No effect of supplementation with vitamin E, ascorbic acid, or coenzyme Q10 on oxidative DNA damage estimated by 8-oxo-7,8-dihydro-2′-deoxyguanosine excretion in smokers\textsuperscript{1–3}

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**ABSTRACT** The protective effect of fruit and vegetables against cancer has been related to their high antioxidant content. However, results from intervention trials have not been conclusive on the protective effect of antioxidant supplementation. In a randomized placebo-controlled trial we investigated the effect of dietary supplementation with antioxidants on a biomarker of oxidative DNA damage with mechanistic relation to carcinogenesis. One hundred forty-two smoking men aged 35–65 years were randomly assigned to one of the following seven treatments for 2 mo: 100 mg \(\alpha\)-tocopherol acetate plus 250 mg slow-release ascorbic acid twice a day \((n = 20)\), 100 mg \(\alpha\)-tocopherol acetate twice a day \((n = 20)\), 250 mg ascorbic acid twice a day \((n = 21)\), 250 mg slow-release ascorbic acid twice a day \((n = 21)\), 30 mg coenzyme Q10 in oil three times a day \((n = 20)\), 30 mg coenzyme Q10 as granulate three times a day \((n = 20)\), or placebo twice a day \((n = 20)\). The trial outcome was the urinary excretion rate of 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodG)—a repair product of oxidative DNA damage. Two months of supplementation did not result in significant changes in the urinary excretion rate of 8-oxodG in any group. The lack of effect of antioxidant supplementation on the excretion rate of 8-oxodG, despite substantial increases in plasma antioxidant concentrations, agrees with the results from recent large intervention studies with cancer as an endpoint. The cancer-protective effect of fruit and vegetables seems to rely not on the effect of single antioxidants but rather on other anticarcinogenic compounds or on a concerted action of several micronutrients present in these foods. *Am J Clin Nutr* 1997;65:503–7.

**KEY WORDS** Oxidative DNA damage, antioxidant supplementation

**INTRODUCTION** The possible cancer-preventing effect of antioxidants in diet or as supplements has been the subject of extensive epidemiologic studies founded on basic research that has proposed oxidants as an important pathogenic factor in carcinogenesis (1–3). Results from the many epidemiologic studies are inconclusive with regard to the protective effects of the specific micronutrients, but reviews of > 200 case-control and longitudinal studies conclude that consumption of a diet rich in vegetables and fruit is associated with reduced cancer risk (4–6). The cancer-protective effect of vegetables and fruit has been related to their high content of antioxidants such as ascorbic acid, \(\alpha\)-tocopherol, and \(\beta\)-carotene, but the effect could also be caused by other substances in fruit and vegetables (7). Thus, the results from two large intervention trials with antioxidants have not been conclusive with respect to the cancer-protective effects of antioxidant supplementation (8, 9).

Epidemiologic studies and primary prevention trials with cancer disease as an endpoint require immense resources and a long study period. The use of a biomarker with a mechanistic relation to carcinogenesis as an intermediate endpoint allows us to conduct experiments within a much shorter period of time. The urinary excretion rate of a repair product of oxidative DNA damage, 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodG), is considered a biomarker of the rate of oxidative DNA damage (10–12). The 8-oxodG concentration of a urine sample can be measured by HPLC (10, 13). Using this method our laboratory showed earlier that smokers excrete ≈50% more 8-oxodG in urine than do nonsmokers (13). This finding agrees with the hypothesis of an increased level of oxidative stress in smokers. Indeed, decreased concentrations of plasma antioxidants were found in smokers compared with nonsmokers, suggesting a lower intake and/or a higher rate of turnover of antioxidants in smokers (14–17).

In this randomized placebo-controlled trial we investigated the effect of supplementation of smokers with three different antioxidants, \(\alpha\)-tocopherol acetate, ascorbic acid, and coenzyme Q10, on the urinary excretion rate of 8-oxodG, a biomarker of oxidative stress with a presumed relation to carcinogenesis.

