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Effects of Vitamins E and C Supplementation on Hepatic Glutathione Peroxidase Activity and Tissue Injury Associated with Ethanol Ingestion in Malnourished Rats

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ABSTRACT

Background: Oxidative stress has been associated with tissue injury in alcoholic liver disease. Although this close association is well known, whether prevention of oxidative stress retards tissue injury has not been thoroughly investigated.

Objective: The aim of this study was to determine the effects of supplementation with vitamins E and C on antioxidant enzyme status and histologic changes in hepatic tissue in a rat model of alcoholic liver disease.

Methods: This 8-week, blinded, controlled study was conducted at the Department of Internal Medicine, Trakya University, Edirne, Turkey. Weanling albino female protein-deficient Wistar rats weighing ~200 g were randomly assigned to 1 of 6 groups: (1) liquid diet + ethanol + vitamin E 15 mg/kg PO (LDetvitE); (2) liquid diet + ethanol + vitamin C 10 mg/kg PO (LDetvitC); (3) liquid diet + ethanol + vitamin E 15 mg/kg + vitamin C 10 mg/kg PO (LDetvitEC); (4) liquid diet + ethanol (LDet); (5) liquid diet + isocaloric sucrose (LDS); and (6) normal diet (control). The primary end point of the study was to determine whether antioxidant vitamin E/C combination therapy prevents development of hepatic fibrosis (ie, cirrhosis in a period of 1 year). After being euthanized at week 8, the rats were weighed, and their livers and spleens were weighed. Hepatic tissue specimens were histopathologically assessed according to the Brunt system. Hepatic tissue glutathione peroxidase, superoxide dismutase, and catalase activities were determined. Biochemical tissue collagen concentrations were measured to determine the presence of hepatic fibrosis.

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Results: Seventy-two rats were included in the study (mean [SE] weight, 205 [21] g) (12 rats per group). Initially planned to last 48 weeks, the study was terminated at 8 weeks due to the death of 3 rats in each group (except the LDS group and control group). The relative liver weight was significantly lower in the LDetvitEC group compared with that in the LDet group (mean [SE], 3.7% [0.5%] vs 4.8% [0.9%]; $P < 0.01$). Mean (SE) hepatic tissue glutathione peroxidase activity was significantly reduced in the LDet-treated rats compared with controls (1.2 [0.2] vs 2.6 [0.3] U/mg protein; $P < 0.001$). The groups that received supplementation with vitamin E, vitamin C, and vitamins E and C combined had significantly more hepatic glutathione peroxidase activity (mean [SE], 2.1 [0.5], 2.5 [0.2], and 2.6 [0.7] U/mg protein, respectively) compared with the LDet group (1.2 [0.2] U/mg protein) (all, $P < 0.001$). No significant between-group differences in hepatic superoxide dismutase or catalase activities were found. Compared with controls (14.5 [1.9] μg collagen/mg protein), the mean (SE) histologic hepatic collagen concentration was significantly higher in all groups (19.2 [1.2], 19.5 [3.3], 18.5 [3.0], 25.9 [3.3], and 21.6 [1.5] μg collagen/mg protein in the LDetvitE, LDetvitC, LDetvitEC, LDet, and LDS groups, respectively; $P < 0.01$, $P < 0.01$, $P < 0.05$, $P < 0.001$, and $P < 0.001$, respectively). Compared with the LDet group, the mean hepatic collagen concentration was significantly lower in the LDetvitE, LDetvitC, and LDetvitEC groups ($P < 0.01$, $P < 0.05$, and $P < 0.01$, respectively). The LDetvitEC group had a significantly lower mean (SE) hepatic inflammatory score compared with the LDet group (0.8 [0.1] vs 1.3 [0.2]; $P < 0.05$). The LDetvitEC group had a significantly lower mean (SE) hepatic necrosis score compared with that in the LDet group (1.5 [0.2] vs 2.4 [0.3]; $P < 0.05$).

Conclusions: The results of this study in protein-deficient rats fed with a high-fat liquid diet suggest that supplementation with vitamin E, vitamin C, and a combination of vitamins E and C was associated with decreased ethanol-induced hepatic glutathione peroxidase activity and hepatic fibrosis, and that supplementation with vitamins E and C might have attenuated the development of hepatomegaly and hepatic necroinflammation, whereas this result was not found in the group given a liquid diet and ethanol in this 8-week study. (*Curr Ther Res Clin Exp.* 2006;67:118–137)

Key words: alcohol, necrosis, inflammation, fibrosis, vitamin E, vitamin C, glutathione peroxidase.

INTRODUCTION

Underlying mechanisms for the development of hepatic fibrosis in alcoholic liver disease have not been thoroughly reported. Although the presence of oxidative stress is well known in alcoholic liver disease, whether it contributes to progressive hepatic fibrosis, resulting in cirrhosis, is debatable.[1] Antioxidants are currently not recommended in the treatment of patients with alcoholic liver disease.[2] Studies performed to demonstrate the effectiveness of antioxidant therapies on prevention or treatment of alcoholic liver disease are

not sufficient. In 2 clinical studies with population numbers of 25 and 67, respectively, patients with alcoholic hepatitis were evaluated for 3 and 12 months, respectively.[3,4] In these controlled studies, vitamin E supplementation did not provide improvement of hepatic laboratory parameters, mortality, or hospitalization rates of decompensated alcoholic cirrhotics. However, these patients did not take vitamins before the initiation of alcohol consumption and no histopathologic examinations were performed before or after the study period.

