

Vitamin E and Vitamin C Treatment Improves Fibrosis in Patients With Nonalcoholic Steatohepatitis

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OBJECTIVE: Nonalcoholic steatohepatitis (NASH) is a common cause of liver disease. Although usually indolent, this disease can progress to cirrhosis in some patients. There is currently no proven medical therapy for the treatment of NASH. The aim of our study was to evaluate the efficacy of combination α -tocopherol (vitamin E) and vitamin C in reducing histologic inflammation and fibrosis.

METHODS: This was a prospective, double-blind, randomized, placebo-controlled trial with a total enrollment of 49 patients; 45 patients completed the study. All patients were randomized to receive either vitamins E and C (1000 IU and 1000 mg, respectively) or placebo daily for 6 months, based on their initial histologic diagnosis of NASH. Additionally, all patients were given standard weight-loss counseling and encouraged to follow a low fat diet (<30 fat g/day). The pre- and posttreatment liver biopsies were reviewed by a single pathologist, who was blinded to the patient's medication. Biopsies were scored based on a modification of the scoring system published by Brunt *et al.* (Am J Gastroenterol 1999; 94:2467–74). A score of 0–4 was possible for fibrosis, and a score of 0–6 was possible for inflammation and hepatocyte degeneration and necrosis. In addition, body mass index, glycohemoglobin, lipids, and liver enzymes were followed throughout the study.

RESULTS: Forty-five patients completed 6 months of therapy without significant side effects. Vitamin treatment resulted in a statistically significant improvement in fibrosis score ($p = 0.002$). No changes were noted in inflammation with treatment.

CONCLUSIONS: Vitamin E and vitamin C, in the doses used in this study, were well tolerated and were effective in improving fibrosis scores in NASH patients. No improvement in necroinflammatory activity or ALT was seen with this combination of drug therapy. A larger, multicenter,

longer-term trial with vitamin E and vitamin C seems to be warranted. (Am J Gastroenterol 2003;98:2485–2490. © 2003 by Am. Coll. of Gastroenterology)

INTRODUCTION

Nonalcoholic steatohepatitis (NASH) is one of the most common forms of liver disease and is frequently associated with clinical conditions such as obesity, type II diabetes mellitus, hyperlipidemia, and hypertension. Recent data suggest that the prevalence of this disease is increasing as the incidence of diabetes and obesity increase (1). Several studies have now demonstrated that this is not a benign condition: 7–17% of patients with NASH will progress to cirrhosis over time (2–4).

Based on present concepts of pathogenesis, oxidative stress likely is involved in the progression of disease from steatosis to NASH and potentially cirrhosis. It has been shown that chronic oxidative stress, generated through the oxidation of cytotoxic free fatty acids, can lead to upregulation of cytokines (5), induction of the liver cytochrome P450 enzyme 2E1, and depletion of hepatic antioxidant concentrations (6). In addition, enhanced lipid peroxidation leads to the generation of byproducts, such as 4-hydroxynonenal and malonaldehyde, which have been shown to further enhance cytokine stimulation and are involved in hepatic stellate cell activation (7), leading to fibrogenesis and enhanced extracellular matrix protein deposition. Seki *et al.* (8) have recently shown that lipid peroxidation products are elevated in NASH patients, occur more prominently in zone 3 of the liver parenchyma, and correlate directly with increasing necroinflammatory activity and fibrosis.

Given that oxidative stress potentially acts as a catalyst for progression of fatty liver disease to NASH, previous treatment trials aimed at decreasing oxidative stress have been performed. Recently, a small study with the antioxidant vitamin E (300 mg/day) showed improvement in inflammation and fibrosis on follow-up liver biopsy in a majority of NASH patients over a 12-month period (9). We

choose to further investigate the possible beneficial effects of antioxidant therapy in NASH by performing a double-blind, placebo-controlled trial using vitamins E and C. We added ascorbic acid to our protocol because evidence suggests that this vitamin enhances regeneration of oxidized vitamin E (10).

