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SERUM AND MACULAR RESPONSES TO ANTIOXIDANT SUPPLEMENTATION VERSUS A CAROTENOID-RICH DIETARY INTERVENTION IN THE ELDERLY

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ABSTRACT: *The aim of this study was to observe responses of serum antioxidants, oxidative stress biomarkers, and macular carotenoid pigments to antioxidant supplements or dietary intervention in a single-masked, randomized, pilot clinical study of elderly subjects receiving antioxidant supplements or a dietary intervention. Methods: From a pool of ninety-eight community volunteers, forty-eight male and female subjects (age 65-85) with the lowest baseline serum lutein + zeaxanthin levels were selected and randomly assigned to receive for 12 weeks one of two different antioxidant supplements or a diet rich in fruits and vegetables containing approximately matched levels of four classes of carotenoids: carotenes, the xanthophylls lutein and zeaxanthin, and lycopene. Forty-six completed the study. Both supplements and diet also were rich in vitamins C and E. Outcome measures were changes from baseline: 1) in serum levels of antioxidant micronutrients (vitamins C and E, lutein, zeaxanthin, and β carotene); 2) in levels of indicators of oxidative stress: serum lipid peroxides (LPO) and urinary 8-hydroxydeoxyguanosine (8-OHdG); and 3) macular pigment, measured by heterochromatic flicker photometry. Results: Interventions to promote eye health by either diet or supplementation showed consistent serum responses, with substantial improvements within twelve weeks. Serum lutein and vitamin C increased for all groups ($p < 0.05$), and lipid peroxides decreased for all subjects ($p < 0.05$); nonetheless, mean macular pigment did not increase significantly during the designated timeframe. Conclusion: Supplementation of elderly individuals potentially at risk for AMD with two different types of antioxidant formulations exhibited positive serum responses similar to a dietary intervention containing approximately comparable levels of carotenoids. This pilot study indicates that the ocular supplements tested in this study elicited responses in serum parameters similar to daily consumption of four servings of carotenoid-rich fruits and vegetables. The study also provides useful criteria for the design of larger-scale and longer-term studies of antioxidant supplementation in an elderly population potentially at risk for AMD.*

KEYWORDS: Antioxidants, Lutein, Macula, Oxidative stress, Serum carotenoids, Zeaxanthin

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INTRODUCTION

Age-related macular degeneration (AMD) is the leading cause of blindness in adults over the age of 65 in developed nations (Age-related Eye Disease Study (AREDS), 2001; Klaver et al., 1998). Treatments for AMD, especially for the "wet" or exudative form, have improved dramatically in recent years, but a large percentage of AMD patients still end up with severe visual loss. Therefore, much research has focused on prevention through identification of modifiable risk factors, such as diet, smoking, and light exposure. Among dietary risk factors, antioxidant status has been inversely correlated with risk for advanced AMD and age-related cataracts (AREDS, 2001; Klaver et al., 1998; Seddon et al., 1994; Delcourt et al., 1999; and Gale et al., 2001), although not all researchers have confirmed this relationship (Mares-Perlman et al., 1995; Smith et al., 1999; and Taylor et al., 2002).

In order to resolve these disparities, the National Eye Institute (NEI) organized a large-scale, prospective, clinical trial conducted by the Age-Related Eye Disease Study (AREDS) group to determine the effects of antioxidant intervention on the progression of AMD (AREDS, 2001). The trial established that individuals in the two most advanced AMD categories supplemented with a combination of zinc and antioxidants had an approximately 25% reduction in risk for progressing to advanced AMD (AREDS, 2001). The trial, however, did not address all of the dietary antioxidants that may affect macular health. The xanthophyll carotenoids lutein (L) and zeaxanthin (Z), found in dark green leafy vegetables, and orange or yellow fruits and vegetables, have been linked epidemiologically and anecdotally with a decreased risk for AMD (AREDS, 2001; Seddon et al., 1994; Moeller et al., 2000; Mares-Perlman et al., 1999; Schlach et al., 1999; Snodderly et al., 1995; Landrum et

al., 2001; and Schlach et al., 2002). Lutein and zeaxanthin are selectively concentrated in the macula where their capacity, observed *in vitro*, to filter blue light (Junghans, et al., 2001) and remove reactive oxygen species (Sujak et al., 1999) is believed to protect sensitive retinal tissues.

Research has shown that levels of macular pigment can be increased gradually in responders when lutein intake is increased in young healthy individuals, either via a nutritional supplement (Landrum et al., 1997; Berendschot et al., 2000) or increased consumption of foods high in lutein (Hammond et al., 1997). Comparatively little research has been done to assess the physiological effects of supplementation in the elderly with lutein alone or in combination with other antioxidant vitamins and minerals, although several recent investigations have provided support for the epidemiological projections (Bone et al., 2003; Nelson et al., 2003; Richer et al., 2004), and a recent review summarizes the current understanding (Bernstein et al., 2003).

