β-carotene alters vitamin E protection against heme protein oxidation and lipid peroxidation in chicken liver slices

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This study investigated the interaction between dietary β-carotene and vitamin E in protecting against heme protein oxidation and lipid peroxidation initiated by incubation of liver slices with ferrous sulfate. Male leghorn chicks were fed a basal diet deficient in β-carotene and vitamin E for 3 weeks. Beta-carotene (0, 30, and 100 mg/kg) and vitamin E (0, 50 mg/kg) were added to the diet using a 3 × 2 factorial arrangement of treatments. Analysis of variance showed the following significant effects. Vitamin E protected against both heme protein oxidation and lipid peroxidation as measured by thiobarbituric acid reactive substances ($P \leq 0.001$). Liver slices from vitamin E adequate chicks had less hemichrome and produced fewer thiobarbituric acid reactive substances than liver slices from vitamin E-deficient chicks. Addition of β-carotene to the diets did not protect chicks significantly against either hemichrome formation or the production of thiobarbituric acid reactive substances ($P \leq 0.001$). Supplementation of the vitamin E-adequate diet with 100 mg β-carotene/kg diet resulted in substantially lower retention of hepatic vitamin E ($P \leq 0.001$). (J. Nutr. Biochem. 5:479–484, 1994.)

Keywords: chicks; vitamin E; β-carotene; heme protein oxidation; lipid peroxidation

Introduction

Oxidative stress is implicated in the pathology of cancer, atherosclerosis, ischemic reperfusion injury, inflammation, and aging.[1] The susceptibility of humans and animals to oxidative damage is dependent on the balance between oxidative stress and antioxidant defense capacity. The nutritional status of various antioxygenic nutrients is an important protective factor against oxidative damage. The most widely accepted role of vitamin E is its ability to scavenge free radicals, delaying or preventing the chain reaction that occurs in lipid peroxidation.[2] There is also increasing evidence that β-carotene may function as an important singlet oxygen and free radical scavenger.[3] The free radical scavenging activity of β-carotene is enhanced at oxygen partial pressures characteristic of tissues.[3] The free radical scavenging ability of β-carotene is due to the delocalizing of an unpaired electron in the conjugated double bond system of β-carotene. A dietary requirement for β-carotene has not been established. In addition, little is known about the interaction between dietary vitamin E and β-carotene and how they function together in protection against oxidative stress.

This study investigated the interaction between vitamin E and β-carotene in protection against lipid peroxidation and heme protein oxidation. The chicks were fed vitamin E-deficient or vitamin E-adequate diets with or without the addition of β-carotene. Following incubation with ferrous sulfate, liver slices were assayed for production of thiobarbituric acid reactive substances (TBARS), hemichrome, and other products of heme protein oxidation. A new method developed in this laboratory, the heme protein spectral analysis program (HPSAP)[4,5] was used to monitor liver slices for the production of oxidation products of heme proteins. HPSAP is a computer-based spreadsheet program using Lotus 123 (Lotus Development Corp., Cambridge, MA, USA).

Methods and materials

Animals and diets

Male white leghorn 1-day-old chicks were obtained from a local hatchery. Chicks were weighed and randomly assigned to one of six dietary groups (six chicks in each treatment). The average initial weight of the chicks was 33.9 ± 2.5 g. Chicks were raised in heated, thermostatically controlled starter brooders with raised wire floors. The basal diet was purchased from Dyets Inc. (Bethlehem,

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PA USA) and is presented in *Table 1*. The basal diet was adequate for growing chicks in all nutrients except vitamin E. The basal diet contained no β-carotene or other carotenoid. The basal diet contained 0.3 mg vitamin E/kg diet, a measurement of the trace amount present in the stripped corn oil. Vitamin E (α-tocopherol succinate) and pure β-carotene were added separately to the diet at 0 and 50 mg/kg diet and 0, 30, and 100 mg/kg diet, respectively. A 2 × 3 factorial arrangement of treatments was used. Chicks had free access to food and deionized water.

