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Effects of β-carotene, vitamin C and E on antioxidant status in hyperlipidemic smokers

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Abstract

Smoking can accelerate the consumption of the stored antioxidant vitamins and increase the oxidative stress in the hyperlipidemic patients. The study investigated the effects of combined β-carotene, vitamin C, and vitamin E on plasma antioxidant levels, erythrocyte antioxidative enzyme activities, and LDL lipid peroxides. Male hyperlipidemic smokers (35–78 years old) were randomly divided into two antioxidant supplemented groups: intervention 1 (I1, n = 22) (15 mg β-carotene/day, 500 mg vitamin C/day, and 400 mg α-tocopherol equivalent/day) and intervention 2 (I2, n = 20) (30 mg β-carotene/day, 1000 mg vitamin C/day, and 800 mg α-tocopherol equivalent/day). After 6-week supplementation, plasma β-carotene, vitamin C, vitamin E, and erythrocyte glutathione levels increased significantly by 200%, 98%, 129%, and 39%, respectively, in the I1 group, and by 209%, 216%, 197%, and 32%, respectively, in the I2 group. Plasma Fe^{+2} concentrations and Fe^{+2}/Fe^{+3} decreased significantly in both groups. Except erythrocyte glutathione peroxidase activity in the I1 group, erythrocyte catalase, glutathione peroxidase, and superoxide dismutase activities increased significantly in both groups. Lipid peroxides in LDL decreased significantly by 56% and 72% in the I1 and I2 groups, respectively. However, the levels of plasma iron, erythrocyte glutathione, and LDL lipid peroxides, and the activities of erythrocyte antioxidative enzymes did not differ between two groups. In conclusion, combined antioxidant supplements increased plasma antioxidant levels and antioxidative enzyme activities, and lowered LDL lipid peroxides in male hyperlipidemic smokers. Higher dosage of the supplements did not have an additive effect. © 2002 Elsevier Science Inc. All rights reserved.

Keywords: β-Carotene; Vitamin C; Vitamin E; Antioxidative enzyme; Hyperlipidemic smokers

1. Introduction

It has been demonstrated that smoking may cause an increase in oxidative stress and an imbalance in antioxidant nutrient intake and status [1–5]. Significantly lower plasma β-carotene, vitamin C, and vitamin E levels and increased oxidative damage were observed in smokers than in non-smokers [3–10]. Recently, many studies have shown that antioxidant nutrient supplementation, especially β-carotene, vitamin C, and vitamin E, are effective in protecting the oxidation of DNA, LDL, and protein against oxidative damage by smoking in vitro [11,12] and in vivo [13–20].

Beta-carotene is highly lipid soluble, and carried largely in LDL-cholesterol (75%) and HDL-cholesterol fractions (25%) [21]. It functions as an efficient singlet oxygen quencher and as a radical-trapping antioxidant at low oxygen pressure to reduce the extent of nuclear damage and to inhibit lipid peroxidation induced by enzymatic sources of oxy radicals, such as xanthine oxidase system [22]. Ascorbic acid is a water-soluble peroxidation chain-breaking antioxidant. It reacts directly not only with superoxide, hydroxyl radicals, and singlet oxygen but also with tocopheroxy radicals, resulting in the regeneration of tocopherol [23]. Tocopherol is the principal lipid-soluble chain-breaking antioxidant in plasma and on the membrane of the tissues, and acts as the predominant antioxidant in the LDL particle by trapping peroxyl free radicals. Supplementation with β-carotene [11,13], vitamin C [12] or vitamin E [14–17] alone, or combined these antioxidants [17–20] in

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vitro or in vivo has shown to protect oxidative damage from smoking.

Combined vitamin C and vitamin E had better defensive ability against oxidative damage compared with single vitamins [23,24]. An in vitro study also showed that mixtures of β-carotene, vitamin C, and vitamin E exhibited greater protective effects on oxidative damage than individual vitamin in human lung cells under hypoxic condition [25]. Thus, this study further determined the effect of combined various dosages of three major antioxidant vitamins, β-carotene, vitamin C, and vitamin E, on plasma antioxidant levels, erythrocyte antioxidative enzyme activities, and lipid peroxidation in male hyperlipidemic smokers who are at high risk of developing coronary heart disease.

