Note

Dose Dependent Elevation of Plasma Tocotrienol Levels and Its Effect on Arterial Compliance, Plasma Total Antioxidant Status, and Lipid Profile in Healthy Humans Supplemented with Tocotrienol Rich Vitamin E

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Summary Tocotrienols are a class of vitamin E reported to be potent antioxidants, besides having the ability to inhibit the HMG-CoA reductase enzyme. This study assessed the effects of 3 doses of tocotrienol-rich vitamin E (TRE) on plasma tocotrienol isomer concentration, arterial compliance, plasma total antioxidant status (TAS), aortic systolic blood pressure (ASBP), serum total cholesterol (TC) and low density lipoprotein cholesterol (LDL-C) in healthy males. Methodology: This randomised, blinded end-point, placebo-controlled clinical trial with a parallel design involved 36 healthy male subjects who took either an oral placebo or TRE at doses of 80, 160 or 320 mg daily for 2 mo. Baseline and end-of-treatment measurements of vitamin E concentration, arterial compliance [assessed by aortic femoral pulse wave velocity (PWV) and augmentation index (AI)], ASBP, plasma TAS, serum TC and LDL-C were taken. Results: Baseline tocotrienol isomer concentrations were low and not detectable in some subjects. Upon supplementation, all TRE-treated groups showed significant difference from placebo for their change in α, γ and δ tocotrienol concentrations from baseline to end of treatment. There was a linear dose and blood level relationship for all the isomers. There was no significant difference between groups for their change in PWV, AI, plasma TAS. ASBP, TC or LDL-C from baseline to end of treatment. Groups 160 mg (p=0.024) and 320 mg (p=0.049) showed significant reductions in their ASBP. Group 320 mg showed a significant 9.2% improvement in TAS. Conclusion: TRE at doses up to 320 mg daily were well tolerated. Treatment significantly increased α, δ, and γ tocotrienol concentrations but did not significantly affect arterial compliance, plasma TAS, serum TC or LDL-C levels in normal subjects.

Key Words tocotrienol-rich vitamin E, δ,γ and α-tocotrienol plasma concentration, arterial compliance

All the large observational and interventional studies on the cardiovascular (CVS) effects of vitamin E assessed the tocopherol class of vitamin E. Tocotrienols (T3), another class of vitamin E are found in high concentrations in palm oil and rice bran. The 4 isomers of T3 are the α, β, γ and δ-T3. Tocotrienols differ from tocopherol in their hydrocarbon tail; T3 has unsaturated isoprenoid tail while tocopherols have a saturated one. Although both scavenge the chain propagating peroxyl radicals, some in-vitro studies suggested T3 to have higher antioxidant potency than tocopherol (1, 2). One pharmacological action of T3 which is not present with tocopherol is its ability to suppress 3-hydroxy-3-methyl-glutaryl CoA (HMG-CoA) reductase activity. This property has been associated with a reduction in plasma cholesterol in animal studies (3–5) and appears to be supported by some human studies (6–8). Therefore it is conceivable that T3 may produce better CVS effects, as opposed to that reported with tocopherol vitamin E in recent large trials.

There are limited clinical studies on T3; most are small studies. Available clinical studies have reported their effects on lipid profile (6–12) or progression of atheroma in patients with carotid stenosis (13) while some are Pharmacokinetic related studies (14, 15). Tomeo et al. had provided promising preliminary data that TRE supplementation led to regression in carotid atherosclerosis among hyperlipidemic patients (13).

Arterial stiffness/compliance is a useful index of vascular health. Arterial stiffening has been shown to be reversible in studies with several dietary interventions including hormone replacement (16), fish oil (17) and soy isoflavones (18). Mottram et al. (19) have shown improved systemic arterial compliance with tocopherol

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vitamin E; however, we have failed to show that \( \alpha \)-tocopherol improves aortic stiffness in healthy menopausal women (20). The mechanism whereby nutrients such as antioxidants affect arterial compliance rapidly probably predominantly involves vascular functional improvement rather than structural mechanisms. Vitamin E has been shown to prevent endothelial dysfunction in animal studies (21). The endothelium produces nitric oxide/endothelium derived relaxing factor (EDRF) that helps maintain vascular relaxation. Free radicals can inactivate EDRF (22); supplementation with powerful antioxidants may scavenge these radicals, thus protecting the physiological action of EDRF. In this study we will assess the effect of TRE on PWV, which reflects aortic compliance. The effect of TRE on AI, as obtained from pulse wave analysis (PWA) will indicate systemic arterial compliance.