The liver is rich in antioxidant enzymes (eg, glutathione peroxidase, superoxide dismutase, catalase) that can metabolize reactive oxygen species.[5] Antioxidant enzymes eliminate superoxide anions and hydroperoxides that can oxidize cellular substrates. However, these enzymes also exert protective effects by scavenging the oxidized forms of the nonenzymatic antioxidants vitamin E, vitamin C, and glutathione.[6] In a study in a rat model by Schisler and Singh,[7] rats given ethanol (4 rats at 5% ethanol; 4 rats at 15% ethanol) for 14 days had decreased mean (SE) hepatic activities of total glutathione peroxidase (0.38 [0.04] μmol · mg/min protein; $P < 0.05$) and superoxide dismutase (0.43 [0.02] U/mg protein; $P < 0.05$) compared with those of matched controls (17.36 [1.50] and 0.71 [0.06], respectively). Hepatic catalase activity was not affected by chronic ethanol treatment in rats.

Vitamin E deficiency might be one of several factors causing oxidative stress in alcoholic liver disease. Studies in alcoholic patients[8] and in rats fed ethanol[9,10] have found significantly reduced vitamin E concentrations in serum and hepatic tissue. In the study by Bjorneboe et al,[8] the effect of heavy alcohol consumption on serum concentrations of vitamin E was studied in 13 alcoholics and 19 control subjects. Of the 13 alcoholics, 6 had a serum concentration of α-tocopherol below the lower limit of reference (14 μmol/L) and mean serum concentration of α-tocopherol was reduced by 37% compared with controls ($P < 0.002$). The effects of chronic ethanol feeding (3 weeks) on hepatic lipid peroxidation and vitamin E levels were investigated in the rats fed low or adequate amounts of dietary vitamin E.[10] Hepatic lipid peroxidation was significantly increased after chronic ethanol consumption in rats receiving a low-vitamin E diet. This indicated that dietary vitamin E is an important determinant of hepatic lipid peroxidation induced by chronic ethanol feeding. Low dietary vitamin E and ethanol consumption significantly reduced hepatic α-tocopherol content, and the lowest hepatic α-tocopherol was found in the rats receiving a combination of low vitamin E and ethanol. Furthermore, ethanol consumption caused a marked increase of hepatic α-tocopheryl quinone, a metabolite of α-tocopherol by free radical reactions. Therefore, ethanol consumption causes a marked alteration of vitamin E metabolism in the liver and the combination of ethanol consumption with a low-vitamin E intake results in a decrease of hepatic α-tocopherol content, which renders the liver more susceptible to free radical attack. The reduction in vitamin E concentration might have been due to malabsorption and low intake.[10] In a separate study in a rat model by Odeleye et al,[11] vitamin E supplementation was associated with normalized (hepatic tissue vitamin E

levels in nmol/g wet liver [SE]: normal rats, 143.74 [19.85]; ethanol group, 89.66 [14.32]; ethanol + vitamin E, 118.62 [27.93] [no statistical difference between normal and ethanol + vitamin E group]) vitamin E concentration in (n = 5 rats per group) ethanol-fed rats.

The present study used vitamin E because it is the primary fat-soluble, chain-breaking antioxidant that protects lipid bilayers in the liver.[6] We also studied another antioxidant, vitamin C, which plays a scavenger role, particularly in plasma, cytosol, and other aqueous compartments.[12] Supplementation with a combination of vitamins E and C might result in a synergistic effect by deoxidizing and regenerating vitamin E.[13,14] The present study aimed to investigate the effects of enzymatic antioxidant defense systems and their response to supplementation with vitamins E and/or C in alcoholic hepatic injury in protein-deficient rats.

MATERIALS AND METHODS

This 8-week, blinded, controlled study was conducted at the Department of Internal Medicine, Trakya University, Edirne, Turkey. The study protocol was approved by the ethics committee of the medical faculty at the university.

Animals and Diet

Weanling albino female protein-deficient Wistar rats weighing ~200 g were housed in cages with stainless steel wire tops and 12-hour light–dark cycles at a mean (SD) ambient temperature of 21°C (1°C) and a mean (SD) humidity of 55% (5%). Rats were labeled with numbers, and corresponding numbers were picked from a box by a blinded member of the study team, randomly assigning the rats to 1 of 6 groups: (1) liquid diet + ethanol + vitamin E 15 mg/kg PO (LDetvitE)*; (2) liquid diet + ethanol + vitamin C 10 mg/kg PO (LDetvitC)†; (3) liquid diet + ethanol + vitamin E 15 mg/kg + vitamin C 10 mg/kg PO (LDetvitEC); (4) liquid diet + ethanol (LDet); (5) liquid diet + isocaloric sucrose (LDS); and (6) normal diet (control).

The daily dose (15 mg/kg) of vitamin E selected, previously used by Montilla et al,[15] was considerably higher than the normal daily needs of rats established by the National Research Council[16] (18 mg/kg). Normally, rats do not require a dietary source of vitamin C. Enzymatic synthesis of this vitamin can occur via glucuronolactone or gluconolactone in the rat liver. Ascorbic acid might help protect against peroxidation and spare vitamin E. It has been found to decrease expired pentane, used as a marker for lipid peroxidation.[17] Administration of vitamins E and C was started on the first day of the study.