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SUBJECTS AND METHODS

Study subjects

The study was conducted in accordance with the Declaration of Helsinki. The protocol was approved by the local ethics committee and signed informed consent was obtained from all subjects. Subjects were recruited by newspaper advertisements. Inclusion criteria were age 35–65 y and smoking > 10 cigarettes/d. Exclusion criteria were regular antioxidant intake, regular intake of acetylsalicylic acid, regular intake of any other drug with antioxidative properties, severe obesity [body mass index (in kg/m²) > 31], insulin-dependent diabetes, severe disease such as cancer, severe cardiovascular disease, or other disease with poor short-term prognosis, any condition limiting the mobility of the subject enough to make trial visits impossible, other disease or condition that would worsen the adherence to the measurement or treatment, and participation in any other trial involving other investigational products within the past 3 mo. Inclusion and exclusion criteria were checked at a screening visit. Subjects had to withdraw from the study if they consumed < 70% of the planned supplement, or experienced concomitant illness such as fever, vomiting, or diarrhea within the last week of the trial.

Randomization and intervention

The study was conducted as a phase I/IIa, block-randomized, single-blind, parallel, placebo-controlled trial testing three types of antioxidants in various formulations. The trial consisted of a screening visit, a baseline visit at which the test product was dispensed, followed by a 2-mo outpatient treatment period, and ended in a termination visit. All study visits took place at the Research Institute of Public Health, University of Kuopio, Finland. At the baseline visit eligible subjects were randomly assigned to one of four different treatment blocks according to a computer-generated randomization list. Each treatment block except the placebo block consisted of two types of treatment. Thus, the 142 subjects were divided into a total of seven treatment groups each containing 20 (or 21) subjects and designed as follows: Block 1: treatment with 100 mg d-α-tocopheryl acetate plus 250 mg slow-release ascorbic acid twice daily (group 1A, n = 20) or 100 mg d-α-tocopheryl acetate twice daily (group 1B, n = 20). Block 2: treatment with 250 mg ascorbic acid twice daily (group 2A, n = 21) or 250 mg slow-release ascorbic acid twice daily (group 2B, n = 21). Block 3: treatment with 30 mg coenzyme Q10 in oil three times daily (group 3A, n = 20) or 30 mg coenzyme Q10 as a granule three times daily (group 3B, n = 20). Block 4: placebo tablets twice daily (group 4, n = 20). The test products were supplied by Ferrosan A/S, Soeborg, Denmark.

To ensure that ≥ 70% of the planned supplements were taken, the remaining tablets were counted for each subject at the termination visit. The investigator had information on the subject’s block assignment but was blinded to the identity of the treatment within each block. However, the investigator knew the identity of the subjects receiving placebo because there were only placebos in that block. No adverse events related to any of the treatments were observed during the study.

Assessments

At the baseline visit and at the termination visit 2 mo later blood samples were collected after subjects fasted overnight from 2200 the previous evening. Subjects brought urine collected during the preceding 24 h. The baseline visit took place just before the start of the intervention and termination blood samples were taken ~8 h after the last supplement was taken.

The urinary concentration of 8-oxodG in 24-h urine was determined by HPLC with electrochemical detection according to a method published earlier (13). Samples were analyzed twice in pairs from each subject in random order. Intra- and interassay CVs were 10% and 13% in the relevant range, respectively.

Fasting blood samples were used for determination of plasma concentrations of ascorbic acid and dehydroascorbic acid (18), α-tocopherol (19), and coenzyme Q10 (20) by HPLC. Total ascorbate concentrations were calculated as ascorbic acid plus dehydroascorbate concentrations.