Given the likely importance of xanthophylls in maintaining macular health, it would be helpful to investigate them in a manner similar to the AREDS trial. Before such a trial should be undertaken, however, an adequate dose of lutein, the approximate timeframe for tissue response, as well as the appropriate mixture and chemical forms of supplementary antioxidants, need to be evaluated to determine bioavailability since nutrients that act in one manner when given alone, may act differently when ingested with other nutrients (Groff et al., 1999). Also, it is important to monitor tissue response to lutein supplementation in the macula noninvasively during a clinical trial because serum response to supplementation does not always correlate well with macular levels (Landrum et al., 1997; Berendschot et al., 2000; Hammond et al., 1997). We compared the efficacy in an elderly population of two dosage forms of ocular antioxidant supplements containing different amounts of lutein and zeaxanthin and varying complements of alternate antioxidant micronutrients, relative to a dietary intervention rich in carotenoids from four daily servings of fruits and vegetables. The evaluated responses included serum carotenoid levels, macular pigment density, antioxidant micronutrients, and biomarkers of oxidative stress. Since supplements offer a low-risk source of antioxidants – free of contamination from heavy metals, organics, pesticides and bacterial load – the question to be resolved was whether the particular matrix (vegetables themselves, dosage form, or supplement source) influenced the nutritional and prophylactic value for an elderly population.

METHODS

This study was a single-masked randomly assigned, clinical trial of the responses of serum and macular pigment to dietary supplementation. Initial values of each group served as its own control. We tested two prototype antioxidant supplements formulated to promote eye health but differing in composition, stability, and bioavailability (Table 1). Our control group did not take supplements but consumed a diet rich in carotenoid-containing fruits and vegetables. Although, a fourth group receiving only a placebo or no intervention would have been ideal, we found in a previous study (Nelson et al., 2003) that the presence of such a group proved to be a severe barrier to subject recruitment, so it was omitted. The three groups were assigned the following regimens: 1) a supplement capsule at two per day (Capsule group; manufactured by Natural Alternatives International, San Marcos, CA); 2) a supplement tablet at two per day (Tablet group; manufactured by J. B. Laboratories, Holland, MI); and 3) a dietary intervention designed to provide levels of carotenoids (β-carotene, xanthophylls, and lycopene) similar to those provided by the supplements (Diet group). Lutein and zeaxanthin in the supplements were derived primarily from marigolds and provided as beadlets of free alcohol (FloraGlo® Lutein, Kemin Health, Des Moines, IA). Monitored responses included serum and urine levels of oxidative stress, serum levels of antioxidants including carotenoids, vitamins C and E, and macular pigment.

Table 1. Supplement Content for Capsule and Tablet Formulations*

	Units	Supplement Capsule		Supplement Tablet	
		per capsule	per day	per tablet	per day
Lutein and Zeaxanthin[1]	mg	3	6	2	4
β- Carotene	IU	2,000	4,000	3,300	6,600
Lycopene	mg	0.25	0.50	-	-
Vitamin C (ascorbic acid)	mg	45	90	200	400
Vitamin E (α-tocopherol)	IU	9	18	75	150
Vitamin B-1 (thiamin)	mg	1.125	2.25	-	-
Vitamin B-2 (riboflavin)	mg	1.3	2.6	5	10
Vitamin B-3 (niacinamide)	mg	10	20	-	-
Vitamin B-6 (pyridoxine)	mg	1.5	3	-	-
Vitamin B-12	mg	0.0005	0.001	-	-
Folic Acid	mg	0.1	0.2	-	-
Biotin	mg	0.075	0.15	-	-
Pantothenic Acid	mg	7.5	15	-	-
Zinc acetate	mg	-	-	30	60
Copper	mg	-	-	2	4
Manganese	mg	-	-	5	10
Selenium	mg	0.025	0.050	0.02	0.04
Grape Seed Extract	mg	5	10	-	-
Spinach Leaf Powder[2]	mg	50	100	-	-
Green Tea Extract	mg	50	100	-	-
Bilberry extract	mg	20	40	-	-
Blueberry Fruit Powder	mg	25	50	-	-
Lemon Powder	mg	125	250	-	-
Peach fruit powder	mg	20	40	-	-

[1] Contains 95 ± 2 % lutein and 5 ± 2 % zeaxanthin according to the manufacturer's specifications.
[2] Supplies approximately 30 micrograms of lutein per capsule.
* Subjects in the diet group consumed approximately 6 mg lutein, 0.6 mg zeaxanthin, 10 mg lycopene, and 8 mg of β-carotene for 5 of 7 days per week.