To develop a model of established alcoholic cirrhosis, the rats were fed a liquid diet, the content of which was similar to that of alcoholic patients—severely deficient in protein and rich in fat (principally comprising a combination of

*Trademark: Evigen® (Aksu Farma, Istanbul, Turkey).
†Trademark: Redoxon® (Roche Müstahzarları Sanayi S.A., Istanbul, Turkey).

mashed potatoes and mayonnaise), providing sufficient calories (38% of total calories from fat, 37% from ethanol, 22% from complex carbohydrates, and 3% from protein), and mixed with ethanol to produce alcoholic liver disease—as previously described by Bosma et al.[18] The diet was prepared daily (by A.R.S. and U.N.B. with fresh food and ingredients) in a kitchen by mixing all of the ingredients using a kitchen mixer. The rats in the control group had free access to standard rat chow and water. All of the groups were fed their particular diets ad libitum.

This study was initially designed to last 48 weeks but was terminated early (at week 8) due to death in some of the animals. At the end of the study, the rats were weighed and euthanized. The liver and spleen of each rat were also weighed. Hepatomegaly was defined as liver wet weight of >3 g per 100 g rat body weight.[19]

Hepatic Tissue Sampling

The left, middle, and right lobes of each liver were explored by a nonblinded investigator (U.N.B.); 1.5 cm^3 of tissue was randomly sampled from one of six 10 × 5 × 5 mm slices of liver (ie, 1 slice from the anterior and distal ends of each of the 3 hepatic lobes). Each slice was cut in half, so that it measured 5 × 5 × 5 mm. One half of each slice was stored for 3 months at –86°C and was subsequently used to determine tissue superoxide dismutase, catalase, and glutathione peroxidase activities. The other half of each slice was fixed in 10% buffered formalin. The paraffin embedding was preceded by dehydration and clearing. First, water from the tissues was removed by dehydration, done with a graded series of ethanol:water (70% to 100% ethanol) mixtures. The ethanol was then replaced by the solvent xylene miscible with embedding medium (paraffin). The cleared fragments were blocked into paraffin for subsequent biochemical analysis to detect collagen concentration.

Tissue Antioxidant Enzyme Activities

All enzyme activities were determined after hepatic tissue was homogenized with phosphate-buffered saline at a pH of 7.4. Total (copper-zinc and manganese) superoxide dismutase activity was determined according to the method of Sun et al,[20] which is based on the inhibition of nitroblue tetrazolium (NBT) reduction by the xanthine-xanthine oxidase system as a superoxide generator. One unit of superoxide dismutase was defined as the amount of enzyme needed to achieve 50% inhibition in the NBT reduction rate. The specific activity was expressed in units per milligram of protein. The catalase activity was measured according to Aebi[21]; a method based on the determination of the rate constant (k/s) of hydrogen peroxide decomposition rate at 240 nm. The results were expressed as the rate constant per milligram homogenate protein. Glutathione peroxidase activity was measured according to Lawrence and Burk,[22] by monitoring the oxidation of reduced nicotinamide adenine dinucleotide phosphate hydrogen (NADPH) at 340 nm. Enzyme units were expressed as the number of micromoles of NADPH oxidized per minute and calculated using the extinction coefficient of NADPH at 340 nm (6.22×10^{-3} mol · cm/L). Results were reported as units per milligram of protein. The protein content of tissue samples was

determined by the method of Lowry et al,[23] using bovine albumin as the standard. The investigators (N.A. and K.K.) who measured enzyme activity were blinded to treatment assignment.

Biochemical Hepatic Collagen Concentration Determination

Hepatic collagen concentration was assayed using the colorimetric method described by Lopez-De Leon and Rojkind.[24] Collagenous protein was stained using Sirius red (36554–8, 2610-10-8; Aldrich Chemical, Deisenhofen, Germany) and noncollagenous proteins were stained using fast green (14280; Merck KGaA, Darmstadt, Germany). Liver slices 15 μm thick were taken from each paraffin block and layered on glass slides. The paraffin was removed from the slices after incubation with xylol, xylol:ethanol (1:1), ethanol, water:ethanol, and water. The slides were stained with a saturated solution of picric acid in distilled water containing 0.01% fast green. The sections were not exposed to light and were incubated at room temperature (20°C) for 15 minutes. They were rinsed until the eluent was free of color. They were then stained with a saturated solution of picric acid in distilled water containing 0.04% fast green and 0.1% Sirius red and incubated in the dark at room temperature (20°C) for 30 minutes. The samples were rinsed and transferred to a test tube containing 1 mL of 0.1% sodium hydroxide in absolute methanol (1:1). The tubes were gently mixed until the color was completely eluted. Absorbance of the eluted color was read using a spectrophotometer (V-50 UV-VIS, Jasco Inc., Easton, Maryland) at 540 and 630 nm.

Maximum absorbance of fast green occurs at 630 nm and Sirius red at 540 nm. Fast green exhibits a small absorbance (7.78%) at 540 nm. This percentage of interference remains constant despite concentration. Because Sirius red has no absorbance at 630 nm, it does not interfere with noncollagenous protein determination.[24,25]

Collagen content was calculated using the following formulas[25]:

Collagen (μg)/Total protein (mg) = Collagen (μg)/(Collagen [mg] + Noncollagenous protein [mg])

Collagen (mg) = ([Absorbance at 540 nm (%) − 7.78%] + Absorbance at 630 nm [%])/37

Noncollagenous protein (mg) = Absorbance at 630 nm (%)/3

where 37 and 3 were determined as color equivalences per milligram of Fast green and Sirius red, respectively.

The investigators (O.T. and N.G.) were blinded to treatment assignment while measuring collagen concentration.

