

## AN ABSTRACT OF THE THESIS OF

Chia-Lin Lee for the degree of Master of Science in Nutrition and Food Management presented on August 14, 2002. Title: Vitamin E and Iron Status in Hemodialysis Patients.

Abstract approved: \_\_\_\_\_

Maret G. Traber

The present study investigated whether vitamin E supplementation reduced oxidative stress in erythrocytes and improved vitamin E status in patients undergoing hemodialysis (HD). Plasma and erythrocyte  $\alpha$ -tocopherol, plasma ascorbic acid, and iron status were determined in 11 regular HD patients prior to and post-dialysis, before and during oral supplementation of vitamin E, 400 IU daily for two months. HD patients were categorized into two groups according to their plasma ascorbic acid levels. We found that only the vitamin C sufficient group ( $>40 \mu\text{M}$ , Group I) had reliable measurements of erythrocyte  $\alpha$ -tocopherol concentrations before vitamin E supplementation. In Group I prior to dialysis, erythrocyte  $\alpha$ -tocopherol concentrations increased in response to vitamin E supplementation from  $6.7 \pm 0.7 \mu\text{mol/L}$  packed cells to  $9.8 \pm 0.6 \mu\text{mol/L}$  packed cells ( $p < 0.04$ ). Moreover, there was a positive correlation ( $p < 0.001$ ) between plasma and erythrocyte  $\alpha$ -tocopherol levels in Group I subjects. Additionally, vitamin E supplementation significantly increased hematocrits ( $39.9\% \pm 1.9\%$  to  $42.3\% \pm 1.6\%$ ,  $p < 0.004$ ) post-dialysis only in Group I subjects. On the contrary, there was no change in hematocrits during vitamin E supplementation in the

vitamin C deficient group ( $<40 \mu\text{M}$ , Group II). With respect to measures of iron status and recombinant human erythropoietin (rHuEPO) dose, no differences between before and during vitamin E supplementation were observed in two groups of patients. In summary, our data suggest that oral vitamin E supplementation protected erythrocytes from oxidative stress and improved vitamin E status in HD patients, but only in patients with adequate vitamin C status.

Vitamin E and Iron Status in Hemodialysis Patients

by  
Chia-Lin Lee

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APPROVED:

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Major Professor, representing Nutrition and Food Management

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Head of the Department of Nutrition and Food Management

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Dean of the 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.

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Chia-Lin Lee, Author

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## 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. All assisted in the interpretation of data. Kylie S. Smith assisted in the analyses of plasma vitamins E and C.

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# Vitamin E and Iron Status in Hemodialysis Patients

## INTRODUCTION

Oxidative stress is enhanced in patients with end stage renal disease (ESRD) undergoing hemodialysis (HD). It may result from increased free radical production and decreased antioxidant levels. The erythrocyte membrane is rich in polyunsaturated fatty acids and erythrocytes contain hemoglobin that can be a source of redox-active iron. Thus, erythrocytes are one of the targets of most oxidative processes. Erythrocytes from HD patients have several metabolic modifications, including a defect in the hexose monophosphate shunt that results in increased superoxide production, and an impaired enzymatic antioxidant defense system, leading to poorer protection against reactive oxygen species (ROS) (2-4). In addition, decreased erythrocyte levels of non-enzymatic antioxidants such as  $\alpha$ -tocopherol have been reported (5-8). All of these factors can contribute to increased lipid peroxidation, which enhances the susceptibility of erythrocytes to damage or rupture (7, 9). When hemolysis occurs, redox-active iron is released, and it may initiate more lipid peroxidation damaging other erythrocytes, shortening their life span, and increasing susceptibility to anemia.

Vitamin E is known as the most important lipophilic antioxidant in human plasma. Increasing evidence suggests that vitamin E administration reduces the oxidative damage associated with uremia and HD. Oral vitamin E supplementation in HD patients reportedly increases erythrocyte osmotic resistance, increasing hematocrits, decreasing anemia (6, 10). Also, Lim et al. (11) reported body iron status is related to the levels of lipophilic antioxidants. They suggested that elevated body iron stores might decrease the levels of lipophilic antioxidants due to

increasing the oxidative consumption of antioxidants. Therefore, in this study, we investigated whether oral vitamin E supplementation can improve vitamin E status and correct anemia in HD patients.

## **HYPOTHESIS**

Vitamin E supplementation may reduce oxidative stress in erythrocytes of HD patients and improve their vitamin E status.

## **SPECIFIC AIMS**

### **Aim 1. Determine Current Vitamin E and Iron Status in HD Patients.**

Erythrocyte and plasma levels of  $\alpha$ -tocopherol, plasma ascorbic acid, and iron parameters (serum iron, total iron binding capacity (TIBC), and plasma ferritin) were measured before and after a dialysis session on two occasions to establish baseline vitamin E and iron status in twelve HD patients.

### **Aim 2. Supplement Patients with Vitamin E and Reevaluate Vitamin E and Iron Status.**

HD patients were supplemented daily with 400 IU vitamin E for two months. Erythrocyte and plasma levels of  $\alpha$ -tocopherol, plasma ascorbic acid, and iron parameters before and after a dialysis session were reassessed following one and two months of supplementation.

## LITERATURE REVIEW

### OXIDATIVE STRESS IN HEMODIALYSIS PATIENTS

Several lines of evidence suggest that HD patients are subject to increased oxidative stress (12-14). Oxidative stress is defined as an imbalance between oxidant production and antioxidant defenses (15).

ROS are reactive molecules, which could affect the integrity of virtually all biomolecules, including lipids, proteins, and nucleic acids, ultimately leading to cell damage (14). Oxidative damage results from production of a large variety of deleterious ROS, derived mainly from superoxide anion ( $O_2^{\bullet-}$ ).  $O_2^{\bullet-}$ , a by-product of the respiratory chain, is continuously produced at a low rate in the internal mitochondrial membrane. Enzymatic processes such as NADPH oxidase complex also can overproduce  $O_2^{\bullet-}$  (9).

Antioxidant defenses include a complex network of non-enzymatic factors, such as vitamin E, vitamin C, carotenoids, and others; and enzymatic systems, such as catalase, superoxide dismutase, and glutathione peroxidase. Both types of scavengers can quench free radicals.

There are three categories of factors that may cause oxidative imbalance in HD patients (14). These are 1) uremia-associated metabolic abnormalities, 2) HD-associated procedure, and 3) adjuvant drug treatment, and are discussed below.

## **Uremia-associated Metabolic Abnormalities**

### *The Alterations of Reactive Oxygen Species (ROS) Production*

Oxidative stress may exist long before initiation of maintenance dialysis therapy in uremic patients. Tepel et al. (13) found by measuring the intracellular ROS in lymphocytes from uremic patients that both spontaneous and phorbol myristate acetate (PMA)-induced ROS production were increased. Ward and McLeisch (16) also showed that the polymorphonuclear (PMN) leukocyte oxidative burst is enhanced in uremic patients. Thus, uremic toxins may play a role in the induction of oxidative stress.

### *Impaired Antioxidant System*

The antioxidant system is impaired in uremic patients and gradually altered with the degree of renal failure. Ceballos-Picot et al. (12) found the total level of glutathione (GSH) and plasma glutathione peroxidase (GSH-Px) activities were significantly decreased in HD patients, and there were positive correlations between creatinine clearance and plasma levels of GSH and GSH-Px. They showed that the disturbances of the antioxidant system gradually increased with the degree of renal failure and were exacerbated by dialysis.

### *Abnormalities of The Nitric Oxide (NO) Control System*

Uremia is also associated with abnormalities in the nitric oxide (NO) systems. NO contributes to the regulation of blood pressure and host defense (15). Vallance et al. (17) and Arese et al. (18) found that chronic renal failure and the uremic state lead to the accumulation of various endogenous inhibitors of the NO systems. Uremia causes impaired NO synthesis, and thus, may contribute to hypertension and immune dysfunction.

### **Hemodialysis (HD)-associated Procedure**

#### *Antioxidants Lost in the Dialysate*

HD is a non-selective process, which clears solutes based on molecular weight and sieving properties of the membrane and protein bound capacity. It uses a highly permeable membrane (cut-off point of 20-25 KD) that induces the losses of waste products (uremic toxins) and some essential substances (glucose, amino acids and vitamin C) (19). Thus, increased dialysis efficiency also enhances antioxidant losses, and thereby impairs oxygen radical scavenging capacity.

#### *Bioincompatibility of the Dialysis System*

Bioincompatibility of the dialysis system plays an important role in the production of ROS (9) and can increase the oxidative stress of HD patients. HD-related oxidative stress relies on two major components of dialysis systems. One

component that increases ROS is the dialysis membrane. Cristol et al. (20) demonstrated that the complement-activating membrane (such as cuprophane) is responsible for free oxygen radical production via leukocyte (PMN and monocytes) activation. The other component is the microbiological contamination and the pyrogen content of dialysate. Endotoxin contaminants of dialysate play a major role in the production of ROS during HD (9). Furthermore the presence of endotoxin (lipopolysaccharide, LPS) in the dialysate may activate blood monocytes or macrophages through the dialysis membrane contributing to cytokine release and free oxygen radical production (19).

### **Adjuvant Drug Treatment**

In order to correct the uremia syndrome generated by chronic renal failure, HD patients are given several drugs. Two medications may strongly affect the oxidative status of patients.

One of the treatments is the intravenous administration of iron. In HD patients, the dose recommended for iron supplementation leads to an over-saturation of transferrin (21). High percentage transferrin saturation was found to be associated with the presence of redox-active iron. Redox-active iron can cause hydroxyl radical formation by the Fenton reaction in the presence of hydrogen peroxide (see below). Thus, iron administration strongly enhances oxidative stress in HD patients (22).

The other medication that may cause oxidative stress is the correction of anemia by recombinant human erythropoietin (rHuEPO). Erythropoietin (EPO) prescribed to correct anemia has been suspected in worsening the oxidative status

of HD patients. Chen et al. (23) found that EPO enhanced formyl-methionyl-leucyl-phenylalanine (FMLP) - stimulated  $O_2^{\cdot-}$  production by PMNs from HD patients, both in vivo and in vitro.  $O_2^{\cdot-}$  may be responsible for the increased oxidative stress in this group of patients.

