STUDY ON THE EFFECT OF *NIGELLA SATIVA* OIL TOWARDS OXIDIZED LOW DENSITY LIPOPROTEIN UPTAKE BY PRIMARY HUMAN MONOCYTES/MACROPHAGES

BY

DR. MAHAYA BINTI CHE MAT

A dissertation submitted in partial fulfillment of the requirements for the Degree of Master of Pathology

(Chemical Pathology)

UNIVERSITI SAINS MALAYSIA

2011

ACKNOWLEDGEMENT

ACKNOWLEDGEMENTS

IN THE NAME OF ALLAH, THE MOST MAGNIFICIENT, THE MOST MERCIFUL

Praises is to ALLAH, without whose grace this dissertation would not have been completed. I would like to take this opportunity to thank my supervisor, Dr. Shahrul Bariyah Sahul Hamid, currently working in Advanced Medical and Dental Institute; co-supervisors Dr. Md. Azman Seeni Mohamed from Dental School and Dr. Wan Zuraida Wan Abdul Hamid from Immunology Department; Universiti Sains Malaysia for their guidance, encouragement and concern throughout my work. My supervisor had opened doors for me, giving me an invaluable experience to the world of dedicated scientists.

I would also like to acknowledge the Head of Chemical Pathology Department, Head of Immunology Department, Head of Pathology Department, Head of Anatomy Department, Dean of Pusat Pengajian Sains Pergigian and all their staffs for giving me opportunity to work in their laboratories and also helping me throughout my works.

I wish to thank my lecturers from Department of Chemical Pathology HUSM for giving their critics and advice. A special thank to Dr. Azriani Abd. Rahman on the advice of the statistical data analysis. I also would like to thank Mr. Jamarudin Mat Asan, Mr. Tan Koh Chung and Ms. Muniira Muhamad for their technical assistances, and to all my colleagues in whom I would like to thank for their encouragement and support.

Last but not least, my deepest appreciation is to my husband, Dr. Fauzi bin Che Hussin, for all the love, support and encouragement that he has given me during this trying times, all the years together and also for years to come. I am also thankful to my beloved mother, Puan Maimunah binti Yaacob and my wonderful sisters for their love and handful support To both my lovely children, Muhammad Azfar Izzat and Hurin Izzati, thank you for the patience and tolerance during completion of this works. Thank you, may Allah bless us all.

TABLE OF CONTENT

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

CAD – Coronary Artery Disease

LDL – Low Density Lipoprotein

HDL – High Density Lipoprotein

ACS – Acute Coronary Syndrome

NCEP – National Cholesterol Education Program

ATP – Adult Treatment Panel

VLDL – Very Low Density Lipoprotein

LDL-R – Low Density Lipoprotein Receptor

ACAT – AcylCoA:Cholesterol Acyl Transferase

SR-A – Scavenger Receptor Class A

VSMCs – Vascular Smooth Muscle Cells

ROS – Reactive Oxygen Species

GRAS – Global Risk Assessment Scoring

TNF-α – Tumor Necrosis Factor alpha

M-CSF – Macrophage-Colony Stimulating Factor

MCP-1 – Monocyte Chemotactic Protein-1

HIV – Human Immunodeficiency Virus

MAb – Monoclonal Antibody

$CuSO_4$ – Copper Sulphate

PE - Phycoerythrin

FACS – Fluorescent-activated Cell Sorter

MFI – Mean Fluorescence Intensity

PBS – Phosphate Buffer Saline

PMA – Phorbol 12-Myristate 13-Acetate

EDTA – Ethynyldiamine tetraacetic acid

DMSO – Dimethyl Sulfide

LPDS – Lipoprotein Deficient Serum

FCS – Fetal Calf Serum

MNC – Mononuclear cells

TQ - Thymoquinone

COX-1 – Cyclooxygenase-1

COX-2 – Cyclooxygenase-2

1L-6 – Interleukin-6

Cu^{2+} - Copper ion

RPMI – Roswell Park Memorial Institute

CCE – Counterflow Centrifuge Elutriation

ABSTRAK

KAJIAN KESAN PENGGUNAAN MINYAK *NIGELLA SATIVA* KE ATAS PENGAMBILAN LIPOPROTEIN TEROKSIDA BERKETUMPATAN RENDAH OLEH SEL MONOSIT/MAKROFAJ MANUSIA