Animal studies reported that T\(_3\) administration to hypertensive rats significantly increased plasma total antioxidant status (TAS) (23). Peng et al. (24) have shown that even healthy subjects could improve their TAS when supplemented with antioxidants. The measure of plasma TAS considers the cumulative action of antioxidants present in plasma and body fluids (25).

To date there is limited data on the dose-blood level effect of T\(_3\), and no published reports on the effect of TRE on arterial health and plasma TAS in humans. There appears to be no clear guideline on dosages to be used in humans; dosages used by prior clinical studies had varied from 40 to 250 mg daily. There are also doubts about the ability of T\(_3\) to be absorbed and remain in systemic circulation and thus exert its biological effects, as some human and animal studies had failed to detect T\(_3\) even on supplementation (4, 13, 26). In order to study these effects of TRE without their being affected by pathological processes, this study was conducted in normal subjects. Thus this study aims to assess the effects of 3 doses of TRE compared to a placebo, on T\(_3\) blood levels, arterial compliance and plasma TAS in normal subjects. The effect of TRE on serum TC and LDL-C, aortic systolic blood pressure (ASBP) and tolerability of subjects to the doses used were also assessed.

**Materials and Methods**

This study is a randomised, blinded end-point, placebo-controlled clinical trial with a parallel design involving 36 males. The study protocol was approved by the Ethics Committee of Universiti Sains Malaysia. Subjects were accepted into the study if they were between the ages of 21 and 30 y, not on chronic medication including dietary supplements and agreed to sign the informed consent form. Subjects were divided into 4 groups, which were placebo, and groups receiving TRE at doses of 80, 160 and 320 mg daily for 2 mo. All subjects underwent medical examination; they were excluded if they had hypercholesterolemia, history of CVS diseases or allergies to vitamin E or palm oil. All were non-smokers who did not consume alcohol throughout the study duration. Only males were chosen due to the possible confounding effect of the menstrual cycle on arterial compliance.

Tocotrienol-rich vitamin E capsules were supplied by the Golden Hope Plantations Bhd. Malaysia. The individual components of vitamin E isomers in each capsule (%) were \( \alpha -T_3 \) (34.6%), \( \gamma -T_3 \) (24.63%), \( \delta -T_3 \) (15%) and \( \alpha -tocopherol \) (26.2%). Placebos were prepared by the Hospital Pharmacy from inert corn flour. Three doses of TRE were chosen, a low dose of 80 mg daily, a middle-range dose of 160 mg daily and a high dose of 320 mg daily. Study capsules were taken with food at breakfast, as it had been shown that maximum concentration and area under the concentration curve values for T\(_3\) in the fed state were higher compared to those of the fasting state (15). Compliance to study medication was monitored by pill counting and measuring plasma vitamin E levels. Subjects attended study sessions in the afternoon 6 h after taking their capsules, fasting from breakfast. Study measurements (arterial compliance, BP, bloods for TAS, vitamin E concentrations and serum TC and LDL-C) were performed at baseline, before supplementation, and after 2 mo of taking study medications.

Plasma T\(_3\) (\( \alpha \), \( \delta \) and \( \gamma \)) and \( \alpha -tocopherol \) concentrations were quantified using high performance liquid chromatography by a method previously described (27). Plasma TAS was determined using a commercially available kit from Randox Laboratories Ltd. (UK). In this assay, 2,2-azino-bis-(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS) was incubated with metmyoglobin (as peroxidase) and hydrogen peroxide (\( \text{H}_2\text{O}_2 \)), resulting in the formation of a radical cation, \( \text{ABTS}^- \), the concentration of which was measured by absorbance. The degree of suppression for the formation of the radical was proportional to the antioxidant concentration in the tested plasma. The coefficient of variation (CV) for this measurement was <2%. TC was measured using standard procedures at the Universiti Sains Malaysia Hospital Laboratory.

Low density lipoprotein cholesterol was determined directly using the L-Type LDL-C test kit (Wako Pure Chemical Industries, Ltd., Japan) and thus quantification did not rely on the TC, triglycerides, HDL-C levels or Friedman’s formula (28). Firstly, test samples were mixed with the first reagent which contains polyanion and amphotheric surfactant (binds and protects LDL-C from enzyme reaction) cholesterol oxidase (CO), cholesterol esterase (CHE) and a catalase from bovine liver. The non-LDL cholesterol reacts with CO and CHE to produce \( \text{H}_2\text{O}_2 \), which is decomposed to water by the catalase. The second reagent was then added, containing 4-aminopyrpyrine, a peroxidase and sodium azide. This addition removes the LDL-C protecting agent from LDL-C, causing the CHE and CO to react with LDL-C producing \( \text{H}_2\text{O}_2 \) which, together with the salt and peroxidase, produces a colour complex, the absorbance of which can be measured. This assay has a CV of less than 5%.

