AN ABSTRACT OF THE THESIS OF

Kylie Sherée Smith for the degree of Master of Science in Nutrition and Food Management presented on June 11, 2002. Title: Vitamins E and C in Patients with End-Stage Renal Disease Undergoing Hemodialysis.

Abstract approved: ____________________________

Maret G. Traber

Patients with end-stage renal disease undergoing hemodialysis have a high incidence of oxidative stress-related diseases. This study evaluated oxidative stress and inflammatory markers in patients undergoing hemodialysis before and during vitamin E supplementation. Blood samples were obtained before and after dialysis during two separate dialysis sessions to establish baseline measurements. For the next two months, subjects consumed 400 IU RRR-α-tocopherol daily. At one month and two months of supplementation, blood samples were also obtained before and after dialysis. Circulating concentrations of α- and γ-tocopherols and their metabolites (carboxyethyl-hydroxychromans, α- and γ-CEHCs), vitamin C, and uric acid were determined by HPLC with electrochemical detection. C-reactive protein (CRP), tumor necrosis factor-α (TNF-α), and interleukin-6 (IL-6) were measured using standard clinical assays. F₂-isoprostanes were evaluated using an enzyme immunoassay. Dietary vitamins E and C were assessed using two 24-hour recalls. In response to vitamin E supplementation, plasma α-tocopherol concentrations increased from 18 ± 1.7 μM to 31 ± 5.4 μM (p<0.0001), while γ-tocopherol concentrations decreased from 2.8 ± 1.0 μM to 1.7 ± 0.6 μM (p=0.001).
Additionally, serum vitamin E metabolites increased, α-CEHCs from 68 ± 20 pmol/ml to 771 ± 161 (p<0.0001) and γ-CEHC from 837 ± 161.8 pmol/ml to 1136 ± 225.9 (p=0.0083). Both CEHCs are well above reported normal values (p<0.0001). Dietary antioxidants (vitamins E and C) were low in most subjects; thus, plasma ascorbic acid levels were low in most subjects, but high in a few, resulting a wide range of responses (88 ± 84 μM). Nonetheless, ascorbic acid concentrations decreased significantly after dialysis to 33 ± 34 μM (p=0.0124), but were unaffected by vitamin E supplementation. Indeed, many parameters decreased significantly by dialysis but were unchanged by vitamin E supplementation, including plasma concentrations of uric acid and TNF-α. Both IL-6 and F₂-isoprostane concentrations were elevated in the subjects but were unaffected by either vitamin E supplementation or dialysis. CRP increased significantly after dialysis (p=0.0161, ANOVA main effect), but in the vitamin E supplemented subjects CRP concentrations were slightly lower before dialysis, but increased following dialysis (p=0.0041, ANOVA interaction). Taken together, the data suggest that there is a complex relationship between chronic inflammation and oxidative stress. Longer supplementation with vitamin E might be necessary in order to observe beneficial effects.
Vitamins E and C in Patients with End-Stage Renal Disease Undergoing Hemodialysis

by

Kylie Sherée Smith

A THESIS

Submitted to

Oregon State University

in partial fulfillment of the requirements for the degree of

Master of Science

Presented June 11, 2002
Commencement June 2003

APPROVED:

______________________________
Major Professor, representing Nutrition and Food Management

______________________________
Department Head of Nutrition and Food Management

______________________________
Dean of Graduate School

I understand that my thesis will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my thesis to any reader upon request.

______________________________
Kylie S. Smith, Author
ACKNOWLEDGEMENTS

This study was funded by a grant from the Good Samaritan Hospital Foundation John C. Erkkila, M.D. Endowment for Health and Human Performance and the Linus Pauling Institute. The vitamin E capsules were a gift from the Archer Daniels Midland Inc. in Decatur, Illinois. Vitamin E standards were gifts from James Clark of Cognis Nutrition and Health, LaGrange, IL.
CONTRIBUTION OF AUTHORS

Dr. Maret G. Traber was involved in the design, analysis, and writing of this thesis. Scott W. Leonard was involved in the method design for the study. Dr. Jim Ridlington was involved in data collection and analysis. Drs. Ishwarlal Jialal and Sridevi Devaraj were involved in the analysis of plasma cytokines. All assisted in the interpretation of data. Leslie Meyer assisted in the analysis of vitamin E metabolites.
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>INTRODUCTION</td>
<td>1</td>
</tr>
<tr>
<td>Hypothesis</td>
<td>1</td>
</tr>
<tr>
<td>Specific Aims</td>
<td>1</td>
</tr>
<tr>
<td>AIM 1: Determine current oxidative stress and inflammatory status of renal patients</td>
<td>1</td>
</tr>
<tr>
<td>AIM 2: Supplement patients evaluated in AIM 1 with vitamins E and reassess oxidative stress and inflammatory status</td>
<td>2</td>
</tr>
<tr>
<td>LITERATURE REVIEW</td>
<td>3</td>
</tr>
<tr>
<td>Introduction</td>
<td>3</td>
</tr>
<tr>
<td>Oxidative Stress and Lipid Peroxidation</td>
<td>4</td>
</tr>
<tr>
<td>Oxidative Stress and Inflammation</td>
<td>4</td>
</tr>
<tr>
<td>Atherosclerosis as an Inflammatory Disease</td>
<td>6</td>
</tr>
<tr>
<td>Vitamin E</td>
<td>7</td>
</tr>
<tr>
<td>Antioxidant properties and structures</td>
<td>7</td>
</tr>
<tr>
<td>Vitamin E Absorption, Lipoprotein Transport and Plasma Regulation</td>
<td>8</td>
</tr>
<tr>
<td>Vitamin E Metabolites</td>
<td>9</td>
</tr>
<tr>
<td>Vitamin E Non-Antioxidant Functions</td>
<td>9</td>
</tr>
<tr>
<td>Renal Patients and Vitamin E</td>
<td>10</td>
</tr>
<tr>
<td>VITAMINS E AND C IN PATIENTS WITH END-STAGE RENAL DISEASE UNDERGOING HEMODIALYSIS</td>
<td>12</td>
</tr>
<tr>
<td>Abstract</td>
<td>12</td>
</tr>
<tr>
<td>Introduction</td>
<td>14</td>
</tr>
<tr>
<td>Section</td>
<td>Page</td>
</tr>
<tr>
<td>------------------------------------------------------------------------</td>
<td>------</td>
</tr>
<tr>
<td>Materials and Methods</td>
<td>15</td>
</tr>
<tr>
<td>Subjects</td>
<td>15</td>
</tr>
<tr>
<td>Materials</td>
<td>16</td>
</tr>
<tr>
<td>Methods</td>
<td>16</td>
</tr>
<tr>
<td>Analytical Techniques</td>
<td>18</td>
</tr>
<tr>
<td>Statistical Analysis</td>
<td>20</td>
</tr>
<tr>
<td>Results</td>
<td>21</td>
</tr>
<tr>
<td>Dietary Vitamins C and E</td>
<td>21</td>
</tr>
<tr>
<td>Plasma α-Tocopherol and γ-Tocopherol Concentrations</td>
<td>21</td>
</tr>
<tr>
<td>Plasma α-CEHC and γ-CEHC Concentrations</td>
<td>24</td>
</tr>
<tr>
<td>Plasma Ascorbic Acid and Uric Acid Concentrations</td>
<td>24</td>
</tr>
<tr>
<td>Plasma Markers of Inflammation</td>
<td>27</td>
</tr>
<tr>
<td>Plasma F₂-Isoprostane Concentrations</td>
<td>30</td>
</tr>
<tr>
<td>Discussion</td>
<td>33</td>
</tr>
<tr>
<td>CONCLUSIONS</td>
<td>36</td>
</tr>
<tr>
<td>BIBLIOGRAPHY</td>
<td>37</td>
</tr>
<tr>
<td>APPENDICES</td>
<td>43</td>
</tr>
</tbody>
</table>
# LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Dietary Vitamins C and E</td>
<td>22</td>
</tr>
<tr>
<td>2. Plasma α- and γ-tocopherols</td>
<td>23</td>
</tr>
<tr>
<td>3. Serum α- and γ-CEHC</td>
<td>25</td>
</tr>
<tr>
<td>4. Plasma Ascorbic Acid</td>
<td>26</td>
</tr>
<tr>
<td>5. Plasma Uric Acid Concentrations</td>
<td>28</td>
</tr>
<tr>
<td>6. Markers of Inflammation</td>
<td>29</td>
</tr>
<tr>
<td>7. Changes in Plasma F₂-Isoprostanes During Dialysis</td>
<td>31</td>
</tr>
<tr>
<td>8. Free Plasma F₂-Isoprostanes</td>
<td>32</td>
</tr>
</tbody>
</table>
# LIST OF APPENDICES

<table>
<thead>
<tr>
<th>Appendix</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. Vitamin E: Chain Breaking Antioxidant</td>
<td>45</td>
</tr>
<tr>
<td>B. Cytokine Cascade</td>
<td>46</td>
</tr>
<tr>
<td>C. Free Radical Attack on Arachidonic Acid</td>
<td>47</td>
</tr>
<tr>
<td>D. Naturally Occuring Tocopherols</td>
<td>48</td>
</tr>
<tr>
<td>E. Vitamin E Metabolites</td>
<td>49</td>
</tr>
<tr>
<td>F. OSU Committee for the Protection of Human Subjects-Approval</td>
<td>50</td>
</tr>
<tr>
<td>F. Good Samaritan Hospital Institutional Review Board-Approval</td>
<td>51</td>
</tr>
<tr>
<td>G. Consent Form</td>
<td>52</td>
</tr>
<tr>
<td>H. Adverse Effect Form</td>
<td>55</td>
</tr>
</tbody>
</table>
Vitamins E and C in Patients with End-Stage Renal Disease Undergoing Hemodialysis

INTRODUCTION

HYPOTHESIS

Inflammation associated with renal disease is caused by oxidative stress. Antioxidant administration may confer health benefits by decreasing inflammatory responses.

