UNIVERSITI PUTRA MALAYSIA

ROLE OF VITAMIN D AND E IN MODULATING GLUCOSE UPTAKE AND INSULIN SENSITIVITY IN INSULIN-RESISTANT NEURONAL CELLS

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By

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September 2017

Chairman : Mohd Sokhini Bin Abd Mutalib, PhD
Faculty : Medicine and Health Sciences

Alzheimer Disease (AD) has been recognized as a metabolic disease with considerable progressive derangements in brain glucose utilization and responsiveness to insulin. Altered expression of multiple players of insulin signal transduction cascade has led to the qualification of AD as a brain-specific form of diabetes. The aims of this study were to develop an insulin-resistant cell culture model in SK-N-SH neuronal cell line by prolonged exposure to insulin in serum-free medium and to determine the effect of vitamin D and E on insulin signaling. Insulin resistant model is developed via prolonged exposure with 100nM, 150nM, 200nM and 250nM of insulin in serum-free medium. The mRNA expression of insulin signaling markers that involved in glucose transport and Alzheimer's markers were measured to validate the development of cellular insulin resistance using real-time PCR while AKT phosphorylation was evaluated using ELISA. Insulin treatment significantly reduced the IR, PI3K, GLUT4 and GLUT 3 markers while reduction in AKT phosphorylation and activation of GSK3β were impaired. Further validation on the cellular insulin resistance via glucose uptake assay demonstrated a 15% reduction of insulin resistant upon treatment with 250nM insulin. Determination of vitamin D (10ng/mL and 20ng/mL) and E (200ng/mL) or with both vitamins that involved in alteration of insulin signaling markers and glucose uptake in insulin resistance model were done by measuring the similar markers again. Improvement in insulin signaling pathway was observed upon treatment with vitamin D alone with significant increase in the level of IR, PI3K, GLUT4, GLUT3, glucose uptake as well as AKT phosphorylation while GSK3β and TAU decreased significantly. Meanwhile treatment with vitamin E and combination of both vitamins showed no significant changes in all insulin signaling pathway and Alzheimer's markers. Contrarily, significant increase in glucose uptake was recorded. Further analysis on the oxidative stress showed vitamin D and E displayed a positive effect in reducing

the ROS level. The potential of vitamin E in reducing oxidative stress is postulated to improve signaling pathway leading to improvement of glucose uptake. In conclusion, overall findings indicate that insulin resistance can be developed in SK-N-SH neuronal cell line. Vitamin D and E may demonstrate as an agent that slows the progression of Alzheimer disease caused by insulin resistance in the brain.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia Sebagai memenuhi keperluan untuk ijazah Sarjana Sains

PERANAN VITAMIN D DAN E DALAM MODULASI PEGAMBILAN GLUKOSA DAN KESENSITIFAN INSULIN DI DALAM RINTANGAN INSULIN SEL NEURON

Oleh

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Penyakit Alzheimer (AD) telah dikenali sebagai suatu penyakit metabolik yang mempunyai gangguan progresif terhadap penggunaan glukosa dan tindak balas insulin di dalam otak. Pengubahan terhadap ekspresi insulin signal transduksi telah menyebabkan Alzheimer dikenali juga sebagai penyakit diabetik spesifik di dalam otak. Tujuan kajian ini adalah untuk menjadikan sel neuron sebagai model rintangan insulin. Model rintangan insulin di dalam sel neuron dibuat dengan mendedahkan sel kepada kepekatan insulin yang berbeza di dalam media bebas serum. Sel didedahkan kepada kepedatan insulin sebanyak 100nM,150nM,200nM dan 250nM. Ekspresi penanda isyarat insulin mRNA yang terlibat di dalam pengangkutan glukosa (IR, PI3K, GLUT 3 dan GLUT 4) dan penanda Alzheimer (GSK3β) telah dikaji menggunakan kaedah '*Real-Time PCR'* untuk melihat kejayaan model rintangan insulin di dalam sel dan fosforilasi AKT diukur menggunakan teknik ELISA. Keputusan menunjukan pengurangan ekspresi IR, PI3K, GLUT4 dan GLUT 3 manakala penurunan didalam aktiviti fosforilasi AKT dan peningkatan di dalam GSK3β ekspresi.Mengukur kadar pengambilan gula di dalam sel juga telah dibuat bagi mengesahkan berlakunya rintangan isulin dan gangguan terhadap pengambilan gula di dalam sel dan penurunan sebanyak 15% dicatatkan. Dalam kajian ini, kesan vitamin D (10ng/mL dan 20ng/mL) dan E (200ng/mL) atau kedua-duanya terhadap pengubahan penanda isyarat insulin dan pengambilan glukosa di dalam sel telah diuji dengan mengukur semula semua penanda isyarat yang terlibat. Keputusan menunjukkan peningkatan penanda isyarat insulin iaitu IR, PI3K, GLUT 4, GLUT 3, peningakatan didalam AKT fosforilasi dan pengurangan gen GSK3β dan TAU apabila dirawat dengan vitamin D. Sementara itu, kesan rawatan oleh vitamin E dan kedua-dua vitamin menunjukkan tiada perubahan didalam penanda isyarat insulin dan penanda alzheimers. Sebaliknya, peningkatan didalam pengambilan gula telah dicatat. Pengukuran menggunakan spesies reaktif oxygen (ROS) telah dilakukan