## **ANEMIA IN HD PATIENTS**

Anemia is one of the dialysis-related pathologies that may be considered a side effect of long-term HD. Approximately 67 % of HD patients have a hematocrit less than 30% (19, 24). The primary cause of anemia in HD patients is insufficient EPO production by diseased kidneys. Additional factors that may cause or contribute to anemia include: shortened erythrocyte survival, severe hyperparathyroidism, blood loss, deficiencies of iron, and others (25). Moreover, oxidative stress may exacerbate the anemia. Chronic hemolysis, caused by increased membrane lipid peroxidation, may be the cause of shortened erythrocyte survival (26). In addition, increased ROS may alter signal transduction from the cellular oxygen sensor to the EPO gene. Thus, ROS, especially hydrogen peroxide ( $H_2O_2$ ), could inhibit EPO synthesis in the kidneys (27).

## **ERYTHROCYTE**

Anemia is defined as an insufficient number of erythrocytes per volume of blood. The major erythrocyte function is to exchange oxygen and carbon dioxide between the lungs and body tissues. To effectively combine with oxygen, the erythrocyte must contain a normal amount of the red protein pigment - hemoglobin, the amount of which depends on the iron levels in the body (28).

The plasma membrane of the erythrocyte contains lipids and proteins. It is selectively permeable and encloses the cytoplasm. Forty percent of the total fatty acids in erythrocyte membrane are polyunsaturated fatty acids. Therefore, membranes are one of the preferential targets of most oxidative processes. Furthermore, erythrocytes contain hemoglobin, which consists of four heme-globin subunits. Hemoglobin could act as iron donor to participate the Fenton reaction (see below) (29, 30). When hemoglobin was exposed to an excess of hydrogen peroxide in vitro, heme-globin subunits were degraded and released both heme and iron ions (31). Both heme and iron ions could stimulate lipid peroxidation, and iron ions could cause hydroxyl radical formation from hydrogen peroxide. Therefore, when heme, iron and hemoglobin are released from erythrocyte, they become potentially damaging molecules (15).

Erythrocytes from HD patients have been reported to have a variety of metabolic defects that could cause enhanced oxidative stress (2-4). A defect in the hexose monophosphate shunt could lead to increased production of free radicals (3). Canestrari et al. (2) and Weinstein et al. (4) demonstrated that the activity of glutathione peroxidase and catalase were decreased, and the levels of the superoxide dismutase were increased, suggesting that enzymatic antioxidant defense mechanisms were impaired. Additionally, vitamin E was also low in erythrocytes from HD patients (5-8). All of these abnormalities could contribute to increased erythrocyte lipid peroxidation and promote erythrocyte hemolysis.

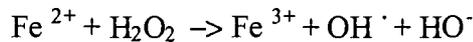
## **IRON**

Iron, a metal, is essential to the human body. It has the capacity to accept and donate electrons readily, interconverting between ferric ( $\text{Fe}^{3+}$ ) and the ferrous

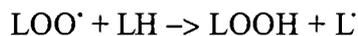
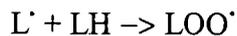
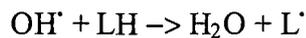
(Fe<sup>2+</sup>) forms (32). With this capacity, iron becomes a useful component of cytochrome, oxygen binding protein, and some enzymes. However, iron can also become hazardous for the organism.

### **Iron and Oxidative Stress**

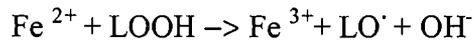
Free iron, non-transferrin bound iron, was shown to be redox-active (31). Redox-active iron catalyses free radical reactions causing single-electron (radical) transfers. It damages tissues by participating in the Fenton chemistry, catalyzing the formation of hydroxyl radicals (OH<sup>·</sup>) (15).



The hydroxyl radical, in contrast to the other oxygen products, reacts at extremely high rates with almost every type of molecule found in the living cells. It is also a powerful stimulator of lipid peroxidation, and can initiate this reaction by abstracting a hydrogen atom from polyunsaturated fatty acids (LH) (33).



When lipid hydroperoxides (LOOH) accumulate, free iron may initiate lipid peroxidation. This reaction of lipid hydroperoxides destroys membrane structure and function.



Since free iron is an active promoter of free radical reactions, it is toxic to living cells. Biological systems have developed several ways to prevent iron-mediated oxidative stress. There are two major mechanisms: 1) to sequester free iron in a protein-bound form and 2) to prevent an excess of iron storage by regulating iron absorption.

### **Free Iron and Iron Binding Proteins**

The major iron-binding proteins in the body are transferrin, ferritin and hemosiderin. Under normal conditions, there is never an appreciable concentration of free iron (or iron chelated by low molecular weight compounds) (32). Any released free iron is immediately chelated in cells by compounds such as citrate, or adenosine diphosphate. Additionally, iron ions circulate bound to plasma transferrin, and accumulate within cells in the form of ferritin (33).

The iron-binding protein of plasma is transferrin, which is an 80-KD protein with 2 iron-binding sites. Transferrin has a high affinity for iron (III), and transports iron in the circulation. Normally, plasma transferrin is only about 30% saturated with iron, and is, therefore, unlikely to release free iron into a solution (32).

Iron is stored in the form of ferritin and hemosiderin. The ferritin molecule has the capacity to sequester a vast quantity of iron (4500 atoms of iron per mole

protein), and the ability to maintain it in a soluble, nontoxic form. Ferritin can prevent excessive intracellular accumulation of free iron, although in the case of substantial loss of blood, it is an important supply of iron for accelerated erythropoiesis (32).

Ferritin is composed of an apoprotein shell, and has a molecular weight of approximately 480 KD. Ferritin synthesis occurs in all cells of an organism, but primarily in hepatocytes and macrophages. High concentrations of ferritin are present in the liver, spleen and bone marrow. It is also found in low concentrations in plasma and urine (33).

Hemosiderin, a water-insoluble degradation product of ferritin, is usually found in lysosomes (33). The hemosiderin protein shells have partly disintegrated, allowing the iron to aggregate (34). The iron content is variable but generally higher than that of ferritin. In a normal human with adequate iron status, most of storage iron is present as ferritin, but with increasing iron accumulation the proportion present as hemosiderin increases (33).

However, under specific circumstances, such as oxidative stress, or in the presence of suitable reactants such as ascorbate, free iron can be released from these iron-binding proteins (35).

### **Iron Status and Anemia**

Various measurements can be used for the assessment of iron status. The major measurements for HD patients include: Hemoglobin concentrations, hematocrit, and iron parameters (serum iron, TIBC, transferrin saturation (TSAT), and serum ferritin) (25).

Hemoglobin and hematocrit are the most commonly used values for indicating the presence of anemia (36). Hemoglobin concentration indicates the amount of hemoglobin per unit of blood, and hematocrit represents that the erythrocyte proportion of the total blood volume.

Serum ferritin reflects the total body iron stores. It is the most sensitive test available for detecting iron deficiency (36). However, ferritin is also an acute phase protein. Therefore, when infections or inflammatory disease occurs, the concentrations are increased (34). Serum iron, TIBC, and transferrin saturation are particularly useful for differentiating between nutritional iron deficiency and iron deficits arising from chronic infections, inflammation, or chronic neoplastic diseases (37). Both serum iron and transferrin saturation can reflect the amount of iron immediately available for hemoglobin synthesis. If either of them is low, iron deficiency may be present. TIBC provides an estimate of serum transferrin. Therefore, when body iron is insufficient, TIBC increases.

Generally, the anemia in HD patients is normocytic and normochromic. In this type of anemia, serum iron and TIBC are low, and transferrin saturation tends towards the low end of the normal range (37). About 25% of HD patients have iron deficiency anemia, which can cause microcytosis. In this case, serum iron, transferrin saturation, and serum ferritin are low and TIBC is increased (25).

## VITAMIN E

### Vitamin E Structures and Nomenclature

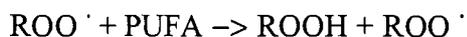
Vitamin E is an antioxidant that inhibits membrane lipid peroxidation. Vitamin E occurs naturally in eight different compounds, which fall into two classes: tocopherols, characterized by phytyl side chain, and tocotrienols, characterized by an unsaturated side chain. Each class is composed of four homologs. Homologs in each class are designated as  $\alpha$ ,  $\beta$ ,  $\gamma$  and  $\delta$  (38). Among these forms, the human body prefers *RRR*- $\alpha$ -tocopherol (39).

The synthetic form of  $\alpha$ -tocopherol is present in fortified foods and in vitamin supplements. It contains eight different stereoisomers as a result of the three chiral centers in the phytyl tail. Four of the stereoisomers are in the *2R*-stereoisomeric form (*RRR*, *RSR*, *RRS* and *SRR*), and four are in the *2S*-stereoisomeric form (*RSS*, *SSR*, *SRS*, and *SSS*). Generally, the *2R*-forms have a higher biologic activity than *2S*-forms; the *2R*-forms equivalent activity to *RRR*- $\alpha$ -tocopherol (40).

### Antioxidant Activity

Vitamin E is a chain breaking antioxidant that prevents the propagation of free radical reactions. It protects polyunsaturated fatty acids (PUFA) within phospholipids of biological membranes and in plasma lipoproteins (39). Vitamin E effectively reacts and terminates carbon-centered radicals and peroxy radicals, which may be generated in the body from reaction between organic compounds and

other radicals, especially hydroxyl radicals (36). A peroxy radical (ROO<sup>·</sup>) reacts with PUFA to form hydroperoxide and another peroxy radical causing a chain reaction.



Peroxy radicals react 1000 times faster with vitamin E than with PUFA. So, in the presence of vitamin E, the peroxy radical reacts with the vitamin E-hydroxyl group to form the organic hydroperoxide and the tocopheroxyl radical. In this way, vitamin E acts as a chain-breaking antioxidant, preventing the further autoxidation of lipids (39).

