Modification of lymphocyte DNA damage by carotenoid supplementation in postmenopausal women

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ABSTRACT

Background: Oxidative stress has been implicated in the pathogenesis of chronic diseases related to aging such as cancer and cardiovascular disease. Carotenoids could be a part of a protective strategy to minimize oxidative damage in vulnerable populations, such as the elderly.

Objective: Our aim was to determine the protective effect of carotenoids against DNA damage.

Design: A randomized, double-blind, placebo-controlled intervention study was conducted. Thirty-seven healthy, nonsmoking postmenopausal women aged 50–70 y were randomly assigned to 1 of 5 groups and were instructed to consume a daily dose of mixed carotenoids (β-carotene, lutein, and lycopene; 4 mg each), 12 mg of a single carotenoid (β-carotene, lutein, or lycopene), or placebo for 56 d. Plasma carotenoid concentrations were analyzed by using HPLC, and lymphocyte DNA damage was measured by using a single-cell gel electrophoresis (comet) assay.

Results: At day 57, all carotenoid-supplemented groups showed significantly lower endogenous DNA damage than at baseline (P < 0.01), whereas the placebo group did not show any significant change. Significantly less (P < 0.05) endogenous DNA damage was found as early as day 15 in the mixed carotenoid (P < 0.01) and β-carotene (P < 0.05) groups.

Conclusions: The results indicate that carotenoid supplementation decreases DNA damage and that a combination of carotenoids (4 mg each of lutein, β-carotene, and lycopene), an intake that can be achieved by diet, or a larger dose (12 mg) of individual carotenoids exerts protection against DNA damage.

KEY WORDS Mixed carotenoids, lutein, β-carotene, lycopene, DNA damage, comet assay, elderly women

INTRODUCTION

Oxidative stress has been implicated in the pathogenesis of chronic diseases related to aging such as cancer and cardiovascular disease (1). Epidemiologic studies have shown that a high fruit and vegetable intake is associated with a lower risk of such chronic diseases (2–5). It is probable that carotenoids found in fruit and vegetables act as a major group of antioxidants, thereby preventing damage from harmful reactive oxygen species, which are continuously produced in the body from normal cellular functions and from exogenous sources (6). Carotenoids have also been reported to modulate the gene expression of steroid targets and inflammatory markers in animals (7) and to prevent UVA radiation–induced gene regulation in human cells (8).

Dietary supplementation with antioxidants, such as carotenoids, can be a part of a protective strategy to minimize the oxidative damage in vulnerable populations, such as the elderly. Carotenoids have in vitro antioxidant activity at physiologic oxygen tensions (9). However, this antioxidant effect of carotenoids is still controversial in vivo (10, 11). It should be pointed out that the metabolism and functions of carotenoids may differ in vivo compared with in vitro systems. For example, antioxidant nutrients can interact with each other during gastrointestinal absorption and metabolism (12–15). Therefore, it is important to determine the biological actions of single carotenoids compared with those of mixed carotenoids in vivo.

The single-cell microgel electrophoresis assay (ie, the comet assay) was developed to detect DNA single or double strand breaks. Broken DNA fragments result in an increased migration in electrophoresis to form a diffuse DNA area, which resembles a comet tail after staining (16). Pool-Zobel et al (17, 18) reported that supplementation of the diet of healthy persons with tomato, carrot, or spinach significantly reduced the number of endogenous strand breaks in lymphocyte DNA. Given that these foods also contain various phytonutrients other than carotenoids, it is difficult to ascertain if the DNA protection was because of the carotenoids. Currently, no human studies exist on the protective role of a combination of the major dietary carotenoids, ie, lutein, β-carotene, and lycopene, against DNA strand breaks. The present study was conducted to compare the protective effect of...
single carotenoid with that of mixed carotenoid supplementation for 8 wk against DNA damage in elderly women.

SUBJECTS AND METHODS

Subjects

Forty nonsmoking postmenopausal women aged 50–70 y were enrolled in the present study. All study subjects were in good health, as determined by a medical history questionnaire, physical examination, and normal results of clinical laboratory tests. All study subjects fulfilled the following eligibility criteria: 1) no history of cardiovascular, hepatic, gastrointestinal, or renal disease; 2) no alcoholism, no smoking, and no exogenous hormone use; 3) no supplemental vitamin or carotenoid use for >6 wk before the start of the study; and 4) plasma carotenoid concentrations <200% of the National Health and Nutrition Examination Survey (NHANES) III median concentration at screening, which excluded women with extreme carotenoid diets. The study protocol was approved by the Institutional Review Board of Tufts-New England Medical Center and Tufts University Health Sciences, and written informed consent was obtained from each study subject.

