EFFECT OF COMMERCIALLY AVAILABLE VITAMIN E PREPARATIONS ON ARTERIAL COMPLIANCE AND SELECTED CARDIOVASCULAR PARAMETERS

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by

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ABSTRAK

KESAN PENYEDIAAN VITAMIN E KOMERSIAL KE ATAS KETEGANGAN SALURDARAH DAN PARAMETER KARDIOVASKULAR TERPILIH

Vitamin E adalah antioksidan larut lemak yang dilaporkan mempunyai sifat antioksidan yang kuat. Ia adalah antioksidan pemecah rantai utama pada tisu manusia, membran dan plasma. Vitamin E boleh dibahagikan kepada dua famili iaitu tokoferol dan tokotrienol, setiap famili pula boleh dibahagikan kepada empat isomer yang berbeza iaitu alfa, gama, delta dan beta tokotrienol dan tokoferol. Matlamat tesis PhD ini adalah untuk menyiasat dengan lebih lanjut beberapa isu yang masih tidak diketahui tentang penggunaan vitamin E bagi kesihatan vaskular.

Kajian pertama tesis ini bermatlamat untuk menilai kesan alfa tokoferol pada komplians / ketegangan salurdarah, iaitu satu indeks kesihatan salurdarah, dalam sekumpulan subjek yang mempunyai risiko kardiovaskular yang tinggi tetapi belum mempunyai penyakit salurdarah. Kajian ini adalah kajian klinikal secara rawak, rabun dua belah, bersilang serta berkawalan plasebo melibatkan 20 wanita putus haid [menopaus] yang tidak mempunyai masalah perubatan. Sukarelawan diberi secara rawak sama ada plasebo atau 400 IU tokoferol setiap hari selama sepuluh minggu, sebelum rawatan disilangkan untuk sepuluh minggu lagi. Setiap lima minggu, wanita tersebut akan menghadiri sesi kajian pada waktu petang di mana pengukuran komplians salurdarah, tekanan darah dan kepekatan vitamin E plasma diambil. Purata umur wanita bagi kajian ini adalah 54.59±1.22 tahun. Selepas 10 minggu rawatan, kepekatan vitamin E plasma adalah 24.22±2.1 μg/ml dan 11.89±0.68 μg/ml masing-masing apabila diberi vitamin E dan placebo ($p<0.001$). Tidak ada perbezaan bermakna antara nilai kelajuan gelombang denyutan [PWV] diperolehi selepas sepuluh minggu rawatan dengan tokoferol berbanding dengan placebo, nilai PWV ialah 9.04±0.29 m/s melawan

9.14±0.29 m/s. Begitu juga, tiada perbezaan dilihat bagi tekanan darah sistolik dan diastolik di antara plasebo dan vitamin E pada akhir minggu ke 10.

Dua kajian klinikal melibatkan tokotrienol telah dijalankan, bertujuan untuk mengkaji kesan dua persedian vitamin E kaya tokotrienol [TRE] yang berbeza terhadap komplians salurdarah manusia. Matlamat utama kajian tocotrienol yang pertama adalah untuk mengkaji kesan tiga dos persediaan biasa TRE terhadap komplians salurdarah yang dinilai menggunakan parameter PWV. Kajian ini adalah kajian klinikal rawak, rabun ukuran akhir [*blinded end point*] dengan kawalan plasebo berbentuk selari melibatkan 36 sukarelawan lelaki yang sihat. Pengukuran lain yang diambil adalah nilai '*augmentation index*' [AI], jumlah status antioksidan plasma [TAS], kepekatan vitamin E plasma, jumlah kolesterol [TC] dan lipoprotin kolesterol kepadatan rendah (*low density lipoprotein cholesterol)* [LDL-C] darah. Sukarelawan dibahagikan kepada empat kumpulan dan diberikan sama ada plasebo atau TRE pada dos 80 mg, 160 mg atau 320 mg setiap hari selama dua bulan. Persediaan tokotrienol yang digunakan mengandungi 34.56%, 24.63%, 15.00% dan 26.17% alfa, gama, delta tokotrienol dan alfa tokoferol masing-masing. Purata umur sukarelawan adalah 23.3±0.25 tahun. Tiada perbezaan bermakna dilihat di antara kumpulan dalam perubahan PWV dan AI [ANOVA, p=0.467 dan p=0.092] akibat rawatan. Tiada juga perbezaan di antara kumpulan bagi parameter sampingan lain yang diukur. Walau bagaimanapun, kumpulan 160 mg menunjukkan pembaikan yang kecil tapi bermakna dalam parameter AI selepas rawatan berbanding dengan sebelum rawatan. Kapsul TRE yang digunakan dalam kajian ini boleh diterima dengan baik oleh sukarelawan.

Kajian klinikal tokotrienol yang kedua bermatlamat mengkaji kesan tiga dos persediaan khas vitamin E kaya tokotrienol [SF-TRE], [didakwa boleh meningkatkan penyerapan tokotrienol] ke atas komplians salurdarah. Kajian ini adalah kajian rawak,

rabun ukuran akhir dengan kawalan placebo, berbentuk selari melibatkan 36 sukarelawan lelaki yang sihat. Pengukuran lain yang diambil ialah AI, TAS plasma, kepekatan vitamin E plasma, paras TC dan LDL-C darah. Sukarelawan dibahagikan kepada empat kumpulan, setiap kumpulan diberi samada plasebo atau SF-TRE secara oral pada dos 50 mg, 100 mg atau 200 mg setiap hari selama dua bulan. Persediaan SF-TRE yang digunakan mengandungi 23.54%, 43.16%, 9.83%, 23.5% alfa, gama, delta tokotrienol dan alfa tokoferol masing-masing. Purata umur sukarelawan ialah 23.9±0.39 tahun. Tiada perbezaan bermakna antara kumpulan dalam perubahan PWV dengan rawatan, perubahan setiap kumpulan masing-masing adalah -0.06±0.29, -0.44±0.20, -0.77±0.19 dan -0.65±0.14 m/s untuk kumpulan plasebo, 50 mg, 100 mg dan 200 mg masing-masing [ANOVA, p=0.117]. Walau bagaimanapun, bagi kumpulan 100 mg dan 200 mg, terdapat pembaikan bermakna dalam PWV selepas rawatan dibandingkan sebelum rawatan [p=0.007 dan p=0.002]. Analisis varians [ANOVA] untuk perubahan AI selepas rawatan menunjukkan perbezaan bermakna atas sempadan [*borderline*] dengan nilai p=0.048. Perubahan nilai AI dengan rawatan adalah 2.22±1.54, -6.59±2.84, -8.72±3.77 dan -6.27±2.67 masing-masing bagi kumpulan plasebo, 50 mg, 100 mg dan 200 mg. Analisis 'post hoc' menunjukkan nilai p atas sempadan pada 0.076 antara kumpulan placebo dan 100 mg. Semua kumpulan yang dirawat dengan SF-TRE menunjukkan pembaikan bermakna bagi parameter AI selepas rawatan dibandingkan dengan sebelum rawatan. Tiada perubahan bermakna antara kumpulan bagi parameter lain yang diukur. Persedian SF-TRE yang digunakan dapat diterima dengan baik oleh sukarelawan.