Peranan sel monosit dan makrofaj di dalam proses aterogenesis telah banyak dilaporkan. *Nigella sativa*, sejenis herba yang mempunyai banyak kebaikan, digunakan di dalam kajian ini untuk melihat kesan antilipid herba tersebut. Oleh itu, fokus kajian ini adalah untuk menentukan kesan penggunaan minyak *Nigella sativa* ke atas perkembangan monosit kepada makrofaj melalui pengambilan lipoprotein teroksida berketumpatan rendah. Sel monosit manusia telah diasingkan dengan kaedah pemencilan negatif "Dynabead MyPure" kit dan sel yang diperolehi dikenalpasti melalui pewarnaan Wright. Sel kemudiannya dikultur pada suhu 37°C dalam keadaan 5% gas karbon dioksida sehingga 90% konfluen sebelum kajian dimulakan. Sel telah diplat dan dibasuh sebelum dirawat menggunakan lipoprotein teroksida berketumpatan rendah (10 μg/ml) atau kombinasi rawatan lipoprotein teroksida berketumpatan rendah (10 μg/ml) dan minyak *Nigella sativa* (72 μg/ml). Perkembangan sel-sel direkodkan untuk setiap 24 jam selama 3 hari dengan menggunakan mikroskop beza-fasa. Pewarnaan oil red O pula digunakan untuk melihat pengumpulan lipid di dalam sel. Pengesanan antigen di permukaan sel, CD11b juga telah dilakukan menggunakan kaedah analisa "flow cytometry" untuk mengkaji perubahan bentuk monosit kepada makrofaj pada sel yang dibekalkan dengan lipoprotein teroksida berketumpatan rendah (20 μg/ml) dan gabungan lipoprotein teroksida berketumpatan rendah (20 μg/ml) dan minyak *Nigella sativa* (72 μg/ml). Hasil kajian mendapati penggunaan minyak *Nigella sativa* memberikan kesan pengurangan saiz

ABSTRACT

STUDY ON THE EFFECT OF *NIGELLA SATIVA* OIL TOWARDS OXIDIZED LOW DENSITY LIPOPROTEIN UPTAKE BY PRIMARY HUMAN MONOCYTE/ MACROPHAGE

Monocytes and lipid-laden macrophages have been reported to play various roles in atherogenesis. *Nigella sativa*, a natural product with various medical benefits was used in the study to evaluate its antilipid effects. The focus was to elucidate the effects of *Nigella sativa* oil on the progression of monocyte-derived macrophages growth *in vivo* via the effect on uptake of oxidized LDL. Human monocytes were isolated using magnetic beads Dynabead MyPure Negative Isolation Kit and identified with Wright staining. The cells were grown at 37°C and 5% CO_2 saturation for 5 days until 90% confluence prior to treatment. The cells were then plated in 24-well plate and washed before treated with ox-LDL (10 μg/ml) or combination of ox-LDL (10 μg/ml) and (72 μg/ml) *Nigella sativa* oil. The growth progression was monitored every 24 hours for 3 days using phase-contrast microscopy. The oil red O staining was used to visualize intracellular lipid deposition. Detection of surface marker antigen CD11b was done with flow cytometry analysis to compare monocyte-to-macrophage defferentiation in the cells supplemented with oxidized LDL alone (20 μg/ml) and in combination of oxidized LDL (20 μg/ml) and *Nigella sativa* oil (72 μg/ml). It was found *Nigella sativa* oil caused significant growth reduction on monocyte and macrophage growth especially 24 to 48 hours after treatment ($p<0.001$). The mean sizes were significantly different between treatment with oxidized LDL alone and combination treatment for both monocytes and macrophages ($p<0.001$). The delayed growth pattern was more in macrophage groups compared to monocytes in the timeline

studied. There were less oil red O staining in cells treated with combination of oxLDL and *Nigella sativa* compared to those treated with oxLDL alone. Flow cytometry analysis showed reduced expression of mean fluorescence intensity in cells treated with combination of 60% *Nigella sativa* oil and oxidized LDL compared to cells supplemented with native LDL and oxidized LDL alone. These preliminary findings indicate the progression of macrophage to foam cells could possibly be controlled with *Nigella sativa* oil treatment at specific level.

1. INTRODUCTION

1. INTRODUCTION

1.1 Coronary Artery Disease and Risk Factors

Coronary artery disease (CAD) has continued to be the leading cause for the world's morbidity and mortality. Ministry of Health of Malaysia declared coronary artery disease as the leading cause of hospital admission and non-accidental deaths for the last 10 years (Chin *et al.*, 2008). Summary of data collected from 1994 to 2001 showed heart disease accounted for 14% to 16% of the principal cause of death in government hospitals in Malaysia (Zambahari, 2004).