### **Interaction with Other Antioxidants**

When vitamin E intercepts a radical, a tocopheroxyl radical is formed (40). It can be reduced to generate tocopherol by interactions with reductants, which serve as hydrogen donors (AH):

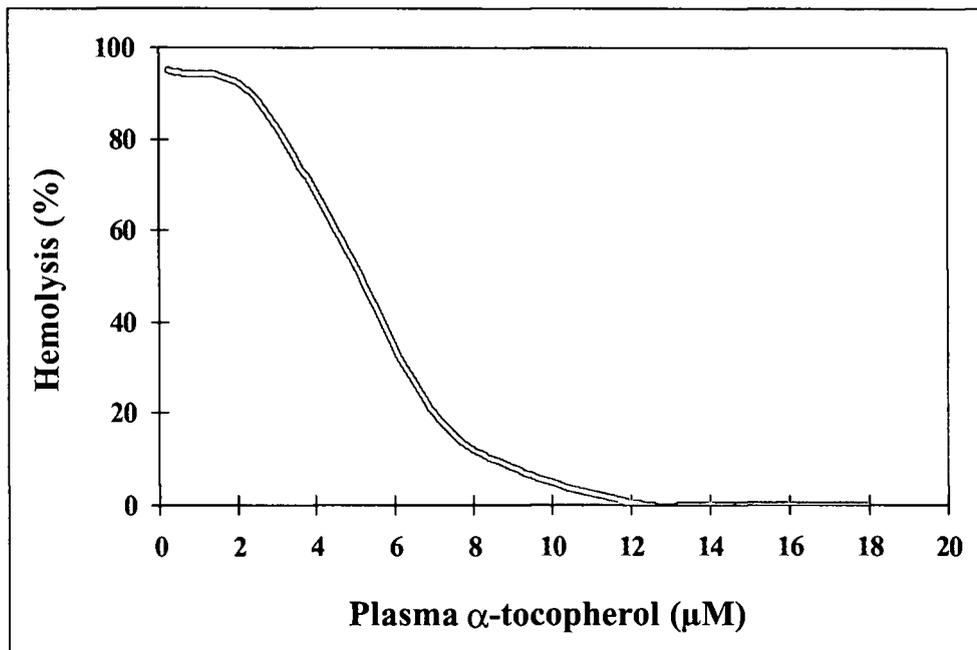


Vitamin C, thiols and glutathione have shown to regenerate tocopherol from the tocopheroxyl radical in vitro (39).

## Erythrocytes and Vitamin E Requirements

Vitamin E is the major and probably the only lipid-soluble free radical chain-breaking antioxidant in human blood plasma and erythrocytes. Vitamin E in erythrocytes is mostly localized in the membrane (41). Erythrocyte  $\alpha$ -tocopherol concentrations (1.16 - 4.64  $\mu\text{mol/L}$  packed cells) are approximately 15-25 % the concentration of the blood plasma (approximately 20  $\mu\text{mol/L}$ ), and erythrocyte  $\gamma$ -tocopherol concentrations in ranges from 0.23 to 0.93  $\mu\text{mol/L}$  packed cells (42).

Vitamin E is essential for the maintenance of normal erythrocyte function. Increased sensitivity of erythrocytes to oxidative stress is an index of vitamin E deficiency (43). Several studies showed that erythrocytes from vitamin E-deficient patients undergo abnormal hemolysis when exposed to hydrogen peroxide in vitro. Farrell et al. (44) suggested that there was a threshold vitamin E concentration in erythrocytes below which antioxidant protective activity was virtually exhausted, and the erythrocyte life span was significantly decreased (**Figure 1**). These abnormalities could be corrected by vitamin E supplementation.



**Figure 1. Relationship of peroxide-induced hemolysis and plasma  $\alpha$ -tocopherol level. (Source: Adapted from Farrell P. M. et al. J Clin Invest, 1977.)**

The 2000 Recommended dietary allowance (RDA) values for vitamin E are based on the relationship between erythrocyte hemolysis induced by hydrogen peroxide, plasma  $\alpha$ -tocopherol concentrations and vitamin E intakes (40). An intake of 12 mg vitamin E per day could maintain plasma  $\alpha$ -tocopherol levels at 12  $\mu\text{M}$ , which was sufficient to prevent hemolysis. The current vitamin E RDA for both men and women is 15 mg (35  $\mu\text{mol}$ ) / day  $\alpha$ -tocopherol. It applies only to the intake of *RRR* - $\alpha$ -tocopherol from food and the *2R*- stereoisomeric forms of  $\alpha$ -tocopherol that occur in fortified foods and supplements. Because other naturally occurring forms of vitamin E ( $\beta$ -,  $\gamma$ -, and  $\delta$ -tocopherols and the tocotrienols) are not

converted to  $\alpha$ -tocopherol by humans and are recognized poorly by the  $\alpha$ -tocopherol transfer protein ( $\alpha$ -TTP) in the liver, these forms do not meet the human vitamin E requirement.

The tolerable upper intake level (UL) for adults is set at 1000 mg/day of any form of supplemental  $\alpha$ -tocopherol based on the adverse effect of increased tendency to hemorrhage (40).

### **Vitamin E and Iron**

Vitamin E can help to protect against iron-induced cellular damage. Chow et al. (45) reported increased formation of lipid oxidation products in rats fed large doses of iron, especially those animals receiving a vitamin E-deficient diet. Whittaker et al. (46) indicated that increasing dietary vitamin E intake prior to increased iron intake could prevent lipid peroxidation. These investigators suggested that the protective effects of vitamin E did not involve enhanced elimination of excess of iron. The benefit was mainly due to the capacity of vitamin E to trap peroxy radicals, which were produced by redox-active iron through the Fenton reaction.

### **SUMMARY**

Oxidative stress is enhanced in patients with end stage renal disease undergoing HD. It may result from increased free radical production and decreased antioxidant levels. Several studies suggest that increased oxidative stress may contribute to the development of dialysis-related pathologies, such as anemia and

cardiovascular diseases, which are the leading causes of morbidity and mortality in HD patients.

Redox-active iron is also involved in oxidative stress. It can initiate lipid peroxidation by generating hydroxyl radicals through the Fenton reaction, and cause damage to tissues and cell membranes. The erythrocyte membrane is one of the preferential targets of most oxidative processes. Lipid peroxidation in the erythrocyte membrane increases its susceptibility to hemolysis. When hemolysis occurs, hemoglobin, which as one source of redox-active iron, is released, and may initiate more lipid peroxidation to damage the erythrocyte, shorten its life span, and leading to anemia.

Vitamin E is known as the most important lipophilic antioxidant in human plasma. Increasing evidence suggests that vitamin E administration reduces the oxidative damage associated with uremia and HD. Gallucci et al. (47) found erythrocytes from HD patients have increased levels of malonyldialdehyde, and decreased levels of vitamin E due to lipid peroxidation. These oxidation products increase erythrocytes' susceptibility to damage or rupture, thereby increasing hemolysis and anemia. Oral vitamin E supplementation in HD patients reportedly increases erythrocyte osmotic resistance, increasing hematocrits, decreasing anemia (6, 10).

The present study investigated whether oral vitamin E supplementation can improve vitamin E status and correct anemia in HD patients. We measured erythrocyte and plasma  $\alpha$ -tocopherols, iron parameters (serum iron, TIBC and plasma ferritin), and plasma ascorbic acid prior to and post-dialysis in 11 renal dialysis patients before and during supplementation of vitamin E (400 IU) daily for two months.

## VITAMIN E AND IRON STATUS IN HEMODIALYSIS PATIENTS

Chia-Lin Lee<sup>1</sup>, James W. Ridlington<sup>1</sup>, Scott W. Leonard<sup>2</sup>, and Maret G. Traber<sup>1,2</sup>

<sup>1</sup>Department of Nutrition and Food Management, <sup>2</sup>Linus Pauling Institute, Oregon State University, Corvallis OR 97331.

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 investigated whether vitamin E supplementation reduced oxidative stress in erythrocytes and improved vitamin E status in hemodialysis (HD) patients. Plasma and erythrocyte  $\alpha$ -tocopherol, plasma ascorbic acid, and iron status were determined prior to and post-dialysis, before and during oral supplementation of vitamin E, 400 IU daily for two months. HD patients were categorized into two groups according to their plasma ascorbic acid levels. Only the vitamin C sufficient group ( $>40 \mu\text{M}$ , Group I) had reliable measurements of erythrocyte  $\alpha$ -tocopherol concentrations prior to vitamin E supplementation. In Group I prior to dialysis, erythrocyte  $\alpha$ -tocopherol concentrations significantly ( $p < 0.04$ ) increased during vitamin E supplementation. Also, a positive correlation ( $p < 0.001$ ) between plasma and erythrocyte  $\alpha$ -tocopherol levels in Group I was observed. Moreover, vitamin E supplementation significantly ( $p < 0.004$ ) increased hematocrits post-dialysis from  $39.9 \% \pm 1.9 \%$  to  $42.3 \% \pm 1.6 \%$  only in this group. On the contrary, there was no change in hematocrits during vitamin E supplementation in the vitamin C deficient group ( $<40 \mu\text{M}$ , Group II). Both iron status parameters and recombinant human erythropoietin (rHuEPO) doses were not different before and during vitamin E supplementation in the two groups of patients. In conclusion, our data suggests that oral vitamin E supplementation protected erythrocytes from oxidative stress and improved vitamin E status in HD patients, but only in patients with adequate vitamin C status.

## INTRODUCTION

Enhanced oxidative stress has been reported in renal patients undergoing hemodialysis (HD) (12-14). The oxidative stress is presumably caused by uremia-associated metabolic abnormalities (9) and bioincompatibility (16) of dialysis membranes, and associated inflammation. All of these factors could increase generation of reactive oxygen species (ROS) and decrease antioxidant protection. Increased oxidative stress may contribute to the development of dialysis related pathologies, such as anemia and cardiovascular diseases, which are the leading causes of morbidity and mortality in HD patients (19).