β-Carotene and vitamin E analysis

Saponified liver samples were extracted with petroleum ether, and β-carotene was measured spectrophotometrically at 450 nm. Total carotenoid values were expressed as μg *trans*-β-carotene equivalents/g tissue.

Vitamin E analysis was done at the Vitamin and Mineral Core Laboratory, University of California, Davis, using the methods of Bieri et al.[6] and Driskell et al.[7]

Preparation and spectrophotometric measurement of tissue slices

During days 18 to 23 of dietary treatment, individual chicks from each dietary group were weighed and decapitated. The livers were immediately dissected, thoroughly rinsed, and immersed in ice-cold Krebs-Ringer phosphate buffer, pH 7.4. Tissue slices were prepared as previously described.[8] Approximately 300 mg of tissue slices was placed in glass serum bottles with 5 mL of O_2 equilibrated Krebs-Ringer phosphate buffer, 10 mmol/L glucose, and O_2 in the headspace. Immediately before incubation, ferrous sulfate was added to the serum bottles at a final concentration of 0, 0.05, 0.25, and 0.5 μmol/L. Tissue slices were incubated for 1 hr in a 37° C, shaking water bath (180 rpm). After incubation, serum bottles were placed on ice. Approximately 50 mg of tissue was dried with filter paper and placed in a spectrophotometer cell (5.5 mm i.d. and 2 mm light path). The cell was placed as close as possible to the photoreceptor of the spectrophotometer to reduce light scattering of the tissue. Parafilm (four layers) was used in the background cell to subtract most of the absorbance caused by the turbidity of the sample.

The absorbance spectrum of each sample was obtained with a Beckman Du-50 spectrophotometer. The samples were scanned from 500 nm to 640 nm, and the absorbance versus wavelength at 5 nm intervals was automatically recorded.

Analysis of absorbance spectra

Absorbance spectra of tissue heme proteins were analyzed using HPSAP. The program contains literature values of micromolar extinction coefficients from 500 to 640 nm in 5 nm increments of heme compounds known to exist in liver tissue. The heme protein spectra included in the program are oxyhemoglobin, deoxyhemoglobin, methemoglobin, ferrylhemoglobin, hemichrome, combined mitochondrial cytochromes, and combined microsomal cytochromes. Micromolar extinction coefficients for reduced and oxidized mitochondrial and microsomal cytochromes are calculated according to the ratio of these compounds in chicken liver. The HPSAP also contains a turbidity factor, which previous research[4] shows to be linear, with a slope of −1.3 under the experimental conditions used in the study.

To determine concentrations of heme proteins, experimental data for each 5 nm over the 500 to 640 nm range were entered into the HPSAP. Quantitative determination of the heme compounds in the sample was achieved by a successive approximations fit between the experimental and theoretical spectra. The theoretical spectrum is based on the assumption that heme compounds closely obey Beer's law. The HPSAP records and subtracts from the absorbance of the sample any contribution due to turbidity in the sample and calculates the mole percent for the individual heme compounds present in the sample. Results are expressed as percent hemichrome, a major oxidation product.

Table 1 Diet composition

Ingredient	g/kg diet
Soy protein	200
DL-methionine	4
Corn starch	424
Dextrose	200
Cellulose	50
Tocopherol stripped corn oil	50
Salt mix #236001	60
Vitamin mix	10
Choline bitartrate	2

Lipid peroxidation in tissue slices

Lipid peroxidation was assessed by measuring the concentration of TBARS. Liver homogenate was prepared by homogenizing the tissue slices in the incubation media. Homogenization was conducted using a motor-driven tissue homogenizer. Aliquots of liver homogenates were immediately frozen for subsequent analysis. Samples were analyzed for TBARS as previously described.[9] Protein concentration was analyzed using the Bio-Rad dye reagent (Bio-Rad Laboratories, Richmond, CA USA). Malondialdehyde (MDA) standards were prepared from 1,1,3,3 tetramethoxypropane. TBARS values were normalized to protein concentration and expressed as mmol/L TBARS (MDA equivalents)/mg protein.

