Original Article

Effects of gamma-tocopherol supplementation on thrombotic risk factors

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Objective: The antioxidant activity of vitamin E is derived primarily from alpha-tocopherol (α-T) and gamma-tocopherol (γ-T). Results of epidemiological studies have demonstrated an inverse relationship between vitamin E intake and coronary disease. However, the results of clinical trials using α-T are equivocal. We determined the effect of 5 weeks of 100 mg/d or 200 mg/d γ-T supplementation on thrombotic markers such as platelet reactivity, lipid profile and the inflammation marker C-reactive protein (CRP).

Methods and results: Fourteen healthy subjects consumed 100 mg/day while 13 consumed 200 mg/d of γ-T and 12 received placebo (soybean capsules with less than 5 mg/d γ-T) in a double-blinded parallel study design. Fasting pre and post dose blood samples were analysed. Blood γ-T concentrations increased significantly (p<0.05) relative to dose during the intervention period. Both groups receiving active ingredients showed significantly lower platelet activation after supplementation (p<0.05). Subjects consuming 100 mg/d γ-T had significantly decreased LDL cholesterol, platelet aggregation and mean platelet volume (MPV) (p<0.05). Little effect of γ-T was observed on other parameters.

Conclusions: These data suggest that γ-T supplementation may have a permissive role in decreasing the risk of thrombotic events by improving lipid profile and reducing platelet activity.

Key Words: gamma tocopherol, platelets, lipids, CRP, thrombotic risk factors

INTRODUCTION

Vitamin E is an essential nutrient, the main lipid soluble antioxidant, and plays a significant role in protecting biological membranes and lipoproteins from oxidative damage caused by free radicals.[1-3] Vitamin E is not a single compound with at least four tocopherols (α, β, δ and γ-tocopherol) and four tocotrienols (α, β, δ and γ-tocotrienol) known.[4] The antioxidant activity of vitamin E is derived primarily from α-tocopherol and γ-tocopherol, of which α-tocopherol is most biologically active and the predominant form found in blood. In contrast, the predominant form of vitamin E found in food is γ–tocopherol.[5] The dietary intake of γ-tocopherol is at least two times that of α-tocopherol in Western diets, while the concentrations of α-tocopherol in human blood are generally four times higher than those of γ-tocopherol.[5]

An inverse relationship has been found between acute coronary events and antioxidant vitamin E intake. For example, epidemiological studies have shown that vitamin E (α-T and γ-T) is associated with reduced number of ischemic cardiac events in patients with documented coronary artery disease.[6] Tocotrienols have also been shown to be beneficial by attenuating the formation of atherosclerotic lesions and decreasing serum cholesterol effects in animal and *in vitro* studies but results in humans have been inconclusive.[7-9] The results of large clinical trials examining effect of Vitamin E (tocopherols) on cardiovascular diseases have been equivocal.[10] Supplementation with large amounts of α-tocopherol (1,200 IU per day) has been shown to decrease blood concentrations of γ-tocopherol.[11] Gamma tocopherol has been found to be more effective than mixed tocopherol in protecting against certain specific types of oxidative damage.

Saldeen *et al* [12-13] showed an antithrombotic effect of vitamin E (γ-T and α-T) on thrombus formation, with more pronounced effect with gamma-tocopherol. These workers also demonstrated that platelet aggregation was inhibited more potently with a mixed tocopherol preparation (100 mg γ-tocopherol, 40 mg δ-tocopherol, and 20 mg α-tocopherol) than with α-tocopherol alone and attributed this observation to increased nitric oxide (NO) release, endothelial constitutive nitric-oxide synthase (ecNOS) activation, and superoxide dismutase (SOD) protein content in platelets in response to mixed tocopherol.[13]

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In a placebo controlled double-blind study, we determined the effect of 5 wk of supplementation with γ-T at different doses (100 mg/d or 200 mg/d) on thrombotic risk factors, including platelet aggregation and activation. The effect of supplementation on plasma lipids and inflammation marker CRP was also investigated.