2. Materials and methods

2.1. Subjects

Forty-two male hyperlipidemic (plasma total triglycerides (TG) $>$2.29 mmol/L, total cholesterol (TC) $>$6.20 mmol/L or HDL-cholesterol (HDL-C) $<$0.90 mmol/L) smokers ($\geq$5 cigarettes/day for at least 10 years) of 35 to 78 years old were recruited from the patients of Taipei Medical University Hospital and Taipei Municipal Wan Fang Hospital. Subjects were selected by interviewing their motivation, commitment to completing the experiment, dietary habits, and medical history, and by laboratory examination of their plasma lipids and general health conditions by the investigators and physicians. Exclusion criteria include secondary hyperlipidemia, diabetes, kidney disease, acute or chronic inflammatory diseases, abnormal body mass index (BMI, $<$20 or $>$30), drastic body weight changes ($>$2.5 kg in last 1 month), vegetarian diet, dietary intake with extreme polyunsaturated fatty acid/saturated fatty acid ratio of greater than 3:1, drug abuse, the habit of alcohol, coffee or tea drinking ($>$3 cups/day) in last 3 months, or dietary supplementation of high-dosage vitamins, minerals or antioxidants ($>$200% Recommended Dietary Allowances) in last 3 months. If the medication was with an antioxidant effect, the physicians were suggested to change the medication two weeks before the beginning of the experiment. Qualified subjects were asked for consenting to attend this study and signing the agreement. During the experimental period, subjects maintained their medication, physical activity, and regular life style. The study was performed in accordance with the regulations of the ethics committee of Taipei Medical University Hospital.

2.2. Antioxidant supplements

This study was randomized and single-blinded. Qualified individuals were randomly divided into two supplemented groups: intervention 1 (I1, n = 22) (15 mg β-carotene/day, 500 mg vitamin C/day, and 400 mg α-tocopherol equivalent (α-TE)/day) or intervention 2 (I2, n = 20) (30 mg β-carotene/day, 1000 mg vitamin C/day, and 800 mg α-TE/day). The subjects were informed of their assigned group. The investigators did not know which supplements the subjects were given. The subjects were given antioxidant supplements in the tablet forms in the unlabeled brown bottles for 21 days by the dietitians at week 0. At week 3, another 21-day antioxidant supplements were given to the subjects, when they came back for physical examination, dietary assessment, and biochemical analyses. One antioxidant tablet contained 7.5 mg β-carotene, 250 mg L-ascorbate, and 298 mg D,L-α-tocopheryl acetate, which was manufactured by the investigators in Purzer Pharmaceutical Co. Ltd (Taoyuan, Taiwan, R.O.C.). After weight-crush test, the hardness of the tablet was 5.5 kg. The tablet was dissolved in water within 18.4 min. The I1 group was asked to consume two tablets within 30 min after the morning meal, and the I2 group was asked to consume two tablets within 30 min both after the morning and the evening meals for 6 weeks. Subjects were allowed to maintain their usual body weight and interviewed for their dietary intake every three weeks to monitor the dietary intake and to assess compliance by the investigators.

2.3. Dietary assessment

Three 24-h dietary recalls, including 2 weekdays and 1 weekend, were randomly done by the trained investigators at week 0, 3, and 6, respectively. The 24-h recall with at least two eating-out meals was excluded. Dietary intake was analyzed by food composition analysis software (November 1997 edition, provided by Dr. Wen-Harn Pan from the Institute of Biomedical Sciences, Academia Sinica, Taipei, Taiwan, R.O.C.).

2.4. Biochemical analyses

2.4.1. Blood sample preparation

Overnight fasting blood samples (10 mL) were drawn by venipuncture into the tubes containing heparin or EDTA (only for lipid peroxidation analysis) from each subject at week 0 (baseline), week 3 (mid-point), and week 6 (end-point). Blood samples were centrifuged at 2,500 $\times$ g for 15 min to separate plasma from whole blood. The erythrocyte pellets were collected for further reduced glutathione and enzyme analyses.

