AN INVESTIGATION OF THE NEUROPROTECTIVE EFFECTS OF PALM VITAMIN E TOCOTRIENOLS USING WHITE MATTER LESIONS AS THE HUMAN STUDY MODEL

by

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DEDICATIONS

This thesis is dedicated to the Almighty God for giving me the strength to complete my PhD, my beloved wife, Dr. Shubashini Gnanasan for her continuous support and inspiration, my adorable sons, Prakaladhan and Sithaarthan who are always there to cheer me up and to my wonderful parents, Mr. L. Gopalan and Madam M. Sathiamah for their everlasting encouragement and blessings on me. Thank you.

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ABBREVIATIONS

ALP	Alkaline Phosphatase
ALT	Alanine Transaminase
Apo B	Apolipoprotein B
AST	Aspartate Aminotransferase
BMI	Body Mass Index
CCA	Common Carotid Artery
CRT	Creatinine Kinase
DSA	Digital Subtraction Angiography
GGT	Gamma Glutamyl Transpeptidase
HDL	High Density Lipoprtein
Hs- CRP	Highly Sensitive C Reactive Protein
ICA	Internal Carotid Artery
LDL	Low Density Lipoprotein
LP (A)	Lipoprotein A
MRA	Magnetic Resonance Angiography
MRI	Magnetic Resonance Imaging
T3	Mixed Tocotrienols
TBARS	Thiobarbituric Acid Reactive Substance
TC	Total Cholesterol

TG	Triglyceride
USM	Universiti Sains Malaysia
WML	White Matter Lesion

PUBLICATIONS

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SATU KAJIAN KESAN NEUROPROTEKTIF VITAMIN E TOKOTRIENOL MINYAK SAWIT MENGGUNAKAN KECEDERAAN ZON PUTIH OTAK SEBAGAI KAJIAN MODEL MANUSIA

ABSTRAK

Satu kajian telah dijalankan untuk menilai aktiviti neuroprotektif campuran tokotrienol menggunakan manusia yang mempunyai kecederaan zon putih otak [White matter lesion (WML)]. WMLs adalah manifestasi penyakit salur darah kecil cerebral.

Di peringkat awal kajian, kesan anti arterogenik campuran tokotrienol di kalangan sukarelawan yang mempunyai paras kolesterol sederhana tinggi telah dinilai melalui pengimejan arteri karotid menggunakan Magnetic Resonance Angiography (MRA). Tiada sesiapapun daripada 50 sukarelawan pertama yang menjalani pengimejan tersebut mengalami stenosis arteri karotid. Oleh itu, bahagian kajian ini telah ditamatkan.

Fasa kajian seterusnya, prevalens WML dikalangan populasi yang mempunyai paras kolesterol sederhana tinggi sekitar bahagian utara semenanjung Malaysia telah dikenalpasti. Daripada 350 sukarelawan yang menjalani pengimejan, 161(46%) mempunyai WML, di mana 65 (18.6%) adalah lelaki dan 96 (27.4%) adalah wanita. Kajian ini juga telah menunjukkan peluang untuk mendapat WMLs adalah 2 kali ganda (30.6%) dalam lingkungan umur 50 keatas berbanding 15.4% dalam kumpulan umur 50 kebawah.

Dibahagian kajian neuroprotektif, kajian rawak rabun dua pihak plasebo terkawal telah dijalankan. Sukarelawan yang diambil adalah ≥ 35 tahun yang mempunyai risiko kardiovaskular dan menjalani Magnetic Resonance Imaging (MRI) otak. Mereka dikategorikan sebagai WML positif (MRI +ve) jika ada WML pada awal kajian.

Sukarelawan tanpa WML pada awal kajian akan diklasifikasikan (MRI -ve). Dalam kohort MRI-ve, 120 sukarelawan dirawakkan untuk menerima sama ada 200 mg campuran tokotrienol atau plasebo dua kali sehari dan disusuli selama setahun. Daripada 113 sukarelawan yang melengkapkan kajian, 19 daripada kumpulan campuran tokotrienol dan 9 dalam kumpulan plasebo membentuk WML baru. Walaubagaimanapun, purata pembentukan volum WML baru selepas setahun adalah lebih rendah dikalangan kumpulan campuran tokotrienol (8%) berbanding placebo (47%) dengan kesignifikan statistik $p = 0.048$.

Dibahagian akhir kajian, 121 sukarelawan yang disahkan WMLs (MRI+ve) telah dirawakkan untuk menerima 200 mg campuran tokotrienol atau plasebo dan disusuli selama 2 tahun. Berdasarkan analisa 'per protocol'(88 sukarelawan) dan 'intention to treat'(121 sukarelawan) purata volum WML kumpulan placebo meningkat selepas 2 tahun, namun kumpulan yang menerima campuran tokotrienol kekal tidak berubah. Purata perubahan volum WML diantara dua kumpulan adalah tidak signifikan pada akhir tahun pertama ($p = 0.150$) tetapi signifikan pada akhir tahun kedua untuk analisa 'per protocol' dan 'intention to treat'($p = 0.019$ dan $p = 0.018$). Dengan itu, kajian ini telah menunjukkan bahawa campuran tokotrienol boleh melemahkan perkembangan WMLs.

