FAX COVER SHEET

To: J.S. Coombes, MD 011-617-3365-6877

Ann-Marie Gaillius

From: ________________

Jan. 11, 2006

Date: ________________

No. of pages including this one: 7

Re: CATH Msc. # 11109

Dear Dr. Coombes:

Our records indicate that this paper was mailed to you on July 19th, and to date we have not heard from you.

I have enclosed a copy of the paper, and a reprint request form. I ask that you go over this and get back to me as soon as possible, regarding any changes you feel need to be made.

I thank you for your assistance and I apologize for the delay in this request.

If you have any questions, please feel free to contact me.

Best Wishes,
Westminster Publications, Inc.

[Signature]

Ann-Marie Gaillius
Publications Coordinator
anngailius@westminsterpublications.com
Vitamin E and α-Lipoic Acid Supplementation Increase Bleeding Tendency via an Intrinsic Coagulation Pathway

Susan A. Marsh, PhD and Jeff S. Coombes, PhD*

School of Human Movement Studies, The University of Queensland, Brisbane, Australia

Summary: Vitamin E and α-lipoic acid are potent nutritional antioxidants, and when used together, their antioxidant capabilities are improved as α-lipoic acid recycles vitamin E. Supplementation of vitamin E has been shown to prolong platelet aggregation but the effects of vitamin E and α-lipoic acid supplementation on bleeding tendency have yet to be reported. Young, male rats consumed either control diet (n=5) or vitamin E and α-lipoic acid-supplemented diet (n=5) for 14 weeks. Activated partial thromboplastin time (APTT) and prothrombin time (PT) were measured as markers of intrinsic and extrinsic coagulation pathways respectively in addition to lipid peroxidation (malondialdehyde). Supplementation significantly prolonged APTT (23.8±1.5 vs 31.4±1.25, p<0.05) compared to the control diet; however, there was no significant difference in PT (27.8±1.5 vs 26.6±0.96, p>0.05). While vitamin E was increased (p<0.05), there was no significant difference in plasma levels of malondialdehyde (p>0.05). Dietary supplementation of vitamin E and α-lipoic acid increases bleeding tendency via inhibition of the intrinsic coagulation pathway with no change in markers of lipid peroxidation. Such supplementation could benefit patients with cardiovascular disease who exhibit elevated levels of coagulation and oxidative stress.

Key Words: Vitamin E—α-lipoic acid—Platelet—Coagulation—Oxidative stress.

The consumption of dietary antioxidant supplements is widespread; however, several antioxidants have potent non-antioxidant effects that may either benefit or hinder diagnosis and subsequent treatments. Vitamin E is a potent lipid-soluble, chain-breaking antioxidant that is also known to increase the incidence of hemorrhagic disorders and inhibit platelet aggregation (1–6). Similarly, α-lipoic acid is a potent antioxidant that also has several known non-antioxidant properties, particularly in the prevention and treatment of diabetes (7) and has been shown to inhibit platelet activation (8). When given together, these antioxidants operate synergistically as vitamin E is recycled by dihydrolipoic acid, the reduced form of α-lipoic acid (9); however, the effects of combined supplementation on bleeding tendencies are unclear. We have previously shown that combined supplementation of vitamin E and α-lipoic acid improves cardiac performance during post-ischemia repertusion in older rats (10), and this may have been mediated by changes in coagulation in addition to the observed decrease in oxidative stress. Therefore, combined antioxidant supplementation of vitamin E and α-lipoic acid may prove beneficial in the prevention and treatment of various thrombolytic-related disorders in addition to reducing the elevated levels of reactive oxygen species (ROS) production seen in cardiovascular diseases. The purpose of this study was to examine changes in bleeding tendency following the combined supplementation of vitamin E and α-lipoic acid in healthy rats.

Address correspondence and reprint requests to Jeff S. Coombes, PhD, School of Human Movement Studies, The University of Queensland, Brisbane Q 4072, Australia; e-mail: jcoombes@hms.uq.edu.au.
MATERIALS AND METHODS

Animals
This experiment was approved by the University of Queensland Animal Ethics Committee in accordance with National Health and Medical Research Council guidelines. Ten young, male Wistar rats, aged 4 weeks (Central Animal Breeding House, The University of Queensland, Australia), were randomly assigned to receive either a control diet (n=5) or an antioxidant-supplemented diet (n=5) for 14 weeks. Animals that received the antioxidant diet were fed the same rat chow as the non-supplemented groups with 1000 IU vitamin E/kg diet (d-α-tocopheryl succinate, Covitol 1185, Cognis, Melbourne, Australia) and 1.6 g α-lipoic acid/kg diet (Lipoeo, Cognis, Melbourne, Australia) added. The dietary antioxidants used in this study were based on the results of previous studies. Machlin and Gabriel showed that the consumption of 1000 IU/kg diet dl-α-tocopherol by rats yielded plasma concentrations of vitamin E equivalent to those found in humans following daily supplementation of 400 mg/day α-tocopherol (11). Similarly, supplementation of 1.65 g/kg diet α-lipoic acid in rats prevents symptoms of vitamin E deficiency (12). Rats were housed two to three per cage, maintained on a 12/12 hour light/dark cycle, and provided with rat chow and tap water ad libitum.