Redox-active iron is also involved in oxidative stress. It can initiate lipid peroxidation by generating hydroxyl radicals through the Fenton reaction, and cause damage to tissues and cell membranes (15). The erythrocyte membrane is rich in polyunsaturated fatty acids and erythrocytes contain hemoglobin that can be a source of redox-active iron. Thus, erythrocytes are one of the targets of most oxidative processes. Erythrocytes from HD patients have several metabolic modifications, including a defect in the hexose monophosphate shunt that results in increased superoxide production, and an impaired enzymatic antioxidant defense system, leading to poorer protection against ROS (2-4). In addition, decreased erythrocyte levels of non-enzymatic antioxidants such as  $\alpha$ -tocopherol have been observed (5-8). All of these factors can contribute to increased lipid peroxidation, which enhances the susceptibility of erythrocytes to damage or rupture. When hemolysis occurs, redox-active iron is released, and it may initiate more lipid peroxidation damaging other erythrocytes, shortening their life span, and increasing susceptibility to anemia.

Vitamin E is known as the most important lipid soluble antioxidant in human plasma. It protects erythrocyte membranes against lipid peroxidation (40). Therefore, erythrocyte  $\alpha$ -tocopherol deficiency can amplify oxidative membrane damage and lead to erythrocyte hemolysis (48). Additionally, vitamin E can protect against iron-induced cell damage. Iron-overload induced lipid peroxidation can be prevented by increasing the dietary vitamin E prior to iron intake (45, 46, 49).

Vitamin E administration may reduce the oxidative damage associated with uremia and HD. Ono (10) reported that vitamin E supplementation significantly increased erythrocyte  $\alpha$ -tocopherol concentrations and hematocrits, suggesting that vitamin E supplementation has clinical benefits in correcting anemia in HD patients. Lubrano et al. (26) reported that vitamin E supplementation decreased levels of malonyldialdehyde (MDA) and increased osmotic resistance of erythrocytes. Also, Lim et al. (11) reported body iron status is related to the levels of lipophilic antioxidants. They suggested that elevated body iron stores might decrease the levels of lipophilic antioxidants due to increasing the oxidative consumption of antioxidants.

The present study investigated whether oral vitamin E supplementation can improve vitamin E status and correct anemia in HD patients. We measured erythrocyte and plasma  $\alpha$ -tocopherols, iron parameters (serum iron, total iron binding capacity (TIBC) and plasma ferritin), and plasma ascorbic acid prior to and post-dialysis in 11 renal dialysis patients before and during supplementation of vitamin E (400 IU) daily for two months.

## DESIGN AND METHODS

### Subjects

The present study is part of a larger study to investigate oxidative stress and inflammation in HD patients (50). The protocol was approved by the Oregon State University Institutional Review Board for the protection of Human Subjects (Appendix A: Human Subject's Committee Approval). Each subject was given both a presentation and a consent form that described the experimental procedure and supplementation schedule. The consent form was signed and dated by both the subject and the investigator (Appendix B: Consent Form). The inclusion criteria for acceptance into this study were: (a) subjects free of acute infection or hepatic disease; (b) subjects who did not take large doses of antioxidant supplements (such as vitamins C, E, selenium) or herbal supplements; (c) subjects who did not have excessive alcohol consumption (routine consumption of more than 3 alcoholic beverage servings per day or more than 10 per week).

The characteristics of subjects are given in **Table 1**. Twelve subjects (5 females and 7 males) who were undergoing HD were recruited from Good Samaritan Hospital Dialysis Unit in Corvallis, Oregon. The cause for their renal failure was hypertension (n=3), diabetes nephropathy (n=5), polycystic kidney disease (n=1), or other causes (n=3). Three subjects were moderate smokers (fewer than 10 cigarettes per day). Of the 12 patients admitted to the study, one (subject #10) died as a result of a heart attack; therefore this subject's data are not included in the analyses. One other subject (#4) transferred to a different dialysis unit, so values for iron parameters are not available for this subject.

**Table 1. Subjects' Characteristics**

<b>Subjects</b>	<b>Gender</b>	<b>Age</b>	<b>Height</b>	<b>Weight</b>	<b>BMI</b>	<b>EPO</b>	<b>Iron</b>
<b>#</b>		<b>(yr)</b>	<b>(cm)</b>	<b>(kg)</b>			<b>Supplements</b>
1	F	54	163	68.4	26	Yes	Yes
2	F	66	168	77.1	28	Yes	Yes
3	F	60	163	72.6	28	Yes	Yes
4	M	53	173	81.7	27	Yes	No
5	F	42	152	77.6	33	Yes	Yes
6	F	70	163	49.0	19	Yes	Yes
7	M	73	188	79.4	23	Yes	No
8	M	67	168	59.0	21	Yes	No
9	M	75	178	72.6	23	Yes	No
11	M	73	173	113.4	38	Yes	No
12	M	81	178	70.3	22	Yes	Yes
<b>Mean</b>	--	<b>64</b>	<b>170</b>	<b>74.6</b>	<b>26</b>	--	--
<b>± SD</b>	--	<b>± 11</b>	<b>± 9</b>	<b>± 16.0</b>	<b>± 6</b>	--	--

Subjects were instructed to consume their usual diets throughout the study. Only 2 subjects (#8 and #9) routinely consumed a vitamin supplement, Nephrovite, before and during study.

All subjects had been undergoing HD for at least 6 months and were dialyzed with polysulfone capillary dialyzer three times per week; each session lasted 4 hours. Dialyzers were not reused. All subjects received subcutaneous

recombinant human erythropoietin (rHu-EPO) at a mean dose of  $2955 \pm 1619$  Unit (range 500 to 6,000 U) three times per week. Six subjects received parenteral iron supplementation (100 and 50 mg, Venofer, Vifor, Gallen, Switzerland) once a week.

## **Study Protocol**

### *Baseline Study*

Before any intervention, the vitamin E and iron status of the patients were evaluated. Blood samples of each patient were removed from their dialysis tubing before and after dialysis on Day 0 and Day 14 of the study. Approximately 8 mL of blood was obtained and collected into ethylene diamine tetraacetic acid (EDTA) tubes and Heparin tubes (Becton Dickinson). Samples for iron status were drawn by the hospital staff on one occasion during the baseline period.

### *Vitamin E Administration*

Patients were instructed to consume one 400 IU *RRR*- $\alpha$ -tocopherol (Archer Daniels Midland Company, Decatur, IL) capsule daily with dinner for 60 days. To improve compliance, vitamin E capsules were provided in a package indicating the date to consume each pill. Blood samples were obtained at 30 days and 60 days after the start of supplementation. Samples for iron status were drawn by the hospital staff on two occasions during the supplementation period.

### *Diet Records and Supplement Intakes*

A 24-hour dietary recall was used to obtain dietary and supplement intakes of each patient before and during vitamin E supplementation. On the Medical History form (Appendix C), patients identified their current supplement use. The 24-hour recall was analyzed by ESHA's food processor (Salem, Oregon). These data established the dietary antioxidant intakes of the subjects during the study. rHu-EPO therapy for each patient was also recorded.

### *Blood Sample Preparation*

Plasma and erythrocytes were separated from blood by centrifugation at 2,000 RPM, 4 °C for 15 minutes. Upon removal of the plasma, erythrocytes were washed three times with 5 ml phosphate buffered saline (PBS) containing 0.05% of EDTA at pH 7.4. Following the final wash, the erythrocytes were resuspended in PBS and the hematocrit of the final suspension was measured. Samples were stored at - 80 °C prior to analysis (51).

## **ANALYTICAL TECHNIQUES**

### **Hematologic and Iron Parameters**

Circulating levels of total iron and TIBC were determined on three occasions prior to dialysis by the Good Samaritan Hospital staff using standard laboratory methods.

Transferrin saturation index was calculated according to the following formula: Saturation (%) = Serum iron / TIBC x 100.

Plasma ferritin was determined by using a commercial kit - Ferritin MAb from the ICN Diagnostics, Inc. (Costa Mesa, CA). The kit uses a solid phase immunoradiometric assay based on monoclonal anti-ferritin antibody immobilized to the wall of a polystyrene tube, and polyclonal anti-ferritin antibody labeled with  $^{125}\text{I}$  in the liquid phase. Ferritin was captured from 25 $\mu\text{l}$  serum between monoclonal anti-ferritin antibodies and the radiolabeled polyclonal anti-ferritin tracer. Unbound  $^{125}\text{I}$ -anti-ferritin antibody was removed by decanting the reaction mixture and washing tubes. The ferritin concentration was proportional to the radioactivity (measured by using a gamma counter) present in the tube after the washing step. Data were calculated as described by the kit instructions.

### **Analysis of Plasma and Erythrocyte Tocopherols**

Plasma or erythrocyte tocopherols were extracted by using a modification of the method by Hatam et al. (52) and analyzed by high-performance liquid chromatography (HPLC) with electrochemical detection according to Podda et al. (53). Briefly, 100  $\mu\text{l}$  plasma or 200  $\mu\text{l}$  washed erythrocytes of known hematocrit was transferred to a 10-ml screw cap test-tube containing 2 ml of 1% ascorbic acid in 100 % ethanol and water to take the sample to 1 ml, and then mixed well. After addition of 0.3 ml saturated potassium hydroxide, samples were incubated 30 minutes in a 70 °C water bath. After cooling on ice, 25  $\mu\text{l}$  butylated hydroxy toluene (BHT) and 1 ml 1% ascorbic acid in water were added, the samples mixed, then extracted with 2 ml hexane. An aliquot of hexane (1.6 ml) was transferred

into a conical tube, dried under nitrogen, and the residue resuspended in 100 $\mu$ l ethanol: methanol, 1:1. The vitamin E contents were analyzed by using the HPLC system (Shimadzu, Kyoto, Japan) consisting of a SCL-10A system controller, a LC-10ADVP HPLC series isocratic pump, a SIL-10A auto-injector, a Beckman Ultrasphere (ODS C-18 column, 4.6 mm i.d., 25 cm, 5  $\mu$ m particle size) with a Waters Spherisorb ODS guard column and a LC-4C amperometric detector with a glassy carbon electrode (Bioanalytical Systems, Lafayette, IN). The mobile phase was a mixture of methanol: water (99:1%, v/v) and 0.1% (w/v) lithium perchlorate. Each run lasted 7 minutes. Peak areas were integrated by using Shimadzu 4.2 class - VP automated software program (Columbia, MD) and tocopherol amounts estimated by comparison to known amounts of authentic compounds.