dan vitamin D dan E menunjukkan kesan positif terhadap pengurangan kepekatan ROS. Potensi pengurangan tekanan oksidatif melalui perangkap radikal bebas oleh vitamin E berkemungkinan membaiki isyarat insulin dan membaiki pengambilan glukosa. Kesimpulannya, semua hasil dapatan menunjukkan bukti yang mengaitkan pembentukan model rintangan insulin dengan menggunakan sel neuron. Vitamin D dan E menunjukkan kesan rawatan yang memperlahankan perkembangan penyakit Alzheimer yang disebabkan oleh rintangan insulin di dalam otak.

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I certify that a Thesis Examination Committee has met on 19 September 2017 to conduct the final examination of Amirah Salwani bt Zaulkffali on her thesis entitled "Role of Vitamin D and E in Modulating Glucose Uptake and Insulin Sensitivity in Insulin-Resistant Neuronal Cells" 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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This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfilment of the requirement for the degree Master of Science.The members of the Supervisory Committee were as follows:

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Signature: ______________________

Name of Member of Supervisory Committee: Dr Sharifah Sakinah Syed Alwi

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LIST OF ABBREVIATIONS

%	Percentage
±	More or less
µg	Microgram
µl	Microlitre
2-DG	2-deoxyglucose
AD	Alzheimer's disease
ANOVA	Analysis of variance
ATP	Adenosine Triphosphate
Aβ	beta-amyloid
AβPP	amyloid- beta precursor protein
cDNA	complementary DNA
CNS	Central Nervous System
CO_2	Cabon Dioxide
CSF	Cerebrospinal fluid
DCF	Dichlorodihydrofluorescein
DCFH-DA	Dichlorodihydrofluorescin diacetate
DEPC	Diethyl pyrocarbonate
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
ER	Endoplasmic Reticulum
ERK/MAPK	Extracellular signal–related kinase/mitogen-activated protein kinase
FASD	Fetal alcohol spectrum disorder
FBS	Fetal Bovine serum
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GLUT 3	Glucose transporter Type 3

GLUT 4	Glucose Transporter Type 4
GSK3β	Glycogen synthase kinase 3 beta
IGF	insulin-like growth factor
iNOS	inducible nitric oxide synthase
IR	Insulin Receptor
IRS 2	Insulin receptor substrate 2
kDA	kiloDalton
KRPH	Krebs-Ringer-Phosphate-HEPES
MEM	Minimal Essential medium
mL	Mililiter
mRNA	Messenger Ribonucleic acid
MTT	3-(4,5-dimethylthiazol-2-yl)-diphenyltetrazolium bromide
NFT	neurofibrillary tangles
Ng	Nanogram
NIRKO	neuronal specific insulin receptor knockout
nM	nanoMolar
NTC	No template control
ºC	Degree Celcius
PBS	Phosphate-buffered saline
PI3K	Phosphatidylinositol-4,5-bisphosphate 3-kinase
PKC	Protien kinase C
RFU	Relative fluorescence units
RNA	Ribonucleic acid
ROS	Reactive oxygen species
Rpm	Revolutions per minutes
RT-PCR	Reverse Transcription-polymerase chain reaction
SiRNA	Small interfering RNA
SKNSH	Human Neuroblastoma Cell line (ATCC HTB- II)

ssDNA	Single-stranded deoxyribonucleic acid
T1DM	Type I Diabetes Mellitus
T2DM	Type II Diabetes Mellitus
TAE	Tris-acetate-EDTA
TBE	Tris-borate-EDTA
TRF	Tocotrienol Rich Fraction
UVB	Ultraviolet B
VDR	Vitamin D Receptor
VDR-RXR	Vitamin D receptor-retinoic acid x-receptor complex

CHAPTER 1

INTRODUCTION

1.1 Background of study

About 3.5 million (17.5%) percent of Malaysian citizens aged 18 years and above have Diabetes (National Health and Morbidity Survey 2015). It has been discover that a decrease in insulin level is not the only causal factor of diabetes. On the other hand, cell insensitivity to insulin occurred even more common in relevance to diabetes, hence advocating the concept of insulin resistance among the scientist and medical experts (Himsworth, 1939).

Insulin resistance and defective insulin signaling have been firmly established as the fundamental characteristics of Type II Diabetes Mellitus (T2DM). Interestingly, these characteristics also happen to appear in chronic neurodegenerative disorder Alzheimer's disease (AD) (de la Monte and Wands, 2008). Postmortem brain studies revealed that molecular, biochemical, and signal transduction abnormalities in AD are virtually identical to those that occur in T1DM and T2DM (de la Monte &wands, 2008; de la monte 2012). Earlier, a study has observed lower cerebrospinal fluid (CSF) insulin levels and reduced insulin-mediated glucose disposal in AD patients when compared with healthy control subjects (Craft et al.,1998).

