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Moderate Antioxidant Supplementation Has No Effect on Biomarkers of Oxidant Damage in Healthy Men with Low Fruit and Vegetable Intakes 1–3


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ABSTRACT The link between high fruit/vegetable intake and reduced chronic disease may be partly explained by antioxidant protection. To determine the effect of moderate antioxidant intake on biomarkers of oxidant damage, we assessed in vivo lipid and protein oxidation in 77 healthy men whose typical diet contained few fruits and vegetables (mean of 2.6 servings/d). The 39 nonsmokers and 38 smokers, age 20–51 y, were given a daily supplement (272 mg vitamin C, 31 mg all-rac-α-tocopherol, and 400 μg folate acid), or placebo, for 90 d with their usual diet. Blood and urine were taken at baseline and the end of the study for determination of lipid peroxidation products, including F2-isoprostanes, and protein carbonyls. Urine thiobarbituric acid reactive substances (TBARS) was the only oxidant damage marker that was significantly higher in smokers compared to nonsmokers (P<0.05). Supplementation increased plasma ascorbate and tocopherol, but had no effect on the oxidant biomarkers. In healthy young men, the endogenous antioxidant defense system and a modest intake of dietary antioxidants are adequate to minimize levels of in vivo oxidant damage such that they cannot be differentiated by current methods. J. Nutr. 133: 740–743, 2003.

KEY WORDS: antioxidant • biomarkers • human • oxidant damage • fruits and vegetables

Epidemiological studies show that increased consumption of fruits and vegetables is associated with a decreased risk of chronic degenerative diseases including cardiovascular disease and cancer (1,2). Antioxidants in fruits and vegetables may decrease risk of disease by modulating DNA damage, lipoprotein oxidation, platelet aggregation, leukocyte adhesion and vascular function (3).

Although many antioxidant supplementation studies have been conducted, the effect of antioxidant nutriture on disease risk remains uncertain. Reported antioxidant supplementation studies often involve healthy well-nourished individuals who already maintain a high antioxidant status before intervention, and who frequently are supplemented with pharmacological rather than nutritional amounts of the antioxidant vitamins. It has been suggested that studies of antioxidants and disease risk should include more subjects with a low intake or poor status of antioxidant nutrients, to increase the likelihood of detecting an impact of the intervention (4,5). To pursue this, we designed an antioxidant intervention study that would include subjects that are primarily low fruit/vegetable eaters, and provide them with a modest antioxidant supplement that would approximate the antioxidant intake of high fruit/vegetable eaters with respect to vitamins C and E. Smokers were included in the study population because they are likely to have more oxidant stress, a lower antioxidant vitamin intake and thus may show a greater benefit from an antioxidant intervention (6). Because the focus of the study is on antioxidant effects, providing antioxidants in supplement form instead of fruits and vegetables eliminates other constituents in fruits and vegetables that could confound the study results.

Because oxidative damage has been strongly linked to chronic disease, we measured biomarkers of in vivo oxidative damage to lipids and protein, before and after 90 d of antioxidant supplementation. The study objective was to determine whether a moderate supplement of antioxidant vitamins given to men eating less than the recommended 5–9 daily servings of fruits and vegetables would alter biomarkers of oxidant damage associated with disease risk.

MATERIALS AND METHODS

Subjects and study design. Approximately 200 men were screened for the study between March 1996 and April 1997 at the USDA Western Human Nutrition Research Center (WHNRC).5

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1 Reference to a company or product name does not imply approval or recommendation of the product by the U.S. Department of Agriculture to the exclusion of others that may be suitable.

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5 Abbreviations used: BMI, body mass index; DNPH, dinitrophenylhydrazine; ELISA, enzyme-linked immunosorbent assay; HHHQ, Health Habits and History
Presidio of San Francisco, CA (7,8). The men were recruited from the San Francisco Bay area, were primarily Caucasian, between the ages of 20–51 y and within 90–130% of ideal body weight (9). Telephone prescreening assessed suitability to meet study criteria and approximate fruit and vegetable intake. The men completed the Block food frequency questionnaire to evaluate their usual dietary habits (11). Fruit juice, tomato products, fried potatoes, legumes, soy products and mixed dishes containing vegetables were counted as fruits and vegetables. Individuals were considered smokers if they smoked 10 or more cigarettes/d, and were positive for the urine cotinine test (> 500 μg/L). Exclusion criteria for this study included any of the following: consumption of > 3.5 servings/d of fruits and vegetables as assessed in the telephone screening, poor health (as assessed by a nursing coordinator and physician), a positive test for infectious disease or illicit drug use or dietary supplement use within the last 6 months. The study was approved by the University of California, Berkeley, Committee for Protection of Human Subjects. All subjects signed informed consent before entering the study.