2.4.2. Antioxidant levels

Plasma β-carotene [26], L-ascorbate [27], and α-tocopherol [28] concentrations were measured by reverse-phase high performance liquid chromatography (HPLC) (Jasco PU-980 Intelligent HPLC Pump, Jasco AS-950 Intelligent Sampler, Jasco UV-975 Intelligent UV/VIS Detector, Jasco PF-920 Intelligent Fluorescence Detector, Jasco Cor., Tokyo, Japan) using a 4 $\times$ 125 mm RP-18E column (5 μm, Merck 50734 Lichrocart 125–4 HPLC-cartridge, Merck

Taiwan Ltd., Taipei, Taiwan, R.O.C.). Plasma β-carotene concentration was measured at 470 nm under the mobile phase of methanol and toluene (3:1). Plasma L-ascorbate concentration was measured using an UV detector at 254 nm under the mobile phase of 40 mmol/L sodium acetate, 0.54 mmol/L sodium EDTA, 7.5% (vol/vol) methanol, and 1.5 mmol/L tetrabutylammonium hydroxide; pH 4.75. Plasma α-tocopherol concentration was measured using a fluorescence detector with excitation at 288 nm and emission at 340 nm under the mobile phase of methanol and toluene (3:1). The data were analyzed by SISC-LAB [32] chromatographic analysis software (Scientific Information Service Cor., Taipei, Taiwan, R.O.C.).

The concentration of reduced glutathione (GSH) in erythrocytes was measured spectrophotometrically at 400 nm using a commercial kit (Calbiochem 354102, Calbiochem-Novabiochem Cor., La Jolla, CA) [29].

The concentrations of free and total iron were measured spectrophotometrically by the method of Xu et al. [30]. Protein in plasma was precipitated with 10% (vol/vol) trichloroacetic acid (TCA), and removed by centrifugation. The supernatant was extracted twice with 0.5 ml diethyl ester to remove excess TCA. The supernatant was mixed with 0.25 mmol/L of 1,10-phenanthroline, which forms a reddish orange complex with ferrous ion (Fe^{+2}). The concentration of free ferrous ion was determined spectrophotometrically at 510 nm. The total iron concentration was measured spectrophotometrically described above after adding 284 mmol/L ascorbic acid to completely reduce ferric (Fe^{+3}) to ferrous ion. The concentration of ferric ion was obtained by subtracting ferrous ion concentration from total iron concentration.

2.4.3. Erythrocyte antioxidative enzyme activities

Catalase activity was determined spectrophotometrically at 240 nm by the methods of Lück [31]. The activity of catalase is expressed as unit k/mg protein, and k is the rate constant of a first-order reaction. Glutathione peroxidase (GPx) activity was measured spectrophotometrically at 340 nm by the methods of Paglia and Valentine [32] using a commercial kit (RS 504, RANDOX Laboratories Ltd., Antrim, UK). The activity of GPx is expressed as U/mg protein. One unit of GPx activity is defined as 1 μmol NADPH oxidized/min. Superoxide dismutase (SOD) activity was determined spectrophotometrically at 525 nm by the methods of Nobet et al. [33] using a commercial kit (Calbiochem 574600, Calbiochem-Novabiochem Cor.). The activity of SOD is expressed as unit/mg protein. One SOD unit is defined as the activity that doubles the autooxidation background in the absence of SOD (Calbiochem, Calbiochem-Novabiochem Cor.). Protein content in erythrocytes was measured by the modified method of Lowry et al. using a Bio-Rad DC protein assay kit (Bio-Rad Laboratories, Hercules, CA) [34].

Table 1
Characteristics and clinical data of the subject prior to antioxidant vitamin supplementation[1]

	Intervention 1 (n = 22)	Intervention 2 (n = 20)
Age (years)	56.6 ± 11.8	57.8 ± 12.8
Smoking (cigarettes/day)	16.1 ± 8.2	12.8 ± 6.6
Body mass index (kg/m^2)	26.3 ± 4.0	26.5 ± 3.5
Systolic pressure (mmHg)	142 ± 14	134 ± 12
Diastolic pressure (mmHg)	91 ± 10	84 ± 9
Triglycerides (mmol/L)	2.90 ± 2.11	2.82 ± 2.44
Total cholesterol (mmol/L)	5.89 ± 1.35	5.68 ± 1.22
LDL-cholesterol (mmol/L)	3.51 ± 1.20	3.27 ± 1.00
HDL-cholesterol (mmol/L)	1.02 ± 0.39	1.00 ± 0.29

[1] Values are mean ± SD. The data did not differ significantly ($P > 0.05$) between the groups by Student's *t* test.