Kesimpulannya, pengambilan 400 IU Vitamin E selama sepuluh minggu meningkatkan kepekatan alfa tokoferol plasma tetapi tidak memberi kesan pada komplians salurdarah bagi wanita putus haid. Bagi vitamin E kaya tocotrienol, semua

kumpulan yang dirawat dengan persediaan TRE dan SF-TRE menghasilkan peningkatan bermakna bagi kepekatan alfa, gama dan delta tocotrienol dalam plasma. Persediaan TRE biasa tidak memberi kesan terhadap komplians salurdarah dalam sukarelawan lelaki sihat. Secara keseluruhannya, SF-TRE juga tidak menunjukkan kesan bermakna ke atas komplians salurdarah. Walau bagaimanapun, terdapat arah kecenderungan [*trend*] terhadap pembaikan komplians salurdarah seperti yang dicadangkan oleh nilai bermakna atas sempadan [*borderline significance*] yang dilihat untuk pengukuran AI dengan SF-TRE. Pembaikan bermakna selepas rawatan, berbanding dengan sebelum rawatan [pembaikan dalam kumpulan] juga dilihat bagi semua kumpulan yang dirawat bagi parameter AI. Kajian lanjut kesan TRE terhadap ketegangan salurdarah pada pesakit yang mempunyai masalah kardiovaskular yang nyata adalah dicadangkan.

ABSTRACT

EFFECT OF COMMERCIALLY AVAILABLE VITAMIN E PREPARATIONS ON ARTERIAL COMPLIANCE AND SELECTED CARDIOVASCULAR PARAMETERS

Vitamin E is a potent lipid soluble antioxidant. It is the principal chain breaking antioxidant in human tissues, membrane and plasma. Vitamin E comprised of two families, the tocopherols and tocotrienols, each family is further divided into the alpha, gamma, delta and beta isomers. This PhD thesis aimed to address some of the unresolved issues on vitamin E use in vascular health.

For tocopherol, a randomised, crossed over, double blind, placebo controlled clinical trial involving 20 healthy post menopausal women was conducted to assess the effect of alpha tocopherol on arterial compliance, an index of vascular health in a group of high cardiovascular risk subjects who has no overt vascular disease. Subjects were randomised to either placebo or tocopherol 400 IU daily for ten weeks, before being crossed over for treatment for another ten weeks. At intervals of 5 weeks, subjects attended afternoon sessions where measurements of arterial compliance, blood pressure and plasma vitamin E level were taken. Mean age of these women were 54.59±1.22 years. After 10 weeks treatment, plasma vitamin E level was 24.22±2.1 μg/ml and 11.89±0.68 μg/ml respectively with vitamin E and placebo ($p < 0.001$). There was no significant difference in pulse wave velocity [PWV] after ten weeks treatment with tocopherol compared to placebo, PWV values being 9.04±0.29 m/s versus 9.14±0.29 m/s respectively. Similarly, no difference in systolic and diastolic blood pressures was seen between placebo and vitamin E at the end of ten weeks.

For tocotrienols, two clinical trials were conducted. The first study was a randomised, placebo controlled, blinded end point clinical trial with a parallel design involving 36 healthy male subjects. This study aimed to determine the effects of a

normal preparation of tocotrienol rich vitamin E [TRE] on the primary parameter, arterial compliance as assessed by aortic femoral PWV. Other measurements taken were augmentation index [AI], plasma total antioxidant status [TAS], plasma vitamin E levels, serum total cholesterol [TC] and low density lipoprotein [LDL-C]. Subjects were randomised to four treatment, either placebo or TRE at doses of 80 mg, 160 mg, or 320 mg mixed tocotrienol daily for two months. The TRE contained 34.56%, 24.63%, 15.00% and 26.17% respectively of alpha-tocotrienol, gamma-tocotrienol, delta tocotrienol and alpha-tocopherol. Mean age of subjects were 23.28±0.25 years. There were no significant differences between the groups in their change in PWV and AI with treatment [ANOVA, p=0.467 and p=0.092 respectively]. There were also no significant differences between groups in other measurements taken. Group 160 mg however, showed a small but significant improvement in AI after treatment compared to baseline. The TRE capsules used in this study were well tolerated by subjects.

The second clinical trial on TRE aims to determine the effect of three doses of a special formulation of tocotrienols (SF-TRE) [claimed to enhance tocotrienol absorption] on arterial compliance. This study was a randomised, placebo controlled, blinded end point clinical trial with a parallel design involving 36 healthy male subjects. Other measurements taken were AI, plasma TAS, plasma vitamin E levels, serum TC and LDL-C. Subjects were grouped into four groups, each group were prescribed either placebo, or SF-TRE at doses of either 50 mg, 100 mg, or 200 mg tocotrienols daily for two months. The SF-TRE contained 23.54%, 43.16%, 9.83%, 23.50% respectively of alpha, gamma, delta tocotrienol and alpha tocopherol. Mean age of subjects were 23.86±0.39 years. There were no significant differences between groups in their change in PWV with treatment; change for each group being -0.06±0.29, -0.44±0.20, -0.77±0.19 and -0.65±0.14 m/s respectively for groups placebo, 50 mg, 100 mg and

200 mg [p=0.117]. However, groups 100 mg and 200 mg showed significant improvement in PWV after treatment compared to baseline [p=0.007 and p=0.002]. Analysis of variance [ANOVA] for change in AI treatment was of borderline significance at p=0.048, change for groups placebo, 50 mg, 100 mg and 200 mg being 2.22±1.54, -6.59±2.84, -8.72±3.77 and -6.27±2.67 respectively. However, post-hoc analysis showed a borderline p value of 0.076 between groups placebo and 100 mg. All treated groups showed significant improvement in AI after treatment compared to baseline. There were no significant differences between groups in the other parameters measured. The SF-TRE used was well tolerated by subjects.