Treatment with α-tocopheryl acetate (group 1B) resulted in a mean increase in plasma α-tocopherol concentrations of 87% (95% CI: 73%, 102%; n = 20), whereas treatment with α-tocopheryl acetate plus ascorbic acid in slow-release formulation (group 1A) resulted in a mean increase in plasma α-tocopherol concentrations of 30% (95% CI: 19%, 42%; n = 19). Thus, treatment with α-tocopheryl acetate alone yielded a plasma α-tocopherol concentration about three times higher than the combination formulation of α-tocopheryl acetate plus slow-release ascorbic acid. Treatment with ascorbic acid in slow-release formulation (group 2B) and in usual formulation (group 2A) resulted in mean increases in plasma total ascorbate concentrations of 53% (95% CI: 32%, 73%; n = 17) and 30% (95% CI: 13%, 48%; n = 17), respectively, whereas treatment with α-tocopheryl acetate plus slow-release ascorbic acid (group 1A) resulted in a 41% (95% CI: 29%, 54%; n = 16) increase in plasma total ascorbate concentrations. Coenzyme Q10 supplementation as a granulate (group 3B) or oil (group 3A) resulted in mean increases in plasma coenzyme Q10 concentrations of 168% (95% CI: 132%, 203%; n = 20) and 169% (95% CI: 128%, 210%; n = 19), respectively. There were no significant changes in plasma concentrations of total ascorbate, α-tocopherol, and coenzyme Q10 in the placebo or nontreated groups.

The discrepancy between the plasma α-tocopherol concentrations yielded by the two different formulations of α-tocopheryl acetate (groups 1A and 1B) was probably caused by the formulation of the combined tablet with α-tocopheryl acetate plus slow-release ascorbic acid. Therefore, group 1A was excluded from data analysis. Baseline plasma concentrations of α-tocopherol, total ascorbate, and coenzyme Q10 were similar in the six groups: 1B, 2A, 2B, 3A, 3B, and 4 and tested by analysis of variance (ANOVA; data not shown).

Statistics

All subjects from groups 1B, 2A, 2B, 3A, 3B, and 4 who completed both baseline and termination measurements were included in statistical analysis. The baseline characteristics were compared between the groups by one-way ANOVA. The change in 8-oxodG excretion rate was evaluated by a repeated-measures ANOVA. Results are presented as means and 95% CIs for the changes in the 8-oxodG excretion rate in each group. The computer program STATISTICA (version 5.0; StatSoft, Inc, Tulsa, OK) was used for the analyses.
RESULTS

One hundred sixteen subjects—20 in group 1B, 18 in group 2A, 20 in group 2B, 19 in group 3A, 20 in group 3B, and 19 in group 4 (the placebo group) collected urine samples both at baseline and at study termination. Subjects randomly assigned to the six treatment groups (1B, 2A, 2B, 3A, 3B, and 4) were similar with respect to age, body weight, daily cigarette consumption, and baseline 8-oxodG excretion rate (tested by ANOVA) as shown in Table 1.

There were no significant changes in the 24-h urinary 8-oxodG excretion in any of the treatment groups or the placebo group after 2 mo of antioxidant intervention (P > 0.05; Table 1). Data analysis including group 1A gave similar results. The limits of the 95% CIs for the changes in 8-oxodG excretion rate ranged from −34% to 26%. A reduction in 8-oxodG excretion rate approached significance in both the placebo group and in the vitamin E–treated group.

The SD of the repeated measurement of 8-oxodG excretion rate in the placebo group, i.e., the intraindividual CV, was 37%. In an earlier study we found an intraindividual CV of 22% for the repeated measurement of 8-oxodG excretion rate in eight nonsmokers (21). The intraindividual variation in the urinary 8-oxodG excretion rate may be higher in smokers than in nonsmokers. The mean (± SD) baseline 8-oxodG excretion rate was 42.43 ± 19.90 nmol/24 h, which was comparable with the rate found in another study in which the excretion rate of 8-oxodG was measured in male smokers (22).