Sample size

The number of study subjects needed to obtain significance was determined using a power analysis (SPSS, 2.03 ed.) with a projected difference of 40% in serum responses for plasma lutein, zeaxanthin, and lipid peroxides (LPO) from baseline, an alpha of 0.05, and a power of 0.80. The analyses were performed on reference means and standard deviations for a population of individuals over the age of 65 and were based on existing literature data for the second most important key indicator, serum lutein levels, since not enough data existed to utilize the most critical indicator, the level of carotenoids in the macula. Fifteen test subjects per group were determined to be sufficient to detect a 40% change in serum lutein levels from baseline.

Sample selection

Our intent was to select for the study individuals potentially at risk for macular degeneration based upon serum carotenoid levels. Ninety-eight volunteers were recruited from a local geriatric population (ages 65-85). These subjects were recruited using advertisements, flyers, a Moran Eye Center volunteer database, and word of mouth. Subjects' fasting blood samples were prescreened for serum lutein and zeaxanthin levels. Prescreening assays were performed by an outside reference laboratory (Genox, Baltimore, MD). The 48 subjects with the lowest combined serum levels of lutein and zeaxanthin (L + Z) were selected for the full 12-week study. The study was approved by the University of Utah Institutional Review Board (IRB #8752-01), and informed consent was obtained from each subject.

Exclusion criteria included subjects continuing on supplements, cholesterol-lowering medications that affect lipid metabolism (*e.g.*, Cholestyramine), or lipid-binding medications (*e.g.*, Orlistat). Subjects who were taking 3-hydroxy-3-methylglutaryl (HMG)-CoA reductase inhibitors, such as Lipitor, were not excluded from the study. People with renal disease were excluded. Subjects on medications were required to be stable for at least 5 weeks prior to the study. Subjects were allowed to take calcium and other non-antioxidant supplements. Subjects with poor pupillary dilation or with significant cataracts, exudative AMD, geographic atrophy, or diabetic macular edema, were also excluded.

Data Acquisition

Following overnight fasting, 10 ml of antecubital venous blood was withdrawn from each of the 98 volunteers. The blood was collected in serum-separator tubes, refrigerated, and centrifuged at 1500 x g for 15 minutes. Serum was pipetted in 1.5 mL aliquots into four cryotubes and stored frozen at –80°C until they were shipped to reference laboratories on dry ice for analysis.

Serum samples were analyzed for lutein (L) and zeaxanthin (Z) concentrations. Forty-eight subjects with the lowest combined L + Z levels, the fraction of the population presumed to be at greatest risk, were selected for the full 12-week study since current methodology used to assess macular pigment levels does not differentiate between L and Z.

The selected subjects were randomly assigned to three different groups using a computerized randomization program, and then each group was randomly assigned to receive either one of two ocular antioxidant formulations or to take part in the dietary intervention. The supplements differed with respect to the number of antioxidants, the amounts of antioxidants, the amounts of lutein and zeaxanthin, and the number of other excipients that may have been added (Table 1). The dietary intervention was designed to provide levels of lutein and zeaxanthin comparable to the supplements, 25-30 mg of total carotenoids per day. Patients selected daily from each of four categories of carotenoid, and thereby approximated the amounts of β-carotene, lutein and zeaxanthin received from the supplements. Selections from Category 1 (green fruits and vegetables) provided an average of 6 mg of lutein and 0.2 mg of zeaxanthin, from Category 2 (red fruits and vegetables) an average of at least 10 mg of lycopene, from Category 3 (yellow fruits and vegetables) an average of 0.4 mg of zeaxanthin, and from Category 4 (orange fruits and vegetables) an average of 8 mg of β-carotene per serving.

After a 2-week washout period, subjects brought a 24-hour urine collection to the Moran Eye Center. The urine samples were pipetted into 4 different 1 mL cryotubes and frozen at -80°C until they were shipped for analysis. Genox Laboratories (Baltimore, MD) analyzed serum and urine samples. Subjects had their visual acuity checked and received a dilated eye exam from an ophthalmologist. Subjects also had their macular pigment measured via heterochromatic flicker photometry (HFP) if their visual acuity was at least 20/50.

Subjects then received 12-weeks worth of pills if they were in a supplement group. Subjects were instructed to take the pills twice daily with food to maximize absorption. Notebooks were provided so that subjects could keep track of when they took their pills. If one pill was missed, subjects were instructed to take two pills the next time. If an entire day's worth of pills were missed, subjects were instructed to skip them and continue as normal from that point on. They were also instructed to record any fruits or vegetables that they ate for the entire study in these notebooks. All subjects kept detailed 3-day food records immediately prior to and after completing the study to ascertain what foods they were habitually eating and to confirm that there were no major changes in dietary habits over the course of the study beyond the fruit and vegetable intervention.