SPECIFIC AIMS

AIM 1: Determine current oxidative stress and inflammatory status of renal patients.

Plasma antioxidants (ascorbic acid, α- and γ-tocopherols), vitamin E metabolites (α- and γ-CEHCs), and a marker of lipid peroxidation (F₂-isoprostanes) were measured before and after a dialysis session on two occasions to establish baseline oxidative stress status in twelve renal dialysis patients. Inflammatory markers, including TNF-α, IL-6, and CRP were assessed before and after dialysis.
AIM 2: Supplement patients evaluated in AIM 1 with vitamins E and reassess oxidative stress and inflammatory status.

The patients were supplemented daily with vitamin E (400 IU) for two months. Oxidative stress and inflammation status parameters described above before and after a dialysis session were reassessed following one month and two months of supplementation.
LITERATURE REVIEW

INTRODUCTION

Renal patients are particularly at risk for developing chronic diseases (1). Diabetes and a lifetime of poor dietary and lifestyle habits are likely causes of kidney failure (2). Kidney failure patients undergo high physiological stress, and the inability of the patient to excrete waste results in accumulation of metabolic byproducts, reactive oxidative species (ROS), and oxidation products. The presence of these toxic, reactive chemicals in the blood stream increases the risk for developing heart disease, cancer, diabetes, Alzheimer's disease, and other chronic diseases (3). The prospect for the patient to develop these chronic diseases is intensified by lack of compliance with dialysis procedures administered three times a week.

An association between acute-phase response and increased prevalence of carotid plaques suggests that there is a relationship between inflammation and atherosclerosis (3, 4). In particular, elevated levels of C-reactive protein (CRP) have been reported in patients with chronic renal failure (3, 5, 6). Bistrian (5) indicated that increased levels of tumor necrosis factor-α (TNF-α) before dialysis suggest that this is an important risk factor in atherogenesis.

Another correlation was drawn between the elevated levels of oxidized LDL, a major component of atherosclerotic lesion, and chronic renal failure (3, 4).
Stenvinkel et al. (3), and Handelman et al. (6) suggest an association between inflammation and oxidative stress.

**OXIDATIVE STRESS AND LIPID PEROXIDATION**

Physiological and pathological pathways generate reactive oxygen species. At rest, the body continuously produces reactive oxygen species (ROS) (7). These oxygen-containing labile molecules include hydrogen peroxide, superoxide, and hydroxyl radicals. Normally, ROS are produced within the bounds of antioxidant defenses. Under increased stress, including inflammation, chemically or physically-induced damage, and nutritional imbalances, the body begins to produce excessive amounts of ROS, leading to lipid peroxidation, nucleic acid oxidation, and protein oxidation.

Lipid peroxidation of mono- and polyunsaturated fatty acids result in the loss of an electron to form a lipid radical, which can be transformed to a lipid peroxyl radical in the presence of molecular oxygen (7). Lipid peroxyl radicals can attack other unsaturated lipids forming more lipid radicals as well as lipid hydroperoxide. This continuing cycle is known as the radical chain reaction. See Appendix A: Vitamin E: Chain Breaking Antioxidant.

**OXIDATIVE STRESS AND INFLAMMATION**

Physiological stress initiates the release of ROS from monocytes and macrophages. Increasing levels of TNF-α and interleukin-1 (IL-1) produced by monocytes/macrophages, activate the cytokine cascade (5). Systemic inflammatory
response (SIR) results, and in turn, performs a pathological role in inflammatory diseases, including atherosclerosis and some forms of cancer.

The cytosine activation also produces interleukin-6 (IL-6), which is primarily responsible for acute-phase protein synthesis in the liver (5). Many of the reactants produced, including CRP and fibrinogen, are beneficial for a short term. Yet, prolonged production of acute-phase proteins can be harmful. See Appendix B: Cytokine Cascade.

In the American diet where linoleic acid contributes between 8-10% of caloric intake, the biosynthesis of arachidonic acid is favored (8). Dietary linoleic acid is converted to arachidonic acid. Excessive intakes of linoleic acid may increase oxidative stress and the pathophysiological actions that occur as a result (8). Free-radical attack on arachidonic acid will initiate a series of reactions to produce F2-isoprostanes. These compounds can be as markers of oxidative stress (1, 8). Unlike other oxidative stress markers, F2-isoprostanes are chemically stable and not vulnerable to further attack by free radicals (1). See Appendix C: Free Radical Attack on Arachidonic acid Produces Isoprostanes.

There may be a link between inflammation and oxidative stress. Handelman et al. (6) reported elevated, yet inconsistent, esterified F2-isoprostanes in hemodialysis patients. Variations may be explained by a chronic oxidant stress from frequent dialysis or other circumstances outside of the dialysis procedure itself. Elevations in inflammatory markers such as CRP, IL-6, and TNF-α in dialysis patients has been reported (3, 5, 6), but responses have not been consistent. Handelman et al. (6) reported that while patients with ESRD have elevated CRP, some patients had values similar to the control group (p<0.02). Similar results were found with plasma F2-isoprostane values. However, CRP was significantly
correlated with elevated F2-isoprostanes ($p=0.015$), suggesting chronic inflammation is a possible contributor to oxidative stress.

**ATHEROSCLEROSIS AS AN INFLAMMATORY DISEASE**

Atherosclerosis is an inflammatory disease and a precursor to plaque rupture and thrombosis (9). Low density lipoproteins (LDL), at high concentrations in the bloodstream, infiltrate the endothelial membrane of the arterial wall. In the intimal lining, LDL become oxidized (10). Oxidation initially has little effect on the protein portion of the molecule known as apolipoprotein B (apoB), the LDL is only minimally oxidized (mmLDL) (11). The mmLDL acts as a signal to recruit monocytes from the bloodstream. The trapped monocytes differentiate becoming macrophages. Macrophages have scavenger receptors that recognize the oxidized apoB on the LDL molecule. These receptors differ from the LDL receptors in that they are not down regulated, resulting in unregulated cholesterol uptake. The macrophage fills with cholesterol forming foam cells that accumulate in the intimal lining. Highly oxidized LDL is toxic to macrophages, causing the macrophages die, and the ensuing necrosis magnifies the inflammatory response. The dead macrophages, leave behind lipid droplets that are consumed both by macrophages and by smooth muscle cells forming new foam cells (12). The lesion grows into the adventitia of the artery wall until it can no longer expand. Plaque rupture may occur after calcification, leading to platelet aggregation, thrombosis and eventually a myocardial infarction (13).
VITAMIN E

Antioxidant properties and structures

Vitamin E is a fat-soluble nutrient mostly known for its antioxidant properties. The lipophilic nature of vitamin E allows for its transportation in plasma lipoproteins and partitions to membranes and fat-storage sites. Its presence in plasma lipoproteins and in membrane phospholipids helps to protect polyunsaturated fatty acids stops free radical chain reactions.

Vitamin E acts as a chain-breaking molecule, thereby preventing the autoxidation of lipids (7). Peroxyl radicals react with vitamin E faster than polyunsaturated fatty acids. The peroxyl radical and the phenolic hydroxyl group of α-tocopherol react to form hydroperoxide and a tocopheroxyl radical. Reduction of the tocopheroxyl radical is dependent on the presence of other antioxidants. Vitamin C acts to keep vitamin E in the reduced state so that it may continue to scavenge free radicals and prevent tissue damage (14, 15).

There are eight forms of vitamin E, each having its own antioxidant capabilities: α-tocopherol, β-tocopherol, γ-tocopherol, δ-tocopherol, α-tocotrienol, β-tocotrienol, γ-tocotrienol, and δ-tocotrienol (16). The tocopherols have saturated side chains and the tocotrienols have unsaturated side chains. Foods of animal origins contain mainly α-tocopherol, whereas γ-tocopherols are found in high concentrations in many vegetable oils, such as corn and soybean oils, and also in margarine (16, 17).

The potential benefits of vitamin E as a protector against oxidative stress allowed for the RDA to be increased in the year 2000 to 15 mg (18). Alpha-
tocopherol is synthesized for use in supplements, and the racemic mixture of RRR, RSR, RRS, RSS, SRS, SRR, and SSR is commonly sold as dl-α-tocopherol (19). Only 2R-stereoisomers of α-tocopherol are included in the RDA as they are the only forms of α-tocopherol that can be maintained in the plasma (18). The biological activity of vitamin E depends on the stereospecificity of the tocopherol transfer protein (18, 21). Only RRR-α-tocopherol (d-α-tocopherol) is found naturally. In racemic vitamin E supplements, only 12.5% is found in this natural form (20). See Appendix D: Naturally Occurring Tocopherols.