Study design

A research dietitian instructed the subjects to exclude foods rich in carotenoids and to limit fruit and vegetable intake to 2 servings/d of light colored fruit and vegetables for 2 wk before starting the study and during the entire study to maximize the blood response to the low doses of carotenoid supplementation. The subjects were randomly assigned to receive one of the following treatments: 1) placebo; 2) 4 mg/d each of lutein, β-carotene, and lycopene; 3) 12 mg lutein/d; 4) 12 mg β-carotene/d; or 5) 12 mg lycopene/d. On sampling days 1, 15, 29, and 43, the subjects were provided with a 2-wk supply of supplements. The subjects were instructed to take the carotenoid supplements with their first meal of the day; the meal was to contain ≥10 g fat to facilitate absorption of the carotenoid supplement. The carotenoid supplements were supplied by BASF AG (Ludwigshafen, Germany) as hard gelatin capsules. β-Carotene and lycopene were synthetic and lutein was of natural origin. To fill the capsules, the carotenoids were formulated as dry powders (beadlets) in such a way that the carotenoids were embedded in a matrix of hydrocolloid and sucrose. For mixed carotenoids, these beadlets contained a mixture of lutein, β-carotene, and lycopene (ie, a multicore structure). Dietary compliance, which included counting any remaining pills and checking the compliance calendar, 3-d food records, and food-frequency questionnaires, was measured biweekly. The research dietitian at the Jean Mayer US Department of Agriculture-Human Nutrition Research Center on Aging at Tufts University interviewed the study subjects on each sampling day.

Fasting (12-h) blood samples were collected in evacuated containers containing EDTA at baseline and at days 15, 29, 43, and 57, and lymphocytes were isolated immediately after collecting the samples and cryopreserved until the single-cell gel electrophoresis analysis. Plasma samples were divided into aliquots and stored at −80 °C for subsequent analyses of carotenoids.

Plasma carotenoid and tocopherol analysis

_all-trans_-β-Carotene (type IV), α-carotene, and lycopene standards were purchased from Sigma Chemical Co (St Louis, MO). Lutein was purchased from Kemin Industries (Des Moines, IA). All HPLC solvents were obtained from JT Baker Chemical (Phillipsburg, NJ) and were filtered through a 0.45-μm membrane filter before use.

Plasma carotenoid concentrations were measured by an HPLC system as previously described, with minor modifications (19). Plasma samples (200 μL) were extracted with 2 mL chloroform: methanol (2:1, by volume) followed by 3 mL hexane. Samples were dried under nitrogen and resuspended in 75 μL ethanol: methyl tert-butyl ether (2:1, by volume), of which 25 μL was injected onto the HPLC. The HPLC system consisted of a Waters 2695 Separation Module (Waters Instruments, Milford, MA), 2996 Photodiode Array Detector (Waters Instruments), a Waters 2475 Multi A Fluorescence Detector (Waters Instruments), a C30 carotenoid column (3 μm, 150 × 3.0 mm; YMC, Wilmington, NC), and a Waters Millenium 32 data station (Waters Instruments). The mobile phase was methanol:methyl tert-butyl ether:water (85:12.5:3, by volume, with 1.5% ammonium acetate in water; solvent A) and methanol:methyl tert-butyl ether:water (8:90:2, by volume, with 1% ammonium acetate in water; solvent B). The gradient procedure was reported earlier (19). The results were adjusted by an internal standard containing echinenone and retinyl acetate. The interassay CV (n = 25) was 4% and the intraassay CV was 4% (n = 9). Recovery of the internal standard averaged 97%.

Single-cell gel electrophoresis analysis (comet assay)

Lymphocyte separation

Lymphocytes were separated immediately after the blood samples were collected. Lymphocytes were isolated by density gradient sedimentation (Histopaque 1077; Sigma Diagnostics) and frozen in a mixture containing 50% fetal calf serum, 40% culture medium (RPMI 1640; Sigma Diagnostics), and 10% dimethyl sulfoxide at a freezing rate of −1 °C/min to a final temperature of −80 °C before storage in liquid nitrogen.

