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The short-term effects of antioxidant and zinc supplements on oxidative stress biomarker levels in plasma: a pilot investigation

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Abstract

Purpose—To determine if short-term Age-Related Eye Disease Study (AREDS) antioxidant and zinc supplementation affects biomarkers of oxidative stress, possibly serving as a predictor of their efficacy.

Design—Prospective interventional case series

Methods—Nineteen subjects, 12 with intermediate or advanced age-related macular degeneration (AMD) (AREDS categories 3 or 4) and 7 non-AMD controls, were admitted to the Vanderbilt General Clinical Research Center and placed on a controlled diet for 7 days. Antioxidant and zinc supplements were stopped two weeks prior to study enrollment. Dietary supplementation with 500 mg vitamin C, 400 IU vitamin E, 15 mg β -carotene, 80 mg zinc oxide, and 2 mg cupric oxide per day was instituted on Study Day 2. Blood was drawn on Study Days 2 and 7, and plasma concentrations of cysteine (Cys), cystine (CySS), glutathione (GSH), isoprostane (IsoP), and isofuran (IsoF) were determined.

Results—Short-term AREDS supplementation significantly lowered mean plasma levels of CySS in participants on a regulated diet ($p = 0.034$). No significant differences were observed for Cys, GSH, IsoP, or IsoF. There were no significant differences between AMD patients and controls.

Conclusions—This pilot interventional study shows that a 5-day course of antioxidant and zinc supplements can modify plasma levels of CySS, suggesting that this oxidative stress biomarker could help predict how likely an individual is to benefit from AREDS supplementation. Further, CySS may be useful for the evaluation of new AMD therapies, particularly those hypothesized to affect redox status.

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Introduction

Age-related macular degeneration (AMD) is the leading cause of irreversible blindness in older individuals in the Western world. Approximately 1.75 million people in the United States over the age of 40 have the sight-threatening advanced stages of the disease, and this number is projected to approach 3 million by 2020.¹ The ability to predict which patients are most likely to progress to advanced AMD has the potential to impact disease management significantly, promoting the best possible clinical outcome.

A wide range of evidence supports the involvement of oxidative stress in the development and progression of AMD. Established risk factors for AMD, such as aging, smoking, and light exposure, have been shown to contribute to cumulative cellular oxidative injury.² The Age-Related Eye Disease Study (AREDS), a multicenter, randomized clinical trial, demonstrated that supplementation with antioxidants (vitamin C, vitamin E, and β -carotene) and zinc slowed progression to advanced AMD.³ Additionally, high dietary intake of antioxidants (particularly carotenoids) has been associated with lower AMD prevalence⁴ and incidence.⁵

Measurement of thiol metabolites and lipid peroxidation products in plasma allows quantification of an individual's redox status. The thiolated amino acid cysteine (Cys) and the Cys-derived antioxidant glutathione (GSH) are oxidized to their respective disulfides, cystine (CySS) and glutathione disulfide (GSSG). We have previously shown that the redox potentials (E_h) of Cys/CySS and GSH/GSSG become more oxidized with two major risk factors for AMD, aging⁶ and smoking.⁷ In a previous case-control study, we found that the mean plasma level of CySS was higher in AMD patients than in controls.⁸ We also demonstrated the long-term effect of antioxidant and zinc supplementation on thiol biomarkers of oxidative stress in two AREDS ancillary studies. After five years, supplemental antioxidants decreased the Cys/CySS redox potential and increased plasma levels of the reduced thiol Cys,⁹ and supplemental zinc decreased plasma levels of the oxidized thiol CySS.¹⁰

Plasma levels of the lipid peroxidation products F₂-isoprostanes (F₂-IsoPs) and isofurans (IsoFs), generated by the non-enzymatic free radical-catalyzed peroxidation of arachidonic acid, provide a comprehensive and reliable approach to in vivo evaluation of lipid-related oxidative stress.^{11, 12} The formation of F₂-IsoPs and IsoFs is regulated by oxygen tension such that IsoF production becomes more favored as oxygen concentration increases.¹¹ Elevated levels of these biomarkers have been reported in association with disease risk factors such as smoking¹³ and multiple systemic diseases.^{12, 14-18} Our previous work suggested a trend towards significance for the association of mean plasma levels of IsoFs with presence of AMD,⁸ but the effects of antioxidant and zinc supplements on F₂-IsoPs and IsoFs remain unknown.

