UNIVERSITI PUTRA MALAYSIA

PROPERTIES OF ASIATICOSIDE IN SUPPRESSING HYPERPERMEABILITY IN HUMAN UMBILICAL VEIN ENDOTHELIAL CELLS INDUCED BY INTERFERON-GAMMA

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Impairment of endothelial barrier function by vasoactive agents or inflammatory mediators promotes endothelial hyperpermeability causing the development of pathological conditions such as tissue oedema. The changes of barrier structure caused gaps formation thus promote vascular leakage of plasma fluid and macromolecules. Interferon-gamma is a well-known pro-inflammatory mediator which disturbed barrier integrity thus promotes hyperpermeability on endothelial cell. Several studies have reported that asiaticoside; a triterpenoid derived from *Centella asiatica* (local - pegaga) exhibits good anti-oxidant properties, wound healing, immunomodulatory, as well as anti-inflammatory effects on several inflammatory models. In order to elucidate the potential properties of asiaticoside on endothelial barrier preservation, its actions on Human Umbilical Vein Endothelial Cell (HUVEC) hyperpermeability induced by interferon-gamma was assessed. It has been found that interferon-gamma caused an increased in endothelial permeability, determined by the flux of fluorescein isothiocyanate-dextran through *in vitro* permeability assay. Pre-treatment of asiaticoside to interferon-gamma-activated HUVEC significantly ($p < 0.05$) attenuated endothelial hyperpermeability. Moreover, it was clearly demonstrated that asiaticoside helps in restoration of nitric oxide; a mediator which regulates barrier integrity and permeability regulator, back to its basal state after impairment by interferon-gamma. p38 Mitogen Activated Protein Kinase signalling pathway is believed to be involved in nitric oxide production. Thus, it was clearly clarified that p38 Mitogen Activated Protein Kinase phosphorylation, conducted via western blot analysis was deactivated after treatment with asiaticoside. For further clarification, cyclooxygenase-2 level as well as prostaglandin E_2 release in interferon-gamma-activated HUVEC was also studied. Interferon-gamma has increased cyclooxygenase-2 level and prostaglandin E_2 release but also stabilized by asiaticoside. These results suggest that asiaticoside regulated endothelial barrier preservation via p38 Mitogen Activated Protein Kinase phosphorylation and nitric oxide restoration. Our results suggest that asiaticoside may become an important remedy in endothelial barrier preservation induced by interferon-gamma.

Keywords: Hyperpermeability, interferon-gamma, asiaticoside, prostaglandin E_2, cyclooxygenase 2, p38 mitogen activated protein kinase.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk Ijazah Master Sains

POTENSI ASIATICOSIDE DI DALAM SUPRESI HIPERKEBOLEHTELAPAN PADA SEL ENDOTHELIAL VENA UMBILIKAL MANUSIA YANG DIARUH OLEH INTERFERON-GAMMA