Conclusion: Supplementary vitamin E for ten weeks at 400 IU daily increased plasma level of alpha tocopherol but has no effect on arterial compliance in healthy post menopausal women. For tocotrienol rich vitamin E, treatment with all groups treated with TRE and SF-TRE produced significant elevations of alpha, gamma and delta tocotrienols. The conventional preparation of TRE did not have an effect on arterial compliance in healthy male subjects. Overall, the SF-TRE also did not show significant effect on arterial compliance. However, there was a trend towards improvement in arterial compliance as suggested by the borderline significance value observed for the measurement of AI with SF-TRE. Significant within group improvement were also observed for all treated groups compared to baseline. Future studies to investigate the effect of TRE on arterial stiffness in patients with clinically manifest cardiovascular disease is suggested.

CHAPTER 1

INTRODUCTION

1.1. INTRODUCTION

Cardiovascular diseases, in particular coronary artery disease [CAD] are the most important cause of morbidity and mortality in developed countries [Tunstall-Pedoe *et al.,* 1994]. In Malaysia, a developing country, cardiovascular diseases is the commonest cause of mortality in government hospitals, accounting for 24.5% of all deaths for the year 1998. Coronary artery disease is the major cause of these deaths [National Heart Association of Malaysia, 2001]. Stroke is another devastating cardiovascular illness and is the most common cause of severe disability in Malaysian adults [Academy of Medicine of Malaysia and Ministry of Health Malaysia, 2000]. The average incidence of stroke is about 2:1,000 population, this risk increases with age such that after the fifth decade, the incidence doubles with every decade of life. Every year about 2,500 deaths and 12,000 stroke discharges are recorded in government hospitals

Atherosclerotic plaque formation has been reported to be the underlying basis for CAD, besides contributing to the occurrence of stroke. The plaque consists of a collection of variable amounts of cholesterol, smooth muscle cells, fibrous tissue and inflammatory cells in the intima, separated by fibrin cap from the flowing blood in the arterial lumen [Yusoff, 2002]. The plaque often grows with time, gradually narrowing the coronary lumen. This progressively decrease and reduce the blood supply to myocardial tissues supplied by the coronary artery. When a critical narrowing of the

coronary lumen eventually occurs, [with approximately 70% diameter reduction], myocardial ischemia occurs, manifested clinically as angina pectoris. Initially, angina occurs on exertion, because it is on exertion that the blood supply, restricted in a fixed manner, is not able to provide the increased demand for blood supply to nourish tissues requiring more energy during exertion.

Later, acute myocardial infarction and unstable angina may occur, as a result of plaque rupture and abrupt coronary occlusion due to acute thrombus formation which is made worse by a coronary stenosis. Inflammation has recently been reported to further contribute towards plaque formation and rupture [Greaves and Channon, 2002; Shah, 2000].

Free radicals are widely reported to contribute to the development of atherosclerotic plaque and vascular diseases [Parthasarathy *et al.,* 1999; Gackowski *et al.,* 2001; Juliano, 2001]. Free radicals are molecules with one or more unpaired electrons, which are usually highly reactive towards bio molecules. Free radicals are involved in the formation of oxidised low density lipoprotein [LDL-C], which contributes to the formation of atherosclerotic plaques. Oxidised LDL-C, not un-oxidised LDL-C, is engulfed by intimal macrophages, transforming them into foam cells which develop into fatty streaks, the precursors of the atherosclerotic plaque. Foam cell formation also leads to endothelial injury and dysfunction. This further facilitates passage of LDL-C from the flowing blood into the intima accelerating the process of vascular damage [Steinberg *et al.,* 1989]. In atherosclerosis, oxidised LDL-C accumulates in the vascular wall [Yla-Herttuala *et al.,* 1989], where it is cytotoxic [Morel *et al.,* 1983] and chemotactic for monocytes [Quinn *et al.,* 1987] leading to the

accumulation of vascular inflammatory cells and perhaps, the production of free radical that can inactivate endothelium derived nitric oxide [Stouffer *et al.,* 2002].

The body has a number of mechanisms to control the production of radical oxygen species [ROS] and to limit or repair damaged tissues. In healthy individuals, the antioxidant system defends tissues against free radical attack. The body's antioxidants may be endogenously produced or derived from the diet. These antioxidants may be present in cells, including their membranes or in extracellular fluids, which may be hydrophilic such as ascorbic acid, or hydrophobic such as vitamin E.

Antioxidants may act at several different stages in the oxidative sequence, for example it can remove ROS, scavenge initiating free radicals, break the chain reaction of an initiated sequence or scavenge and quench singlet oxygen [Young and Woodside, 2001]. Because of the contribution of free radicals to plaque formation and vascular diseases, and the ability of antioxidants to neutralise these radicals, the relationship between antioxidants and cardiovascular incidence is intriguing to many. This prompted a lot of work in this field, looking at the relationship between plasma antioxidant levels and cardiovascular diseases, and the effect of antioxidant supplementations to cardiovascular diseases.

The most abundant lipid soluble antioxidant in human plasma is vitamin E [Burton *et al.,* 1983] and the oxidation of LDL-C in vitro is limited by vitamin E [Reaven *et al.,* 1993]. Thus vitamin E has received much attention for its role in cardiovascular and vascular pathology. *In vitro* studies had shown that increased resistance to oxidation of LDL-C was achieved when they were enriched with an

antioxidant, such as vitamin E [Esterbauer *et al.*, 1991; Jialal and Grundy, 1992; Abbey *et al.*, 1993; Reaven *et al.*, 1993; O'Byrne *et al.*, 2000]. This was supported by animal studies, where it had been shown that vitamin E reduce plaque formation and lead to plaque stabilisation [Black *et al.*, 2000, Qureshi *et al.*, 2001a].

Supplementary vitamin E is effective in reducing the progression of atherosclerosis in subjects with previous coronary artery bypass graft surgery not treated with lipid-lowering drugs [Kritchevsky *et al.*, 1995]. Large prospective cohort studies have demonstrated that intake of vitamin E from certain foods and supplements were associated with a reduced risk of major coronary events in postmenopausal women and subjects above the age of forty [Rimm *et al.*, 1993; Stampfer *et al.*, 1993; Kushi *et al.*, 1996]. A secondary prevention study in patients with CAD showed a 77% reduction in nonfatal myocardial infarction can be achieved with 400-800 IU alpha tocopherol daily over a median follow up period of 510 days [Stephens *et al.*, 1996]. However, results from more recent large interventional clinical trials [Rapola *et al.*, 1997; GISSI-Prevenzione Investigators, 1999; The Heart Outcomes Prevention Evaluation Study Investigators, 2000; Collins *et al.*, 2002] had not shown benefits conferred by vitamin E supplementation, suggesting that vitamin E has no role in protecting against cardiovascular events.

Despite numerous work conducted on vitamin E, there are, however, some unanswered questions on the role of vitamin E in cardiovascular diseases. This thesis consists of three randomised clinical trials, designed to address some of the unresolved issues of vitamin E and its vascular effect. Why is there a need to re-look at vitamin E and vascular protection?

