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Vitamin E supplementation in the mitigation of carbon tetrachloride induced oxidative stress in rats[1]

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Abstract

Oxidative stress is implicated in the pathophysiology of a number of chronic diseases including atherosclerosis, diabetes, cataracts and accelerated aging. The aim of this study was to elucidate the protective role of vitamin E supplementation when oxidative stress is induced by CCl_4 administration, using the rat as a model.

Rats were fed diets for four weeks either with or without dl-α-tocopherol acetate supplementation. Half of the rats (n = 9) from each of the diet groups were then challenged with CCl_4 at the completion of the four week diet period. Plasma levels of 8-iso-$PGF_{2\alpha}$, antioxidant micronutrients and antioxidant enzyme activities were measured to examine changes in oxidative stress subsequent to the supplementation of dl-α-tocopherol in the diet.

Plasma α-tocopherol (vitamin E) concentrations were higher for the groups supplemented with dl-α-tocopherol acetate, however the supplemented diet group that was subsequently challenged with CCl_4 had significantly lower ($p < 0.001$) plasma α-tocopherol concentration than the dl-α-tocopherol acetate diet group that was not challenged with CCL_4. Total plasma 8-iso-$PGF_{2\alpha}$ concentration was elevated in diet groups challenged with CCl_4, however, the concentration was significantly lower ($p < 0.001$) when the diet was supplemented with dl-α-tocopherol acetate. The antioxidant enzymes were not influenced by either dietary α-tocopherol manipulation or by the inducement of oxidative stress with CCl_4. Plasma concentrations of trans-retinol (vitamin A) were reduced by CCl_4 administration in both the dl-α-tocopherol acetate supplemented and unsupplemented diet groups.

The results of this study indicate that dl-α-tocopherol acetate supplementation was protective of lipid peroxidation when oxidative stress is induced by a pro-oxidant challenge such as CCl_4.

Keywords: Oxidative stress; α-tocopherol supplementation; 8-iso-$PGF_{2\alpha}$; Carbon tetrachloride; Rats; Retinol

1. Introduction

Lipid peroxidation, caused by oxidative modification due to the presence of free radicals, is thought to be one of the major pathways of disease initiation and proliferation. Vitamin E is thought to be a major chain breaking lipid soluble antioxidant. For example, in LDL particles, in terms of molar ratios, Vitamin E is the major lipophilic antioxidant (approximately 6 molecules of α-tocopherol per LDL particle) while all other antioxidants are present in much smaller quantity [1]. In humans Vitamin E supplementation has been shown to decrease the susceptibility of LDL to oxidation ex vivo [2]. In ApoE-deficient mice Vitamin E supplementation suppressed isoprostane generation and reduced atherosclerosis [3]. Vitamin E therefore appears to have a major role in prevention of LDL oxidation and thus atherosclerosis. The epidemiological evidence linking Vitamin E status and the incidence of cardiovascular disease and cancer in the literature leans towards low plasma levels of Vitamin E and increased incidence of cardiovascular disease and cancer [4]. In an effort to support the epidemiological data clinical trials involving Vitamin E supplementation have provided mixed results, and detailed knowledge of the action of Vitamin E *in vivo* with respect to oxidative stress is scarce. This is largely due to a lack of techniques which adequately provide an index of *in vivo* lipid peroxidation, an inability to clearly identify high risk groups, and a lack of reliable measures to determine the *in vivo* effectiveness of interventions [5].

One of the major reasons for this lack of information is the difficulty in measuring free radicals directly *in vivo.* For example the reaction rate of the OH- radical is very quick (rate constant of 10^9 $M^{-1}sec^{-1}$), it reacts with the next mol-

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ecule it meets and therefore measuring the amount of the free radical formed is difficult [6]. Most methods are either too non-specific or not sensitive enough to give reliable results [7]. Isoprostanes are molecules formed by the non-enzymatic oxygenation of arachidonic acid by free radicals. They are a stable and reliable marker of lipid peroxidation, and therefore can be used as an *in vivo* marker of oxidative stress [8]. Elevated levels of isoprostanes have been observed in a variety of conditions where oxidative stress is enhanced [8,9]. Elevated isoprostane concentrations have been demonstrated in studies where disease states related to elevated oxidative stress have been present [10,11] and in situations where antioxidants, for example Vitamin E and Selenium have been deficient [12].