AN INVESTIGATION OF THE NEUROPROTECTIVE EFFECTS OF PALM VITAMIN E TOCOTRIENOLS USING WHITE MATTER LESIONS AS THE HUMAN STUDY MODEL

ABSTRACT

A study was conducted to evaluate the neuroprotective activity of mixed tocotrienols using human volunteers with white matter lesion (WML). WMLs are regarded as manifestations of cerebral small vessel disease.

Initially, the anti atherogenic effect of mixed tocotrienols was also evaluated in mildly hypercholesterolemic volunteers via imaging the carotid arteries using Magnetic Resonance Angiography (MRA). Of the first 50 consecutive volunteers who undergone the carotid artery imaging, none of them were detected having carotid artery stenosis and hence this part of the study was discontinued.

In the next phase of the study, the prevalence of white matter lesion (WML) in a local hypercholesterolemic population in north –west peninsular Malaysia was determined. Of the 350 research volunteers who were imaged, 161 (46%) of them had WMLs, of which 65 (18.6%) were males and 96 (27.4%) females. The study also demonstrated that the chances of developing WMLs doubled (30.6%) in the age group of above 50 compared to 15.4% in the age group of below 50.

In the neuroprotective part of the study, a randomized double-blind placebo-controlled trial was conducted. Volunteers recruited were ≥ 35 years with cardiovascular risk factors and undergo Magnetic Resonance Imaging (MRI) of the brain. They were categorized as WML positive (MRI +ve) if they were present with WML at baseline.

Volunteers without WMLs at baseline were classified as (MRI -ve). In the MRI –ve cohort, 120 volunteers were randomized to receive either 200 mg mixed tocotrienols or placebo twice daily and were followed up for 1 year. Out of 113 volunteers who completed the study, 19 in the mixed tocotrienols and 9 in the placebo group developed new WMLs. However, the mean volume of the WMLs developed after 1 year was lesser in the mixed tocotrienol treated group (8%) compared to placebo (47%) with a statistical significance of $p = 0.048$.

In the final part of the study, 121 volunteers with MRI confirmed WMLs (MRI +ve) were randomized to receive 200 mg mixed tocotrienols or placebo twice daily and were followed up for 2 years. According to per protocol (88 volunteers) and intention-to-treat (121 volunteers) analyses, the mean WML volume of the placebo group increased after 2 years, whereas that of the mixed tocotrienol supplemented group remained essentially unchanged. The mean WML volume change between the two groups was not significantly different ($p = 0.150$) at the end of 1 year but was significant at the end of 2 years for both per protocol and intention-to-treat analyses ($p = 0.019$ and $p = 0.018$). Thus, the present study found that mixed tocotrienols can attenuate the progression of WMLs.

CHAPTER 1: INTRODUCTION

1.1. White Matter Lesion

1.1.1. Introduction

White matter lesions (WMLs) are areas of increased signal intensity detected on T2-weighted magnetic resonance imaging (MRI) scans. These lesions are common among older adults (Brickman, Schupf, Manly, & et al., 2008)(F.-E. de Leeuw et al., 2001) and are thought to reflect small vessel vascular disease and neurodegeneration of nerve bundles. WMLs are frequently observed in individuals with advanced age, hypertension, prior ischemic stroke and other cerebrovascular risk factors (Hénon, Godefroy, Lucas, Pruvo, & Leys, 1996; Kalaria & Erkinjuntti, 2006; Leys et al., 1999; Räihä, Tarvonen, Kurki, Rajala, & Sourander, 1993; Wiszniewska, Devuyst, Bogousslavsky, Ghika, & van Melle, 2000). The white matter of the brain accounts for the 60% of the total brain volume. It includes major commissural tracts, the cortical association fibers, and all the cortical afferent and efferent fibers. White matter consists of nerve fibers, supporting cells, interstitial space and vascular structures. Axons are major component of white matter and it is enveloped by myelin with two types of neuroglia; oligodendrocytes and astrocytes. Myelin acts as an insulator of axons and its structure facilitates rapid transmission of impulses (Valk & van der Knaap, 1989). The role of white matter is in information transmission to connect various grey matters (neuron bodies) which is mainly responsible for information processing.