Firstly, the above mentioned large intervention randomised studies were conducted on high risk populations; in populations with known pre-existing vascular diseases or those with history of diabetes or hypertension [Rapola *et al.,* 1997; GISSI Prevenzione Investigators, 1999; The Heart Outcomes Prevention Evaluation Study Evaluation Investigators, 2000; Collins *et al.*, 2002]. The observation that vitamin E did not confer cardiovascular protection in those studies may be because the trials were conducted relatively late in the disease process. The protective effects of dietary vitamin E may be more evident earlier in the disease process; at a time when pre-atherosclerotic vascular changes first become manifest and are more likely to be susceptible to modifications. The effects of vitamin E on cardiovascular events, in lower risk populations have not been well studied. One major problem of conducting a cardiovascular outcome study in a low risk group is the very large sample size needed for such a study. This is because of the low expected incidence of cardiovascular events in this group. Measuring effect of vitamin E on arterial compliance is one way to assess its effect on arterial function before vascular disease is established. This is because arterial compliance had been shown to be dependent on endothelial function [Ramsey et al., 1995]. The endothelium produces endothelium derived relaxing factor [EDRF] that helps maintain vascular relaxation which maintains vascular compliance and decreases arterial stiffness. The endothelium may also cause changes in smooth muscle tone that affects arterial compliance. Endothelial dysfunction is an early precursor for vascular damage. Changes in endothelial function had been shown to predate the development of pathological vascular states [Anderson, 1999]. Reduced arterial compliance is also associated with coronary artery disease [Dart *et al.,* 1991] as well as with various cardiovascular risk factors including age, arterial blood pressure [Dart and Qi, 1995], diabetes [McVeigh *et al.,* 1994], and LDL cholesterol levels [Tanaka *et al.,* 1998].

Arterial stiffness is a strong predictor of cardiovascular events. In the elderly patients, it is a strong predictor of cardiovascular mortality [Meaume *et al.*, 2001]. In hypertension, arterial compliance was shown to be an independent predictor of both cardiovascular and all cause mortality [Laurent *et al.*, 2001]. The same is true for type 2 diabetes and individuals with impaired glucose tolerance [Cruickshank *et al.*, 2002].

Thus the effect of tocopherol on arterial compliance in healthy post menopausal women formed the first randomised clinical trial of this thesis. Menopausal women were chosen as they are known to have higher risk of cardiovascular events compared to pre-menopausal women. Healthy menopausal women were chosen as these women have not had established vascular pathology, thus enabling us to study the effect of tocopherol on vascular function in a high risk cardiovascular group before vascular disease manifestation.

Secondly, there are two classes of commercially available vitamin E in the market, tocopherol and tocotrienol. All the above observational and large randomised clinical trials used tocopherol as the vitamin E. This may be due to tocopherol being the vitamin E that was first discovered in 1922 [Evans and Bishop, 1922]. Furthermore, tocopherol vitamin E is much more easily available in the Western parts of the world where most of these studies were done. They can be obtained from vegetable and seed oil sources such as soybean, safflower and corn, sunflower seeds, nuts, whole grains and wheat germ. These sources of vitamin E predominantly contain alpha tocopherol with insignificant amounts of tocotrienol. It was only recently that tocotrienol was discovered. Tocotrienols are the major vitamin E in palm oil and rice bran; these two

sources contain tocotrienol as the predominant vitamin E. The palm oil is the highest source of natural tocotrienol [Watkins *et al.*, 1999; Packer *et al.*, 2001].

Tocotrienols had been reported *in vitro*, to have higher antioxidant properties compared to alpha-tocopherol [Serbinova *et al.*, 1991; Suzuki *et al.*, 1993]. Tocotrienol was also reported to have another potentially beneficial property in reducing cardiovascular diseases, the potential to lower serum lipid levels. *In vitro* [Pearce *et al.*, 1992, Parker *et al.*, 1993] and experimental studies [Qureshi *et al.*, 1991a; Qureshi *et al.*, 1996; Qureshi *et al.*, 2001a] had shown that tocotrienols inhibit 3 hydroxy-3-methylglutyryl-coenzyme A reductase [HMG-CoA reductase], the enzyme responsible for *in vivo* cholesterol synthesis. These two properties of tocotrienol, potent antioxidant and lipid lowering potential, may give a more favourable effect on arterial health and cardiovascular events compared to tocopherol.

When literature search was first commenced for this thesis [approximately four years ago], a lot was not known on tocotrienols. Most studies conducted on tocotrienols were *in vitro* or animal work. There were minimal human studies on tocotrienols, which will be reviewed in Chapter 2. Certainly there are no large randomised trials on tocotrienols. There are no studies on the effect of tocotrienol rich vitamin E on cardiovascular events. Most human clinical studies with tocotrienols looked at the lipid lowering effect of tocotrienols. Even so, the doses that had been used in these clinical studies vary widely, ranging between 40 mg/day to 240 mg/day with no clear guidelines on the doses of tocotrienols to be used. Although the effect of tocotrienols on lipid peroxidation in humans had been reported [Tomeo *et al.*, 1995; Ismail et *al.*, 2002],

there were no human studies that assessed the effect of tocotrienols on the plasma total antioxidant status, i.e the ability of plasma to quench generation of free radicals.

Thus the second clinical trial of this thesis investigated a few unanswered issues on tocotrienol by looking at the effects of different doses [low, medium and high] of palm tocotrienol rich vitamin E [TRE] on selected cardiovascular parameters with the primary end point being arterial compliance. Free radicals had been shown to inactivate endothelium derived relaxing factors [EDRF], the substance that causes vasorelaxation thus helping to maintain vascular compliance. Antioxidants may help in quenching these free radicals, preserving the effect of EDRF on the vessel compliance. Other parameters such as the effects of graduated doses of TRE on steady state blood levels of vitamin E, plasma antioxidant and lipid profile were also measured. Adverse effects and tolerability of subjects to the doses used were also studied, as 320 mg daily of TRE was the highest dose reported so far in the literature at that time.

The absorption of tocotrienols, being fat soluble vitamins, were known to be highly variable and dependent on the physiological processes in the stomach and small intestines. The absorption of fat soluble vitamins are induced by food, especially fat intake [Yap *et al.*, 2001]. Only with sufficient food and fat intake will there be sufficient pancreatic juice and bile secreted to emulsify the tocotrienols for satisfactory absorption. Since factors like food and fat intake tend to be variable, the absorption of tocotrienols will also be variable. A special formulation of tocotrienol rich vitamin E [SF-TRE] which claimed to be able to provide 200-300% higher absorption than the normal preparation was launched fairly recently. These capsules were reported to be able to self emulsify to form a readily absorbable form of tocotrienol in order to increase

tocotrienol bioavailability and thus their blood levels. Theoretically, tocotrienol absorption from this formulation will not be dependent on the physiological mechanisms of the stomach like pancreatic juice and bile secretion. Thus its absorption should be less affected by food intake. Therefore the third clinical trial of this thesis was conducted to assess if the special formulation tocotrienols [SF-TRE] has enhanced pharmacodynamic effect consequent to increased blood levels. The pharmacodynamic effects of interest are those on cardiovascular parameters similar to the second clinical trial.