MATERIALS AND METHODS

We conducted a prospective, randomized, double-blind, placebo-controlled trial at our institution from August, 2000 until June, 2002. This study received approval from the Brooke Army Medical Center institutional review board before enrollment, and all patients gave written informed consent to participate in this study. Inclusion criteria included all patients with a clinical and histologic diagnosis of NASH who were 18 yr of age or older and had a liver biopsy within the past 6 months for elevated aminotransferases. The patients must have had well compensated liver disease with the following parameters at study entry: Hb values of at least 12 g/dl for women and 13 g/dl for men, white blood cell count of greater than 3,000/mm³, neutrophil count of greater than 1,500/mm³, platelets greater than 70,000/mm³, serum albumin greater than 3 g/dl, and a serum creatinine less than 1.4 mg/dl. Exclusion criteria included 1) other causes for chronic liver disease, to include hepatitis B and C, hereditary hemochromatosis, α -1 antitrypsin deficiency, Wilson's disease, or autoimmune liver disease, 2) use of drugs associated with steatohepatitis, such as tamoxifen, steroids, chloroquine, or amiodarone, 3) prior surgical procedures, such as gastroplasty, jejunoileal or jejunocolic bypass, 4) evidence of decompensated liver disease, such as a history of or the presence of ascites, bleeding varices, or hepatic encephalopathy, 5) pregnancy, 6) total parenteral nutrition within the past 6 months, 7) a history of organ transplant, 8) other conditions that have been known to cause NASH or worsen the disease, and 9) a history of alcohol consumption of greater than 10 g per day. Patients and family members were questioned extensively about the use of alcohol at the time of the study, as well as in the past.

The primary endpoint of the study was to determine the efficacy of combination vitamin E and vitamin C treatment in reducing hepatic inflammation and necrosis and/or fibrosis in patients with NASH. The null hypothesis was that there was no relationship between treatment and the differences in the histologic score after treatment. The alternative hypothesis was that vitamins E and C would improve the histologic score by 1 point in 40% of the subjects, whereas the placebo would result in a 1-point improvement in 5% of the subjects. To detect a 35% difference in efficacy with a power of 80% and a level of confidence of 95%, 18 subjects were required in each study group. A goal of 25 patients enrolled in each arm of the study was planned to allow for dropout.

A total of 49 patients met criteria for enrollment and, after consultation with the principal investigator, agreed to par-

Table 1. Group Characteristics

	Placebo Group (n = 22)	Vitamin Group (n = 23)	P value
Age	50.2	52.5	NS
Gender (Female)	11 (50%)	14 (61%)	NS
BMI kg/m ²	30.8	34.7	p = 0.01
Hyperlipidemia	18 (82%)	18 (78%)	NS
Diabetes Mellitus	5 (23%)	14 (61%)	p = 0.02
Hypertension	12 (55%)	13 (57%)	NS
ALT (U/L)	109	92.3	p = 0.35
AST (U/L)	80.2	63.2	p = 0.25
Total Cholesterol (mg/dl)	212.7	202.2	NS
Triglycerides (mg/dl)	227.5	206.9	NS
Glycohemoglobin, %	5.8*	6.2	NS
Fasting Serum Glucose (mg/dl)	124	126	NS
Fibrosis	1.3	1.8	p = 0.11
Stage 3-4	4 patients	6 patients	NS
Necro-inflammatory activity	3.0	3.1	NS

All values for continuous variables reported as means.

* Data calculated on 21 patients.

ticipate in the study. Each patient was then randomized according to a computer-generated randomization table, designed by the Brooke Army Medical Center pharmacy, to receive either the combination of vitamin E 1000 IU and vitamin C 1000 mg per day or placebo. This randomization table was kept by the pharmacy where the vitamins or placebo were to be obtained by the patient. The patients were assigned to either the vitamin group or the placebo group, based on the coded randomization table, so that only the pharmacist knew which intervention the patient was receiving. The pill containers were coded with a similar number and had the same appearance. Compliance was determined based on pill diaries the patients kept and returned on completion of the study. Baseline biochemical data were obtained (Table 1) and included fasting glucose and lipid profiles, glycohemoglobin, and liver enzymes. Body mass index (BMI) was also calculated. Hyperlipidemia was defined as a total cholesterol or triglyceride level greater than 200 mg/dl. All patients in both groups were given the same 1600-calorie diet and written exercise plan, as outlined by the National Institutes of Health and the National Heart, Lung and Blood Institute (available at www.nhlbi.nih.gov/health/public/heart/obesity/lose_wt). Biopsy specimens were then coded and scored according to the scoring system of Brunt *et al.* (11), with modifications (Table 2). Both the principal investigator and pathologist were blinded as to the patient's intervention.