1.1.2. **Etiology of White Matter Lesion**

Based on clinicopathological studies, WMLs can be classified into three different categories which includes punctate, periventricular, and confluent WMLs and showed that at least one of these is due to small vessel disease (Fazekas et al., 1993). This affects arteries of 150μm in diameter and is generally associated with hypertension, diabetes, or both. The major vascular pathological finding is the one that Fisher in the 1950s termed "segmental arterial wall disorganization" (Fisher, 1982) also widely known by others as lipohyalinosis (Ogata, 1999), where the loss of the arterial architecture consists of whorls, tangles or wisps of more or less fine connective tissue that entirely replaces the vessel wall and obliterates the normal vascular coats. The general outline of the vessel in less destructive lesions is preserved and the disintegrating wall consists in a loose meshwork of collagenous strands separated by empty interstitial clefts (Fisher, 1968). The foremost consequences of lipohyalinosis are small-vessel wall thickening and luminal narrowing which leads to arteriolosclerosis (Leonardo Pantoni & Julio H. Garcia, 1997; Wardlaw, Sandercock, Dennis, & Starr, 2003). The axonal damage that occurs within the confluent lesions is however variable, which involves demyelination with preservation of axonal integrity to complete axonal disruption with loss of axonal function (Fazekas et al., 1988). As a result of the degeneration of the neurons, axonal disruption leads to functional loss of the affected network, while demyelination causes only slowing of stimulus speed conduction, hence at least in some cases WMLs may lead to reduced efficiency of the affected network, but with preserved function. On the other hand, imaging-pathological studies have shown that punctate lesions can be related with variable pathological findings ranging from no detectable

pathology at all to enlargement of perivascular spaces (Fazekas, et al., 1993). In the case of discontinuation of the ependymal lining which results in permeation of water into the axons and chronic edema of the white matter adjacent to the ventricles, the condition is known as periventricular caps and halo but with no axonal damage (Fazekas, et al., 1993). Reduced blood flow was also evident in WMLs. Cerebral blood flow was lower in WMH areas relative to normal appearing white matter, which in turn, was lower than grey matter. Regions with consistently lower cerebral blood flow across individuals were more likely to appear as WMLs (Brickman et al., 2009).

1.1.3. **Clinical Implication of White Matter Lesions**

White matter lesions are more common and widely detectable in patients with cardiovascular risk factors and symptomatic cerebrovascular disease (Launer, 2004). The presence of WMLs can be attributed to several factors. The Rotterdam Scan Study (Sarah E. Vermeer et al., 2003) and Sydney Stroke Study (Wen & Sachdev, 2004) documented that individuals with white matter lesions have an increased risk of clinical stroke. In addition to that, larger white matter lesions can actually represent subclinical brain infarct (Marshall, Bradley, Marshall, Bhoopat, & Rhodes, 1988). Subcortical silent brain infarction which was presented as periventricular hyperintensity and focal WMLs is associated with the presence cardiovascular risk factors such as hypertension, diabetes and retinal artery sclerosis (Kobayashi, Okada, Koide, Bokura, & Yamaguchi, 1997). Incidence of severe WMLs at baseline is an independent predictor of future stroke while patients with initially mild WML may develop subsequent stroke as the WML progresses (Yamauchi, Fukuda, & Oyanagi, 2002). Similar findings were also reported that cerebral WMLs predict both ischemic strokes and myocardial infarctions in patients with established atherosclerotic disease (Gerdes et al., 2006). Silent brain infarcts, marked periventricular hyperintensity and distinct subcortical white matter lesions are important risk factors of clinical stroke. Moreover, silent brain infarcts and marked periventricular hyperintensities increase the risk of mortality (Bokura et al., 2006).

WMLs also contribute to the cognitive impairment of the patients. In the Rotterdam Study (M. M. B. Breteler et al., 1994) elderly individuals with WMLs were associated with lower scores on test of cognitive function and were significantly associated with subjective mental decline. WMLs and periventricular hyperintensity were significantly

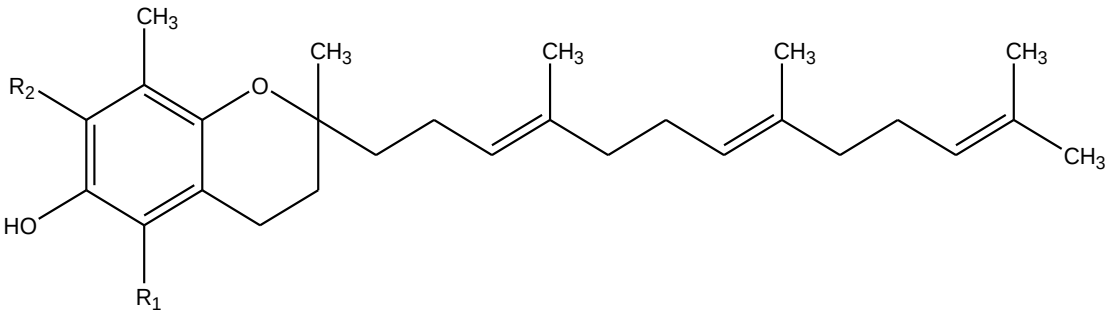
more extensive in dementia with lewy bodies than in controls in patients with Alzheimers and vascular dementia. Presence of frontal WMLs was associated with higher depression scores (Barber et al., 1999). Severity of dementia is directly correlated with the amount and size of WML in the periventricular and subcortical regions of the brain (Targosz-Gajniak, Siuda, Ochudło, & Opala, 2009b).

