HUVEC (Huot *et al.,* 1998; Kevil *et al*., 2000). Kevil *et al*. (2000) demonstrated that HUVEC treated with H_2O_2 at the concentration of 500 μM for 3 hr were resulted in hyperpermeability in correlation to ERK1/2 activation and

disorganization of occludin and ZO-1 at the cell-to-cell contact sites. Huot *et al.* (1998) had also shown that peroxide administration caused an early increase in ERK1/2 activity, and a more prolong increase in p38 MAPK activity. It was interesting that pretreatment of the ECV304 monolayer with quercetin for 30 min significantly attenuated permeability changes from H_2O_2 exposure. This observation was correlated to an apparent normal immunofluorescent staining for occludin and ZO-1 in quercetin pretreated group. In addition, quercetin was able to prevent a significant loss of occludin and ZO-1 exprssion upon challenging ECV304 with H_2O_2.

The protective effect of quercetin may be involved with an alteration of MAPK activities. In addition to the structural disorganization of tight junction complexes, activation of MAPK pathway could lead to downregulation of occludin expression, consequently the barrier permeability increased (Chen *et al*., 2000). In this study, quercetin affected the activities of MAPK in particular ways. The results demonstrated that quercetin had no significant effects on MAPK activities in the normal ECV304 cells. However, quercetin appeared to potentiate H_2O_2-induced stimulation of ERK1/2 activities, whereas the opposite effect was found in p38 activities. The anti-active MAP kinase antibody revealed that an increased p38 activity resulting from H_2O_2 exposure for 4 hr could be prevented by quercetin. Taken together, these results suggested that activation of p38 was involved in the regulation of ECV304 permeability. There were different viewpoints about the involvement of p38 and ERK1/2 activations in H_2O_2-induced hyperpermeability. Some studies suggested that ERK1/2 phosphorylation was a key regulatory pathway in the maintenance of tight junction integrity against H_2O_2 treatment (Huot *et al.,* 1998; Kevil *et al*., 2000; Fischer *et al*., 2005). On the contrary, others reported that p38 MAPK, but not ERK, involved with the barrier dysfunction caused by H_2O_2 (Usatyuk and Natarajan, 2004). These differences may be involved in several factors such as cells type, culture condition, and time to exposure.

The results in this study demonstrated that the protective effects of quercetin against H_2O_2-induced disruption of tight junction integrity were possible related to the reduction of phosphorylated p38 MAP. However, the molecular targets of quercetin were not yet identified. It had been hypothesized that H_2O_2 activated the membrane G-protein coupled receptor in order to initiate signaling cascades that led to the hyperpermeability in BBMECs (Fischer *et al*., 2005). The receptor activation was resulted from the oxidation mechanism at the cysteine-rich region of target proteins. In addition, N-acetylcysteine (NAC) which was a known anti-oxidative agent could prevent the H_2O_2-induced hyperpermeability of BBMECs (Fischer *et al*., 2005). Hence, it was possible that quecetin, which was a known antioxidant flavonoid compound, exerted protective actions through the anti-oxidative mechanism in similar manner to threat of NAC. It was noteworthy that in this experiment quercetin was washed out prior to the addition of H_2O_2. After 30 min of pretreatment, quercetin could permeate through the plasma membrane and elicited its actions. The protective effects of quercetin in this study were not likely from the neutralization of H_2O_2 in culture medium. Further studies to clarify the anti-oxidative mechanism to protect tight junction modulation might be of interest.

In addition to antioxidant activity, quercetin had been known for its endothelium-dependent vasorelaxation effect which occurred through the NO-cGMP pathway (Chi *et al*., 1999; Lee and Cheng, 2003). Nitric oxide was an important signaling molecule involving with several physiological and pathological conditions. It had been reported that NO levels were correlated with paracellular permeability of endothelial barrier (Johns *et al*., 1987; Hampl *et al*., 1995; Nathan, 1992). For example, Parenti *et al.* (1998) suggested that nitric oxide and cGMP contributed to the VEGF-dependent ERK1/2 activation. In addition, exposure to H_2O_2 induced a rise of intracellular Ca^{2+} which was a key activator of nitric oxide synthase (NOS) (Johns *et al.,* 1987; Hampl *et al.,*1995). In this study, quercetin had no significant effect on NO release from ECV304, whereas H_2O_2

led to a greater production of NO. Apparently, combination of quercetin and H_2O_2 resulted in a reduction of NO, in comparison with H_2O_2-treated group. These findings were in agreement with previous reports which demonstrated the inhibitory effects of quercetin against accumulation of nitrite in rat hepatocyte treated with IL-1β. These might be due to its ability to suppress iNOS gene expression (Susana *et al.,* 2005). Moreover, the study of Malladi *et al.* (2004) demonstrated that activation of NF-κB and MAPK signaling pathways resulted in increased iNOS expression and NO production. In the present study, the western blot analysis revealed that cell exposed to quercetin prior to the addition of H_2O_2 caused a reduction of phosphorylated p38 MAPK. Hence, it was possible that the increased NO production observed in ECV304 treated with H_2O_2 was associated with the expression of MAPK, in particular p38 activity. This hypothesis needed further investigation.