Statistical methods

The statistical package SAS (SAS Institute Inc., Cary, NC USA) was used to analyze all data. All results were expressed as means ± standard deviation. Data were analyzed using ANOVA. When significant P values were obtained, the method of least square means was used to find significant differences ($P \leq 0.05$) between treatment means.

Simulation modeling of heme protein oxidation and lipid peroxidation

To test the hypothesis that the oxidation processes seen in this research follow the general relationship, oxidation products = oxidation processes/antioxidants,[10] simulation models were developed. For liver tissue the model of heme protein oxidation (HPO) is shown in Equation 1.

$$\text{HPO} = \frac{[\text{HPO components}] \times \log [\text{Fe}]}{[\text{vit E}] + 0.5\,[\beta\text{-carotene}]} + \text{basal HPO.} \quad (1)$$

For liver tissue the model for lipid peroxidation (LP) is shown in Equation 2.

$$\text{TBARS} = \frac{[\text{LP components}] \times \log [\text{Fe}]}{[\text{vit E}] + 0.5\,[\beta\text{-carotene}]} + \text{basal LP.} \quad (2)$$

Oxidation components are a composite of factors involved in the oxidations that are not otherwise expressed in the equations. To test the model, the means of the experimental values were used. When the vitamin E concentration was below the detection limit of 4 μg/g *(Table 2)*, it was approximated as 4 μg/g because the

Table 2 Hepatic vitamin E and β-carotene concentration

Diet	Dietary		Liver	
	Vitamin E (IU/kg)	β-carotene (mg/kg)	Vitamin E (μg/g liver)	β-carotene (μg/g liver)
EO, CO	0	0	ND*	0.6 ± 0.4[a]
EO, C30	0	30	ND*	2.4 ± 1.0[b]
EO, C100	0	100	ND*	7.3 ± 2.9[c]
E50, CO	50	0	28 ± 5[b]	1.1 ± 0.4[a]
E50, C30	50	30	25 ± 5[b]	3.0 ± 1.1[b]
E50, C100	50	100	9 ± 3[a]	3.4 ± 0.9[b]

Values with different superscrips are significantly different at $P \leq 0.05$.
*Concentration of hepatic vitamin E was below the detection limit of 4 μg/g.

stripped, corn oil always has some tocopherol, and the basal diet analyzed as 0.3 mg vitamin E/kg diet. Beta-carotene antioxidant effectiveness compared with vitamin E is low and was approximated at 0.5. Because of its concentration *(Table 2)*, β-carotene usually provides much less antioxidant protection than vitamin E.

Results

Effects of dietary treatment on hepatic vitamin E concentration

Chicks fed a vitamin E-deficient diet had a low hepatic concentration of vitamin E at all concentrations of β-carotene *(Table 2)*. In chicks fed a vitamin E-adequate diet, hepatic concentration of vitamin E was dependent on the concentration of β-carotene in the diet. Increasing β-carotene from 0 to 30 mg/kg diet in vitamin E-adequate chicks resulted in a slight but not significant decrease ($P \leq 0.05$) in hepatic vitamin E. Increasing β-carotene to 100 mg/kg diet in chicks fed a vitamin E-adequate diet resulted in a significant 68% reduction in hepatic vitamin E.

Effects of dietary treatment on hepatic β-carotene concentration

Hepatic retention of β-carotene was dependent on the concentration of dietary vitamin E and β-carotene. In chicks fed a vitamin E-deficient diet, increasing dietary β-carotene from 0 to 30 mg/kg diet resulted in a significant increase to fourfold in hepatic retention of total carotenoids *(Table 2)*. Increasing the concentration of dietary β-carotene from 30 to 100 mg/kg diet in vitamin E deficient chicks, resulted in an increase to threefold in hepatic carotenoids. In chicks fed a vitamin E adequate diet, increasing β-carotene from 0 to 30 mg/kg diet, resulted in an increase to 2.7 fold in liver carotenoids. However, increasing β-carotene further to 100 mg/kg diet did not significantly increase liver carotenoids in vitamin E-adequate chicks.

