

UNIVERSITI PUTRA MALAYSIA

MODULATION OF LIPIDOMIC MARKERS IN OXIDATIVE STRESS NEURAL-DERIVED EMBRYONIC STEM CELL CULTURES WITH VITAMIN E SUPPLEMENTATION

AFIFAH BINTI ABD JALIL

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By

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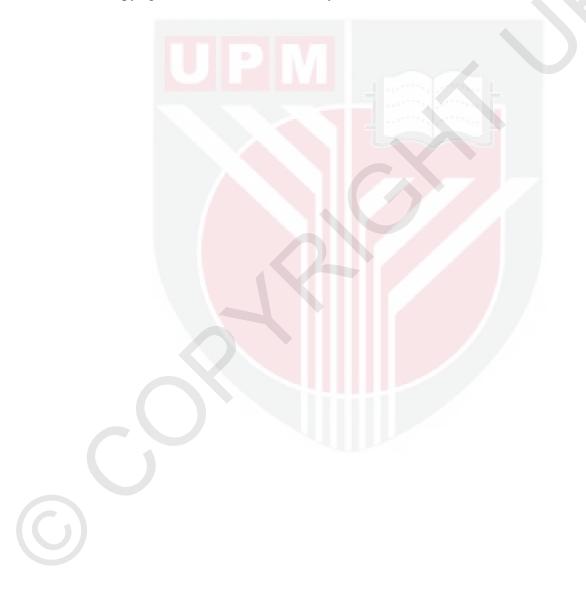
Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfillment of the Requirements for the Degree of Master of Science

May 2017

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Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfillment of the requirement for the Degree of Master of Science

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AFIFAH BINTI ABD JALIL

May 2017

Chairman: Huzwah Binti Khaza'ai, PhDFaculty: Medicine and Health Sciences

Abnormal lipid metabolism is strongly related to the pathogenesis of Alzheimer's disease (AD). Apolipoprotein E (APO E) is the major apolipoprotein in the CNS that has a role in cholesterol transport. Oxidative stress brain has reduced capacity for neuronal delivery of cholesterol suggesting a defect in cholesterol delivery for neuronal repair mechanism contribute to AD progression. Glutamate is the main excitatory neurotransmitter in the CNS, which can be excitotoxic at high concentration. Vitamin E has been shown to possess potent antioxidant and neuroprotection activities. It has two potent antioxidant isomers which are tocopherol and tocotrienol. In this present study, the effects of Tocotrienol Rich Fraction (TRF) from palm oil and alpha-Tocopherol (α-TCP) in modulating lipidomic markers in oxidative stress neural-derived embryonic stem (ES) cell cultures were elucidated. Transgenic mouse ES cell line (46C) was differentiated into neural lineage by induction with retinoic acid in vitro. The cells were then exposed to oxidative stress by a significantly high concentration of glutamate. Reactive oxygen species (ROS) measurement was done upon glutamate excitotoxicity and recovery processes by vitamin E were determined. Gene expression of glutamate receptors (NMDA and Kainate receptors), neuron-specific enolase (NSE), lipidomic markers including APO E, low density related protein (LRP) and 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMGCR) were elucidated using real-time PCR. The result reveals downregulation of NMDA, Kainate receptor, NSE and HMGCR upon posttreatment with different concentration of TRF and α -TCP in oxidative stress neural-derived 46C cells, a sign of neurorecovery process. Treatment of vitamin E also reduced the concentration of ROS to 33.05% and 57.2% upon 300ng/mL of TRF and α-TCP treatment respectively, in glutamate-induced oxidative stress neural cells which indicated that vitamin E is one of the potent antioxidants. In conclusion, TRF and α -TCP have protective and antioxidant properties against glutamate toxicity in neural-



derived ES cell and have the possibility to develop into potential treatment agents for AD.