Due to the long duration of the study, subjects in the diet group were asked to eat fruits and vegetables from the lists only 5 of the 7 days per week. They were also given notebooks to record their participation in the study, and were instructed to record which foods they chose from the lists, how they were prepared, how much they ate and which days they were "off diet." Subjects were also asked to record any other fruits or vegetables they ate during this time. Subjects received phone calls every 3 weeks during the study to encourage compliance and to check for any problems.

At the end of the 12 weeks, subjects returned to the Moran Eye Center. They brought another 24-hour urine collection and again had blood drawn. They also turned in their notebooks of food intake records at this time. Subjects had their macular pigment measured again by HFP.

Sample analysis

Serum was analyzed by the reference laboratory (Genox, Baltimore, MD) for lutein, zeaxanthin, α? and β-carotene, β-cryptoxanthin, lycopene, α? and γ-tocopherol, vitamin C, and LPO. Urine was analyzed for creatinine and 8-OHdG. The hydrophobic carotenoids and tocopherols were measured together. One-hundred mL of serum were taken from each sample. Proteins in the serum were denatured with ethanol. The carotenoids and tocopherols were extracted with hexane and analyzed by reverse-phase HPLC with an isocratic mobile phase. A photodiode array was used to measure carotenoids, and tocopherols were detected with fluorescence (Hammond et al., 1997; Clinton et al., 1996; Dorgan et al., 1998; Khachik et al., 1995; Murakashi et al., 1992).

Vitamin C and urine creatinine were analyzed using a spectrophotometric assay performed on a robotic chemical analyzer (McGown et al, 1982; Bowers et al., 1980).

Urine samples were clarified if necessary by centrifuging urine at 2,000 x g for 10 minutes (for opaque samples only; clear samples were not centrifuged) prior to analysis for 8-OHdG. A primary 8-OHdG monoclonal antibody was added to a solution composed of either sample or standard. That solution was added to a microtiter plate to which 8-OHdG had been bound. The primary antibody bound competitively to either 8-OHdG in solution or to 8-OHdG bound to a microtiter plate. Higher concentrations of 8-OHdG in solution led to reduced binding of the primary antibody to the 8-OHdG on the plate. The amount of bound primary antibody was quantified colorimetrically by reacting it with a secondary antibody that could be activated with a reagent. The kit is available commercially (8-OHdG ELISA Kit GC, Genox laboratories Baltimore, Maryland 21230).

Lipid hydroperoxides (LPO) were analyzed at Genox using a commercial test kit. Briefly, in the presence of hemoglobin, lipid hydroperoxides were reduced to hydroxyl derivatives (lipid alcohols) and the 10-*N*-methylcarbamoyl-3,7-bis (dimethylamino)phenothiazine (MCDP) chromogen was oxidatively cleaved to form methylene blue in an equal molar reaction. Lipid peroxides were quantitated colorimetrically by measuring the methylene blue at 675 nm.

Macular Pigment Measurements

Snodderly et al. have described the Heterochromatic Flicker Photometry (HFP) psychophysical method of measuring macular pigment at considerable length (Snodderly et al., 1999). Briefly, a stimulus of short-wavelength light close to the absorbance peak of macular pigment (460 nm) is alternated with middle-wavelength light (560 nm), which is not absorbed by the macular pigment. The flickering stimulus is focused on the foveal center where macular pigment reaches its maximum concentration, and then to the parafovea where macular pigment is at a very low level. The subject can adjust the relative luminance of one light source, eliminating the flicker. The difference between the foveal and parafoveal sensitivities to blue light is used as a measurement of macular pigment density (Snodderly et al., 1999). HFP can yield reproducible results and show good retest reliability (Snodderly et al., 1999). However, some subjects find the task of HFP difficult to perform, particularly those with poor visual acuity (Snodderly et al., 1999; Curran-Celantano et al, 2002). The instrument used in these studies was a Bone and Landrum flicker photometer, model number FPM302'. The foveal spot size was 1.5° with a flicker rate of 32 Hz, and the peripheral reference point was at 7° with a flicker rate of 15 Hz.

Statistical Analysis

Data were analyzed using SPSS for Windows, version 10.0 (SPSS, Chicago, IL). Parametric statistical tests were used to analyze these data. Power analyses were conducted using Sigma Stat for Windows, version 2.03. A paired t-test was used to determine changes from baseline within groups, and an analysis of covariance (ANCOVA) was used to assess the significance of differences between groups, with pre-test scores put in as the covariate (Green et al., 2000). A two-way ANCOVA was used to determine differences between groups for males and females and for those with AMD and those without, with the pre-test scores put in as a covariate (Green et al., 2000). Alpha was adjusted using Holm's Sequential Bonferroni Procedure to correct for multiple comparisons of the data (Green et al., 2000). Pearson's r test was used to assess relationships between variables (Green et al., 2000).