Histopathologic Examination

Necroinflammatory activity grading and fibrosis staging were determined using the Brunt system (**Table I**).[26] The pathologists (S.A. and K.K.) were blinded to treatment assignment while reading the results.

Table I. Nonalcoholic steatohepatitis grading and staging (Brunt scoring system).[26]

	Grading					
Score	Semiquant	Steatosis	Ballooning	Lobular Inflammation	Portal Inflammation	Staging
1	Mild	<33%, usually macro	Rare, zone 3	Rare acute and occasional chronic	None or mild	Zone 3 perivenular perisinusoidal/pericellular fibrosis, focal or extensive
2	Moderate	Any degree, usually mixed	Obvious, zone 3	PMNs associated with ballooned hepatocytes	Mild to moderate	As above with focal or extensive periportal fibrosis
3	Severe	>66%, panacinar	Marked, predominantly zone 3	Frequent acute and chronic	Mild to moderate	Bridging fibrosis, focal or extensive
4	–	–	–	–	–	Cirrhosis

PMNs = polymorphonuclear leukocytes.

Statistical Analysis

Data were expressed as mean (SE). The Shapiro-Wilks test was used to assess the normality of continuous data. For all variables, the statistical differences between groups were tested using 1-way analysis of variance for normally distributed data (relative liver, spleen, and body weights and glutathione peroxidase, superoxide dismutase, and catalase activities). The nonparametric Kruskal-Wallis test was used for nonnormally distributed data (biochemical hepatic collagen concentration, inflammatory activities, necrosis grade, and fibrosis stage).[27] Multiple comparisons were made using the Tukey post hoc multiple comparisons test for normally distributed data, and the nonparametric Tukey post hoc multiple comparisons test for nonnormally distributed data.[28] Statistical analysis was performed with SPSS version 11.0 (SPSS Inc., Chicago, Illinois). $P < 0.05$ was considered statistically significant. A power of the study analysis was not made.

RESULTS

Seventy-two rats were included (mean [SE] weight, 205 [21] g) (12 rats per group). Three rats in each group (except the LDS group and control group) died from empyema and/or deep neck infection, as confirmed on autopsy, before week 8 and were excluded from the analysis. Because of these deaths, the study was shortened from the intended 48 weeks to 8 weeks.

Although body weight was statistically similar between all treatment groups at the beginning of the study, the rats in the LDet group weighed significantly less than those in the control group at 8 weeks (mean [SE], 169 [28] vs 231 [32] g, respectively; $P < 0.001$) (**Table II**). When we compare baseline body weights with week 8 and those who were excluded from the analysis, only the control group showed a statistically significant weight gain compared with baseline ($P < 0.01$) and only the LDet group lost weight compared with baseline ($P < 0.05$) (Wilcoxon 2-related samples test).

Significant differences in regard to relative liver weight were only present between the LDet group and the control group (4.8% [0.9%] vs 3.6% [0.3%]; $P < 0.01$) and between the LDet group and the LDetvitEC group (4.8% [0.9%] vs 4.1% [0.6%]; $P < 0.01$). Mean relative spleen weights were statistically similar between all 6 study groups.

On microscopic examination of the liver sections, hyperemia, cellular swelling, sinusoidal dilatation, and necrosis were found in the LDet group (**Figure 1A–C**). In the rats administered LDetvitEC, cellular damage was less compared with that in the LDet group or absent (**Figure 1D–F**).

The groups treated with vitamin supplementation had significantly more glutathione peroxidase activity compared with that in the LDet group (mean [SE], 2.1 [0.5], 2.5 [0.2], and 2.6 [0.7] U/mg protein in the LDetvitE, LDetvitC, and LDetvitEC groups, respectively, vs 1.2 [0.2] U/mg protein in the LDet group; all, $P < 0.001$) (**Table III** and **Figure 2**). Hepatic tissue glutathione peroxidase activ-

Table II. Total body weight and relative liver and spleen weights in rats at 8 weeks. Values are mean (SE).

Parameter	LDetvitE (n = 9)	LDetvitC (n = 9)	LDetvitEC (n = 9)	LDet (n = 9)	LDS (n = 12)	Control (n = 12)
Total body weight, g						
Baseline	204 (16)	199 (10)	201 (15)	208 (31)	200 (21)	214 (29)
Week 8	207 (31)	200 (15)	194 (30)	169 (28)*†	207 (22)	231 (32)‡
Relative liver weight, %	4.0 (0.3)	4.1 (0.6)	3.7 (0.5)§	4.8 (0.9)‖	4.2 (0.4)	3.6 (0.3)
Relative spleen weight, %	0.37 (0.08)	0.39 (0.12)	0.37 (0.22)	0.31 (0.13)	0.32 (0.10)	0.29 (0.04)

LDetvitE = liquid diet + ethanol + vitamin E 15 mg/kg PO; LDetvitC = liquid diet + ethanol + vitamin C 10 mg/kg PO; LDetvitEC = liquid diet + ethanol + vitamin E 15 mg/kg + vitamin C 10 mg/kg PO; LDet = liquid diet + ethanol; LDS = liquid diet + isocaloric sucrose.
*$P < 0.001$ versus control.
†$P < 0.5$ versus baseline.
‡$P < 0.01$ versus baseline.
§$P < 0.01$ versus LDet.
‖$P < 0.01$ versus control.