The purpose of this study was to determine the effects of short-term AREDS antioxidant and zinc supplementation using two independent markers of oxidative stress: thiol redox metabolites and lipid peroxidation products. The ability to predict which patients will not respond well to antioxidant and zinc therapy, the current standard of care for early and intermediate AMD, may allow physicians to modify follow-up schedules such that high-risk patients are monitored more closely to achieve the best possible visual outcome.

Methods

Study Participants

For this pilot interventional study, individuals over the age of 55 were recruited from the Retina Division at the Vanderbilt Eye Institute using the inclusion/exclusion criteria of the ARED Study.³ Cases were diagnosed with intermediate or advanced AMD (AREDS categories 3 or 4)³ and were required to have at least intermediate drusen in both eyes. Controls showed no clinical signs of AMD. Priority was given to spouses, as these individuals would tend to have similar dietary and environmental exposures. Exclusion criteria included the presence of any retinopathy other than AMD, active uveitis or ocular infection, any ocular surgery within the 60 days prior to enrollment, and current or previous participation in a clinical trial utilizing an investigational drug or treatment within 30 days prior to the study start date. Patients with diabetes mellitus were excluded given the potential role of oxidative stress in the pathogenesis of diabetic complications.^{19, 20} All participants had a dilated eye exam performed by an experienced retina specialist (PS) during the study week. Disease status was confirmed by high-resolution fundus photography. Fifty-degree fundus images were examined by a masked retina specialist (PS) for the presence or absence of the following AMD-related findings: drusen, RPE changes, neurosensory retinal detachment, pigment epithelial detachment, sub- and/or intra-retinal exudation (hemorrhage and/or lipid), choroidal neovascularization, and fibrovascular tissue. AMD patients were then classified by disease stage using the AREDS criteria.³ Smoking history was obtained from all participants. The risks and benefits of supplementation, including the association between long-term beta-carotene intake and lung cancer, were carefully explained to all patients who wished to participate.

Controlled Diet

Six meal plans with varying caloric loads were designed to accommodate study participants with different caloric needs. Daily kilocalorie (kcal) intake ranged from 1600 to 2600 kcal per day, increasing in 200 kcal increments. All meal plans were controlled for dietary antioxidant intake, including $\pm 10\%$ of the recommended daily amount (RDA) of vitamins A, C, and E for females aged 51-70 years: 700 $\mu\text{g/day}$ vitamin A, 75 mg/day vitamin C, 15 mg/day vitamin E. The macronutrient distribution was 27-30% fat, 55-60% carbohydrates, and 15-20% protein. No juice was allowed in the study diet. A sample diet plan is included as Supplemental Material.

Prior to Admission Day, the resting energy expenditure (REE) was calculated for each patient using the Mifflin-St. Jeor Equation. The REE was multiplied by an appropriate activity factor to estimate caloric needs, and the appropriate calorie level was assigned.

Stay at the General Clinical Research Center

The 6 AMD patients who were taking AREDS supplements prior to study enrollment were instructed to discontinue all antioxidant and zinc supplements two weeks prior to admission to the Vanderbilt General Clinical Research Center (GCRC). Throughout the duration of their 7-day stay at the GCRC, patients received a controlled diet, as described in detail above. Patients were allowed to leave the facility but required to eat all meals at the GCRC during the study week. The nutritionist confirmed that the patients were receiving the appropriate amount of food every day. Three patients requested an increase in calorie level during the study week. Each patient's progress was monitored daily by a study physician.