**Vitamin E Absorption, Lipoprotein Transport and Plasma Regulation**

Tocopherols are incorporated into chylomicrons and are transported with them through the lymph to the hepatic circulation (22). In the liver, only α-tocopherol is preferentially secreted into the plasma in VLDL. During VLDL lipolysis, α-tocopherol is transferred to all of the circulating lipoproteins. The lipoproteins transport α-tocopherol to the tissues.

The human body prefers the RRR-α-tocopherol to all other forms of vitamin E, a result of the function of the hepatic α-TTP (tocopherol transfer protein) (16, 23). The ratio of α- to γ-tocopherol is 1:5 in the diet and 10:1 in the human body. Although γ-tocopherol is absorbed from the diet and taken up by the liver, it is not preferentially secreted into the plasma by the liver (23). The failure of post-absorptive packaging of the γ-tocopherol reduces its physiological availability.
While in the LDL molecule, α-tocopherol protects the molecule from oxidation, which could lead to oxidative stress and the chronic diseases that result (24).

Vitamin E has no specific storage site, although it is found in large concentrations in the adipose tissue.

**Vitamin E metabolites**

Human urine contains vitamin E metabolites of α–tocopherol (α–CEHC) and of γ–tocopherol (γ-CEHC). Both of these metabolites result from truncations of the phytol tail; they are not a result of vitamin E antioxidant activity (24). Surprisingly, γ-CEHC is a natriuretic factor (25), while α–CEHC is not (26).

Swanson et al. (27) have estimated that ~50% of γ–tocopherol is converted to γ–CEHC, while a study by Traber et al. (28) demonstrated that only a small percent of α–tocopherol is converted to α–CEHC. It was also shown that more all rac compared with RRR-α–tocopherol is converted to α–CEHC (28). See Appendix E: Vitamin E Metabolites.

**Vitamin E Non-Antioxidant Functions**

Vitamin E may also play a role in inflammation and platelet function (29). Alpha-tocopherol inhibits platelet adhesion, aggregation, thrombin, and release reactions, but also down-regulates the expression of adhesion molecules, which decrease the adhesion of monocytes to the endothelium (29-33). Resulting benefits
of both these actions by α-tocopherol show a potential mechanism for decreasing lesion volume and neurological impairment from stroke (34).

For hemodialysis patients, supplemental vitamin E could be recommended to prevent inadequate dietary intakes. Studies show that higher doses of α-tocopherol result in greater plasma concentrations (4, 35). The study on the secondary prevention with antioxidants of cardiovascular disease in end-stage renal disease (SPACE) demonstrated that vitamin E supplemented hemodialysis patients had significantly fewer primary and secondary cardiovascular disease endpoints and fewer myocardial infarctions than those in the placebo group (4).

RENA L PAT IENTS AND VITAMIN E

Oxidative stress in end-stage renal patients may result from decreased plasma antioxidants, increased oxidation of VLDL and LDL, increased activation of oxidative processes in leukocytes, or increased platelet aggregation (36, 37). Prevailing evidence demonstrating that oxidative stress is associated with increased cardiovascular morbidity and mortality allow for antioxidant research opportunities.

Supplementation with vitamin C in renal patients is strongly cautioned, however, due to increasing evidence of oxalate formation (37). Ascorbic acid forms oxalate can be excreted in normal healthy people but renal patients are often unable to excrete waste through urine. The accumulation of oxalate in the plasma may increase its deposition into body tissues, including the liver, kidney, and cardiovascular system.
Alpha-tocopherol's potential benefits in renal patients relate to the decrease in oxidative stress and inflammation. The SPACE trial demonstrated a significant decrease in LDL oxidation following supplementation with 800 IU of vitamin E a day for 2 years (4). Researchers from that study concluded that the inhibition of proatherogenic events is attributed to the high-dose supplemental vitamin E. Another study showed that supplementation of α-tocopherol in renal patients increases the vitamin E content of lipoproteins, thereby enhancing lipoprotein protection from oxidation (36).

We determined the current oxidative stress status of renal patients by measuring plasma antioxidants (ascorbic acid, α- and γ-tocopherols), vitamin E metabolites (α- and γ-CEHC), and a marker of lipid peroxidation (F_2-isoprostanes) before and after a dialysis session on two occasions to establish baseline oxidative stress status. Inflammatory markers (TNF-α, IL-1, IL-6, and CRP) were assessed before and after dialysis. Then, patients were supplemented daily with vitamin E (400 IU) for two months. Oxidative stress and inflammation status parameters described above before and after a dialysis session were reassessed following one month and two months of supplementation.
VITAMINS E AND C IN PATIENTS WITH END-STAGE RENAL DISEASE UNDERGOING HEMODIALYSIS

Kylie Sherée Smith¹, James W. Ridlington¹, Scott W. Leonard², Sredevi Devaraj³, Ishwarlal Jialal³, and Maret G. Traber¹,²,⁴

¹Department of Nutrition and Food Management, ²Linus Pauling Institute, Oregon State University, Corvallis OR 97331, ³Center for Human Nutrition and Division of Clinical Biochemistry and Human Metabolism, University of Texas Southwestern Medical Center, Dallas TX 75235, and the ⁴Department of Internal Medicine, University of California, Davis, School Of Medicine, Sacramento, California 95817

Address for Correspondence:
Maret G. Traber, Ph.D.
Department of Nutrition and Food Management
Linus Pauling Institute
571 Weniger Hall
Oregon State University
Corvallis, OR 97331-6512
maret.traber@orst.edu
ABSTRACT

This study evaluated oxidative stress and inflammatory markers in patients undergoing hemodialysis before and during vitamin E supplementation. On two occasions prior to, and at one and two months of supplementation (400 IU RRR-α-tocopherol daily), blood samples were obtained before and after dialysis. In response to vitamin E supplementation, plasma α-tocopherol concentrations increased from 18 ± 1.7 μM to 31 ± 5.4 μM (p<0.0001), while gamma-tocopherol concentrations decreased from 2.8 ± 1.0 μM to 1.7 ± 0.6 μM (p=0.001). Serum vitamin E metabolites also increased, α-CEHCs increased from 68.5 ± 20 pmol/ml to 771 ± 160.9 (p<0.0001), while γ-CEHCs increased from 837 ± 161.8 pmol/ml to 1136 ± 225.9 (p=0.0083). Dietary antioxidants (vitamins E and C) were low in most subjects; thus, plasma ascorbic acid levels were low in most subjects, but high in a few, resulting a wide range of responses (88 ± 84 μM). Nonetheless, ascorbic acid concentrations decreased significantly after dialysis to 33 ± 34 μM (p=0.0124), but were unaffected by vitamin E supplementation. Both IL-6 and F2-isoprostane concentrations were elevated in the subjects but were unaffected by either vitamin E supplementation or dialysis. CRP increased significantly after dialysis (p=0.0161, ANOVA main effect), but in the vitamin E supplemented subjects CRP concentrations were slightly lower before dialysis, but increased following dialysis (p=0.0041, ANOVA interaction). Taken together, the data suggest that there is a complex relationship between chronic inflammation and oxidative stress.
INTRODUCTION

Renal patients are particularly at risk for developing chronic diseases (1). Diabetes and a lifetime of poor dietary and lifestyle habits are likely causes of kidney failure (2). Kidney failure patients undergo high physiological stress, and the inability of the patient to excrete waste results in accumulation of metabolic byproducts, reactive oxidative species (ROS), and oxidation products. The presence of these toxic, reactive chemicals in the blood stream increases the risk for developing heart disease, cancer, diabetes, Alzheimer's disease, and other chronic diseases (3). The prospect for the patient to develop these chronic diseases is intensified by lack of compliance with dialysis procedures carried out three times a week.

An association between acute-phase response and increased prevalence of carotid plaques suggests that there is a relationship between inflammation and atherosclerosis (3, 4). In particular, elevated levels of C-reactive protein (CRP) have been reported in patients with chronic renal failure (3, 5, 6). TNF-\( \alpha \) concentrations were also elevated prior to dialysis suggesting that this is an important risk factor in atherogenesis (5).

Another correlation was drawn between the elevated levels of oxidized LDL, a major component of atherosclerotic lesion, and chronic renal failure (3, 4). Stenvinkel et al (3), and Handelman et al. (6) suggest that an association between inflammation and oxidative stress.

Therefore, we determined the current oxidative stress status of renal patients by measuring plasma antioxidants (ascorbic acid, \( \alpha \)- and \( \gamma \)-tocopherols), vitamin E metabolites (carboxyethyl-hydroxychromans (\( \alpha \)- and \( \gamma \)-CEHC), metabolites of \( \alpha \)-
and γ-tocopherols, respectively), and a marker of lipid peroxidation (F2-isoprostanes) before and after a dialysis session on two occasions to establish baseline oxidative stress status. Inflammatory markers, including TNF-α, IL-6, and CRP, were assessed before and after dialysis. Then, patients were supplemented daily with vitamin E (400 IU) for two months. Oxidative stress and inflammation status parameters described above before and after a dialysis session were reassessed following one month and two months of vitamin E supplementation.