RESULTS

Subject Characteristics

Information from the selected 48 subjects was initially collected with respect to age, gender, incidence of eye disease, incidence of diabetes, height, weight, body mass index (BMI), percent body fat measured by a bioelectric impedance analyzer (Tanita Corporation, Arlington Heights, IL), smoking behavior, and iris color. Table 2 compares the characteristics of the individuals participating in the three treatment groups. The Diet group was significantly heavier and had a higher BMI. There were no other statistically significant imbalances between the groups.

Table 2. Subject Characteristics

	Capsule(n = 16)	**Tablet**(n = 16)	**Diet** (n = 16)
Males	7	10	7
Females	9	6	9
Age (Mean ± SD)	70.7 ± 5.4	72.5 ± 4.1	71.1 ± 5.6
Age range (years)	20	20	20
Current smokers	0	0	1
Eye color			
Blue	4	3	9
Green/Hazel	4	7	4
Brown	8	6	3
Patients with AMD (dry, wet)	4 (4, 0)	3 (1, 2)	3 (3, 0)
Patients with diabetes	1	5	2
Pseudophakic patients	4	5	4
Height (cm)	172.5 ± 10.1	174.0 ± 10.9	175.0 ± 7.1
Weight (kg)	80.4 ± 13.6	80.6 ± 16.4	89.0 ± 16.9[1]
Body Mass Index (BMI)	26.9 ± 3.4	26.8 ± 4.1	29.1 ± 5.1[1]
Percent body fat	33.2 ± 9.2	30.8 ± 8.5	34.7 ± 11.7

[1] Diet group participants significantly different than the other two groups, $p < 0.01$.

Of the 48 subjects selected for the study of the two supplements and the carotenoid-rich diet, 46 (96%) completed the full 12-week intervention. One subject (assigned to the Tablet group) suffered a progression in his pre-existing AMD within the first few weeks, and he decided to seek medical attention rather than continue participation in the study. The other subject (assigned to the Diet group) suffered a fall and broke her humerus, and she was unable to complete study procedures.

Compliance with the interventions, as measured by the individuals' records of consumption and serum carotenoid responses, was high for all treatments. Both supplement groups reported an average of 95% compliance with the tablet or capsule regimens. The compliance for the diet group amongst the four categories was high: 94% in the green category, 119% in the red, 96% in the yellow, and 103% compliance in the orange category. (Percentages greater than 100% indicate that the subjects consumed more than the required amounts of fruits and vegetables.)

The Diet group consumed significantly more servings of fruits and vegetables than the other subjects, 4.8 servings per day, compared to 2.8 servings per day ($p < 0.001$). However, the Diet group consumed less total L + Z on average per day (2.5 mg) than the supplement groups when supplement amounts were included along with their dietary intakes as determined from the food intake diaries (Capsule = 6.9 mg/d, Tablet = 4.9 mg/d) ($p < 0.01$). While the Diet group exhibited excellent compliance, their daily selections of foods containing L + Z did not achieve the goal of 6 mg/d.

Serum carotenoids

The mean serum levels and standard deviations for carotenoids pre- and post- intervention for all groups are listed in Table 3. Serum lutein levels were increased for all groups, regardless of the intervention ($p < 0.05$) with a somewhat greater response observed in the Tablet group; however, zeaxanthin levels were not significantly increased for any group. Combined lutein and zeaxanthin (L + Z) levels did not increase significantly pre to post for any group, but were significantly increased ($p < 0.05$) when all subjects were combined (Total). The Tablet group doubled its β-carotene level in response to supplementation, while responses were more modest in the other two groups. Only the Diet group exhibited significant elevations in lycopene and in β-cryptoxanthin.

Antioxidant vitamins

The effects of the interventions with vitamin C and the tocopherols also are listed in Table 3. All groups had a significant increase in serum vitamin C levels. This was observed both within groups ($p < 0.01$) and for all subjects ($p < 0.01$). Only the Tablet group showed any significant increases in α-tocopherol ($p < 0.01$). The Tablet group was also the only group to show a significant decrease in γ-tocopherol levels ($p < 0.05$).

Table 3 Serum Levels of Carotenoids and Antioxidant Vitamins Pre- and Post- Intervention (mg/mL)

