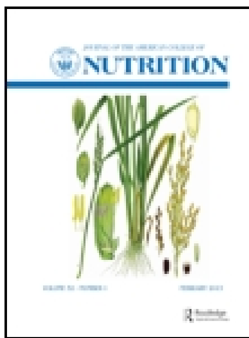

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Original Paper

The Effect of a Low Carotenoid Diet on Malondialdehyde-Thiobarbituric Acid (MDA-TBA) Concentrations in Women: A Placebo-Controlled Double-Blind Study

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Key words: carotenoids, human, oxidative damage, malondialdehyde

Objective: The purpose of the study was to evaluate the effect of a low carotenoid diet (83 μg β-carotene) on malondialdehyde-thiobarbituric acid (MDA-TBA) concentrations of nine pre-menopausal women.

Methods: Subjects lived on the metabolic research unit of the Western Human Nutrition Research Center (WHNRC), where diet, exercise and other activities were controlled. Five subjects (Group C, control group) consumed a low carotenoid diet and received an additional 0.5 mg/day of β-carotene while four subjects (Group P, placebo group) received only the low carotenoid diet during days 1 to 60 (period 1). All subjects received 0.5 mg/day of β-carotene during days 60 to 100 (period 2), plus three capsules/day mixed carotenoid supplement (Neo-Life Company) during study days 100 to 120. Changes in MDA-TBA concentrations were analyzed during the study periods and between the groups.

Results: At the start of the study (day 1), no significant difference in the MDA-TBA concentration was observed between the control (Group C) and the placebo (Group P) subjects. During period 1 (days 2 to 60), when Group P subjects consumed the low carotenoid diet without supplementation, the MDA-TBA values for Group P rose markedly and were significantly ($p<0.05$) higher than the MDA-TBA values for Group C subjects who were receiving carotenoid supplementation. During period 2 (days 60 to 100) when both groups received carotenoid supplementation, the MDA-TBA values of Group P subjects were significantly ($p<0.05$) reduced to the point where they were similar to the MDA-TBA values for Group C subjects.

Conclusions: These findings provide evidence to support the beneficial effects of carotenoids in preventing lipid peroxidation in the cells. Further studies are needed to identify the exact mechanism by which carotenoids prevent lipid peroxidation and the amount needed for normal activity.

INTRODUCTION

β-carotene is one of the most prevalent carotenoids found in foods. Traditionally, nutritionists considered β-carotene the carotenoid with the greatest importance because of its provitamin A activity. However, recent evidence has shown that β-carotene has additional biochemical functions that cannot be attributed to vitamin A. These functions include singlet oxygen quenching ability and antioxidant activity. β-carotene can also scavenge free radicals directly by acting as an antioxidant [1–5]. Carotenoids serve as a source of lipid-soluble antioxidants which are important in protecting lipid membranes against free radical damage.

Free radicals formed during normal or pathological biological processes can attack lipid membranes and initiate a chain reaction resulting in lipid peroxidation, leading to cell and

Abbreviations: MDA-TBA=malondialdehyde-thiobarbituric acid, HPLC=high performance liquid chromatography.

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tissue injury. This prompted researchers to investigate methods to limit damage from free radicals. Recently investigators studying free radical damage, have found that high-intake of carotenoid-rich foods decreases the risk of various diseases, including cancer, atherosclerosis, and aging [6–8]. Based on the association between the intake of carotenoid-rich foods and reduced risk of various types of chronic diseases including cancers, β-carotene has been proven to be an important antioxidant in humans [3,9–14].

For years, scientists used lipid peroxidation as an index of oxidative stress in biological systems [14–16]. Lipid peroxidation is one of the most important organic expressions of oxidative stress where unsaturated lipids, such as arachidonic acid, undergo a reaction with oxygen free radicals to yield lipid hydroperoxides. The consequence of lipid peroxidation is degradation of the membrane's polyunsaturated fatty acids, with a subsequent disorganization of membrane structure and disturbance in membrane function.

To get a clearer picture of lipid peroxidation in humans, scientists have described many chemical and physical methods to assess different chemical stages of the oxidative degradation of unsaturated fatty acids [17–20]. Methods used to assess metabolites of lipid peroxidation include measurement of malondialdehyde (MDA), ultraviolet (UV) absorption of conjugated dienes, fluorometry of lipofuscin-like substances, and measurement of ethane and pentane formations [1]. To date, because of its simplicity and reliability, assessment of MDA has become one of the most commonly used technique to measure degree of oxidative damage in biological samples [15,20,21].