The buffer used to “wash” erythrocytes did not include vitamin C (or other antioxidants), which could prevent oxidation during erythrocyte isolation. Therefore, in subjects with low plasma ascorbic acid concentrations, erythrocyte  $\alpha$ -tocopherol may have been oxidized prior to analysis during erythrocyte sample preparation in the baseline study.

### **Analysis of Plasma Ascorbic Acids**

For vitamin C analysis, 50  $\mu$ l 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 by using paired-ion reverse-phase HPLC coupled with electrochemical

detection (54). Ascorbic acid standards (in 1mM diethylene-triaminepenta-acetic acid (DTPA) in PBS) were analyzed before and after each of samples. Results are expressed as  $\mu\text{mol/l}$  plasma.

## **STATISTICAL ANALYSES**

Data are expressed as means  $\pm$  SE. Analysis of variance (ANOVA) with repeated measures was used to determine if statistically significant differences occurred for each parameter between the periods before and during supplementation, prior to and post-dialysis, on the two occasions during baseline and vitamin E supplementation. Student paired t-test was used to compare if there were significant difference between parameters within the same group. Pearson's correlation was used to determine the relationships between concentrations of parameters. All data were analyzed by using SPSS statistical software (SPSS Inc., Chicago, IL) and p value less than 0.05 was considered to be statistically significant.

## RESULTS

### Dietary Intakes

Summaries of each individual's dietary vitamin E, vitamin C, and iron before and during vitamin E supplementation are shown in **Table 2**. Despite wide variability between and within subjects, vitamin E intakes were similar on the two occasions. Vitamin C and iron intakes were found to meet the current recommended dietary allowances (RDAs), respectively, which are vitamin C for 90 mg for men and 75 mg for women and 8 mg iron for both men and women (34, 40).

### Erythrocyte and Plasma $\alpha$ -Tocopherols, Relationship to Ascorbic Acid

Erythrocyte  $\alpha$ -tocopherol concentrations were used to assess the adequacy of vitamin E status in these HD subjects. Unfortunately, erythrocyte  $\alpha$ -tocopherol concentrations are extremely susceptible to oxidation during sample handling. During the baseline study, erythrocyte  $\alpha$ -tocopherol concentrations were found to range from 0.7 to 9.9  $\mu\text{mol/L}$  packed cells (**Table 3**). Only those subjects whose plasma ascorbic acid were above 40  $\mu\text{M}$  appeared to have erythrocyte  $\alpha$ -tocopherol concentrations that were reliable ( $>\sim 4.0$   $\mu\text{mol/L}$  packed cells), likely due to the technical difficulties with the assay in subjects with low plasma ascorbic acid, as discussed in Methods. Therefore, the subjects were categorized into two groups according to their plasma ascorbic acid levels prior to dialysis during the baseline study (**Table 3**). The plasma ascorbic acid levels in the vitamin C sufficient group

**Table 2. Individuals' dietary intakes.** Shown are dietary vitamin E, vitamin C, and iron before and during vitamin E supplementation.

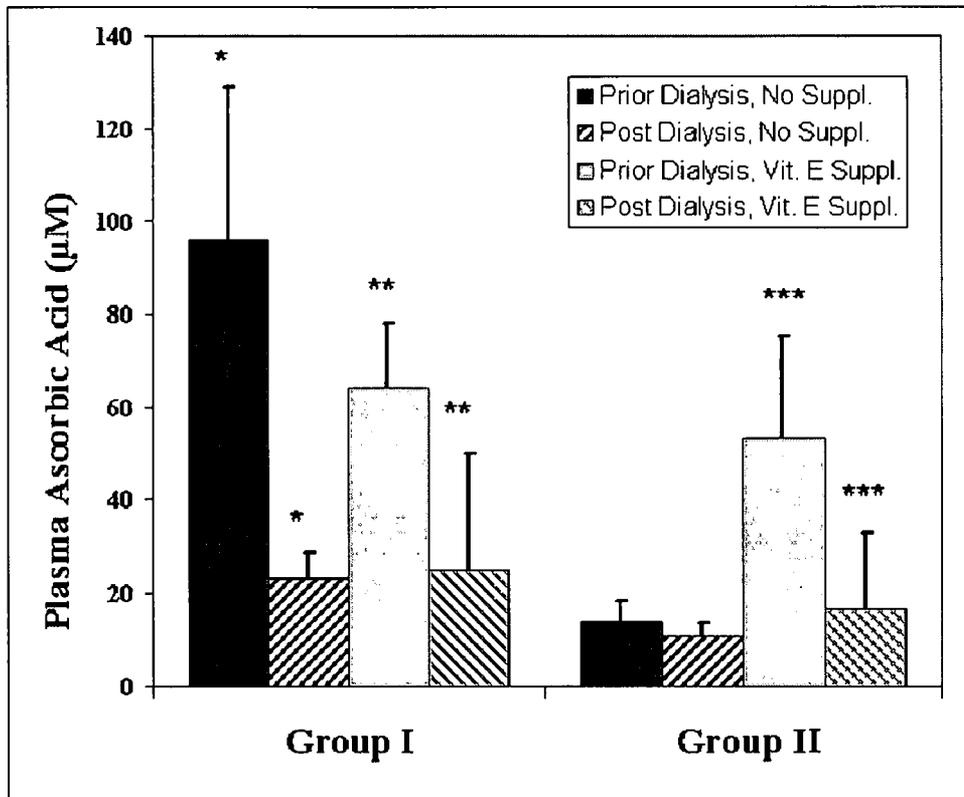
Subject No.	Vitamin E (mg/day)		Vitamin C (mg/day)		Iron (mg/day)	
	Before	During	Before	During	Before	During
1	8.8	2.7	45	22	28.2	18.9
2	12.7	16.0	105	21	25.3	20.6
3	7.8	6.1	409	41	21.4	8.1
4	16.2	2.6	454	269	26.5	12.5
5	3.9	1.2	108	1.1	16.5	6.9
6	6.9	3.4	47	38	14.7	21.8
7	10.0	3.7	127	112	42.8	37.3
8	8.4	8.1	123	79	28.0	31.9
9	18.5	7.9	338	143	26.5	28.5
11	17.8	21.6	317	47	24.6	22.0
12	38.2	10.6	115	41	38.7	9.8
<b>Mean</b>	13.6	7.6	199	74	26.7	19.8
<b>± SE</b>	± 3.0	± 2.0	± 47	± 24	± 2.6	± 3.1

**Table 3. Erythrocyte  $\alpha$ -tocopherol ( $\mu\text{mol/L}$  packed cells) and plasma ascorbic acid ( $\mu\text{M}$ ) concentrations before vitamin E supplementation.**

	Subject #	Erythrocyte		Plasma	
		$\alpha$ -tocopherol		Ascorbic acid	
<b>Dialysis</b>					
		Prior	Post	Prior	Post
<b>Vitamin C sufficient group (Group I, N=6)</b>	2	5.1	1.2	43.7	10.3
	3	7.0	No Sample	52.3	14.5
	4	3.5	1.5	76.3	18.0
	8	9.9	8.4	45.6	9.9
	9	8.4	7.8	308.8	56.2
	11	6.1	6.5	48.9	30.1
	<b>Mean</b>	<b>6.7</b>	<b>5.1</b>	<b>95.9</b>	<b>23.2</b>
	<b><math>\pm</math> SE</b>	<b><math>\pm</math> 0.7</b>	<b><math>\pm</math> 1.1</b>	<b><math>\pm</math> 33.2</b>	<b><math>\pm</math> 5.6</b>
<b>Vitamin C deficient group (Group II, N=5)</b>	1	1.0	1.1	36.7	20.7
	5	1.0	1.0	5.1	6.9
	6	0.7	0.8	3.9	1.5
	7	1.1	No Sample	10.8	5.1
	12	1.2	0.8	13.1	18.8
		<b>Mean</b>	<b>1.0</b>	<b>0.9</b>	<b>13.9</b>
	<b><math>\pm</math> SE</b>	<b><math>\pm</math> 0.1</b>	<b><math>\pm</math> 0.0</b>	<b><math>\pm</math> 4.2</b>	<b><math>\pm</math> 2.7</b>

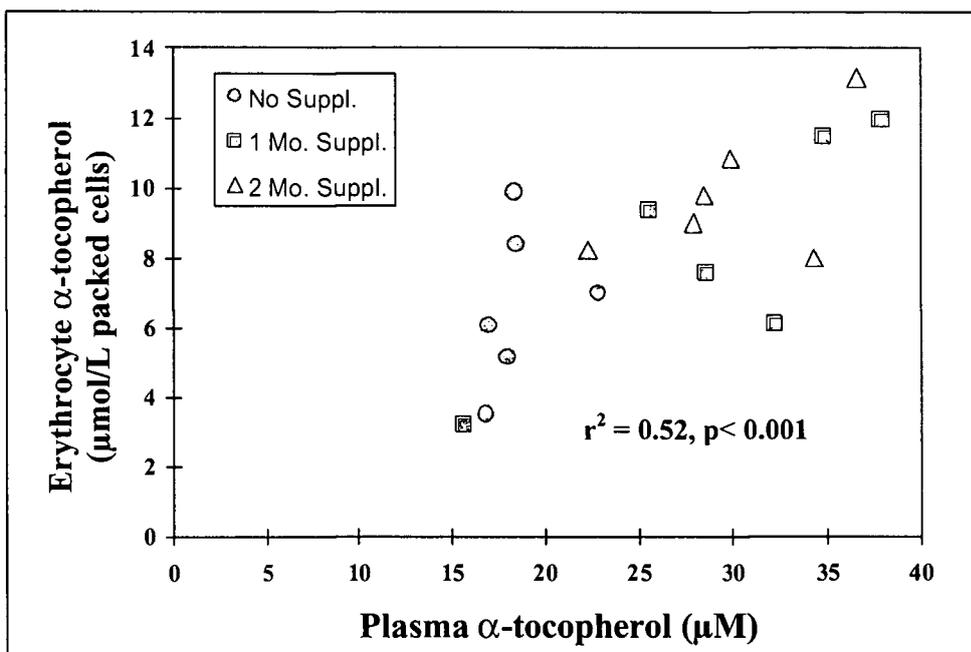
(Group I) were above 40  $\mu\text{M}$ , while Group II subjects' ascorbic acid concentrations were below 40  $\mu\text{M}$ . In Group I, ascorbic acid concentrations were significantly higher than those of Group II (main effect,  $p < 0.03$ ). In both groups, dialysis significantly decreased plasma ascorbic acid concentrations (main effect,  $p < 0.002$ , **Figure 2**). Vitamin E supplementation had no effect on plasma ascorbic acid concentrations.