The first 48 hours on a regulated diet served to normalize baseline parameters. Dietary supplementation with the AREDS formula of 500 mg vitamin C, 400 IU vitamin E, 15 mg β -carotene, 80 mg zinc oxide, and 2 mg cupric oxide per day was instituted on Study Day

2.³ Blood (19 ml) was drawn at 4:00 P.M. with a 23-gauge butterfly needle on Days 2 and 7 for measurement of plasma biomarkers of oxidative stress.

Plasma Biomarker Measurement

For the measurement of plasma thiol metabolites, 1.5 ml blood was immediately transferred to a microcentrifuge tube containing 0.5 ml of serine-borate preservation solution, which has been demonstrated to protect against auto-oxidation.²¹ Following centrifugation to remove blood cells, 200 μ l of supernatant was transferred to another microcentrifuge tube containing 200 μ l of 10% perchloric acid, 0.2 M boric acid, and 10 μ M γ -glutamyl-glutamate (internal standard). Samples were frozen at -80°C until derivatization with dansyl chloride. Plasma Cys, CySS, and GSH were measured by high-performance liquid chromatography (HPLC).^{8, 21} Levels of GSSG were below the detection limit for the majority of specimens. All patients with available biomarker measurements were included in data analyses.

For the measurement of lipid peroxidation metabolites, two 4-ml blood collection tubes containing 7.2 mg K₂ EDTA each were centrifuged at 4°C to remove blood cells, and 2 ml supernatant from each tube was transferred to one of two 15 ml conical tubes, which were immediately frozen at -80°C and not thawed prior to analysis.^{22, 23} Samples were analyzed for F₂-IsoP and IsoF concentration by the Vanderbilt University Eicosanoid Core Laboratory using gas chromatography/negative-ion chemical ionization mass spectrometry (GC/NICI-MS) as described previously.^{8, 24}

After the addition of the internal standard [²H₄]-15-F_{2t}-IsoP (8-iso-PGF_{2 α}), plasma samples were applied to a C18 Sep-Pak cartridge (Waters, Milford, MA) followed by a silica Sep-Pak cartridge (Waters). Initial separation of IsoPs, PGF_{2 α} , and IsoFs from other lipid metabolites was achieved by thin layer chromatography (TLC) in a solvent system of chloroform:methanol (93:7, v/v), and compounds migrating in the region $\pm$ 1 cm of the PGF_{2 α} standard were collected from the TLC plate.

Following TLC purification, GC/NICI-MS was carried out on an Agilent 5973 Inert Mass Selective Detector coupled with an Agilent 6890n Network GC system (Agilent Labs, Torrance, CA) and interfaced with an Agilent computer. GC was performed using a 15 m by 0.25 μ m (film thickness) DB-1701-fused silica capillary column (J and W Scientific, Folsom, CA). The column temperature was programmed to increase from 190°C to 300°C at 20°C per minute. Levels of endogenous F₂-IsoPs and IsoFs in each biological sample were calculated from the ratio of intensities of the ions m/z 569 (IsoPs) or m/z 585 (IsoFs) to m/z 573. Validation of this assay has shown precision of $\pm$ 6% and accuracy of 94% in biological fluids.²²⁻²⁴

Data Analysis

Descriptive statistics for all demographic and clinical variables were calculated. Biomarker levels before (Day 2) and after (Day 7) five days of AREDS supplementation were compared in AMD patients only, controls only, and all participants using two-tailed paired t-tests. Linear regression models adjusting for age, gender, and AMD status were fitted to each biomarker independently. All analyses were performed with Microsoft Excel and R (www.r-project.org). For all statistical analyses, p < 0.05 was considered to be significant.

Results

Nineteen Caucasian participants, including 12 patients with intermediate or advanced AMD and 7 non-AMD controls, were enrolled in this study. Four AMD patients had intermediate AMD, six had neovascular AMD, and two had geographic atrophy and no neovascular

AMD. The mean age of all participants was 74.7 years, and 42.1% of all participants were female. The mean body mass index (BMI) was 25.1, and one control patient was a current smoker. Detailed demographics of the study population are presented in Table 1.