MATERIALS AND METHODS

Subjects and dietary intake

After obtaining approval from RMIT Human Research Ethics Committee, 42 healthy volunteers were recruited. All the subjects were between 20 to 40 years old with no known medical history. None were on any form of medication for the duration of the study. Exclusion criteria included heavy drinking, smoking, and taking anti-inflammatory medication and antioxidant supplements or antioxidant rich food interfering with platelet function in the 2 wk prior to study entry. None of the subjects had a history of bleeding disorders, diseases of the circulatory system or diabetes. Two subjects withdrew from the study due to time constraints while one subject was withdrawn due to difficulty in phlebotomy. No subjects used any platelet inhibitors during study.

Dietary γ-tocopherol supplementation was achieved through consumption of tablets after signed informed consent was obtained from all participants. The subjects were separated into 3 groups and randomly assigned to one of the treatment or placebo groups. Fourteen healthy subjects (7 male and 7 female) consumed 100 mg/day of γ-T while 13 (7 male and 6 female) consumed 200 mg/d of γ-T and 12 (5 male and 7 female) received colour and flavour-matched placebo (soybean capsules with less than 5 mg/d γ-T) for 5 wks. Tama Biochemical Company Ltd Japan supplied all γ-tocopherol and placebo capsules.

Subjects were required to complete a food frequency questionnaire over a 7-day period before commencing dietary supplementation, to confirm study participants were not consuming antioxidant rich foods or antioxidant supplements.

Fasting blood samples were collected for baseline assessment prior to the study and after 5 wks of supplementation. All the procedures were followed in accordance with RMIT HREC guidelines.

Experimental Trial

On the morning of an experiment subjects reported to the laboratory between 0700-0800 h after a 12-14 h overnight fast. A resting blood sample was obtained for total platelet count, whole blood platelet aggregation, ATP release from platelet granules, mean platelet volume (MPV), flow cytometry for platelet activation marker, total cholesterol, high density lipoprotein (HDL) cholesterol, low density lipoprotein (LDL) cholesterol and triacylglycerols. CRP and serum α- & γ-tocopherol concentrations were also evaluated.

Laboratory methods

Blood collection. A total of 20 mL of venous blood was collected using a vacutainer adapter and 21 gauge vacuettes (Greiner bio-one GmbH, Kremsmünster, Austria) on two occasions. Blood was collected into 2 mL tripotassium ethylene-diamine-tetra acetic acid EDTA (1.8mg/mL), 8 mL tri-sodium citrate (3.8%) and 10 mL SST (serum separator tubes) tubes (Greiner bio-one GmbH, Kremsmünster, Austria). The EDTA tube was collected before the tri-sodium citrate tube to avoid collecting platelets activated by venipuncture. Care was taken to ensure minimal specimen handling and agitation. All blood samples collected were tested according to the protocols described subsequently.

Platelet function tests. Platelet count and MPV were measured by using whole blood collected in EDTA-containing tubes with the use of a Beckman Coulter A^c.T 5diff CP analyser (Coulter Corporation, Miami, FL, USA). Performance of the analyser was validated using Coulter Calibrator and Controls Plus.

Whole blood platelet aggregation was measured with an impedance aggregometer (Chrono-Log Corp, Philadelphia) equipped with MacLab software (ADInstruments Pty, Ltd, Castle Hill, Australia) for data quantitation and analysis. This method has been described previously.[14] Calibrations for impedance and ATP release were performed daily before analysing blood samples. Briefly citrated whole blood was diluted with saline (1:1), 100 uL chrono-Lume reagent was added, sample was then incubated, and mixed with agonists 2 µg collagen/mL [Chrono-Log Corp] [1 mmol arachidonic acid (AA)/L was used to stimulate platelets for repeating the aggregation as a check for anti-inflammatory intake], and aggregation was recorded for 6 min. ATP release from platelets reacted with luciferin-luciferase in the Chrono-Lume reagent and luminescence was measured, at 650nM by photomultiplier tube (PMT) built in the aggregometer, simultaneously with platelet aggregation.