In summary, this thesis is composed of 3 randomised, placebo controlled clinical trials to address some of the unknown issues on the role of vitamin E in vascular health. The first study investigates the effect of a commercially available alpha tocopherol daily on arterial compliance in healthy postmenopausal women, a group known to have less compliant [stiffer] arteries compared to pre-menopausal women. The second clinical trial investigates the effect of varying doses of a marketed normal preparation of TRE on arterial compliance in healthy volunteers. The third clinical trial also investigates the effect of varying doses of a self emulsifying preparation of TRE on arterial compliance in healthy volunteers. Secondary parameters such as plasma total antioxidant status, serum total cholesterol and low density lipoprotein levels were also performed in the second and third clinical trials as secondary end points. Tolerability and adverse effects of these formulations were also investigated. Plasma vitamin E levels were performed to ensure compliance and absorption of the vitamin E by study subjects. This is to confirm that any effects seen during treatment were due to the vitamin E consumption.

1.2. AIMS OF THIS THESIS

1. To assess the effect of natural alpha tocopherol on arterial compliance in post menopausal women without overt vascular pathology.

2. To assess the effect of three doses of normal preparation of tocotrienol rich vitamin E [low, medium and high] on arterial compliance in healthy volunteers. Secondary outcomes are plasma total antioxidant status, serum total cholesterol and low density lipoprotein levels.

3. To assess the effect of three doses [low, medium and high] of a self emulsifying formulation of tocotrienol rich vitamin E on arterial compliance in healthy volunteers. Secondary outcomes are plasma antioxidant status, serum total cholesterol and low density lipoprotein levels.

CHAPTER 2

LITERATURE REVIEW

2.1 INTRODUCTION TO FREE RADICALS

Free radicals are molecules with one or more unpaired electrons, which are usually highly reactive towards bio molecules [Abuja and Albertini 2001]. Free radicals can react with cell components causing damage. Free radicals formed in the body are the result of metabolic reactions (such as mitochondrial electron transport chain and oxidative enzyme reactions) as well as exposure to environment toxins such as ultraviolet light, cigarette smoke, ionising radiation and other pollutants. Among the main free radical species which occur in the human body include superoxide radicals, hydroxyl radicals, nitric oxide radical and peroxyl radicals. Free radicals modify endogenous molecules (such as proteins, lipids and deoxyribonucleic acid) when the unpaired electron of the free radical removes an electron from the target molecule. In some cases, the product of this initial oxidation reaction will cause subsequent oxidation of other molecules, thus propagating an oxidation chain reaction [Young and Woodside 2001]. The relative effect of a free radical depends on the type that is generated and the biological tissue in which the reaction takes place [Stouffer *et al*., 2002]. Free radicals have been suggested to be either directly or indirectly involved in a wide variety of clinical disorders such as atherosclerosis [Yla-Herttuala *et al*., 1989], cancer, reperfusion injury, diabetes and Parkinson's disease among others [Knight, 1995; Abuja and Albertini, 2001].

2.1.1 Free radicals and cardiovascular diseases

There are three mechanisms proposed by which oxidative damage from free radicals could contribute to cardiovascular diseases especially coronary heart disease. The first is through direct damage that free radicals may cause to cells and molecules. The second is through its role on endothelium dependent vasorelaxation. The third recently proposed mechanism is via participation of free radicals in specific cell signalling pathways.

With regard to the molecular and cell damage caused by free radicals, a major area of interest is the oxidative modification of LDL-C. This has been proposed as a possible explanation for the relationship between LDL-C and atherosclerosis [Steinberg *et al.*, 1989]. Low density lipoprotein cholesterol is a collection of cholesterol, cholesterol esters and triglycerides surrounded by a matrix of phospholipids and apolipoproteins. Free radicals have the capability to oxidise LDL-C, resulting in numerous changes that could potentially contribute to atherogenesis [Parthasarathy *et al.*, 1999]. Support for involvement of oxidised LDL-C in atherogenesis came from the demonstration that LDL-C extracted from atherosclerotic plaques formed *in vivo* is similar to LDL-C that was oxidatively modified *in vitro* [Yla-Herttuala *et al*., 1989].

Oxidised LDL-C had been shown to accelerate several steps in atherosclerosis including endothelial damage, monocytes / macrophage recruitment, rapid uptake of oxidised LDL-C by monocytes / macrophage scavenger receptors, alteration in vascular tone and induction of growth factors. Oxidised LDL-C affects growth, proliferation, and death of cells in the arterial wall [Stouffer *et al.*, 2002]. Oxidised LDL-C is

recognised by scavenger receptors on the macrophages. The macrophages take up the oxidatively modified LDL-C in an unregulated manner causing accumulation of large amounts of lipids from oxidised LDL-C in the macrophages. These lipid rich macrophages are eventually converted into foam cells, which are the hallmark of the atherosclerotic fatty streak. This early fatty streak is the precursor lesion that subsequently leads to the development of the intermediate and the final complicated lesion of atherosclerosis.

In addition to LDL-C, oxidative modification of other molecules such as nitric oxide is also thought to impact on cardiovascular diseases. An endothelium derived relaxing factor [EDRF], identified as nitric oxide [NO] is an important substance involved in the local control of vascular tone and platelet adhesion to the endothelial surface. Atherosclerosis had been shown to be associated with abnormalities in endothelium dependent arterial relaxation that may, in part result from the effects of oxidised LDL-C on endothelium derived NO. Modified LDL-C inhibits receptor mediated endothelium dependent arterial relaxation [Kugiyama *et al*., 1990] and degrades endothelium derived NO directly [Chin *et al.,* 1992]. A variety of free radicals can also interact with and degrade nitric oxide produced by the endothelial wall, thereby impairing endothelium dependent vasorelaxation [Cai and Harrison, 2000]. Loss of coronary vasorelaxation function had been correlated with a worse prognosis for cardiovascular events [Schachinger *et al*., 2000].