MATERIALS AND METHODS

Subjects

The Oregon State University and the Good Samaritan Hospital, Corvallis, Institutional Review Boards for the Protection of Human Subjects approved the protocol for this study (Appendix F). Twelve subjects undergoing renal dialysis were chosen from Good Samaritan Hospital Dialysis Unit in Corvallis, Oregon. Each subject provided signed consent. (Appendix G: Consent to Participate in a Research Study on Antioxidants and Renal Patients.)

Patients with good compliance with dialysis procedures were selected for study. Inclusion criteria also included stable body weight (between 80% and 130% ideal body weight) and willingness to maintain normal activity patterns. Prior to the study, patients did not consume large doses of antioxidant supplements (vitamin C, vitamin E, and carotenoids), did not have a resting blood pressure above 160/105 mm Hg, did not have excessive alcohol consumption (routine consumption
of more than 3 alcoholic beverage servings per day or more than 10 per week), and
did not have a fasting blood glucose concentration greater than 7.77 mmol/l
(140mg/dl). Subject characteristics are shown in Table 1. On the Diet/Medical
History form, patients identified their current supplement use. Some subjects were
taking Nephrovite and were instructed to continue taking it as usual. Nephrovite
contains 60 mg of ascorbic acid.

One adverse event occurred during our study. Before the third blood draw,
one of our subjects died from a heart attack while under hospital care.
Investigation into the subject's death and discussion with the subject's physician
indicated that vitamin E supplementation was not likely to have increased the risk
of a heart attack. See Appendix H: Adverse Effect Form.

Materials

RRR-α-tocopherol capsules were a gift from the Archer Daniels Midland
Inc. in Decatur, Illinois.

Methods

To evaluate the oxidative stress and inflammatory status of the subjects,
blood was obtained on two occasions prior to intervention. Approximately 8 ml of
blood was obtained from dialysis tubing and collected into ethylene diamine tetra
Table 1. Subject Characteristics

<table>
<thead>
<tr>
<th>Subject</th>
<th>Age (y)</th>
<th>Weight (kg)</th>
<th>Height (cm)</th>
<th>Gender</th>
<th>Cause of Renal Failure</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>54</td>
<td>47.7</td>
<td>162.6</td>
<td>F</td>
<td>Diabetes</td>
</tr>
<tr>
<td>2</td>
<td>66</td>
<td>80.0</td>
<td>167.6</td>
<td>F</td>
<td>Adverse Reaction to Medication</td>
</tr>
<tr>
<td>3</td>
<td>60</td>
<td>72.7</td>
<td>162.6</td>
<td>F</td>
<td>Diabetes</td>
</tr>
<tr>
<td>4</td>
<td>53</td>
<td>81.8</td>
<td>122.7</td>
<td>M</td>
<td>Diabetes</td>
</tr>
<tr>
<td>5</td>
<td>42</td>
<td>77.7</td>
<td>152.4</td>
<td>F</td>
<td>Hypertension</td>
</tr>
<tr>
<td>6</td>
<td>70</td>
<td>49.1</td>
<td>163.8</td>
<td>F</td>
<td>Hypertension</td>
</tr>
<tr>
<td>7</td>
<td>73</td>
<td>79.5</td>
<td>188.0</td>
<td>M</td>
<td>Hypertension</td>
</tr>
<tr>
<td>8</td>
<td>60</td>
<td>59.1</td>
<td>167.6</td>
<td>M</td>
<td>Polycystic</td>
</tr>
<tr>
<td>9</td>
<td>75</td>
<td>72.7</td>
<td>177.8</td>
<td>M</td>
<td>Hypertension</td>
</tr>
<tr>
<td>10</td>
<td>73</td>
<td>113.6</td>
<td>172.7</td>
<td>M</td>
<td>Diabetes</td>
</tr>
<tr>
<td>11</td>
<td>81</td>
<td>70.5</td>
<td>177.8</td>
<td>M</td>
<td>Unknown</td>
</tr>
</tbody>
</table>

Mean: 64.3 (y) 73.1 (kg) 169.6 (cm) 5F/6M

± Std. Dev.: 11.6 (y) 18.0 (kg) 9.6 (cm)
acetic acid (EDTA) tubes (Becton Dickinson) before and after dialysis on Day 0 and Day 14 of the study. Subjects were then instructed to consume one 400 IU RRR-α-tocopherol supplement (Archer Daniels Midland Company, Decatur, IL) daily with dinner for 60 days. EDTA blood samples were obtained at 30 days and 60 days after the start of supplementation. Blood sampling was identical as described above for baseline studies.

Dietary and supplement intakes were assessed using two 24-hour recalls. Subjects were asked to identify their dietary intakes in a 24-hour recall before supplementation. The 24-hour recalls were analyzed by ESHA’s food processor (Salem, Oregon). Data obtained from the analysis was used to establish dietary antioxidant intake of the study subjects.

Analytical Techniques

The plasma samples were analyzed for vitamin E, vitamin C, F₂-isoprostanes, α- and γ-CEHCs, and markers of inflammation (IL-6, TNF-α, CRP). For vitamin C analysis, 50 μl of the freshly drawn, EDTA plasma was mixed with an equal volume of chilled 5% (wt/vol) metaphosphoric acid in 1 mM diethylenetriamine pentaacetic acid (made fresh daily) and centrifuged to remove the precipitated proteins. A portion of the supernatant was frozen at -80°C until day of analysis-within 2 weeks of sample collection. Plasma ascorbic acid was measured using paired-ion reverse-phase HPLC coupled with electro-chemical detection (38). Ascorbic acid standards (in 1mM diethylene-triaminepenta-acetic acid (DTPA) in PBS) were analyzed before and after each of samples. Results are expressed as μmol/l plasma.
Plasma α- and γ-tocopherols were measured by high pressure liquid chromatography (HPLC) using electrochemical detection according to the method of Podda et al. (39), with the exception that only the isocratic mobile phase was used for the HPLC system. Plasma tocopherol concentrations are expressed as μmol/l.

For measurements of CEHCs, plasma was converted into serum as described by Bersot et al. (40). To 1 ml of plasma, 10 μl of 1.2 NIH units/μl thrombin was added. After 30 minutes the tube was centrifuged and the serum removed. Serum α- and γ-CEHCs were extracted as described by Stahl et al. (41) and measured by high pressure liquid chromatography (HPLC) with electrochemical detection according to Lodge et al (42). Serum metabolites are expressed as pmol/ml.

An enzyme immunoassay (EIA) from Cayman Chemical (Ann Arbor, MI) was used to measure plasma isoprostanes. Plasma (1 ml) was frozen in liquid nitrogen and stored at -80°C until time of analysis. To 1 ml of thawed plasma, 9 ml water, pH 3.0, was added, along with 25 μl of 3H-labeled isoprostanes. Samples were purified on C18 SPE Cartridges (Sep-Pak, Waters). Free isoprostanes were eluted with ethyl acetate/methanol (1:1), dried under nitrogen and resuspended in EIA buffer; an aliquot was counted with a Liquid Scintillation Counter to determine percent recovery. The remaining sample was aliquoted onto an ELISA plate (Cayman Kit) in quadruplicate wells, the plate incubated 10-12 hours at room temperature, then developed with Ellman’s Reagent and read with a Molecular Devices SpectraMax 190 Microplate Reader (PerSeptive Biosystems CytoFluor®); wavelength 405 nm. Data were analyzed according to kit instructions.
Cytokines IL-6, TNF-α, and CRP were measured using immunoassay as reported previously by Devaraj et al (43). Both IL-6 and TNF-α values are reported as pg/ml and CRP values are reported as mg/l.

**Statistical Analysis**

Pre- and post-supplementation parameters were compared with ANOVA with repeated measures. Statview (SAS Institute, Cary, NC) statistical software will be used for most analyses, particularly PROC MIXED for analysis of variance models. Data are reported as mean ± standard deviation.
RESULTS

Dietary Vitamins C and E

The subjects undergoing dialysis did not consume many antioxidant-rich foods. Median vitamin C intakes were 106.1 mg/d while mean intakes were 136.6 ± 132.6 mg/d (Figure 1) with values ranging from 1.1 to 454. Based on the two 24-hour recalls, 41% of the subjects consumed less than 75% of the RDA for vitamin C (75 mg for women and 90 mg for men), and 77% of the subjects consumed less than 150 mg/d.

Mean vitamin E intakes were 10.6 ± 8.4 mg/d while median intakes were 8.2 mg/d with values ranging from 1.2 to 38. 68% of subjects consumed less than 75% of the RDA for vitamin E (15 mg α-tocopherol).