Since plasma ascorbic acid is lost during dialysis, blood samples obtained prior to dialysis were used for comparison of erythrocyte  $\alpha$ -tocopherol concentrations before and during vitamin E supplementation. Subjects in the vitamin C sufficient group (Group I) had erythrocyte  $\alpha$ -tocopherol concentrations of  $6.7 \pm 0.7$   $\mu\text{mol/L}$  packed cells. These concentrations increased significantly ( $p < 0.04$ ) with 1 and 2 months of vitamin E supplementation ( $8.3 \pm 1.1$   $\mu\text{mol/L}$  packed cells and  $9.8 \pm 0.6$   $\mu\text{mol/L}$  packed cells, respectively). Furthermore, in Group I subjects, there was a positive correlation between erythrocyte and plasma  $\alpha$ -tocopherol concentrations ( $r^2 = 0.52$ ,  $p < 0.001$ ; **Figure 3**).



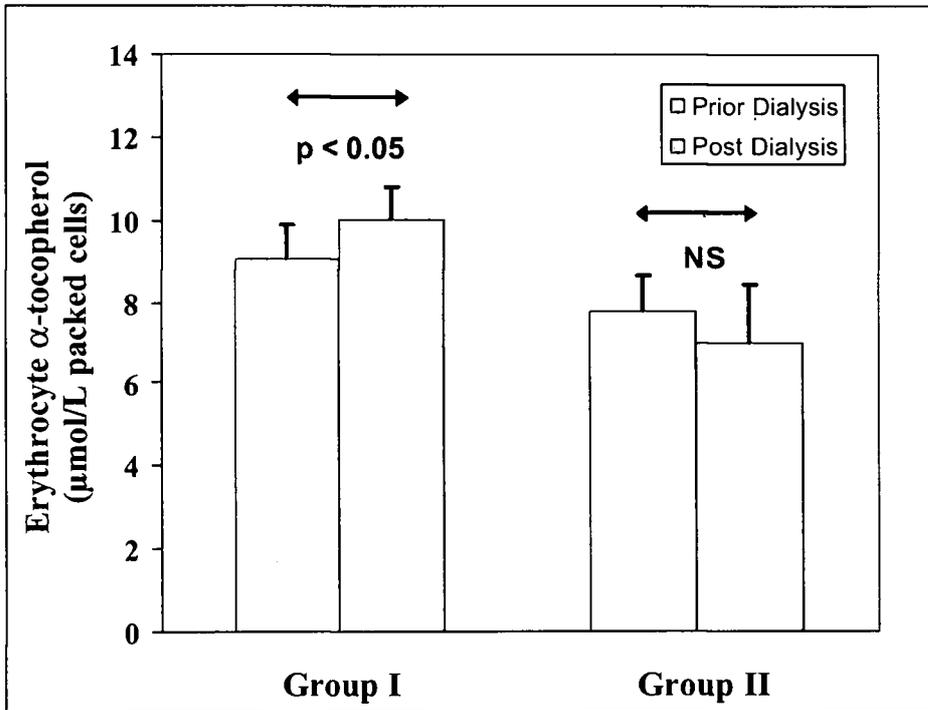
**Figure 2. Plasma ascorbic acid concentrations in Groups I and II.**

\*, Post-dialysis significantly ( $p < 0.01$ ) different from prior to dialysis in Group I before vitamin E supplementation. \*\*, Post-dialysis significantly ( $p < 0.01$ ) different from prior to dialysis in Group I during vitamin E supplementation. \*\*\*, Post-dialysis significantly ( $p < 0.005$ ) different from prior to dialysis in Group II during vitamin E supplementation.



**Figure 3. Relationship of erythrocyte and plasma  $\alpha$ -tocopherol concentrations in Group I.**

During vitamin E supplementation, both prior to and post-dialysis erythrocyte  $\alpha$ -tocopherol concentrations were examined. There was a significant ( $p < 0.04$ ) interaction between groups and dialysis. In Group I, erythrocyte  $\alpha$ -tocopherol concentrations increased significantly ( $p < 0.05$ ) following dialysis. In contrast, there was no change in erythrocyte  $\alpha$ -tocopherol concentrations between levels prior to and post-dialysis in Group II (**Figure 4**).

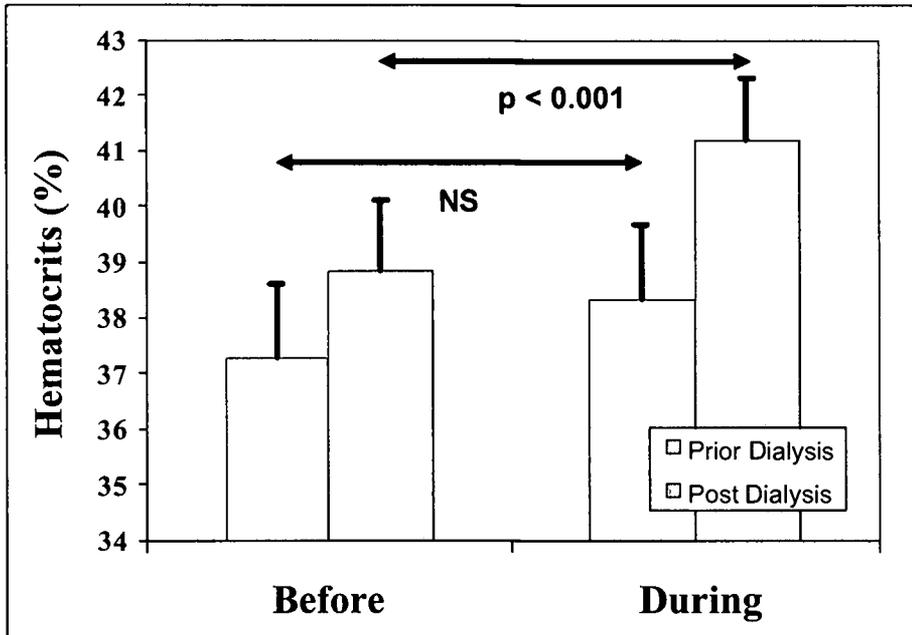


**Figure 4. Erythrocyte  $\alpha$ -tocopherol concentrations during vitamin E supplementation in Groups I and II.**

Shown are erythrocyte  $\alpha$ -tocopherol concentrations prior to and post-dialysis during vitamin E supplementation. Erythrocytes  $\alpha$ -tocopherol concentrations post-dialysis significantly increased only in Group I subjects. There was no difference in Group II.

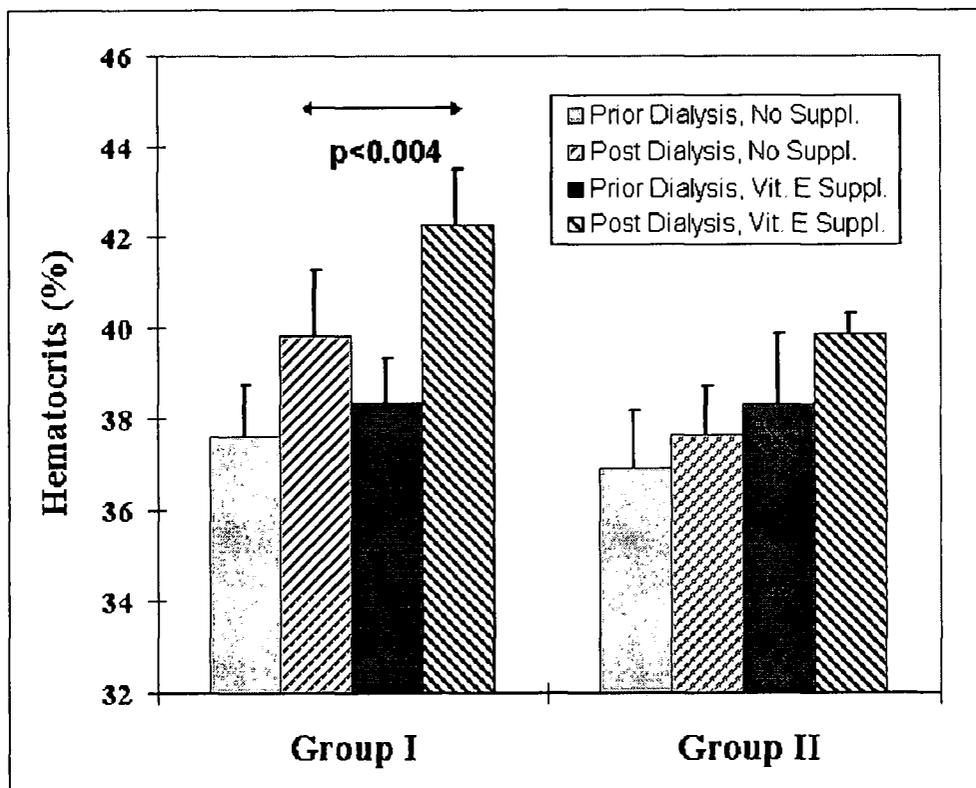
## Hematocrits

Erythrocytes are highly susceptible to hemolysis if they contain insufficient  $\alpha$ -tocopherol (40); therefore, we also measured hematocrits. In response to vitamin E supplementation, hematocrits post-dialysis increased ( $p < 0.001$ ; **Figure 5**). However, dialysis also increased hematocrits (main effect,  $p < 0.02$ ). The groups were found to respond differently post-dialysis to vitamin E supplementation (**Figure 6**). Group I hematocrits post-dialysis is significantly increased from  $39.9\% \pm 1.9\%$  to  $42.3\% \pm 1.6\%$  ( $p < 0.004$ ). On the contrary, Group II hematocrits were unchanged by either vitamin E supplementation or by dialysis. These data suggest that sufficient plasma ascorbic acid (Group I), as well as vitamin E, is needed for erythrocyte membrane protection.



