Gamma-Tocopherol and Docosahexaenoic Acid Decrease Inflammation in Dialysis Patients

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Objective: Increased cardiovascular risk in hemodialysis patients may be related to augmented oxidative stress and inflammation, for which no proven beneficial therapies are available.

Study Design: We examined the effects of gamma tocopherol and docosahexaenoic acid (DHA) administration on inflammation and oxidative stress markers in hemodialysis patients in a randomized, double-blinded, placebo-controlled, clinical trial. Active treatment consisted of capsules containing gamma tocopherol (308 mg) and DHA (800 mg).

Setting: Outpatient dialysis center.

Patients: Seventy maintenance hemodialysis patients.

Main Outcome Measures: Plasma concentrations of interleukin-6 (IL-6) and protein carbonyl content were determined by enzyme-linked immunosorbant assay. C-reactive protein was measured by nephelometry. The F2 isoprostanes were measured by gas chromatography-mass spectrometry. Erythrocyte DHA content was measured by gas chromatography.

Results: Sixty-three patients were enrolled, and 57 completed the study. No serious adverse events were attributed to either active treatment or placebo. In the treatment group, but not in the placebo group, there were significant decreases in IL-6 (21.4 ± 3.5 to 16.8 ± 3.7 pg/mL), white blood cell (WBC) count (7.4 ± 0.3 to 6.9 ± 0.4 10^3/L), and neutrophil fraction of WBCs (4.8 ± 0.3 to 4.4 ± 0.3 10^3/L), at P < .05 for all. There were no significant changes in plasma concentrations of CRP, F2 isoprostanes, or carbonyls in either group.

Conclusion: Thus, gamma tocopherol and DHA are well-tolerated and reduce selected biomarkers of inflammation in hemodialysis patients. Larger randomized, clinical trials will be required to determine if gamma tocopherol and DHA can reduce cardiovascular complications in hemodialysis patients.

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jects with and without renal failure, but do not consistently lower plasma interleukin-6 levels. Two recent studies of antioxidative agents in dialysis patients reported reduced cardiovascular events, without a significant effect on overall mortality. Neither study reported on the effects of antioxidants on biomarkers of inflammation or oxidative stress. We and others recently reported that renal failure alters the metabolism of α-tocopherol and γ-tocopherol. An earlier study suggested that administration of mixed tocopherols may reduce biomarkers of acute-phase inflammation in hemodialysis patients. The purpose of the present study was to investigate the effects of a formulation containing γ-tocopherol and docosahexaenoic acid (DHA), nutrients with potential synergistic antioxidative and anti-inflammatory properties, on biological markers of inflammation and oxidative stress in hemodialysis patients. Docosahexaenoic acid is an anti-inflammatory agent, but is highly prone to degradation by reactive oxygen species (ROS). The potential synergy between these agents involves the protection of DHA from ROS-mediated degradation by concurrently administered γ-tocopherol, affording the DHA greater net anti-inflammatory effect per unit dose administered.

Subjects and Methods

Patients

Between March–October 2002, 70 patients undergoing maintenance hemodialysis were recruited from dialysis centers located in and around Portland, Maine, and screened for entrance into the study. Candidates were included if they were 18–75 years of age with a body mass index <35 kg/m², and if they had normal hepatic function and white blood cell (WBC) count, and were excluded if they had clinically significant electrocardiogram abnormalities, a chronic inflammatory condition, cancer in the previous 5 years except for skin cancer, a recent bacterial infection, if they took vitamin E supplements >60 IU/day, vitamin C >500 mg/day, or anti-inflammatory medications (except for aspirin <325 mg/day), and if they were participants in a concurrent or recent intervention study or were pregnant or anticipated becoming pregnant over the course of the study. Sixty-three of 70 patients screened (90%) met the entrance criteria and enrolled into the study. Subjects continued all medications, and only changed medications and dosage during the course of the study as prescribed by their physician. All study subjects routinely received intravenous erythropoietin for treatment of anemia. All patients received dialysis using high-flux polysulfone dialysis membranes, and dialyzers were not reused during this study. The study protocol was approved by the Maine Medical Center Research Institutional Review Board. All subjects provided written, informed consent.