As per our previous studies, aortic compliance was determined using the carotid femoral PWV (20, 29, 30). Carotid femoral PWV gives the velocity of the pulse wave along the carotid-femoral segment; recorded by placing a pressure sensitive probe on the carotid fol-
lowed by the femoral pulsations, and comparing the time delay at both sites against a simultaneously measured QRS complex (from the electrocardiography). This measurement was performed using the Sphygmocor (PWV Medical Pty Ltd., Australia); the repeatability of the measurements assessed as intra-observer CV was 2.01%. A higher PWV indicated lower arterial compliance and vice versa.

Pulse wave analysis using the Sphygmocor was used to assess ASBP and AI non-invasively (31). Radial artery pressure waveform was recorded non-invasively by anplation tonometry using a micromanometer (Millar for Atcor Medical, US). A validated integral transfer function is used to derive the aortic pressure waveform from the radial waveform, enabling the aortic pressures and AI to be measured. As a pressure wave travels from the heart towards the periphery, it is reflected when there is a change in vascular impedance. The contribution of wave reflection to central arterial pressure can be assessed by examining the pressure waveform, expressed as the AI. Intra-observer variability of this measurement in this study was 2.02%.

Statistical analysis was computed using the SPSS software (version 12). Baseline values between groups were analysed by one-way ANOVA. Outcome was primarily measured as change in a parameter as a result of treatment, computed as the difference between baseline and end-of-treatment values. This change was compared between groups using one-way ANOVA and post-hoc analysis. The effect of treatment within a group was analysed using paired t-test. Non parametric equivalent tests were used where appropriate. Results are presented as mean±standard error of the mean (SEM). A p value of less than 0.05 with α set at 0.05 was taken as the significance level.

Results
All subjects completed the study; overall compliance was good at 98% by pill counting. Study medications were well tolerated with no serious adverse events recorded. Mean age, body mass index, TC, SBP and mixed T3 concentration at baseline were not significantly different between groups, being 23.3±0.3 y, 22.8±0.6 kg/m², 4.6±0.1 mmol/L and 120.5±1.5 mmHg and 34.3±9.6 ng/mL respectively.

Plasma vitamin E levels

Figure 1a, 1b, 1c and 1d show δ, γ and α-tocopherol concentrations at baseline and at the end of treatment for each group. All TRE-treated groups showed significant rises in their δ, γ and α-T3 levels from baseline to the end of 2 mo. Delta T3 before supplementation was very low, being only detectable in 10 subjects. Change in δ-T3 concentration was obtained by subtracting the baseline from the end-of-treatment con-
Table 1. Baseline and end of treatment values for pulse wave velocity (PWV), augmentation index, plasma total antioxidant status (TAS), serum total cholesterol, low density lipoprotein cholesterol and aortic systolic blood pressure (SBP) for each study group.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Placebo</th>
<th>80 mg TRE</th>
<th>160 mg TRE</th>
<th>320 mg TRE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma TAS (mmol/mL)</td>
<td>1201.00 ± 35.65</td>
<td>1258.75 ± 34.87</td>
<td>1187.78 ± 27.07</td>
<td>1287.78 ± 39.47</td>
</tr>
<tr>
<td>PWV (m/s)</td>
<td>7.83 ± 0.27</td>
<td>7.73 ± 0.17</td>
<td>7.43 ± 0.23</td>
<td>7.52 ± 0.14</td>
</tr>
<tr>
<td>Augmentation index (%)</td>
<td>97.4 ± 3.21</td>
<td>96.9 ± 2.36</td>
<td>92.4 ± 3.36</td>
<td>95.3 ± 2.06</td>
</tr>
<tr>
<td>Total cholesterol (mmol/L)</td>
<td>4.85 ± 0.18</td>
<td>5.07 ± 0.24</td>
<td>4.62 ± 0.28</td>
<td>5.00 ± 0.34</td>
</tr>
<tr>
<td>Low density lipoprotein (mmol/L)</td>
<td>3.00 ± 0.13</td>
<td>3.02 ± 0.17</td>
<td>2.92 ± 0.29</td>
<td>3.18 ± 0.34</td>
</tr>
<tr>
<td>Aortic SBP (mmHg)</td>
<td>104.9 ± 2.00</td>
<td>104.3 ± 2.00</td>
<td>97.6 ± 1.00</td>
<td>96.1 ± 1.51</td>
</tr>
</tbody>
</table>