Carotenoids & Vitamins	Capsule (n=16) (Mean ± SD)		Tablet (n=15) (Mean ± SD)		Diet (n=15) (Mean ± SD)		Total (n=46) (Mean ± SD)	
	Pre-	Post-	Pre-	Post-	Pre-	Post-	Pre-	Post-
Lutein (L)	0.11 ± 0 .04	0.16 ± 0.08[1]	0.11 ± 0.04	0.22 ± 0.13[1]	0.12 ± 0.03	0.16 ± 0.16[1]	0.11 ± 0.04	0.18 ± 0.10[1]
Zeaxanthin (Z)	0.04 ± 0.02	0.03 ± 0.01	0.04 ± 0.01	0.04 ± 0.02	0.05 ± 0.02	0.04 ± 0.02	0.05 ± 0.02	0.04 ± 0.02
L + Z	0.15 ± 0.05	0.19± 0.10	0.16 ± 0.05	0.26 ± 0.14	0.17 ± 0.05	0.20 ± 0.08	0.16 ± 0.05	0.22 ± 0.11[1]
Lycopene	0.13 ± 0.07	0.10 ± 0.06	0.15 ± 0.11	0.15 ± 0.08	0.14 ± 0.06	0.18 ± 0.07[1,3]	0.14 ±0.08	0.14 ± 0.08
α-Carotene	0.07 ± .05	0.09 ± 0.07	0.09 ± 0.09	0.11 ± 0.08	0.14 ± 0.18	0.19 ± 0.16	0.10 ± 0.12	0.13 ± 0.12[1]
β-Carotene	0.20 ± 0.11	0.26 ± 0.11	0.30 ± 0.20	0.62 ± 0.53[1,2]	0.29 ± 0.20	0.34 ± 0.19	0.26 ± 0.19	0.40 ± 0.36[1]
β-Cryptoxanthin	0.06 ± 0.04	0.06 ± 0.03	0.07 ± 0.06	0.07 ± 0.05	0.06 ± 0.03	0.12 ± 0.06[1,4]	0.06 ± 0.04	0.08 ± 0.05[1]
Vitamin C	8.13 ± 1.1	9.10 ± 0.97[1]	8.32 ± 1.2	9.92 ± 1.6[1,2]	7.99 ± 0.61	8.83 ± 0.65[1]	8.15 ± 0.10	9.28 ± 0.12[1]
α-Tocopherol	12.5 ± 4.7	10.8 ± 2.2	13.6 ± 7.1	16.6 ± 7.6[1,2]	11.5 ± 2.5	10.4 ± 1.9	12.5 ± 5.1	12.6 ± 5.3
γ-Tocopherol	1.49 ± 0.93	1.33 ± 0.55	1.53 ± 0.53	1.12 ± .47[1]	1.45 ± 0.58	1.45 ± 0.55	1.49 ± 0.69	1.30 ± 0.53

[1] Indicates a significant difference between Pre- and Post- values, $p < 0.05$.
[2] Indicates a significant difference between Tablet and the other two groups, $p < 0.05$.
[3] Indicates a significant difference between Diet and Capsule, $p < 0.01$.
[4] Indicates a significant difference between Diet and the other two groups, $p < 0.01$.

Table 4. Levels of Oxidative Stress Biomarkers Pre- and Post- Intervention

	Capsule (n = 16) (Mean ± SD)		Tablet (n = 15) (Mean ± SD)		Diet (n = 15) (Mean ± SD)		Total (n = 46) (Mean ± SD)	
	Pre-	Post-	Pre-	Post-	Pre-	Post-	Pre-	Post-
8-OHdG (ng/mgCRE)	0.14 ± 0.07	0.14 ± 0.08	0.13 ± 0.08	0.11 ± 0.09	0.14 ± 0.10	0.13 ± 0.10	0.14 ± 0.08	0.13 ± 0.09
LPO (μmol/L)	1.42 ± 0.24	1.29 ± 0.25[1]	1.31 ± 0.45	1.25 ± 0.35	1.33 ± 0.44	1.22 ± 0.36	1.40 ± 0.38	1.25 ± 0.32[1]

[1] Indicates a significant difference between Pre- and Post- values, $p < 0.05$.
Abbreviations: 8-hydroxydeoxyguanosine (8-OHdG); lipid peroxides (LPO); CRE (creatinine).

Oxidative stress indicators

The effects of the various treatments on urinary 8-OHdG and serum LPO, both indicators of oxidative stress are listed in Table 4. No significant change in 8-OHdG was observed for any group or the total population. A significant decrease in LPO was observed for all subjects combined ($p < 0.05$); however, only the Capsule group showed a significant decrease within the group ($p < 0.05$).

Macular pigment levels

No discernible differences in macular pigment were observed from pre- to post- intervention for any group (Table 5). No other significant differences were found when the data were examined by gender or by a person's AMD status. In part, this was a consequence of the limited number of subjects capable of performing the measurements reproducibly.

DISCUSSION

Based on epidemiology, it has long been suspected that AMD is in part a disease of oxidative stress, and recent research has supported that linkage (Bernstein et al., 2001; Beatty et al., 2000). The AREDS study is the first large clinical intervention to provide compelling evidence for recommending antioxidants–a combination of zinc, copper, vitamin C, vitamin E, and β-carotene–as the standard of care for patients at high-risk for progression to advanced AMD (AREDS, 2001), but it certainly is not the last study to explore nutrients capable of delaying progression of AMD. Other antioxidants need to be tested, and formulations need to be optimized to improve the success of nutritional interventions. Smaller pilot studies can serve to assist in the identification of ingredients and their amounts that may be capable of improving efficacy of the original AREDS composition, and identifying surrogate noninvasive and minimally invasive biomarkers indicative of a protective response to antioxidant interventions. These pilot studies can provide guidance for the design of the next generation of large-scale interventional trials against AMD.