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

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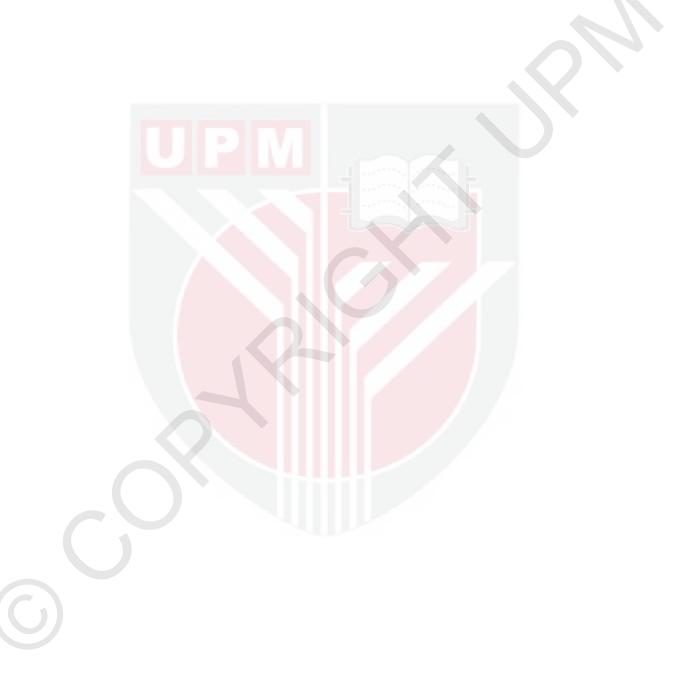
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Metabolisma lipid yang tidak normal adalah sangat berkaitan dengan patogenesis penyakit Alzheimer (AD). APO E adalah apolipoprotein utama dalam CNS yang berperanan dalam pengangkutan kolesterol. Tekanan oksidatif di dalam otak telah mengurangkan kapasiti penghantaran kolesterol kepada neuron dimana kerosakan dalam mekanisme penghantaran kolesterol untuk pembaikan neuron menyumbang kepada perkembangan AD. Glutamat adalah neurotransmiter yang merupakan perangsang utama dalam CNS dan boleh menjadi toksik pada kepekatan yang tinggi. Vitamin E telah terbukti mempunyai aktiviti antioksidan dan pelindung neuron yang berkesan. Ia mempunyai dua isomer antioksida iaitu tokoferol dan tokotrienol. Dalam kajian ini, kesan fraksi kaya tokotrienol (TRF) di ekstrak daripada kelapa sawit dan α-Tokoferol (α-TCP) dikaji dalam modulasi penanda lipidomik pada tekanan oksidatif neural yang telah diferensiasi daripada sel ES. Tikus transgenik embrionik sel stem (46C) telah diferensiasi kepada sel neural dengan pengaruh asid retinoik in- vitro .Selsel ini kemudiannya didedahkan kepada tekanan oksidatif dengan kepekatan glutamate yang agak tinggi. Pengukuran spesies reaktif oksigen (ROS) telah dilakukan sebaik proses eksitotoxisiti glutamat dan pemulihan dengan vitamin E telah ditentukan. Ekpresi gen reseptor glutamat (reseptor NMDA and Kainate), "neuronspecific enolase" (NSE) dan penanda lipidomik termasuk APO E, "low density related protein" (LRP) dan "3-hydroxy-3-methylglutaryl-coenzyme A reductase" (HMGCR) telah dijelaskan menggunakan Real time-PCR. Hasil ujikaji mendedahkan penurunan ekpresi NMDA, Kainate reseptor, NSE dan HMGCR dengan pasca-rawatan TRF dan α- TCP dengan kepekatan yang berbeza dalam tekanan oksidatif sel neural, yang menandakan proses neuroperlindungan sedang berlaku. Rawatan vitamin E juga mengurangkan kepekatan ROS kepada 33.05% dan 57.2% setiap satu setelah dirawat dengan 300ng/ml TRF dan α-TCP, dalam sel neural dibawah tekanan oksidatif glutamat yang menunjukkan vitamin E adalah salah satu antioksidan yang kuat.