Heme oxidation in liver slices

Raising the concentration of iron in the incubation media resulted in increases in heme protein oxidation in all six dietary groups *(Figure 1)*. The extent of heme protein oxidation was also modified by the dietary concentrations of vitamin E and β-carotene. Thirty milligrams of dietary β-carotene/kg diet did not substantially affect heme protein oxidation of vitamin E-deficient liver slices incubated with 0.05 and 0.25 μmol/L iron. However, in vitamin E-deficient liver slices incubated in 0.5 μmol/L iron, increasing dietary β-carotene from 0 to 30 mg/kg diet resulted in significantly less heme protein oxidation.

In vitamin E-deficient liver slices incubated in 0.05 and 0.25 μmol/L iron, the addition of 100 mg/kg dietary β-carotene did not substantially affect the level of heme protein oxidation compared with that of 30 mg/kg dietary β-carotene. However, in the incubation of vitamin E-deficient liver slices with 0.50 μmol/L iron, 100 mg β-carotene/kg diet significantly increased heme protein oxidation over that of liver treated in the same way from vitamin E-deficient chicks fed 30 mg β-carotene/kg diet.

In vitamin E-adequate chicks, 30 mg β-carotene/kg diet did not significantly raise heme protein oxidation.

With all concentrations of iron used in chicks fed a vitamin E-adequate diet and 100 mg β-carotene/kg diet, heme oxidation in liver was significantly elevated compared with that of liver slices from chicks fed a vitamin E-adequate diet containing 0 or 30 mg β-carotene/kg diet.

Within each dietary group subjected to the same concentration of iron, liver slices from vitamin E-adequate chicks had significantly less heme protein oxidation than liver slices from vitamin E-deficient chicks, with or without β-carotene in the diet.

Effects of dietary treatment on lipid peroxidation (TBARS)

TBARS increased in all dietary groups as the concentration of iron in the incubation media was raised *(Figure 2)*.

β-carotene did not affect the concentration of TBARS in liver incubated with iron from vitamin E-deficient chicks except in slices incubated with 0.5 mmol/L iron. Supplementation of vitamin E-deficient chicks with 30 mg β-carotene/kg diet resulted in a significant decline in the concentration of TBARS in liver incubated in 0.5 μmol/L iron.

In iron-incubated liver slices from vitamin E-adequate chicks, increasing the concentration of dietary β-carotene from 0 to 30 mg had no significant effect on TBARS. Increasing β-carotene from 30 to 100 mg/kg diet in vitamin E adequate chicks resulted in a significant increase in TBARS of liver slices by 52% (0 μmol/L iron), 34% (0.05 μmol/L iron), 40% (0.25 μmol/L iron), and 21% (0.5 μmol/L iron).

Within each dietary group undergoing incubation with the same concentration of iron, liver slices from vitamin E-adequate chicks fed 30 mg β-carotene/kg diet or no dietary β-carotene had markedly lower TBARS than liver slices from vitamin E-deficient chicks with 30 mg β-carotene/kg diet or no dietary β-carotene. When 0, 0.05, or 0.25 μmol/L iron was the inducer, liver slices from vitamin E-adequate chicks fed 100 mg β-carotene/kg diet had nonsignificantly decreased TBARS compared with those from vitamin E-deficient chicks fed 100 mg β-carotene/kg diet. With 0.5 μmol/L iron as the oxidant, TBARS decreased markedly in vitamin E-adequate chicks that received 100 mg β-carotene/kg diet compared with vitamin E-deficient chicks fed 100 mg β-carotene/kg diet. The means of percent hemichrome *(Figure 1)* show a significant correlation of $r = 0.96$, $P < 0.001$ with TBARS *(Figure 2)*.