The above data clearly demonstrated that white matter hyperintensities as detected on MRI is closely related to vascular events of the brain and in certain instances represent subclinical infarcts. Moreover, the degenerative changes of the small vessel salso contribute towards the mental and cognitive decline of the patients.

1.2. Vitamin E

1.2.1. **Tocotrienol**

Vitamin E consists of eight isomers comprising four tocopherol and four tocotrienol isomers, which share similar structural features of a chroman head and a 16-carbon phytyl chain. Both tocopherols and tocotrienols are designated as α, β, γ and δ, depending on the number and positions of methyl groups on the chroman ring (Qureshi & Qureshi, 1993). The difference between tocopherols and tocotrienols lie mainly in the former having a saturated phytyl chain, while the latter is unsaturated, with 3 double bonds at 3', 7' and 11' positions (A. Kamal-Eldin & L. A. Appelqvist, 1996).Figure 1.1 shows the general structure of α - tocotrienol. While tocopherols are generally present in nuts and common vegetable oils, natural sources of tocotrienols are quite limited, with palm oil and rice bran oil containing the highest concentrations of tocotrienols in nature (Tan, 1989).



Tocotrienols	R1	R2
α-tocotrienol	CH_3	CH_3
β-tocotrienol	CH_3	H
γ-tocotrienol	H	CH_3
δ-tocotrienol	H	H

Figure 1.1: Chemical structure of tocotrienols

1.2.2. **Neuroprotective Properties**

Much investigation has been carried out to study the neuroprotective properties of tocotrienols. Of special significance is the study by (Khanna et al., 2005) which demonstrated that tocotrienols introduced into a neuronal cell at nanomolar concentrations could prevent glutamate-induced death of neuron cells in mice. The underlying mechanism behind the protective properties is through the inhibition of c-Src kinase (Sen, Khanna, Roy, & Packer, 2000)and 12-lipoxygenase (Khanna et al., 2003). It was also shown that the degeneration of the nerve cells was due to induced stress mimicking clinical situations such as ischemia. Therefore, the findings of Khanna et al. (2005) implicated that tocotrienols might have a role in the prevention of degenerative diseases involving the nervous system. Khanna et al. (2005) showed that tocotrienols have in vitro neuroprotective properties. In addition, tocotrienol-supplemented rats showed more protection against stroke-inducedinjury compared with matched controls (Khanna, et al., 2005). In another study, mongrel canines fed with tocotrienol-enriched supplementation significantly attenuated ischemic stroke induced lesion volume. Furthermore, it prevented loss of white matter fiber tract connectivity, improved cerebrovascular collateral circulation to the ischemic mid cerebral artery territory during mid cerebral artery occlusion(Rink et al., 2011). In another study, subjects supplemented with self emulsifying preparation of tocotrienol rich vitamin E showed a trend towards improvement of arterial compliance which would enable better elasticity and sustainability of the blood vessels in the event of stroke (Rasool, Rahman, Yuen, & Wong, 2008).

1.2.3. **Anti-atherogenic Properties**

The development of atherosclerosis involves a multitude of factors both genetic and environmental in nature and these include elevated levels of cholesterol especially LDL, apolipoprotien B and lipoprotein Lp(a)**,** oxidation of LDL-cholesterol, adhesion molecule expression and monocytic cell adherence. The anti-atherogenic activity of α-tocopherol has been linked to its ability to prevent the oxidation of LDL (Esterbauer, 1991; Salonen, Yia-Hertuala, & Yamamoto, 1992). Nevertheless, this theory remains controversial with some studies failing to show association between plasma α-tocopherol levels and reduced mortality from cardiovascular disease(Hense, Stender, Bors, & Keil, 1993). Despite the numerous studies carried out on α-tocopherol, the relationship between tocotrienols and atherosclerosis was investigated only recently. These studies demonstrated various benefits of tocotrienols ranging from improving lipid profiles [reduced total and LDL cholesterol, apolipoprotein B and lipoprotein Lp(a)], lowering of thromboxane B2 and platelet factor 4 levels to reduce LDL oxidation (Qureshi et al., 1995; Qureshi, Bradlow, Salser, & Brace, 1997; Qureshi, Qureshi, Halser-Raspacz, et al., 1991; Tomeo, Gellar, Watkins, Gapor, & Bierenbaum, 1995)

1.2.4. **Hypocholesterolemic effect of tocotrienols**

Epidemiological studies have convincingly shown that plasma cholesterol is a major risk factor in the development of atherosclerosis as well as cardiovascular diseases such as myocardial infarction and coronary heart disease. A high plasma cholesterol concentration is associated with a higher risk of cardiovascular diseases (Goldbourt, Holtzman, & Nuefeld, 1985). Therefore, reduction of plasma cholesterol is essential in lowering the risk (Iso, 1989) and hence decreasing the mortality associated with cardiovascular diseases. Reduction of cholesterol can be achieved through restriction of cholesterol intake (Chait et al., 1993) and administration of hypocholesterolemic agents. The ability of the tocotrienols or tocotrienol rich fraction from palm oil to reduce plasma cholesterol is a subject of much investigation (Qureshi, et al., 1995).