Oleh

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Kemerosotan fungsi halangan endothelial oleh ejen vasoaktif atau pengantara keradangan menggalakkan hiperkebolehtelapan sel endothelial yang menyebabkan pembangunan keadaan patologi seperti rangkaian edema. Perubahan struktur halangan menggalakkan kebocoran vaskular cecair plasma dan makromolekul. Interferon-gamma terkenal sebagai mediator pro-radang yang mengganggu integriti halangan lalu menggalakkan peningkatan ketelapan pada sel-sel endothelial. Beberapa kajian telah melaporkan bahawa asiaticoside; salah satu daripada triterpenoids yang diperolehi daripada *Centella asiatica* (tempatan - pegaga) mempamerkan ciri-ciri anti-oksida yang baik, penyembuhan luka, peningkatan system imun, serta kesan-kesan anti-radang pada beberapa model radang. Dalam usaha untuk menjelaskan potensi asiaticoside pada pemeliharaan sistem halangan endothelial, tindakannya terhadap peningkatan ketelapan HUVEC yang diaruh oleh interferon-gamma telah dinilai. Didapati bahawa interferon-gamma menyebabkan peningkatan ketelapan endothelial yang ditentukan berdasarkan pengaliran fluorescein isothiocyanate-dextran melalui kaedah *'in vitro'* assay kebolehtelapan. Pra-rawatan asiaticoside untuk interferon-gamma-aktifkan HUVEC mempunyai kesan signifikasi ($p<0.05$) dimana peningkatan ketelapan endothelial dapat dilemahkan. Selain itu, ia jelas menunjukkan bahawa asiaticoside membantu dalam pemulihan oksida nitrik; mediator yang mengawal integriti halangan dan pengawalatur ketelapan, kembali kepada keadaan basal selepas terjejas oleh interferon-gamma. Laluan isyarat p38 Mitogen Diaktifkan Protein kinase dipercayai terlibat dalam pengeluaran nitrik oksida. Oleh itu, ia jelas menunjukkan bahawa pemfosforilan p38 Mitogen Diaktifkan Protein Kinase telah dinyahaktifkan selepas rawatan dengan asiaticoside melalui kaedah blot western. Untuk penjelasan lanjut, tahap cyclooxygenase-2 serta prostaglandin E2 diterbitkan oleh interferon gamma-aktifkan HUVEC juga telah dikaji. Interferon-gamma telah meningkatkan aras cyclooxygenase-2 dan penghasilan prostaglandin E_2 tetapi juga mampu distabilkan oleh asiaticoside. Keputusan ini menunjukkan bahawa asiaticoside memelihara sistem halangan endothelial melalui pemfosforilan p38 Mitogen Diaktifkan Protein kinase dan pemulihan oksida nitrik. Keputusan kami mencadangkan bahawa asiaticoside boleh menjadi satu ubatan yang penting dalam pemeliharaan sistem halangan endothelial yang diaruh oleh interferon-gamma.

Kata kunci: Peningkatan ketelapan, interferon-gamma, asiaticoside, prostaglandin E_2, cyclooxygenase-2, p38 Mitogen Diaktifkan Protein Kinase

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I certify that a Thesis Examination Committee has met on _____ to conduct the final examination of NurNabila Huda Khairudin on her thesis entitled "Potential Properties of asiaticoside in Suppression of Hyperpermeability in Human Umbilical Vein Endothelial Cell induced by Interferon-gamma" in accordance with the Universities and Universiti College Act 1971 and the Constitution of the Universiti Putra Malaysia [P.U. (A) 106] 15 March 1998. The committee recommends that the student be awarded the Master of Science.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

AA	Arachidonic acid
BCA	Bicinchoninic acid
BSA	Bovine serum albumin
Ca2+	Calcium ion
COX	Cyclooxygenase
cNOS	Constitutive nitric oxide synthase
DAN	2,3-diaminonaphthalene
DMSO	Dimethyl sulfoxide
ECL	Enhanced chemiluminescence
ECM	Endothelial cell media
EDTA	Ethylenediaminenetetraacetic acid
END-D Cells	Mouse vascular endothelial cells
ELISA	Enzyme-linked Immunosorbent Assay
eNOS	Endothelial nitric oxide synthase
ERK	Extracellular signal-regulated kinases
FBS	Fetal bovine serum
FDA	Food & drug administration
FITC	Fluoresence isothiocyanate
GPCR	G protein coupled receptors
HPLC	High performance liquid chromatography
HUVEC	Human umbilical vein endothelial cell
HO	Heme oxygenase
ICAM-1	Intercellular adhesion molecule-1
IFN-α	Interferon-alpha
IFN-β	Interferon-beta
IFN-γ	Interferon-gamma
IFN-ω	Interferon-omega
IFN-τ	Interferon-tau
IL	Interleukin
iNOS	Inducible nitric oxide synthase
IP3	Inositol 1,4,5-triphoshate
JNK	C-jun N terminal kinase
LNAME	L-NG-Nitroarginine methyl ester
LPS	Lipopolysaccharide
MAF	Macrophage-activating factor
MAPK	Mitogen activated protein kinase
MHC	Major histocompatibility complex
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NaOH	Sodium hydroxide
nNOS	Neuronal nitric oxide synthase
NO	Nitric oxide
NK cell	Natural killer cell
NSAIDs	Non-steroidal anti-inflammatory Drugs
PAF	Platelet-activating factor
PAMP	Pathogen-associated molecular pattern
PBS	Phosphate buffered saline
PGE_2	Prostaglandin E_2
PKC	Protein kinase C