Design

This was a randomized, double-blinded, placebo-controlled, clinical trial to evaluate the efficacy of γ-tocopherol and DHA in decreasing plasma concentrations of biomarkers of inflammation and oxidative stress. Subjects were randomly assigned to receive six soft-gel capsules per day of either active treatment (two capsules providing a total dose of 308 mg of γ-tocopherol, 13 mg of α-tocopherol, and 11 mg of β- and δ-tocopherols, from Incon, Batavia, IL, and four capsules providing a total dose of 800 mg of DHA from Martek, Columbia, MD), or matching placebos (containing a total of 3 g of high-oleic sunflower oil), for a period of 8 weeks. The DHA oil used for this formulation contained 4 mg of α-tocopherol per gram. Randomization was performed by using stratified, permuted blocks of size four with the presence of diabetes mellitus as the stratum. The blocks of size four were chosen to allow a maximum imbalance of two in the number of diabetics in each group. Blood samples were collected twice at baseline (each at 4 weeks apart), and values were averaged to minimize the regression-toward-the-mean phenomenon. Blood sampling was repeated at 4 weeks and at 8 weeks after study initiation. Subjects, site staff, and investigators were blinded to treatment assignment. Compliance was monitored by pill counting by a research coordinator at each clinic visit.

Measurements

Blood samples were drawn into ethylenediamine tetracetic acid to assess the following markers of inflammation and oxidative stress: CRP, IL-6, leukocytes, albumin, prealbumin, F₂ isoprostanes, and protein carbonyls. High-sensitivity
CRP was determined using nephelometry. Interleukin-6 levels were determined by an enzyme-amplified sensitivity immunoassay (Biosource, Nivelles, Belgium). Complete blood counts were determined with an EPEX Coulter Counter (Miami, FL). Prealbumin was determined using an autoanalyzer. The F2 isoprostanes were measured as previously described. Plasma protein carbonyl concentration was determined by enzyme-linked immunosorbent assay (Zenith Technology, Dunedin, New Zealand). The erythropoietic index, a surrogate measure for erythropoietin (EPO) resistance defined as EPO dose/hematocrit (Hct), was employed in this study.

Red blood cell DHA content was measured as an objective indicator of adherence. Red cells were purified from whole blood via centrifugation (1600 × g for 10 min), and frozen at −70°C. Red-cell total lipids were extracted by the method of Bligh and Dyer, whereby amounts of chloroform, methanol, and water were mixed with the sample, ultimately resulting in a lipid-rich chloroform layer. The chloroform layer was then taken to near-dryness under nitrogen (to protect unsaturated fatty acids), and then derivatized into fatty-acid methyl esters via commercially available boron trifluoride (10%) in excess methanol. Resulting fatty-acid methyl esters were then extracted, concentrated, and injected into an automated gas chromatograph (Shimadzu GC17 [Shimadzu Scientific Instrument, Columbia, MD], utilizing a 50m Quadrex free fatty-acid phase coating [Quadrex Corporation, Woodbridge, CT]) with flame ionization detection, as previously described. Fatty-acid peaks were determined by comparison to authentic fatty-acid methyl ester standards (NuChek Prep, Elysian, MN), using chromatographic software. Nitrogen was blanketed on samples throughout the analysis, to prevent oxidation of highly unsaturated fatty acids. The percentage of total fatty-acid weight for each fatty acid (wt%) was calculated (Dr. Douglas Bibus, Lipid Technologies, Austin, MN).

Statistical Analysis

The primary outcome followed in this study was change in plasma concentrations of IL-6. We hypothesized that the administration of tocopherol and DHA would decrease plasma concentrations of IL-6 by at least 15%. Our preliminary data show an average plasma IL-6 concentration of 20.17 ± 4.37 pg/mL (mean ± SEM) in patients initiating maintenance hemodialysis treatment. In the present study, we anticipated finding similar values at baseline. Under these circumstances, a sample size of 30 in the treatment group would provide 94% power to detect a 15% difference in IL-6 concentrations, i.e., a first-condition mean of 20 pg/mL and a second-condition mean of 17 pg/mL, assuming a standard deviation of the differences of 4.5 pg/mL, using a paired t test approach and a two-sided 0.05 significance level. It should be noted that this particular pilot study was not powered to show between-group differences.