Statistics

The independent variables were treatment (placebo vs vitamins E and C) and time (before vs after treatment). The dependent variable was histologic score (11). The histologic score classified the disease by inflammation and fibrosis. We used a two-factor analysis of variance (treatment, time) followed as needed by independent and paired *t* tests cor-

Table 2. Outline for Grading and Staging of Liver Biopsy Specimens

Grading for necroinflammatory activity: (combined score of inflammation and hepatocyte degeneration and necrosis)

Inflammation

0 None

1 Scattered, rare intra-acinar neutrophils. Variable intra-acinar lymphocytes. Little to no portal inflammation

2 Intra-acinar neutrophils more prominent but without significant associated necrotic hepatocytes. Intra-acinar chronic inflammation also noted. Mild to moderate portal inflammation

3 Intra-acinar neutrophils prominent and associated with increased hepatocyte degeneration/necrosis. Intra-acinar chronic inflammation also noted. Mild to moderate portal inflammation

Hepatocyte Degeneration/Necrosis

0 None

1 Occasional ballooning degeneration of hepatocytes, limited to zone 3. No hepatocyte necrosis

2 Obvious ballooning degeneration of hepatocytes, most marked in zone 3. May have rare necrotic hepatocytes

3 Ballooning degeneration of hepatocytes with hepatocyte disarray. Degeneration may be pan-acinar. Hepatocyte necrosis more prominent than in two with associated inflammatory cells

Staging for fibrosis

0 None

1 Zone 3 perisinusoidal/pericellular fibrosis; focally or extensively present

2 Zone 3 perisinusoidal/pericellular fibrosis with focal or extensive periportal fibrosis

3 Zone 3 perisinusoidal/pericellular fibrosis and portal fibrosis with focal or extensive bridging fibrosis

4 Cirrhosis

rected for multiple comparisons. BMI, ALT, and AST were evaluated in the same way as the histologic scores. If histograms or the Kolmogorov-Smirnov test revealed that the data were not normally distributed, the results were confirmed with a nonparametric test. All *p* values are two-tailed and considered significant if less than 0.05.

RESULTS

A total of 45 patients completed the study. Twenty-three patients were randomized to receive vitamins E and C; 22 patients received placebo. Four patients did not complete the study, two in each group. Three of the four patients did not wish to have follow-up liver biopsies, and one patient moved away before completion of the study. These patients were not included in the analysis.

Table 1 lists the baseline characteristics of the two randomized groups. The two arms of the study were balanced for all parameters, except BMI (*p* = 0.012) and the number of diabetic patients (*p* = 0.022), which was higher in the vitamin group.

Figure 1 illustrates the difference in BMI between treatment groups before and after treatment. In addition to the difference in BMI between groups at initiation of treatment,

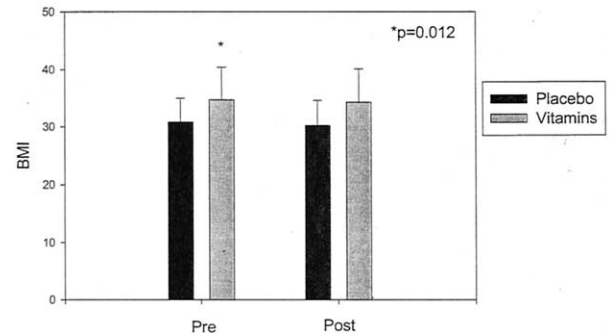


Figure 1. Difference in BMI between and within vitamin and placebo groups, pre- and posttreatment. BMI is represented in kg/m². There is a statistically significant difference between the placebo group and the vitamin group before treatment.

the placebo group had a statistically significant difference (30.8 vs 30.2) between pre- and posttreatment (*p* = 0.03). This difference is relatively small and probably not clinically significant.