Blood Collection and Haematologic Analyses
Animals were killed via an intraperitoneal injection of sodium pentobarbital (100 mg/kg), and a thoracotomy was performed under anesthesia. Sodium pentobarbital has been shown to have no effect on either intrinsic or extrinsic coagulation pathways (13). Before cessation of blood flow, blood was collected from the inferior vena cava into EDTA, sodium citrate, or lithium heparin. Platelet counts were determined immediately in EDTA samples using an automated counter (VetScan HMT, Abaxis, Union City, CA). Citrated samples were centrifuged at 1500 g for 10 minutes at 4°C and the plasma was frozen at −20°C until analysis for prothrombin time (PT) and activated partial thromboplastin time (APTT), basic indicators of coagulation status (14). PT and APTT were then determined in duplicate using the Simplastin Excel S and Automated APTT kits, respectively (Organon Teknika, Durham, NC) according to the manufacturer's instructions. Briefly, PT was the time taken for detection of a fibrin clot following the addition of 200 μL Simplastin Excel S reagent to 100 μL citrated plasma. PT reflects the capacity of the extrinsic or tissue factor coagulation pathway and is sensitive to factors II, V, VII, and X (14,15). APTT was determined by incubating 100 μL sample with 100 μL automated APTT reagent for 5 minutes at 37°C and recording the time taken for clot detection following the addition of 100 μL 25 mM CaCl₂. APTT is sensitive to factors II, V, VII, IX, X, XI, and XII and is indicative of the function of the intrinsic coagulation pathway (15,16).

Lipid Peroxidation
Plasma levels of malondialdehyde (MDA) were determined by high performance liquid chromatography (HPLC) according to the method of Sim and associates (17). Heparinized whole blood samples were centrifuged at 1500 g for 10 minutes at 4°C and frozen at −80°C until assay. Samples were thawed on ice and hydrolyzed in 1.3 mM NaOH to a final concentration of 1 mM NaOH, incubated for 60 minutes at 60°C, and cooled on ice for 5 minutes. Proteins were precipitated by the addition of 35% perchloric acid, cooled on ice for 5 minutes, and centrifuged for 5 minutes at 14000 g. The supernatant was added to 2,3-dinitrophenylhydrazine (DNPH) and incubated for 10 minutes at room temperature in the dark. The aqueous phase was extracted with hexane and evaporated with the dry extract reconstituted in mobile phase containing 45% acetonitrile and 0.2% glacial acetic acid. MDA concentrations were determined at 310 nm using HPLC (Shimazu) with a Lichrochrom C18 column (250 x 4 mm, 5 μm; Merck, Darmstadt, Germany) with a flow rate of 1 mL/min and 9.8 MPA backpressure. MDA aliquots of appropriate concentrations were used as external standards. The coefficient of variation for this assay is less than 5% in our hands.

Plasma Vitamin E
Concentrations of vitamin E (α-tocopherol) were determined in plasma with reverse phase HPLC using the liquid-liquid extraction method of Taibl and Nicotra (18). Briefly, proteins were precipitated and lipids extracted in a single step by incubation with an ethanol chloroform mixture (3:1 v/v). After separation of the precipitated protein, the 20 μL supernatant was injected onto a Lichrochrom C18 column (250 x 4 mm, 5 μm; Merck, Darmstadt, Germany) with a flow rate of 1 mL/min and 9MPa backpressure and analyzed using fluorometric detection. Stock so-
VITAMIN E AND \( \alpha \)-LIPOIC ACID SUPPLEMENTATION INCREASE BLEEDING

In our hands, the coefficient of variation for this assay is less than 2%. Plasma levels of \( \alpha \)-lipoic acid were not measured because preliminary data in our laboratory indicated \( \alpha \)-lipoic acid was undetectable in both control and supplemented animals. This finding is consistent with previous studies and occurs as \( \alpha \)-lipoic acid is rapidly converted to various metabolites and maximal plasma concentration is reached less than 1 hour after supplementation.

**Statistical Analysis**

Values reported are means ± SEM. Data were analyzed using an independent t-test. Significance was established at a p value less than 0.05.

**RESULTS**

APTT was significantly prolonged (23.6 ± 1.5 vs 31.4 ± 1.2, p < 0.05) following supplementation of vitamin E and \( \alpha \)-lipoic acid (Table 1) whereas PT was unchanged (27.8 ± 1.5 vs 26.6 ± 0.9, p > 0.05). There were no significant differences (p > 0.05) in body weight or platelet numbers. Plasma levels of vitamin E were significantly increased in the supplemented rats (25.1 ± 2.3 vs 36.9 ± 3.8 \( \mu \)M, p < 0.05); however, there were no significant differences in plasma levels of MDA (p > 0.05).