Kesimpulannya, TRF dan α -TCP mempunyai sifat pelindung dan antioksidan terhadap ketoksikan glutamat dalam sel-sel neural yang diperolehi daripada ES sel. Ini menunjukan vitamin E berpotensi menjadi ejen rawatan untuk AD.



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I certify that a Thesis Examination Committee has met on 15 May 2017 to conduct the final examination of Afifah binti Abd Jalil on her thesis entitled "Modulation of Lipidomic Markers in Oxidative Stress Neural-Derived Embryonic Stem Cell Cultures with Vitamin E Supplementation" 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 Master of Science.

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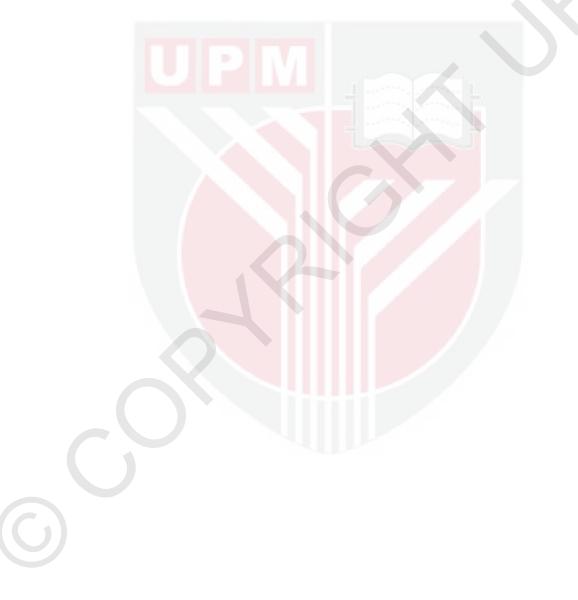
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LIST OF ABBREVIATIONS

4-HNE	4-Hydroxynonenal
ABC A1	ATP-binding cassette transporter subtype A1
ACAT	Acetyl coenzyme A-cholesterol
AD	Alzheimer's disease
AGEs	Glycoxidation end products
AMPA	α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
ANNOVA	Analysis of variance
APO B	Apolipoprotein B
APO C1	Apolipoprotein C subtype 1
APO E	Apolipoprotein E. Gene: Apo-E
APO E2	Apolipoprotein E allele 2
APO E3	Apolipoprotein E allele 3
APO E4	Apolipoprotein E allele 4
APO ER2	Apolipoprotein E receptor 2
APO J	Apolipoprotein J
APP	Amyloid precursor protein
ATP	Adenosine triphosphate
ATRA	All-trans retinoic acid
Αβ	Beta-amyloid
BAC	Bacterial artificial chromosome
BMP	Bone morphogenetic protein
BSA	Bovine serum albumin
Ca ²⁺	Calcium ion
Caspase	Cysteine-aspartic proteases

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	cDNA	complementary DNA
	CE	Cholesterol ester
	CO_2	Carbon dioxide
	Cq	Quantitation cycle
	CSF	Cerebrospinal fluid
	DAPI	4',6-diamidino-2-phenylindole
	DCF	2', 7' –dichlorofluorescein
	DCFH-DA	2',7' –dichlorofluorescin diacetate
	DEPC	Diethyl pyrocarbonate
	DMEM	Dulbecco's Modified Eagle Medium
	DMSO	Dimethyl sulfoxide
	DNA	Deoxyribonucleic acid
	Е	Efficiency
	EAA	Excitatory amino acid
	EAAC1	Excitatory amino acid carrier 1
	EAAT	Excitatory amino acid transporter
	EAAT1	Excitatory amino acid transporter 1
	EAAT2	Excitatory amino acid transporter 2
	EAAT3	Excitatory amino acid transporter 3
	EAAT5	Excitatory amino acid transporter 5
	EB	Embryoid body
	EBM	Embryoid body medium
	EDTA	Ethylenediaminetetraacetic acid
	eGFP	Enhanced green fluorescent protein
	ELISA	Enzyme-linked immunosorbent assay
	ER	Endoplasmic reticulum