Cryopreserved lymphocyte recovery

Cells were recovered by submersion in a 37 °C water bath until the last trace of ice was melted. The cells were transferred to prechilled 50% RPMI 1640 medium and 50% fetal calf serum and were centrifuged at 200 × g for 5 min at 4 °C. The cells were resuspended in cold phosphate buffered saline and checked for viability (typically ≥95% viability) and cell number (typically 1 × 10⁶ cells/mL). For each subject, the lymphocytes at the 5 time points (days 1, 15, 29, 43, and 57) were recovered on the same day and time.

Alkaline single-cell gel electrophoresis

DNA strand breaks were measured in lymphocytes with the alkaline single-cell gel electrophoresis assay, ie, the comet assay, (20) with minor modifications. Endogenous DNA damage and hydrogen peroxide-challenged DNA damage were measured by exposing the agarose embedded with cells to either phosphate buffered saline alone or hydrogen peroxide (10 μmol/L) in phosphate buffered saline for 10 min.

Quantitation of DNA damage

DNA damage was measured by visual image analysis (21). The comet-like images were classified visually into 5 categories.
FIGURE 1. Visual classification of DNA damage, according to the relative proportion of DNA in the tail (scores 0–4), obtained by single-cell gel electrophoresis. Score 0 represents undamaged cells, and score 4 represents the most heavily damaged cells.

(0–4) by a blinded observer according to appearance, which resulted from the relative proportion of DNA in the tail, as shown in Figure 1. At least 100 cells were counted and categorized to avoid any selection bias. The percentage DNA in the tail [(2.5 × cells) + 12.5 × cells] of 30 × cells + 60 × cells + 90 × cells]/2 cells] was calculated to express the amount of DNA damage.

Statistical analysis

Values are expressed as means ± SEMs. Three subjects dropped out during the study because of personal reasons. When the randomization code was uncovered after completing the biochemical analyses, there were 6 subjects in the placebo group, 8 in the mixed carotenoid, lutein, and lycopene groups, and 7 in the β-carotene group. A repeated-measures analysis of variance with a post hoc Dunnett’s multiple comparison test was used to measure the effect of carotenoid supplementation on plasma carotenoid concentrations and to measure the endogenous DNA damage for each day with respect to day 1. The differences between the groups for each time point were measured by a one-way analysis of variance with Tukey’s multiple comparison. A paired t test was used to calculate the effect of low fruit and vegetable diets for 2 wks (day −14 compared with day 1). When an equal variance test failed, a Mann-Whitney rank sum test was used.

The mean (±SEM) concentration levels of α-tocopherol, ascorbic acid, uric acid, and characteristics of the study subjects are presented in Table 2. No significant difference in major antioxidant nutrients and lipid profiles was observed between the groups at baseline (day 1).

Plasma lutein concentrations were significantly increased on day 15 in the mixed carotenoids and lutein groups (P < 0.01), so that the values were 228% and 514%, respectively, of the baseline values (Figure 2). These concentrations were maintained throughout the study period. No significant increases in plasma lutein concentrations were observed in the placebo, β-carotene, and lycopene groups throughout the intervention period. The statistical analyses were performed with SIGMASTAT (version 11.5; SPSS Inc, Chicago, IL).

RESULTS

Changes in plasma carotenoid concentrations after low fruit and vegetable diets and carotenoid supplementation

The low fruit and vegetable diets the subjects consumed for 2 wk before starting the intervention resulted in decreases in plasma carotenoid concentrations, as shown in Table 1. Two weeks on a low fruit and vegetable diet reduced the subjects’ plasma carotenoid concentrations of lutein, β-carotene, and lycopene by 62%, 64%, and 58%, respectively (day −14 compared with day 1).

The mean (±SEM) baseline concentrations of α-tocopherol, lutein, β-carotene, and lycopene groups throughout the intervention period. The

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<td>Changes in plasma carotenoid concentrations in all the study participants after low fruit and vegetable diets for 14 d*</td>
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<td>Carotenoid</td>
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<td>Lycopene (µmol/L)**</td>
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* All values are x ± SEM; n = 37.
** A Mann-Whitney rank sum test was performed when an equal variance test failed.
1,2 Significantly different from day −14 (paired t test): *P < 0.001, **P < 0.01.

