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Protective Effects of Vitamins C and E on the Number of Micronuclei in Lymphocytes in Smokers and their Role in Ascorbate Free Radical Formation in Plasma

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Cigarette smoke is widely believed to increase free radical concentrations causing subsequent oxidative processes that lead to DNA damage and hence, to several diseases including lung cancer and atherosclerosis. Vitamin C is a reducing agent that can terminate free-radical-driven oxidation by being converted to a resonance-stabilized free radical. To investigate whether short-term supplementation with the antioxidants vitamin C and E decreases free-radical-driven oxidation and thus decreases DNA damage in smokers, we determined the frequency of micronuclei in lymphocytes in 24 subjects and monitored the electron paramagnetic resonance signal of ascorbate free radical formation in plasma. Further parameters comprised sister-chromatid exchanges and thiobarbituric acid-reactive substances. Twelve smokers and twelve non-smokers took 1000 mg ascorbic acid daily for 7 days and then 1000 mg ascorbic acid and 335.5 mg RRR-α-tocopherol daily for the next 7 days. Baseline concentrations of both vitamins C and E were lower and baseline numbers of micronuclei were higher ($p < 0.0001$) in smokers than in non-smokers. After 7 days of vitamins C and E, DNA damage as monitored by the number of micronulei was decreased in both, smokers and non-smokers, but it was more decreased in smokers as indicated by fewer micronuclei in peripheral lymphocytes ($p < 0.05$). Concomitantly, the plasma concentrations of vitamin C ($p < 0.001$) as well as the ascorbate free radical ($p < 0.05$) were increased. The corresponding values in non-smokers, however, did not change. Our findings show that increased ascorbate free radical formation in plasma after short-term supplementation with vitamins C and E can decrease the number of micronuclei in blood lymphocytes and thus DNA damage in smokers.

Keywords: ascorbic acid, ascorbate free radical, α-tocopherol, EPR, micronuclei

Abbreviations: EPR, electron paramagnetic resonance; SCE, sister-chromatid exchanges; TBARS, thiobarbituric acid-reactive substances; BN, binucleated cells

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INTRODUCTION

There is increasing evidence that free radical driven oxidation induced by cigarette smoke is an important early event in several diseases including lung cancer and atherosclerosis.[1],[2] One line of evidence is that the gas phase of cigarette smoke contains numerous compounds of free radicals that are involved in oxidation processes.[3],[4] Church and Pryor[5] identified a quinone/hydroquinone complex, which is held as a polymer in the tarry matrix of cigarette smoke and may reduce molecular oxygen, thus producing superoxide and hydroxyl radicals. Furthermore, EPR studies showed that the black pigmentation in lungs of smokers is due to the accumulation of iron stores in ferritin and in hemosiderin.[6] Although this Fe^{3+} is unreactive, it can be easily reduced by several agents, such as the hydroquinone radical. This oxidative potential of cigarette smoke may be a cause of the increased DNA damage seen in smokers, as indicated by the increased number of sister-chromatid exchanges in circulating human blood lymphocytes.[7]

Since antioxidants are believed to have a scavenger activity in free radical driven oxidation processes, this DNA damage might be decreased by antioxidants, such as ascorbic acid and α-tocopherol, which are carried in the blood stream.[8],[9] Highly reactive radicals, such as superoxide, hydroxyl and peroxyl radicals, reacting rapidly with ascorbate that is being oxidized by donating electrons forming the resonance-stabalized and fairly unreactive ascorbate free radical.[10] Frei et al.[11] have shown a decrease in α-tocopherol after the complete consumption of ascorbate during free-radical-mediated oxidation showing the demand for antioxidants. The sequential consumption of these antioxidants was also shown by detection of their free radicals in a biological system by the use of EPR spectroscopy.[12]

We therefore asked whether short-term supplementation of vitamin C and vitamin E decreases free-radical-driven oxidation and thus decreases DNA damage in smokers. To answer these questions, we carried out a pilot study on small well-defined groups of smokers and non-smokers, half of whom received vitamin C supplement daily for 7 days and then vitamins C and E for the next 7 days. We assessed free-radical-driven oxidation by using EPR spectroscopy to measure ascorbate free radical formation in plasma and DNA damage by using the cytokinesis-block method to count the number of micronuclei in peripheral blood lymphocytes.