Recently, there is growing research interest in investigating the role of free radicals in cellular signalling pathways involved in cardiovascular disease processes [Griendling *et al*., 2000]. This includes the cell capacity to exert control over intra and

extra cellular oxidative stress through the specific production and metabolism of free radicals. Because many of these signal transduction pathways play a role in cardiovascular physiology, this provides another possible link between oxidative stress and cardiovascular disease. For example free radicals may be involved in activating protein kinase C in vascular smooth muscle cells. Protein kinase C activation had been implicated in vascular disease due to diabetes [Tesfamariam *et al*., 1991] and oxidised LDL-C [Ohgushi *et al*., 1993]. Free radicals may also be involved in influencing leucocyte adhesion to endothelial cell, monocyte transmigration, and oxidant mediated toxicity. The redox sensitive pathways control a wide range of cellular functions, including the expression of cell surface markers, cellular growth and apoptosis.

2.1.2 Antioxidants

Antioxidants are defined as substances or compounds that significantly delays, prevents or reduce oxidation of that substrate [Abuja and Albertini, 2001]. There are generally three categories of antioxidants; antioxidant enzymes, chain breaking antioxidants and preventive antioxidants [Maxwell and Lip, 1997; Young and Woodside, 2001].

Antioxidant enzymes catalyse reactions involved in the conversion of free radicals to oxygen and water. They include the catalase, glutathione peroxidase and superoxide dismutase. The chain breaking antioxidants prevent propagation of oxidative chain reactions by terminating free radicals or the reactive products of molecules that have been damaged by free radicals. Lipid soluble chain breaking antioxidants include vitamin E, beta carotene, vitamin A (for which beta carotene is the precursor), the

flavonoids, and ubiquinol-10 (the reduced form of coenzyme Q10). Water soluble chain breaking antioxidants include vitamin C, uric acid, bilirubin bound to albumin, and the thiol groups of plasma proteins such as albumin. The preventive antioxidants are the metal binding proteins that function to sequester free iron or copper to prevent production of the hydroxyl radical from other free radicals. These include ferritin, transferrin, lactoferrin and ceruloplasmin [Stouffer *et al*., 2002].

In the plasma, major antioxidant defences include vitamin C, protein thiols, bilirubin, urate and alpha tocopherol. Plasma also contains the 'preventive' antioxidants, ceruloplasmin and transferrin, the iron scavenging proteins whose contribution to the total antioxidant capacity is to prevent iron availability [Young and Woodside 2001].

Because of the role free radicals are believed to contribute to the pathogenesis of atherosclerosis and cardiovascular diseases, antioxidant represent a promising, but yet unproven means of decreasing atherosclerosis and coronary artery disease. Postulated mechanisms whereby antioxidants could protect against cardiovascular diseases were derived from basic research demonstrating ability of antioxidants to inhibit oxidation of LDL-C [Abbey *et al*., 1993], atherosclerosis development and progression [Black *et al.*, 2000; Qureshi *et al*. 2001b] and preventing endothelial dysfunction [Anderson *et al*., 1994; Stewart-Lee *et al.,* 1994; Keaney *et al*., 1996]. Because antioxidant vitamins are part of the body's nutrient needs, and are considered safe substances, they have generated a lot of interest as supplements that could potentially be beneficial in cardiovascular health.

Vitamin E is an antioxidant vitamin that has received a lot of attention as a possible means of preventing and / or reducing cardiovascular diseases. This partly stems from the fact that it is the principal lipid soluble chain breaking antioxidant in human tissues, membrane and plasma [Machlin, 1991; Traber and Sies, 1996]. It is also a major lipid soluble chain breaking antioxidant that prevents the propagation of free radical reactions in membranes and lipoproteins [Azzi, 2004]. It was also reported to be the predominant antioxidant in the LDL-C particle [Esterbauer *et al*., 1991a].

2.2 INTRODUCTION TO VITAMIN E

Vitamin E, in the form of alpha tocopherol was discovered in 1922 by Evans and Bishop, as a factor essential for reproduction [Evans and Bishop, 1922]. Lack of this factor in rats caused 'infertility' in male rats and increased risk of miscarriage / abortion in female rats. The name tocopherol then came from the Latin word 'tokos' (childbirth) and 'phorein' (to bring forth). The suffix 'ol' was added at the end to indicate the phenolic nature of the substance [Kamal Eldin and Appelqvist, 1996].

Nowadays, the term vitamin E is used as the collective name for the eight naturally occurring molecules [four tocopherols and four tocotrienols] which qualitatively exhibit the biological activity of alpha tocopherol. Vitamin E occurs in nature in at least eight different isoforms: alpha, gamma, delta and beta tocopherols and alpha, delta, gamma and beta tocotrienols [Packer *et al*., 2001]. The eight isomers of vitamin E share some important traits. Firstly, all have a head or chroman ring in technical term. Secondly, all have a 'tail', which is called the phytyl tail for tocopherols.

Thirdly, all have the hydroxyl group, which is the active group on the head of the molecule. Tocotrienols differ from the corresponding tocopherols only in their aliphatic tail. Tocopherols have a phytyl side chain attached to their chromanol nucleus, whereas the tail of tocotrienol is unsaturated and forms an isoprenoid chain. The tocotrienol tail has three double bonds while the tocopherol tail has none. The gamma, delta and beta isoforms of tocotrienols and tocopherol differ by the number [alpha has 3, beta and gamma have 2, while delta has 1] and position of their methyl groups on the chromanol nucleus [Traber *et al*., 1997; Packer *et al*, 2001]. The structures of tocopherol and tocotrienols are illustrated in figure 2.1

2.2.1. Source and requirements of vitamin E

Lipid rich plant products and vegetable oils are the main natural sources of vitamin E. These include nuts, wheat germ, sunflower, safflower, palm oils, rice bran and others. Tocopherols are present in polyunsaturated vegetable oils and in the germ of cereal seeds. Tocotrienols are found in high concentrations and is the predominant vitamin E in palm oil, barley and rice bran [Theriault *et al*., 1999]. Approximately 70% - 75% percent of the vitamin E content in palm oil consists of tocotrienol isomers, while the rest are alpha tocopherol. Palm oil was reported to be the richest source of tocotrienols in nature [Tan, 1989]. Other cooking oils such as corn oil, soy and sunflower oils are good sources of tocopherol but contain no tocotrienols.