The 97 men that completed screening were grouped by smoking status (48 nonsmokers and 49 smokers) and randomized into either placebo or supplement groups with stratification of the groups for age, body mass index (BMI) and alcohol consumption. The supplement/placebo intervention was conducted in double-blind fashion. Of the 97 men that began the study, 20 dropped out because of noncompliance with the protocol or for personal reasons, leaving 77 men (39 nonsmokers and 38 smokers, age 20–51 y) that completed the 90-d intervention period.

The supplement and look-alike placebo were custom prepared and donated by Pharmavite Corp. (Mission Hills, CA). The supplement contained by analysis 272 mg ascorbic acid, 31 mg all-rac-α-tocopherol acetate and 400 μg folic acid. The design of the supplement was based on Food and Nutrition Board estimates of intakes of these nutrients that would be provided by a diet with > 5 combined servings of fruits/vegetables daily (12). The subjects were required to take the supplement or corresponding placebo daily for 90 d while continuing with their usual diet and smoking habits. Compliance was monitored by monthly telephone consultations and a log in which subjects recorded daily supplement consumption and food intake. Information supplied by the log was reviewed to confirm that the men complied with the study requirements to consume the supplements and maintain their usual dietary pattern. Additionally, the men were asked to return their pill bottles and any remaining pills were counted. Overall study compliance was 95% and did not differ between the treatment groups.

**Table 1**

Baseline characteristics of healthy men receiving placebo or antioxidant supplement

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Nonsmokers (n = 39)</th>
<th>Smokers (n = 38)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, y</td>
<td>34.0 ± 7.6</td>
<td>35.6 ± 9.2</td>
</tr>
<tr>
<td>Body mass index, kg/m²</td>
<td>25.4 ± 4.3</td>
<td>25.2 ± 4.4</td>
</tr>
<tr>
<td>Smoking, cigarettes/d</td>
<td>0</td>
<td>23 ± 15</td>
</tr>
<tr>
<td>Vitamin C intake, mg/d</td>
<td>77.5 ± 29.1</td>
<td>76.9 ± 36.1</td>
</tr>
<tr>
<td>Plasma ascorbate, μmol/L</td>
<td>41.4 ± 25.1</td>
<td>27.0 ± 20.0*</td>
</tr>
<tr>
<td>Urine thiobarbituric acid intake, mg/d</td>
<td>9.6 ± 2.8</td>
<td>11.4 ± 5.7</td>
</tr>
<tr>
<td>Beta-carotene intake, mg/d</td>
<td>1.7 ± 0.8</td>
<td>2.0 ± 1.1</td>
</tr>
<tr>
<td>Fruit/vegetable intake, servings/d</td>
<td>2.6 ± 1.0</td>
<td>2.7 ± 1.5</td>
</tr>
</tbody>
</table>

1 Values are means ± SD for healthy men; data from Lykkesfeldt et al. (7). * Different from nonsmokers, P < 0.01.

2 α-Tocopherol equivalents, mg.

Baseline characteristics and plasma ascorbate concentrations are detailed in **Table 1**. These data were reported previously (7), but are included again here because they are important for interpreting the present results on oxidant damage measures. Smokers did not differ from nonsmokers in age, body size and fruit/vegetable or antioxidant intakes, but had significantly lower plasma ascorbate concentrations.

Baseline values for oxidant damage biomarkers for nonsmokers and smokers are presented in **Table 2**. Values were not different between the two groups, except urine TBARS was higher in the smokers. Values for plasma ascorbate and oxidant damage measures at the beginning and end of the study by treatment and smoking status are shown in **Table 3**. Except for urine TBARS being higher in smokers (P < 0.05), two-way ANOVA models showed no significant differences in oxidant damage measures between treatment groups, or in the effect of treatments over time.