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<td>Anthropometric and biochemical characteristics of the study groups at baseline (day 1)*</td>
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* All values are x ± SEM.
1 Significantly different from placebo group, P < 0.01 (one-factor ANOVA with Dunnett’s multiple comparison test).
concentrations of plasma β-carotene were significantly increased in the mixed carotenoids and β-carotene groups after 15 d of supplementation (P < 0.01) so that concentrations were 146% and 387%, respectively, of the baseline concentrations. The placebo-, lutein-, and lycopene-supplemented groups did not show any significant increases in plasma β-carotene concentrations (Figure 3). Plasma lycopene concentrations were significantly increased in the lycopene group after 15 d of supplementation (P < 0.01), whereas the placebo, lutein, and β-carotene groups had significantly lower plasma lycopene concentrations during the intervention period than at baseline (P < 0.05) (Figure 4). The plasma lycopene concentrations of the mixed carotenoids group did not significantly change during the intervention period.

### Endogenous and hydrogen peroxide–induced DNA damage after carotenoid supplementation

The effects of carotenoid supplementation on DNA damage are shown in Table 3. Endogenous DNA damage at baseline was significantly higher in the β-carotene group than in the placebo group (P < 0.05). Compared with day 1, the amount of endogenous DNA damage was significantly decreased by day 15 in the mixed carotenoid (P < 0.01) and β-carotene (P < 0.05) groups and by day 57 in all of the carotenoid-supplemented groups (P < 0.01). The placebo group did not show any significant change in endogenous DNA damage throughout the intervention period. When DNA was challenged by hydrogen peroxide (10 μmol/L for 10 min), there were no significant time-by-treatment interaction and no changes over time or across treatment groups (Table 3).

### DISCUSSION

Epidemiologic evidence continues to accumulate that diets high in fruit and vegetables are associated with a reduced risk of chronic diseases, such as cardiovascular disease (5, 22–25). Carotenoids found in fruit and vegetables may have biological actions to prevent such diseases. It has been reported that supplementation with 24 mg β-carotene/d for 12 wk, or an equal amount of a carotenoid mixture containing lutein, β-carotene, and lycopene, ameliorates UV radiation–induced erythema in
In accordance with studies conducted by others (10, 28–31), plasma lutein, β-carotene, and lycopene concentrations were significantly and selectively increased to mean values of 0.90, 1.47, and 1.07 μmol/L, respectively, after 15 d of supplementation with 12 mg/d of lutein, β-carotene, or lycopene. Other carotenoid concentrations were either maintained or were lower than baseline concentrations. However, note that these increased carotenoid concentrations were much higher than 90% of those obtained in NHANES III (lutein, 0.67; β-carotene, 0.91; lycopene, 0.70 μmol/L) for the same age, sex, and ethnic group (50–70 y, non-Hispanic white women; n = 1017) as our study subjects. The plasma concentrations reached by supplementation with 12 mg individual carotenoids are generally not attained by diet alone. On the other hand, plasma carotenoid concentrations in the mixed carotenoid group, which received 4 mg each of lutein, β-carotene, and lycopene, reached 0.40, 0.50, and 0.57 μmol/L on day 15 for lutein, β-carotene, and lycopene, respectively. These concentrations are well within the 50th–75th percentile range of the values from NHANES III for the same age, sex, and ethnic group and are not difficult to attain with a diet that contains the recommended intakes of fruit and vegetables. Because of the low dose of the lycopene supplement compared with the usual lycopene intake of our study subjects, lycopene concentrations did not significantly increase with 4 mg lycopene supplementation in the mixed carotenoid-supplemented group.

Oxidative DNA damage is an ongoing process of aging (32–35) and is elevated in patients with disease (36). However, intervention trials that involved increased fruit and vegetable intakes have shown mixed results regarding DNA damage; ie, the studies either show decreased oxidative DNA damage (18, 28, 37–44) or no effect on DNA damage and repair (45).