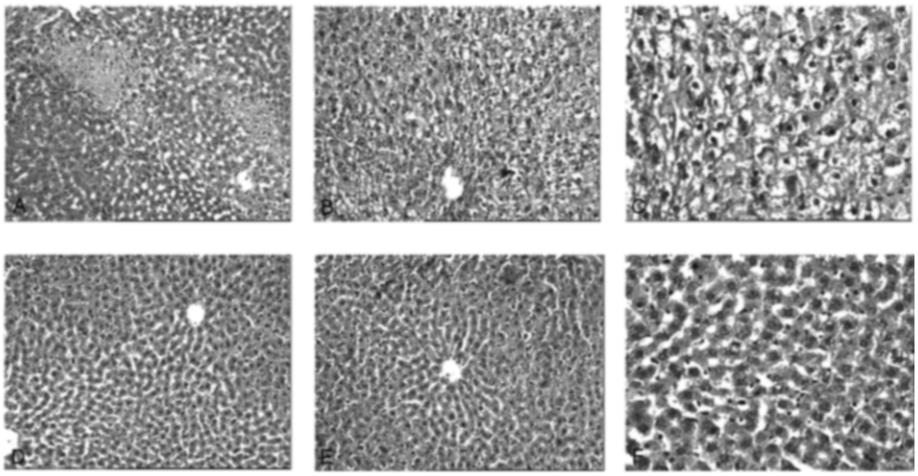

Figure 1. Histologic changes in rats receiving ethanol without supplementation with vitamin E or C, showing (A) widespread hepatic necrosis, (B) hydropic degeneration of hepatocytes, and (C) sinusoidal dilatation. Preservation of hepatic structure in rats administered combined vitamins E and C supplementation, showing (D) no necrosis or (E, F) hydropic degeneration. (Hematoxylin and eosin; magnification, ×100 [A, B, D, E] or ×200 [C, F].)

ity was significantly less in the LDet group compared with that in the control group (mean [SE], 1.2 [0.2] vs 2.6 [0.3] U/mg protein; $P < 0.001$). No significant differences in hepatic tissue superoxide dismutase or catalase enzyme activities were found between any of the groups (**Table III**).

The mean (SE) hepatic collagen concentrations were significantly higher in the LDetvitE, LDetvitC, LDetvitEC, LDet, and LDS groups compared with that in the control group (19.2 [1.2], 19.5 [3.3], 18.5 [3.0], 25.9 [3.3], and 21.6 [1.5] μg/mg protein, respectively, vs 14.5 [1.9] μg/mg protein; $P < 0.01$, $P < 0.01$, $P < 0.05$, $P < 0.001$, $P < 0.001$, respectively) (**Table III** and **Figure 3**). Despite having higher hepatic collagen concentrations when compared with that of the control group, the LDetvitE, LDetvitC, and LDetvitEC groups had significantly (25%) lower hepatic collagen concentrations compared with the LDet group as measured using the biochemical method ($P < 0.01$, $P < 0.05$, and $P < 0.01$, respectively). No statistically significant differences in mean hepatic total biochemical collagen concentrations were found between the groups treated with vitamin supplementation.

The histologic inflammation score was found to be significantly lower in the LDetvitEC group when compared with those in the LDetvitE, LDetvitC, and LDet groups (0.8 [0.1] vs 1.5 [0.2], 1.4 [0.2], and 1.3 [0.2], respectively; all, $P < 0.05$) (**Table IV**). The mean (SE) necrosis score was significantly lower in the LDetvitEC group compared with the LDet group ($P < 0.05$). Differences in fibrosis scores were not statistically significant between any of the groups.

Table III. Hepatic tissue antioxidant enzyme activities and collagen concentration in rats at 8 weeks. Values are mean (SE).

Enzyme	LDetvitE (n = 9)	LDetvitC (n = 9)	LDetvitEC (n = 9)	LDet (n = 9)	LDS (n = 12)	Control (n = 12)
Glutathione peroxidase activity, U/mg protein	2.1 (0.5)*	2.5 (0.2)*	2.6 (0.7)*	1.2 (0.2)†	1.8 (0.4)‡	2.6 (0.3)
Superoxide dismutase activity, U/mg protein	6.4 (0.7)	6.4 (0.6)	6.8 (0.9)	7.7 (1.1)	7.0 (1.1)	6.8 (0.2)
Catalase activity, k/mg protein	1.8 (0.6)	2.1 (0.5)	2.1 (0.6)	1.7 (0.7)	2.0 (0.4)	2.3 (0.4)
Hepatic collagen concentration, µg/mg protein	19.2 (1.2)‡§	19.5 (3.3)‡‖	18.5 (3.0)§¶#	25.9 (3.3)†	21.6 (1.5)†	14.5 (1.9)

LDetvitE = liquid diet + ethanol + vitamin E 15 mg/kg PO; LDetvitC = liquid diet + ethanol + vitamin C 10 mg/kg PO; LDetvitEC = liquid diet + ethanol + vitamin E 15 mg/kg + vitamin C 10 mg/kg PO; LDet = liquid diet + ethanol; LDS = liquid diet + isocaloric sucrose.
*$P < 0.001$ versus LDet.
†$P < 0.001$ versus control.
‡$P < 0.01$ versus control.
§$P < 0.01$ versus LDet.
‖$P < 0.05$ versus LDet.
¶$P < 0.05$ versus control.
#$P < 0.05$ versus LDS.