Vitamin E is included as one of the body's essential nutrient which must be obtained either from dietary intake or as a supplement. The recommended daily dietary

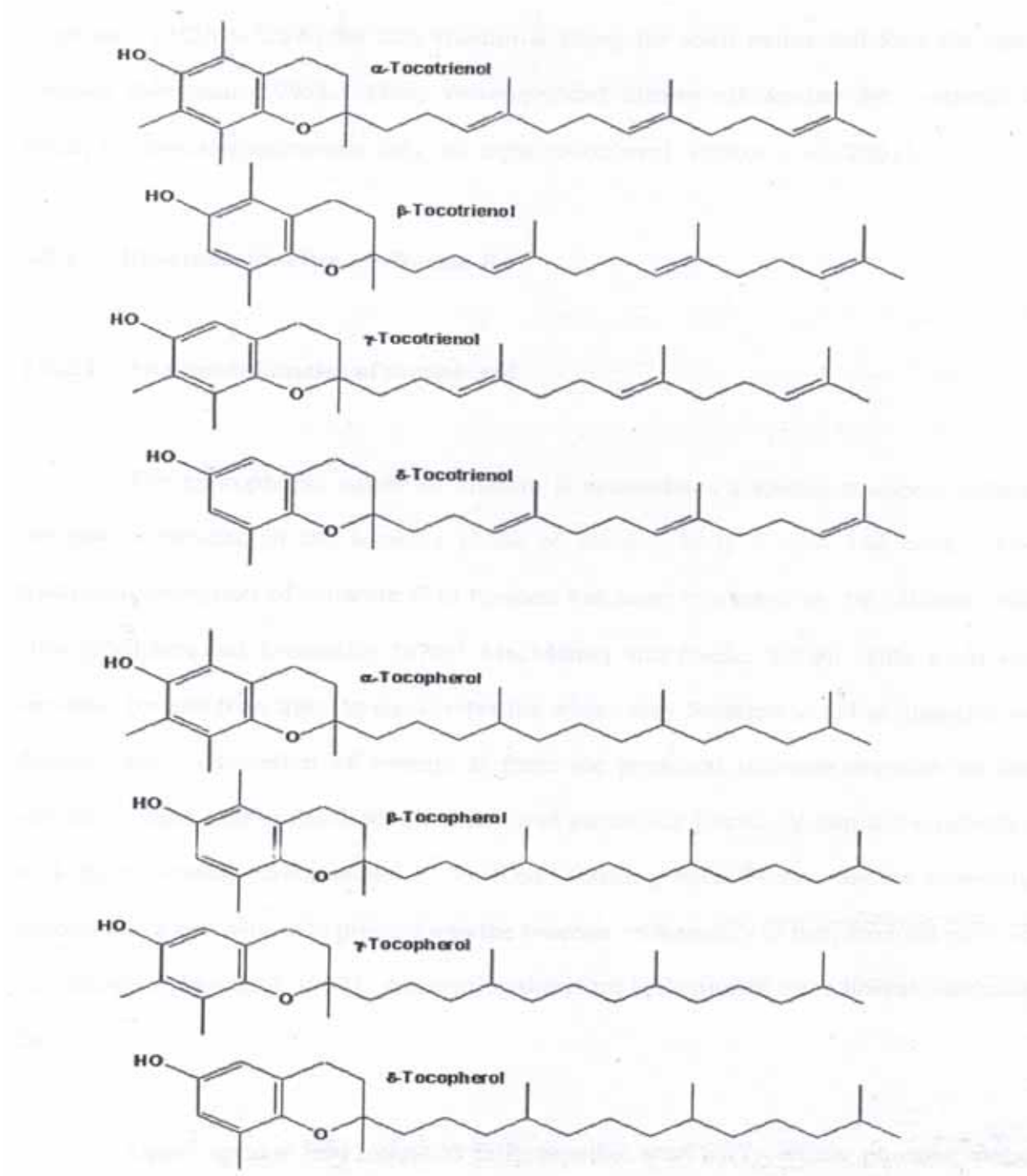

Figure 2.1

The molecular structures of the different isomers for tocopherol and tocotrienol.

allowance [RDA – USA] for this vitamin is 10 mg for adult males and 8 mg for adult females [Meydani, 1995]. These recommended dietary allowances for vitamin E define the human requirement only for alpha tocopherol [Packer *et al*., 2001].

2.2.2 Pharmacokinetics of vitamin E

2.2.2.1 Pharmacokinetics of tocopherol

The hydrophobic nature of vitamin E necessitates a special transport system for this substance in the aqueous phase of plasma, body liquids and cells. The fractional absorption of vitamin E in humans has been estimated to be between 60-70% [Kelleher and Losowsky 1970; MacMahon and Neale, 1970]. Bile acids are secreted by the liver into the small intestine where they function to aid in digestion of dietary fat. Absorption of vitamin E from the proximal intestine depends on the amount of lipid / fat in the food, bile acids and pancreatic juices. Vitamin E emulsifies with lipid soluble components in the food forming micelles that can be passively absorbed in a non saturable process into the mucosa cells mainly at the proximal parts of the intestine [Meydani, 1995]. Absorption therefore is facilitated by a liberal intake of fat.

Upon uptake into intestinal cells, together with triglycerides, phospholipids, cholesterol and apolipoproteins, vitamin E are reassembled into chylomicrons by the Golgi apparatus in the mucosal cells [Traber *et al*., 1986]. Chylomicrons are excreted by exocytosis into the lymphatic system to reach the blood [Kayden and Traber, 1993; Ikeda *et al.,* 1996]. Chylomicron lipolysis, facilitated by the enzyme lipoprotein lipase, allows part of vitamin E to be distributed to tissues. The remaining chylomicron

remnants deliver the remaining alpha tocopherol to the liver. Inside the liver, vitamin E is incorporated into liposomes. Uptake of vitamin E into liposomes is non specific, but there is a specific transport protein to alpha tocopherol, that is the α-tocopherol transfer protein [α –TTP] which is sized at 32 kDA. Alpha–TTP transports alpha tocopherol from liposomes into lipoproteins especially Very Low Density Lipoprotein [VLDL] before the liver cells secrete VLDL into the blood stream for distribution into other organs [Stocker and Azzi, 2000]. VLDL helps transport and delivers alpha tocopherol to peripheral cells [Bjorneboe *et al*., 1990].

Secretion of alpha tocopherol in VLDL can lead to the enrichment of all circulating lipoproteins with alpha tocopherol [Kayden and Traber, 1993, Bjorneboe *et al.*, 1990]. Upon secretion into the plasma, the VLDL is catabolised by the lipoprotein lipase and returned to the liver, while the remainder are converted in the circulation to LDL-C. Alpha tocopherol that is secreted from the liver in VLDL can have alternative fates. Some of the alpha tocopherol can be transferred to High Density Lipoprotein cholesterol [HDL-C] during lipolysis, some can travel with the VLDL core during conversion to LDL-C, and some can return to the liver as VLDL remnants [the intermediate density lipoprotein]. Thus, it can be appreciated here that the plasma transport of tocopherol is mainly by plasma lipoproteins especially via LDL-C and HDL-C in association with plasma surface components. In terms of mass distribution of tocopherol among lipoproteins in humans, the percentages of tocopherol transported by the respective lipoproteins had been reported to be VLDL 19%, intermediate density lipoprotein [IDL] 3%, LDL-C 42% and HDL-C 36%. However, plasma vitamin E is in a constant state of flux between the lipoproteins. The transport of vitamin E from peripheral tissues to the liver has not been studied extensively.

