Title: Gamma tocopherol supplementation prevents exercise induced coagulation and platelet aggregation

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Abstract: INTRODUCTION: Strenuous exercise in sedentary individuals increases the acute risk of atherothrombotic events and depletes endogenous antioxidants. Supplementation with the antioxidant tocopherol ameliorates the risk; however it is unclear whether α-tocopherol, γ-tocopherol or a combination of both is more efficacious. We therefore measured the effects of γ-α- and mixed tocopherol supplementation on blood thromboinflammatory markers after an acute bout of endurance exercise. MATERIALS AND METHODS: 36 healthy, sedentary volunteers were randomly assigned to a 6 week antioxidant supplementation regimen of either low dose (300 mg every other day) or high dose (300 mg per day) γ-tocopherol, low dose (400 IU every other day) or high dose (400 IU per day) α-tocopherol, a combination (alternating 400 IU α-tocopherol or 300 mg γ-tocopherol per day), or a soy placebo control. Volunteers exercised at 70% of peak O2 uptake for one hour, or until volitional fatigue before and after intervention and laboratory parameters including full blood examination, platelet aggregation, lipid profile and inflammation markers measured. RESULTS: γ-tocopherol ameliorated the exercise-induced hyper-coagulable and hyper-aggregatory states following exercise, reducing collagen induced platelet aggregation below baseline (89% of baseline, p<0.01). Lipid profile, inflammation markers or platelet count were not affected. CONCLUSION: Chronic supplementation with γ-tocopherol, but not α-tocopherol prevented exercise-induced platelet aggregation and hyper-coagulability. Furthermore, supplementation with high dose γ-tocopherol in combination with exercise significantly reduced collagen-induced platelet aggregation below pre-exercise levels. Therefore γ-tocopherol in the absence of high dose α-tocopherol may enhance the beneficial effects of exercise on haemostatic and cardiovascular function.
Original article

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ABSTRACT

INTRODUCTION: Strenuous exercise in sedentary individuals increases the acute risk of atherothrombotic events and depletes endogenous antioxidants. Supplementation with the antioxidant tocopherol ameliorates the risk; however it is unclear whether α-tocopherol, γ-tocopherol or a combination of both is more efficacious. We therefore measured the effects of γ-, α- and mixed tocopherol supplementation on blood thromboinflammatory markers after an acute bout of endurance exercise. MATERIALS AND METHODS: 36 healthy, sedentary volunteers were randomly assigned to a 6 week antioxidant supplementation regimen of either low dose (300 mg every other day) or high dose (300 mg per day) γ-tocopherol, low dose (400 IU every other day) or high dose (400 IU per day) α-tocopherol, a combination (alternating 400 IU α-tocopherol or 300 mg γ-tocopherol per day), or a soy placebo control. Volunteers exercised at 70% of peak O₂ uptake for one hour, or until volitional fatigue before and after intervention and laboratory parameters including full blood examination, platelet aggregation, lipid profile and inflammation markers measured. RESULTS: γ-tocopherol ameliorated the exercise-induced hyper-coagulable and hyper-aggregatory states following exercise, reducing collagen induced platelet aggregation below baseline (89% of baseline, p<0.01). Lipid profile, inflammation markers or platelet count were not affected. CONCLUSION: Chronic supplementation with γ-tocopherol, but not α-tocopherol prevented exercise-induced platelet aggregation and hyper-coagulability. Furthermore, supplementation with high dose γ-tocopherol in combination with exercise significantly reduced collagen-induced platelet aggregation below pre-exercise levels. Therefore γ-tocopherol in the absence of high dose α-tocopherol may enhance the beneficial effects of exercise on haemostatic and cardiovascular function.

Keywords: platelets, platelet aggregation, exercise, γ-tocopherol

Abbreviations: Cardiovascular disease (CVD), reactive oxygen species (ROS), reactive nitrogen species (RNOS), prothrombin time (PT), activated partial thromboplastin time (APTT), C-reactive protein (CRP), creatine kinase (CK), full blood examination (FBE), platelet count (Plt), γ-tocopherol (γT), α-tocopherol (αT), nitric oxide (NO), D-dimers (DD), nitric oxide synthase (NOS), oxidatively modified LDL (ox-LDL), von Willebrand factor (vWF), haematocrit (Hct), high density lipoprotein cholesterol (HDL), low density lipoprotein cholesterol (LDL)
Cardiovascular disease (CVD) is the leading cause of morbidity and mortality in western world [1], with platelet hyperactivity and oxidative stress considered major contributory factors for the development, acuity and severity of CVD [2]. An acute bout of strenuous exercise in sedentary individuals acutely increases cardiovascular risk [3] by increasing oxidative stress [4], promoting coagulation [5] and platelet aggregation [3]. The mechanism(s) by which acute exercise promotes thromboinflammatory risk are multifactorial [6-11] and include increased platelet and coagulation factor concentration associated with dehydration and splenic or hepatic release [3, 5, 12] and enhanced activation of platelets by adrenaline [13, 14]. Increased generation of reactive oxygen species (ROS) [12, 14], and depleted antioxidant capacity [8, 15-18] also contributes to activation of platelets and the coagulation cascade. Therefore, dietary antioxidants such as vitamin E (tocopherol) may be important in attenuating exercise associated cardiovascular risk.