Previous work [13] suggested that low carotenoid diet increased susceptibility to oxidative damage in humans. However, a potential problem with that study was that all the subjects were fed the low carotenoid diet at the same time; there was no control group. Therefore, we have completed a second study, which was a placebo-controlled, double-blind study of carotenoid depletion in pre-menopausal women.

MATERIALS AND METHODS

Subjects

Twelve healthy, normal-weight pre-menopausal women, aged 23 to 43 years, were selected to participate in the study. Because of the difficulty of recruiting and retaining participants, two subjects who were on a different schedule from the main group and one other individual did not complete the study. Participants lived in the metabolic research unit (MRU) of the United States Department of Agriculture (USDA), Western Human Nutrition Research Center (WHNRC), Presidio of San Francisco, CA, for 120 days. The study was restricted to women because the communal living arrangements of the Center make mixed sex studies impractical. Procedures for this study were approved by the Human Subjects Review Committee of the University of California, Davis, and the USDA Review Committee at Tufts University and conformed to Helsinki Conference guidelines for research on human subjects.

Research Design

Subjects were randomly assigned to one of two groups (Fig. 1), the placebo group (group P) and a control group (group C). Group selection was done in a double-blind fashion. The chief dietitian divided the subjects into groups using a random number generator and was the only one who knew which subjects were in the placebo and the control groups, respectively. In addition, she had the responsibility of making sure the assigned subjects received the placebo or the β-carotene capsule. Four subjects (group P) were fed a low carotenoid diet containing 83 μg/day of carotenoids (approximately 90% as β-carotene, based on HPLC analysis) plus carotene-free placebo beadlets (lot 312581, Hoffmann LaRoche Inc., Nutley, NJ) for 60 days. The other five subjects (group C), were fed the same low carotenoid diet supplemented with 0.5 mg β-carotene/day (Dry Carotene beadlets, lot 14240; Hoffmann LaRoche Inc.) for 60 days. The placebo and the β-carotene beadlets are identical in composition and formulation with the exception of β-carotene. Dry β-carotene beadlets (10%) composed of beadlets of β-carotene compounded with gelatin, sucrose, food starch and peanut oil. Ascorbyl palmitate and α-tocopherol were added as antioxidants. During study days 60 to 100, all subjects received the diet plus 0.5 mg β-carotene/day from dry beadlets. During the final 20 days (days 100 to 120) of the study, both groups were supplemented with 0.5 mg β-carotene/day from dry beadlets plus carotenoids from three capsules/day of mixed Carotenoid Complex® (Neo-Life Company of America, Fremont, CA; Table 1).

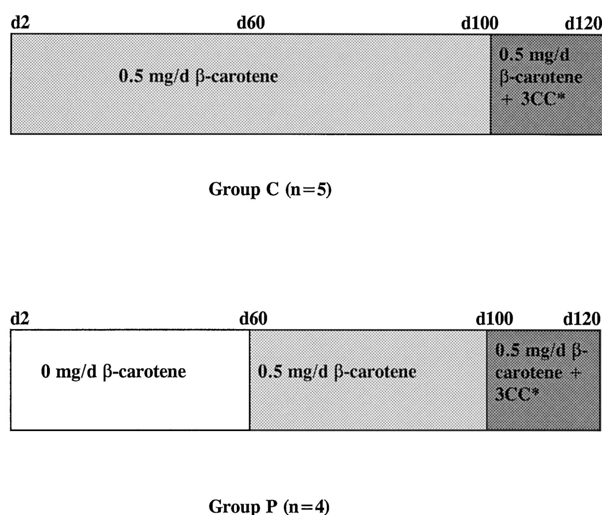

Fig. 1. 3CC*=three capsules of Carotenoid Complex® from Neo-Life Company of America.

Table 1. Measured Carotenoid Concentrations of Carotenoid-Complex®[1] Capsule

Carotenoid type	mg/capsule	mg/d supplement
α-carotene	0.466	1.393
β-carotene	1.102	3.306
β-cryptoxanthin	0.039	0.117
Lutein/zeaxanthin	0.497	1.491
Lycopene	0.221	0.663

[1] Neo-Life Company of America, Fremont, CA.