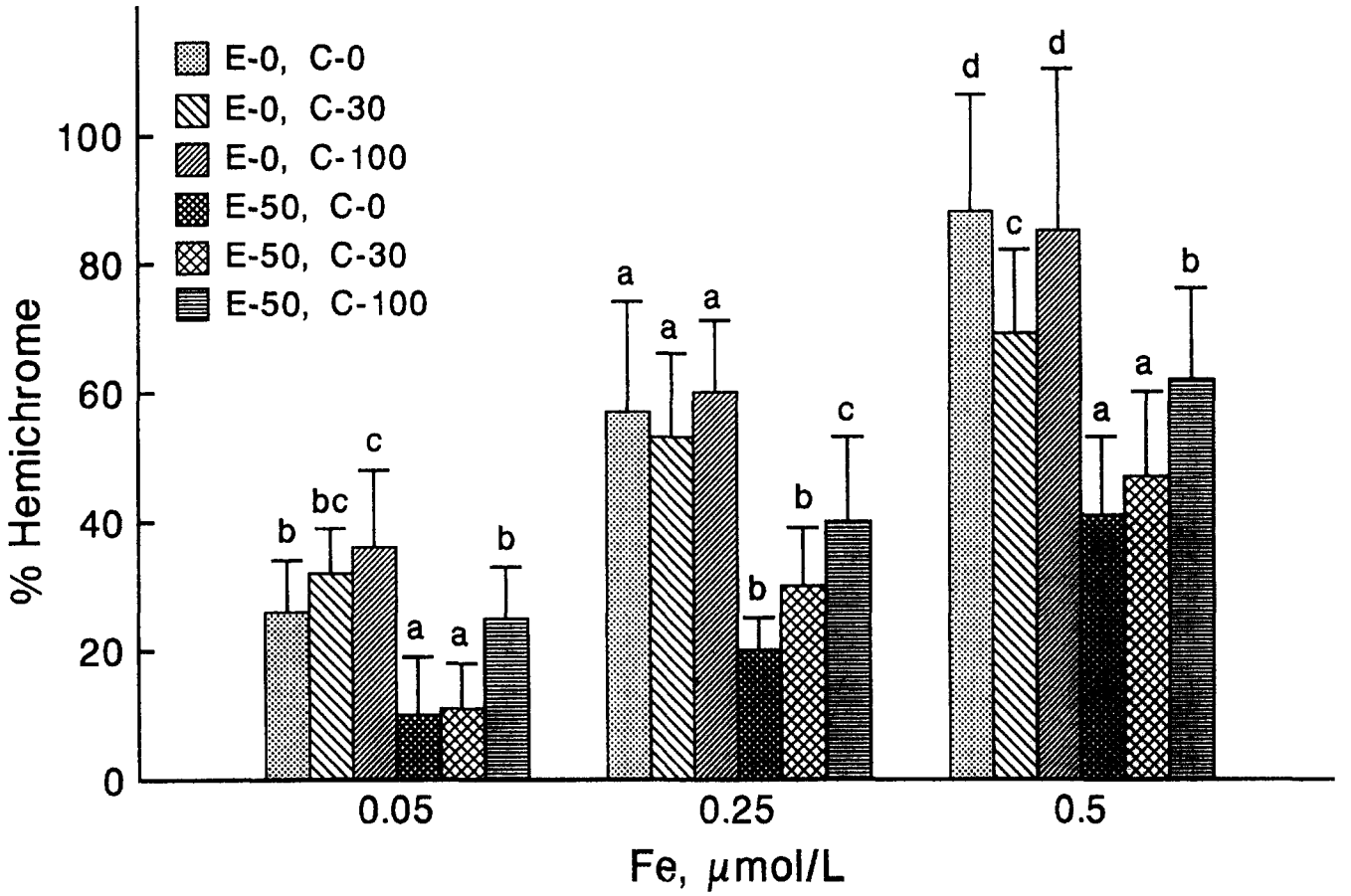

Figure 1 Effect of diet and iron on the oxidation of heme proteins expressed as percent hemichrome produced in liver slices in 1hr incubation. Bars show percent hemichrome means ± standard deviation. Dietary vitamin E (E) and β-carotene (C) are given in *Table 2*. Percent hemichrome with different superscripts within the same iron concentration are significantly different at $P \leq 0.05$.