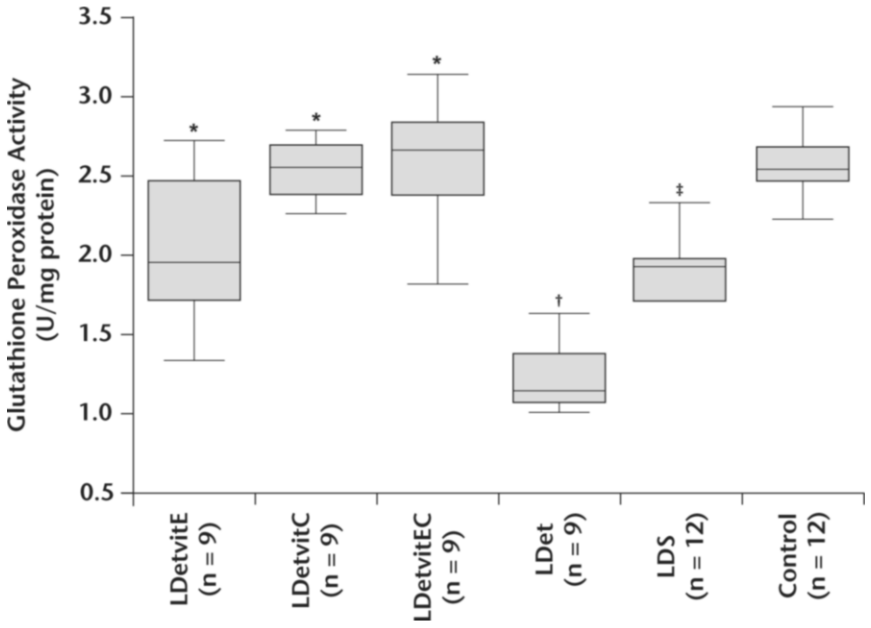

Figure 2. Glutathione peroxidase activity in each treatment group. LDetvitE = liquid diet + ethanol + vitamin E 15 mg/kg PO; LDetvitC = liquid diet + ethanol + vitamin C 10 mg/kg PO; LDetvitEC = liquid diet + ethanol + vitamin E 15 mg/kg + vitamin C 10 mg/kg PO; LDet = liquid diet + ethanol; LDS = liquid diet + isocaloric sucrose. *$P < 0.001$ versus LDet; †$P < 0.001$ versus control; ‡$P < 0.01$ versus control (all comparisons; nonparametric Tukey test).

DISCUSSION

The results of this study in a rat model suggest that long-term ethanol administration reduced glutathione peroxidase activity in rats with severe malnutrition. Supplementation with vitamins E and/or C was associated with attenuation of the ethanol-induced decrease in hepatic glutathione peroxidase activity (LDetvitE and LDetvitC groups, $P < 0.001$ vs LDet group), decreased hepatocellular inflammation (LDetvitE and LDetvitC groups, $P < 0.05$ vs the control group) and necrosis (LDetvitE and LDetvitC groups, $P < 0.05$ and $P < 0.01$ vs the control group, respectively), retardation of alcoholic fibrosis (as demonstrated by biochemical liver collagen content determination), and reduced hepatomegaly (LDetvitEC, $P < 0.01$ vs LDet)—the most common clinical manifestation of alcoholic liver disease. Body weight loss was not found in the groups receiving vitamin E and/or C.

Hepatic metabolism of ethanol inherently produces reactive oxygen species, including superoxide anion, hydroxyethyl, and hydroxyl radicals.[5] Free radicals can damage a variety of intracellular compounds by attacking unsaturated lipids in cellular membranes, and can initiate a lipid peroxidation chain reaction. Membrane fluidity increases, and calcium homeostasis is disturbed, lead-

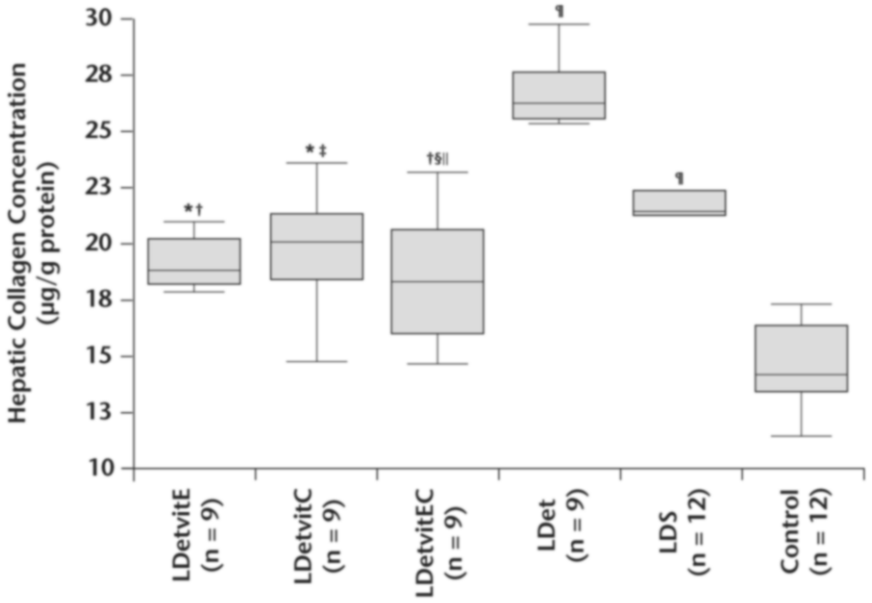

Figure 3. Effects of treatments on hepatic collagen concentration of malnourished rats measured using a biochemical method. LDetvitE = liquid diet + ethanol + vitamin E 15 mg/kg PO; LDetvitC = liquid diet + ethanol + vitamin C 10 mg/kg PO; LDetvitEC = liquid diet + ethanol + vitamin E 15 mg/kg + vitamin C 10 mg/kg PO; LDet = liquid diet + ethanol; LDS = liquid diet + isocaloric sucrose. *$P < 0.01$ versus control; †$P < 0.01$ versus LDet; ‡$P < 0.05$ versus LDet; §$P < 0.05$ versus control; ‖$P < 0.05$ versus LDS; ¶$P < 0.001$ versus control (all comparisons; nonparametric Tukey test).