Dietary supplementation with α-tocopherol for prevention of cardiovascular events has yielded inconsistent results in clinical trials [19-23]. It is possible that α-tocopherol supplementation causes inhibition of dietary γ-tocopherol uptake, which has greater antioxidant capacity [15, 24-29], via competition for transport proteins, resulting in the lower than expected benefit [19, 23, 29-31]. Plasma γ-tocopherol concentration is inversely correlated with coronary heart disease [32, 33]. We therefore sought to test the hypothesis that high vitamin E supplementation with high concentrations of γ-tocopherol will ameliorate the platelet activating effects of oxidative stress produced by strenuous exercise in sedentary individuals better than α-tocopherol alone, or mixed supplementation.

**MATERIALS AND METHODS**

Thirty seven healthy and sedentary volunteers between 18-55 years old gave informed consent to participate in the study, 36 completed the treatment regimen. Institutional Human Research Ethics Committee approval and notification of the Therapeutic Goods Administration was performed. All participants were healthy, non-smokers with no history of medical conditions and not on any medications or other dietary supplements for the duration of the study. Sedentary volunteers were identified by VO₂max less than 40 (range 32.3 ± 6.4) mL/kg/min [34]. A flow chart for subject screening, enrolment and study participation is outlined in Figure 1.
Subjects were randomized to one of 6 single-blind oral 6-week supplement regimens, outlined in Figure 2, and compliance verified by leftover capsules count. A food frequency questionnaire ensured no excess of antioxidant rich foods during the study period and standardized diet-influenced variation in results between groups.

On the morning of the experiment subjects reported to the laboratory between 06.00 and 10.00 am. Resting blood sample was obtained for:

a) Full blood examination (FBE) that revealed a total platelet count (Plt) and haematocrit (Hct)

b) Platelet aggregation analysis

c) Coagulation studies including prothrombin time (PT), activated partial thromboplastin time (APTT) and quantitative D-Dimers

d) Lipid profile that revealed total cholesterol, high density lipoprotein cholesterol (HDL), low density lipoprotein cholesterol (LDL) and triacylglycerols

e) Inflammation markers that included C-reactive protein (CRP) and creatine kinase (CK)

A total of 22 mL of venous blood was collected at each time point using a vacutainer adapter and 21 gauge vacuettes. Blood was collected into 2 mL tri-potassium ethylene-diamine-tetra acetic acid (EDTA 1.8mg/ml), 4 mL tri-sodium citrate (3.8%) and 8 mL serum separator tubes. Care was taken to ensure minimal specimen handling and agitation.

FBE was performed on EDTA anticoagulated whole blood using Beckman Coulter Ac.T 5diff analyser (Coulter Corporation, Miami, FL, USA). The performance of the analyser was evaluated using Coulter Calibrators and Controls Plus.

Whole blood platelet aggregation was assessed with an impedance aggregometer (Chrono-Log Corp, Ph, USA) equipped with MacLab software (ADInstruments PTY, LTD, Castle Hill, AUS) for data quantification and analysis according to the manufacturer’s instructions. Briefly, within 15 minutes of collection, citrated whole blood, was diluted in 1:1 in pre-warmed saline, 2 μg/mL collagen (Chrono-Log Corp, ph, USA) was added and aggregation was recorded for 6 minutes and maximum impedance recorded.

Coagulation parameters PT, APTT and D-dimer were performed on ACL Futura using appropriate Hemosil reagents (Instrumentation Laboratory, IL Company, Lexington, USA) according to
manufacturer’s instructions. Prothrombin time and APTT testing was performed in duplicates and an average result recorded. Results for PT (sec) and APTT (sec) were validated using appropriate controls (either Hemosil or Citrol from Dade Behring, Marburg, Germany) and DD results using Hemosil DD- calibrators and controls.

Lipid profile and inflammation markers were tested at a diagnostic laboratory service (Gribbles Pathology, Melbourne, Australia) on Dimension XL (Dade Behring) biochemistry analyser. All reagents including controls and calibrators were specific for Dimension instrument and are produced by Dade Behring.

The results were analysed using GraphPad Prism 5.01. Exercise-induced changes before and after supplementations were expressed as the percentage of deviation from a baseline level. The means of those changes before and after supplementation were compared using a paired t-test. Significant change was indicated by p<0.05 for the null hypothesis.