PKG	Protein kinase G
PVDF	Poly-vinylidenedifluoride
RIPA Buffer	Radioimmunoprecipitation assay buffer
ROS	Reactive oxygen species
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
TBS	Tris-buffered saline
Th cell	T Helper cell
TLC	Thin layer chromatography
TNF-α	Tumour Necrosis Factor-α
$[Ca^{2+}]_i$	Intracellular Calcium ion concentration
U/mL	Units per ml
µM	Micromolar
µM/mL	Micromolar per millilitre
µg/mL	Microgram per millilitre
µL	Mircolitre
ng/mL	Nanogram per millilitre
µm	Micrometer
mM	Milimolar
Mg	Miligram

CHAPTER 1

INTRODUCTION

1.1 General introduction

Vascular tissue is a fundamental effector when there is body's tissues damaged due to infections and trauma. The vessels will start to release fluid due to high permeability and increases blood flow in order to stop and repair the damage. Endothelial hyperpermeability marks the occurrence of inflammatory vascular oedema. Inflammation is the reaction or response of living tissue when there is infection and injury caused by physical trauma, noxious chemicals, or microbiologic agents take place. Inflammation is a normal, protective response and one of the body's efforts to inactivate or destroy invading organisms, remove irritants and directly set the stage of tissue repair – healing. When healing process is completed, these inflammatory processes usually cease. However, if assisted repair mechanism is poorly conducted, chronic inflammatory disorder can be developed which leads to persistent tissue damage by leucocytes, lymphocytes or collagen (Nathan, 2002) and further promotion to scarring and loss of organ function (Ricciotti & FitzGerald, 2011).

Inflammation encompasses a complex cascade of reaction at the peripheral receptor terminals. It works on two ways; primary (acute) and secondary (chronic) pathways. Typically, primary pathway focuses on detoxification and repair while secondary pathway works on protection or body defence process. Inflammation can be produced with the aid of pro-inflammatory cytokines such as Interferon-gamma (IFN-γ), Tumour Necrosis Factor (TNF), Interleukin (IL)-8 as well as neuropeptide bioactive substance, Substance P (SP) which contribute to neurogenic inflammation. SP in turn may act on target cells in periphery such as mast cells, immune cells, as well as vascular smooth muscle (Richardson & Vasko, 2002; Walsh et al., 1995) thus evoking inflammatory peripheral effects such as vasodilatation, plasma extravasation (Lembeck & Holzer, 1979) and leukocyte activation (O'Connor et al., 2004). This process appears to contribute in the pathogenesis of numerous diseases including psoriasis, asthma, fibromyalgia, eczema, multiple chemical sensitivity and migraine (Richardson & Vasko, 2002). IFN-γ is pleiotropic cytokine that possessses anti-tumour (Schroder, Hertzog, Ravasi, & Hume, 2004), anti-proliferative and antiviral activity (Dinarello, 2000; Pawliczak et al., 2005). IFN-γ may also boost TNF activity and induced nitric oxide (NO) formation. Therefore, IFN-γ is considered as a pro-inflammatory cytokine (Dinarello, 2000) and since the last decades, cytokine-based therapy has brought in much attention to researchers (Ng et al., 2015).

When there is disruption or sudden disturbance of endothelial layer during pathological conditions, the injury may lead to endothelial cell damage, contraction of adjacent endothelial cell, plasma extravasation (Granger, 2010) and subsequently cause chronic inflammatory diseases (Javanmard & Dana, 2012). Endothelial gaps causing endothelial cell to become highly permeable to proteins and plasma fluids, which eventually leads to oedema and swelling (Fong et al., 2015). Endothelial cells become the main targets of the occurrence of adverse effects as they are routinely exposed to circulating immune cells and effector molecules of immunity after responding to stimuli (Javanmard & Dana, 2012). In the present study, Human Umbilical Vein

Endothelial Cell (HUVEC) was used as *in vitro* model system to study whether IFN-γ-induced endothelial hyperpermeability can be attenuated by asiaticoside which is one of the components found in *Centella asiatica*.