MATERIALS AND METHODS

Study Subjects

The study subjects were 12 healthy smoking volunteers and 12 healthy non-smoking volunteers aged 19–33 years with a body mass index between 18 and 25 kg/m^2. Six smokers and six non-smokers were men; the other six smokers and six non-smokers were women. Exclusion criteria were cardiovascular diseases, hyperlipidemia, hypercholesterolemia (> 250 mg/dl), insulin-dependent diabetes, cancer or any other severe illness. In addition, those who were already supplementing their diet with antioxidants were not allowed to participate.

Chemicals and Solutions

Metaphosphoric acid, hexadecyltrimethyl-ammonium bromide, orthophosphoric acid, butylated hydroxytoluene and 2-thiobarbituric acid were of highest grade commercially available and were purchased from Sigma, USA or Merck, Germany. RPMI-1640 medium, fetal calf serum and phytohaemagglutinin for cell culture purposes were from Life Technologies, USA and penicillin/streptomycin from Bio Whittaker, USA. RRR-α-tocopherol was obtained from Hermes, Germany. For SCE and micronuclei

assays bromodeoxyuridine, colcemid solution and cytochalasin B from Serva, Germany and bisbenzimide (33258) from Hoechst, Germany were used. Enzymatic assays (colorimetric test Chol MPR 1) for the determination of cholesterol concentration in blood plasma were purchased from Roche Diagnostics, Mannheim, formerly Boehringer Mannheim, Germany.

Blood sample preparation

EDTA-plasma was isolated from 10 ml of fresh whole blood from each volunteer by centrifugation (1039 × *g*, 4 °C, 15 min) and separated into aliquots. For ascorbic acid analysis, 400 µl plasma samples were stabilized by vortexing with 600 µl of a solution containing 1 mol/l metaphosphoric acid and 5 mmol/l Na_2EDTA (20 s). After microcentrifugation (16000 × *g*, 22°C, 10 min) all samples were immediately frozen and stored in the dark at –78 °C. For cell culture, 10 ml heparinised fresh whole blood was used. The blood cells were cultured for 71 hours in RPMI-1640 medium supplemented with 10 % fetal calf serum, 1 % penicillin/streptomycin and 200 µl phytohaemagglutinin.

Study Design

To determine whether short-term supplementation with vitamins C and vitamin E can decrease free-radical-driven oxidation and thus decrease DNA damage in smokers, we gave six smokers (three men, three women) and six non- smokers (three men, three women) who had not fasted 1000 mg ascorbic acid daily for 7 days and then 1000 mg ascorbic acid and 335.5 mg RRR-α-tocopherol daily for the next 7 days. The average cigarette consumption of the female smokers was 20 and of the male smokers 30 cigarettes per day. Blood was taken by venipuncture from the 24 subjects at 9:00 a.m. on day 0, 7, 14 and 21, corresponding to baseline, 7 days of vitamin C, 7 days of vitamins C and E and washout. On each day plasma concentrations of vitamins C and E were determined. To measure ascorbate free radical formation, we examined the blood plasma of all subjects by EPR spectroscopy. DNA damage was assessed by counting the number of micronuclei in peripheral blood lymphocytes by the micronuclei-test. In addition we measured the frequencies of SCEs in circulating lymphocytes and the levels of TBARS in plasma.