**Figure 5. Hematocrits in all subjects.**

Shown are the hematocrits in all subjects before and during vitamin E supplementation. Vitamin E supplementation significantly increased hematocrits post-dialysis. No change was found prior to dialysis during supplementation. Dialysis had effect (main effect,  $p < 0.02$ ) on hematocrits before and during vitamin E supplementation.



**Figure 6. Hematocrits in Groups I and II.**

Vitamin E supplementation significantly increased post-dialysis hematocrits only in Group I subjects. No changes were found prior to and post-dialysis in Group II subjects.

### Iron Parameters and EPO doses

Hematocrits could change depending on iron status; therefore, various iron parameters were measured. Plasma ferritin is a marker of inflammatory responses. As shown in **Table 4**, plasma ferritin concentrations were more than 2 standard deviations above normal values at all of the times measured in HD subjects (1). Moreover, ferritin concentrations increased with dialysis ( $p < 0.02$ ). There were no significant differences in plasma ferritin concentrations in response to vitamin E supplementation, or between Groups I and II (data not shown).

**Table 4. Plasma ferritin concentrations ( $\mu\text{g/L}$ ) in all subjects (N=11)\*.**

	Normal Value**	No Vitamin E Supplement	1 month Supplement	2 months Supplement
Prior to dialysis	15 ~ 300	439 $\pm$ 76 <sup>a</sup>	381 $\pm$ 58 <sup>b</sup>	412 $\pm$ 68 <sup>c</sup>
Post-dialysis		498 $\pm$ 89 <sup>a</sup>	466 $\pm$ 75 <sup>b</sup>	435 $\pm$ 78 <sup>c</sup>

\* Values are the means  $\pm$  SE.

\*\* Normal ranges for adult men and post-menopausal women (1).

<sup>a,b,c</sup> Significantly different from prior dialysis session ( $p < 0.02$ ).

In contrast, as shown in **Table 5**, both serum iron and TIBC were 2 standard deviations lower than normal values (1). Serum iron, transferrin saturation, and TIBC were unchanged by vitamin E supplementation and were similar between groups.

**Table 5. Serum iron parameters and EPO dosage in all subjects (N=11)\*.**

Parameters	Normal Value**	No Vitamin E Supplement	1 month Supplement	2 months Supplement
Serum Iron ( $\mu\text{mol/L}$ )***	14 ~ 35	9.2 $\pm$ 1.0	8.5 $\pm$ 0.9	9.4 $\pm$ 0.8
TIBC ( $\mu\text{mol/L}$ )***	45 ~ 72	33.0 $\pm$ 1.9	33.4 $\pm$ 1.0	33.7 $\pm$ 1.1
Transferrin Saturation (%)***	20 ~ 55	28.2 $\pm$ 3.4	26.1 $\pm$ 3.1	28.3 $\pm$ 2.1
rHuEPO Dose (unit)	--	2955 $\pm$ 512	3227 $\pm$ 672	3136 $\pm$ 765

\* Values are the means  $\pm$  SE.

\*\* Normal ranges for adult men and post-menopausal women (1).

\*\*\* Data were averaged only from 10 subjects. (Subject #4 was not included.)

Abbreviations: TIBC, Total iron binding capacity; rHuEPO, Recombinant Human EPO.

EPO is routinely given to HD subjects to maintain erythrocyte production. No significant differences were found in EPO dose between Groups I and II. In addition, vitamin E supplementation had no significant effect on the level of EPO therapy.

## DISCUSSION

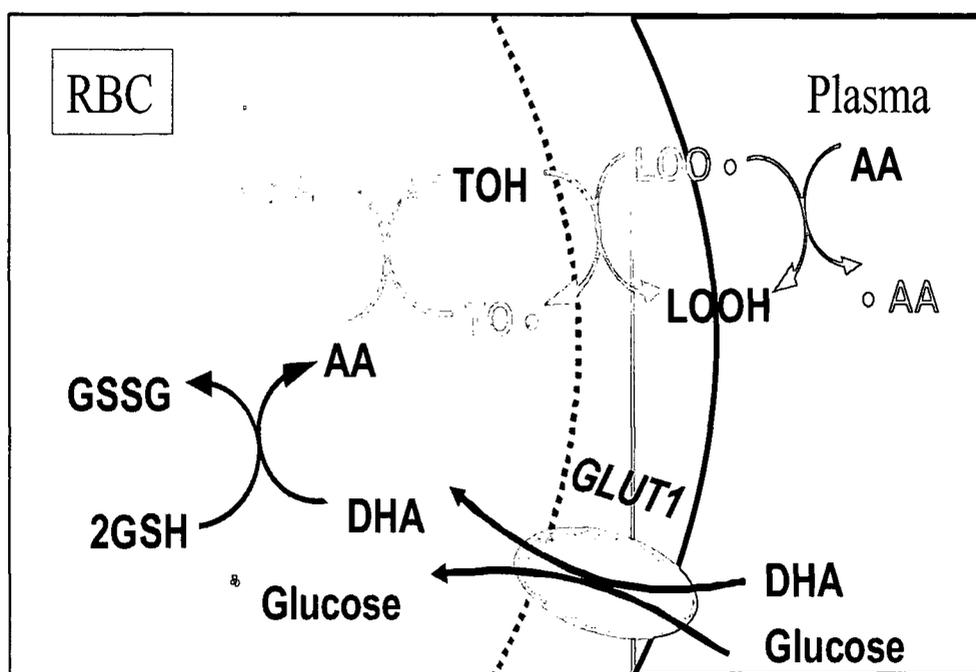
Vitamin E is the most potent lipid-soluble antioxidant protecting erythrocyte membranes against oxidative damage (40); therefore, HD patients were supplemented with vitamin E for 2 months. HD patients responded to vitamin E supplementation with an increase in post-dialysis hematocrit values (**Figure 5**). Importantly, our study showed that these protective effects also required sufficient plasma ascorbic acid.

### Protection and Recycling of $\alpha$ -Tocopherol in Erythrocyte Membranes

Tappel et al. (55) first proposed that vitamin C in the water-phase could regenerate membrane vitamin E from the vitamin E radical, thereby maintaining an active vitamin E pool. Indeed, several studies in variety of in vitro systems have shown subsequently that vitamin C spares or recycles membrane  $\alpha$ -tocopherol (56-60). The mechanism of such recycling has been generally accepted to be a reduction of the  $\alpha$ -tocopheroxyl radical at the water-lipid interface of the membrane bilayer (61). This interaction between vitamins E and C likely protects erythrocyte membranes (**Figure 7**).

HD subjects with low plasma ascorbic acid levels (Group II) had erythrocytes that oxidized easily, even during sample preparation. Van den Berg et al. (62) suggested that high plasma ascorbic acid levels maintained low erythrocyte  $\alpha$ -tocopherol levels in an unoxidized state. Furthermore, Chen et al. (63) reported that vitamin C acts as an antioxidant to inhibit lipid peroxidation in vivo, even during iron overload. Only Group I HD patients had reliable erythrocyte

$\alpha$ -tocopherol concentrations in the baseline study. Moreover, only Group I demonstrated increased erythrocyte  $\alpha$ -tocopherol concentrations after dialysis during vitamin E supplementation (**Figure 4**). These data suggest that erythrocytes from Group II subjects hemolyze readily because their plasma ascorbic acid concentrations were so low (**Table 3**).



**Figure 7.**  $\alpha$ -Tocopherol recycled on erythrocyte membrane by ascorbic acid. Abbreviations: RBC, Human erythrocyte; DHA, Dehydroascorbic acid; AA, Ascorbic Acid; TOH,  $\alpha$ -Tocopherol; LOOH, Lipid hydroperoxide. •, Free radicals. GSH, Glutathione, GSSG, ox-Glutathione.

Enhanced peroxidation can induce the polymerization of phospholipids and high molecular weight proteins in erythrocytes membrane, thereby increasing

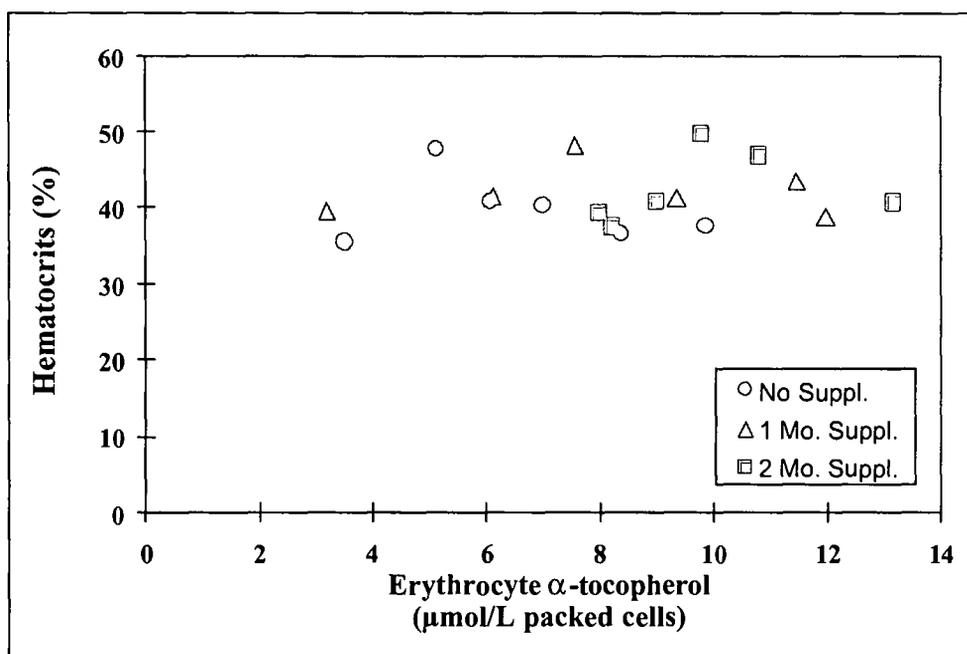
membrane rigidity, making them less deformable (64). Due to hemoconcentration during dialysis, erythrocytes also face osmotic stress, which further increases their susceptibility to hemolysis. Vitamin E administration was reported by Lubrano et al. (26) to improve erythrocyte osmotic resistance by decreasing lipid peroxidation of erythrocyte membranes in HD patients. In our study during vitamin E supplementation, patients with adequate vitamin C status had increased erythrocyte  $\alpha$ -tocopherol levels post-dialysis. These data suggest that not only vitamin E, but also adequate vitamin C is required to reduce oxidative stress. And it further improves osmotic resistance in erythrocytes.