In this study the effect of Vitamin E supplementation was examined in a model of induced oxidative stress using isoprostanes as the *in vivo* marker of lipid peroxidation to examine the effectiveness of vitamin E in reducing induced oxidative stress.

2. Materials and methods

Thirty six female weanling Wistar rats (Animal Resource Center, Murdoch WA, Australia) weighing between 102-136g (114 ± 1.34) were randomly assigned to two diet groups of 18 rats in each group. The rats were fed semi-synthetic, nutritionally adequate diets with (7g α-tocopherol/Kg diet) or without (approximately 55mg α-tocopherol/200g oil occurring naturally in the oil) added α-tocopherol supplement. The rats were fed ad libitum for four weeks. The animals were fed fresh food daily and weighed twice a week. The rats were individually caged in polycarbonate cages with high-topped wire lids and housed in a facility with alternating 12 hr light/dark cycles. The University of Newcastle's Animal Care and Ethics Committee approved the study.

The basal diet (Glen Forrest Stockfeeders, Perth WA, Australia) contained sucrose (44g/100g), starch maize (18g/100g), cellulose (7g/100g), casein (25g/100g), methionine (0.38g/100g), AIN 93G mineral mix (4.2g/100g), and AIN 93 G vitamin mix (1.2g/100g) providing 1.5 mg/100g of α-tocopherol. Sunola oil (20% W/W) served as the fat source in the diet. The fatty acid profile of the oil was 8.7% as saturated fat, 80.8% as monounsaturated fat of which 80.1% was C18:1n-9, 10.1% omega 6 fatty acids predominantly C18:2n-6, and small amounts of omega 3 fatty acids (0.2%).

At the completion of the four week feeding regime, half the animals in each diet group (n = 9) were administered a sublethal dose of the CCl_4 purity 99.94% (Merck, Victoria, Australia) (2mLs/Kg body weight in a 1:1 dilution with corn oil) by gavage to induce lipid peroxidation [13], the remaining animals were given corn oil only by gavage. After four hours these animals were anesthetized with isoflurane (Veterinary Medical Supplies, Newcastle, NSW, Australia), a heart puncture performed and the animal was euthanaised using CO_2. The animals not administered with CCl_4 were also anesthetized with isoflurane, a heart puncture performed and the animals' euthanaised using CO_2. Blood was collected in ethylenediaminetetraacetic acid (EDTA) coated tubes with reduced glutathione added (1mg/ml blood) and centrifuged at 3000 rpm, at 4°C for 10 min. The plasma and the Red Blood Cells (RBC) pellet were separated for analysis and stored at -70°C, for analysis at a later date.

2.1. Antioxidant and micronutrient analysis

Plasma levels of vitamins A and E were determined by reverse phase High Performance Liquid Chromatography (HPLC) using a program variable wavelength UV-visible detector [14]. Samples were thawed, mixed with ethanol to precipitate proteins, vortexed, and reconstituted with hexane and injected into the HPLC column (lab-packed Whatman ODS 3 (5micron) 300 x 3.5 mm ID). The eluting solvent had a flow rate of 1 ml/min. Vitamin A was measured at 310 nm and Vitamin E was measured at 450nm. Plasma levels of zinc and selenium were analyzed by Inductively Coupled Plasma-Mass Spectrometry (ICP-MS). Samples were diluted in an ammonium EDTA based diluent in a Quantitative Application; platinum and rhodium were used as internal standards in the diluent. Calibration was by Addition Calibration in a pooled plasma base.

3. 8-iso-$PGF_{2\alpha}$ assay

An aliquot of plasma was taken and a known amount of tritiated prostaglandin ($PGF_{2\alpha}$) (Amersham, Arlington Heights, IL, U.S.A.) was added, to determine recovery rate after the purification procedure. Ethanol was added to precipitate proteins and the sample was centrifuged at 1500 rpm, at 4°C for 10 min. The supernatant was decanted and an equal volume of 15% KOH was added, then incubated at 40°C for one hour to cleave the esterified isoprostane molecules. After incubation the pH was lowered to 3 by dropwise addition of dilute HCl (Caymen Chemical, Ann Arbor, MI, U.S.A.). The sample was first passed through a C18 Sep Pak reverse phase cartridge (Waters, Milford, MA, U.S.A.), and eluted with ethyl acetate: heptane (1:1). The sample was further purified by passing through a Silica Sep Pak cartridge (Waters Milford, MA, U.S.A.) and eluted with ethyl acetate: methanol (1:1). The solvent was evaporated using N_2, and the sample reconstituted in assay buffer. A small amount of the sample was then analyzed with an 8-isoprostane enzyme immunoassay Kit (Cayman Chemical, Ann Arbor, MI, U.S.A.). Absorbance values were measured using a plate reader at 405nm wavelength, and the raw data corrected using the recovery rates.