	ES cell	Embryonic stem cell
	ESM	Embryonic stem cell medium
	FACS	Fluorescence-activated cell sorting
	FBS	Fetal bovine serum
	FC	Free cholesterol
	FITC	Fluorescein isothiocyanate
	g	Gram
	GABA	Gamma-aminobutyric acid
	GAD	Glutamic acid decarboxylase
	GAPDH	Glyceraldehyde 3-phosphate dehydrogenase. Gene: GAPDH
	gDNA	Genomic deoxyribonucleic acid
	GFAP	Glial fibrillary acidic protein
	GFP	Green fluorescent protein
	GLAST	Glutamate-aspartate transporter
	GLT	Glutamate transporter
	GLT-1	Glutamate transporter 1
	GluK1	Glutamate receptor, kainate 1 (gene)
	GluN1	Glutamate receptor, NMDA-1 (gene)
	GLUT	Glucose transporter
	GLUT 1	Glucose transporter 1
	GLUT 2	Glucose transporter 2
	GLUT 3	Glucose transporter 3
	GLUT 4	Glucose transporter 4
	GMEM	Glasgow modified essential medium
	gp-130	Glycoprotein-130
	GSK-3β	Glycogen synthase kinase 3 beta

	H ₂ O	Water
	HDL	High-density lipoprotein
	HMGCR	3-hydroxy-3-methyl-glutaryl-coenzyme A reductase. Gene: <i>HMGCR</i>
	HNF-3a	Hepatocyte nuclear factor 3α
	HT-22	Hippocampal neuronal cell line
	IC	Inhibitory concentration
	ICC	Immunocytochemistry
	ICM	Inner cell mass
	IGF	Insulin-like growth factors
	IgG	Immunoglobulin G
	IgG2b	Immunoglobulin G subtype 2b
	iGluR	Ionotropic glutamate receptor
	JAK	Janus kinase
	K ⁺	Potassium ion
	kD	Kilodalton
	LDL	Low-density lipoprotein
	LIF	Leukemic inhibitory factor
	LPL	Lipoprotein lipase
	LRP	Low-density lipoprotein receptor-related protein
	LRP-1	Low-density lipoprotein receptor-related protein 1. Gene: Lrp-1
	MEF	Mouse embryonic fibroblasts
	MEM	Minimum Essential Medium
	mg	Milligram
	mGluR	Metabotropic glutamate receptor
	mL	Millilitre

	mM	Millimolar
	MTP	Mitochondrial permeability transition pore
	MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide
	mV	miliVolt
	Na ⁺	Sodium ion
	NADPH	Nicotinamide adenine dinucleotide (reduced form)
	NaOH	Sodium hydroxide
	NFT	Neurofibrillary tangles
	ng	Nanogram
	NLC	Neural –like cell
	nm	nanometer
	nM	Nanomolar
	NMDA	N-methyl-D-aspartate
	NO	Nitric oxide
	NPC	Neural precursor cell
	NSE	Neuron-specific enolase. Gene: NSE
	NTC	Non-template control
	ORF	Open reading frame
	PA 6	Stromal cell lines
	pA/pF	picoamperes per picofarad
	Pac	Puromycin N-acetyl-transferase
	pax 3	Phosphatidylinositol 3-Kinase
	PBS	Phosphate buffered saline
	PC 12	Pheochromocytoma cell line
	PD	Parkinson's diseases
	PDL	Poly-D-Lysine