2.4.4. Plasma and LDL lipid peroxides

The concentration of lipid peroxides in plasma and LDL was assessed colorimetrically at 586 nm by a commercial kit (Calbiochem 437634, Calbiochem-Novabiochem Cor.) [35]. LDL (density 1.063 kg/L) was isolated from plasma by density gradient ultracentrifugation at 121,000 × g (Beckman Opitma™ TLX Ultracentrifuge, Beckman Instruments (Taiwan) Inc., Taipei, Taiwan, R.O.C.), 4°C overnight [36]. LDL fraction was subsequently dialyzed and desalted in phosphate buffered saline. Protein concentration of LDL was measured by the modified method of Lowry et al. [34]. Plasma or LDL fraction (200 μL) was mixed with 650 μL of Reagent 1 (7.7 mmol/L N-methyl-2-phenylindole in 75% acetonitrile and 25% methanol) and 150 μL of Reagent 2 (15.4 mol/L methanesulfonic acid) at 45°C for 40 min. The levels of malondialdehyde (MDA) and 4-hydroxy-2(E)-nonenal (4-HNE), the end products derived from peroxidation of polyunsaturated fatty acids and related esters, were measured at 586 nm.

Table 2
Daily dietary intake of the subjects during the experimental period[1]

	Intervention 1 (n = 22)	Intervention 2 (n = 20)
Energy (MJ)	8.16 ± 1.82	9.56 ± 3.02
(kcal)	1954 ± 435	2286 ± 722
Carbohydrate (g)	269.3 ± 65.1	268.9 ± 80.7
(% energy)	55.1	47.1
Fat (g)	65.5 ± 19.1	76.9 ± 24.6
(% energy)	30.1	30.3
Protein (g)	72.4 ± 16.2	84.2 ± 31.5
(% energy)	14.8	14.7
Vitamin A (μg RE)	1247 ± 995	2179 ± 2788
Vitamin C (mg)	124.2 ± 110.7	104.5 ± 103.4
Vitamin E (mg α-TE)	13.55 ± 5.36	16.76 ± 7.92
Iron (mg)	11.68 ± 3.33	16.60 ± 11.48

[1] Values are mean ± SD. Dietary intake did not differ significantly ($P > 0.05$) between the groups by Student's *t* test.

Table 3
Plasma β-carotene, vitamin C, vitamin E, and erythrocyte reduced glutathione concentrations of the subjects before and after antioxidant vitamin supplements[1]

μmol/L	Intervention 1			Intervention 2		
	Week 0 (n = 22)	Week 3 (n = 22)	Week 6 (n = 20)	Week 0 (n = 20)	Week 3 (n = 20)	Week 6 (n = 18)
β-Carotene	0.20 ± 0.11[a]	0.39 ± 0.19[b]	0.60 ± 0.28[c]	0.32 ± 0.15[a,b]	0.63 ± 0.22[c]	0.99 ± 0.43[d]
Vitamin C	31.6 ± 8.3[a]	44.2 ± 12.3[b]	62.5 ± 12.3[c]	31.4 ± 9.5[a]	60.4 ± 17.2[c]	99.3 ± 23.7[d]
Vitamin E	18.8 ± 5.4[a]	32.5 ± 9.1[b]	43.1 ± 14.0[c]	23.9 ± 7.6[a]	47.5 ± 13.4[c]	71.1 ± 19.1[d]
Glutathione	220.3 ± 116.9[a]	289.7 ± 84.1[b,c]	306.8 ± 109.4[c]	237.0 ± 80.3[a,b]	299.4 ± 69.8[c]	312.0 ± 87.0[c]

[1] Values are mean ± SD. Values in each row not sharing the same superscript differ significantly ($P < 0.05$) by Fisher's least significant difference test.