The assay was validated by adding known amounts of 8-isoprostane to aliquots of purified plasma, the concentration of these samples was determined using the EIA kit. A

strong correlation (0.99) was obtained between the known amounts of pure 8-isoprostane added and the concentration determined by EIA. The antiserum used in the assay has 100% cross-reactivity with 8-isoprostane, 20% with 8-isoprostane $F_{3\alpha}$, 0.2% with $PGF_{2\alpha}$, $PGF_{3\alpha}$, PGE_1 and PGE_2 and 0.1% with 6-keto-$PGF_{1\alpha}$. Detection limit is 4 pg/ml (Caymen Cayman Chemical, Ann Arbor, MI, U.S.A.).

3.1. Glutathione peroxidase

Whole blood samples were collected into EDTA tubes, and centrifuged at 8500g, at 4°C for 10 min. The plasma was discarded and the cells were washed in 10 volumes of cold buffer (50mM TRIS-HCL, pH 7.5, containing 5 mM EDTA, and 1mM dithiothreitol), centrifuged at 8500g, at 4°C for 10 min and the supernatant discarded. RBC were lysed by 4 volumes of cold deionized water and again centrifuged at 8500g, at 4°C for 10 min. Supernatant was collected and stored at -70°C for analysis. RBC cellular glutathione Peroxidase (GSHPx) activity was measured using a GPx-340 colorimetric assay (Bioxytech; OXIS International, Portland, OR, U.S.A.), on a spectrophotometer at room temperature for 3-5 min at A340 nm wavelength to obtain values in units per millilitre. The hemoglobin (Hb) concentration of the sample was measured before the extraction procedure using Kit no. 525 for total Hb (Sigma), on a spectrophotometer at room temperature at A 540 nm wavelength, to allow the GSHPx activity to be expressed as units per milligram of Hb.

3.2. Superoxide dismutase

The RBC pellet was thawed and 4 volumes of cold deionized water added and vortexed. Ice cold extraction reagent (ethanol/chloroform, 62.5/37.7 (v/v)) added to an aliquot of the suspension and vortexed, then centrifuged at 3000 g, at 4°C for 5 min. The upper aqueous phase is used for analysis. The erythrocyte Zn/Cu SOD was analyzed using a SOD-525 Spectrophotometric assay kit (Oxis Health Products, Inc, Portland, OR, U.S.A.), on a spectrophotometer at room temperature for 3–5 min at A 525 nm wavelength, to obtain values in units per milliliter (Oxis). The hemoglobin (Hb) concentration of the sample was measured before the extraction procedure using Kit no. 525 for total Hb (Sigma), to allow the Zn/Cu SOD activity to be expressed as units per milligram of Hb.

3.3. Fatty acid analysis

Erythrocyte pellets were collected and stored in an EDTA coated tube at -70°C. The samples were thawed and the cells lysed and membranes soluablilized using the method of Tomoon et al (1984). The erythrocyte membranes were then methylated using the method of [15]. A small portion of the suspended membrane was added to a methanol: Toluene (4:1) mixture containing an internal standard and BHT. Acetyl chloride was added and incubated at 100°C for one hour. Potassium carbonate is added to stop the reaction and the mixture is centrifuged at 3000 rpm, at 5°C for 10 min. Fatty acid methyl esters in Toluene phase were analyzed using 30m x 0.25mm (DB-225) fused carbon silica column, coated with cyanopropylphenyl as previously [16]. The injector and detector port temperature is 700°C. The oven temperature begins at 170°C for two minutes then increases 10°C per minute to 220°C and this is maintained throughout the run time of 30 min. The sample fatty acid peaks were identified by comparison with authentic standard mixture.

Plasma samples were methylated using the same method as for the erythrocyte membranes by the method [15] and analyzed on the GC using the same conditions described above. A known amount of C21:0 was added to the methanol: toluene mixture and was used as the internal standard. This enabled quantitative analysis of plasma fatty acids expressed as microgram per mL.