DISCUSSION

In the present study supplementation for 2 mo with the classic dietary antioxidants ascorbic acid [250 mg twice daily; Nordic recommended dietary allowance (RDA) 60 mg/d (23)], α-tocopherol (100 mg twice a day; Nordic RDA 10 mg/d), and coenzyme Q10 (30 mg three times a day), resulting in substan-
tial increases in plasma antioxidant concentrations, did not change the urinary excretion rate of the DNA repair product 8-oxodG. We showed earlier that smokers excrete ~50% more 8-oxodG in urine than do nonsmokers (13) and Verhagen et al (24) reported recently that in nonsmokers, a 28% reduction in the 8-oxodG excretion rate can be achieved by intake of 300 g Brussels sprouts/d. The present study was designed to detect a similar effect. As indicated by the limits of the 95% CIs of the mean changes in 8-oxodG excretion rate, such an effect can be excluded. A smaller effect might have been present but not detected. However, the reduction in 8-oxodG excretion rate was more pronounced for the placebo group than for the supplemented groups.

The development of a validated noninvasive method for measuring the urinary concentration of 8-oxodG by HPLC (10, 13) allows investigation of the effect of intervention on a biomarker of the rate of in vivo oxidative DNA damage. The oxidation rate of DNA is high—estimated to > 10^3 oxidative hits of DNA per cell per day (25). In vivo, 8-oxodG in DNA is extensively repaired by nucleotide excision or the formamidopyrimidine-DNA glycosylase enzyme resulting in the two products, 8-oxodG or the corresponding base 8-oxoguanine, respectively (10, 26-31). Studies with repair-deficient cell lines suggest that nucleotide excision is an important pathway for 8-oxodG in humans (32). In addition, digestion of damaged DNA from cell renewal and mitochondrial turnover will liberate 8-oxodG. Oxidized nucleosides and nucleotides from the cellular pool may be incorporated into DNA and lead to mutations (33, 34) unless cleaned up by an enzyme that hydrolyses the phosphates of 8-oxodG triphosphate with high affinity and 8-oxodG as a putative end product (35). Animal experiments have shown that injected 8-oxodG is readily excreted unchanged into the urine whereas 8-oxodG in the diet or oxidation of deoxyguanosine during excretion does not contribute (10, 27, 36). In urine samples 8-oxodG is stable for ≥ 6 y at −20 °C (12). Thus, although the exact relative importance of

### TABLE 1

Baseline characteristics of subjects and changes in the excretion rate of 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxodG) after intervention

<table>
<thead>
<tr>
<th>Group</th>
<th>1B-Vitamin E</th>
<th>2A-Vitamin C</th>
<th>2B-Slow-release</th>
<th>3A-Coenzyme</th>
<th>3B-Coenzyme</th>
<th>4-Placebo</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(n = 20)</td>
<td>(n = 18)</td>
<td>(n = 20)</td>
<td>(n = 19)</td>
<td>(n = 20)</td>
<td>(n = 19)</td>
<td>(n = 116)</td>
</tr>
<tr>
<td>Baseline</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age (y)</td>
<td>47.9 ± 5.8</td>
<td>45.2 ± 7.8</td>
<td>44.8 ± 8.2</td>
<td>44.4 ± 4.2</td>
<td>46.1 ± 8.2</td>
<td>45.2 ± 7.4</td>
<td>45.6 ± 7.0</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>83.6 ± 10.9</td>
<td>78.3 ± 10.8</td>
<td>79.2 ± 12.8</td>
<td>81.5 ± 8.6</td>
<td>81.0 ± 10.1</td>
<td>78.5 ± 9.7</td>
<td>80.4 ± 10.5</td>
</tr>
<tr>
<td>Cigarettes/d</td>
<td>20.5 (12-30)</td>
<td>22.9 (15-35)</td>
<td>18.6 (10-35)</td>
<td>22.6 (13-40)</td>
<td>23.6 (12-40)</td>
<td>21.5 (11-40)</td>
<td>21.6 (10-40)</td>
</tr>
<tr>
<td>8-oxodG excretion rate (nmol/24 h)</td>
<td>40.58 ± 16.37</td>
<td>39.81 ± 17.93</td>
<td>36.55 ± 16.31</td>
<td>44.15 ± 22.56</td>
<td>40.20 ± 20.13</td>
<td>53.68 ± 23.13</td>
<td>42.43 ± 19.90</td>
</tr>
<tr>
<td>Change in 8-oxodG excretion rate (nmol/24 h)</td>
<td>–3.78</td>
<td>4.04</td>
<td>–0.53</td>
<td>1.14</td>
<td>3.75</td>
<td>–8.50</td>
<td>–0.67</td>
</tr>
<tr>
<td>(%)</td>
<td>(–9.4, 1.8)</td>
<td>(–2.3, 10.4)</td>
<td>(–5.4, 4.4)</td>
<td>(–7.7, 9.9)</td>
<td>(–2.5, 10.0)</td>
<td>(–18.1, 1.1)</td>
<td>(–3.5, 2.1)</td>
</tr>
</tbody>
</table>