**DISCUSSION**

Recent studies support the combined use of the antioxidants vitamin E and \( \alpha \)-lipoic acid to decrease oxidative stress and improve cardiac performance. This is the first study to examine the combined effects of vitamin E and \( \alpha \)-lipoic acid supplementation on bleeding tendencies. Our results indicate that combined supplementation of vitamin E and \( \alpha \)-lipoic acid prolongs APTT with no change in PT or lipid peroxidation.

Inhibition of platelet activation by various antioxidant supplementation regimens has been reported previously; however, this effect is not likely to be caused by the antioxidant properties of these compounds as Freedman and colleagues demonstrated that butylated hydroxytoluene, a potent lipid-soluble antioxidant, does not affect platelet aggregation. Interestingly, while different combinations of antioxidants were used by Salonen and colleagues and Mehta and colleagues, both contained vitamin E, a known inhibitor of platelet aggregation. These effects appear to be dose-dependent and an increased incidence of hemorrhagic disorders has been reported when vitamin E is administered in large doses. The mechanism of vitamin E in the inhibition of platelet activation is unclear, but an increased requirement for vitamin K or inhibition of protein kinase C activity may be likely targets.

**TABLE 1.** Body Weight, Hematologic Parameters, Vitamin E, and Lipid Peroxidation After 14 Weeks Consumption of Control Diet or Antioxidant Supplemented Diet

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control Diet</th>
<th>Antioxidant Diet</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (g)</td>
<td>474 ± 12</td>
<td>512 ± 20</td>
</tr>
<tr>
<td>APTT (s)</td>
<td>23.8 ± 1.5</td>
<td>31.4 ± 1.1*</td>
</tr>
<tr>
<td>PT (s)</td>
<td>27.8 ± 1.5</td>
<td>26.6 ± 0.9</td>
</tr>
<tr>
<td>Platelet count (x10^9/L)</td>
<td>690 ± 59</td>
<td>622 ± 43</td>
</tr>
<tr>
<td>Vitamin E (( \mu )M)</td>
<td>25.1 ± 2.3</td>
<td>36.9 ± 3.8*</td>
</tr>
<tr>
<td>Malondialdehyde (( \mu )M)</td>
<td>20.9 ± 1.2</td>
<td>18.4 ± 0.7</td>
</tr>
</tbody>
</table>

Values are mean ± SEM. *p < 0.05 compared to control diet.
Few studies have examined the effect of α-lipoic acid on platelet activation; however, Ford and colleagues (8) reported significant decreases in fibrinogen, factor VII, and von Willebrand factor following 2 weeks of α-lipoic acid supplementation in both diabetic and non-diabetic rats. This could be due to the ability of α-lipoic acid to regulate NF-κB activity (30), which in turn regulates endothelial tissue factor and finally, factor VII (8). Because tissue factor is an activator of the extrinsic coagulation pathway, it is possible that the effects seen in the current study were solely the result of vitamin K rather than α-lipoic acid; however, confirmation of these findings are beyond the scope of this experiment.

We also reported no significant decrease in malondialdehyde despite a significant increase in vitamin E levels in antioxidant supplemented rats. These results are similar to those of Meagher and colleagues (31), who reported no change in markers of lipid peroxidation following vitamin E supplementation in healthy human adults. Because increases in reactive oxygen species (ROS) production are typically only observed in subjects with pathologic conditions such as coronary heart disease or diabetes, it is unlikely that antioxidant supplementation in healthy individuals would provide any beneficial antioxidant effects. Indeed, a basal level of ROS production is necessary for various cell signaling pathways (32); thus, excessive ingestion of antioxidants may have a detrimental effect on such functions in healthy subjects. Moreover, as we have shown an increase in bleeding tendency in healthy rats following a moderate level of vitamin E and α-lipoic acid supplementation, we suggest that caution should be used with moderate to large doses of these antioxidants in a healthy population because excessive and uncontrolled bleeding may result.

In summary, this is the first study to show that dietary supplementation of vitamin E and α-lipoic acid prolongs clotting time via inhibition of an intrinsic coagulation pathway. Because patients with coronary heart disease typically exhibit increased levels of oxidative stress together with decreased antioxidant enzyme activities and increased platelet aggregation (33), we suggest that supplementation of moderate levels of vitamin E and α-lipoic acid could provide both antioxidant and anti-coagulant effects that could benefit these patients.

ACKNOWLEDGMENTS

We gratefully acknowledge the technical advice and assistance of Gary Wilson. The antioxidant supplements used in this study were a generous gift from Herron Pharmaceuticals. This study was funded by The University of Queensland.

REFERENCES