The plasma unsaturated index (or double bond index) was calculated by multiplying the amount (μg/ml) of unsaturated fatty acid by the number of double bonds in the fatty acid, the values then summed to provide a number which represents the fatty acid unsaturated index [17].

3.4. Statistics

Normally distributed data is presented as mean $\pm$ SEM. Results were analyzed using Minitab version 12 for Windows (Minitab Inc., State college, PA, USA). Data tested for normality using the Anderson Darling normality test and for homogeneity of variance. Statistical comparisons were performed using ANOVA followed by Tukey multiple comparison test, with significance level of 0.05. Differences were considered significant when $p < 0.05$.

4. Results

At the completion of the study the average body weight increase for rats in both diet groups did not differ significantly (87 $\pm$ 1.7g; $P = 0.2$) indicating that the addition of α-tocopherol to the diet had no adverse influence on growth rate. The animals in the two different diet groups consumed similar amounts of food (19 $\pm$ 0.4g) throughout the study period.

The quantitative analysis of the plasma fatty acids is detailed in Table 1. The control and Vitamin E supplemented diet groups that were not administered CCl_4 were comprised predominantly of the fatty acid C18:1n-9 (729.8 $\pm$ 101.2 μg/ml; 634.0 $\pm$ 69.4 μg/ml respectively). The control and the Vitamin E group rats that were administered the CCl_4 showed reduced concentrations of the fatty acid C18:1n-9 compared to the groups that were not administered with CCl_4 (159.2 $\pm$ 20.3 μg/ml; 164.5 $\pm$ 17.9 μg/ml respectively, $P < 0.001$). This was also true for the fatty acid

Table 1
The fatty acid composition of plasma (μg/mL) in rats from all the diet groups (n=9)

Diet Fatty acid	Control	Control + CCl_4	Vitamin E	Vitamin E +CCl_4	P value
C16:0	191.0 ± 14.4[a]	79.0 ± 15.5[b]	171.6 ± 13.7[b]	102.2 ± 6.9[b]	<0.001
C16:1n-7	10.1 ± 1.6	13.3 ± 10.0	9.6 ± 1.0	4.1 ± 0.8	
C18:0	269.6 ± 16.l[a]	163.7 ± 19.9[b]	234.7 ± 12.7[a]	192.8 ± 12.5[b]	<0.001
C18:1n-9	729.83 ± 101.2[b]	159.2 ± 20.3[b]	633.95 ± 69.4[a]	164.5 ± 17.9[b]	<0.001
C18:1n-7	30.4 ± 2.2	20.8 ± 12.2	24.5 ± 1.8	10.4 ± 1.9	
C18:2n-6	139.6 ± 12.3[a]	36.4 ± 4.0[b]	130.6 ± 11.5[a]	44.7 ± 4.8[b]	<0.001
C20:1n-9	3.63 ± 1.1	—	—	—	
C20:2n-6	7.5 ± 0.7	—	4.4 ± 1.0	—	
C20:4n6	305.4 ± 27.4	248.5 ± 21.3	245.6 ± 10.7	256.5 ± 22.7	
C22:6n-3	10.5 ± 2.2	9.4 ± 0.5	6.7 ± 1.6	8.8 ± 0.7	
C24:0	17.3 ± 2.8	11.5 ± 3.1	11.7 ± 2.6		
Total FA	1751.0 ± 163.6[a]	772.0 ± 63.1[b]	1496.4 ± 113.1[a]	814.5 ± 52.8[b]	<0.001
Σ SFA	493.3 ± 31.5[a]	177.4 ± 28.1[b]	430.2 ± 29.0[a]	309.8 ± 19.0[c]	<0.001
Σ MUFA	776.4 ± 104.2[a]	75.4 ± 15.9[b]	669.9 ± 72.2[a]	183.2 ± 19.3[b]	<0.001
Σ n-6 PUFA	453.8 ± 36.0[a]	300.2 ± 273[b]	380.6 ± 17.6[a,b]	301.2 ± 23.7[b]	≤0.001
Σ n-3 PUFA	27.63 ± 6.74[a]	10.7 ± 2.3[b]	15.67 ± 1.5[a]	20.3 ± 2.5[a]	<0.01
Unsaturated index	2437.5 ± 240.3[a]	1358.2 ± 132.2[b]	1996.1 ± 112.1[a,b]	1393.7 ± 97.4[b]	<0.001

[a] Values are reported as average ± SEM.
[b] Values with common superscripts are significantly different.