Simulation modeling of heme protein oxidation and lipid peroxidation

Figures 3 and 4 show correlations between values for simulated and experimental heme protein oxidation expressed as hemichrome and between values for simulated and experimental TBARS. These correlations include the major variables of the study: oxidative damage to proteins and lipids; catalysis of oxidation by iron; and protection, mainly by vitamin E, and, to a very small extent, by β-carotene.

Discussion

Hemoglobin is susceptible to oxidative damage that results in structural and functional changes, as the results in *Figure 1* show. In the presence of a prooxidant, hemoglobin is oxidized to methemoglobin.[11] If the globulin structure is destabilized or a suitable ligand is available, methemoglobin is converted to hemichrome.[12] Hemichromes are unstable, precipitate readily, and are the main constituent of Heinz bodies.[13]

In this study, chicks fed a vitamin E-deficient diet had significantly higher heme oxidation and lipid peroxidation than vitamin E-adequate chicks. Data from the present iron incubation studies showed that in comparison with vitamin E adequate chicks, vitamin E-deficient chicks had significantly higher hemichrome formation in tissue slices regardless of the level of dietary β-carotene.

These findings are corroborated by studies reported in the literature. Vitamin E protects against oxidative damage in both liver slices[14] and red blood cells.[15] Heinz body formation after exposure to H_2O_2 vapor is significantly higher in erythrocytes isolated from vitamin E-deficient patients than in erythrocytes isolated from vitamin E-adequate patients.[16] Previous studies showed an increase in distorted and contracted erythrocytes in vitamin E-deficient premature infants.[17] The major physiological role of α-tocopherol is as a chain-breaking antioxidant, protecting the cell membrane from lipid peroxidation.[2]

Adding β-carotene to the vitamin E-deficient or -adequate diet did not protect isolated tissue slices significantly from either lipid peroxidation or heme protein oxidation. However, in vitro studies show protection by β-carotene against lipid peroxidation induced by singlet oxygen[18] and free radical initiators.[19] β-carotene and vitamin E inhibit conjugated diene and malondialdehyde formation by microsomal lipids exposed to the lipid-soluble radical initiator, azo-bis-isobutyronitrile, in a dose-dependent manner.[20]