Interestingly, ALT demonstrated a statistically significant improvement posttreatment in the placebo group (*p* = 0.007). No differences were noted in the treatment group (Fig. 2). This difference was further analyzed statistically for an association with a drop in weight within the placebo group, and no association was found. Additionally, no differences were noted between groups or within groups for AST.

Evaluation of the histologic data demonstrated no statistically significant differences in inflammation/necrosis score between the vitamin group and placebo group or within the vitamin or placebo groups (Fig. 3). Additionally, no statistically significant difference in fibrosis was noted between the vitamin and placebo groups. However, significant improvement in fibrosis (analysis of variance, *p* = 0.002) was noted within the group that received vitamins E and C but not in the placebo group (Fig. 4). This was confirmed by the Wilcoxon signed rank test (*p* = 0.005). Two patients (8.7%) in the vitamin E and C group had a 2-point improvement in fibrosis over the 6-month period, and nine patients (39.1%)

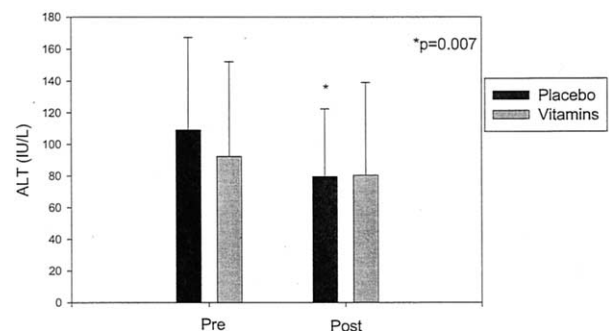


Figure 2. Difference in ALT levels between and within vitamin and placebo groups, pre- and posttreatment. There is a statistically significant difference in ALT before and after treatment in the placebo group. No significant differences were noted in the vitamin group.

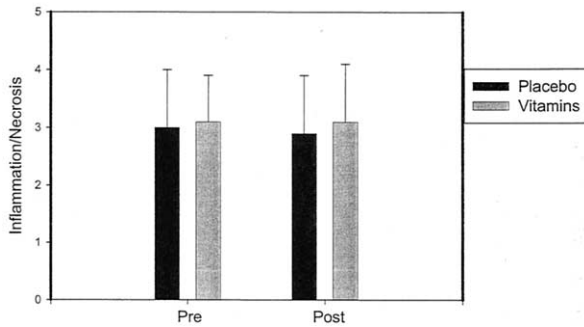


Figure 3. Combined inflammation/necrosis score in the vitamin and placebo groups pre- and posttreatment. Inflammation did not change in the placebo or vitamin group with time.

had a 1-point improvement. Eleven patients (47.8%) remained unchanged, and one patient (4.3%) had a 1-point worsening in fibrosis (Fig. 5a). In the placebo group, there were no significant differences noted with respect to time ($p = 0.16$). One patient (4.5%) had a 2-point improvement, eight patients (36.4%) had a 1-point improvement, 10 patients (45.5%) were unchanged, and three patients (13.6%) worsened in their fibrosis stage (two patients by 1 point, and one patient by 2 points) over the 6-month study (Fig. 5b). Intraobserver liver pathology variability data were also calculated by coded slide reanalysis by the same pathologist several months later, and no statistically significant differences were noted for fibrosis: there were 32 out of 37 (86.5%) ties for the two readings at different time intervals 6 months apart.

Further analysis was done to assess for an association between antecedent fibrosis stage and subsequent response to vitamin E and C treatment. There was no significant difference by convention ($p > 0.05$), but there was a trend toward a greater improvement in the patients with a more severe fibrosis score ($p = 0.097$).