Determination of ascorbic acid

Plasma concentrations of ascorbic acid were determined using a modified reversed phase HPLC system equipped with a PED 300 Pulsed Electrochemical Detector (Biometra, Germany) and a C_{18}-column Grom-Sil 120 Amino-2 PA (particle size 5 µm, 250 × 4 mm, Grom, Germany) operated at 0.5 ml/min.[13] In brief, metaphosphoric acid-treated plasma samples were thawed and the upper phases were vortexed with methanol/H_2O (2/8). Then all proteins were removed by microcentrifugation (16000 × *g*, 22°C, 5 min) and 20 µl of the upper phase was injected into the HPLC system. As the mobile phase, 10 % methanol in double-distilled water containing 10 mmol/l hexadecyltrimethyl-ammonium bromide, 10 mmol/l NaCl, 0.6 mmol/l Na_2EDTA and 140 mmol/l acetic acid was used. The pH was adjusted to 4.0 with 4 mol/l NaOH.

Determination of α-tocopherol

Plasma concentrations of α-tocopherol were determined using a modification of an HPLC method previously described.[14] In brief, 200 µl human plasma samples were extracted twice with 500 µl hexane after precipitation of the proteins with 200 µl ethanol. The hexane phases were separated, dried under a continuous stream of nitrogen, and subsequently redissolved in 200 µl hexane. α-Tocopherol was separated on a 250 × 4 mm Merck LiChrosphere CN

column using hexane as the eluent. The detection was performed by UV (293 nm, UV/VIS Detector 432, Kontron, Germany)

EPR measurements

EPR spectra were recorded at 293 K using a Bruker ESP 300E spectrometer, equipped with a Bruker TE_{102} (ER 4102ST) cavity, operating at 9.6 GHz with a 100 kHz modulation frequency. A microwave power of 6.3 mW was typically employed. For EPR analysis, plasma samples were thawed at room temperature in the dark and 60 µl were immediately transferred to a flat cell. No exogenous oxidation catalysts were added. The total radical concentration was determined by measuring the peak-to-peak amplitude. Mean values for each study subject were obtained by multiple analysis of duplicate plasma samples. The ascorbate free radical concentration was standardized by using 4-hydroxy-2,2,6,6-tetramethylpiperidine-1-oxyl. After double integration, 10000 a.u. of the signal height corresponded to 0.144 µmol/l ascorbate free radical. Due to the construction of the flat cell and a special holder as well as fixed instrumental conditions, the precision of the method varied in a small range relative to the boundary of the interindividual differences.[15] Instrumental settings were modulation amplitude: 1 G, receiver gain: 4×10^5, conversion time: 82 ms, time constant: 20.5 ms, number of scans: 8.

Micronuclei-test

The number of micronuclei in peripheral blood lymphocytes was determined using the original cytokinesis-block method as described by Fenech and Morley[16]. Briefly, cytochalasin B was added to lymphocyte cultures 27 hours prior to harvest at a final concentration of 3 µg/ml After 71 hours of cultivation time, cells were incubated by mild hypotonical treatment (10 min, 50 mmol/l KCl, 37 °C) and fixed twice in ice-cold methanol / acetic acid (10/1). The slides were dried and stained with Giemsa solution. A total of 4000 binucleated cells per subject with visible cytoplasm was checked for micronuclei on days 0, 7, 14 and 21 according to the criteria of Countryman and Heddle[17].

Analysis of sister-chromatid exchanges

The frequency of SCEs in lymphocytes was determined according to Perry and Wolff[18]. Briefly, bromodeoxyuridine was added at a final concentration of 3 µg/ml to the blood cell cultures 24 hours after culture setup. For metaphase preparation, 200 µl 0.001 % colcemid solution was added to all cultures 2 hours prior to harvesting. Finally, cells were treated hypotonically (20 min, 37.5 mmol/l KCl, 37 °C), fixed three times in ice-cold methanol / acetic acid, and dropped onto clean glass slides. Slides were prepared and stained by the fluorescence plus giemsa technique, which consists of staining with bisbenzimide, rinsing, irradiation by a sunlamp (UV 254 nm), buffer incubation, and staining with Giemsa solution. After this procedure, the two sister-chromatids of each chromosome present a different staining which allows quantitative evaluation of SCE frequency. Analysis was performed in 50 metaphases per subject on days 0, 7 and 14.