Unlike tocopherols, tocotrienols have been reported to inhibit cholesterol synthesis by suppressing 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMG-CoAR) activity. This effect was ascribed to the unique ability of the isoprenoid side-chain to increase cellular farnesol, a mevalonate-derived product. Farnesol in turn down-regulates HMG-enzyme (Correll, Ng, & Edwards, 1994; Goldstein, 1990). The different isomers of tocotrienols exhibit varying degrees of cholesterol lowering activity. *In vitro* models have identified γ- and δ-tocotrienol to be more potent than α-tocotrienol in suppressing cholesterol biosynthesis (Pearce, Parker, Deason, Qureshi, & Wright, 1992).

A number of studies using animal models (Khor & Chieng, 1997; Qureshi, Qureshi, Halser-Raspacz, et al., 1991)and human volunteers (Qureshi, et al., 1995; Qureshi, et al., 1997; Qureshi, Qureshi, Wright, et al., 1991; Tan, Khor, Low, Ali, & Gapor, 1991)have demonstrated the cholesterol-suppressive action of tocotrienols. However, there are

studies that show supplementation of tocotrienols had no marked favorable effects on the serum lipoprotein profiles in human volunteers with elevated cholesterol levels (Mensink, van Houwelingen, Kromhout, & Hornstra, 1999; Mustad, Smith, Ruey, Edens, & DeMichele, 2002; Wahlqvist et al., 1992). Nevertheless, Qureshiet al. (1996) reported that the relative alpha-tocopherol to the tocotrienol content of more than 30% tend to attenuate the cholesterol lowering activity of the tocotrienols. In this regard, it should be noted that the preparation used in the study by Wahlqvistet al. (1992) and Mensinket al. (1999), had an alpha-tocopherol to tocotrienols ratio of approximately 4:6 and 3:7 respectively, and might indeed be the reason for the observed negative response. In view of the conflicting results, more controlled studies using human volunteers need to be carried out to establish the beneficial effects of tocotrienol on cholesterol profiles.

1.2.5. **Hypo-apolipoprotein B effect of tocotrienols**

Apolipoprotein B (Apo-B), the major structural component of VLDL (very low density lipoprotein) and LDL, has been recognized as an independent risk factor for the development of premature coronary artery disease (CAD) (Albers, Brunzell, & Med., 1989). Apo-B appears to be directly involved in the atherosclerotic process as lowering Apo-B levels in CAD subjects has resulted in the regression of atherosclerosis (Brown, Albers, & Fisher, 1990). Tocotrienols have also been shown to reduce plasma apolipoprotein B levels in hypercholesterolemic subjects (Qureshi, et al., 1995; Qureshi, Qureshi, Wright, et al., 1991). It is believed that tocotrienols lower plasma Apo-B levels partly by up-regulating LDL receptors in the liver, which facilitate the clearance of LDL-ApoB from the blood stream (Parker, Pearce, Clark, Gordon, & Wright, 1993). It has been suggested that tocotrienols *in vitro*, in particular γ-tocotrienol, increase the intracellular proteolytic degradation of Apo-B which in turn alter the assembly of VLDL with core lipids and its secretion from the liver (Theriault, Wang, Gapor, & Adeli, 1999; Wang, Theriault, Gapor, & Adeli, 1998). In short, both the increased clearance rate of LDL and the decreased production rate of VLDL cause a reduction of the plasma Apo-B levels. Nevertheless, the beneficial reduction of apoB by tocotrienols merit further investigation and confirmation.

1.2.6. **Hypo- lipoprotein Lp (a) effect of tocotrienols**

Lipoprotein (a) [Lp(a)], a plasma lipoprotein whose structure resembles that of LDL, has been found to be the strongest predictor of coronary heart disease (Maher & Brown, 1995). Elevated plasma levels of Lp(a) is considered atherogenic. When modified by lipid peroxidation, it is taken up by macrophages. As a result, cholesterol is deposited into macrophages, which forms foam cells within the atherosclerotic lesion (Naruszewicz, Selinger, & Davignon, 1992). However, Lp(a) association with atherosclerosis is believed to be due to its interference with plasminogen activation associated with thrombosis (Miles, Fless, Levin, Scanu, & Plow, 1989). Lowering of Lp(a) has not been successful with diet and lipid lowering drugs. However, Qureshiet al. (1997) have demonstrated that a novel tocotrienol rich fraction from rice bran oil enriched with didesmethyl-tocotrienol (no methyl groups on the chromanol ring) decreased plasma Lp(a) levels. This finding also merits further long-term clinical studies to verify their beneficial effect in prevention of artherosclerosis.