C18:2n-6, and the saturated fatty acids C16:0 and C18:0. The control and Vitamin E diet groups without the CCl_4 had higher concentrations of C18:2n-6 (139.6 ± 12.3 μg/ml; 130.6 ± 11.5 μg/ml) than did the control and Vitamin E supplemented group that was administered CCl_4 (36.4 ± 4.0 μg/ml; 44.7 ± 4.8 μg/ml, P <0.001). This pattern of reduced fatty acid concentration due to CCl_4 administration is repeated with the C16:0 and C18:0. There was no significant difference in the unsaturated index of the plasma fatty acids when compared by diet groups.

The erythrocyte pellet membrane fatty acid results are outlined in Table 2. Similarly to the plasma fatty acid data, the C18:1n-9 fatty acid is the predominant fatty acid in the diet groups without CCl_4 administration (15.8%; 15.4%). The administration of CCl_4 reduced the concentration of this fatty acid in the RBC pellet (13.3%; 12.6% respectively). There was a significantly higher concentration of C18:1n-9 in the groups that were not administered CCl_4 than in the groups that were administered CCl_4 (13.3%; 12.6% respectively). Again this result is also repeated for the C18:2n-6 fatty acid. There were significant differences in the C14:0 (elevated in control group p <0.05) and C16:0 (elevated in the vitamin E and vitamin E +CCl4 p <0.05) and C24:1n-9 between the control and the Vitamin E supplemented diet groups. There was no significant difference in the unsaturated index of the RBC pellet when comparing diet groups.

Plasma 8-iso-$PGF_{2\alpha}$ concentrations (Figure 1) were elevated in both diet groups after CCl_4 administration. This elevation was significant in the control diet group that was administered CCl_4 (1450 ± 225 μg/ml, P <0.001) compared to the other diet groups (control without CCl_4: 359 ± 65; Vitamin E supplemented with: 410 ± 72 μg/ml; and without: 776 ± 111 μg/ml the administration of CCl_4).

Plasma Vitamin E concentration (Figure 2) was highest in the Vitamin E supplemented diet group that was not administered CCl_4 (64.5 ± 5.17μmol/L). The concentration of vitamin E was reduced after administration of CCl_4 in both diet groups, however the reduction was only significant in the vitamin E diet group (control + CCl_4: 31.56 ± 1.5 μmol/L; Vitamin E + CCl_4: 44.89 ± 2.32 μmol/L; P <0.001). There was little difference in the change in plasma Vitamin E concentration between the Vitamin E diet group compared to the control diet group after CCl_4 administration.

Plasma Vitamin A concentrations (Figure 3) were reduced by the administration of CCl_4 in both the control and the Vitamin E supplemented diet groups.

There were no significant differences in the levels of activity of GSHPx and SOD, this is also true for the concentration of plasma Selenium. However, there was a significant reduction in Zinc concentrations in the Vitamin E supplemented diet group that was not administered CCl_4 (15.0 ± 0.5, P <0.05) compared with the control diet group that was administered CCl_4 (Table 3).

5. Discussion

This study involved the supplementation of the diet with Vitamin E to investigate the role of Vitamin E in an *in vivo* model of induced oxidative stress. Non-enzymatic, free radical catalyzed peroxidation of arachidonic acid to form 8-iso-$PGF_{2\alpha}$ was used as an *in vivo* marker of oxidative stress [13,18], and CCl_4 was used to initiate lipid peroxidation [13].

Vitamin E is a lipid soluble vitamin capable of scavenging free radicals. *In vitro* studies involving induction of

Table 2
Fatty acid composition of the rat RBC membranes (%) in all diet groups (n=9)