* Indicates significant change (p<0.05) at the end of treatment compared to baseline.

centration. There were significant differences between the placebo and all treated groups in this change. TRE-treated groups also had higher Δ-T1 levels compared to the placebo after treatment (p=0.0001). γ-T3 concentrati ons could be detected in 17 subjects at baseline. Significant difference was seen between the placebo and all TRE-treated groups for their change in γ-T1 from baseline to end of treatment (p=0.005, 0.0001 and 0.001 respectively for groups 80, 160 and 320 mg). All TRE-treated groups had significantly higher γ-T1 levels compared to the placebo after treatment (p=0.002, 0.001 and 0.002 respectively). Baseline α-T1 was not detectable in 13 subjects. TRE treated groups showed a higher α-T1 concentration compared to the placebo after 2 mo (p=0.005, p<0.0001 and p=0.001 respectively). There was also a significant difference between groups 80 and 160 mg (p=0.002) and between groups 80 and 320 mg (p=0.009). Significant differences for change during treatment between the placebo and all treated groups (p=0.019, 0.0001 and 0.001 respectively) were also observed. There were also significant differences between groups 80 and 160 mg (p=0.004) and between groups 80 and 320 mg for this change (p=0.005).

Plasma α-tocopherol concentration could be detected in all subjects at baseline, the mean concentration was 8.60±0.23 μg/mL. Groups 80 mg (p=0.025) and 320 mg (p=0.001) showed significant rises in their α-tocopherol concentration from baseline to end of treatment. However, there was no significant difference between groups for their change in α-tocopherol from baseline to end of treatment, although there was a trend of increasing α-tocopherol level. Significant difference was only seen between the placebo and 320 mg group in their α-tocopherol concentration at 2 mo (p=0.024).

Pharmacodynamic results

Table 1 shows PWV, AI, ASBP values and plasma TAS, TC and LDL-C levels at baseline and after 2 mo treatment for each group. There was a small but significant 5.4% reduction (p=0.013) in AI with the 160 mg group at the end of 2 mo compared to baseline. However, differences between groups for their change in AI with treatment were not significantly different. The 160 and 320 mg groups showed significant falls in ASBP from baseline (p=0.024, p=0.049 respectively) after 2 mo’ treatment. However, there was no significant difference between groups for their change in ASBP with treatment.

The 320 mg group produced a small but significant 9.2% increase for plasma TAS compared to baseline (p=0.024). However, there was no significant difference between groups for change in TAS due to treatment. Similarly, change in TC and LDL-C at the end of 2 mo compared to baseline was not significantly different between groups. The placebo group did not show any significant changes in any parameters at the end of treatment compared to baseline.

Discussion and Conclusion

This clinical trial assessed the effects of 3 doses of TRE on plasma vitamin E levels, arterial compliance, plasma TAS, aortic SBP, serum TC and LDL-C in healthy subjects. Generally the capsules were well tolerated up till doses of 320 mg daily for 2 mo, with no adverse effects necessitating drug withdrawal. Plasma T1 concentration before supplementation was low, especially for δ-tocotrienol where only 27.8% of subjects had detectable levels. Baseline γ and α-T3 were detectable in 52.8% and 63.9% of subjects. After 2 mo, all treated groups showed significant rises in their δ, γ and α-T1 concentrations. Significant difference for the change in concentration from baseline to end of treatment was seen between the placebo and all treated groups for all isomers. For all the doses, significantly higher levels of δ, γ and α-T1 were achieved at 2 mo compared to the placebo. For α-tocopherol, baseline concentration was
detectable in all subjects. Only the 320 mg group showed higher \( \alpha \)-tocopherol levels compared to the placebo. There was no significant difference between groups for their change in \( \alpha \)-tocopherol with treatment.

After supplementation, the highest concentration of T3 isomer was for \( \alpha \)-T3\(_s\), followed by \( \gamma \) and the lowest was \( \delta \)-T3. This corresponds with the preparation being reported to contain the highest percentage of \( \alpha \)-T3\(_s\) (34.6\%), followed by \( \gamma \)-T3 (24.6\%) and the least, \( \delta \)-T3 (15\%). A linear relationship exists between the daily dosage of individual isomers and the concentration achieved; as the dose was increased, the concentrations also increased.

