UNIVERSITI PUTRA MALAYSIA

IN VITRO EFFECTS OF CRYPTOTANSHINONE ON EARLY ATHEROGENIC EVENTS INDUCED BY OXIDIZED LOW-DENSITY LIPOPROTEIN AND TUMOUR NECROSIS FACTOR-α

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By

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

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Chair: Associate Professor Dr. Zuraini Ahmad, PhD

Faculty: Faculty of Medicine and Health Sciences

Development of early atherogenic events involve endothelial cell injury by oxidized low-density lipoprotein (oxLDL) and pro-inflammatory cytokines, such as tumour necrosis factor-α (TNF-α). The injuries of endothelial cells lead to subsequent increase in endothelial permeability and expression of adhesion molecules favouring monocytes' adhesion to endothelium. In addition, the production of nitric oxide (NO), a permeability-regulator, is also impaired in dysfunctioned endothelium. Cryptotanshinone (CTS) is one of the major compounds isolated from the Chinese herb *Salvia milthiorrhiza*, which is found to be effective against cardiovascular diseases. However, the effects of CTS on oxLDL and TNF-α-induced early atherosclerotic events have not been investigated. The aim of this study was to evaluate the anti-atherosclerotic effects of CTS at pre-lesional stage by examining its effects on the endothelial permeability, expression of adhesion molecules and chemokines, restoration of nitric oxide (NO) and adhesion of U937

monocytic cells to human umbilical vein endothelial cells (HUVEC). OxLDL (100 μg/ml) and TNF-α (10 ng/ml) were used to induce endothelial hyperpermeability, to increase expression of adhesion molecules, i.e. vascular cell adhesion molecule-1 (VCAM-1) and intercellular adhesion molecules-1 (ICAM-1), adhesion of monocytes, and to suppress NO. TNF-α was also used to induce the expression of monocyte chemoattractant protein-1 (MCP-1) in HUVEC. The results of MTT assay showed that CTS had no cytotoxic effect to HUVEC up to 10 μM concentration. During oxLDL-induced early atherogenic events, CTS, at 1-10 μM, significantly suppressed the endothelial hyperpermeability and at 2.5 - 20 μM, it significantly reduced the adhesion of monocytes to HUVEC and restored the production of NO. ICAM-1 was significantly suppressed by 2.5 – 10 μM of CTS whereas VCAM-1 expression was suppressed by 1 – 20 μM of CTS. For the events induced by TNF-α, 1 – 20 μM CTS significantly reduced endothelial hyperpermeability, 1 – 10 μM CTS significantly suppressed monocytes' adhesion to HUVEC, the expression of ICAM-1, and at similar range of concentrations, restored NO production CTS, at 2.5 – 10 μM, significantly suppressed the expressions of VCAM-1 and MCP-1 (P<0.05). These findings suggest that CTS may play a role in the prevention of early or pre-lesional stage of atherosclerosis by suppressing increased endothelial permeability and monocytes' adhesion to endothelium. These data indicate that the restoration of NO bioavailability may play a role in reversing the elevated endothelial permeability, and that CTS may attenuate the recruitment of monocytes via the suppression of adhesion molecules and chemokine's expressions.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah master sains

KESAN CRYPTOTANSHINONE PADA TAHAP AWAL ATEROGENIK YANG DIARUH OLEH LIPOPROTIN TEROKSIDA KEPADATAN RENDAH DAN TUMOR NEKROSIS FAKTOR-α *IN VITRO*

Oleh

ANG KOK PIAN

Disember 2010

Pengerusi: Profesor Madya Dr. Zuraini Ahmad, PhD

Fakulti: Fakulti Perubatan dan Sains Kesihatan

Perkembangan tahap awal aterogenik melibatkan kecederaan pada sel endothelia oleh lipoprotein teroksida berdensiti rendah (oxLDL) dan pro-inflamasi sitokin, seperti tumour necrosis factor-α (TNF-α). Kecederaan sel endotelia diikuti oleh peningkatan ketelapan endothelia dan ekspresi molekul adhesi yang menggalakkan perlekatan sel monosit pada sel endothelia. Selain itu, penghasilan nitrik oksida, yang merupakan satu pengawalatur ketelapan, juga terganggu pada sel endotelia yang tidak berfungsi. Cryptotanshinone (CTS) merupakan satu sebatian utama yang diekstrak dari *Salvia milthiorrhiza* yang terbukti berkesan melawan penyakit kardiovaskular. Namun begitu, kesan CTS pada kejadian tahap awal aterogenik yang diaruh oleh oxLDL dan TNF-α masih belum dikaji. Tujuan projek penyelidikan ini ialah untuk menilai kesan anti-aterosklerotik CTS pada tahap awal aterosklerosis dengan mengenalpasti kesannya terhadap ketelapan endotelia, ekspresi molekul adhesi dan kemokin, pemulihan penghasilan nitrik oxida