	PET	Positron emission tomography
	рН	Potential of hydrogen
	PL	Phospholipids
	PS-1	Presenilin-1
	PS-2	Presenilin-2
	PUFA	Polyunsaturated fatty acid
	qPCR	Quantitative polymerase chain reaction
	R ²	Correlation coefficient
	RA	Retinoic acid
	RAR	Retinoic acid receptor
	RFU	Relative fluorescence unit
	RNA	Ribonucleic acid
	RNS	Reactive nitrogen species
	ROS	Reactive oxygen species
	Rpm	Revolutions per minute
	rRNA	Ribosomal ribonucleic acid
	RT	Room temperature
	RT-PCR	Real-time PCR
	RxR	Retinoid X receptor
	SDIA	Stromal-derived inducing activity
	SEM	Standard error mean
	SGG	Salt glucose glycine
	SK-N-S-H	Neuroblastoma
	SOD-1	superoxide dismutase 1
	STAT	Signal transducer and activator of transcription
	TAE	Tris-acetate-EDTA

TBARS Thiobarbituric acid reactive substances

- TBE Tris-borate-EDTA
- TBI Traumatic brain injury
- TRF Tocotrienol-rich fraction
- TUNEL Terminal deoxynucleotidyl transferase dUTP nick end labeling
- UK United kingdom
- USA Unites states of America
- UV Ultraviolet
- α-TCP Alpha-tocopherol

CHAPTER 1

INTRODUCTION

1.1 Background of the study

Vitamin E is a fat-soluble compound with antioxidant properties that exist in eight forms in nature (alpha-, beta-, gamma- and delta-tocopherol and alpha-, beta-, gamma- and delta- tocotrienol) each with its own biological properties (Sen *et al.*, 2006). The difference between tocopherol and tocotrienol is that they have different number and position of methyl groups attached to the aromatic ring (Osakada *et al.*, 2004). In short, tocopherols are saturated forms of vitamin E, whereas tocotrienol is unsaturated and possess an isoprenoid side chain. This variant of vitamin E only occurs at very low levels in nature, with the highest concentration found in palm oil. Currently, there is an increase in interest on Tocotrienol Rich Fraction (TRF) from palm oil. TRF consist of 25% of alpha-tocopherol (α -TCP) and 75% of tocotrienol. TRF of palm oil has been shown to possess potent antioxidant, anticancer, and cholesterol-lowering activities (Khor *et al.*, 2016; Osakada *et al.*, 2004;).

At normal concentrations, glutamate plays a role as a major neurotransmitter in the brain, important for cognition, memory, and learning. However, elevated levels of glutamate can cause overstimulation of glutamate receptor including NMDA, AMPA and kainate receptors that cause an influx of calcium ions in the postsynaptic membrane. High energy in the form of ATP is needed to rectify back to the normal concentrations influx of intracellular calcium ion. The high requirement of energy will cause the mitochondrion to generate more reactive oxygen species (ROS) as a natural byproduct. ROS is a chemically reactive species containing oxygen including peroxides, superoxide, hydroxyl radical and singlet oxygen (Dayem *et al.*, 2010). Generally, it is a byproduct of DNA, amino acid, and lipid oxidation which can cause significant damage to cells. Oxidative stress is term for a condition where the production of ROS is greater than the capacity of the body to reduce oxidation.

Apolipoprotein E (APO E) is the major apolipoprotein in the CNS that has a role in cholesterol transport. Cholesterol is needed by neurons to build up their cellular membranes such as cell membrane of the axons, dendrites, and synapses (Poirier *et al.*,1993). This current study postulates that in an oxidative stressed brain, cholesterol recycling is unable to be performed accurately due to the deterioration of cholesterol delivery mechanism in injured neuronal cells. The understanding of the cholesterol metabolism and it's delivery in the brain and its role in neurodegenerative diseases therefore warrant further investigation. Thus, factors such as APO E, LRP receptor, HMGCR are the lipidomic markers involved in cholesterol homeostasis which became the main interest in this study.