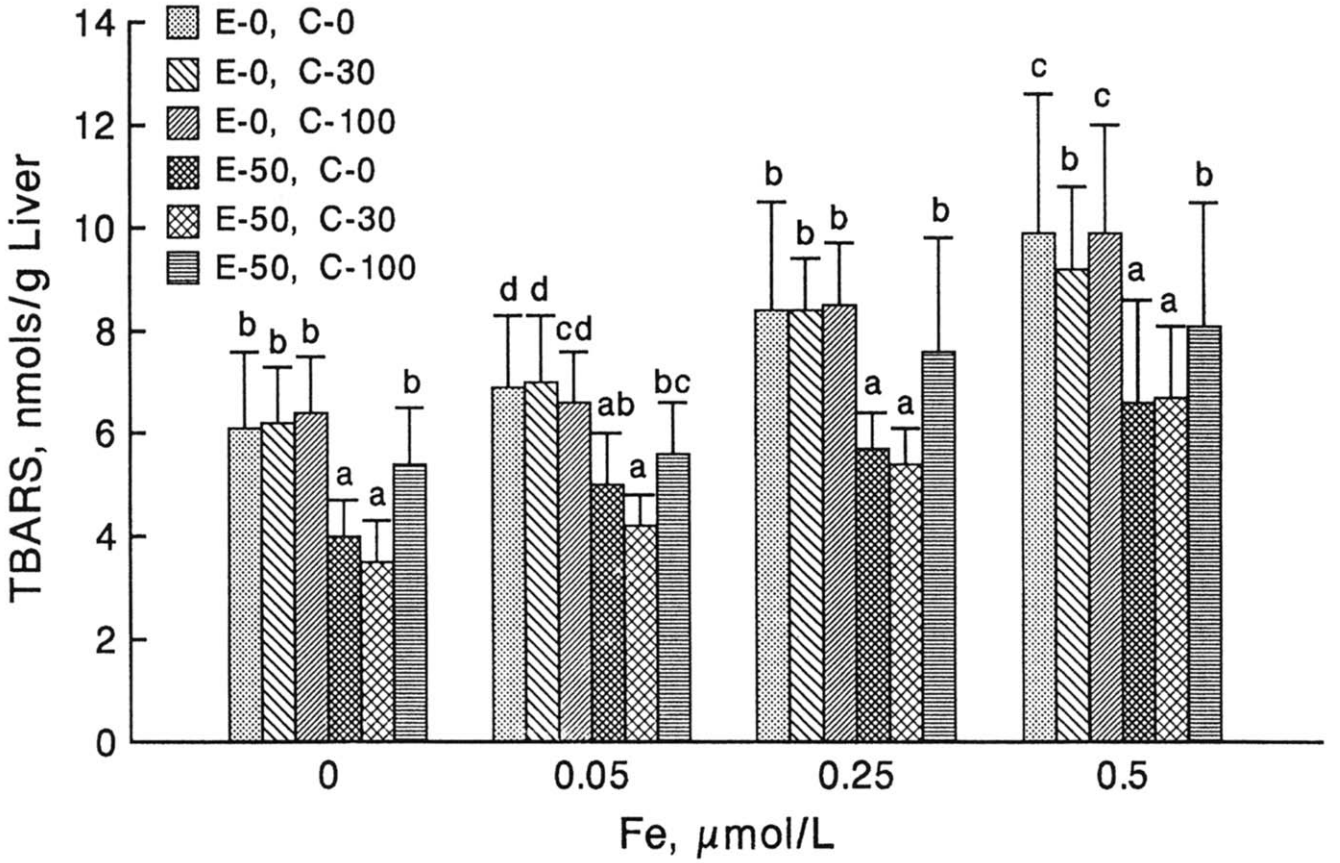

Figure 2 Effect of diet and iron on the TBARS produced by liver slices in 1hr incubation. Bars show TBARS means ± standard deviation. Dietary vitamin E (E) and β-carotene (C) are given in *Table 2*. TBARS with different superscripts within the same iron concentration are significantly different at $P \leq 0.05$.

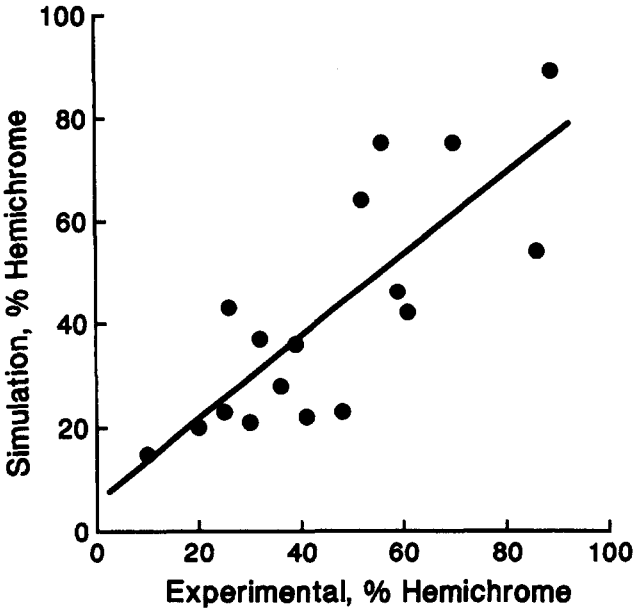

Figure 3 Correlation of simulated and experimental values of heme protein oxidation: $r = 0.81$, $P < 0.001$.