Present study will focused on asiaticoside in restoration of IFN-γ-induced endothelial hyperpermeability on HUVEC and the possible mechanisms involved. Traditional plant remedies can become an important source in providing new, active and potential treatment to cure diseases related to inflammation. *Centella asiatica* (local name – pegaga in Malay) has been utilized for many years in medicinal treatment while asiaticoside; a major triterpenoid saponin component of *Centella asiatica* is believed to possess various antioxidant properties (Pitella et al., 2009), anti-inflammatory activities (Gohil et al., 2010) and contribute to wound healing (Shukla et al., 1999). Apart from the valuable effects of asiaticoside stated above, the beneficial effects of asiaticoside on IFN-γ-induced endothelial hyperpermeability in HUVEC are still insufficiently declared.

This research can provide a very important finding where asiaticoside becomes a novel alternative treatment if it could be an anti-hyperpermeability induced by IFN-γ on HUVEC. Nevertheless, the aim of this study is not only to determine the anti-hyperpermeability ability of asiaticoside, but also the beneficial inhibitory effects of asiaticoside on the underlying mechanisms involved in hyperpermeability. More scientific data are required to explain the biological activities, mechanism of reaction as well as the active components in *Centella asiatica* though a large number of studies reported over the past decades.

1.2 Problem statement and justification

To date, conventional NSAIDs (non-steroidal anti-inflammatory drugs) as well as selective COX-2 inhibitor are associated with small and definite risk of several side effects. There are over 15,000 deaths annually among patients following doctor's prescriptions for NSAIDs and this toxicity is especially noticed in elderly (Odell & Sorgnard, 2008). Furthermore, the use of these drugs offers other weaknesses such as significant side effects, high cost and the route of administration. For example, at least two subcutaneous injections per week are required for an effective drug therapy (Santos, 2004). Other than that, glucocorticoid therapy is also widely used to treat inflammation. Prolonged use of glucocorticoid therapy may induce side effects involving the central nervous system, gastrointestinal tract as well as cardiovascular system (Moghadam-Kia & Werth, 2010). In this perspective, therapeutic agents which derived from natural plant may have positive contributions and benefits with regards to the treatment for inflammatory disorder as well as other diseases with inflammatory origin. Dexamethasone, a well-known glucocorticoids were used to inhibit pro-inflammatory cytokines production as well as other pro-inflammatory mediators as described by Pang & Knox (1997). It was used in the present study as the positive control to define and compare the anti-inflammatory activity of a new potential treatment from natural plant origin, Asiaticoside as well as the possible mechanisms involved.

1.3 Hypothesis

Asiaticoside has no toxic effects and will not cause cell death to HUVEC on tested concentrations. Asiaticoside will significantly attenuate IFN-γ-induced endothelial hyperpermeability in Human Umbilical Vein Endothelial Cell (HUVEC) by improvement of impaired nitric oxide (NO) levels back to its basal states. Asiaticoside also reduces cyclooxygenase-2 (COX-2) enzyme activity and prostaglandin E_2 (PGE_2) release on HUVEC. Asiaticoside aids in deactivation of p38 mitogen-activated protein kinases (MAPK) in Human Umbilical Vein Endothelial Cell (HUVEC).

1.4 Research objectives

1.4.1 General objective

To evaluate the effects of asiaticoside on IFN-γ-induced endothelial hyperpermeability and the possible mechanisms involved in cell model; Human Umbilical Vein Endothelial Cell (HUVEC).

1.4.2 Specific objectives

1. To determine the viability of HUVEC towards asiaticoside.
2. To determine the effects of asiaticoside on IFN-γ-induced endothelial hyperpermeability in HUVEC.
3. To determine the effects of asiaticoside on nitric oxide (NO) levels, cyclooxygenase-2 (COX-2) levels, and prostaglandin E_2 (PGE_2) release in activated HUVEC.
4. To determine the effects of asiaticoside on p38 mitogen-activated protein kinases (MAPK) phosphorylation in activated HUVEC.

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