UNIVERSITI PUTRA MALAYSIA

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

NURNABILA HUDA BINTI KHAIRUDIN

FPSK(M) 2017 75
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By

NURNABILA HUDA BINTI KHAIRUDIN

Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfilment of the Requirements for the Degree of Master of Science

NOVEMBER 2016
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Abstract of thesis presented to the senate of Universiti Putra Malaysia in fulfilment of the requirement for the degree of Master of Science

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NOVEMBER 2016

Chair: Enoch Kumar Perimal, PhD
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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 E2 release in interferon-gamma-activated HUVEC was also studied. Interferon-gamma has increased cyclooxygenase-2 level and prostaglandin E2 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

NURNABILA HUDA BINTI KHAIRUDIN

NOVEMBER 2016

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Kemerosotan fungsi halangan endothelial oleh ejen vasoaktif atau pengantara keradangan mengalakkan hiperkebolehtelapan sel endothelial yang menyebabkan pembangunan keadaan patologi seperti rangkaian edema. Perubahan struktur halangan mengalakkan kebocoran cecair plasma dan makromolekul. Interferon-gamma terkenal sebagai mediator pro-radang yang mengganggu integriti halangan lalu mengalakkan 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-oksidan yang baik, penyembuhan luka, peningkatan sistem 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 signifikan (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 oksida nitrik. 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 E2 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 E₂, cyclooxygenase-2, p38 Mitogen Diaktifkan Protein Kinase
ACKNOWLEDGEMENT

In the name of Allah the most gracious and the most merciful, Alhamdulillah thanks to Allah s.w.t whom with His willingness of giving me the opportunity to complete this research project with flying colours.

First and foremost, I would like to express my gratitude and appreciation to my late supervisor, Allahyarhamah Associate Professor Dr. Zuraini Ahmad for her guidance and patience throughout this research project. Without her support, advice and unceasing encouragement, this research project and thesis wouldn’t have been done and written. I’m very grateful and it’s my pleasure to have the chances to work under her supervision. She was understanding person that always give positive motivation in order to succeed in live. I also want to express my heartiest appreciation to my current supervisor, Dr. Enoch Kumar Perimal for his guidance and valuable advice for me to complete this research project, as well as hearties thanks to my co-supervisor, Dr Yong Yoke Keong for his guidance.

Special thanks go to my respective seniors and lab mates in Physiology Research Laboratory for their guidance, advices and kind assistance during my research experiments. I apologize for any unintended inconvenience cause. Not to forget, deepest thanks and appreciation to my lab-mates Mrs Nurfarahdilla and Mr. Shaari who had helping me a lot in completing my project as well as they’re being the one who always supports me in every hard phase and moments.

I am also grateful and would like to give my appreciations to the lecturers and staff from Department of Biomedical Science and Physiology Laboratory for their willingness and assistance every time when needed especially in providing many facilities in Lab. They are also supportive and always get ready whenever I need to use the lab during my late nights experiment as well as during holidays. Not to forget, a special thanks to my dearest friends who always support and be there whenever I’m facing the hard time and provides me an endless support in many ways during my research stage.

Last but not least, I also would like to express my gratitude to the one that raise me, provides me an endless love, support me physically and mentally at every time and everywhere, encouragement and their prayers to my beloved parents; Mr Khaifurudin Hassan and Mrs Noranita Salamat. I don’t think I could ever complete my Master Degree and fulfil my dream without them.
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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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 Ethylenediaminetetraacetic 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 Fluorescence 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-triphosphate
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
PGE2 Prostaglandin E2
PKC Protein kinase C
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<td>Protein kinase G</td>
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<tr>
<td>PVDF</td>
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<td>RIPA Buffer</td>
<td>Radioimmunoprecipitation assay buffer</td>
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<td>ROS</td>
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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 possesses 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 E2 (PGE2) 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 E2 (PGE2) 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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