Measurement of TBARS

The measurements of TBARS in human blood plasma were carried out as recently described.[19] Briefly, tubes containing 200 µl plasma samples and 200 µl 0.2 mol/l orthophosphoric acid were vortexed with 25 µl butylated hydroxytoluene (10 s, 5.2 mmol/l butylated hydroxytoluene in the reaction mixture). The reaction mixture was then vortexed with 25 µl 0.11 mol/l 2-thiobarbituric acid reagent, incubated at 90 °C (45 min) and then cooled down to room temperature. TBARS were extracted with

500 µl butanol, and 50 µl saturated NaCl solution was added. The tubes were microcentrifuged in an Eppendorf centrifuge (11600 × *g*, 22°C, 60 s) and 250 µl of the upper butanol phase were measured by UV in a 96-well multititer plate. TBARS concentrations were calculated using the difference in absorption at two wavelengths (535 nm and 572 nm, UNICAM UV 2 spectrophotometer, UK).

Statistical analysis

Statistical analysis was done with one-way ANOVA using the MicroCal Origin version: 4.1, Microcal Software, Inc., MA, USA.

RESULTS

Baseline plasma concentrations of vitamins C and E tended to be lower in smokers than in non-smokers and vitamin C concentrations were much lower in male smokers than in female smokers (Table I, II). After supplementation with vitamins C (day 7) and E (day 14) for 7 days, plasma concentrations of both antioxidants were substantially increased in smokers (Tables II, III). In contrast, non-smokers had no such increases. After 7 days of washout (day 21), plasma concentrations of ascorbic acid and α-tocopherol returned to baseline but the decrease was greater in non-smokers. There were no changes in control groups. Plasma concentrations of α-tocopherol paralleled those of cholesterol and were lower in female smokers than in female non-smokers (Table I).

The characteristic spectrum of the ascorbate free radical in blood plasma consisted of a doublet with a coupling constant of $A_H = 1.83$ G (data not shown). Baseline concentrations of this radical species were lower in male smokers than in female smokers as well as in male non-smokers (Table I). After supplementation with vitamins C and E, ascorbate free radical formation increased in smokers, as shown by the EPR signal amplitude, but not in non-smokers (Fig. 1a and b). After washout, ascorbate free radical concentrations declined to baseline values in smokers and to below baseline in non-smokers. In addition, after 7 days of vitamin C, the ratio of ascorbate free radical / ascorbic acid decreased substantially, by 44 %, in smokers but not in non-smokers ($p < 0.01$, data not shown). Values in control groups did not change.

TABLE I Baseline data for all subjects

Variable	*female non-smokers*	*female smokers*	*male non-smokers*	*male smokers*
Ascorbic acid [µmol/l]	88.1 ± 5.9	79.1[a] ± 9.0	69.9[b] ± 10	36.7[a,b] ± 9.4
α-Tocopherol [µmol/l]	23.0 ± 2.3	20.1 ± 3.0	25.3 ± 1.8	22.0 ± 3.1
Cholesterol [mg/dl]	223[c] ± 16	167[c] ± 15	235 ± 20	206 ± 13
α-Tocopherol / cholesterol [µmol/l / mg/dl]	0.103 ± 0.005	0.119 ± 0.011	0.11 ± 0.008	0.105 ± 0.009
Ascorbate free radical concentration [nmol/l]	452 ± 35	460[h] ± 27	433[i] ± 21	328[h,i] ± 31
Micronuclei	2.92[d] ± 0.19	4.54[d] ± 0.49	3.04[e] ± 0.30	5.58[e] ± 0.45
Sister chromatid exchanges	4.98[f] ± 0.14	6.69[f] ± 0.18	5.07[g] ± 0.13	6.36[g] ± 0.24
TBARS [µmol/l]	0.42 ± 0.04	0.41[j] ± 0.04	0.47 ± 0.04	0.62[j] ± 0.07

Data are mean ± SEM for 6 subjects and p values relate to comparison of one variable as indicated:
$p < 0.05$: b, c, d, i, j;
$p < 0.01$: a, h;
$p < 0.001$: e, g;
$p < 0.0001$: f.