RESULTS

Exercise resulted in a 3.5% increase in haematocrit due to exercise-induced hypohydration and haemoconcentration (pre-exercise 0.426, post-exercise 0.441, p<0.01). However, platelet count increased by 12% (pre-exercise 252 x 10^9/L, post-exercise 282 x 10^9/L). When corrected for haemoconcentration, exercise caused an 8% increase in platelet count (p<0.01). This was not significantly different between supplement treatment groups.

Exercise increased collagen induced platelet aggregation for all volunteers prior to supplementation (117% of pre-exercise aggregation, p < 0.05). Following supplementation, exercise continued to cause a significant increase in collagen induced platelet aggregation in those taking soy placebo (110% of baseline, p < 0.05). However supplementation with low dose γ-tocopherol ameliorated this increase (95% of pre-exercise baseline, p = 0.56), while high dose of γ-tocopherol resulted in a decrease in aggregation below the baseline level after exercise (89% or pre-exercise baseline, p < 0.05) and was significantly lower than the soy control (110% vs 89%, p = 0.05 unpaired) (Figure 3). Supplementation with α-tocopherol or combined α-tocopherol and γ-tocopherol did not reduce the exercise induced increase in collagen induced platelet activation.
Exercise caused a decrease in APTT to 88% of the baseline value (p < 0.05); this was ameliorated by supplementation with low concentrations of γ-tocopherol (96% of the baseline value), while a similar trend existed for high doses of γ-tocopherol (94% to 98% of baseline) this was not statistically significant (Figure 4). There was no effect on APTT with α-tocopherol or placebo (data not shown).

Vitamin E supplementation and exercise did not have any effect on lipid profile, PT, DD nor inflammation markers, all of which remained normal (data not shown).

DISCUSSION

The novel finding of the present study was that six weeks supplementation with γ-tocopherol, but not α-tocopherol or a mixture of the two prevented exercise-induced platelet activation and hyper-coagulability. Furthermore, supplementation with high dose γ-tocopherol in combination with exercise reduced collagen-induced platelet aggregation below the pre-exercise levels. Therefore γ-tocopherol in the absence of high dose α-tocopherol may enhance the beneficial effects of exercise on haemostatic and cardiovascular function by inhibiting, rather than increasing, collagen induced platelet aggregation. Accordingly, supplementation with γ-tocopherol, and avoidance of α-tocopherol supplementation, may be recommended in sedentary individuals planning to commence on a program of strenuous exercise.

The results of previous studies have demonstrated that strenuous exercise causes increased platelet aggregability, while moderate exercise has little or no effect of platelet activation and aggregation [3, 34]. Our findings suggest that the undesirable platelet-stimulating effect of strenuous exercise may be modulated to resemble the desirable effects of moderate exercise by supplementing with γ-tocopherol, but not α-tocopherol.

The mechanism by which γ-tocopherol supplementation exerts its effect on exercise induced platelet function is not clear, in part because the mechanism by which exercise in sedentary people induces platelet activation is not clear. Some studies have suggested that increased shear stress may contribute to elevated platelet activation, while the others have suggested catecholamine release [14]. Our results show that platelet counts increase after exercise due to both haemoconcentration resulting from dehydration, and release of platelets from sequestration organs in response to catecholamine release, consistent with previous findings [35, 36]. However, tocopherol supplementation in any isoform was not capable of preventing the increase in platelet
count associated with strenuous exercise. Therefore these results suggest that tocopherol supplementation does not impact on dehydration or catchecolamine response.

Exercise increases oxygen radical production, lipid peroxidation and production of oxidatively modified LDL that decreases platelets ability to generate NO, which is associated with adverse cardiovascular outcomes. One possible mechanism by which γ-tocopherol may reduce collagen induced platelet aggregation after exercise below the baseline levels could be related to NO production. γ-tocopherol is known to increase NO and NOS activity as well as increase eNOS protein expression [27, 32, 37, 38]. Furthermore, chronic moderate exercise enhances biosynthesis of eNOS, resulting in higher plasma NO concentration [39, 40], and resulting in NO-induced inhibition of exercise induced platelet activation. This cumulative effect of supplementation with recurrent bouts of exercise on NO levels may have not only overcome the free radical production associated with acute exercise, but preconditioned the platelets to increased NO production with exercise, this resulting in exercise inhibiting, rather than augmenting, platelet activation.

Both γ- and α- isoforms of tocopherol compete for uptake using the same transport proteins,[19, 23, 29-31]. Therefore supplementation with α-tocopherol may prevent uptake of dietary γ-tocopherol or effectiveness of γ-tocopherol supplementation by out competing γ-tocopherol for transport proteins [15, 24-29]. This explains why our findings show that γ-tocopherol did not inhibit exercise induced platelet activation when supplemented in combination with α-tocopherol, but is able to inhibit exercise induced augmentation of collagen induced platelet activation only when supplemented alone.