Alpha-TTP gene mutation results in low serum and cell alpha tocopherol. Thus, the two factors needed for realising an adequate level of alpha tocopherol in the body are dietary availability and the expression of liver α-TTP. Alpha-TTP is also expressed in tissues including the trophoblast region of placenta. The α-TTP probably plays an important role in supplying the vitamin to the foetus, and explains the foetal resorption occurring in rats fed a vitamin E deficient diet. Plasma phospholipid transfer protein facilitates the exchange of tocopherol between LDL-C and HDL-C. Alpha tocopherol that is bound to lipoprotein is transported from the blood into cells with the help of the scavenger receptor B1 [SR-B1] [Kayden and Traber, 1993]. In the cells, alpha tocopherol is placed into cell membrane components including mitochondria and the endoplasmic reticulum [Machlin, 1991].

Dimitrov [1991] had reported that after single administration of alpha tocopherol, the plasma concentrations peaked at between 12 to 24 hours. With chronic administration for 28 days, doses at 440 IU, 880 IU and 1200 IU resulted in a steady state that occurred by days four to five of supplementation. This was consistent with the reported plasma half life of alpha tocopherol being approximately 20 hours. Dimitrov also reported that the plasma elevation of alpha tocopherol was affected by dietary fat intake. Individuals consuming a high fat diet showed significantly greater plasma alpha tocopherol concentrations compared with those fed a low fat diet. This is explained by alpha tocopherol being a fat soluble vitamin, thus its absorption will be affected by dietary lipids and bile in the gastrointestinal tract.

2.2.2.2. Pharmacokinetics of tocotrienol

The fate of supplemented tocotrienols and the relationship between its intestinal absorption, blood levels and tissue distribution is still not well studied [Watkins *et al*.,1999]. Some investigators even had difficulties in detecting plasma tocotrienols before and after supplementation. For example Khor *et al*. [1995] reported that guinea pigs treated with different dosages of tocotrienols for six days had only alpha tocopherol and no tocotrienols in their serum. Tomeo *et al.* [1995] did not manage to detect plasma tocotrienols after supplementation with 240 mg daily of TRE to patients with carotid stenosis and hypercholesterolemia. Hayes *et al.* [1993] also reported that in fasting humans, the plasma tocotrienol concentration was not increased significantly after tocotrienol supplementation.

In studies using thoracic duct cannulated rats, Ikeda *et al*. [1996] showed that, similar to alpha tocopherol, alpha, delta and gamma tocotrienols were transported via the lymphatic system after oral absorption. This is because they are too lipophilic to be absorbed via the hepatic portal vein. They also reported that alpha tocotrienol was preferentially absorbed compared with delta and gamma tocotrienols. Hayes *et al*. [1993] suggested that tocotrienols are transported non-specifically like any lipid soluble compound, most likely being incorporated with triglyceride in the core of the triglyceride rich chylomicrons. Once transported to the adipose tissue, a modest level of tocotrienols especially gamma tocotrienols appears to be deposited and stored with the triglycerides, presumably to be released during lipolysis. Unlike alpha tocopherol, which has a cytosolic binding protein in the liver to sequester and enhance their

conservation and re-secretion into lipoproteins, no similar transport / binding protein have been found as yet for tocotrienols.

The pharmacokinetics and bioavailability of alpha, gamma and delta tocotrienols under fed and fasted conditions were studied in eight healthy volunteers [Yap *et al*., 2001] after a single dose of 300mg mixed tocotrienols composed of approximately 56.1%, 29.4%, 14.4% and 31.5% respectively of gamma, alpha and delta tocotrienol and alpha tocopherol. Absorption of tocotrienols was markedly increased and also more consistent when taken with food. Peak plasma concentrations for alpha, gamma and delta tocotrienol were reported to be achieved at 4.3 hrs after dosing for alpha and gamma tocotrienol and at 3.9 hours after dosing for delta tocotrienol [Yap *et al*., 2001]. Plasma concentrations of all tocotrienols increased markedly when dosed with food; mean maximum concentration [Cmax] and area under the curve [AUC] values for all tocotrienol isomers for the fed state were higher compared with the values of the fasted state. This is because absorption of fat soluble vitamins in general, requires emulsification by bile salts before transportation across the gut mucosa. A high fat diet causes stimulation of bile secretion, which may thus explain the increased absorption of tocotrienol in the fed state. Mean elimination half life of alpha tocotrienol, gamma and delta tocotrienols were 4.4, 4.3 and 2.3 hours respectively. These are between 4.5 – 8.7 fold shorter than that for alpha tocopherol, which have a half life of about twenty hours. There were no significant differences in the plasma half life of the tocotrienol isomers when supplementation was given in the fed or fasted state. The volume of distribution of tocotrienol [Vd] appeared to be relatively big, which the authors reported may be indicative of either incomplete absorption or extensive redistribution from the blood or both.

Plasma transport of tocopherol is mainly by plasma lipoproteins, especially LDL-C and HDL-C. However, the exact plasma transport of tocotrienol is not well elucidated and may differ from tocopherol. Hayes *et al*. [1993] reported that there appears to be a lack of tocotrienols in LDL-C or HDL-C. They reported that the concentration of tocotrienols in platelets was only about 3-5% that of the alpha tocopherol, although the platelet concentration of tocotrienols doubled after supplementation with palm vitee [tocotrienol enriched palm capsules] in humans. The increase in tocotrienols in platelets was greater than the percentage of increase observed for tocopherols. Suarna *et al*., [1993] however contradicted Hayes study. Suarna reported that after dietary supplementation with mixed tocotrienols, alpha and gamma tocotrienols were actually detected in circulating lipoproteins. Suarna's study had used a sensitive method of detecting tocotrienols that was electrochemical detection following chromatographic separation.

There is currently no data in humans on tissue accumulation of tocotrienols. In hamsters, tissue concentrations of tocotrienols were typically much lower than tocopherol (1:180 on average) following palm vitee supplementation. The exception is in the adipose tissue where the tocotrienol to tocopherol ratio in supplemented hamsters was greater than 1 [Hayes *et al*., 1993]. The adipose tissue was the only tissue where the tocotrienol level exceeds that of tocopherol concentration.

2.2.3. Storage of vitamin E.

Vitamin E, as a fat soluble vitamin distributes throughout the body. The human body stores about 40 mg of vitamin E for every kilogram of body weight, where