Major independent risk factors for coronary artery disease (CAD) are advancing age, elevated blood pressure, elevated serum total and low density lipoprotein cholesterol levels, low serum high density lipoprotein cholesterol level, diabetes mellitus, and cigarette smoking (Grundy *et al.*, 1999, Wilson, 1994). Levels of low density lipoprotein and high density lipoprotein; habits of smoking and lacking physical activity; diabetes mellitus and hypertension are all considered as modifiable risk factors, which can be modified to prevent CAD.

An assessment done indicated that age, education, current smoking, diabetes, hypertension and elevated serum cholesterol levels were significant risk factors for ischemic heart disease mortality among all race and sex group of 10,766 subjects studied including black and white, men and women, for the period from 1971 to 1992 in United States of America (Chang *et al.*, 2001). Within the period stated, 1401 (13.0%) of subjects died from ischemic heart disease. In patients presenting with acute coronary syndrome (ACS), half of them had diabetes, hyperlipidemia, smoking and

hypertension which are considered as traditional cardiovascular risk factors (Chin *et al.*, 2008).

The importance of low density lipoprotein (LDL) cholesterol measurement has been stated in the National Cholesterol Education Program Adult Treatment Panel III (NCEP ATP III) guidelines. NCEP ATP III is the latest report of the NCEP guidelines and measurement of LDL cholesterol had been identified as its first step of risks assessment followed by high density lipoprotein (HDL) and serum triglyceride (TG) measurements. Lowering LDL cholesterol has been the target for management. LDL cholesterol level of < 100 mg/dL is defined as optimal for the whole population (Pasternak, 2002). NCEP ATP III was a detailed and evidence-based approach to both diagnosis and management in hypercholesterolemia and the guidelines emphasized on the patient's risk of developing coronary artery disease. The ATP III guideline also emphasized on the therapeutic lifestyle changes (TLC) such as benefits of healthy diet, weight control and exercise especially in patients with lifestyle risk factors such as obesity, sedentary lifestyle and elevated plasma glucose, as its risk-lowering strategy (Stone *et al.*, 2005).

1.2 Atherosclerosis

Atherosclerosis is closely related to development of coronary artery disease (Libby, 2005). It is a result from a combination of abnormalities in lipoprotein metabolism, oxidative stress, chronic inflammatory process and tendency to thrombosis. Atherosclerosis is characterized by a multifocal, smoldering disease of medium-sized

and large arteries fuelled by lipids resulting in the formation of fatty and fibrous lesion in the arterial wall (Ross, 1999). The concept that atherosclerosis is an inflammatory disease has been widely accepted. In the process, there is accumulation of intra- and extracellular lipids, foam cell formation, proliferation of smooth muscle cells and accumulation of connective tissue components. The earliest event in atherogenesis appears to be endothelial cell dysfunction that can be caused by various insults such as hypertension, diabetes, smoking, dyslipidemia and hyperhomocysteinemia (Libby, 2002). After endothelial cell dysfunction, mononuclear cells that initially loosely attach to the endothelium, adhere firmly afterwards and diapedese into the subendothelial space. During the atherosclerotic process, numbers of cytokines and growth-regulatory molecules were formed and released from the cells in the lesions, which consist of endothelial cells, smooth muscle cells, macrophages and T-lymphocytes (Ross, 1993).

The earliest visible lesion of atherosclerosis is the fatty streak, an aggregation of cholesterol-loaded macrophages or foam cells within the arterial wall (Nagy *et al.*, 1998). Fatty streak formation is widely accepted as a precursor of clinically significant and complicated atherosclerotic lesions later on (Steinberg and Witztum, 1990). With time, the fatty streak progressed into a fibrous plaque, the hallmark of established atherosclerosis. The lesion will ultimately evolve to contain large amounts of lipids and become unstable that can lead to thrombotic occlusion of the overlying artery (Crowther, 2005). If the progression of the process is rapid as in the case of plaque rupture accompanied by superimposed thrombosis, it may caused devastating acute coronary and cerebrovascular events such as unstable angina, myocardial infarction, stroke or sudden death (Patel *et al.*, 2008).