Additional aliquots of citrated whole blood diluted in modified tyrode's buffer were activated with 2 µg/mL collagen, and then incubated in the dark with monoclonal antibodies, phycoerythrin conjugated CD41 (Immunotech, Marseille; which was used to identify platelets because it has specificity for the glycoprotein IIb portion of the glycoprotein IIb-IIIa antigen present on resting and activated platelets), fluorescein isothiocyanate conjugated CD62p (Immunotech; an activation-dependent antibody directed against P-selectin, a component of the α-granule membrane of resting platelets that becomes expressed on the platelet surface membrane upon activation), or one of the isotype controls, immunoglobulin G_1 (IgG_1). Samples were fixed with paraformaldehyde to prevent artifactual *in vitro* platelet activation. Modified tyrode's buffer terminated the fixation, and samples were analysed on an EPICS Elite flow cytometer (Coulter Electronics) equipped with a 15-mW argon laser, at an excitation of 488 nm. The fluorescence of fluorescein isothiocyanate and phycoerythrin was detected by using 525 nm and 575 nm band pass filters respectively. Activated platelets were defined as CD41-positive events that expressed P-selectin. The data are reported as a proportion of maximum CD62P expression.

Lipid screening and CRP Analysis. Lipids and CRP were analysed on the automated Olympus AU2700 biochemical analyser. The instrument was validated by calibrating and running controls for each parameter. Enzymatic col-

orimetric methods were used for measuring total cholesterol (based on cholesterol oxidase method), HDL cholesterol (determined using anti human-beta-lipoprotein antibody binding to lipoproteins other than HDL) and triacylglycerols (based on a method with glycerol blank).

LDL cholesterol was calculated, using following Friedwald formula[15]:

LDL Cholesterol = Total cholesterol – [HDL Cholesterol + VLDL],

Where, VLDL = triacylglycerols/5

CRP was measured using immuno-turbidimetric method using anti-human CRP antibodies to form insoluble aggregates.

Serum alpha and gamma-tocopherol concentration. The Shimadzu HPLC system (Kyoto, Japan) used in this study consisted of a chromatograph LC-10AD and spectrofluorophotometer detector (RF-551) equipped with a 12 µL LC flow cell. *γ-Tocopherol, dl-α*-Tocopherol and *dl-α*-tocopheryl acetate were detected at excitation λ=298 nm, emission λ=325 nm, recorded by a CR6A Chromatopac Recorder /Integrator.

For the analysis serum samples in ethanol (1:1) were treated with petroleum ether solvent (2:1) before a reversed-phase C-18 column, with a mobile phase of Acetonitrile: Dichloromethane: Methanol (7:2:1), at a flow rate of 1.0 ml/min was employed. Quantitation of these compounds was achieved using linear calibration curves constructed from the peak area versus the concentration of the standard compound. Before starting a sample run, external standards, followed by an internal QC (plasma frozen and allocated into multiple vials on day one from a normal subject) were checked.

Statistical analysis

Data are presented as means ± SDs. The data analyses were performed using a SPSS version 11.5 program (SPSS Corporation, Chicago IL, USA). Effects of different Vitamin E (γ-tocopherol) doses (dose effect) on the parameters were analysed using GLM repeated measures of ANOVA adjusted for baseline values. Changes between pre- and post treatment in same dose (treatment effect) were using paired t-test. *p* values <0.05 were considered significant.

RESULTS

Compliance was good, as assessed by the number of pills returned and measure of serum α and γ-tocopherol

Table 1. Serum Gamma (γ-) tocopherol and alpha (α-) tocopherol

	<5mg G-T (Placebo n=12)		100mg G-T (n=14)		200mg G-T (n=13)		*p*-value*		
	Pre	Post	Pre	Post	Pre	Post	Treatment	Dosage	T*D
γ-tocopherol (mg/mL)	5.3±3.3	5.4±2.3	5.3±3.5[b]	16.8±7.2[a]	5.4±4.1[b]	30.1±20.7[a]	0.00	0.00	0.00
α-tocopherol (mg/mL)	26.7±5.6	26.7±5.0	24.9±8.2	22.4±5.4	22.8±7.5	24.1±9.8	0.94	0.25	0.31

Each parameter was analysed using GLM repeated measures of ANOVA; [a,b]Significant difference within groups (Paired-Samples t-test).

