Macular Pigment Optical Density is Related to Blood Glutathione Levels in Healthy Individuals

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Purpose. To assess the relationship between macular pigment optical density (MPOD) and blood markers for antioxidant defense in otherwise healthy volunteers.

Methods. Forty-seven healthy volunteers were subjected to blood analysis to detect the level of circulating glutathione in its reduced (GSH) and oxidized (GSSG) forms. The level of MPOD was measured using heterochromatic flicker photometry. Systemic blood pressure (BP) parameters, heart rate (HR), body mass index (BMI), and plasma levels of total, HDL, and LDL cholesterol and triglycerides (TGs) were also determined.

Results. A simple correlation model revealed that the level of MPOD correlated significantly and positively with both GSH ($P < 0.001$) and t-GSH ($P < 0.001$) levels but not with those of GSSG ($P > 0.05$). Age, sex, systemic BP parameters, HR, BMI, and plasma levels of cholesterol and TGs did not have any influence on either MPOD or glutathione levels (all $P > 0.05$). In addition, a forward stepwise multiple regression analysis showed MPOD to have a significantly and independent correlation with GSH levels ($\beta = 0.63$; $P < 0.001$).

Conclusions. In otherwise healthy older individuals, there is a positive correlation between local and systemic antioxidant defense mechanisms. (*Invest Ophthalmol Vis Sci.* 2011;52: 5029–5033) DOI:10.1167/iovs.11-7240

Among environmental, nutritional, and genetic risk factors involved in the etiology of AMD, high levels of oxidative stress, a harmful state defined by the presence of pathologic levels of reactive oxygen species (ROS) relative to the antioxidant defense, have been said to play a role.[1–3] In healthy subjects, local retinal protection against free radicals is aided by the presence of macular pigment (MP), which is comprised of the carotenoid lutein and its isomers zeaxanthin[4] and meso-zeaxanthin.[5] It has been suggested that these xanthophylls play a similar role in humans as in plants—that is, as antioxidants and screeners of high-energy blue light.[6] In this way, MP may prevent light-initiated oxidative damage to the retina and therefore protect against subsequent AMD.[7] Indeed, the absorbance spectrum of MP peaks at 460 nm and it is thought to act as a broadband filter, reducing the sensitivity of the macular region to short wavelength light, which is most damaging in the 440 to 460 nm range.[8,9] In addition, the fact that lutein and zeaxanthin have been found in higher concentrations in the rod outer segments of the perifoveal retina than the peripheral retina lends support to their proposed protective role against AMD.[10] These carotenoids are able to quench singlet oxygen (a potent oxidant),[11] scavenge ROS,[12] limit the peroxidation of membrane phospholipids,[13] and reduce lipofuscin formation.[14]

In addition to local retinal damage, high levels of oxidative stress also induces vascular changes that confer a background for circulatory disturbances in the systemic macro- and microcirculation that are also present in AMD patients.[15,16] Indeed, it has been shown that in addition to ocular vascular complications, AMD patients are at higher risk for developing coronary heart disease (CHD) and stroke[17,18]; moreover, they also are more likely to have a decreased survival rate compared to the general age-matched population.[19] It has been shown that AMD patients have lower levels of circulating glutathione (GSH), a major low-molecular weight antioxidant peptide[20]; in addition to a direct vascular effect, this deficiency could also result in low bioavailability of the vasodilatory molecule nitric oxide (NO)[21] and endothelial dysfunction, a condition known to precede both metabolic and cardiovascular diseases but also ocular circulatory disturbances.

It has been reported that dietary intake of lutein and zeaxanthin augument the level of MP with a possible positive effect on AMD prevention and prognosis.[22,23] In addition, carotenoid-rich food intake has a positive influence on systemic antioxidant status in healthy persons.[24] The aim of the present study was to determine whether there is a relationship between local and systemic antioxidant defense markers in older volunteers free of either ocular or systemic diseases.

Materials and Methods

Study Sample

Healthy subjects recruited by advertising at Aston University, Birmingham, United Kingdom were considered for inclusion in this prospective study. Ethical approval was sought from the local ethics committee, and written informed consent was obtained from all participants before enrolling into the study. The study was designed and conducted according to the principles of the Declaration of Helsinki.