ing to cellular necrosis and cellular death.[29] Glutathione peroxidase provides a second line of defense to destroy the peroxides before they damage membranes.[5] The effects of free radicals on the liver might be amplified when ethanol reduces antioxidant defenses. In a study of a pig model of alcoholic liver disease by Zindenberg-Cherr et al,[30] a decrease in glutathione peroxidase activity was seen and might have been related to the effect of free radicals in inactivating glutathione peroxidase. In our study, superoxide dismutase and catalase activities were not significantly altered, but glutathione peroxidase activity was prevented to decrease in 3 of the groups given vitamin supplementation compared with the LDet group (LDetvitE, LDetvitC, and LDetvitEC, $P < 0.001$ vs LDet). Hyperemia, cellular swelling, sinusoidal dilatation, and necrosis were observed in the livers of ethanol-fed rats and were likely associated with impaired glutathione peroxidase activity. Compared with LDet, supplementation with combined vitamins E and C was associated with normalized glutathione peroxidase activity and attenuation of hepatocellular inflammation and necrosis ($P < 0.05$,

Table IV. Histologic necroinflammatory and fibrosis activity scores according to the Brunt system[26] in rats at 8 weeks. Values are mean (SE).

Score Type	LDetvitE (n = 9)	LDetvitC (n = 9)	LDetvitEC (n = 9)	LDet (n = 9)	LDS (n = 12)	Control (n = 12)
Inflammation	1.5 (0.2)*	1.4 (0.2)*	0.8 (0.1)†	1.3 (0.2)	1.3 (0.2)	0.9 (0.1)
Necrosis	2.0 (0.3)*	2.0 (0.2)‡	1.5 (0.2)§	2.4 (0.3)‖	2.3 (0.3)‡	1.0 (0.2)
Fibrosis	0.1 (0.1)	0.1 (0.1)	0.1 (0.1)	0.4 (0.2)	0.3 (0.2)	0.1 (0.1)

LDetvitE = liquid diet + ethanol + vitamin E 15 mg/kg PO; LDetvitC = liquid diet + ethanol + vitamin C 10 mg/kg PO; LDetvitEC = liquid diet + ethanol + vitamin E 15 mg/kg + vitamin C 10 mg/kg PO; LDet = liquid diet + ethanol; LDS = liquid diet + isocaloric sucrose.
*$P < 0.05$ versus control.
†$P < 0.05$ versus LDetvitE, LDetvitC, and LDet.
‡$P < 0.01$ versus control.
§$P < 0.05$ versus LDet.
‖$P < 0.001$ versus control.

$P < 0.05$, and $P < 0.05$, respectively). The administration of ethanol, vitamin E, or vitamin C was not associated with changes in catalase and superoxide dismutase activities. Whether this unaltered enzyme activity stemmed from the effect of the liquid diet needs to be clarified.

Hepatic fibrosis is a highly integrated cellular response to tissue injury induced by various factors (eg, viruses, autoimmune activity, metabolism, alcohol ingestion).[31] Activated perisinusoidal hepatic stellate cells become myofibroblast-like and secrete excessive type I collagen, the major extracellular matrix protein deposited in hepatic tissue during fibrogenesis.[32] Alcohol intake might contribute to stellate cell activation in several ways. Elevated hepatic malondialdehyde concentration might contribute to hepatic fibrosis by directly inducing collagen secretion from stellate cells by reduction-oxidation–sensitive transcription factors (eg, activator protein-1 and nuclear factor-κB), and paracrine stimulation of stellate cells through the release of transforming growth factor (TGF)-β from Kupffer cells.[33–35] Vitamin E supplementation has been associated with the inhibition of lipid peroxide-induced collagen synthesis in cultured fibroblasts and stellate cells.[36,37] It is suggested that this inhibition was caused by decreased fibrogenic cytokine TGF-β concentrations.[36] In our study, no significant differences in hepatic total biochemical collagen concentrations were found between the groups treated with vitamin supplementation on light microscopy, suggesting that combination therapy with vitamins E and C was as effective as vitamin E or vitamin C administered alone in reducing hepatic fibrosis. However, we also measured collagen concentration using a biochemical method more sensitive than microscopy; that method found that supplementation with vitamins C and E was accompanied by a significant reduction in collagen concentration (LDetvitE, LDetvitC, and LDetvitEC groups, $P < 0.01$, $P < 0.05$, and $P < 0.01$ vs LDet group, respectively; $P < 0.01$, $P < 0.01$, and $P < 0.05$ vs controls, respectively). These findings suggest that preventing a decrease in antioxidant enzyme activity results in decreased hepatic fibrosis. Vitamin E acetate was not found to accumulate at crucial sites of cell injury, such as the mitochondria, or to partition into organelle phospholipid bilayers.[38] However, vitamin C is thought to regenerate vitamin E,[13,14] possibly allowing for its accumulation at crucial sites.