### **Erythrocyte and Plasma $\alpha$ -Tocopherol in HD Patients**

Before supplementation with vitamin E, although plasma  $\alpha$ -tocopherol concentrations (**Figure 3**) in HD patients were at the low end of normal (40), it was difficult to assess whether or not erythrocyte  $\alpha$ -tocopherol concentrations were normal. Assessment was confounded because 1) there is wide variability in the literature values of normal subjects, 2) HD patients' erythrocytes tended to hemolyze readily, especially in subjects with low plasma ascorbic acid concentrations, and 3) the assay is technically difficult and subject to in vitro oxidation.

Erythrocyte  $\alpha$ -tocopherol deficiency can amplify the oxidative membrane damage and cause erythrocytes to hemolyze (48). It remains uncertain how much erythrocyte  $\alpha$ -tocopherol is necessary to protect cell membranes. From our results (**Figure 8**), there was a wide range of erythrocyte  $\alpha$ -tocopherol concentrations at similar hematocrits, and no correlation was found between these parameters. Thus,

we cannot identify the cutoff values for adequate erythrocyte  $\alpha$ -tocopherol concentrations for maintenance of normal hematocrits. However, approximately 4  $\mu\text{mol/L}$  packed cells or higher  $\alpha$ -tocopherol concentrations were associated with normal hematocrits. Increases in erythrocyte  $\alpha$ -tocopherol concentrations during vitamin E supplementation are known to prevent hemolysis (43).



**Figure 8. Relationship of erythrocyte  $\alpha$ -tocopherol and hematocrits.**

No correlation was found between erythrocyte  $\alpha$ -tocopherol concentrations and hematocrits prior to dialysis before and during vitamin E supplementation in Group I subjects.

In Group I subjects, who had adequate ascorbic acid status, vitamin E supplementation increased both plasma and erythrocyte  $\alpha$ -tocopherol

concentrations. Indeed, a strongly significant positive correlation was found between plasma and erythrocyte  $\alpha$ -tocopherol concentrations, when data from before and during supplementation was evaluated (**Figure 3**). Several investigators (51, 65, 66) have observed that vitamin E supplementation in healthy subjects increased both plasma and erythrocyte  $\alpha$ -tocopherol concentrations. Cheeseman et al. (66) reported a positive correlation between erythrocyte and plasma  $\alpha$ -tocopherol concentrations in normal subjects. Ono (10) reported that supplementation of HD patients with 600 mg vitamin E for two months doubled plasma and tripled erythrocyte  $\alpha$ -tocopherol concentrations. These studies taken together with ours suggest vitamin E supplementation increased both plasma and erythrocyte  $\alpha$ -tocopherol concentrations.

Erythrocyte  $\alpha$ -tocopherol concentrations were reported to be low in HD patients, even with normal plasma  $\alpha$ -tocopherol levels (10, 67). Taccone-Gallucci et al. (68) indicated that this discrepancy might be due to impaired  $\alpha$ -tocopherol transfer from plasma to the erythrocytes. However, our findings do not confirm this assumption, as there was a significant positive correlation between plasma and erythrocytes during vitamin E supplementation in Group I (**Figure 3**). These data suggest that vitamin E could be transported from plasma to erythrocytes efficiently in HD patients. However, Group II subjects, who had low plasma ascorbic acid levels and nearly normal plasma  $\alpha$ -tocopherol concentrations, had erythrocyte  $\alpha$ -tocopherol concentrations that were too low to be reliably measured. It appears that erythrocyte  $\alpha$ -tocopherol was oxidized during analysis. Thus, diminished erythrocyte  $\alpha$ -tocopherol concentrations resulted mainly from increased consumption of vitamin E in the face of enhanced oxidative stress.

## **Vitamin E Has a Beneficial Effect on Anemia in HD Patients**

The causes of anemia in HD patients are multiple, but the primary causes include insufficient production of EPO by the diseased kidney, iron deficiency and shortened erythrocyte survival (25). rHuEPO, which stimulates the bone marrow to produce erythrocytes, is used in the treatment of the anemia. HD patients are monitored monthly and the rHuEPO dose adjusted depending on the hematocrits and/or hemoglobin concentrations (25). Both Turi et al. (67) and Inal et al. (69) reported that vitamin E supplementation in HD patients decreased the rHuEPO dosage and improved its therapeutic effect. All of our subjects were undergoing EPO therapy; however, no change in the rHuEPO dose with vitamin E supplementation was noted (**Table 5**).

Iron is essential for hemoglobin formation. Inadequate iron status leads to decreased erythrocyte generation. Adequate iron stores are needed before rHuEPO therapy is initiated (25). All of our HD patients had low serum iron and TIBC, while their plasma ferritin levels were high. These data indicate that the amount of iron immediately available for erythropoiesis was deficient in all subjects (**Tables 4 & 5**). Although some patients with serious iron deficiency received iron supplements by injection, no differences were found in iron parameters before and during vitamin E supplementation in two groups. Combined with the results of EPO dosage, these findings indicate that the increased hematocrits observed during vitamin E supplementation were not caused by EPO therapy or iron supplementation.

Shortened erythrocyte survival is another factor causing anemia in HD patients. Decreased erythrocyte life span is commonly attributed to oxidative

hemolysis (2). Our study shows that vitamin E supplementation increased erythrocyte  $\alpha$ -tocopherol concentrations and significantly induced increases in hematocrits. These findings suggest that anemia in HD patients can partly be attributed to insufficient erythrocyte antioxidants, vitamins E and C, which accompany increased rates of lipid and protein oxidation, as well as destruction of membrane function. Therefore, supplementation of antioxidants vitamins E and C may have a beneficial effect on anemia in HD patients.

In summary, this study indicates that vitamin E supplementation increased erythrocyte  $\alpha$ -tocopherol concentrations, and in HD subjects with adequate ascorbic acid status protected erythrocyte membranes from oxidative damage, and increased their hematocrits. Since these beneficial effects required adequate plasma ascorbic acid, it must be emphasized that HD patients do require adequate vitamin C intakes.

## CONCLUSIONS

Vitamin E is the most potent lipid-soluble antioxidant protecting erythrocyte membranes against oxidative damage. However, our study showed that these protective effects also required sufficient plasma ascorbic acid.

In this study, hemodialysis patients with adequate plasma ascorbic acids levels responded to oral vitamin E supplementation by significantly increasing their erythrocyte  $\alpha$ -tocopherol concentrations and hematocrits. Moreover, there was a significant, positive relationship between plasma and erythrocyte  $\alpha$ -tocopherol concentrations. On the contrary, erythrocytes from patients with inadequate plasma ascorbic acid concentrations were easily hemolyzed, even during sample preparation. Moreover, Group II hematocrits did not change during vitamin E supplementation. These results indicate that vitamin C plays an important role in the erythrocyte antioxidant protection by vitamin E. In addition, there were no changes during vitamin E supplementation in iron parameters and EPO dosage of all patients, suggesting that increased hematocrit was a result of vitamin E supplementation. Therefore, supplementation of vitamins E and C could have beneficial effects on anemia in HD patients.

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## **APPENDICES**

## APPENDIX A. HUMAN SUBJECT'S COMMITTEE APPROVAL



OREGON STATE  
UNIVERSITY

### Report of Review by the Institutional Review Board for the Protection of Human Subjects

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

## APPENDIX B. 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.

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#### TITLE OF STUDY

Antioxidants in Patients Undergoing Hemodialysis

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#### 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

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#### 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.

Page 1 of 3

Participant Initials \_\_\_\_\_

Investigator Initials \_\_\_\_\_

## APPENDIX B. 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 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 ( $\alpha$ - and  $\gamma$ -tocopherols)
2. Markers of the damage caused by free radicals and inflammation:  $F_2$ -isoprostanes, ICAM, VCAM, C-reactive protein, interleukins 1 and 6, and tumor necrosis factor- $\alpha$ .

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### 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.

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### BENEFITS

The potential benefit to you would be a decrease in your free radical ( $F_2$ -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.

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### 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 this study.

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### 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 B. CONSENT FORM (Continued)

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**QUESTIONS**

If you have any questions about the research study or specific procedures, please contact Kylie Smith or Jam 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 C. PATIENTS MEDICAL HISTORY FORM

### PATIENT MEDICAL HISTORY FOR RESEARCH ON VITAMIN E AND ANTIOXIDANTS

Date                      Patients Code Number                      Age      Sex      Race      Ht      Wt      BP  
 Procedure (scheduled and actually performed, if different)

Procedure Findings/Outcome

History of Present Illness/Past Medical History/Review of Systems  
 CV

Pulm

Neuro

Endocrine

Liver

Renal

GI

Hematology

Other

Exercise/ Activity

Medications (prescription, OTC, or herbal) and nutritional supplements, with time of last dose

Allergies or Adverse Drug Reactions

Diet (typical and recent, if different)

Everything ingested in 12 hours prior to procedure, & time ingested

Past Surgical and Anesthetic History

Family History

Labs

Signature of Researchers