Plasma levels of thiol metabolites (Cys, CySS, and GSH) and lipid peroxidation products (IsoP and IsoF) were measured in participants before and after five days of AREDS supplementation. The mean baseline level of CySS in the plasma of all participants after two days on a controlled diet was $59.55 \mu\text{M} \pm 11.97 \mu\text{M}$. After five days of supplementation with antioxidants and zinc, mean plasma CySS had decreased to $55.58 \mu\text{M} \pm 9.99 \mu\text{M}$, representing a significant 6.7% reduction in plasma CySS ($p = 0.034$, Table 2). AREDS supplementation lowered plasma CySS in 12 of 18 study participants, including 8 of 11 AMD patients and 4 of 7 controls. The individual changes in CySS ranged from $-17.71 \mu\text{M}$ to $+7.65 \mu\text{M}$. None of the other oxidative stress markers demonstrated a significant change pre- and post-treatment with antioxidants and zinc. Biomarker levels on Days 2 and 7 are presented in Table 2. The potential influence of age, gender, and AMD status on the magnitude of change in biomarker level was examined by linear regression. In our cohort, these variables did not significantly affect the difference between Day 2 and Day 7 levels for any of the five biomarkers.

Mean plasma levels of the five biomarkers were not significantly different between AMD patients and controls on Day 2 or Day 7. To determine whether AMD patients and controls responded differently to AREDS supplements, we compared the change in biomarker level (Day 2 minus Day 7) in cases v. controls. Mean change in plasma CySS level after supplementation was $-3.96 \mu\text{M} \pm 7.31 \mu\text{M}$ in all participants, $-4.45 \mu\text{M} \pm 7.40 \mu\text{M}$ in AMD patients only, and $-3.21 \mu\text{M} \pm 7.67 \mu\text{M}$ in controls only. No significant difference for the change in any biomarker was detected between AMD patients and controls.

Discussion

This pilot study demonstrates that antioxidant and zinc supplements can reduce blood plasma oxidation, as measured by the thiol metabolite CySS in plasma, after only five days of use. While supplementation is beneficial overall, not all individuals are protected from AMD progression. It is possible that plasma CySS levels could be used to identify individuals who are less likely to benefit from antioxidant and zinc therapy for the prevention of advanced AMD.

Our previous work has indicated that thiol redox status can be modified with antioxidant and zinc therapy. Previously, we have shown that the Cys/CySS pool is responsive to long-term treatment with antioxidants⁹ and zinc.¹⁰ Additionally, human RPE cells treated with zinc demonstrated increased GSH levels.²⁵ F₂-IsoPs have also been shown to decrease significantly in response to 2-4 months of vitamin C or vitamin E supplementation.^{26, 27}

The results of this study suggest that the effects of supplemental antioxidants and zinc are apparent shortly after starting supplementation. However, only two thirds of the patients (regardless of AMD status) showed a reduction in CySS. We hypothesize that the patients whose plasma CySS decreases after five days of supplementation are more likely to experience less systemic oxidation over time and thus less likely to progress to advanced AMD. This hypothesis will need to be confirmed in a longitudinal study linking short-term and long-term data in the same individuals.

We have previously described age-related oxidation evident in the Cys/CySS pool.⁶ Elevated levels of F₂-IsoPs have also been associated with aging.²⁸ In the present study, age did not demonstrate a significant effect on the change in plasma concentration of any biomarker following treatment with AREDS supplements. Interestingly, Cys/CySS redox

has been identified as a useful biomarker for other systemic diseases related to aging and oxidative stress, such as cardiovascular and inflammatory diseases.²⁹⁻³¹

This pilot study is limited by its sample size. We have previously reported elevated mean plasma CySS levels in 77 AMD patients compared to 75 controls before adjustment for age.⁸ In the current study, mean plasma CySS levels did not differ significantly between AMD patients and controls, but these levels trend in the expected direction, with higher mean values in AMD patients (62.96 μ M on Day 2 and 58.51 μ M on Day 7) than in controls (54.18 μ M on Day 2 and 50.98 μ M on Day 7). This study only allows us to draw conclusions about the effects of five days of AREDS supplementation on the measured biomarkers. It is possible that a longer study period and/or a different supplement composition would yield significant changes in other oxidative stress biomarkers. Analysis of short-term versus long-term response to AREDS supplementation, in conjunction with AMD progression data, could be used to assess whether short-term response predicts long-term protection against AMD progression.