This study aims to elucidate the protective role of vitamin E against glutamate toxicity and to understand how vitamin E is involved in modulating lipidomic markers, antioxidant activity and neurons-specific enolase expression in accomplishing the neurorecovery. It is expected that both forms of vitamin E (TRF and α -TCP) would have a neuroprotective effect against oxidative stress in the brain.

1.2 Problem statement

Neurodegenerative diseases are considered one of the major problems in our aging society as it can be serious and life-threatening. Prevalence of these diseases is increasing yearly; however, there is a lack of effective therapies or specific drug to treat this disease. Current medication only alleviates symptoms, relieves pain and helps to improve patients' quality of life. A high concentration of glutamate that contributes to oxidative stress in CNS is believed to reduce the capacity of cholesterol delivery to neuronal cells. Failure of the repair mechanism may be one of the factors contributing to the progression of neurodegenerative diseases such as AD. Thus, free radical scavenger compounds such as vitamin E in the form of TRF and α -TCP are the great interest knowing its protective properties is well documented against oxidative stress. Therefore, this study was designed to evaluate the potency of both isomers of vitamin E in protecting neural-derived embryonic stem (ES) cells from glutamate induced oxidative stress and modulating lipidomic markers for repair mechanism of the cells.

1.3 Research objective

1.3.1 General objective

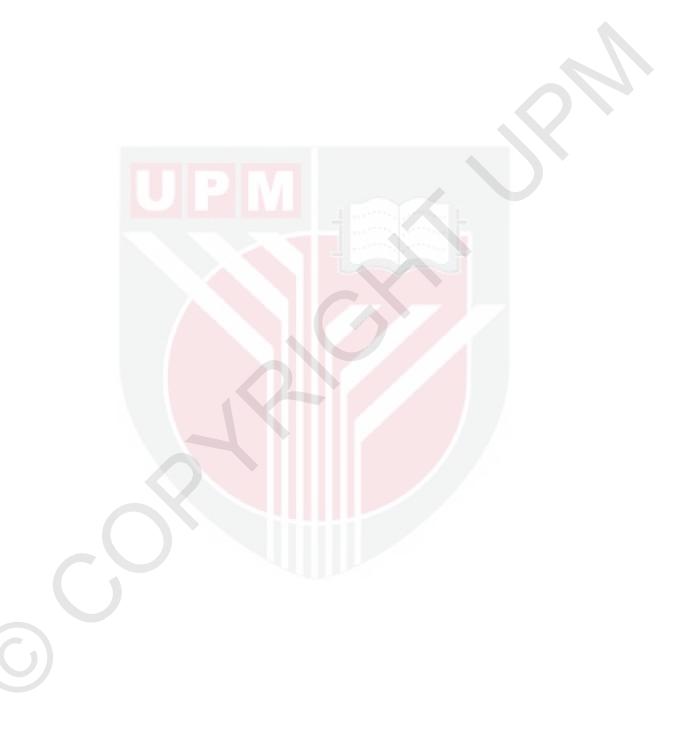
To elucidate modulation of lipidomic markers in oxidative stress neural- derived ES cell upon supplementation of vitamin E.

1.3.2 Specific objective

- To differentiate transgenic mouse embryonic stem (ES) cell line (46C) into neural commitment and confirmation with immunocytochemistry.
- To determine the dose response and time course of glutamate toxicity in neuralderived 46C cells by using MTT assay.
- To develop *in-vitro* oxidative stress model by using glutamate in neural- derived 46C cells.
- To determine the ROS activity as a neural oxidative stress marker upon glutamate toxicity and the role of TRF and α -TCP as an antioxidant.
- To assess the gene expression of glutamate receptors (NMDA and kainate receptor), NSE and lipidomic markers (APO E, HMGCR, and LRP-1) upon glutamate toxicity and the recovery process after supplementation with TRF and α -TCP.

1.4 Hypothesis

TRF and α -TCP are able to overcome oxidative stress hence, modulating lipidomic markers in *in-vitro* oxidative stress model.



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