Plasma α-Tocopherol and γ-Tocopherol Concentrations

Prior to vitamin E supplementation, most subjects had low plasma vitamin E concentrations (Figure 2). The dialysis procedure had no effect on vitamin E concentrations either before or during supplementation. Plasma α-tocopherol concentrations increased with vitamin E supplementation from 18 ± 1.7 μM to 31 ± 5.4 μM (p<0.0001), while gamma-tocopherol concentrations decreased from 2.8 ± 1.0 μM to 1.7 ± 0.6 μM (p=0.001).
Figure 1. Dietary Vitamins C and E

Shown are the dietary vitamin C and E intakes in subjects before vitamin E supplementation. The circle indicates mean, the line median, the box 90% interval and the vertical lines the range of values.
Figure 2. Plasma α- and γ-tocopherols

Shown are the plasma concentrations in subjects before and during vitamin E supplementation. No statistically significant differences were found before and after dialysis irrespective of supplementation status, so these data were averaged for each subject. The averages for each subject were used to generate the mean ± SD shown.
Plasma \( \alpha \)-CEHC and \( \gamma \)-CEHC Concentrations

Prior to vitamin E supplementation, serum \( \alpha \)-CEHC concentrations were decreased by dialysis from 68 ± 20 pmol/ml and 57 ± 18 pmol/ml \((p<0.0007)\). Vitamin E supplementation increased serum \( \alpha \)-CEHC concentrations significantly \((Figure 3. \ p<0.0001)\). These concentrations were decreased significantly \((771 ± 161 \text{ pmol/ml and } 682 ± 140 \text{ pmol/ml}) \) \((Figure 3. \ p=0.0007)\). Unlike serum \( \alpha \)-CEHC concentrations, \( \gamma \)-CEHC concentrations were unchanged by dialysis. They were, however, increased with vitamin E supplementation \((Figure 3, \ p=0.0080)\). Prior to vitamin E supplementation, \( \gamma \)-CEHC concentrations before and after dialysis were 837 ± 162 pmol/ml and 835 ± 142, respectively; during vitamin E supplementation, they were 1136 ± 226 pmol/ml and 1187 ± 243, respectively.

Plasma Ascorbic Acid and Uric Acid Concentrations

Water-soluble plasma antioxidants, ascorbic acid and uric acid, were also measured. Plasma ascorbic acid concentrations were variable, and some subjects were at sub-optimal concentrations. Four subjects had plasma ascorbic acid levels less than 40 \( \mu \text{M} \) prior to dialysis. All but one subject’s plasma ascorbic acid concentrations were depleted to less than 40 \( \mu \text{M} \) after dialysis. The ascorbic acid concentrations were significantly \((p=0.0124)\) decreased by dialysis from 88 ± 84 \( \mu \text{M} \) and 33 ± 34 \((Figure 4)\). During vitamin E supplementation, ascorbic acid concentrations before and after dialysis were 59 ± 54 \( \mu \text{M} \) and 21 ± 12, respectively.
Figure 3. Serum α- and γ-CEHC

A shows serum α-CEHC concentrations before and after dialysis, before and during supplementation. B shows serum γ-CEHC concentrations before and during supplementation. The averages for each subject were used to generate the mean ± SE shown.
Figure 4. Plasma Ascorbic Acid

Shown are the plasma concentrations prior to and post-dialysis in subjects before and during vitamin E supplementation. The circle indicates mean, the line median, the box 90% interval and the vertical lines the range of values.
Plasma uric acid levels were within the normal range (423 ± 84 μM) in the subjects and following dialysis decreased significantly (103 ± 21 (p<0.0001, ANOVA main effect)) (Figure 5). During vitamin E supplementation, uric acid concentrations before and after dialysis were 325 ± 63.8 μM and 104 ± 54, respectively. Uric acid concentrations were decreased significantly after vitamin E supplementation (p=0.0003).

**Plasma Markers of Inflammation**

Plasma CRP, TNF-α, and IL-6 concentrations are shown in Figure 6.

CRP increased significantly after dialysis (p=0.0161, ANOVA main effect). CRP concentrations before and after dialysis were 9.4 ± 9.7 mg/l and 10.4 ± 11.2, respectively. During vitamin E supplementation, CRP concentrations before and after dialysis were 8.1 mg/l ± 8.0 and 9.6 ± 9.6, respectively. Thus, CRP concentrations were slightly lower before dialysis in the vitamin E supplemented subjects, but increased following dialysis irrespective of vitamin E supplementation status (p=0.0041, ANOVA interaction).

TNF-α decreased significantly after dialysis (p=0.0098). Prior to vitamin E supplementation, TNF-α concentrations before and after dialysis were 4.1 ± 0.8 pg/ml and 3.8 ± 0.9 respectively. During vitamin E supplementation before and after dialysis, they were 4.1 ± 0.9 pg/ml and 3.5 ± 1.1, respectively.

Neither dialysis nor vitamin E supplementation affected IL-6 concentrations. Before vitamin E supplementation, IL-6 concentrations before and after dialysis were 20.4 ± 16.6 pg/ml and 19.0 ± 15.3, respectively. During vitamin
Figure 5. Plasma Uric Acid Concentrations

Shown are the plasma concentrations prior to and post-dialysis in subjects. No statistically significant differences were found between the two testing periods, so these data were averaged for each subject for the with and without vitamin E supplementation trials. The averages for each subject were used to generate the mean ± SD shown.
Figure 6. Markers of Inflammation

Shown are the plasma concentrations prior to and post-dialysis in subjects before and during vitamin E supplementation.
E supplementation, the concentrations before and after dialysis were 19.3 ± 13.3 pg/ml and 17.7 ± 13.8, respectively.

**Plasma F\(_2\)-Isoprostane Concentrations**

Plasma F\(_2\)-Isoprostanes were not significantly affected by dialysis or by vitamin E supplementation (Figure 7). F\(_2\)-Isoprostanes were elevated in the subjects, with concentrations of 1088 ± 1041 pg/ml before dialysis and 728 ± 447 pg/ml after dialysis (Figure 8). All but one subject consistently had plasma isoprostanes above 200 pg/ml.
Plasma F₂-Isoprostanes were not significantly affected by dialysis or by vitamin E supplementation. Diagram A shows individual data from the 11 subjects prior to vitamin E supplementation, and B shows the same subjects after 60 days of vitamin E supplementation.
Free plasma F$_2$-Isoprostanes were elevated with concentrations of 1077.6 ± 180.1 pg/ml before dialysis and 733.5 ± 124.2 pg/ml after dialysis. All but one subject consistently had plasma isoprostanes above 200 pg/ml.
DISCUSSION

This study assessed oxidative stress in hemodialysis patients through measurements of plasma antioxidants (\(\alpha\)-and \(\gamma\)-tocopherol, ascorbic acid, and uric acid), vitamin E metabolites (\(\alpha\)-and \(\gamma\)-CEHCs), and a marker of lipid peroxidation (free plasma F\(_2\)-isoprostanes). Plasma \(\alpha\)-tocopherol levels significantly increased with vitamin E (\(\alpha\)-tocopherol) supplementation, doubling plasma concentrations in all but one subject. During supplementation, plasma \(\gamma\)-tocopherol concentrations fell, as was previously reported by Handelman et al. (44). These data confirm that the subjects consumed the vitamin E supplements.

Vitamin E metabolites have been suggested by Brigelius-Flohé's laboratory (24, 45) to be a marker of vitamin E adequacy. They observed that urinary \(\alpha\)-CEHC increased when vitamin E supplements were consumed. We found in all subjects that serum \(\alpha\)- and \(\gamma\)-CEHC concentrations increased with supplementation. Serum vitamin E metabolites in renal dialysis patients were significantly higher (\(p<0.0001\) for \(\alpha\)-CEHC) than previously reported by Stahl et al. (41), who found in normal subjects that \(\alpha\)- and \(\gamma\)-CEHCs increased with 500 IU RRR-\(\alpha\)-tocopherol supplementation from 5-10 pmol/ml to 200 for \(\alpha\)-CEHC and from 50 pmol/ml to 80 for \(\gamma\)-CEHC. We found that that prior to vitamin E supplementation, \(\alpha\)-CEHC concentrations were \(-69\) pmol/ml and increased to 771, \(\gamma\)-CEHC were at 837 and increased to 1136. Neither plasma tocopherols nor serum \(\gamma\)-CEHCs were affected by dialysis, and \(\alpha\)-CEHCs only changed minimally. Plasma CEHC levels did not continually increase over the course of the supplementation with vitamin E, suggesting that urinary excretion may not be the only route for eliminating vitamin E metabolites from the blood.
Dialysis has a dramatic effect on water soluble components in the blood. Uric acid levels were normal in all subjects before dialysis (46), but decreased significantly after dialysis. Plasma ascorbic acid concentrations varied widely in the subjects and diminished significantly after each dialysis session. These findings are in part due to the wide variation in intakes as well as the depletion during dialysis.

Dietary vitamin C intakes in some subjects were quite low. The values are comparable to ones reported by Kalantar-Zadeh et al. (47) who investigated dietary intakes of hemodialysis patients using the Block Food Frequency Questionnaire. Hemodialysis patients consume low amounts of vitamin C compared to the RDA and to normal controls. Dietary vitamin E intakes in our subjects were also lower than the RDA and similar to results found by Kalantar-Zadeh et al. and were similar to those in normal subjects (47).