In conclusion, exposure to the low concentration of H_2O_2 may not cause severe cellular injury, leading to cell death. Certainly, H_2O_2 could damage cellular function and membrane integrity by interacting with macromolecules as well as by activating a number of signaling pathways. Endothelium/epithelium barriers could be primary targets of H_2O_2 assaults, leading to functional abnormality associated with the collapse of tight junction structure. As a result, changed in paracellular solute permeability was observed in correlation with disorganization of junctional proteins including occludin and ZO-1. This study was the first investigation to demonstrate the protective effect of quercetin, an edible flavonoid, against H_2O_2-mediated tight junction disruption and hyperpermeability of endothelial barrier. Pretreatment the barrier with quercetin prior to H_2O_2 exposure prevented the loss of expression and dislocalization of occludin and ZO-1, which were regulatory proteins in tight junction assembly. Although the molecular targets of quercetin had not been fully understood, it was very likely that quercetin exerted its protective action through suppression of MAP kinase activities, especially p38 MAPK. In

addition, it was possible that quercetin prevented the oxidative activation of membrane receptor induced by H_2O_2, resulting in inhibition of the subsequent signaling cascades which were involved with regulation of tight junction assembly.

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APPENDICES

APPENDIX A

PREPARATION OF REAGENTS

M199 1,000 ml

1. Dissolve the following ingredients in 1,000 ml of distilled water.

1.	M199	1	pack
2.	$NaHCO_3$	2.2	g

2. Adjust pH of this solution to 7.2-7.4 by adding 1 N HCl or 1 N NaOH.
3. Sterile by filtration through 0.22 µm acrocap filter.
4. Store in a refrigerator at 4 °C (Stable for 1 month).
5. Mix with 10% fetal bovine serum (FBS) + 1% penicilin-streptomycin before used (89 ml of medium + 10 ml of FBS + 1 ml Pen-streptomycin).

Preparation of 0.25% Trypsin, 0.038%EDTA in PBS (100 ml)

1. Dissolve 0.25 g of trypsin and EDTA 0.038 g in phosphase buffer saline solution (PBS) and adjust volume to 100 ml
2. Sterile by syring filtration through 0.22 µm
3. Store in a refrigerator at 4 °C.

Preparation of phosphate buffer saline solution (PBS) 1000 ml

1. Dissolve the following ingredients in 1,000 ml of distilled water.

Potassium chloride	0.20 g
Sodium chloride	8.00 g
Potassium phosphate monobasic (anhydrous)	0.20 g

Sodium phosphate dibasic (anhydrous) 1.15 g

2. Adjust pH of this solution to 7.2-7.4 by adding 1 N HCl or 1 N NaOH.
3. Sterile using autoclave (121°C, 20 min)
4. Store in a refrigerator at 4°C

Preparation of Acrylamide gel

Solution A : acrylamide (50%) Dissolve the following ingredients in 100 ml of distilled water

0.8 g methylene bis acrylamide

49.2 g acrylamide

Solution B : 4X Separating buffer (Main gel) 100 ml

(75 ml 2 M Tris HCl, pH 8.8 + 4 ml 10% SDS + 21 ml DDW)

Solution C : 4X Stacking buffer (Top gel) 100 ml

(25 ml 2 M Tris HCl, pH 6.8 + 4 ml 10% SDS + 71 ml DDW)

APS (ammonium persulfate) 10% APS in DDW

TEMED (N, N, N, N-tetramethylenediamine)

Solution A, B, APS, and TEMED stored at 4 °C

Solution C stored at room temperature

APS prepared every week

The gel apparatus and spacers (1.5 mm thick) were assembly.

1. **Preparation of separating gel**

To make two plates of 12% acrylamide gel, the ingredients of separating gel are

Solution A 2.4 ml

Solution B 2.5 ml

DDW 5.1 ml

All of the ingredients were thoroughly mixed. The mixture was supplemented with 50 µl APS and 10 µl TEMED, then thoroughly mixed and immediately pour the gel between the glass plates. Before gel polymerization was complete, 0.1% SDS in DDW was layered on the top of the separating gel (5 mm thick). Leave the separating gel until the gel completely polymerized approximately 20-30 min.