2.5. Statistical analysis

All data are expressed as mean ± SD. Data were analyzed by Student's *t* test or two-way analysis of variance to determine the supplement dosage and time effects using SAS (version 6.12, SAS Institute Inc., Cary, NC). Fisher's least significant difference test was used to make post-hoc comparisons if the main effect was demonstrated. Statistical significance was assigned at the 0.05 level.

3. Results

Before entering the trial, the subjects in the I1 and I2 groups had similar age, smoking habit, BMI, blood pressure, and plasma lipid profile (Table 1). During the trial, four individuals dropped after week 3. In the I1 group, one individual had a poor appetite, and another one had dramatically increased plasma triglycerides. Two individuals in the I2 group could not finish the trial due to traveling. Neither complaint nor clinical adverse symptoms were observed in others. From interviewing their antioxidant supplementation and dietary intake, no unusual situation was found.

The data of daily dietary intake were shown in Table 2. Because of no significant difference among dietary assessments from different days, the daily average intake of the I1 and I2 groups was used. The intake for energy, macronutrients, vitamin A, vitamin C, vitamin E, and iron did not differ significantly between the groups during the experimental period. The intake for carbohydrate, fat, and protein was 55.1%, 30.1%, and 14.8% energy in the I1 group, and 47.1%, 30.3%, and 14.7% energy in the I2 group.

The concentrations of plasma β-carotene, vitamin C, and vitamin E increased dose- and time-dependently during the 6-week period (Table 3). Before antioxidant vitamin supplementation, the levels of plasma β-carotene, vitamin C, vitamin E, and erythrocyte GSH did not differ significantly between the groups. At week 3 and 6, plasma β-carotene concentrations increased significantly by 95% and 200% ($P < 0.05$) in the I1 group, and by 97% and 209% ($P < 0.05$) in the I2 group. Plasma vitamin C levels elevated significantly by 40% and 98% ($P < 0.05$) in the I1 group, and by 92% and 216% ($P < 0.05$) in the I2 group. Plasma vitamin E concentrations increased significantly by 73% and 129% ($P < 0.05$) in the I1 group, and by 99% and 197% ($P < 0.05$) in the I2 group. Erythrocyte GSH levels increased significantly by 32% and 39% ($P < 0.05$) in the I1 group, and by 26% and 32% ($P < 0.05$) in the I2 group. However, erythrocyte GSH levels did not differ between the groups at week 3 and 6, or between week 3 and 6 within the same group.

Table 4 shows plasma iron concentrations before and after vitamin supplementation. The level of ferrous ion decreased time-dependently ($P < 0.05$) in both groups. However, ferrous ion level did not differ significantly between the groups during the 6-week period. Neither supplement dosage nor time affected the concentrations of ferric ion and total iron. The ratio of ferrous to ferric ion decreased significantly by 33% and 39% ($P < 0.05$) in the I1 and I2 groups, respectively, at week 6 compared with that at week

Table 4
Plasma free and total iron concentrations of the subjects before and after antioxidant vitamin supplements[1]

	Intervention 1			Intervention 2		
	Week 0 (n = 22)	Week 3 (n = 22)	Week 6 (n = 20)	Week 0 (n = 20)	Week 3 (n = 20)	Week 6 (n = 18)
Fe^{2+} (μmol/L)	11.00 ± 2.26[a,b]	9.55 ± 2.12[c]	8.18 ± 1.33[d]	11.79 ± 2.66[a]	9.62 ± 2.56[b,c]	8.44 ± 1.94[c,d]
Fe^{3+} (μol/L)	25.20 ± 8.84	26.80 ± 5.78	25.96 ± 7.26	23.99 ± 7.37	27.32 ± 11.67	28.95 ± 8.26
Fe^{2+}/Fe^{3+}	0.51 ± 0.32[a,b]	0.38 ± 0.14[b,c]	0.34 ± 0.12[c]	0.54 ± 0.22[a]	0.44 ± 0.25[a,b,c]	0.33 ± 0.16[c]
Total iron (μmol/L)	36.20 ± 9.46	36.34 ± 5.69	34.03 ± 7.12	35.78 ± 8.36	36.95 ± 11.21	37.39 ± 8.15

[1] Values are mean ± SD. Values in each row not sharing the same superscript differ significantly ($P < 0.05$) by Fisher's least significant difference test.