TABLE II Determination of ascorbic acid concentrations [μmol/l] on day 0, 7, 14 and 21

Group	*day 0*	*day 7*	*day 14*	*day 21*
Non-smokers control	70.2 ± 8.7	56.4 ± 5.9	66.3 ± 5.4	71.8 ± 8.4
Non-smokers supplemented	87.8 ± 8.4	113 ± 9.8	122^{l} ± 7.0	81.5^{l} ± 9.4
Smokers control	61.0 ± 17	70.5 ± 16	65.8 ± 14	67.9 ± 14
Smokers supplemented	54.8^{k} ± 8.5	122^{k} ± 12	108 ± 14	73.3 ± 8.7

Data are mean ± SEM for 6 subjects and p values relate to comparison of one group as indicated:
$p < 0.01$: l;
$p < 0.001$: k.

TABLE III Determination of α-Tocopherol concentrations [μmol/l] on day 0, 7, 14 and 21

Group	*day 0*	*day 7*	*day 14*	*day 21*
Non-smokers control	22.8 ± 1.9	22.6 ± 1.3	21.4 ± 0.9	21.3 ± 1.2
Non-smokers supplemented	25.5 ± 2.2	29.2^{o} ± 2.2	$38.0^{o,p}$ ± 3.0	25.5^{p} ± 2.0
Smokers control	22.4 ± 3.7	23.2 ± 3.0	21.9 ± 2.1	20.1 ± 2.5
Smokers supplemented	19.6 ± 2.1	21.2^{m} ± 2.1	$35.1^{m,n}$ ± 3.7	23.7^{n} ± 1.7

Data are mean ± SEM for 6 subjects and p values relate to comparison of one group as indicated:
$p < 0.05$: n, o;
$p < 0.01$: m, p.

Baseline values of DNA damage, as indicated by the number micronuclei in peripheral blood lymphocytes, were higher in smokers than in non-smokers and were higher in supplemented groups than in controls (Table I, Fig. 2a and b). After supplementation of vitamins C and E, the number of micronuclei remained quite constant in controls but decreased distinctively in the supplemented subjects (Fig. 2a and b). This large effect was greater in smokers than in non-smokers. After washout, the numbers of micronuclei returned to below baseline but the return was closer in non-smokers. Values in control groups did not change.

Baseline values of SCEs in circulating lymphocytes, another indicator of DNA damage, were higher in smokers than in non-smokers (Table I). Values were unchanged after supplementation with vitamins C and E (Table IV). Baseline TBARS values were higher in male smokers than in female smokers, whereas the males had lower plasma concentrations of vitamin C (Table I). The same trend with high plasma concentrations of ascorbic acid and low values of TBARS was seen in supplemented smokers (days 7, 14; Tables II, V).

TABLE IV SCE frequency on day 0, 7 and 14

Group	*day 0*	*day 7*	*day 14*
Non-smokers control	5.02 ± 0.14	5.41 ± 0.08	5.26 ± 0.14
Non-smokers supplemented	5.04 ± 0.13	5.46 ± 0.15	5.47 ± 0.26
Smokers control	6.85 ± 0.15	6.92 ± 0.19	7.00 ± 0.21
Smokers supplemented	6.20 ± 0.19	6.09 ± 0.19	6.25 ± 0.15

Data are mean ± SEM for 6 subjects.

TABLE V Concentrations of TBARS [μmol/l] on day 0, 7, 14 and 21

Group	*day 0*	*day 7*	*day 14*	*day 21*
Non-smokers control	0.41 ± 0.03	0.40 ± 0.04	0.38 ± 0.03	0.39 ± 0.03
Non-smokers supplemented	0.48 ± 0.04	0.42 ± 0.06	0.42 ± 0.04	0.40 ± 0.02
Smokers control	0.54 ± 0.09	0.45 ± 0.03	0.39 ± 0.02	0.42 ± 0.02
Smokers supplemented	0.49 ± 0.06	0.38 ± 0.02	0.37 ± 0.03	0.52 ± 0.14

Data are mean ± SEM for 6 subjects.