Statistical analyses were performed on the 63 patients enrolled in the study, based on the intention-to-treat approach, where all participants are analyzed in the group to which they were assigned, whether or not they completed the intervention given to the group. This measure was taken to prevent bias caused by the loss of participants, which may disrupt the baseline equivalence established by random assignment, and which may reflect nonadherence to the protocol. Data are reported as means ± SEM or median (interquartile range), unless otherwise noted. Comparisons of one-time data between treatment and placebo were completed using the Student t test or Wilcoxon rank-sum test, for normally and non-normally distributed variables, respectively, and Fisher’s exact test for categorical variables. The Bonferroni test was used to adjust the observed significance level when multiple comparisons were performed. Analyses of changes in continuous variables over time were completed using repeated-measures analysis of variance, with time-points and groups as fixed factors. Once differences were determined, the Scheffé post hoc range test was used in multiple pairwise comparisons to determine which of the means differed. Age, gender, and duration of dialysis were included as covariates. The variables CRP, IL-6, and protein carbonyl were log-transformed for analyses. All tests were two-tailed, and P ≤ .05 was considered statistically significant. Because the erythrocyte membrane fatty-acid data were not normally distributed, the nonparametric Wilcoxon signed rank test was used for these analyses, and median values are reported here. The statistical software SPSS version 13.0 (Chicago, IL) was used for inferential
Results

Patients

Of 63 subjects enrolled, 31 were randomized to the treatment group, and 32 to the placebo group. There were four dropouts in the treatment group, and two dropouts in the placebo group (97% vs. 94% completion rates, \( P = 0.11005 \) NS). The dropouts in the treatment group were attributable to adverse events deemed unrelated to the study drug (upper gastrointestinal bleeding, ruptured appendix, cardiac arrhythmia, or death). The two withdrawals from the placebo group were attributable to withdrawal of consent. No moderate or severe adverse events were attributed to either the active treatment or placebo. However, four mild adverse events were rated as possibly or probably related to the active treatment (loose stools, constipation, upset stomach, and flatulence), and one to the placebo (abdominal cramps and nausea). Overall, there were 33 total adverse events: 20 adverse events in 16 patients in the active treatment group, and 15 events in 11 patients in the placebo group. There were no significant differences in the proportions of patients experiencing an adverse event between groups. Pill counting revealed that three patients returned drug bottles with \( \approx 20\% \) of the test article unused. One subject (active treatment) missed 12 of the last 28 days, a second subject (active treatment) missed 23 of the last 28 days, and a third subject (placebo) missed 22 of the last 28 days.

Baseline Characteristics

The median age was 59.7 ± 10.8 years (range, 33–74 years). Twenty subjects were female, and 36 were diabetic. The median duration of hemodialysis was 20 months (range, 2–192 months), plasma albumin was 3.8 ± 0.27 mg/dL, and prealbumin was 34.3 ± 8.4 mg/dL. Table 1 shows the baseline characteristics and biochemistry for both groups. There were no significant differences between treatment groups for age, duration of hemodialysis, diabetes, Kt/V, blood pressure, albumin, or EPO dose. There were no significant differences in any baseline measures of inflammation or oxidative stress biomarkers based on gender. Diabetes and hypertension were largely responsible for subjects’ renal failure. Baseline characteristics for the seven patients who failed the screening criteria and the six subjects who did not complete the study are listed in Table 2.

Erythrocyte Membrane Fatty-Acid Content

Median red blood cell membrane DHA content increased significantly in the active treatment group.
from 0.84 wt% (range, 0.33–1.52) at baseline to 1.21 wt% (range, 0.82–3.29) at week 8 (P < .005) compared to the placebo group, in which there was no significant change (0.84 wt% [range, 0.30–2.01] at baseline to 0.81 wt% [range, 0.43–2.57] at week 8, P = NS), demonstrating good subject adherence to taking the test article (Fig. 1). Median erythrocyte arachidonic-acid content at baseline was 4.70 wt% (range, 2.48–6.88) in the active treatment group, and 5.24 wt% (range, 3.00–10.26) in the placebo group (P = NS). At week 8, median erythrocyte content of arachidonic acid had not significantly changed in the active treatment group (4.28 wt%; range, 2.31–6.50) or in the placebo group (5.47 wt%; range, 2.98–12.40). There were no significant changes over time in the 31 other identified fatty acids in either treatment group.