In this pilot study the responses recorded changes observed in a 12-week period of supplementation in an elderly population. The main outcome measures were serum increases in carotenoids and antioxidant vitamins and alterations in serum and urinary biomarkers of oxidative stress (LPO and 8-OHdG). All three interventions contained considerably more than the 2 mg/day consumption of lutein and zeaxanthin that might be predicted for an elderly population based upon NHANES III intake data. These amounts were anticipated to increase macular pigment levels, which were monitored noninvasively by heterochromatic flicker photometry.

All three groups reported excellent compliance with the study regimens, and this was mirrored by the positive serum response for many of the nutrients. Serum lutein levels rose in a significant manner in the range of 50-100% in all groups in response to a substantial increase in lutein intake relative to the usual American dietary intake. Although the capsule formulation contained more lutein and zeaxanthin than the tablet formulation, the serum level increased more for individuals consuming the tablet formulation. One inference could be that the slower release of nutrients from the tablet improved bioavailability, and that the type of formulation can influence absorption.

Likewise, β-carotene levels rose in all groups, especially in the Tablet group. Lycopene and β-cryptoxanthin were elevated in the Diet group only, as anticipated since these components were not provided in either the tablet or capsule supplement but were present in the dietary food selection categories. Serum zeaxanthin levels were largely unchanged in all three groups, as expected, since the supplements and the diet provided 0.6 mg or less of this nutrient.

Vitamin C levels also rose significantly for all groups, especially when the Tablet formulation was employed. Vitamin E response was less consistent, however. Only the Tablet group showed a significant increase in serum α-tocopherol, with a small but significant decline in γ-tocopherol. This decline may have resulted from the documented competition of α- and γ-tocopherol for specific binding sites on endogenous binding proteins (Handelman et al., 1985, Hosomi et al., 1997).

Accompanying increases in serum antioxidants, a reduction in two biomarkers of oxidative stress, serum LPO and urinary 8-OHdG, was anticipated. All three groups demonstrated a trend for decreased LPO, and these trends were significant for the Capsule group and for the pooled populations. 8-OHdG levels

were unchanged or slightly decreased but never reached statistical significance. These trends suggest one or both of these oxidative stress biomarkers may be useful to incorporate into larger clinical trials of antioxidant supplements in AMD populations. There is some evidence to suggest that the effects of oxidative stress as measured by 8-OHdG may not decrease significantly as a result of antioxidant intervention unless the subjects are experiencing high levels of pre-existing oxidative stress (Schmidt et al., 2000). Our subjects were generally healthy and free of known oxidative stress such as smoking or renal failure. This work suggests that LPO is probably a better marker of oxidative stress for patients in a non-stressed environment, and also indicates that lipids and membranes may be more susceptible to mild oxidative stress than nucleic acids.

Another factor influencing these results may be that eighty-eight of the ninety-eight original volunteers reported regular use of a variety of supplements containing a variety of antioxidants prior to enrollment, typically for many years, so the required two-week washout period free of antioxidant supplements prior to baseline oxidative stress measurements may have been inadequate for this population. While this washout period is likely to be sufficient for water-soluble antioxidants such as vitamin C, the fat-soluble antioxidants such as carotenoids or vitamin E have longer serum and tissue half-lives and may require considerably longer washout periods in future studies of oxidative stress in the elderly. In a previous study, changes in the placebo control group indicated the removal of antioxidant supplementation may lead to a gradual decrease in markers of oxidative status (Nelson et al., 2003).

A recent small study found clinically normal fellow eyes of patients with exudative AMD had lower levels of macular pigment, assessed by heterochromatic flicker photometry (Beatty et al., 2001). In a larger study of macular carotenoid levels using resonance Raman spectroscopy, AMD patients were observed to have significantly lower levels of lutein and zeaxanthin relative to age-matched control eyes unless they had been consuming high-dose lutein supplements (≥ 4 mg/day) for long periods of time (Bernstein et al., 2002).

In this small study of limited duration, no statistically significant differences in macular pigment could be identified as a consequence of any of the interventions. This may be due in part to inherent limitations of all noninvasive measurement methods for macular pigment which typically have a day to day variability of approximately 10%. More invasive measurement methods such as HPLC have less variability, but their spatial resolution is very limited. The overall conclusions of this study are that any improvements in accuracy and reproducibility of the method for measuring macular pigment would be beneficial, any means of simplifying the measurements or improving patient competence would be constructive, and that the magnitude of the changes in macular pigment may need to be increased for the changes to be observable and significant. The former can be achieved by enhancing the operating characteristics of the device and improving the device-user interface, and the latter can be accomplished by either extending the duration of the study or increasing the daily levels of dietary supplementation.