Finally, when controlling for the presence of diabetes alone, among nondiabetic patients, there were no significant differences in baseline fibrosis or change in fibrosis over the 6-month study period in either group. However, in patients

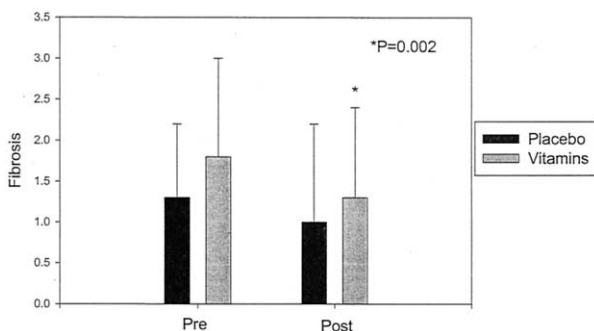
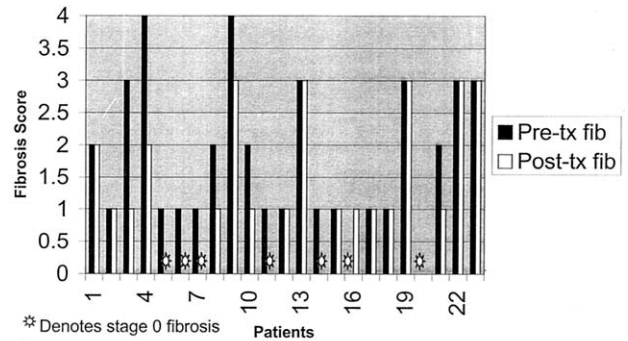
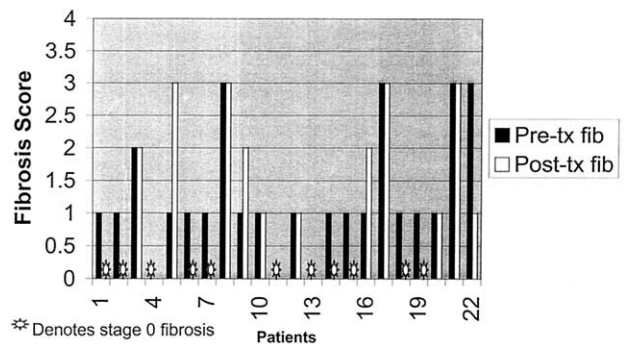


Figure 4. Fibrosis score in the vitamin and placebo groups pre- and posttreatment. Fibrosis improved in the vitamin group over the 6-month course of therapy ($p = 0.002$). The vitamin group did not have a statistically significant improvement.



A



B

Figure 5. (A) Fibrosis change in 23 patients in the vitamin group. Two patients had a 2-point improvement, nine patients had a 1-point improvement, 11 patients had no change, and one patient had a 1-point worsening in fibrosis score over a 6-month study period. (B) Fibrosis change in the 22 patients in the placebo group. One patient had a 2-point improvement, eight patients had a 1-point improvement, 10 patients had no change, and three patients had worsening of their fibrosis scores. Pre-tx fib = prebiopsy fibrosis scores; Post-tx fib = postbiopsy fibrosis scores.

with diabetes (Table 1), more of whom were in the vitamin-treated group, there was more baseline fibrosis ($p = 0.008$) in the vitamin-treated patients, and fibrosis was decreased selectively in this group with treatment ($p = 0.006$). It should be emphasized that only five patients were diabetic in the control group, but the data suggest more benefit in diabetic patients with more fibrosis.

DISCUSSION

NASH is no longer considered a completely benign disease with an indolent course. Data now demonstrate that a significant proportion of NASH patients will progress to cirrhosis over time (12). In fact, recent studies have demonstrated that fibrosis or cirrhosis is evident on initial biopsy in 15%–50% of NASH patients (13). Furthermore, increasing evidence suggests that significant complications of NASH-induced cirrhosis, such as end-stage liver disease necessitating liver transplantation (14, 15) and hepatocellular carcinoma, can occur (16, 17). Moreover, Matteoni *et al.* (18) have suggested that the liver-related mortality in patients with NASH is higher than with other forms of chronic liver

disease. Given this evidence, as well as data supporting an increasing prevalence of this disease in our society (1), it is important that new and effective therapeutic modalities be developed.