Diet Fatty acid	Control	Control + CCl_4	Vitamin E	Vitamin E +CCl_4	P value
C14:0	0.2 ± 0.0[a]	0.1 ± 0.0[b]	0.1 ± 0.0[b]	0.1 ± 0.0[b]	<0.05
C16:0	17.6 ± 0.5[a]	16.2 ± 2.2[a]	15.6 ± 0.2[b]	15.1 ± 1.0[b]	<0.05
C16:1n-7	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	1.7 ± 1.6	
C18:0	22.9 ± 0.4	25.0 ± 3.4	23.1 ± 0.7	22.2 ± 0.8	
C18:1n-9	15.8 ± 0.7[a]	13.3 ± 0.3[b]	15.4 ± 0.3[b]	12.6 ± 0.8[b]	<0.01
C18:ln-7	1.4 ± 0.0	1.5 ± 0.1	1.4 ± 0.0	1.6 ± 0.1	
C18:2n-6	4.8 ± 0.1[a]	4.0 ± 0.8[b]	4.7 ± 0.1[a]	4.0 ± 0.3[b]	<0.05
C20:0	0.1 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.1	
C20:1n-9	0.3 ± 0.0	0.2 ± 0.1	0.3 ± 0.1	0.2 ± 0.0	
C20:2n-6	0.4 ± 0.0	0.3 ± 0.1	0.3 ± 0.1	0.3 ± 0.0	
C20:3n-6	0.3 ± 0.0	0.2 ± 0.1	0.2 ± 0.0	1.9 ± 1.7	
C20:4n-6	26.7 ± 0.5	28.7 ± 10.8	27.8 ± 0.4	28.2 ± 2.0	
C20:5n-3	0.1 ± 0.1	0.1 ± 0.0	—	—	—
C22:0	0.8 ± 0.0	0.8 ± 0.1	0.8 ± 0.1	0.7 ± 0.1	
C22:1n-9	—	—	—	0.1± 0.0	
C22:2n-6	—	—	0.1± 0.0		
C22:5n-3	1.2 ± 0.1	1.4 ± 0.3	1.5 ± 0.2	1.2 ± 0.1	
C22:6n-3	2.6 ± 0.1	2.6 ± 1.6	2.6 ± 0.1	2.8 ± 0.1	
C24:0	1.8 ± 0.1	2.1 ± 0.3	2.1 ± 0.1	2.2 ± 0.1	
C24:1n-9	2.7 ± 0.1[a]	3.0 ± 0.2[a]	3.4 ±0.1[b]	3.4 ± 0.1[b]	<0.005
Unsaturated index	160.4 ± 2.9[a]	165.4 ± 2.9[a,b]	166.4 ± 1.9[a,b]	171.5 ± 1.9[b]	<0.05
Σ SFA	43.4 ± 0.5[a]	44.4 ± 0.5[a]	41.9 ± 0.6[a,b]	40.5 ± 1.6[b]	<0.05
Σ MUFA	20.5 ± 0.7	18.3 ± 0.2	20.8 ± 0.4	19.6 ± 2.0	0.38
Σ n-6 PUFA	32.2 ± 0.5[a]	33.2 ± 0.4[a,b]	33.1 ± 0.5[a,b]	34.5 ± 0.6[b]	<0.05
Σ n-3 PUFA	3.8 ± 0.2	4.1 ± 0.3	4.3 ± 0.3	4.2 ± 0.1	0.54

[a] Values are reported as average ± SEM.
[b] Values without common superscript are significant different.

oxidation have demonstrated a protective role of vitamin E against oxidation. These studies involved the feeding of vitamin E supplemented diets followed by isolation of plasma LDL then induction of oxidation *in vitro* by chemicals such as copper chloride and the measurement of the subsequent lag time for oxidation of LDL. However, direct evidence for the antioxidant role of Vitamin E *in vivo* is rare.

There are a few studies that have examined the effect of vitamin E supplementation *in vivo* [3,12,19]. For example in the study by Pratico et al [20] supplementation with vitamin E reduced isoprostane generation in urine, plasma and vas-

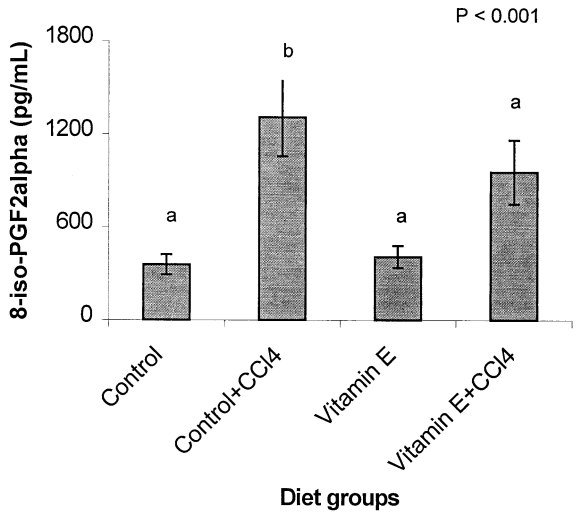

Fig. 1. The values without common superscripts are significantly different n = 9. 8-epi-$PGF_{2\alpha}$ (pg/ml) concentrations in the plasma of rats from all the diet groups following four hours after CCl_4 administration