Table 5. Mean Macular Pigment Density as Measured by Heterochromatic Flicker Photometry (HFP)

	HFP Perceived Optical Density (Mean ± SD			
	Right Eye		Left Eye	
	Pre-	Post-	Pre-	Post-
Capsule (n = 9)	0.37 ± 0.20	0.41 ± 0.23	0.26 ± 0.19	0.37 ± 0.22
Tablet (n = 9)	0.50 ± 0.27	0.39 ± 0.26	0.32 ± 0.14	0.32 ± 0.18
Diet (n = 11)	0.24 ± 0.12	0.22 ± 0.18	0.32 ± 0.16	0.24 ± 0.16
Total (n = 29)	0.35 ± 0.21	0.34 ± 0.21	0.31 ± 0.21	0.31 ± 0.17

The results from Table 5 suggest some guidelines. The global mean of the measured ocular density was approximately 0.3 OD units. In order to distinguish a 25 % change in the level of optical density (a change of 0.075 OD units, or equivalently a change of 25% in macular pigment to which the optical density is linearly related) the standard deviation of each of the groups would have to be smaller than 0.075 OD units, for small groups with about 15 individuals per group. This suggests that for discrimination of the effects of diet, it will be helpful to have groups with reasonably homogeneous initial levels of macular pigment, patients with the ability to generate mean readings for each eye with standard deviations less than about 0.075 OD and with bilateral symmetry such that the difference between mean values for each eye is no larger than this value. Within these guidelines, it is readily perceivable from Table 5 that the consistency of the measurements would need to be increased about three-fold. Other investigators also have been concerned about the importance of the protocol for measuring macular pigment, and a protocol for improving the accuracy of HFP measurements is available on the world-wide-web (Snodderly et al., 2004). Alternative methods for evaluating the level of macular pigment are emerging, and those that are rapid, do not involve pupil dilation, and are objective will offer improved opportunity for resolving the effects of dietary intervention and its value.

Whatever technique is utilized for measuring macular pigment, there will be numerous underlying assumptions and requirements for successful, reproducible, and accurate evaluation of macular pigment. For psychophysical methods, one assumption is that the relative retinal sensitivity to short and middle-wavelength lights is the same in foveal and parafoveal regions, an

approximation improved with proper adjustment of the flicker frequency (Snodderly et al., 1999). Elsner and colleagues have noted irregularities in cone distributions particularly in older subjects who had signs of retinal disease (Elsner et al., 1998), and the implications for accurate measurement of macular pigment are uncertain. A recent study has shown that psychophysical methods also may not provide accurate assessment of macular pigment either when the amount of pigment is at a low level (Delori et al., 2001) or when peripheral macular pigment levels are elevated, as may occur with supplementation (Bhosale and Bernstein, unpublished data). Furthermore, since the most important evaluations probably will be those made in the elderly, the effects of additional sources of scattering and absorption will need to be incorporated into any analysis.

Previous supplementation studies using subjective and objective measurement techniques have generally shown significant increases in macular carotenoid levels in response to higher dose lutein supplementation within three months, but these were always performed in a relatively young population, not in the elderly (Landrum et al.,1997; Berendschot et al., 2000; Hammond et al., 1997). It is possible that the transport processes of carotenoids from the gut to the macula via liver and bloodstream are slower or less efficient in the elderly. Uptake of lutein and zeaxanthin into the macula is likely to be mediated by specific and saturable binding proteins (Yemelyanov, et al., 2001), which may decline with age. An isoform of human glutathione S-transferase (GSTP1) extracted from human macula membrane tissue has been demonstrated to bind zeaxanthin and may enhance the antioxidant function of zeaxanthin within the macula (Bhosale et al. 2003) Antioxidant efficiency within the macula may be a function of both the serum and macular level of lutein and zeaxanthin as well as the activity of the specific transfer proteins.

CONCLUSION

This 12-week pilot study demonstrates that supplementation with two different antioxidant formulations directed toward individuals potentially at risk for visual loss from AMD exhibited positive serum antioxidant responses. These serum antioxidant level and biomarkers of oxidative stress responses were of comparable magnitude to those observed with a dietary intervention enriched in fruits and vegetables containing high levels of carotenoids. Future studies of carotenoid supplementation in the elderly may require longer periods of supplementation and/or higher dosage levels to translate serum increases into clinically significant changes in macular carotenoid levels. Refinements in techniques for the measurement of macular pigment will be beneficial for detecting changes in carotenoid content of the macula. The results from the study reported here indicate the need for longer-term, continuous supplementation with antioxidants and xanthophylls to translate serum responses into ocular benefit.

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