Exclusion criteria were smoking, a history of any chronic systemic disease with presumed abnormal circulating GSH levels, including autoimmune diseases,[25] alcoholic liver disease,[26] cancer,[27] and diabetes mellitus.[28] In addition, subjects were also excluded if they had cardiovascular or cerebrovascular disease, coronary artery disease (heart failure, arrhythmia, stroke, or transient ischemic attacks), inflammatory conditions (rheumatoid arthritis and systemic lupus erythematosus), or were receiving hormone replacement therapy or antioxidant/vitamin/iron supplements.

Potential participants were screened for ocular diseases by an ophthalmologist and patients with ocular diseases, such as cataracts, glaucoma, and AMD, were also excluded from the study. Retinal

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photographs were taken using a digital camera (Zeiss FF 450 Plus Fundus Camera; Carl Zeiss Meditec, Inc, Jena, Germany) and the presence or absence of drusen or pigmentary changes associated with AMD was assessed before inclusion in the study. In addition, potential participants were required to complete a questionnaire on their general health, physical activity, and alcohol consumption. The questionnaire included a detailed investigation on the daily intake of fruit and vegetables and other nutrients.

Investigations

Subjects were instructed to fast after 9 PM on the evenings before being tested. On the morning of the test, subjects were requested to have only a light breakfast, such as toast. They were also asked to avoid any cooked breakfast, meat, cereal, fresh fruits, or fruit juice.[29] In addition, subjects were asked to abstain from caffeinated beverages and chocolate and from alcohol for at least 2 hours before the visit. The height and weight of the participants was measured and the body mass index (BMI) was calculated (as kg/m^2).

Blood Pressure Measurement

Blood pressure (BP) was measured in each subject in the morning between 8 AM and 9 AM with a BP monitor (UA-779; A7D Instruments Ltd., Oxford, UK). In preparation for this measurement, each subject rested in a sitting position for approximately 10 minutes in a quiet room to achieve sufficient mental and physical calm. The systolic BP (SBP) and diastolic BP (DBP) were measured three times (1 minute apart). The average readings for SBP and DBP were then used to calculate the mean BP (MBP) using the formula: MBP = 2/3DBP + 1/3SBP.

Blood Sampling and Analyses

All blood samples were obtained by a qualified phlebotomist between 9 AM and 10 AM.

Routine Tests

Fasting, triglycerides (TG), and total and high-density lipoprotein (HDL) cholesterol levels were measured automatically using a Reflotron Desktop Analyzer (Roche Diagnostics, Welwyn Garden City, UK).

Blood Sampling for GSH Analyses

Seven milliliters of blood was collected in EDTA tubes (to prevent oxidation)[30] by venipuncture to the antecubital vein using a butterfly needle and syringe to avoid hemolysis.[20] The blood (30 μL) was then transferred into centrifuge tubes for the initial processing. GSH was released from the blood cells by protein precipitation and cellular disruption achieved by addition of 33.3 μL of 5-sulfosalicylic acid (SSA), 1 g/mL within 10 minutes from the blood collection.[31] The addition of acid minimizes artifactual sample oxidation and removes interfering protein thiols.

Each sample was then diluted with 936.7 μL sodium phosphate buffer (pH 7.5) and the content of each tube was rapidly centrifuged at 13,000 rpm for 5 minutes. Small amounts (150 μL) of supernatant were collected into clean centrifuge tubes and immediately cooled at −70°C. Samples stored at this temperature are stable for at least 2 months and can be transported on dry ice without deterioration.[32] In our hands, GSH loss is $<$ 5% over this time period.