Table 2. Platelet function tests

	<5mg G-T (Placebo n=12)		100mg G-T (n=14)		200mg G-T (n=13)		*p*-value*		
	Pre	Post	Pre	Post	Pre	Post	Treatment	Dosage	T*D
P-selectin CD62p (%)	47.5±9.7	45.9±15.5	50.0±5.0[a]	45.3±7.1[b]	47.1±8.0[a]	40.3±8.0[b]	0.03	0.32	0.57
Agg/Slope (Ω/Sec)	0.22±0.05	0.21±0.07	0.25±0.08[a]	0.22±0.06[b]	0.22±0.06	0.21±0.08	0.04	0.70	0.20
ATP release (um)	2.01±1.28	1.80±1.07	2.24±1.38	2.68±2.25	2.48±1.64	1.63±0.70	0.51	0.44	0.23
Platelet ($x10^9$/L)	231±47	230±36	237±49	226±48	244±61	236±72	0.19	0.88	0.75
MPV (fL)	8.35±0.69	8.09±0.70	8.62±0.95[a]	8.35±0.75[b]	8.12±0.70	7.99±0.74	0.00	0.32	0.67

Each parameter was analysed using GLM repeated measures of ANOVA; [a,b]Significant difference within groups (Paired-Samples t-test).

Table 3. Lipids and inflammation marker

	<5mg G-T (Placebo n=12)		100mg G-T (n=14)		200mg G-T (n=13)		*p*-value*		
	Pre	Post	Pre	Post	Pre	Post	Treatment	Dosage	T*D
Cholesterol (mmol/L)	4.9±0.9	5.1±1.0	4.9±1.1	4.6±0.8	5.1±1.2	4.4±1.0	0.18	0.70	0.11
LDL (mmol/L)	2.8±0.6	2.9±0.7	3.0±1.0[a]	2.6±0.8[b]	3.1±0.0	2.5±0.8	0.02	1.00	0.08
HDL (mmol/L)	1.5±0.4 [b]	1.7±0.5[a]	1.3±0.4[b]	1.5±0.4[a]	1.3±0.5	1.4±0.4	0.00	0.41	0.06
Triacylglycerols (mmol/L)	1.4±0.9	1.1±0.6	1.1±0.3	1.0±0.4	1.3±0.6	1.2±0.6	0.12	0.62	0.71
C-reactive Protein (mg/L)	2.1±3.5	1.8±2.7	2.6±3.0	3.1±4.5	2.3±2.4	1.9±1.7	0.65	0.67	0.15

Each parameter was analysed using GLM repeated measures of ANOVA.; [a,b]Significant difference within groups (Paired-Samples t-test).

concentrations. No side effects from the active or placebo tablets were reported.

Baseline results for all groups were comparable. Five week of supplementation with 100 mg and 200 mg γ-T resulted in proportionately increased concentrations of serum γ-T ($p<0.05$). The serum level of α-tocopherol did not change after intervention in any group (Table 1).

There was a significant effect of γ-tocopherol treatment on platelet activation: p-selectin (CD62p%) decreased significantly after both doses ($p<0.05$), with little change after placebo (Table 2).

MPV decreased significantly with 100 mg γ-tocopherol treatment ($p<0.05$) but the decrease was not statistically significant for the higher dose. Platelet aggregation also decreased significantly with 100 mg γ-tocopherol ($p<0.05$) though little effect was seen with 200 mg γ-tocopherol or placebo (Table 2).

Gamma-tocopherol treatment resulted in a significant effect on LDL cholesterol. LDL cholesterol was significantly decreased following 100 mg/d of γ-tocopherol supplementation ($p<0.05$) (Table 3).

HDL cholesterol increased significantly with 100 mg γ-tocopherol and placebo. However a similar increase was not found with 200 mg γ-tocopherol suggesting the increase is random and unlikely due to γ-tocopherol treatment (Table 3)

Inflammation marker CRP did not decrease significantly after supplementation with 200 mg γ-tocopherol. There were no significant changes in the serum levels of triacylglycerol, or in the whole blood platelet count and ATP release.