In conclusion, we detected a significant change in the thiol metabolite CySS following short-term AREDS supplementation in participants in a well-controlled setting. This is the first study to examine short-term effects of these interventions and to raise the question of whether short-term response may be predictive of long-term benefit. Our results suggest that this oxidative stress biomarker could predict the efficacy of antioxidant and zinc supplements in individual patients. Further, the concentration of CySS in plasma may be useful for the evaluation of new AMD therapies, particularly those hypothesized to affect redox status.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Involved in study design and conduct (MB, JC, PS); data collection (BS, KR, GM, JC); data management, analysis, and interpretation (MB, MO, PL, CL, GM, JC, PS); manuscript preparation (MB, MO, PS); manuscript review and approval (MB, MO, BS, KR, PL, CL, GM, JC, PS).

All procedures were approved prospectively by the local Institutional Review Board, the Vanderbilt University Human Research Protection Program. Research adhered to the tenets of the Declaration of Helsinki and was conducted in accordance with Health Insurance Portability and Accountability Act regulations. Informed consent was obtained from all participants upon study enrollment. The NCT Registry Number for this clinical trial is NCT00668213.

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Biography

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Table 1

Patient demographics depicting age, gender, smoking status, and previous vitamin supplementation in patients with age-related macular degeneration and controls

Variable	AMD (n = 12)	Controls (n = 7)	All Participants (n = 19)
Age, mean yrs	76.8	71.3	74.7
Gender, n (%) female	5 (41.7%)	3 (42.9%)	8 (42.1%)
Smokers, n (%)	0 (0%)	1 (14.3%)	1 (5.3%)
Supplementation, n (%) yes	6 (50%)	0 (0%)	6 (31.6%)

AMD = age-related macular degeneration; n = number

Table 2

Plasma biomarker levels in patients with age-related macular degeneration and controls before and after oral supplementation with Age-Related Eye Disease Study vitamins

	AMD Patients		Controls		All Participants	
	Mean ± SD	p=	Mean ± SD	p=	Mean ± SD	p=
Cys, μM						
Day 2	6.99 ± 2.93		7.55 ± 2.20		7.20 ± 2.62	
Day 7	7.03 ± 1.72	0.94	8.65 ± 2.92	0.21	7.66 ± 2.33	0.32
CySS, μM						
Day 2	62.96 ± 11.86		54.18 ± 10.81		59.55 ± 11.97	
Day 7	58.51 ± 10.49	0.074	50.98 ± 7.67	0.31	55.58 ± 9.99	0.034
GSH, μM						
Day 2	2.15 ± 0.49		2.34 ± 0.53		2.23 ± 0.50	
Day 7	2.13 ± 0.41	0.63	2.24 ± 0.35	0.68	2.17 ± 0.38	0.55
IsoP, ng/ml						
Day 2	0.038 ± 0.0065		0.052 ± 0.019		0.042 ± 0.012	
Day 7	0.037 ± 0.0063	0.65	0.042 ± 0.013	0.23	0.039 ± 0.0086	0.18
IsoF, ng/ml						
Day 2	0.24 ± 0.20		0.20 ± 0.072		0.23 ± 0.17	
Day 7	0.25 ± 0.29	0.91	0.17 ± 0.048	0.43	0.23 ± 0.25	0.91

AMD = Age-related macular degeneration; Cys = cysteine; CySS = cystine; GSH = glutathione; IsoF = isofuran; IsoP = F₂-isoprostane; SD = standard deviation

Levels of Cys, CySS, and GSH were available at both time points for 18 participants (11 AMD patients and 7 controls). Levels of IsoP and IsoF were available at both time points for 14 participants (10 AMD patients and 4 controls).