1.2.7. **Antioxidant properties of tocotrienols**

The initial step in the pathogenesis of atherosclerosis is believed to be LDL lipid peroxidation, which is followed by a cascade of events leading to the formation of foam cells in the atherosclerotic lesion (Steinberg, Parthasarathy, Carew, Khoo, & Witztum, 1989). Tocotrienols, like tocopherols, are free radical scavengers, which owe their antioxidative activity to their chain-breaking property that neutralizes peroxyl and alkoxyl radicals generated during lipid peroxidation (Burton & Traber, 1990; A. Kamal-Eldin & L. A. Appelqvist, 1996).

Until recently, of all the vitamin E isomers α-tocopherol was generally regarded as the most potent antioxidant protecting against lipid peroxidation. Serbinovaet al. (1991) made remarkable observations that α-tocotrienol possessed 40-60 times higher antioxidant activity than α-tocopherol against Fe^{2+}+ascorbate- and Fe^{2+}+NADPH-induced lipid peroxidation in rat liver microsomal membranes and 6.5 times better protection of cytochrome P-450 against oxidative damage. Their findings also suggested that the higher antioxidant potency was due to α-tocotrienol's higher recycling efficiency from chromanoxyl radicals, its more uniform distribution in membrane bilayer and its stronger disordering of membrane lipids, enabling better interaction of chromanols with lipid radicals. In addition, Kamat and Devasagayam (1995)also reported a significant inhibition of oxidative damage *in vitro* to both lipids and proteins in rat brain mitochondria with γ-tocotrienol showing superior activity compared to the other tocotrienols.

1.2.8. Inhibition of adhesion molecule expression and monocytic cell adherence by tocotrienols

Monocytic adherence to endothelium of blood vessels, mediated by multiple cell adhesion molecules, including ICAM-1, VCAM-1 and E-selectin(Carlos & Harlan, 1994)has been shown to be critical in the development of atherosclerosis. Enhanced over-expression of these surface molecules has been shown to be stimulated by oxidized LDL but down regulated by antioxidants (Khan, Parthasarthy, Alexander, & Medford, 1995). α-tocopherol has been shown to reduce endothelial adhesion molecule expression and monocytic cell adherence (Devaraj, Li, & Jialal, 1996) but the effect of tocotrienols was only recently investigated. Theriault et al. (2002) reported that α-tocotrienol was the most effective form of vitamin E for reducing endothelial expression of adhesion molecules and adhesion to monocytes when they compared 3 forms of vitamin E, α-tocopherol, α-tocopheryl succinate and α-tocotrienol.

In view of the neuroprotective and anti-atherogenic potential of tocotrienol rich fraction through the various mechanisms mentioned above, the protective effects of tocotrienols on progression of sub-clinical white matter lesions and carotid artery stenosis in humans deserve further investigation and confirmation.

1.3. Clinical Evaluations: Basic Principles of Magnetic Resonance Imaging of the Brain

An MRI is a noninvasive approach that produces cross-sectional images of the body. Images of the brain, spine, joints, abdomen, and pelvis are generated using a strong magnetic field and radio waves to produce very clear and detailed computerized images of the inside of the body. Its efficacy as a clinical imaging modality is based primarily upon humans being proton-rich; the tissues are composed of between 70% and 90% water, which is concentrated hydrogen nuclei or protons. MRI images are obtained by measuring how rapidly hydrogen nuclei of different tissues return to their resting energy states after being excited by a strong magnetic field (McRobbie, Moore, Graves, & Prince, 2003).

The properties and amount of water within a tissue can alter drastically with disease or injury; MRI is very sensitive to the former and, therefore, a very sensitive diagnostic modality. MRI images display a better definition between the lesion and the adjacent normal tissue than other imaging modalities (Silvers, 2006). T1 scans are often known as 'anatomy scans', because their images display excellent contrast, and most clearly show the boundaries between different tissues. T2 images take longer to acquire than T1 images. T2 images are often termed 'pathology scans' because collections of abnormal fluid are bright against the darker normal tissue (McRobbie, et al., 2003). On a T_2-weighted scan, water- and fluid-containing tissues are bright and fat-containing tissues are dark. The reverse is true for T_1-weighted images. Damaged tissue tends to develop edema, which makes a T_2-weighted sequence sensitive for pathology, and generally able to distinguish pathologic tissue

from normal tissue. With the addition of an additional radio frequency pulse and additional manipulation of the magnetic gradients, a T_2-weighted sequence can be converted to a FLAIR sequence, in which free water is now dark, but edematous tissues remain bright. This sequence is currently the most sensitive way to evaluate the brain for demyelinating diseases (Rinck, 2012).

MRI of the carotid blood vessels which is also referred as Magnetic Resonance angiography (MRA). The blood vessels in the neck (carotid and vertebral arteries) and brain are frequently studied by MRA to look for areas of constriction (narrowing) or dilatation (widening).

1.4. Scope of the study

The nonexistence of a proper treatment for WMLs has led to the initiation of the current clinical trial which is the first and largest human study to propose a treatment for such condition. The objectives of the study are:

1.4.1. Primary Objective

To assess the neuroprotective properties of tocotrienols supplementation as determined by white matter lesion load on serial MRI.