Table 5
The activities of erythrocyte antioxidative enzymes of the subjects before and after antioxidant vitamin supplements[1]

	Intervention 1			Intervention 2		
	Week 0 (n = 22)	Week 3 (n = 22)	Week 6 (n = 20)	Week 0 (n = 20)	Week 3 (n = 20)	Week 6 (n = 18)
Catalase (unit K/mg protein)	323.1 ± 190.0[a]	386.7 ± 249.3[a,b]	493.1 ± 217.5[b,c]	349.8 ± 142.7[a]	367.2 ± 146.7[a,b]	507.8 ± 114.8[b]
Glutathione peroxidase (U/mg protein)	9.65 ± 5.63[a]	12.97 ± 7.25[a]	15.60 ± 8.88[a,b]	11.04 ± 6.56[a]	18.40 ± 15.93[b]	19.95 ± 10.02[b]
Superoxide dismutase (U/mg protein)	2.63 ± 1.99[a]	4.39 ± 3.29[a]	8.23 ± 8.93[b]	4.87 ± 2.41[a]	5.60 ± 3.76[a,b]	8.64 ± 5.15[b]

[1] Values are mean ± SD. Values in each row not sharing the same superscript differ significantly ($P < 0.05$) by Fisher's least significant difference test.

0. However, the ratio of ferrous to ferric ion was similar between the groups.

The activities of erythrocyte antioxidative enzyme did not differ between the groups before supplementation (Table 5). Catalase activity did not differ at week 3 compared with that at week 0 in both groups. However, catalase activity increased significantly by 53% and 45% ($P < 0.05$) in the I1 and I2 groups, respectively, at week 6. The activity of GPx was similar in the I1 group during the 6-week period. Whereas GPx activity increased significantly by 67% and 81% ($P < 0.05$) in the I2 group at week 3 and 6. The activity of SOD elevated significantly by 213% and 77% ($P < 0.05$) in the I1 and I2 groups, respectively, at week 6. Supplement dosage did not affect the activities of these enzymes.

Table 6 shows the levels of lipid peroxides (MDA + 4-HNE) in plasma and LDL before and after vitamin supplementation. Plasma lipid peroxides did not differ between the groups at week 0 and 3. However, the I2 group had significantly decreased plasma lipid peroxides compared with the I1 group at week 6. Plasma lipid peroxides lowered significantly by 16% ($P < 0.05$) in the I1 group at week 3, and by 17% ($P < 0.05$) in the I2 group at week 6. Lipid peroxides in LDL decreased significantly by 46% ($P < 0.05$) in the I2 group at week 3. At week 6, the I1 and I2 groups lowered significantly LDL lipid peroxides by 56% and 72% ($P < 0.05$), respectively.

4. Discussion

In this study, there was no placebo-controlled group, and the subjects served as their own control to eliminate the variation among the individuals. Because daily dietary intake did not differ significantly between the groups, the effects on antioxidant levels, antioxidative enzyme activities, and lipid peroxidation resulted from the antioxidant vitamin supplements per se.

The dosages of antioxidant vitamin supplements in this study were much higher than the mean of dietary intake in smokers or Recommended Dietary Allowances. Epidemiologic evidence showed that low antioxidant status was associated with increased death from cancer and from atherosclerosis [37,38]. Bronchus (RR = 1.8, $P < 0.05$) and stomach cancers (RR = 2.95, $P < 0.05$), and ischemic heart disease (RR = 1.53, $P = 0.02$) were associated with low plasma carotene levels [37]. The average intake of vitamin C was strongly inversely related to the 25-year stomach-cancer mortality ($r = -0.06$, $P = 0.01$), also after adjustment for smoking and intake of salt or nitrite [38]. A poor vitamin E status was correlated with an increased risk of subsequent cancer [37]. However, an epidemiologic study found that daily vitamin E supplementation (50 mg) could not prevent lung cancer, and daily β-carotene supplementation (20 mg) increased the risk of lung cancer in smokers [39]. Normal subjects ingesting large amount of β-carotene (20–50 mg/day) or vitamin E (100–800 IU/day), or over long period had no carcinogenicity and without side effects, indicating the toxicity of β-carotene and vitamin E is low [39]. Due to lack of a controlled study of vitamin C toxicity in humans, more evidence for the toxicity of vitamin C requires further study.