DISCUSSION

In this study we show that short-term supplementation with vitamins C and E decreases free-radical-driven oxidation processes in smokers, as indicated by an increased ascorbate free radical formation. Due to the higher exposure to oxidative stress, smokers had lower basal plasma concentrations of vitamins C and E and therefore a higher demand for antioxidants. Thus, supplementation with vitamin C and vitamin E for 7 days resulted in a larger increase of plasma concentrations of these vitamins in smokers than in non-smokers. After intake of vitamins C and E the ascorbate free radical level is proportional to the overall rate of ascorbate oxidation, thus indicating continuing oxidative stress in smokers, whereas non-smokers did not show such effects. In addition, we found a large decrease in the ratio of ascorbate free radical / ascorbic acid in smokers after 7 days supplementation with vitamin C. The strong correlation of the plasma concentration of cholesterol and α-tocopherol reflects the integration of this antioxidant into the LDL membranes.[20]

This study also shows that short-term supplementation with vitamins C and E decreases DNA damage, as indicated by larger changes in the frequency of micronuclei in smokers than in non-smokers. Baseline numbers of micronuclei were higher in smokers than in non-smokers. Following vitamin C treatment, the mean values decreased slightly but not significantly in both groups. However, combined vitamin C and E treatment induced a significant micronuclei decrease in smokers. This finding differs from the findings of Podmore et al.[21]. Using levels of 8-oxoguanine and 8-oxoadenine in peripheral blood lymphocytes as markers of DNA damage, Podmore et al.[21] found that supplementation with 500 mg vitamin C per day induced a significant increase in 8-oxoadenine. These conflicting results for vitamin C supplementation may be explained by the very different mutagenic assays used in the two studies. In addition, Levine et al.[22] and Poulsen et al.[23] mentioned that artefactual oxidation may have occurred during lymphocyte isolation and DNA extraction in the study of Podmore et al.[21]. In the present report, in vitro effects can be excluded because spontaneous occurrence of micronuclei in human lymphocytes is rare.[24]

In support of our findings from the micronuclei-test, we measured TBARS as a marker of lipid peroxides, which are potential contributors to background DNA damage.[25] As found for the number of micronuclei, we found a tendency towards decreased concentrations of TBARS after intake of ascorbic acid and α-tocopherol, indicating a protective effect of vitamins C and E. In addition to the number of micronuclei, we used the SCE frequency as an another indicator of DNA damage induced by free radical formation. SCE frequency is especially increased by genotoxic agents that disturb DNA synthesis, whereas the number of micronuclei responds to a broad variety of mutagens, causing clastogenic and aneugenic effects. In our study the number