Plasma F2-isoprostanes are a marker of in vivo oxidative stress. It is surprising that in these patients F2-isoprostanes were not affected by vitamin E supplementation. Concentrations were elevated in the subjects compared to published norms. Individual dialysis sessions produced inconsistent increases and decreases in free plasma F2-isoprostanes. These findings are similar to results published by Handelman et al. (6) where esterified F2-isoprostanes were measured in hemodialysis patients. The values were elevated, yet did not indicate any particular increase or decrease. They did find that higher levels of CRP correlated with higher levels of F2-isoprostanes, indicating a correlation between oxidative stress and inflammation. We, however, found no such correlation (data not shown).
This study also assessed the inflammatory status of hemodialysis patients, using markers (CRP, TNF-α, and IL-6) that have been reported previously in other studies to be decreased by vitamin E supplementation (3, 5, 6). The data in this study suggest that many dialysis patients have chronic inflammation. In particular, interleukin-6 (IL-6) was elevated in the subjects before vitamin E supplementation. There was no significant effect of vitamin E supplementation or dialysis on circulating IL-6 concentrations. Elevations in TNF-α were not observed in this group of subjects. Dialysis had a significant effect on TNF-α, decreasing its concentration. CRP concentrations were borderline high in these dialysis subjects (6, 43) and a significant increase in CRP was observed after dialysis. There was also a significant interaction between supplementation and dialysis, suggesting that supplementation may have decreased CRP levels, but these were subsequently elevated by the dialysis process.

In summary, dialysis patients have low vitamin E and vitamin C intakes, low circulating antioxidants, and high levels of oxidative stress markers. Vitamin E metabolites did not increase continuously over the two months of supplementation suggesting that vitamin E can be metabolized and excreted effectively in these patients. Inflammatory markers are inconsistently elevated suggesting that oxidative stress is not the only regulator of these concentrations. A 60-day supplementation trial with 400 IU vitamin E was unable to decrease markers of oxidation or inflammation.
Patients undergoing hemodialysis are prone to heart disease and chronic inflammation. The overall health of persons with end-stage renal disease undergoing hemodialysis is not improving with current treatment methods. A combination between low dietary antioxidant intake, chronic inflammation, and oxidative stress from the dialysis procedure compound the health problems. Further research investigating the antioxidant needs of hemodialysis patients could improve our knowledge and the patient’s health. Because vitamin E is a potent antioxidant and has been reported to affect some markers of inflammation, it is possible that it may offer health benefits for this group of people.

Low ascorbic acid levels in hemodialysis patients, particularly after dialysis, may limit antioxidant protection. Hemodialysis patients are instructed to consume restrictive diets that prevents fatal fluctuations in plasma potassium, but also prevent them from obtaining a substantial dietary antioxidant intake. It appears prudent to recommend that patients forego eating fruits and vegetables, but instead consume antioxidant supplements.

The limited subject population in our study resulted in high variability in the data, and could likely be a cause for the lack of significance with vitamin E supplementation in inflammatory markers. Future studies of vitamin E supplementation in hemodialysis patients should include a larger group of subjects and a longer period of supplementation. Other investigations should measure oxalate in hemodialysis patients after vitamin C supplementation and correlate oxalate levels with stroke and related heart disease problems.


# LIST OF APPENDICES

<table>
<thead>
<tr>
<th>Appendix</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. Vitamin E: Chain Breaking Antioxidant</td>
<td>45</td>
</tr>
<tr>
<td>B. Cytokine Cascade</td>
<td>46</td>
</tr>
<tr>
<td>C. Free Radical Attack on Arachidonic Acid</td>
<td>47</td>
</tr>
<tr>
<td>D. Naturally Occuring Tocopherols</td>
<td>48</td>
</tr>
<tr>
<td>E. Vitamin E Metabolites</td>
<td>49</td>
</tr>
<tr>
<td>F. OSU Committee for the Protection of Human Subjects-Approval</td>
<td>50</td>
</tr>
<tr>
<td>F. Good Samaritan Hospital Institutional Review Board--Approval</td>
<td>51</td>
</tr>
<tr>
<td>G. Consent Form</td>
<td>52</td>
</tr>
<tr>
<td>H. Adverse Effect Form</td>
<td>55</td>
</tr>
</tbody>
</table>
Vitamin E Chain Breaking Antioxidant

Initiating Event

Carbon-centered Free Radical

Initiation

Propagation

Termination via Antioxidant


Appendix A: Vitamin E: Chain Breaking Antioxidant
Cytokine Cascade

Vascular sources:
Inflamed atheroma
Hypertension
Aortic aneurysms

Primary Inflammation Cytokines:
IL-1, TNF-α

Extravascular sources:
Adipose tissue
Chronic infections

Messenger Cytokine:
IL-6

LIVER
CRP

Blood Vessel

Adapted from Science Vol. 296 pp. 242
Appendix B: Cytokine Cascade
APPENDIX C. FREE RADICAL ATTACK ON ARACHIDONIC ACID

Arachidonate-Isoprostane Pathway

\[
\begin{align*}
\text{CH}_2\text{-O-Fatty Acid} & \\
\text{CH}_2\text{-O-Arachidonate} & \\
\text{CH}_2\text{-O-PO}_3\text{-R}^+ & \\
\quad \cdot\text{O}_2^- \text{ or} \quad R^* & \\
\text{Rearrangement} & \\
\quad \text{CH}_2\text{-O-Fatty Acid} & \\
\text{CH}_2\text{-O-Peroxy Acid} & \\
\text{CH}_2\text{-O-PO}_3\text{-R}^+ & \\
\quad \beta\text{-Cleavage} & \\
\quad \text{CH}_2\text{-O-Fatty Acid} & \\
\text{CH}_2\text{-O-Endoperoxide} & \\
\text{CH}_2\text{-O-PO}_3\text{-R}^+ & \\
& \xrightarrow{\text{Reduction by cellular peroxidases}} \\
\text{Phospholipase A}_2 & \\
& \\
\quad \text{CH}_2\text{-O-Fatty Acid} & \\
\text{CH}_2\text{-O-Isoprostanes} & \\
\text{CH}_2\text{-O-PO}_3\text{-R}^+ & \\
R = (\text{CH}_2)_2\text{N(CH}_3)_3 & \\
\end{align*}
\]

4-hydroxy nonenal
+ other aldehydes

malondialdehyde + 12(S)-HTT

8-isoprostane (8-\text{iso PGF}_2\alpha)
+ other cis-side-chain isomers
+ lyso-phospholipids
APPENDIX D. NATURALLY OCCURRING TOCOPHEROLS

α-Tocopherol

β-Tocopherol

γ-Tocopherol

δ-Tocopherol
APPENDIX E. VITAMIN E METABOLITES

\[ \alpha\text{-tocopherol metabolite} \]
\[ \alpha\text{-CEHC} \]

\[ \gamma\text{-tocopherol metabolite} \]
\[ \gamma\text{-CEHC} \]
APPENDIX F.    OSU COMMITTEE FOR THE PROTECTION OF HUMAN SUBJECTS-APPROVAL

OREGON STATE UNIVERSITY


June 26, 2001

TO: Maret G Traber
   Linus Pauling Institute

COPY: Laura Lincoln

RE: Antioxidants and Patients Undergoing Hemodialysis

The referenced project was reviewed under the guidelines of Oregon State University's institutional review board (IRB), the Committee for the Protection of Human Subjects, and the U.S. Department of Health and Human Services. The IRB has approved your application. The approval of this application expires upon the completion of the project or one year from the approval date, whichever is sooner. The informed consent form obtained from each subject should be retained in program/project's files for three years beyond the end date of the project.

Any proposed change to the protocol or informed consent form that is not included in the approved application must be submitted to the IRB for review and must be approved by the committee before it can be implemented. Immediate action may be taken where necessary to eliminate apparent hazards to subjects, but this modification to the approved project must be reported immediately to the IRB.

Anthony Wilcox, Chair
Committee for the Protection of Human Subjects
Langton 214
anthony.wilcox@orst.edu; 737-6799

Date: 6/26/01
July 9, 2001

The following project has been reviewed by the Institutional Review Board (IRB) utilizing the guidelines of Good Samaritan Hospital's Policy for Research, Investigations, and Clinical Trials.

PROJECT TITLE:
Antioxidants and Patients Undergoing Hemodialysis

PRINCIPAL INVESTIGATOR:
James E. Ridlington, Ph.D.
Maret G. Traber, Ph.D.
Kylie S. Smith (Student)
Oregon State University

HOSPITAL LIAISON:
Mohammed Mohammed, MD
Jackie Chandler, RN/Manager
Samaritan Dialysis Services

COMMITTEE DECISION:
Approval is granted based upon additional clarification and information provided (see attached revised protocol) and review of informed consent specific to the program.

INVESTIGATORS MUST PROVIDE:

1. The original informed consent will be filed in the hospital medical record and a copy of the consent form will be given to the patient.

2. At conclusion of the study, provide the IRB with a report that includes a brief conclusion, number of patients entered at the GSH Site, any details regarding adverse events or outcomes as a result of study participation.