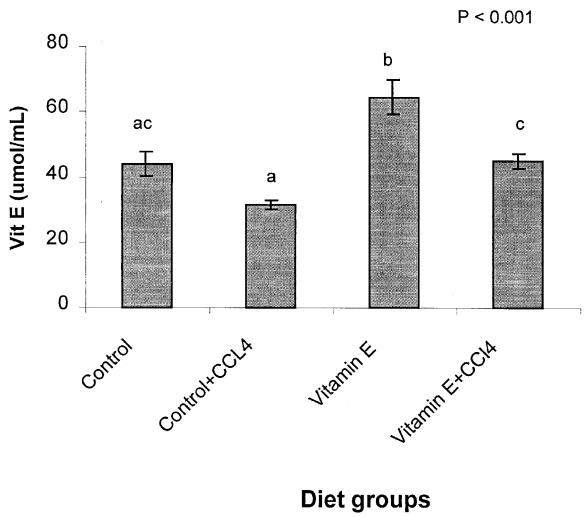

Fig. 2. The values without common superscripts are significantly different n = 9. Vitamin E (μmol/L) concentration in the plasma of rats from all the diet groups following four hours after CCl_4 administration.

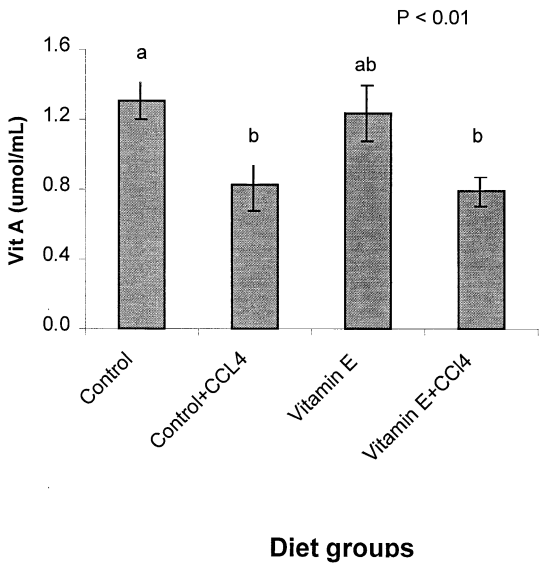

Fig. 3. The values without common superscripts are significantly different n = 9. Vitamin A (μmol/L) concentration in the plasma of rats from all the diet groups following four hours after CCl_4 administration.

cular tissue. Sodregren et al [21] showed that supplementation with vitamin E reduced the concentration of urinary 8-iso-$PGF_{2\alpha}$ and free liver 8-iso-$PGF_{2\alpha}$, however not plasma 8-iso-$PGF_{2\alpha}$or total liver concentration of 8-iso-$PGF_{2\alpha}$.

These results indicate that there is a role for Vitamin E in the reduction of oxidative stress, however that role may not be as clear in basal levels of oxidative stress as when the oxidative stress is induced to mimic the levels experienced in acute or chronic illness. This study provides evidence that the supplementation of Vitamin E causes a substantial reduction in the concentration of plasma total 8-iso-$PGF_{2\alpha}$ after oxidative stress is induced in the rat model by administration of CCl_4.

As expected Vitamin E concentration in the plasma was elevated following supplementation with Vitamin E for four weeks. Administration of CCl_4 reduced Vitamin E concentrations in both the supplemented and the unsupplemented groups indicating increased demand for the Vitamin in situations where oxidative stress is elevated. In fact the Vitamin E concentration in the supplemented group that was administered CCl_4 returned to an amount similar to the unsupplemented control group value. This is direct evidence of the increased utilization of Vitamin E when oxidative stress is induced by CCl_4, and therefore outlines the protective role of Vitamin E in mitigating elevated oxidative stress.