1. ± SD.
2. ± range in parentheses.
3. ± 95% CI in parentheses.
the DNA repair pathways remains to be determined, the urinary excretion rate of 8-oxodG reflects the general average risk of a promutagenic oxidative adduct in DNA of all tissues and organs. The relevance of 8-oxodG as a biomarker of oxidative DNA damage is supported by the observations that the guanine base is a major target of oxidative DNA damage (37, 38) and that the oxidation of guanine residues in DNA is mutagenic (39). The relation to cancer is founded on in vitro experiments showing that oxidation of guanine bases in replicating DNA leads to guanine-thymine substitutions (40). Guanine-thymine substitutions are found to be among the most frequent hot-spot mutations in the p53 suppressor gene in human lung cancers (41) and guanine-thymine transversion in codon 12 of the K-ras oncogene has been shown to result in activation (42).

Ascorbic acid and α-tocopherol were chosen as supplements in this study because of their possible cancer-preventing effects according to epidemiologic studies (43, 44). Coenzyme Q10 is closely related to cellular energy metabolism, which is a prominent endogenous source of reactive oxygen species (45), and has furthermore been suggested as a scavenger of reactive oxygen species (46).

The effect of intervention was studied in smokers, who are exposed to high amounts of oxidants from tobacco smoke (47, 48). Also, concentrations of plasma antioxidants have been found to be lower in smokers than in nonsmokers, indicating a decreased intake and/or a higher turnover of antioxidants in smokers (16). The excretion rate of 8-oxodG was found to be elevated in smokers compared with nonsmokers (13).

Our present finding of no change in the urinary excretion rate of 8-oxodG after antioxidant supplementation is in accordance with the earlier finding from our laboratory that the intake of antioxidant vitamin C, E, and A equivalents estimated from a weighed diet record was not associated with the 8-oxodG excretion rate (13). The urinary 8-oxodG excretion rate was also unchanged after intervention with β-carotene (20 mg/d for 14 wk) in male smokers (22).

As indicated by the results of the various studies mentioned above, the incontestable beneficial effect of a high intake of vegetables and fruit on the risk of cancer (4, 6, 7) may rely not on the effect of single antioxidants but rather on a concerted action of several micronutrients present in these foods. Anticarcinogenic compounds in vegetables and fruit other than the well-known vitamins C, E, and A may play a central role. Phytochemicals have been proposed to be such alternative anticarcinogenic compounds in plants (51). Intervention with 300 g Brussels sprouts/d for 3 wk in five nonsmokers resulted in a significant 28% decrease in the 8-oxodG excretion rate (23). Although the result might have been different in smokers, this finding supports the hypothesis of a chemopreventive role for phytochemicals. Brussels sprouts contain huge amounts of the phytochemicals glucosinolates, which have been shown to have anticarcinogenic effects in animal experiments and epidemiologic settings (51, 52). The nature, chemistry, and disease-preventing effects of the large group of phytochemicals will be a field for future research. A biomarker such as 8-oxodG will represent a valuable tool in the research into the mechanisms linking diet and cancer.

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