James Phelps, MD, Chair, Institutional Review Committee
Good Samaritan Hospital Corvallis
APPENDIX G. CONSENT FORM

LINUS PAULING INSTITUTE

OREGON STATE UNIVERSITY
571 Weniger Hall, Corvallis, Oregon 97331-6512
Telephone 541-737-7977, Fax 541-737-5077

July 12, 2001

CONSENT TO PARTICIPATE IN A RESEARCH STUDY ON
ANTIOXIDANTS IN PATIENTS UNDERGOING HEMODIALYSIS

You have the right to refuse to participate in this study, or to quit this study.

Your medical care will be exactly the same whether or not you volunteer for this study. You may change your mind about being in the study, at any time before or during the study. If you change your mind, we will not remove any additional blood, and we will not do any tests on the blood that we have already removed and your specimens will be destroyed.

CONFIDENTIALITY

We will not disclose your name or any confidential medical information to anyone. We will use code numbers to identify all medical histories, blood specimens, and laboratory results in any publications of our data. We will not disclose any information that might allow anyone to identify you or your lab results.

TITLE OF STUDY

Antioxidants in Patients Undergoing Hemodialysis

INVESTIGATORS, DEPARTMENTS, AND PHONE NUMBERS

1. James Ridlington, Ph.D., Nutrition and Food Management, Oregon State University, telephone number 541-737-8004
2. Maret Traber, Ph.D., Linus Pauling Institute, Oregon State University, telephone number 541-737-7977.
3. Kylie Smith, graduate student, Nutrition and Food Management, Oregon State University, telephone number 541-737-8004

PURPOSE OF THIS RESEARCH

You are asked to participate in a research study on renal patients undergoing hemodialysis therapy. Patients with renal disease may have higher levels of free radicals and inflammation. Some evidence demonstrates that antioxidants may reduce the damaging effects of the free radicals and inflammatory products. We hope to learn more on how certain antioxidants (vitamin E) will affect the levels of free radicals in blood.

PROCEDURES

Dr. Ridlington will interview you after you consent to participate in this study. He will record the information you provide on a Medical History Form. Any values unknown to the subject will be written down for them to inquire to Dr. Mohammed. After obtaining values, they can report them to Dr. Ridlington. The Medical History Form will be numbered by a code so not to identify you, and will only be used for research, and analyzing the results of your laboratory studies. The history form will not be part of your medical record, but this consent form will be a part of your medical record.

Although you will be interviewed and a Medical History Form will be filled out, you might still be excluded from the study for not meeting the criteria on the next page.

Participant Initials _____
Investigator Initials
APPENDIX G.  CONSENT FORM-CONTINUED

Criteria to be in the study:
You must be between 80% and 130% ideal body weight, have a resting blood pressure lower than 160/105mmHg and a fasting blood glucose concentration less than 7.77 mmol/L (140mg/dl).

You must not consume large doses of antioxidant supplements (vitamin C, E, and carotenoids), herbal supplements or phytochemicals. You must not consume more than 3 alcoholic beverage servings per day.

Dietary Recall
We will ask you questions about the food you ate in the past 24-hours on two separate days. The purpose of the dietary recall is to assess how much antioxidants you consume in your diet.

Blood Sampling and Vitamin E Supplementation
The study will occur over a 3-month period. We will remove blood a total of 8 times on four separate occasions. We will remove blood twice on each occasion, before and after your dialysis treatment from your dialysis tubing. The total amount of blood removed on each draw will be 22.5 cc (approximately 1.5 tablespoons, or 22.5 ml of blood). Blood will be removed on Day 0, 14, 44, and 74 of the study.

On day 14 of the study, you will be provided with antioxidant supplements (400 IU of vitamin E). Your doctor already advises you to take Nephrovite, which contains 60 mg of vitamin C. Starting on Day 15, in addition to Nephrovite, you will take one 400 IU vitamin E capsule with your dinner every day for two months. After one month (Day 44 of the study), a blood sample will be removed before and after your dialysis. After the second month (Day 74 of the study), a final blood sample will be removed before and after your dialysis.

You will be asked to collect 4 different 24-hour urine samples on the days before each blood removal (Day -1, 13, 43, and 73). You will be reminded to do this the day before each urine collection. We will provide the appropriate containers for urine collections. You will be asked to return the filled containers at your next dialysis.

We will measure the following substances from your blood:
1. Antioxidants: vitamin C (ascorbic acid), vitamin E (α- and γ-tocopherols)
2. Markers of the damage caused by free radicals and inflammation: F2-isoprostanes, ICAM, VCAM, C-reactive protein, interleukins 1 and 6, and tumor necrosis factor-α.

RISKS
Risks for removing blood for this study are minimal. Since little blood is being removed during the study (3 tablespoons per day), then the risk of anemia is low. There are no risks to urine collection.

BENEFITS
The potential benefit to you would be a decrease in your free radical (F2-isoprostane) and inflammatory damage. The benefits gained from this research will be an understanding of antioxidant requirements in renal patients, as well as your personal antioxidant levels and oxidative stress.

COSTS/COMPENSATION
You will not be paid for participation in this study. The hospital and your doctors will still charge you their regular fees for your dialysis and other medical care, but you will not be charged for any of the expenses of the study.

PRINCIPAL INVESTIGATOR'S DISCLOSURE OF PERSONAL OR FINANCIAL INTERESTS IN THE RESEARCH STUDY AND SPONSOR
Your investigators have NO financial interest in this research.
APPENDIX G. CONSENT FORM - CONTINUED

QUESTIONS
If you have any questions about the research study or specific procedures, please contact Kylie Smith or James Ridlington, Ph.D. (541-737-8004), or Maret Traber, Ph.D. (541-737-7977). If you have any questions about your rights as a participant, please contact the IRB Coordinator, OSU Research Office 541-737-3437 or via email at IRB@orst.edu.

CONSENT
YOUR SIGNATURE, BELOW, WILL INDICATE THAT YOU HAVE DECIDED TO VOLUNTEER AS A RESEARCH SUBJECT AND THAT YOU HAVE READ AND UNDERSTOOD THE INFORMATION PROVIDED ABOVE.

Signature of participant or legal representative__________________________ Date ______________
Subject's Printed name ________________________________________________
Subject's Present address ______________________________________________
Subject's phone number ________________________________________________
Signature of Investigator ______________________________________________ Date ______________

You will be given a signed and dated copy of this form to keep.
APPENDIX H. ADVERSE EFFECT FORM

OREGON STATE UNIVERSITY
INSTITUTIONAL REVIEW BOARD
ADVERSE EVENT FORM

Adverse Event: Any happening not consistent with routine expected outcomes that result in bodily injury and/or psychological, emotional, or physical harm or stress.

Federal Regulations require that all adverse events and/or injuries experienced by research participants be reported to the Institutional Review Board. This report must be completed and submitted to the IRB within 3 calendar days after the awareness of the adverse event. All material must be typed and submitted to the IRB Coordinator, Research Office, 312 Kerr Administration Bldg, Corvallis, OR 97331. Send an e-mail to IRB@orst.edu or call (541) 737-3437 with any questions.

Principal Investigator: James Ridlington, Ph.D., Co-PI Maret G. Traber, Ph.D. E-mail: Ridlingj@orst.edu; maret.traber@orst.edu

Department: NFM, LPI Telephone: 78004, 77977

Project Title: Antioxidants and Patients Undergoing Hemodialysis

Student Name (if any): Kylie Smith E-mail: smithkyl@mailbox.orst.edu

IRB Protocol No. Funding Source: Erkkila Foundation

Current Approval Date: 6/26/01

1. Event Date: 9/20/01

2. Was this a routine expected outcome as described in the protocol and the informed consent document(s)? □ Yes □ No

3. Severity of Event: □ Mild □ Moderate □ Severe □ Life Threatening □ Fatal

4. Is this event related to the research? □ Related □ Possibly Related □ Not Related

□ Probably Not Related □ Not Enough Information to Judge

5. Date of Treatment provided to the participant:

6. Participant's recovery was: □ complete □ moderate □ minimal □ not resolved at this time

7. Research involves a: □ drug □ device □ procedure

8. Name of drug, device, or procedure:

9. Has the Adverse Event been reported to: □ Sponsor, Date of report: 10/1/01 □ PHS, Date of report: □ FDA, Date of report:

ON A SEPARATE SHEET PLEASE PROVIDE THE FOLLOWING INFORMATION IN DETAIL.

9-01 1
APPENDIX H. ADVERSE EFFECT FORM-CONTINUED

1. Description of event (include location).
2. Cause of event.
3. Outcome of event.
4. If the event is Related or Possibly Related to the research, explain what procedures were in place to minimize or reduce this risk?
5. Describe the treatment provided to the participant.

<table>
<thead>
<tr>
<th>CHANGES NECESSITATED BY ADVERSE EVENT/INURY</th>
</tr>
</thead>
<tbody>
<tr>
<td>In your opinion, does this adverse event/injury require a change in the protocol, consent/assent, or information/re-consent provided to the participants?  □ Yes  □ No</td>
</tr>
</tbody>
</table>

If yes, please attach a MODIFICATION REQUEST FORM with this report.

SIGNATURES: I certify that to the best of my knowledge the information presented herein is an accurate reflection of the adverse event.