Plasma total 8-iso-$PGF_{2\alpha}$ concentrations were elevated in rats administered with CCl_4 in both the control and supplemented groups. Increased plasma total 8-iso-$PGF_{2\alpha}$ concentration following CCl_4 administration in the Vitamin E supplemented diet group was not to the same extent as in the unsupplemented group. This suggests protection from oxidative stress by Vitamin E in the Vitamin E supplemented group. Concentration of plasma total 8-iso-$PGF_{2\alpha}$ was not altered by Vitamin E supplementation in rats not administered CCl_4 indicating that although Vitamin E does not provide extra protection to the basal levels of oxidative stress, it may be of greater significance when the animals are challenged with a pro-oxidant. Since there was no significant effect of Vitamin E or CCl_4 on arachidonic acid (C20:4n-6) concentration in the plasma or erythrocyte pellet the difference noted in the plasma total 8-epi-$PGF_{2\alpha}$ concentration can not be attributed to changes in available substrate (C20:4n-6).

Antioxidant trace elements (zinc and selenium) concentrations in plasma were not altered following CCl_4 administration, or by Vitamin E supplementation. Also, Vitamin E supplementation and CCl_4 administration affected neither of the antioxidant enzyme's level of activity (SOD and GSHPx). These results suggest that during induced oxidative stress Vitamin E is preferentially used for protection. This re-emphasis's the potent antioxidant role played by Vitamin E during times of elevated oxidative stress and thus in the prevention of related diseases. In the absence of sufficient concentration of Vitamin E in the diet the antioxidant enzyme activities as well as trace-mineral concentrations would be expected to be adversely effected. This is especially true for selenium [22]. Influence of vitamin E

Table 3
The concentration of plasma antioxidant micronutrients (μmol) and antioxidant enzymes ([29]/mg Hb) in all diet groups (n=9)

Antioxidant micronutrient (μmol/L)	Control	Control+ CCI_4	Vitamin E	Vitamin E + CCI_4	P-value
Zinc	16.44 ± 0.5[a,b]	17.8 ± 0.7[b]	15.0 ± 0.5[a]	16.5 ± 0.5[a,b]	<0.05
Selenium	4.9 ± 0.2	4.8 ± 0.1	4.9 ± 0.1	4.9 ± 0.2	
Superoxide dismutase ([30]/mg Hb)	2.8 ± 0.1	3.0 ± 0.2	3.2 ± 0.2	3.1 ± 0.2	
Glutathione Peroxidase ([12]/mg Hb)	0.5 ± 0.02	0.6 ± 0.04	0.6 ± 0.04	0.6 ± 0.1	

[a] Values are reported as average ± SEM.
[b] Values without common superscript are significant different.

deficiency on antioxidant defense mechanisms therefore merits further investigation.

Four hours after CCl_4 administration saturated fatty acids (C16:0 and C18:0), monounsaturated fatty acids (mainly C18:1n-9) and linoleic acid (C18:2n-6) concentrations were lowered compared to the control group. These results reflect those of Fontana et al [23]. In the experiments by Fontana et al [23] plasma, erythrocyte membrane and liver microsomes were analyzed and decreases in linoleic and arachidonic acid were observed. The administration of CCl_4 affected fatty acid metabolism, which altered tissue fatty acid profiles. In a study by Yasuda et al, [24] increases in dietary PUFA content failed to increase the oxidation products in the liver however, it did result in lowered triacylglycerol level compared to saturated and monounsaturated diet groups. The unsaturated index of the plasma and RBC showed no significant difference due to dietary intake. This is probably due to membrane homeostatic mechanisms which act to keep the unsaturated index of membranes fairly consistent in order to maintain functionality [25].

The vitamin A results in this study show an interesting relationship between this vitamin and oxidative stress. The results indicate that the administration of a pro-oxidant reduces the levels of plasma vitamin A. Vitamin A is not often recognized as one of the main antioxidant vitamins, although there is some evidence in recent literature to suggest otherwise [26,27]. However, CCl_4 is metabolized by the cytochrome P450 enzyme system, specifically the CYP2E1 isoform of the enzyme. The induction of the microsomal enzymes in the liver to metabolize the administered CCl_4 results in the depletion of Vitamin A [28]. In this experiment the reduction in plasma Vitamin A levels occurs regardless of the Vitamin E concentration in the diet, indicating that although Vitamin E protects against lipid peroxidation and therefore the loss of the cytochrome P450 enzymes [28] the result is still the loss of Vitamin A. Further investigation into the role of Vitamin A in oxidative stress and in the induction of microsomal enzymes is required.

In summary the results presented in this paper provide for the first time direct evidence for the antioxidant role played by Vitamin E in situations where oxidative stress is elevated. Similar experiments need to be carried out to determine the antioxidant capacity of other nutrients such as Vitamin C, the carotenoids and the flavanoids [29,30].

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