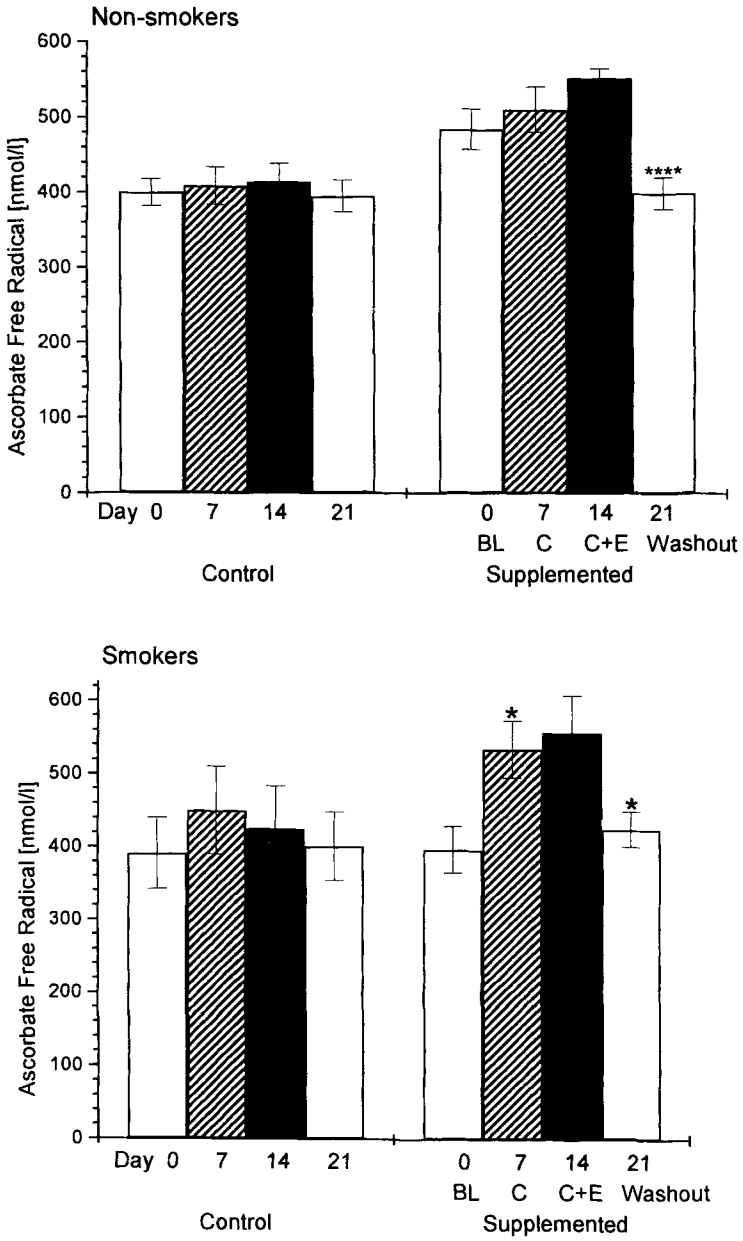

FIGURE 1 Ascorbate free radical concentration as determined by EPR in human plasma in non-smokers (a) and smokers (b) on day 0 (Baseline), day 7 (vitamin C), day 14 (vitamin C + E) and day 21 (Washout). Six smokers and six non-smokers received 1000 mg ascorbic acid daily for 7 days and 1000 mg ascorbic acid and 335.5 mg RRR-α-tocopherol daily for the next 7 days. Control groups (six subjects) received no vitamins. Bars represent mean ± SEM for six subjects. The ascorbate free radical concentration was standardized by using 4-hydroxy-2,2,6,6-tetramethylpiperidine-1-oxyl as described in Materials and Methods. Instrumental settings: modulation amplitude: 1 G, receiver gain: 4×10^5, conversion time: 82 ms, time constant: 20.5 ms, number of scans: 8. * $p < 0.05$, **** $p < 0.0001$ vs. the value 7 days earlier