DISCUSSION

The potentially beneficial effects of vitamin E on cardiovascular disease (CVD) have been intensively investigated in many interventional and epidemiological studies. However, most of these studies focused exclusively on α-tocopherol and the protective effects of α-tocopherol supplementation on CVD is equivocal.[16, 17] Accordingly, we evaluated the effect of pure γ-tocopherol on platelet function, lipid concentrations and inflammation marker in blood. To the best of our knowledge, this is the first study that provides *in vivo* relationship between pure γ-tocopherol and thrombotic risk factors.

Specifically, the present study determined the effect of two doses of γ-T on platelet function, lipid profile and inflammation marker CRP in human subjects. We observed a significant increase in serum γ-tocopherol concentrations after both doses. A significant decrease in platelet activity post 200 mg and 100 mg γ-tocopherol dose was also observed, though the changes between groups were not statistically significant. There was also a significant reduction in MPV, platelet aggregation and LDL cholesterol post 100 mg γ-tocopherol supplementation only.

Serum γ-tocopherol concentrations increased significantly in all subjects in accordance with the concentration of dose, while no change was observed in serum α-tocopherol concentrations. This suggests that any changes observed in this study in the platelet function and lipid profile would have been due to γ-tocopherol ingestion.

Several independent investigations have demonstrated that the blood concentration of γ-tocopherol, not α-tocopherol, was negatively correlated to the incidence of coronary heart disease.[18, 19] Gamma tocopherol is present in the diet at higher levels than α-tocopherol, but most studies show increased α-tocopherol in plasma. [5] Supplementation with large amounts of α-tocopherol was shown to increase the breakdown and decrease blood concentrations of γ-tocopherol, [11, 20] as a result of the function of the hepatic α-tocopherol transfer protein (α-TTP), which preferentially incorporates α-tocopherol into the plasma. [10] Hosomi[7] found that the biological activity of vitamin E analogs correlates to their affinity for α-TTP. Chopra and Bhagavan[21] showed that bioavailability of both natural and synthetic α-tocopherol significantly suppresses the serum γ-tocopherol to the same extent.

Our results could explain the findings of previous epidemiological studies showing inverse correlation between vitamin E intake and incidence of CVD. It has been shown dietary source of vitamin E is much higher in γ-tocopherol [5] compared to α-tocopherol, but all the investigational studies used very high concentration of α-tocopherol, which could have broken down the γ-tocopherol [11] and nullified the effect of vitamin E seen by previous studies. High dose of α-tocopherol reduces

intestinal absorption, cell membrane transport and utilisation of other forms of vitamin E, especially γ-tocopherol. [11,22,23] The hepatic α-TTP has the greatest affinity for α-tocopherol compared to γ-tocopherol and is crucial for the relative percentage of transport of the various forms of vitamin E in the plasma lipoproteins. [5,7,23] This imbalance of α-tocopherol / γ-tocopherol levels in plasma may have significant health consequences. [7, 18]

Liu, Saldeen and Li *et al* [12,13,24,25] have shown in various animal and human studies that mixed tocopherols are more potent in preventing platelet aggregation and have stronger inhibitory effect on lipid per oxidation than α-tocopherol alone. In the current study we used a pure form of γ-tocopherol and our results suggest that γ-tocopherol supplementation had a direct positive effect on platelet activity and LDL cholesterol concentrations in blood of normal healthy people.

Circulating activated platelets are useful markers of local thrombotic events occurring in cardiovascular diseases. We used flow cytometry to detect circulating activated platelets through expression of platelet surface glycoprotein, p-selectin, also known as CD62p. The p-selectin, is rapidly translocated from α-granules inside the platelets to the cell surface on stimulation by physiological agonists. During agonist-induced platelet activation, energy-dependent fusion of both alpha and dense granules with the plasma membrane permits expression of p-selectin, which promote and propagate platelet adhesion to endothelial cells, neutrophils and monocytes.[26]