1.4.2. Secondary Objectives

a) To evaluate the anti-atherogenic effects of tocotrienols supplementation with serial carotid artery MRA.

b) To determine the effects of tocotrienols on blood parameters including total lipid profile (LDL and HDL subfractions), Apo-B, C-reactive protein, antioxidant profile, Lp(a) and lipid peroxidation.

c) To determine the prevalence of WML in a local hypercholesterolemic population in northwest peninsular Malaysia.

CHAPTER 2: MAGNETIC RESONANCE ANGIOGRAPHY OF THE CAROTID ARTERY STENOSIS AMONG MILDLY HYPERCHOLESTEROLEMIC VOLUNTEERS

2.1. Introduction

Carotid arteries are two large blood vessels in the neck that supplies brain with blood. In the presence of carotid artery disease, the arteries become narrow, usually because of atherosclerosis. Atherosclerosis (or arteriosclerotic vascular disease) is a condition where the arteries become narrowed and hardened due to an excessive build up of plaque around the artery wall. The disease disrupts the flow of blood around the body, posing serious cardiovascular and cerebrovascular complications such as myocardial infarction and stroke.

The current gold standard of imaging carotid artery stenosis is digital subtraction angiography (DSA). However, DSA has a risk of morbidity and mortality which includes transient ischaemic attack, minor stroke or a small risk of death (<1%) (Davies & Humphrey, 1993; Hankey, Warlow, & Molyneux, 1990).The other option of conducting the carotid artery imaging is by magnetic resonance angiography (MRA) or duplex ultrasound (DUS). Both methods are non invasive. In a preoperative diagnostic study by Nederkoorn et al. (2002) and a subsequent metanalysis by Nederkoorn et al.(2003), MRA was shown to have a better accuracy than DUS in diagnosing carotid artery stenosis. Thus, MRA of the carotid artery was preferred to assess the presence of stenosis in our subject population. The aim of the study was to evaluate the anti-atherogenic effects of mixed tocotrienols supplementation with serial carotid artery

MRA. A pilot study carried out by Kooyenga et al. (1997b) investigated the protective effects of a tocotrienol and tocopherol enriched palm oil in patients with carotid stenosis. Apart from measuring the various blood atherogenic indicators, they also monitored the changes in the degree of carotid artery stenosis using duplex carotid ultrasonography. After 2 years follow up, they found carotid atherosclerotic regression in 8 and progression in 2 of the 25 patients receiving the palm vitamin E preparation, while none of the control group exhibited regression and 10 of the 25 patients showed progression ($p<0.01$). Moreover, the serum thiobarbituric acid reactive substance (TBARS) decreased for patients in the treatment group ($p<0.05$) while there was no change for patients in the placebo group, indicating that the anti-atherogenic effect of palm vitamin E might be attributed to their antioxidant properties and protection against lipid peroxidation. In view of this encouraging outcome, the MRA of the carotid artery was conducted in this study to assess the anti atherogenic effect of mixed tocotrienols in a mildly hypercholesterolemic volunteers using MRA as a preferred method of imaging.

2.2. Materials and methods

2.2.1. Study Population

Data sets were obtained from the initial 50 consecutively selected volunteers (mean age 48 ± 6.4 years, 27 males and 23 females) scheduled for the brain MRI WML study, were also subjected to undergo the MRA of the carotid artery. Informed consent was obtained from all volunteers, and the study was approved by the Research Ethics Committee for Human Studies of Universiti Sains Malaysia (http://www.crp.kk.usm.my/pages/jepem.htm). The volunteers were recruited if they had one or more of the following criteria; total cholesterol level between 5.2 – 6.2 mmol/L and low-density cholesterol level between 2.6 – 4.2 mmol/L, body mass index (BMI) of more than 25kg/m^2, hypertension (according to Joint National Committee 7 guidelines, 2003) or diabetes mellitus under medical supervision and treatment and their levels are under control. Apart from the above criteria, the volunteers should display normal liver and renal functions. Volunteers were excluded if they have consumed vitamin E supplementation within the past 3 months at the time of recruitment, known history of hypersensitivity to vitamin E, pregnant females, unable to comply to the study protocol, history of drug dependence or drug abuse, undergoing antihyperlipidemic treatment and contraindicated for the brain MRI and MRA screening such as claustrophobia and presence of metal implants in their body. Baseline assessment of demographic profile (age, race, present disease) and clinical parameters (TC, LDL, HDL, Apo B, LP (A), TG, HsCRP, ALT, ALP, AST, CRT, GGT, BMI, fasting glucose and blood pressure) were documented and are shown in table 2.1.