To date, therapeutic options for patients with NASH remain quite limited. The published literature mainly consists of small trials without placebo control or follow-up histopathologic data (12). Furthermore, among most of the therapeutic studies that have been promoted as being beneficial, the liver enzymes improved, but either there was no improvement in histology or follow-up biopsies were not performed. Two previous studies, one in children (19) and one in adults (9), in which vitamin E was used in a cohort of NASH patients, have been published. Lavine (19) used escalating doses of vitamin E, up to 1200 IU/day, in children with nonalcoholic fatty liver disease (NAFLD) and demonstrated improvement in liver-associated enzymes, but liver histology was not assessed. More recently, in a small, uncontrolled, pilot trial, Hasegawa *et al.* (9) demonstrated improvement in fibrosis in 66% of NASH patients who took vitamin E in doses of 300 mg/day for 1 year.

In this prospective, randomized trial of vitamin E and C in patients with histologic evidence of NASH, 6 months of therapy demonstrated a statistically significant, albeit modest, clinical improvement in fibrosis within the vitamin group. Additionally, among the subset of NASH patients with diabetes, there was a significant difference in fibrosis between the placebo and vitamin groups ($p = 0.006$). All patients tolerated the therapy without significant side effects. To decrease the chance of observer variation, the pathologist performed a second independent evaluation of the biopsy specimens, and intraobserver variation was calculated. There was no significant difference in the assessment of fibrosis between the two separate, blinded readings.

Data from animal models support the concept of a decrease in fibrosis seen in the vitamin group. Vitamin E supplementation significantly repleted hepatic glutathione, with a subsequent reduction in measures of oxidative stress (thiobarbituric acid reactive substances) and improvement in hepatic fibrosis, in a mouse model of NASH (20). Furthermore, Parola *et al.* (21) have demonstrated that vitamin E reduces oxidative damage and subsequent collagen deposition in rats exposed to carbon tetrachloride (21).

Further evidence to support a potential beneficial role for vitamin E in NASH is found in studies related to cytokine stimulation of stellate cells. Hepatic stellate cells are primarily responsible for fibrogenesis. Transforming growth factor β (TGF- β), produced in part by Kupffer cells in the perisinusoidal space, seems to have a direct stimulatory effect on stellate cells (22). Studies have demonstrated that TGF- β is upregulated in the presence of fibrosis (23, 24). Vitamin E has been shown in both animal and human studies to decrease TGF- β levels after treatment (9, 25).

Despite a relatively short treatment interval, a significant, albeit small, improvement in fibrosis was evident. Although sampling variation can be seen in assessing fibrosis in nee-

dle biopsy specimens, a similar bias would apply to the placebo group, wherein no statistically significant fibrosis change was seen. Previous studies with short follow-up periods, mainly evaluating the efficacy of weight loss in patients with NAFLD and chronic hepatitis C, have shown no fibrosis improvement, despite improvement in steatosis and liver function tests (26, 27). Interestingly, in our study, there was a lack of significant fibrosis improvement in the placebo group, despite a small weight improvement, whereas the vitamin group demonstrated improvement in fibrosis without associated weight loss. It remains to be seen whether a longer duration of therapy with vitamin E and vitamin C results in continued improvement in fibrosis scores in NASH patients and, more importantly, whether this modest improvement subsequently results in a decrease in the percentage of NASH patients progressing to cirrhosis and possibly hepatocellular carcinoma.

Unexpectedly, we observed a significant drop in ALT in the placebo group. Although the BMI also dropped in this group over time, and data suggest that weight loss improves serum transaminases, the BMI decline in this study is trivial and likely not clinically significant. In fact, statistically there was no association with weight loss and drop in ALT within the placebo group. Furthermore, the drop in ALT did not correlate with improvement in histologic score, consistent with previous studies indicating that serum transaminases correlate poorly within underlying disease activity.

In conclusion, vitamin E and vitamin C supplementation, in doses of 1000 IU and 1000 mg per day, respectively, were well tolerated and seemed to improve hepatic fibrosis scores in NASH patients, in particular those with diabetes. This vitamin treatment did not significantly improve inflammation or ALT levels. Given that this disease likely has multiple pathogenetic mechanisms, to include underlying insulin resistance, polymorphisms in gene expression, and environmental factors, it is possible, and even probable, that the eventual treatment for this disease will be based on multiple modalities (including antioxidants) that interact at multiple sites along the molecular pathway from steatosis to NASH.

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