One possible mechanism by which γ-tocopherol might have reduced platelet activity (i.e. the reduced expression of p-selectin through decreased CD62p binding on platelet surface) could be related to platelet derived nitric oxide (NO) bioactivity. Impaired platelet NO production and decreased bioavailability of NO have been associated with coronary disease states.[27] Platelet-derived NO has been found to inhibit platelet aggregation and reduce platelet recruitment to a growing thrombus.[28] α-tocopherol might increase platelet NO release by its free radical scavenging activity and by preventing its quenching by peroxyl radicals.[29,30] One compensatory mechanism is nitration of γ-tocopherol, through available nitration-prone positions on the chromanol ring of γ-tocopherol .[31,32] and thus depleting NO by reacting with it, which leads to an up-regulation of NO synthesis.[28] Freedman [30] suggested α-tocopherol might also be responsible in balancing NO and super oxide anion (SOD) in human platelets. Saldeen *et al* [13] suggested up-regulation of SOD by both α-tocopherol and γ-tocopherol might be important mechanism for the effect of γ-tocopherol on platelet aggregation, but they found γ-tocopherol to be more potent than α-tocopherol in these effects.

A decrease in platelet activation could possibly be explained in terms of antioxidant effect of γ-tocopherol. Jiang *et al* [33-35] demonstrated cyclooxygenase-2 (COX-2) catalyses the synthesis of prostaglandins through oxidation of arachidonic acid, which are important elements within the inflammatory process as well as platelet activation. They found COX-2 activity is inhibited by γ-tocopherol but not by α-tocopherol.[33-35]

Significant decrease in LDL cholesterol is indicative of improved lipid profile, which may also explain the reduced platelet activity observed in this study. In recent *in vitro* studies, γ-tocopherol has been shown to inhibit lipid per oxidative damage [36] and to trap mutagenic electrophiles [31] more efficiently than α-tocopherol. This finding supports our results demonstrating an improved lipid profile and a trend of reduction in platelet activity post γ-tocopherol supplementation.

Contrary to our findings, Jiang and Ames also provided strong evidence that γ-tocopherol shows anti-inflammatory activities *in vivo* in rats.[35] One of the reasons for that could be that most of baseline results of CRP in this study were normal, around 2 mg/L and so there was not much scope for the values to further go down. It will be more realistic in future to evaluate the effect of γ-tocopherol on populations with higher inflammatory conditions.

In conclusion, the results from the current study suggest that the daily consumption of small amounts of γ-tocopherol, in conjunction with usual dietary intake from mixed food sources may provide protection from oxidative damage and prevent thrombosis. Further cellular research would be valuable in helping to understand the mechanisms behind the biological effects of γ-tocopherol in higher risk populations such as diabetic subjects or those with known cardiovascular disease. Potential synergistic effects between γ-tocopherol and other antioxidants and comparison with α-tocopherol supplementation could also be explored to clarify the role of γ-tocopherol in human health.

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Original Article

Effects of gamma-tocopherol supplementation on thrombotic risk factors

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Gamma-生育醇的補充對血栓危險因子的影響

目的：維生素 E 的抗氧化活性主要衍生自 alpha-生育醇(α-T)及 gamma-生育醇(γ-T)。流行病學研究的結果指出維生素 E 的攝取與冠狀疾病為負相關。然而，採用 α-T 的臨床試驗結果是可議的。我們評估每天補充 100 毫克或 200 毫克的 γ-T5 週對血栓標記，例如：血小板活性、血脂及發炎標記 C 反應蛋白(CRP)的影響，

方法及結果：採用雙盲平行研究設計，14 名健康受試每天攝取 100 毫克的 γ-T，13 名攝取 200 毫克的 γ-T 及 12 名接受安慰劑(大豆膠囊加每天低於 5 毫克的 γ-T)。分析禁食前後血液樣本的劑量。血中 γ-T 上升濃度與介入期的劑量呈現顯著相關(p<0.05)。接受有效成分的兩組，顯示補充之後血小板活性顯著較低(p<0.05)。每天攝取 100 毫克 γ-T 的受試者，LDL 膽固醇、血小板凝集及平均血小板容積(MPV)顯著降低(p<0.05)。γ-T 對其它的參數影響較小。

結論：這些數據指出補充 γ-T 可能透過改善血脂及降低血小板活性，達到降低血栓事件。

關鍵字：gamma-生育醇、血小板、脂質、CRP、血小板危險因子。