UNIVERSITI TEKNOLOGI MARA

ELUCIDATING VASCULAR PROTECTIVE EFFECT AND MOLECULAR MECHANISMS OF THYMOQUINONE IN HOMOCYSTEINE-INDUCED ENDOTHELIAL DYSFUNCTION: *EX VIVO* AND *IN VITRO* MODEL

SITI SARAH BINTI M. SOFIULLAH

Thesis submitted in fulfilment
of the requirements for the degree of
Master of Science
(Medicine)

Faculty of Medicine

January 2024

ABSTRACT

Hyperhomocysteinemia (HHcy) has been linked to an increased risk of cardiovascular diseases. High levels of homocysteine (Hcy) cause damage to endothelial cells by promoting endoplasmic reticulum (ER) stress that increases reactive oxygen species (ROS), and cause endothelial nitric oxide synthase (eNOS) uncoupling, leading to endothelial dysfunction. Thymoquinone (TQ) is the major active ingredient in *Nigella sativa* seeds volatile oil and is shown to have a cardioprotective effect. However, no study evaluated the effect of TQ against Hcy-induced endothelial dysfunction. Thus, this study aims to investigate the effects and mechanisms of TQ in reversing Hcy-induced endothelial dysfunction. Isolated aorta from male Sprague-Dawley (SD) rats were incubated with Hcy (500µM) as inducer for HHcy and co-treated with or without TQ (0.1 µM, 1 µM, and 10 µM), 20 µM Taurine-Conjugated Bile Acid (TUDCA), 100 µM apocynin or 1 mM Tempol as positive control in organ bath to study the endothelium dependent relaxation (EDR) and endothelium independent relaxation (EIR). Additionally, human umbilical vein endothelial cells (HUVECs) were incubated with Hcy (10 mM) and various concentrations of TQ (1 and 10 μM), apocynin (100 μM), TUDCA (100 μM) or H_2O_2 (0.20 mM) to evaluate the cell viability by using a phase contrast microscope and dye exclusion assay. Involvement of endoplasmic reticulum (ER) stress pathway, reactive oxygen species (ROS) and nitric oxide (NO) bioavailability were accessed via immunoassay and fluorescent staining respectively. Our results revealed that Hcy impaired endothelium-dependant relaxation in isolated aorta, as well as induced apoptosis in HUVECs. These effects were reversed by TQ, TUDCA, tempol and apocynin. Treatment with TQ (10 μM) also reduced ROS level, improved NO bioavailability and reduced glucose-regulated protein 78 (GRP78) and nicotinamide adenine dinucleotide phosphate oxidase 4 (NOX4) protein in HUVECs. Taken together, the present results suggest that TQ preserved endothelial function in rat aorta and reduced apoptosis of HUVECs induced by Hcy through the inhibition of ER stress-mediated ROS and eNOS uncoupling, suggesting that TQ could be an adjunct treatment for HHcy.

ACKNOWLEDGEMENT

In the name of Allah, the most Gracious and the most Merciful.

Firstly, I want to express my heartfelt gratitude and appreciation to several persons who without them, this thesis would not be feasible. First and foremost, I would like to thank my supervisors, Dr Choy Ker Woon, AP Dr Suhaila Abd Muid and A/P Dr Dharmani Murugan, for their guidance during my project. They consistently engaged me in various research skills and gave me the opportunity for lab attachments and other conferences.

My deepest gratitude to UiTM Postgraduate Teaching Assistant (UPTA) Scheme under the Institute of Graduate Studies, Geran Insentif Penyeliaan (600-RMC/GIP 5/3 (079/2021), and Geran Penyelidikan Lestari (600-RMC/GPK 5/3 (269/2020) for the financial support. My special thanks to the Institute of Medical Molecular Biotechnology (IMMB), Faculty of Medicine, UiTM Sungai Buloh and Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC) accredited animal experimental unit at the Faculty of Medicine, Universiti Malaya for the great research facilities. Additionally, I would like to express my gratitude to every member of the lab, especially the individuals who worked with cell cultures, for their ongoing advice, encouragement, and perspectives during this project.

Finally, I would like to thank my late grandmother, , my parents, M. Sofiullah Sikder and , my brother, Muhammad Sofian Sikder, and my closest friend, Afif Zharfan, for their prayers, encouragement, and understanding of my keen interest in the subject of research. I would be unable to complete this study and thesis without their assistance. Thank you for always bearing with me through my ups and downs. To those that contributed indirectly to this research, I appreciate your help and kindness very much.

TABLE OF CONTENTS

CHAPTER 1
INTRODUCTION

1.1 Research Background

Hyperhomocysteinemia (HHcy) is a major risk factor for cardiovascular diseases such as atherosclerosis, vascular disease and hypercoagulability states (P. Ganguly & S. F. Alam, 2015). According to the World Health Organization, 17.9 million deaths were reported from cardiovascular disease, accounting for 32% of all global fatalities, and 85% of these deaths are the result of a heart attack or a stroke in 2019 (Mahadir Naidu *et al*., 2019). The earliest sign of atherosclerosis and vascular disease is endothelial dysfunction (Nappi & Avtaar Singh, 2023).

According to several studies, homocysteine (Hcy) negatively impairs endothelial function (Barroso et al., 2016; Da Silva, Barroso, Moura, Castro, & Soveral, 2018; Dubey et al., 2022; Kern et al., 2022; Xun Wu et al., 2019; Z. Zhang et al., 2017a). HHcy may impair endothelial-dependent dilatation through activation of endoplasmic reticulum (ER) stress which elevates oxidative stress and disrupts uncoupling of nitric oxide (NO) synthase activity (X. Wu *et al*., 2019) . Elevated Hcy levels have been demonstrated to cause ER stress by interrupting normal protein folding and processing in the ER, resulting in a build-up of misfolded proteins via activation of unfolded protein response (UPR) (Lindholm, Korhonen, Eriksson, & Kõks, 2017b). UPR mitigates the misfolded protein burden by upregulating ER protein folding capacity through increased production of chaperones such as glucose-regulated protein 78 (GRP78) and downregulating the ER protein load by suppressing protein transcription and translation (Amen, Sarker, Ghildyal, & Arya, 2019). Three parallel signal transduction pathways mediate such response: inositol requiring enzyme 1 (IRE1), RNA-activated protein kinase-like ER kinase (PERK) and activating transcription factor 6 (ATF6) (Oslowski & Urano, 2011).

The UPR activation increases reactive oxygen species (ROS) generation. Several studies have described the function of NADPH oxidase 4 (NOX4) in ROS generation in ER stress-induced endothelial dysfunction (Amanso, Debbas, & Laurindo, 2011; Loughlin & Artlett, 2010; Santos, Tanaka, Wosniak Jr, & Laurindo, 2009). NOX4 activity is controlled by the availability of its cofactor, nicotinamide adenine