2.2.2. **MRA Imaging Procedures**

All patients were imaged on a 1.5T MRI scanner (Model Signa HDx, General Electric, Milwaukee, USA) using a HD 8 ch NV array (In vivo corporation, Pewaukee Wisconsin) radiofrequency coil using specially-designed, phased-array surface coils. The examination included sequences of axial 2D spoiled gradient-recalled echo, T1-weighted images of the carotid artery at 5 to 7 locations, centered on the carotid bifurcation. The imaging parameters were as follows: repetition time (TR), 100 ms; echo time (TE), 3.5 ms; flip, 60°; thickness, 3 mm; gap, 1 mm; field-of-view, 1612 cm; and matrix, 256144. Each acquisition was repeated 10 times, with a repetition interval of 15 seconds. Coincident with the second image in the sequence, 0.1 mmol/kg (0.2 mL/kg) gadolinium-based contrast agent (Omniscan, Amersham Health) was injected at a rate of 2 mL/s via a power injector. After acquisition, one location per patient was selected for analysis. The primary criteria for selection were the presence of a large atherosclerotic plaque and clear delineation of the lumen and outer wall boundaries of the artery. If more than one location met these criteria, the one closest to the bifurcation was selected to facilitate matching with histological specimens. Each selected image sequence was then individually post processed with the Kalman Filtering Registration and Smoothing (KFRS) algorithm, which reduced patient motion and noise in the image sequences. Finally, contours were drawn around the carotid lumen and just inside the outer wall boundary using the first image after contrast agent arrival in the carotid lumen. The outer contour was carefully drawn to exclude the adventitia, thereby limiting the analysis region to the plaque itself. Contrast agent kinetics was analyzed for the plaque region between contours.

2.2.3. **Image Analysis**

One reader (consultant radiologist) with 16 years of experience, blinded to clinical information and results of other diagnostic tests reviewed all contrast-enhanced MR angiographic studies. Patient identifiers were masked. Image analysis was performed and the subjects were randomized and evaluated. The contrast enhanced MR angiograms were the evaluated using Osirix (Rosset, Spadola, & Ratib, 2004) and each left and right internal carotid artery (ICA) was evaluated as an independent unit.

For each study, the reader first chose the projection that demonstrated any stenosis. It is done initially by subjective visual impression. Four measurements were then made: (1) luminal diameter of the normal distal ICA beyond the bulb where the artery wall becomes parallel; (2) luminal diameter at the site of maximal narrowing; (3) luminal diameter of the estimated original width of the artery at the site of maximal narrowing; and, finally, (4) luminal diameter of the proximal disease-free common carotid artery (CCA) where the artery wall becomes parallel. The calculation of percentage stenosis was performed using North American Symptomatic Trial Collaborators (NASCET) method as illustrated in figure 2.1. Stenosis was classified as mild (0% to49%), moderate (50% to 69%), severe (70% to 99%), or complete occlusion (U-King-Im et al., 2004).

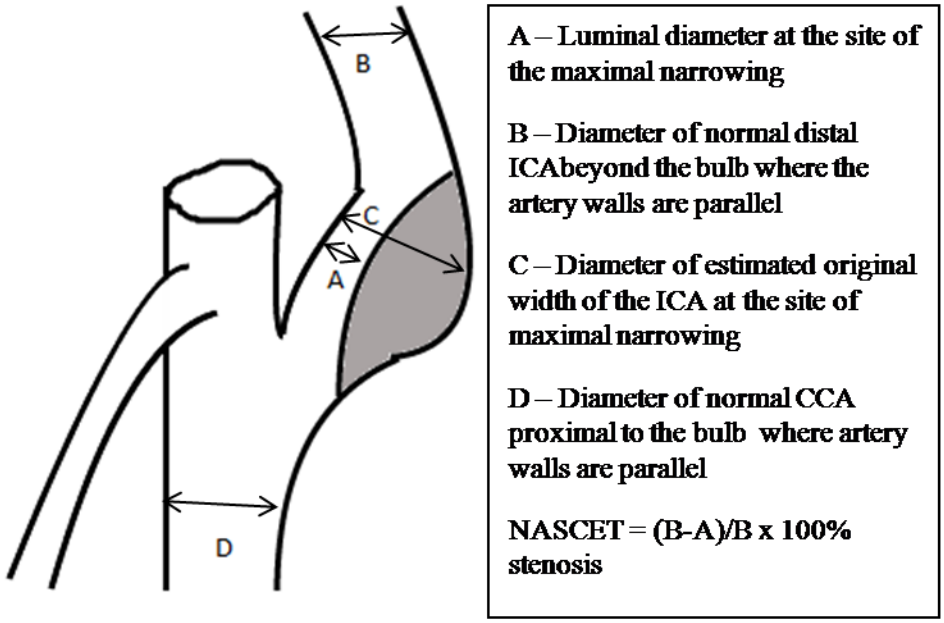


Figure 2.1: Diagram of an ICA stenosis illustrating measurement method (Adapted from North American Symptomatic Carotid Endartectomy Trial (NASCET) Steering Committee 1991)

2.2.4. **Statistical Analysis**

All the statistical analysis were conducted using Statistical Package for Social Sciences (IBM SPSS) software version 19.The homogeneity of the baseline characteristics (table 2.1) between the two groups was assessed using an independent Student's t–test. A chi square test was used to assess the association of disease profile between male and female volunteers (table 2.2). A statistically significant difference was considered at $p < 0.05$.