**Biomarkers of Inflammation and Oxidative Stress**

Plasma IL-6 concentrations decreased significantly at week 4, and remained significantly lower than baseline at week 8 in the treatment group, whereas no significant changes were observed in the placebo group (Fig. 2). Similarly, the total WBC count and the neutrophil fraction of WBCs decreased significantly in the treatment group over the study period, while there were no changes in the placebo group (Table 3). C-reactive protein levels did not change significantly over time in either study group (Fig. 3). There were no differences between groups in plasma protein carbonyl content (data not shown). Plasma F₂ isoprostane levels did not change significantly over time in either study group, although there was a statistically significant difference in F₂ isoprostane levels at baseline (0.148 ±
0.02 for the placebo group, and 0.109 ± 0.01 for the treated group, \( P = .025 \).

**Erythropoietic Index**

The baseline erythropoietin dose in this patient cohort was significantly correlated with plasma F2 isoprostane content (\( R_p = 0.284, \ P = .027 \)), plasma IL-6 levels (\( R_p = 0.356, \ P = .007 \)), and CRP levels (\( R_p = 0.351, \ P = .005 \)). There was also a trend toward correlation with plasma protein carbonyl content (\( R_p = 0.231, \ P = .068 \)). Because inflammation and oxidative stress may affect erythrocyte production and survival, and EPO use may modify oxidative stress biomarkers, we examined the effects of intervention on the erythropoietic index (weekly erythropoietin dose divided by Hct). Table 3 demonstrates that over the 8 weeks of intervention, there was a 27% decline in the erythropoietic index within the treatment group (\( P < .05 \)), whereas there was no significant difference within the erythropoietic index in the placebo group.

**Discussion**

The present study reports on the results of a single-center study examining the effects of a gamma-tocopherol-enriched mixture of tocopherols and the n-3 polyunsaturated fatty acid DHA compared with placebo in hemodialysis patients. The results demonstrate that the active treatment arm was associated with a decrease in plasma IL-6 concentrations as well as total WBC count and the neutrophil component of the WBC count compared with placebo. Based on a previous observational study, the 5-pg/mL mean reduction in IL-6 in the present study, if sustainable, would predict a 22% reduction in risk of mortality. There was also a decrease in the erythropoietic index in the active treatment arm, which supports the concept that erythropoietin resistance is at least partly related to inflammation. However, there was no significant effect observed in serum CRP concentrations, plasma F2 isoprostane, or protein carbonyl content.

Gamma tocopherol and its carboxyethyl hydroxychroman metabolite have antioxidative and anti-inflammatory properties that help to explain the effects observed in our study. Recent data suggest that gamma-tocopherol and its metabolites may have more potent anti-inflammatory properties than alpha-tocopherol, as reviewed in Hensley et al. In vivo, gamma-tocopherol inhibits inflammation-induced protein nitration and ascorbate oxidation, and decreases the proinflammatory prostaglandins and leukotrienes more than does alpha-tocopherol. In a previous study, we demonstrated that gamma carboxyethylhydroxychroman, the predominant metabolite of gamma-tocopherol, accumulates in uremic patients compared with healthy subjects, and appears to have more potent anti-inflammatory effects.