(NO) dan perlekatan sel monosit U937 kepada sel endotelia vena umbilikal manusia (HUVEC). OxLDL (100 μg/ml) dan TNF-α (10ng/ml) digunakan untuk meningkatkan ketelapan endotelia, ekspresi molekul adhesi, i.e. molekul adhesi sel vaskular-1 (VCAM-1) dan molekul adhesi interselular-1 (ICAM-1), adhesi sel monosit dan penurunan penghasilan NO. TNF-α juga digunakan untuk mengaruh peningkatan ekspresi protin kemopenarikan sel monosit-1 (MCP-1) pada HUVEC. Ujian MTT menunjukkan bahawa CTS tidak sitotoksik terhadap HUVEC sehingga tahap kepekatan 10 μM. Semasa kejadian tahap awal aterogenik yang diaruh oleh oxLDL, CTS, dari kepekatan 1-10 μM, menurunkan secara signifikan ketelapan tinggi endothelia, dan dari 2.5-20 μM, ia dengan signifikannya mengurangkan perlekatan sel monosit pada HUVEC dan mengembalikan penghasilan NO yang normal. ICAM-1 dikurangkan secara signifikan oleh 2.5-10 μM CTS, manakala ekspresi VCAM-1 dikurangkan oleh CTS pada kepekatan 1-20 μM. Untuk kejadian aterogenik yang diaruh oleh TNF-α, kepekatan CTS dari 1-20 μM telah dengan signifikannya menurunkan ketelapan tinggi endotelia, CTS pada 1-10 μM pula mengurangkan secara signifikan perlekatan sel monosit pada HUVEC, ekspresi ICAM-1 dan mengembalikan penghasilan NO kepada asal, kepekatan dari 2.5-10 μM, dengan signifikannya menurunkan ekspresi VCAM-1 dan MCP-1 (P<0.05). Penemuan-penemuan ini menunjukkan bahawa CTS mungkin memainkan peranan penting di dalam pencegahan kejadian tahap awal aterogenik dengan mengurangkan kadar ketelapan tinggi endotelia dan perlekatan monosit pada endotelia. Data ini juga menunjukkan pemulihan penghasilan NO yang mungkin memainkan peranan untuk menurunkan kadar ketepalan tinggi endothelia, dan CTS mungkin menghalang perlekatan sel monosit melalui pengurangan ekspresi molekul adhesi dan kemokin.

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I certify that a Thesis Examination Committee has met on 16th December 2010 to conduct the final examination of Ang Kok Pian on his thesis entitled "**In vitro effects of cryptotanshinone on early atherogenic events induced by oxidized low-density lipoprotein (OXLDL) and tumour necrosis factor – α (TNF-α)"** in accordance with the Universities and University Colleges 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 degree of Master of Science (Physiology).

Members of the Thesis Examination Committee were as follows:

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This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfillment of the requirement for the degree of Master of Science. The members of the Supervisory Committee were as follows:

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DECLARATION

I declare that the thesis is my original work except for quotations and citations which have been duly acknowledged. I also declare that it has not been previously, and is not concurrently, submitted for any other degree at Universiti Putra Malaysia or at any other institution.

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ANG KOK PIAN

Date: 16 December 2010

LIST OF FIGURES

LIST OF ABBREVIATIONS

oxLDL	Oxidized low-density lipoprotein
TNF-α	Tumour necrosis factor-α
IL-1β	Interleukin-1β
IFN-γ	Interferon-gamma
CTS	Cryptotanshinone
ICAM-1	Intercellular adhesion molecules-1
VCAM-1	Vascular cell adhesion molecules-1
MCP-1	Monocyte chemoattractant protein-1
HUVEC	Human umbilical vein endothelial cell
NO	Nitric oxide
LOX-1	Lectin-like oxidized LDL receptor-1
AJ	Adherens junction
TJ	Tight junction
GJ	Gap junction
VEGF	Vascular endothelial growth factor
LDL	Low-density lipoprotein
JMD	Juxtamembrane domain
CTD	C-terminal domain
cGMP	Cyclic guanosine monophosphate
eNOS	Endothelial-nitric oxide synthase
iNOS	Inducible-nitric oxide synthase
nNOS	Neuronal-nitrix oxide synthase
JAM-C	Junctional adhesion molecule-C

M-SCF	Macrophage colony stimulating factor
PECAM-1	Platelet endothelial cellular adhesion molecules
JAM-A	Junctional adhesion molecule-A
IgG	Immunoglobulin-G
LFA-1	Lymphocyte function-associated antigen
HMG-CoA	3-hydroxy-3-methylglutaryl-coenzyme A
DMSO	Dimethyl sulfoxide
LSGS	Low serum growth supplement
FBS	Fetal bovine serum
RPMI	Roswell Park Memorial Institute medium
PBS	Phosphate buffered saline
BSA	Bovine serum albumin
BCECF-AM	2',7'-*bis-(*2-Carboxyethyl)-5(6)-carboxyfluorescein acetoxymethyl ester
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
ELISA	Enzyme-linked immunosorbent assay
ATCC	American Type Culture Collection
FITC	Fluorescein isothiocyanate
µM/ml	Micromolar/mililitre
µg/ml	Microgram/militre
µL	Microlitre
ng/ml	Nanogram/mililitre
ROS	Reactive oxygen species

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