2. **Preparating of stacking gel**

Once the separating gel has completely polymerized, 0.1% SDS was remove from the top of the polymerized gel. To make stacking gel, the ingredients are

Solution A 400 µl

Solution C 1 ml

DDW 2.6 ml

All of the ingredients were thoroughly mixed. The mixture was supplemented with 30 µl APS and 5 µl TEMED, then thoroughly mixed and immediately pour the gel between the glass plates. The combs were inserted between the two glass plates of two sets of gel apparatus. The gels were leaved for approximately 30 min to polymerize.

3. Application of samples

Once the stacking gel has completely polymerized, the combs were gently removed. The wells were flushed out thoroughly with running buffer. The clips and sealing tapes were removed and set up the gel chamber. Running buffer was filled out both inner and outer chamber. Before loading samples and protein marker, all air bubbles between layers were removed by gentle rolling the chamber.

Running buffer

To make 1 liter of 1X running buffer, pH 8.3, the ingredients are

Tris	3 g
Glycine	14.4 g
SDS	1 g

All ingredients were dissolved in DDW with continuously stirring. The solution was adjusted volume to 1000 ml.

Transfer buffer

To make 1 liter of 1X transfer buffer, pH 8.1-8.4, the ingredients are

Tris	1.93 g
Glycine	9 g

All ingredients were dissolved in 800 ml DDW with continuously stirring. The solution was adjusted volume to 1000 ml. Before use, the solution was supplement with 200 ml methanol.

Tris-buffer saline. 0.1% Tween 20 (TBST)

To make 500 ml of 10X TBS, the ingredients are

2M Tris-HCl (pH 7.5)	25 ml
NaCl	43.8 g

All ingredients were dissolved in DDW with continuously stirring. The solution was adjusted volume to 500 ml. Before use, the solution was diluted to 1X TBS and supplemented with 1 ml Tween 20.

Sample buffer

To make 50 ml of 5X sample (60 mM Tris HCl (pH 6.8), 2% SDS, 25% glycerol, 14.4 mM β-mercaptoethanol and 0.1% bromophenol blue) for stock solution. All ingredients were dissolved in DDW with continuously stirring. The solution was adjusted volume to 50 ml. 5X sample buffer was aliquot into 1 ml/tube and stored at -20 °C.

Lysis buffer for Western blot analysis

To make 50 ml of lysis buffer (20 mM Tris HCl (pH 7.4), 150 mM NaCl, 1% Triton-X 100, 1% Na deoxycholate, 10mM NaF, 1mM Na orthovanadate). All ingredients were mixed well in DDW. The lysis buffer was aliquot into 1ml/tube and stored at -20°C. Before use, the solution was supplemented with 1 mM Pefabloc and 5 µg/ml aprotinin.

Color reagent for measure the nitric oxide level

Color reagent #1 (1% Sulhanilamide (p-Aminobenzenesulfonamide) in 3N HCl)

Color reagent #2 (0.1% N-(1-Naphthyl) ethylenediamine dihydrochloride in deionized water)

APPENDIX B

TABLES OF EXPERIMENTAL RESULTS

Table 2: The percentage of MTT reduction of cultured ECV304 treated with quercetin for 30 min and 24 hr

Concentration (μM)	% MTT reduction (compared to control)	
	30 min	24 hr
0	100.0 ± 0.00	100.0 ± 0.00
0.1	93.7 ± 2.10	94.0 ± 1.82
1	95.7 ± 0.80	93.9 ± 0.83
10	98.3 ± 1.15	95.2 ± 0.87
100	38.5 ± 1.59*	38.0 ± 3.83*

Each value represented as mean ± SEM of three independent experiments, each performed in triplicate. Asterisks refer significant differences from the control group: *p <0.001 (One-way ANOVA followed by Scheffe's post hoc testing).

Table 3: The percentage of MTT reduction of cultured ECV304 treated with H_2O_2 for 4 hr and pretreatment with quercetin 10 M for 30 min and followed with varies concentration of H_2O_2

Concentration of H_2O_2 (μM)	% MTT reduction (compared to control)	
	w/o quercetin	pretreatment with quercetin 10 μM for 30 min
0	100.0 ± 0.00	101.7 ± 5.50
50	98.5 ± 0.37	96.2 ± 0.83
100	97.1 ± 1.13	99.6 ± 1.49
125	87.9 ± 0.59*	98.1 ± 2.31
250	77.6 ± 2.53*	83.8 ± 1.30*
500	55.8 ± 1.07*	68.6 ± 0.67*
1000	37.7 ± 1.02*	62.3 ± 1.08*

Each value represented as mean ± SEM of three independent experiments, each performed in triplicate. Asterisks refer significant differences from the control group: *p <0.05 (One-way ANOVA followed by Scheffe's post hoc testing).