Previous studies had shown that combined antioxidant vitamin supplements increased significantly plasma antioxidant levels in smokers [8,13,17–20] and nonsmokers [13,

Table 6
Plasma and LDL lipid peroxides of the subjects before and after antioxidant vitamin supplements[1]

	Intervention 1			Intervention 2		
	Week 0 (n = 22)	Week 3 (n = 22)	Week 6 (n = 20)	Week 0 (n = 20)	Week 3 (n = 20)	Week 6 (n = 18)
Plasma malondialdehyde and 4-hydroxy-2(E)-nonenal (μmol/L)	12.88 ± 2.2[a]	10.77 ± 2.88[b,c]	11.60 ± 4.07[a,b]	11.84 ± 3.08[a,b]	11.04 ± 3.40[a,b,c]	9.79 ± 2.80[c]
LDL malondialdehyde and 4-hydroxy-2(E)-nonenal (μmol/L)	496.9 ± 378.4[a,b]	352.5 ± 87.7[b,c]	219.7 ± 87.9[c]	694.1 ± 730.3[a]	371.7 ± 426.2[b,c]	194.5 ± 133.7[c]

[1] Values are mean ± SD. Values in each row not sharing the same superscript differ significantly ($P < 0.05$) by Fisher's least significant difference test.

18,40,41]. The results in our study also indicated that the levels of plasma β-carotene, vitamin C, vitamin E, and erythrocyte GSH increased significantly in hyperlipidemic smokers after 6-week combined antioxidant vitamin supplements. Plasma antioxidant vitamin concentrations in smokers increased significantly after daily supplementation with combined vitamin C (350 mg) and vitamin E (250 mg) [13], vitamin cocktails (272 mg vitamin C, 31 mg D,L-α-tocopheryl acetate, and 400 μg folic acid) [8] or consumption of tomato-based juice supplemented with multi-vitamins (30 mg β-carotene, 600 mg vitamin C, and 400 mg vitamin E) [19]. A long-termed study also showed that healthy smoking men had significantly increased plasma β-carotene, vitamin C, and vitamin E concentrations by 209%, 45%, and 72% after daily supplementation with 30 mg β-carotene, 400 mg vitamin C, 200 mg vitamin E, and 100 μg organic selenium for 3 months [17]. Higher doses of antioxidant vitamin supplements (22.5 mg/day β-carotene, 1500 mg/day vitamin C, and 1200 IU/day vitamin E) for 6 weeks elevated the levels of plasma β-carotene, vitamin C, and vitamin E by 8.9-, 1.6-, and 2.0-fold in smokers, and by 6.6-, 1.6-, and 2.4-fold in nonsmokers [18]. Jialal and Grundy [40] observed that plasma β-carotene, vitamin C, and vitamin E levels elevated by 16.3-, 2.6-, and 4.1-fold in healthy nonsmoking men after daily supplementation with 30 mg β-carotene, 1000 mg vitamin C, and 727 mg vitamin E for 3 months compared with those in the placebo group. Abbey et al. [41] also found that plasma β-carotene, vitamin C, and vitamin E concentrations increased by 400%, 27%, and 55% in healthy nonsmokers after daily supplementation with 18 mg β-carotene, 900 mg vitamin C, and 200 mg vitamin E for 3 months compared with those before supplementation.

Although plasma β-carotene, vitamin C, and vitamin E concentrations increased significantly, plasma ferrous ion level and the ratio of ferrous to ferric ion decreased significantly in hyperlipidemic smokers after antioxidant vitamin supplements. Morrison et al. [42] found a positive association between serum iron and the risk of fatal acute myocardial infarction (RR = 2.18 and 5.53 for males and females, respectively). The formation of reactive oxygen species is related to the redox state of transition metals [43]. A previous study found that the maximal rate of lipid peroxidation occurred when the ratio of ferrous to ferric ion was approximately one [44]. The ratio of ferrous to ferric ion was far away from one after antioxidant vitamin supplements in this study, which may lead to the decreased lipid peroxidation in plasma and LDL. In addition, when ascorbate concentration (>50 μmol/L) was sufficient to reduce all ferric ion to ferrous ion, ascorbate inhibited lipid peroxidation [45]. However, our result showed that plasma ferrous ion level decreased after antioxidant vitamin supplements even the mean of plasma vitamin C level was more than 50 μmol/L in both groups at week 6. Yoshino and Murakami [46] observed flavonoids enhanced autooxidation of ferrous ion, resulting in the inhibition of the formation of reactive oxygen species. It is possible that antioxidant vitamins promote autooxidation of ferrous ion to decrease the ratio of ferrous to ferric ion, and further to reduce the formation of reactive oxygen species, such as superoxide anion produced through the one-electron reduction of oxygen by ferrous ion [47].