While the decrease in aPTT associated with exercise is likely to be large due to haemoconcentration of coagulation proteins associated with exercise related dehydration and oxidative stress resulting from the exertion. Our results show that this appears to have been ameliorated, at least in part, by supplementation with γ-tocopherol, particularly at low doses. This may be due to antioxidant activity of the γ-tocopherol. However, platelet activation has been shown to affect coagulation function by formation of a procoagulant membrane, or by modulating the formation of a procoagulant membrane phenotype on other cells [41]. It is therefore possible that inhibition of platelet activation by γ-tocopherol may have inhibited platelet activation induced shortening of the aPTT, similar to other anti-platelet agents such as clopidogrel[42] or prasugrel[43].

One limitation of the present study were the small group sizes with several outcome measures displaying relatively large inter-individual variation. To address this, data was analysed and presented by comparison to the same individuals baseline (e.g. pre exercise, pre supplementation date) by paired analyses where appropriate. Furthermore, while VO₂max was standardized
between groups, inter-individual differences in fitness level and catecholamine / stress responses may also have affected oxygen radical production and therefore contributed to variability in results. Finally, while platelet aggregometry remains the gold standard for measuring platelet function, it is not clear whether the inhibition of exercise induced platelet aggregation with \( \gamma \)-tocopherol was a result of decreased platelet-fibrinogen binding, platelet degranulation or platelet activation signalling \textit{per se}. Molecular and flow cytometric studies, particularly measurement of NO level and function, may be effective in determining the biochemical mechanism of this potentially beneficial effect.

In conclusion, this is the first study to assess the effect of supplementation of different isoforms of tocopherol on exercise induced parameters of haemostasis. Our findings suggest both that supplementation with \( \gamma \)-tocopherol both prevents exercise induced platelet and coagulation hyperactivity through as yet undetermined mechanisms, and results in inhibition of platelet function below baseline levels following exercise in sedentary individuals. Therefore \( \gamma \)-tocopherol supplementation, but not \( \alpha \)-tocopherol may enhance the beneficial effects of exercise in this population, may be indicated in sedentary individuals embarking on a program of exercise to improve their health.

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REFERENCES


FIGURE LEGENDS
Figure 1. Study protocol recruitment and timeline.

Figure 2. Supplementation regimen groups, sampling and testing protocol.

Figure 3: Collagen induced platelet aggregation. The results are expressed as the means percent of baseline after exercise ±SEM for (A) placebo, (B) low γ-tocopherol and (C) high γ-tocopherol. * indicates p<0.05 vs baseline, † indicates p = 0.05 vs placebo post supplementation and post exercise (unpaired).

Figure 4: aPTT measurement. The results are expressed as the means of percentage of baseline after exercise ±SEM for low γ-tocopherol (A) and high γ-tocopherol (B). Supplementation with low γ-tocopherol dose significantly inhibited APTT decrease following exercise. * indicates p<0.05 vs pre-exercise baseline (paired), † indicates p < 0.05 vs placebo post supplementation and post exercise (unpaired).

CONFLICTS OF INTEREST

This study was supported in part by funding from grant support from GlaxoSmithKline to JH The authors wish to declare no conflict of interest.
**Conflict of Interest Form**

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DAY 1
Participants were given the volunteer screening questionnaire.
Healthy volunteers on no medication were included.
VO₂ max test was performed.
Participants were included if VO₂max was <40 mL/kg/min.

DAY 8
Fasting blood samples were taken.
Participants were asked to exercise at 70% of their maximum for at least 30 min or maximum of one hour.
Second blood sample taken, immediately after exercise.

DAYS 9-51
Randomized to supplementation regimen (see Figure 2)
6 weeks of supplementation

DAY 52
Fasting blood samples were taken.
Participants were asked to repeat pre supplementation exercise.
Second blood sample taken, immediately after exercise.
**Protocol**

**Pre-supplementation**
- Fasting blood tests
- Exercise – 30min - 1hr @ 70% VO₂ max
- Blood testing

**6 weeks supplementation**
- Placebo, 6 volunteers
- Low Gamma (150mg γT+17mg αT, One capsule every second day), 6 volunteers
- Low Alpha (200IU αT), 6 volunteers
- Mix (200IU αT & 150mg γT alternate αT and γT capsules), 5 volunteers

**Post-supplementation**
- Fasting blood tests
- Exercise – 30min - 1hr @ 70% VO₂ max
- Blood testing
Pre Supplementation
Post Supplementation

0
50
100
150
% baseline aggregation after exercise

A

B

C

* †
Pre Supplementation | Post Supplementation

% baseline aPTT after exercise

A

B

* †