Undetectable baseline concentration of some T3 isomers in some subjects was not unexpected and had been reported in prior studies (9, 11, 26). Even upon supplementation with TRE, some human and animal studies failed to detect T3 (4, 13, 26); two reasons may explain this observation. Firstly, T1 may not be detectable in fasting samples. The half lives (T1/2) of T3 are generally short, reported to be between 2.3 and 4.4 h (15); thus their levels may not be detectable after 12 h of fasting. This is supported by a study (32) on hamsters that failed to detect fasting plasma T3, although postprandial levels were detected. Our subjects had their blood levels taken 6 h after ingestion of T3. Secondly, the method used to quantify T3 levels may not have been sensitive enough in those studies. Thus our study confirmed the absorption and presence of individual T3 isomers in the circulation 6 h after oral administration.

We noted large interindividual differences in blood levels of T3 among subjects given the same dosages of TRE, which had also been reported previously (11, 14, 15) despite controlling subjects' food intake, such as that seen with pharmacokinetic studies (14, 15). We also noted considerably higher tocopherol levels compared to T3 despite the tocopherol weight consisting of only about 26\% of the supplemented vitamin E. For the 320 mg dose, the tocopherol concentration was 12 \( \mu \)g/mL, while for total T3, it was only 0.690 \( \mu \)g/mL; thus the concentration of tocopherol was approximately 17\( \times \) higher. A few reasons may contribute to this observation. Firstly, T1 may have lower bioavailability compared to \( \alpha \)-tocopherol. In rats, the absolute bioavailability of \( \alpha \)-T3 was 28\%; those of \( \delta \) and \( \gamma \)-T3 were comparable with a value of about 9\% (33). Although this could be suggested to be attributed to lower T1 absorption, Ikeda et al. had shown that lymphatic recovery of \( \alpha \)-T3 was approximately 2\( \times \) higher than that of \( \alpha \)-tocopherol, while that of \( \gamma \) and \( \delta \)-T3 was intermediate between these two \( \alpha \) forms (34). Thus one explanation for the low bioavailability may be related to the need of vitamin E to be transported with a specific transport system in the circulation as it is a lipid soluble substance. While tocopherol had been shown to have a specific transport system, the \( \alpha \)-tocopherol binding protein (\( \alpha \)-TTP), it is currently not yet known if a separate transport system exists for T3, or whether they share the same transport protein. If so, there will be competition between \( \alpha \)-tocopherol and T1 for this binding protein. Affinity of T3 for \( \alpha \)-TTP may be lower than that of tocopherol; Hosomi et al. had reported that relative affinity of \( \alpha \)-T3 (RRR-\( \alpha \)-tocopherol=100\%) calculated from the degree of competition was 12\% (35). Tocopherol also persists longer than T3 in the plasma. The plasma elimination T1/2 of \( \alpha \), \( \gamma \), and \( \delta \)-T3 were estimated to be 4.4, 4.3 and 2.3 h respectively, compared to that of \( \alpha \)-tocopherol being approximately 20 h. There are, currently, attempts to produce an improved T3 formulation to increase its bioavailability (14). However, in our study, despite having a lower concentration than for tocopherol, the concentration increase with T3 was much higher. With the 320 mg dose, T3 increased by 19\( \times \) compared to baseline as opposed to \( \alpha \)-tocopherol, which increased by only 1.4 times. Thus treatment with the TRE markedly increased T3 levels, with minimal effect on \( \alpha \)-tocopherol levels.

In this study the 3 doses of TRE produced no significant difference from placebo for change in arterial compliance with treatment, as assessed by PWV and AL. However, significant improvement after treatment compared to baseline was observed for systemic arterial compliance, assessed by AL in the 160 mg group. Subjects treated with 160 and 320 mg also showed significant falls in aortic SBP after treatment compared to baseline. The placebo group did not show any change in any of the study parameters. Although TRE had been shown to increase plasma TAS in hypertensive rats (23), our study on normal subjects did not show significant difference between the treated groups and placebo for their change in TAS with treatment, although the 320 mg group showed a small increase after treatment compared to baseline. However, one limitation of this study in terms of TAS measurement was that, although use of dietary supplements were disclaimer, dietary intake of antioxidants such as vegetables, fruits, chocolates and fruit juices was not controlled, as it was not possible for us to control them in non-institutionalised subjects in a 2 mo study. Our study also did not show a significant effect of treatment on serum TC or LDL-C.

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