It has been reported that single and combined antioxidant vitamins have different effects on the antioxidative enzyme activity in healthy subjects and smokers. Single β-carotene (15 mg/day) supplement for 28 days elevated erythrocyte catalase and GPx activities but not total glutathione (reduced and oxidized) levels after depletion of β-carotene for 68 days in young healthy women [48]. Healthy nonsmoking men and women consumed 500 mg vitamin C daily for 2 weeks had increased GSH levels in erythrocytes by nearly 50% after a one-week vitamin C-restricted diet [49]. Brown et al. [50] demonstrated that erythrocyte catalase activity increased in both smoking and nonsmoking men supplemented with vitamin E (280 mg/day) for 10 weeks. However, increased GPx and glutathione reductase activities, and decreased SOD activity and GSH content in erythrocytes were only observed in nonsmoking men supplemented with vitamin E compared with those consumed placebo. A 14-day short-termed study showed that vitamin E supplement (1000 IU/day) had no effect on erythrocyte catalase, GPx, and SOD activities or reduced and oxidized glutathione levels in both male smokers and nonsmokers [51]. Combined antioxidant vitamin supplements (4500 IU vitamin A/day, 750 mg vitamin C/day, and 400 IU vitamin E/day) for 40 days increased erythrocyte GSH content by 173%, and glutathione reductase and GPx activities in uraemic patients with hemodialysis compared with those before supplementation [52]. Hilbert and Mohsenin [18] indicated that smokers had higher SOD activity in bronchoalveolar (BAL) cells after supplementation with combined antioxidant vitamins (22.5 mg β-carotene/day, 1500 mg vitamin C/day, and 1200 IU α-tocopherol/day) for 6 weeks. However, combined antioxidant vitamins had no effects on catalase and GPx activities in BAL cells of the smokers. Because of different subjects with distinct physiological conditions, types of antioxidant supplementation, treatment duration, and measurement tissues, various results were observed in the studies described above.

Similar to our results, previous studies showed that lipid peroxide concentrations were inversely correlated with plasma antioxidant levels in smokers [15,17,19]. A significant reduction in plasma MDA levels was observed in smokers supplemented with vitamin E (200 IU) [15]. Decreased breath pentane excretion and enhanced resistance of LDL to oxidation were also found in smokers after consumption of tomato-based juice supplemented with multivitamins (30 mg β-carotene, 600 mg vitamin C, and 400 mg vitamin E) [19]. Daily supplementation with 30 mg β-carotene, 400 mg vitamin C, 200 mg vitamin E, and 100 μg organic selenium prolonged lag phase of LDL oxidation in smokers [17].

In conclusion, combined antioxidant vitamin supplements improve the overall antioxidant protection capacity and reduce the oxidative stress in male hyperlipidemic smokers by increasing antioxidant levels and antioxidative enzyme activities, and by decreasing lipid peroxidation. The data suggest that antioxidant vitamin supplements increase plasma antioxidant vitamin levels to reserve the consumption of GSH and antioxidative enzymes in erythrocytes, which results in the increases in GSH levels and antioxidative enzyme activities. Additionally, improved antioxidant status after supplementation protects against lipid peroxidation. The present study also revealed that higher doses of antioxidant vitamin supplements had not further improved effects on the antioxidative enzyme system and lipid peroxidation. It is considerable that combined β-carotene (15 mg/day), vitamin C (500 mg/day) and vitamin E (400 mg/day) supplements for 6 weeks can be used in a clinical trial among the hyperlipidemic smokers to eliminate the oxidative stress and damage.

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