Subjects' Diet and Exercise

All meals were consumed under observation, with subjects leaving the facility only with an escort to ensure absolute compliance. Subjects ate natural foods low in carotenoid-rich fruits, vegetables and their juices. Foods were prepared mostly by baking, boiling or microwaving. Canned and frozen foods were bought in manufacturers' lots to ensure that the macro- and micronutrient composition of the diet did not change significantly throughout the study. The percentages of macronutrients in the diet were 55% carbohydrate, 14% protein and 33% fat (with a polyunsaturated/saturated fat ratio of 0.92 by data-base analysis). Four daily meals were served in a 6-day rotational pattern. Mean dietary intake for the subjects is presented in Table 2. Subjects were given daily vitamin and mineral supplements to maintain intake of at least 100% of the US RDA for all nutrients except carotenes (Table 3). Iron was supplemented at a relatively high concentration because participants in our metabolic unit studies often develop low hematocrit and hemoglobin concentrations, presumably because of the amount of blood collected during these studies. Subjects followed a mild exercise program, consisting of two, 2-mile walks per day which corresponded more closely to a free-living life style than a sedentary existence on a metabolic unit.

Table 2. Mean Dietary Intake for Women on Low Carotenoid Diet

Nutrient	Mean±SD
Energy (kcal)	2100.24±1.3
Protein (g)	75.98±3.0
Fat (g)	75.96±0.9
Carbohydrate (g)	287.35±4.2
Cholesterol (mg)	183.60±20.5
Fiber (g)	12.35±4.5
Calcium (mg)	859.02±121.2
Iron (mg)	18.15±6.7
Magnesium (mg)	275.47±45.9
Phosphorus (mg)	1305.84±240.9
Zinc (mg)	9.48±1.3
Copper (mg)	1.68±0.7
Manganese (mg)	5.57±0.7
Potassium (mg)	2562.33±308.5
Sodium (mg)	2737.81±1079.9
Vitamin A (RE)	470.66±187.20
Thiamine (mg)	1.56±0.3
Riboflavin (mg)	1.86±0.1
Niacin (mg)	21.76±4.2
Vitamin B_6 (mg)	1.76±0.4
Vitamin B_{12} (mg)	3.15±0.6
Vitamin C (mg)	86.45±40.8
Vitamin E (mg)	3.09±1.1

Table 3. Micronutrients Intake from Supplement and Diet for Women on Low Carotene Diet

Nutrient	Supplement	Diet	Total	RDA[1] value
Vitamin A (RE)	320	471±184	791±184	800
Vitamin C (mg)	____[2]	86±40	86±40	60
Vitamin E (mg)	11.1	3.1±1.1	14.2±1.1	8
Iron (mg)	18.0	18.2±6.7	36.2±6.7	15
Zinc (mg)	15.0	9.5±1.3	24.5±1.3	12

[1] RDA=Recommended Dietary Allowance.
[2] ____No supplement given.

Sample Collection and Preparation

Thirty ml samples of blood were collected from each subject on days 2, 3, 4, 9, 16, 23, 37, 46, 60, 61, 62, 64, 67, 74, 81, 100, 101, 102, 106, 113, and 120 after an overnight fast. Samples were processed by methods previously reported [13]. Samples were stored at −70°C and delivered in dry ice to Florida International University (FIU), Miami, FL for MDA-TBA analysis.

Determination of MDA-TBA

Plasma samples delivered from WHNRC were thawed and processed immediately for MDA determination by a modification of a previously published method [21]. MDA levels were determined by the MDA-TBA test which is the colorimetric reaction of malondialdehyde and thiobarbituric acid in acid solution. After the formation of the MDA-TBA complex, High Performance Liquid Chromatography (HPLC) was used to assess the concentration of the complex based on a known standard curve. The MDA-TBA standard provided by Dr. Draper (Department of Nutritional Sciences, University of Guelph, Ontario), was prepared by the method of Sinnhuber et al 1958 [22] and was used to calculate the estimated concentration of MDA-TBA.

HPLC Analysis

Analyses of standards and plasma were performed on a Perkin Elmer HPLC equipped with a LDC/Milton Roy recorder (Series 410), SCO detector (V4 absorbance detector) and a C_{18} μBondapak HPLC stainless steel column (3.9×300 mm, 10 μm particle size) with a Direct-connect™ Refillable guard column. The eluate was monitored at 532 nm at an AUFS of 0.005. Dried samples were individually dissolved in 250 μl of HPLC-grade water, and an aliquot of 20 μl was applied to the HPLC column. The mobile phase consisted of acetonitrile (15%) and 0.6% tetrahydrofuran (THF) in 5 mM phosphate buffer (85%).