Table 4: The percentage of tight junction protein expression

Groups	ZO-1 expression	Occludin expression
Control	100.0±0.00	100.0±0.00
H_2O_2 100 µM	69.6±1.75*	71.7±2.48*
H_2O_2 125 µM	71.1±4.54*	68.0±5.18*
Quercetin 10 µM	101.7±3.78	98.4±1.32
Quercetin 10 µM + H_2O_2 100 µM	86.1±3.94	96.9±2.88#
Quercetin 10 µM + H_2O_2 125 µM	89.4±2.82	84.8±7.72

Each value represented as mean ± SEM of three independent experiments, each performed in triplicate. Asterisks refer significant differences from the control group. *p <0.05 (One-way ANOVA followed by Scheffe's post hoc testing).

Table 5: The percentage of the level of ROS in cultured ECV304

Groups	% level of ROS (compared to control)
Control	100.0 ± 0.00
H_2O_2 100 μM	120.9 ± 14.10[a]
H_2O_2 125 μM	129.5 ± 15.36[**]
Quercetin 10 μM	126.9 ± 7.83
Quercetin 10 μM + H_2O_2 100 μM	131.1 ± 10.24[##]
Quercetin 10 μM + H_2O_2 125 μM	122.4 ± 6.47[##]

Each value represented as mean ± SEM of three independent experiments, each performed in triplicate. ** p <0.01 compared with control cultures, [##] p <0.01 compared with H_2O_2 alone, [a] p <0.05 compared with quercetin. (One-way ANOVA followed by Scheffe's post hoc testing).

Table 6: The percentage of the level of nitric oxide in cultured ECV304

Groups	% level of nitric oxide (compared to control)
Control	100.0 ± 0.00
H_2O_2 100 μM	126.8 ± 3.40
H_2O_2 125 μM	141.8 ± 3.91*
Quercetin 10 μM	102.7 ± 1.74
Quercetin 10 μM + H_2O_2 100 μM	107.6 ± 2.39
Quercetin 10 μM + H_2O_2 125 μM	119.9 ± 4.33

Each value represented as mean ± SEM of three independent experiments, each.performed in triplicate. *p <0.05 compared with control cultures.

Table 7: The TEER value of cultured ECV304 cells in Transwell®

Well	Day after seeding cell				
	3	6	9	10	12
1	36.3	78.0	78.7	90.0	95.3
2	15.7	45.0	59.3	77.3	73.3
3	20.0	63.7	108.0	110.7	103.0
4	29.3	70.0	70.3	73.3	76.7
5	26.7	78.3	110.7	131.7	130.0
6	37.3	68.7	76.7	78.3	93.3
mean	27.6	67.3	83.9	93.6	95.3
SD	8.66	12.30	20.81	23.07	20.48
SEM	3.54	5.02	8.49	9.42	8.36

Each value represented as mean ± SEM of four independent experiments, each performed in duplicate.

Table 8: TEER value of ECV304 cell before and after treatment

Treatment group	TEER($\Omega \cdot cm^2$)	
	Before	After
Control	92.75±1.37	89.44±1.16
Quercetin 1 μM	93.54±0.70	90.56±1.45
Quercetin 10 μM	91.89±2.08	89.89±1.79
H_2O_2 100 μM	96.67±0.87	81.57±1.75*
Quercetin 1 μM +H_2O_2 100 μM	94.56±0.84	87.89±1.06*
Quercetin 10 μM +H_2O_2 100 μM	91.22±2.26	89.44±1.26

Each value represented as mean ± SEM of four independent experiments, each performed in duplicate.

VITA

Miss Somrudee Chuenkitiyanon was born on May 9, 1975 in Bangkok, Thailand. She received the Bachelor of Nursing from Mahidol University in 1998. She has worked at the Siriraj Hospital for 5 years. She completed her Master's Degree of Sciences (Pharmacology) from in Interdisciplinary Program in Pharmacology, Graduate School, Chulalongkorn University in 2002 and went to be a lecturer at Faculty of Nursing, Siam University since 2003. She has enrolled the philosophy's degree program in Interdisciplinary Program in Pharmacology, Graduate School, Chulalongkorn University in 2004.