<table>
<thead>
<tr>
<th>Facility Research</th>
</tr>
</thead>
<tbody>
<tr>
<td>Faculty Name</td>
</tr>
<tr>
<td>NA</td>
</tr>
<tr>
<td>Implementer Present During Adverse Event</td>
</tr>
<tr>
<td>Date</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Student Research</th>
</tr>
</thead>
<tbody>
<tr>
<td>Faculty Sponsor</td>
</tr>
<tr>
<td>Kelly Smith</td>
</tr>
<tr>
<td>Student</td>
</tr>
<tr>
<td>Date</td>
</tr>
<tr>
<td>NA</td>
</tr>
<tr>
<td>Implementer Present During the Adverse Event</td>
</tr>
<tr>
<td>Date</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Witness</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sandra Miller</td>
</tr>
<tr>
<td>Date</td>
</tr>
<tr>
<td>10-10-01</td>
</tr>
<tr>
<td>Address</td>
</tr>
<tr>
<td>Los Angeles</td>
</tr>
<tr>
<td>Telephone</td>
</tr>
<tr>
<td>541-737-5075</td>
</tr>
</tbody>
</table>

FOR IRB USE ONLY Signatures below certify review of this report.

<table>
<thead>
<tr>
<th>IRB Chair</th>
</tr>
</thead>
<tbody>
<tr>
<td>Date</td>
</tr>
<tr>
<td>Prior reports of similar events: □ Yes □ No</td>
</tr>
<tr>
<td>Inform all study participants? □ Yes □ No</td>
</tr>
<tr>
<td>Should protocol be revised? □ Yes □ No</td>
</tr>
<tr>
<td>Revised consent form submitted? □ Yes □ No</td>
</tr>
<tr>
<td>Revise consent form? □ Yes □ No</td>
</tr>
<tr>
<td>Initial Review (date and description):</td>
</tr>
</tbody>
</table>

| Copy to Legal Advisor |
| Copy to Vice Provost for Research |
| Report to IRB for review and action |
| Write to investigator with concerns |

9-01 2
APPENDIX H.  ADVERSE EFFECT FORM-CONTINUED

1. Description of event (include location).
   Death

2. Cause of event.
   Unknown as of Oct 1, 2001

3. Outcome of event
   Death

4. If the event is related or possibly related to the research, explain what procedures were in
   place to minimize or reduce this risk?
   Not related

5. Describe the treatment provided to the participant.
   No treatment by investigators. Subject was hospitalized at time of death.
Atten: Maret Traber
Re: Vitamin E Study

October 5, 2001

He suffered acute myocardial infarction.

His cause of death has not relationship to vitamin E that he had been receiving as part of a current study.

If I can be of further assistance, please do not hesitate to call.

Mohammed S. Mohammed M.D.
Internal Medicine/Nephrology
MSM/slcl
October 2, 2001

Ms. Peggy Lowry
Director, Sponsored Projects

Dear Peggy,

Enclosed is additional information to be added to the OSU Institution Review Board Adverse Event Form. I was informed of the subject's death on September 26, 2001 and was aware that the Adverse Event Form had to be returned to your office within 3 days. The cause of death was not available to us until today and so the previous information submitted was rather limited. We have now obtained the death notification with cause of death and enclose it for your records. The physician, Dr. Mohammed Mohammed, has provided this information to us. His phone number is 753-7473.

My opinion as a vitamin E expert is that the death of this subject was not caused by the vitamin E supplements; there is no evidence in the literature that vitamin E causes heart attacks. In a phone conversation with me, Dr. Mohammed confirmed that he concurs with this statement and that the subject had a history of heart disease, including 9 previous heart attacks. Therefore, the one month supplementation with vitamin E was not a factor in the subject's death. Additionally, this is a very high risk population we are studying. One other subject, not enrolled in our study but who was a patient in the renal dialysis unit at the same time as our study, died in the last week. It should be emphasized that the patients attending the dialysis have "end stage renal disease" and are under study because they have high rates of heart disease mortality. I enclose an abstract from a recent paper in The Lancet documenting the beneficial effects of vitamin E in patients with "end-stage renal disease".

Yours sincerely,

Maret G. Traber, Ph.D.
Principal Investigator, Linus Pauling Institute
Associate Professor, Department of Nutrition & Food Management
APPENDIX H. ADVERSE EFFECT FORM-CONTINUED

Addendum to Adverse event form dated October 1, 2001

1. Description of event (include location).
Maret G. Traber, Ph.D. spoke on October 2, 2001 with Dr. Mohammed Mohammed, physician-in-charge of the Samaritan Dialysis unit and co-investigator on this project. Dr. Mohammed informed Dr. Traber that the subject had a history of heart disease, had 9 previous heart attacks and died of an acute myocardial infarction while a patient at Good Samaritan Hospital on September 20, 2001. He stated that it was his opinion that the vitamin E supplement consumed by the patient was not a factor in the patient’s death.

2. Cause of event.
acute myocardial infarction

3. Outcome of event
Death

4. If the event is related or possibly related to the research, explain what procedures were in place to minimize or reduce this risk?
Not related

5. Describe the treatment provided to the participant.
No treatment by investigators. Subject was hospitalized at time of death.
APPENDIX H. ADVERSE EFFECT FORM-CONTINUED


Comment in:
ACP Journal Club 2001 May-Jun;134(3):91


Department of Epidemiology and Preventive Medicine, Sackler Faculty of Medicine, Tel Aviv University, Israel. mboaz@yahoo.com

BACKGROUND: Excess cardiovascular mortality has been documented in chronic haemodialysis patients. Oxidative stress is greater in haemodialysis patients with prevalent cardiovascular disease than in those without, suggesting a role for oxidative stress in excess cardiovascular disease in haemodialysis. We investigated the effect of high-dose vitamin E supplementation on cardiovascular disease outcomes in haemodialysis patients with pre-existing cardiovascular disease.

METHODS: Haemodialysis patients with pre-existing cardiovascular disease (n=196) aged 40-75 years at baseline from six dialysis centres were enrolled and randomised to receive 800 IU/day vitamin E or matching placebo.

Patients were followed for a median 519 days. The primary endpoint was a composite variable consisting of myocardial infarction (fatal and non-fatal), ischaemic stroke, peripheral vascular disease (excluding the arteriovenous fistula), and unstable angina. Secondary outcomes included each of the component outcomes, total mortality, and cardiovascular-disease mortality.

FINDINGS: A total of 15 (16%) of the 97 patients assigned to vitamin E and 33 (33%) of the 99 patients assigned to placebo had a primary endpoint (relative risk 0.46 [95% CI 0.27-0.78], p=0.014). Five (5.1%) patients assigned to vitamin E and 17 (17.2%) patients assigned to placebo had myocardial infarction (0.3 [0.11-0.78], p=0.016). No significant differences in other secondary endpoints, cardiovascular disease, or total mortality were detected.

INTERPRETATION: In haemodialysis patients with prevalent cardiovascular disease, supplementation with 800 IU/day vitamin E reduces composite cardiovascular disease endpoints and myocardial infarction.

Publication Types:
Clinical trial
Multicenter study
Randomized controlled trial
PMID: 11072938 (PubMed - indexed for MEDLINE)
APPENDIX H.  ADVERSE EFFECT FORM-CONTINUED

END STAGE RENAL DISEASE MEDICAL INFORMATION SYSTEM

ESRD DEATH NOTIFICATION

According to the Program Protection Act of 1986, no person is required to respond to a collection of information unless he displays a valid OMB control number. The valid OMB control number for this information collection is 0938-0444. The time required to complete this form: 10 minutes per report, including the time to review instructions, search existing data sources, gather the data needed, and complete and submit the report. This collection of information is required under Title 42 Code of Federal Regulations, Section 405. The submission of false information is punishable under Title 18 United States Code, Sections 1001 and 1078.

1. PATIENT'S LAST NAME [Confidential]
2. PATIENT'S FIRST NAME [Confidential]
3. PATIENT'S MIDDLE INITIAL
4. PATIENT'S SEX a. Male b. Female
5. PATIENT'S DATE OF BIRTH
6. PATIENT'S PLACE OF RESIDENCE
7. PROVIDER NAME AND ADDRESS (CITY AND STATE)
8. PROVIDER NUMBER
9. PLACE OF DEATH (Check one)
   a. Hospital b. Dialysis c. Home d. Other
10. WAS AN AUTOPSY PERFORMED? a. Yes b. No
11. CAUSES OF DEATH (Enter code from list of causes below.)
    a. Primary Cause (2)
    b. Secondary Causes? Yes, Specify
12. FOR ALL DEATHS INDICATE YES/NO
    c. If deceased received a transplant a. Date of most recent transplant
       d. Was kidney functioning (patient not on dialysis) at time of death? Yes No
       e. Did transplant patient resume chronic maintenance dialysis prior to death? Yes No
13. IF DECEASED RECEIVED A TRANSPLANT
14. REMARKS

15. NAME OF PHYSICIAN
16. SIGNATURE OF PERSON COMPLETING THIS FORM


Form HCFA-2746-U3 (8-96) 20/20 09152811+5 S11A4H9 6/21 1002-29-100