Statistical Analysis

The student paired t test was used to compare differences in plasma MDA-TBA concentrations within the sampling periods and between the groups. Comparison of mean values between the two groups was conducted using analysis of variance [23]. Differences associated with $p<0.05$ were regarded as statistically significant. Results are reported as means±SD.

RESULTS

Effects of β-Carotene Depletion and Repletion on MDA-TBA Levels

Data from the study showed no significant difference in MDA-TBA values between groups on the initial day (day 1, baseline) of the study. For group P subjects (subjects who received the placebo beadlets in study period 1 and additional carotenoid supplements in study period 2), there was a significant ($p<0.05$) decrease in MDA-TBA concentration during study period 2 (Table 4). There was no significant change for the control (group C) subjects, even though levels of MDA-TBA decreased as the subjects were supplemented with more carotenoids.

When we compared mean MDA-TBA levels between subjects in groups P and C (Table 5), the only significant difference ($p<0.03$) observed was during period 1 when the control subjects received an additional 0.5 mg/day of β-carotene, while the placebo group received carotene-free placebo beadlets.

DISCUSSION

According to previous studies [19,21], plasma MDA-TBA concentrations in normal humans vary from 0.12 to 1.71 μmol/L. MDA-TBA levels were reported to be higher, up to 3.7 μmol/L in patients with diabetes or stroke [24,25]. In our investigation, MDA-TBA remained in the normal range of 0.15 to 0.69 μmol/L.

In our study, we observed higher levels (for group P subjects) of plasma MDA-TBA during the carotenoid depletion period (period 1) followed by a decrease after repletion with carotenoid. Thus, carotenoid appears to inhibit formation of secondary oxidative products.

Table 4. Mean Levels of MDA-TBA[1] Concentrations during the Two Study Periods for the Two Groups

Group	Day 2 to 60 (Period 1)	Day 60 to 100 (Period 2)
Group P (n=4)	0.252±0.095[b]	0.195±0.061[a]
Group C (n=5)	0.195±0.038[a]	0.187±0.038[a]

[1] Values are means±SD μmol/L

[a,b] Mean values in the same row with different superscripts are significantly different ($p<0.05$).

Table 5. MDA-TBA[1] Changes Between the Two Groups during the Study Periods

Period	Groups		p value
	P	C	
Baseline	0.336±0.071	0.339±0.078	0.953
1	0.410±0.153	0.351±0.063	0.030
2	0.360±0.230	0.341±0.100	0.684

[1] Values are means±SD μmol/L.

We also found that supplementing the placebo group (group P) with low doses of carotenoid had a greater lowering effect on their plasma MDA-TBA levels as compared to the subjects (group C) that were not depleted. Long-term low-dose supplementation (for group C subjects) seemed to have no significant effect on MDA-TBA levels. A possible reason for this could be that the MDA-TBA levels are under some type of feedback control mechanism that helps the body keep MDA-TBA at an optimum level; which has been reported to be necessary for the biosynthesis of prostaglandin [19]. However, there is not enough evidence to support the theory of a feedback mechanism thus far. Our results suggest that the preventive function of carotenoid will be more effective when subjects are in a depleted condition or, are suffering from some forms of disease caused from lipid peroxidation.

A recent study [26], in which humans ate large concentrations of β-carotene, showed little or no improvement in oxidative status. Our studies [13, this report] also show no benefit from supplementing the diet with large amounts of carotenoids, suggesting that carotenoid might not be as beneficial for individuals who are consuming a healthy diet. It is noteworthy that this study suggests that relatively low intakes of β-carotene are required to maintain MDA-TBA levels at normal concentrations. The amount of β-carotene fed to the control group in the study (approximately 0.6 mg/day) is considerably less than the average estimated dietary intake of β-carotene consumed by US adults (Advanced, NHANES III, approximately 2 to 4 mg/day).

CONCLUSION

This study provides evidence to support the hypothesis that carotenoids can significantly decrease levels of plasma MDA-TBA and also supports the beneficial effects of carotenoids in the diet which can prevent lipid peroxidation in the cells. Further studies are needed to identify the exact mechanism by which carotenoids prevent lipid peroxidation. In conclusion, carotenoids are necessary even when the subjects consume a diet that contains RDA levels of "preformed" vitamins A, E and C.

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