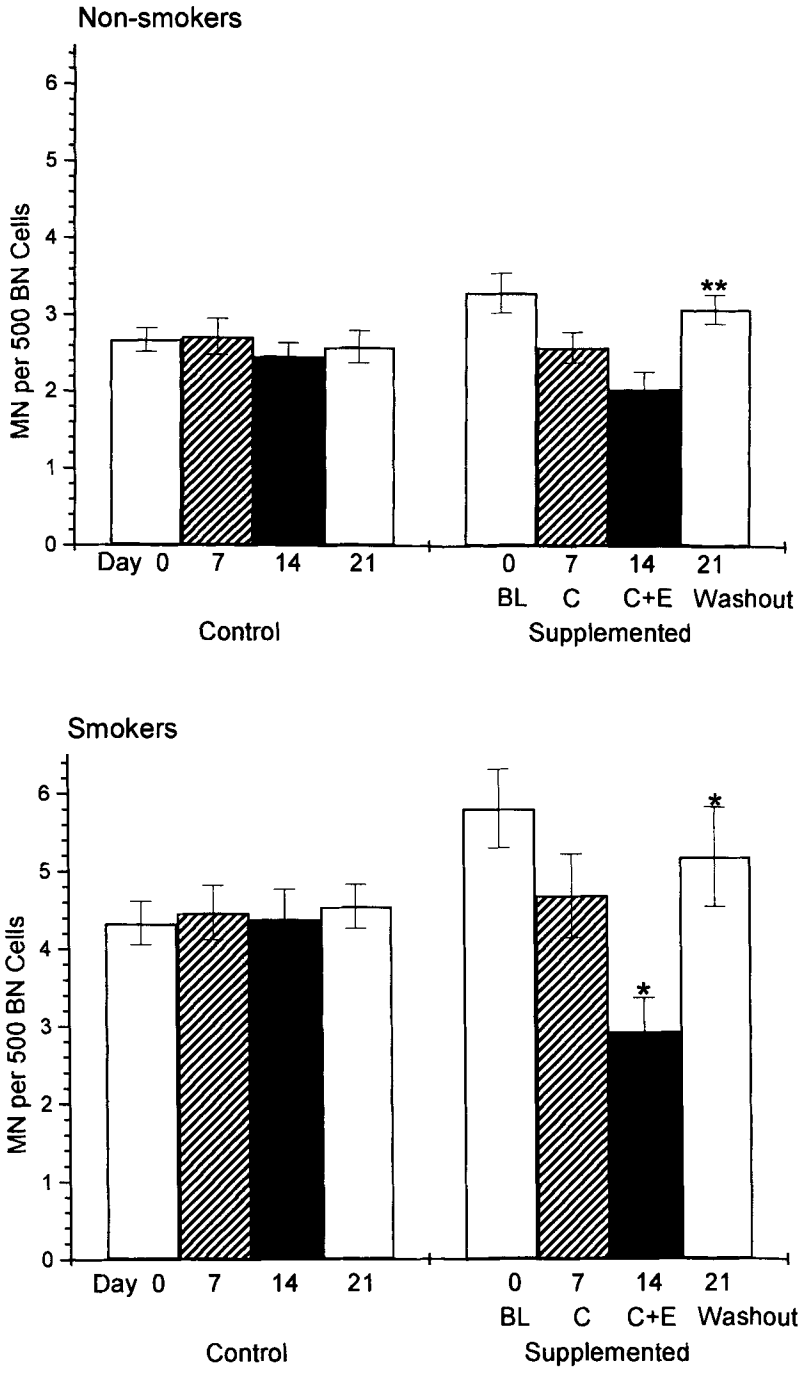

FIGURE 2 The number of micronuclei in peripheral blood lymphocytes per 500 binucleated cells in non-smokers (a) and smokers (b) on day 0 (Baseline), day 7 (vitamin C), day 14 (vitamin C + E) and day 21 (Washout). Bars represent mean ± SEM for six subjects. Conditions of study design are as given in the legend to Fig. 1. * $p < 0.05$, ** $p < 0.01$ vs. the value 7 days earlier

of SCE was significantly higher in smokers than in non-smokers. However, vitamin supplementation did not change SCE frequencies. This result probably indicates that S-phase-dependent DNA damage is not influenced by the vitamins C and E. Our results are consistent with data published by Rensburg et al.[7].

In contrast to Muscat et al.[26], who found a dose-response relationship between smoked cigarettes and increasing levels of cholesterol, we observed higher values of plasma cholesterol for male and female non-smokers. However, the Muenster Heart Study of Cullen et al.[27] found increased mortality at low cholesterol concentrations in smokers but not in non-smokers. This increased mortality was explained by an increase in smoking-related cancer deaths. In our study, the differences that were found between male and female subjects may have been caused in part by the higher average consumption of 30 cigarettes per day of the male smokers in this study.

In summary, short-term supplementation with 1000 mg vitamin C and 335.5 mg vitamin E daily decreased free-radical-driven oxidation and thus decreased oxidative damage to DNA in smokers. Since ascorbate free radical formation is correlated with a decrease of micronuclei, increase of ascorbate free radicals cannot be taken as a harmful effect of vitamin C supplementation. Although our data showed a positive interaction between vitamin C and vitamin E in decreasing micronuclei, no significant effects were found in the EPR signal of ascorbate free radicals after supplementation with both vitamins compared to the sole intake of ascorbic acid. It has to be considered, however, that the presented results are derived from a pilot study with only a limited number of subjects.

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