It is difficult to compare DNA damage between studies because of the discordance of methodologic conditions, such as duration of alkali unwinding, electrophoresis, and visual scoring system (46). The present study applied the visual scoring system, as described by Collins et al (21). Visual scoring data accurately corresponds to the data that is measured by computer image analysis (16, 47). Another potential variable could be the use of fresh compared with stored cells. In the present study, the cryopreserved lymphocytes of each subject that were collected at 5 time points (days 1, 15, 29, 43, and 57) were analyzed at the same time for DNA damage to avoid experimental day-to-day variance. Duthie et al (48) reported that freezing lymphocytes does not increase DNA strand breaks compared with freshly isolated lymphocytes, and that fresh and frozen lymphocytes responded almost identically to hydrogen peroxide treatment.

In our study, there was detectable endogenous DNA damage at baseline (day 1) (11.0 ± 2.3% DNA in the tail), ie, after 2 wk of a low fruit and vegetable diet (<2 servings/d). It is plausible that the deprivation of fruit and vegetables could affect endogenous DNA damage. Furthermore, it has been reported that both endogenous DNA damage (49) and hydrogen peroxide–induced DNA damage (50) are positively associated with age. In addition, increased frequencies of micronuclei and chromosome aberrations with age have been reported, which suggests increased genetic instability with age (51). We also found that our older subjects showed higher endogenous DNA damage than did the younger subjects (data not shown). It is possible that the high endogenous DNA damage in our study subjects because of the low fruit and vegetable diet allowed for a significant improvement in endogenous DNA damage as early as day 15 of supplementation with either 12 mg/d of a single carotenoid or, perhaps more importantly, with a physiologic dose of mixed carotenoids.
The effects of carotenoid supplementation against hydrogen peroxide–induced DNA damage were weaker than were those shown for endogenous DNA damage. It is possible that carotenoids actively protect against endogenous DNA damage in vivo, whereas the protective effects are reduced ex vivo due to the loss of interactions with other antioxidants.

In contrast to the present study in which we found a significant decrease in endogenous DNA damage by supplementation with 12 mg of a single carotenoid, Collins et al (52) reported that there was no effect on endogenous DNA damage in men and women aged 25–45 y after a 12-wk supplementation with 15 mg/d each of α- and β-carotene, lutein, or lycopene in a placebo-controlled parallel study design. However, note that there was an inverse correlation between total serum carotenoid concentrations and oxidized pyrimidines in their study. On the other hand, another study that used lutein, β-carotene, or lycopene supplementation for 1 wk, in the same amounts as those in our study, showed significant increases in DNA repair in younger men and women aged 24–34 y, probably because of an antioxidant effect of carotenoids rather than a direct effect on the repair process (53). Intervention studies that used a combination of antioxidant supplements have consistently shown protective effects against DNA damage in accordance with the present study, even though different antioxidants and dosages were applied [eg, a combination of 100 mg vitamin C, 280 mg vitamin E, and 25 mg β-carotene (54); a combination of 8 mg lycopene, 0.5 mg β-carotene, and 11 mg vitamin C (29); and a combination of 8.2 mg β-carotene, 3.7 mg α-carotene, and 1.75 mg α-tocopherol (10)].

In conclusion, supplementation with a single carotenoid or a combination of carotenoids resulted in beneficial effects against DNA damage in older women. In particular, a physiologic dose of mixed carotenoids was protective against endogenous DNA damage in older women. Additional studies on the effect of physiologic doses of carotenoids, in combination with other antioxidants contained in fruits and vegetables, on oxidative DNA damage are needed to support the role of a diet high in fruit and vegetables in the prevention of chronic diseases such as cardiovascular diseases and cancer.

We thank the volunteers who participated in this study, Hong Wang for her assistance in measuring plasma carotenoids, Paul Jacques for making the NHANES III data available, and the Metabolic Research Unit and Nutrition Evaluation Laboratory staffs at the Jean Mayer US Department of Agriculture-Human Nutrition Research Center on Aging at Tufts University. We also thank the BASF AG for providing the carotenoid supplements. XZ helped with the comet assay, data interpretation, and manuscript preparation. GA set up the comet assay for the study with K-JY and assisted in the interpretation of the results. EJF supervised the plasma carotenoid measurements. HR was responsible for educating and monitoring the study participants and ensuring the compliance of the study participants. KK and HW were responsible for the formulation and safety of the carotenoid supplements. NM was responsible for manufacturing the carotenoid supplements. NIK and RMH helped design the study and interpret the results. K-JY designed and conducted the interventions and supervised the project and manuscript preparation. None of the authors had any advisory board affiliations or financial interest in any organization sponsoring the research.

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