Values for oxidant damage measures showed significant relationships to age but not BMI, plasma ascorbate or number of cigarettes smoked. For all subjects, baseline values of plasma TBA-MDA (P = 0.004, r = +0.326), urine 8-isoprostan (P = 0.033, r = +0.256) and urine TBARS (P = 0.031, r = +0.249) were directly related to age.

**RESULTS**

**Table 2**

Baseline characteristics of healthy men receiving placebo or antioxidant supplement

**Table 3**

Results are presented as means ± SD for healthy men; data from Lykkesfeldt et al. (7). * Different from nonsmokers, P < 0.01.

1 α-Tocopherol equivalents, mg.

Results are presented as means ± SD. Differences were considered statistically significant for the two-tailed P-value < 0.05.
An important objective of the present study was to determine the effects of moderate antioxidant supplementation on oxidant damage in healthy nonsmoking and smoking men who may be in poor antioxidant status because of low consumption of fruits and vegetables. The subjects’ mean intake of fruits and vegetables of 2.6/d was well below the 5–9 servings per day recommended as part of a healthy diet. Baseline plasma antioxidant concentrations (7), a good biomarker of fruit/vegetable intake, were relatively low, the mean of the four treatments by smoking groups, 23 to 42 μmol/L, being in the lower third of the normal range of 23–85 μmol/L (20). Plasma ascorbate and α-tocopherol were increased significantly at T90, indicating that the 90-d supplement treatment was effective in increasing antioxidant status. The study hypothesis was that healthy men eating few fruits and vegetables would show some signs of low antioxidant status, and that levels of oxidant damage biomarkers would be decreased upon moderate antioxidant supplementation. The results of the present study, however, do not verify the study hypothesis, given that the 90-d antioxidant supplementation did not alter oxidant damage measures.

Of the measured oxidant damage markers, only urine TBARS showed a higher level for smokers compared to nonsmokers. The concentrations of urine F2-isoprostanes, a more sensitive and specific measure of lipid peroxidation than TBARS, showed no difference between smokers and nonsmokers, and no effect of antioxidant supplementation, although the intersubject variability was very high. Thiobarbituric acid reacts with other substances besides lipid peroxidation products so the increased urine TBARS in smokers of the present study may reflect the nonspecific nature of the TBARS assay. The present results are in contrast to some other studies that show increased oxidant damage in smokers (21–24). However, the latter studies were not designed to match dietary intakes between nonsmoker and smoker groups, as done in the present study. Hence, the increased oxidant damage levels of smokers seen in some studies may be attributed in part to the poorer diet of smokers, which contains fewer fruits and vegetables (6). The present results are similar to the findings of some other studies of healthy young adults that found little or no differences in oxidant damage markers between nonsmokers and smokers (25,26).

Despite the subjects’ low fruit/vegetable consumption and low-normal baseline plasma ascorbate levels, biomarkers of oxidant damage did not change after 90 d of moderate antioxidant supplementation. It is not likely that the period of supplementation was too short, in that other studies have reported reduction of plasma MDA (21), urine F2-isoprostanes (23,27) and plasma protein carbonyl (28) concentrations after supplementation with antioxidants for periods of 5 d to 10 wk. The studies noted above provided considerably more antioxidants in their supplement (400 mg vitamin C and 100–800 mg vitamin E) than the present study (272 mg vitamin C and 31 mg vitamin E). This may partly explain the lack of effect on oxidant damage markers in the present study.

Another factor that may explain the lack of effect of the supplement was the relatively young age of the subjects. In a

### TABLE 2

Baseline values for biomarkers of oxidant damage in healthy men receiving placebo or antioxidant supplement