1.3 Lipids in blood

The major lipids present in the plasma are fatty acids, triglycerides, cholesterol and phospholipids. Elevated plasma concentrations of lipids, particularly cholesterol (hypercholesterolemia), are causally related to the pathogenesis of atherosclerosis which is responsible for development of cardiovascular disease (Marshall and Bangert, 2008). Hypercholesterolemia is also one of the most studied risk factors for atherosclerosis (Taylor *et al.*, 2000).

Serum cholesterol is carried in the blood by several lipoprotein particles that perform the complex physiologic tasks of transporting dietary and endogenously produced lipids (reviewed in Witztum and Steinberg, 1995). Chylomicrons provide the primary means of transport of dietary lipids (principally triglycerides), while transport of endogenous lipids are by very low density lipoproteins (VLDL), low density lipoproteins (LDL) and high density lipoproteins (HDL).

1.4 Low Density Lipoprotein (LDL)

The low density lipoproteins (LDL) are pseudomicellar, quasi-spherical particles. LDL is the major vehicle of cholesterol transport in human plasma, mainly in the form of cholesteryl esters. LDL particles consist essentially of cholesterol in both esterified and non-esterified forms, phospholipids and minor amounts of triacylglycerols. Apolipoprotein B100, a highly specialized protein of hepatic origin is also one of the LDL components (Chapman *et al.*, 1998).

LDL particles are small enough to cross the endothelium and deliver cholesterol to the tissues. LDL will bind to LDL receptor (LDL-R) and form a complex that will be internalized into the cells and undergoes lysosomal degradation. The apo B100 is hydrolysed to its constituent amino acids and cholestryl ester to free cholesterol. The free cholesterol released is incorporated into membranes, leading to inhibition of the synthesis of new LDL receptors, inhibiting cholesterol synthesis by reducing the synthesis of HMG CoA reductase and promoted the activity of acylCoA:cholesterol acyltransferase (ACAT), which synthesizes cholesterol esters (Olson, 1998). These regulatory events are mediated by a sterol regulatory element binding protein, which monitors the free cholesterol concentration in the cell and adjusts the expression of the cholesterol regulatory genes (Wang *et al.*, 1994). The rise in free cholesterol levels causes reduction in the expression of LDL receptors, inhibition of the enzyme HMG CoA-reductase and enhancement of the activity of ACAT. This will inhibit further accumulation of cholesterol within cells. The turnover of LDL-R occurs partially as a result of recycling receptors that have undergone endocytosis and partially as a result of new synthesis (Goldstein and Brown, 2009).

1.5 Oxidized Low Density Lipoprotein

LDL oxidation has been proven to be associated with atherosclerosis and has been regarded as a key step in atherogenesis (Steinberg, 2002). The accumulation of LDL in the arterial intima is an early event in lesion formation and continues as lesions advance. When LDL particles were trapped in an artery, they undergo a progressive oxidation in a localized microenvironment forming oxidized LDL (Hamilton, 1997). A

numbers of potential oxidant-generating systems that could directly or indirectly target LDL lipids and modified the particles have been investigated, including myeloperoxidase, nitric oxide synthase and lipoxygenases. An in vitro oxidation can be done by using metal ions such as Cu^{2+} and Fe^{2+} (Mertens and Holvoet, 2001). The major components of the arterial wall, endothelial cells, smooth muscle cells and macrophages are also capable to modified LDL and oxidized it to a form recognizable by the scavenger receptors (Steinberg, 1997). Figure 1.1 summarized the event in fatty streak formation, the earliest lesion in atherosclerosis. The contribution of oxidized LDL to the atherosclerotic process is believed to be in the activation of inflammatory events (Berliner and Heinecke, 1996). The oxidatively modified LDL was found to be relatively rich with free cholesterol, protein, triglycerides and oxidized lipids with a diminished content of vitamin E (Avogaro *et al.*, 1988). The oxidized LDL isolated from patients with CAD was characterized by a 1.3-fold higher electrophoretic mobility on agarose gels, 75% reduction of the arachidonate levels and 80% reduction in the linoleic acid levels. Fractions of aldehyde substitution of lysine residues was approximately 30 – 40% of the standard preparations of *in vitro* oxidized LDL, indicating that between 60 and 90 lysine residues in the apoB moiety of oxidized LDL were substituted. These characteristics suggested in vivo oxidized LDL most likely was generated by cell-associated oxidative enzymatic activity in the arterial wall not from extensive metal ion-induced oxidation (Holvoet *et al.*, 1998).