In a search of English-language literature concerning the use of vitamins E and C in alcoholic liver disease using MEDLINE (key terms: *vitamin E*, *vitamin C*, *ethanol* and *hepatic fibrosis*; years: 1967–2005), we found only 2 long-term experimental studies in which the effects of vitamin E supplementation on alcoholic liver disease were assessed. In a study by Nanji et al,[39] 10 rats were fed fish oil and ethanol for 6 weeks. At the end of week 6, ethanol treatment was discontinued and vitamin E treatment was started in 5 rats. Vitamin E was associated with decreased necroinflammation (30 cells/mm^2 vs 15.7 cells/mm^2; $P < 0.05$) and improved lipid peroxidation (thiobarbituric acid reactive substances level 0.74 [0.19] nmol/mg protein vs 0.30 [0.11] nmol/mg protein; $P < 0.01$) compared to those rats not administered vitamin E (n = 5). Our findings were consis-

tent with those of Nanji et al. However, in a study by Sadrzadeh et al,[40] although high-dose vitamin E supplementation was associated with decreased lipid peroxidation, it was not associated with decreased severity of tissue injury. In that study, rats were administered 1200-fold the normal amount of required vitamin E, and whether that high dose contributed to the failure of vitamin E to improve hepatic injury is unclear. Furthermore, the findings from Sadrzadeh et al might be misleading because hepatic fibrosis was assessed using only light microscopy, which is not a sensitive method. Light microscopy is not sensitive enough to quantify or compare subtle fibrotic changes in the liver, especially in alcoholic liver disease models based on such a short period of time, as is the case of both the Sadrzadeh et al study and ours. This is primarily due to the short time period of the study; 2 months is an inadequate amount of time for significant changes to be established and observed. Therefore, we used a more sophisticated and sensitive method to compare the hepatic fibrosis (ie, the determination of collagen content by biochemical method).[41,42] According to the results found with this method, antioxidants effectively reduced hepatic fibrosis in this rat model of alcoholic liver disease.

In a separate study in a rat model of acute ethanol-induced hepatic damage, Ozdil et al[43] found that the use of combined supplementation with vitamin E, vitamin C, and selenium was associated with decreased degenerative changes observed on light and electron microscopy. In human studies,[44,45] the efficacy of combined supplementation with vitamins E and C has been reported in nonalcoholic steatohepatitis (NASH), which is histopathologically similar to alcohol-related hepatitis. In the study by Yakaryilmaz et al,[45] 16 patients with NASH were given vitamin E 800 U/day in 2 divided doses for 6 months. Significant improvements in mean (SD) serum liver enzyme activities were observed at 6 months compared with baseline. Posttreatment liver biopsy was available in 13 patients (81%). Significant improvements in the mean (SD) scores of steatosis (1.46 [0.66] vs 2.43 [0.62]; $P = 0.002$) and necroinflammatory grade (0.84 [0.24] vs 1.31 [0.51]; $P = 0.006$) were observed at 6 months compared with baseline, respectively. However, no significant change was noted in the mean (SD) score of fibrosis stage (0.77 [0.33] vs 1.12 [0.59], respectively). In the second study, which was a prospective, double-blind, randomized, placebo-controlled trial by Harrison et al,[44] 45 patients were randomized to receive either vitamins E and C (1000 IU and 1000 mg, respectively) or placebo QD for 6 months. Vitamin treatment resulted in a statistically significant improvement in fibrosis score ($P = 0.002$). However, they observed no changes in inflammation with treatment. In our study, in contrast to patients with NASH in the study by Harrison et al, necroinflammation was reduced by combined vitamins E and C therapy. The different study designs (humans vs rats) and different diseases (NASH vs alcoholic liver disease) may not explain the controversy between these 2 studies. However, improvement in hepatomegaly observed only in the combination group (LDetvitEC) compared with the LDet group ($P < 0.01$), in association with the improvement of necroinflammation in the liver, suggests that combining

antioxidants might increase the therapeutic effects of each on alcoholic liver disease.

We used the liquid diet described by Bosma et al[18] to simulate the diet of alcoholic patients to achieve an alcoholic cirrhosis model in rats. However, when we used that diet, the rats began to die during the second month of the study due to empyema and deep neck infections related to severe cachexia and malnutrition. Hence, we decided to terminate the study and euthanize the rats at 8 weeks, before the predetermined time period of 48 weeks. The rats used in the study by Bosma et al did not die earlier than expected, possibly because they used a different species of rats than the ones we used, and because the rats were kept in aseptic conditions from birth. Despite its short duration of 8 weeks, ours is the longest-term animal study of the antioxidant effects of vitamins E and C on alcoholic hepatic injury in a rat model based on our MEDLINE search.

In our study, necroinflammation and fibrosis were similar between rats in the LDet and LDS groups, suggesting that the damage might have been associated with the high-fat, protein-deficient liquid diet. However, none of the rats in the LDS group died earlier than expected, in contrast to those in the LDet group. Hepatomegaly, an important clinical finding in alcoholic liver disease, was observed in the LDet group but not in the LDS group. Nonetheless, the addition of a group fed normal diet and ethanol might have allowed for a better comparison of the impact of ethanol on tissue injury. The effects of diet, malnutrition, sex, and genetics on tissue damage in the pathogenesis of alcoholic liver disease still need to be studied.

CONCLUSIONS

The results of this study in protein-deficient rats fed with a high-fat liquid diet suggest that supplementation with vitamin E, vitamin C, and a combination of vitamins E and C was associated with decreased ethanol-induced hepatic glutathione peroxidase activity and hepatic fibrosis, and that supplementation with vitamins E and C might have attenuated the development of hepatomegaly and hepatic necroinflammation, whereas this result was not found in the group given a liquid diet and ethanol in this 8-week study.

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