A cascade of reactions initiated with reduced cell's sensitivity to insulin may explain the lower insulin level in the brain of AD patients. Reduced sensitivity to insulin can result in hyperinsulinemia, which consequently down-regulate the insulin receptors at the blood-brain barrier, impair insulin signaling and reduce the uptake of insulin by the brain. These events later manifest brain inflammation, oxidative stress, alterations in beta-amyloid (Aβ) levels, and cell death (Talbot et al., 2012). Since insulin resistance can be part of the pathogenesis, it appears as a potent accelerator of AD. Despite these facts, the molecular mechanism of insulin resistance in neurons remains largely unknown contrary to that in peripheral tissues like skeletal muscle, liver, and adipose. There are numerous etiologies for insulin resistance, including lipotoxicity, inflammation, endoplasmic reticulum (ER) stress, oxidative stress and hyperinsulinemia (Kaneto et al., 2006). Hyperinsulinemia contributes to insulin resistance and its effects on peripheral tissues have been demonstrated (Kahn et al., 2000). The involvement of neurons in the brain insulin resistance is relatively unclear, since little is known about its molecular mechanism.

Although there were many factors that lead to the development of AD, this study only focuses on insulin resistance as the causal that mimics AD in neuronal cells. It is anticipated that with this new findings, the understanding will enable better identification of suitable remedies that can reverse the condition of insulin resistance in AD.

The role of vitamin D in the pathogenesis and prevention of diabetes has generated good scientific interest. Numerous studies have shown a relationship between vitamin D status and the risk of diabetes or glucose intolerance. It has been shown that the prevalence of hypovitaminosis D was higher in diabetic patients than in non-diabetic people (Cigolini et al., 2006).

Vitamin D plays an important role in improving insulin resistance and the T2DM by affecting either insulin sensitivity or β-cell function or both (Chiu et al., 2004). Vitamin D can stimulate the expression of the insulin receptor, and hence improving insulin responsiveness for glucose transport into the cells (Maestro et al., 2000).Vitamin E may also boost insulin sensitivity and decrease diabetes risk by reducing oxidative stress to the cell (Manning et al., 2004). Through *in vitro* and in animal models of diabetes, it was found that antioxidants, improve insulin sensitivity (Scott et al., 2004). As vitamin E exhibits anti-oxidant property, it has been shown to improve the insulin sensitivity both in *in- vitro* and animal models of diabetes (Houstis et al., 2000)

Overall, this study is aimed to clarify the involvement of vitamin D and E in improving insulin resistance that potentially interferes in insulin signaling cascade at gene expression level.

1.2 Problem Statement

Neurodegenerative diseases are considered as one of the major problems in our aging society. Indeed, it can be serious and life-threatening. Prevalence of these diseases is growing 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. Insulin resistance and defective insulin signaling have been firmly established as the fundamental characteristics to appear in chronic neurodegenerative disorder Alzheimer's disease (AD) (de la Monte and Wands, 2008). Furthermore, alteration of insulin pathway may provide an oxidative environment which in turn implicates the onset and the progression of Alzheimer's disease. Vitamin D may modulate insulin sensitivity as a functional vitamin D receptor (VDR) element have been identified in the promoter region of insulin receptor, suggesting that vitamin D may play a role in the regulation of insulin resistance. Free radical scavenger compound such as vitamin E in the form of TRF is of great interest knowing its protective properties are well documented against oxidative stress. Therefore, this study was designed to evaluate the potency of both Vitamin D and E in improving insulin resistance that potentially interferes in insulin signaling pathway that lead to reduce the incidence of Alzheimer's disease.

1.3 Justification of Study

To identify compounds with potential insulin resistance reverting properties and optimizing vitro model that could mimic the state of insulin resistance and reflect the pathophysiological progression of the AD condition. Thus the present study was designed to develop an in-vitro model mimicking insulin resistance using SK-N-SH neuronal cell line and to exploit it for the identification of Vitamin D and E potency as an insulin resistance reverting properties.

1.4 Objectives

1.4.1 General Objective

To investigates the role of vitamin D and E towards insulin resistance in neuronal cells (SK-N-SH) in relation with PI3K-AKT signaling pathway.

1.4.2 Specific Objectives

- To determine cell viability using MTT Assay upon induction with insulin.
- To develop cellular insulin resistance model using SK-N-SH neuroblastoma cell line.
- To validate the cellular insulin resistance model by evaluating the gene expression level on insulin signaling markers involve in glucose transport (Insulin Receptor (IR), PI3K, AKT, GLUT3 and GLUT4), glucose uptake and Alzheimer's markers (GSK3β, TAU).
- To determine the gene expression level of insulin signaling markers, and Alzheimer's markers upon treatment with vitamin D and E under insulin resistance condition.
- To determine the glucose uptake and oxidative stress level upon treatment with vitamin D and E under insulin resistance condition.

1.5 Hypothesis

1. Hyperinsulinemia induces insulin resistance in neuronal cells.
2 Vitamin D and E improve insulin signaling pathway under the condition of insulin resistance leading to improvement of glucose uptake in the brain.

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