<table>
<thead>
<tr>
<th>Biomarker</th>
<th>Non-smokers (n = 38)</th>
<th>Smokers (n = 38)</th>
</tr>
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<tbody>
<tr>
<td>TBA-MDA, μmol/L</td>
<td>0.44 ± 0.11</td>
<td>0.44 ± 0.20</td>
</tr>
<tr>
<td>MDA, nmol/L</td>
<td>104 ± 51</td>
<td>120 ± 65</td>
</tr>
<tr>
<td>Protein carbonyl (DNPH), nmol/mg protein</td>
<td>0.57 ± 0.12</td>
<td>0.53 ± 0.15</td>
</tr>
<tr>
<td>Protein carbonyl (ELISA), nmol/mg protein</td>
<td>0.43 ± 0.21</td>
<td>0.40 ± 0.20</td>
</tr>
<tr>
<td>Urine TBARS, μmol/g creatinine</td>
<td>1.91 ± 0.39</td>
<td>2.19 ± 0.74*</td>
</tr>
<tr>
<td>Urine total isoprostanes, ng/mg creatinine</td>
<td>19.1 ± 16.4</td>
<td>18.4 ± 15.0</td>
</tr>
<tr>
<td>Urine 8-isoprostanes, ng/mg creatinine</td>
<td>0.73 ± 0.66</td>
<td>0.83 ± 0.93</td>
</tr>
</tbody>
</table>

1 Values are means ± SD. * Different from non-smokers, P < 0.05.

### TABLE 3

Plasma ascorbate and biomarkers of oxidant damage in healthy men receiving placebo or antioxidant supplement

<table>
<thead>
<tr>
<th>Biomarker</th>
<th>Placebo</th>
<th>Antioxidant supplement</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Non-smokers (n = 22)</td>
<td>Smokers (n = 20)</td>
</tr>
<tr>
<td>Plasma ascorbate, μmol/L</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>41.7 ± 25.2</td>
<td>35.3 ± 18.8</td>
</tr>
<tr>
<td>90 d</td>
<td>30.4 ± 23.0†</td>
<td>34.0 ± 31.7</td>
</tr>
<tr>
<td>MDA, nmol/L</td>
<td>0.42 ± 0.11</td>
<td>0.36 ± 0.11</td>
</tr>
<tr>
<td>Protein carbonyl (DNPH), nmol/mg protein</td>
<td>0.61 ± 0.13</td>
<td>0.61 ± 0.17</td>
</tr>
<tr>
<td>Total isoprostanes, ng/mg creatinine</td>
<td>1.92 ± 0.35</td>
<td>1.97 ± 0.52</td>
</tr>
<tr>
<td>8-isoprostanes, ng/mg creatinine</td>
<td>18.7 ± 19.5</td>
<td>22.8 ± 14.8</td>
</tr>
<tr>
<td></td>
<td>0.64 ± 0.62</td>
<td>0.74 ± 0.57</td>
</tr>
</tbody>
</table>

1 Values are means ± SD. * Different from baseline, P < 0.05. † Different from non-smokers, P < 0.05. Ascorbate data from Lykkefeldt et al. (7).
sample of 103 healthy men, Liu et al. (29) found significant differences in plasma ascorbate, tocopherol and MDA between smokers and nonsmokers for the cohort of men age > 45 y, but not for the cohort of men < 45 y. This suggests that oxidative stress is handled more effectively by younger than by older men. Consistent with this, values in the present study for plasma TBA-MDA, urine 8-isoprostanes and urine TBARS for all subjects at baseline were directly correlated with age (P < 0.05), and the strength of these relationships was diminished after the supplementation period.

Similar to findings of the present study, other recent antioxidant interventions with young adults have shown little or no effect on biomarkers of oxidant damage (30–34). In a design similar to the present study, van den Berg et al. (34) provided a vegetable/fruit concentrate with high antioxidant capacity for 3 wk to 22 healthy male smokers (age 18–50 y) who typically consume a low fruit/vegetable intake. The intervention increased plasma ascorbate, carotenoids and antioxidant capacity: the first National Health and Nutrition Examination Survey of markers of oxidant damage to lipids (MDA, F2-isoprostane), protein carbonyls, DNA (Comet assay) and several functional measures.

Overall, the conclusion drawn from the present study is that moderate antioxidant supplementation of healthy young men, including smokers and those with low fruit/vegetable intakes, does not significantly reduce in vivo oxidant damage. In healthy young adults, the endogenous antioxidant defense system and a modest amount of dietary antioxidants is apparently adequate to minimize levels of in vivo oxidant damage such that they cannot be differentiated by current methods. Some evidence from this and previous studies suggests this may not be true for older adults. The hypothesis that high fruit/vegetable intakes provide additional protection against oxidant damage associated with chronic disease requires further testing in other population groups, and with more sensitive and/or selective biomarkers of oxidant damage.

ACKNOWLEDGMENTS

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LITERATURE CITED