We chose to augment the potential anti-inflammatory effect of gamma-tocopherol administration with the addition of the polyunsaturated fatty acid DHA. Docosahexaenoic acid is a marine-derived omega-3 fatty acid \( \text{C}_{22:6}\)-n-3. Dietary fish consumption and fish-oil supplementation are antiatherogenic, and reduce the risk of sudden death in those with and without a history of cardiovascular disease. The mechanism of this effect is relatively independent of the classic lipoprotein risk factor, and appears to occur through modulation of inflammation or stabilization of mycardiocyte membranes via effects on ion channels. Eicosapentaenoic acid and DHA can replace membrane arachidonate, reducing the substrate for proinflammatory eicosanoids. Recent novel anti-inflammatory eicosanoids and docosanoids formed by eicosapentaenoic acid (EPA) and DHA via a 15-lipoxygenase-like enzyme were described. Docosahexaenoic acid is a cyclooxygenase-2 inhibitor and reduces IL-6 production from endothelial cells. The selective effectiveness of DHA on IL-6 may explain the positive results obtained in IL-6, but not in CRP. Dwyer et al. noted that polymorphisms in the promoter region of the arachidonate 5-lipoxygenase correlate with both CRP and atherosclerosis risk, which can be modulated by dietary intake of arachidonate or DHA. Docosahexaenoic acid is also a prime target for destruction by ROS. It is therefore desirable to provide DHA in the context of highly effective antioxidant therapy.

In this study, active treatment resulted in an increase in erythrocyte DHA content, but no significant change in arachidonate content. This is somewhat surprising, because the administration of DHA is commonly associated with a decline in erythrocyte membrane arachidonate.
content. Furthermore, while there are no recognized standards for normal erythrocyte membrane arachidonate or DHA content, the levels of erythrocyte arachidonate and DHA observed in this study in dialysis subjects were substantially lower than the levels recently reported in healthy subjects. This is consistent with earlier reports in uremic subjects who also exhibited lower arachidonate levels, although a recent study showed comparable levels of DHA in HD patients vs. healthy controls. Also of note, there was no elevation of Mead acid (20:3n-9), despite the low levels of membrane arachidonate, indicating that these subjects did not manifest classic essential fatty-acid deficiency. These data suggest that there may be reduced endogenous synthesis of arachidonate and DHA in hemodialysis subjects, presumably because of the decreased functionality of desaturase and elongase enzymes. Alternatively, accelerated destruction of these labile membrane components in a high oxidative stress environment could contribute to lower arachidonate concentration. Because membrane polyunsaturated fatty-acid content affects membrane fluidity and cell signaling, these findings are striking and worthy of further investigation.

The active treatment agents and the placebo were well-tolerated in this study. There were no serious adverse events deemed related to study agents in either treatment group. Overall in the study, there was one death in 120 observed months of treatment (annualized mortality rate, 10%), consistent with expected mortality in this patient population. Similarly, there was no significant difference in the rate of mild adverse events possibly or probably related to therapy between the treatment groups. These data are similar to those in two previous clinical trials using antioxidant therapy in a hemodialysis population. Thus, antioxidant therapy appears to be relatively safe and well-tolerated in the dialysis population.

There are a number of limitations to this study. Despite randomization, there was an imbalance in gender distribution between treatment groups. Although slight differences in the content and distribution of alpha-tocopherol on the basis of gender have been reported, most interventional studies demonstrated similar pharmacologic and biologic effects of tocopherols in men and women. In univariate and multivariate analysis, gender was not a predictor of inflammatory or oxidative stress biomarkers in this study. Thus it is unlikely that the difference in gender distribution in this study contributed substantially to the outcome. Moreover, we did not observe a significant difference in outcome of inflammatory biomarkers within treatment groups (data not shown). The sample size in this study was relatively small. The study duration was relatively short, and it is not clear that the anti-inflammatory effects observed in the active treatment arm could be sustained for a longer duration.

In conclusion, our results show that the provision of gamma-tocopherol and DHA was associated with a significant reduction in plasma IL-6 levels, total WBC counts, and the neutrophil component of WBC counts in the active treatment arm compared with placebo. The provision of gamma-tocopherol and DHA was generally well-tolerated, with no reports of serious adverse events deemed related to the study agents. These data suggest that a combination of antioxidants and fish oils may have efficacy in reducing acute-phase inflammation in maintenance hemodialysis patients. Although the pathogenesis of inflammation in dialysis patients is not well-understood, the close association of markers of inflammation with subsequent risk for morbidity and mortality suggests that therapies that can lower inflammation might have beneficial effects on outcomes. Furthermore, therapies that can improve the efficacy of erythropoietic-stimulating agents may be cost-effective adjuncts for the treatment of anemia in kidney disease. However, the findings in this study must be considered preliminary, and larger prospective trials will be required to definitively answer these questions.

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