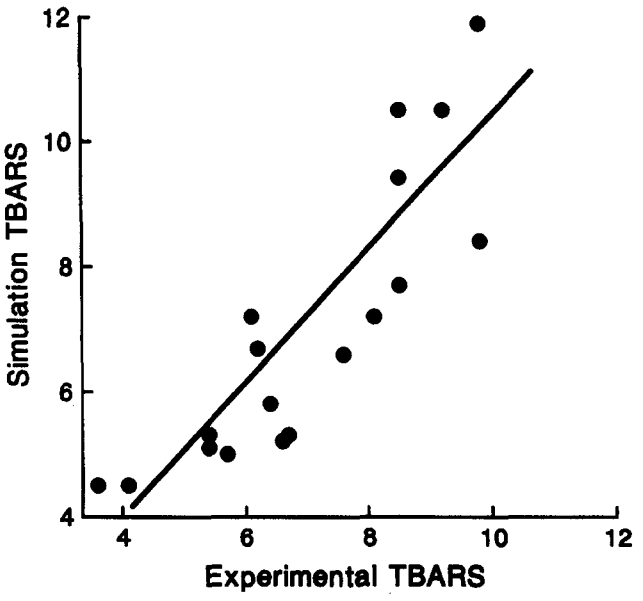

Figure 4 Correlation of simulated and experimental values of TBARS; $r = 0.86$, $P < 0.001$.

The effects of β-carotene on oxidative damage observed in this study may be explained by the decreased retention of hepatic α-tocopherol in the presence of β-carotene. β-carotene could have altered absorption on binding of vitamin E in the liver. Previous studies[21] showed a decrease in hepatic retention of vitamin E when high concentrations of β-carotene were consumed. Reduction of plasma and liver vitamin E concentration results when rats are fed 48 and 480 mg β-carotene/kg diet.[21] Human volunteers fed 800 mg of α-tocopherol for 16 weeks have a doubling of plasma α-tocopherol and a moderate but significant decrease in plasma carotenoids.[22] Our data suggested an interaction between dietary β-carotene and α-tocopherol on the retention of these two compounds in the liver. α-Tocopherol and β-carotene may compete with each other for absorption. High concentrations of vitamin A interfere with vitamin E absorption in chicks.[23] In addition, both vitamin E[24] and β-carotene[25] are bound to lipoproteins when transported in the blood. Vitamin E and β-carotene may compete with each other for binding sites on the lipoprotein molecule. In contrast to the results of our study, Mayne and Parker[26] found that β-carotene, when fed to chicks at a relatively high level of 0.5 g/kg of diet, increased vitamin E in liver twofold to fourfold. Also, Mayne and Parker[27] found that canthaxanthin, when fed to chicks at 0.5 g/kg diet, increased vitamin E in hepatic membrane twofold. These increases in hepatic vitamin E may be related to the high level of carotenoids fed. Before recommending consumption of diets or supplements high in β-carotene to the general public, further research is needed to determine the effects of dietary β-carotene on absorption, transport, and retention of vitamin E.

Simulation modeling and various empirical correlations are applied in nutritional studies. The models used in the present study included the major variables of the study. The simulation also helped to rationalize the interpretation of the present results and suggested that various factors involved in the oxidation processes can be included in a model to give a broad perspective of oxidant and antioxidant interactions.

There are two main conclusions that come from this study. First, β-carotene and vitamin E in the diet had competing effects on the concentration of the other in the liver. Second, vitamin E was strongly protective against oxidation in liver slices measured by heme protein oxidation and TBARS.

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