GSH Assay

The total GSH levels (t-GSH) were assessed by the GSH reductase-DTNB (5.5 dithiobis-2-nitrobenzoic acid) recycling procedure, as described in previous studies.[30,33,34] Standards were prepared from 0 to 80 μM in 20-μM increments using 10 mM GSH solution. The standards contained the same final concentrations of SSA as the samples. To each well of a 96-well plate, NADPH (0.3 mg/mL), dissolved in 150 μL of 125 mM sodium phosphate with 6.3 mM EDTA pH 7.5, also known as daily buffer, 50 μL of DTNB solution, and 25 μL of standards or samples were added in quadruplicate and the plate was incubated at 37°C for 3 minutes. Finally, 25 μL of GSH reductase (GSR) was added to the previous mixture and the plate was read at 410 nm using a 96-well plate reader. A standard curve of GSH was then generated using a linear regression program (Microsoft Excel; Microsoft, Redmond, WA) as previously reported.[34]

GSSG Assay

GSSG levels were assessed using a GSH reductase-DTNB recycling assay.[30] The reagents used in this assay were those already described above for GSH assay and, in addition, triethanolamine (TEA) and 2-vinyl pyridine (2-VP). TEA prevents a high local pH and oxidation, while 2-VP is used for derivitization of GSH. GSSG standards were prepared from 0 to 10 μM in 1-μM increments; 100 μL of standards and samples were transferred into separate centrifuge tubes and 2 μL 2-VP was added to each tube. TEA was then used to adjust the pH of the standards/samples to pH 7.5. The assay was carried out as for GSH assay described above. Finally, a standard curve of GSSG was then generated using a linear regression program (Microsoft Excel) as previously reported.[34]

The GSH levels [t-GSH – (2 × GSSG)] and the redox index (defined as the GSH/GSSG ratio) were then calculated.

Macular Pigment Optical Density Measurement

Macular pigment optical density (MPOD) was determined using the MPS 9000 (also known as the M:Pod and the QuantifEYE; Topcon House, Berkshire, UK). This device measures MPOD using heterochromatic flicker photometry, in which subjects respond to the appearance of flicker as the alternation rate is decreased at 6 Hz per sec from a starting level of 60 Hz.[35] Because this is above the critical flicker fusion frequency for the test conditions, subjects do not perceive any flicker initially. A sequence of blue-green ratios is used and these are inverse-yoked to ensure that overall luminance remains constant. The device determines each observer's sensitivity to flicker before the main part of the test. This technique is well documented,[36] and the reliability of this particular instrument has also been assessed by our group.[37]

The eye not being tested was occluded and participants wore their habitual refractive correction (a trial frame and lenses were used when necessary). The central target is a 1° circular stimulus composed of blue (465 nm) and green (530 nm) LEDs. For the foveal (central) test, the observer looked directly at the stimulus while the alternation rate between the blue and green was ramped down from 60 Hz. At the point when they first detected flicker, the observer pressed a response button and this plotted a point on a graph that was visible to the operator on a computer screen. Once the flicker had been perceived, the process started again. The first five responses were used to ascertain the flicker sensitivity of the subject. The observer is asked to respond to a series of green-blue ratios until a V-shaped curve is plotted on the computer screen. The minimum point on the curve corresponds to equiluminance of the blue and green lights. The process was then repeated for the peripheral test, where the subject's gaze was directed to a larger red target, 8° eccentric from the central spot. The difference between the central and peripheral minima is used by an internal algorithm to calculate the MPOD. Each subject was given a practice run of the test, and the results were screened such that if an appropriate V-shaped curve was not obtained, the test was repeated.

Statistical Analysis

Statistical analysis was performed using Statistica for Windows (version 9.0; StatSoft Inc., Tulsa, OK). Data are expressed as mean ± SD. Pearson's correlation test and a forward stepwise multiple regression analysis were performed to test the relationship between the measured variables. P values $<$ 0.05 were considered statistically significant.

Results

Sixty-one healthy subjects with similar dietary habits were selected for inclusion. However, after the evaluation of the

TABLE 1. Characteristics of the Study Participants

Age, y (± SD)	Sex, F:M	SBP, mm Hg (± SD)	DBP, mm Hg (± SD)	BMI, kg/m^2 (± SD)	Total Cholesterol, mmol/L (± SD)	HDL Cholesterol, mmol/L (± SD)	LDL Cholesterol, mmol/L (± SD)	TG, mmol/L (± SD)
50 ± 9	29:18	118 ± 13	73 ± 9	25.20 ± 4.15	4.46 ± 0.82	1.29 ± 0.33	2.61 ± 0.80	1.23 ± 0.62

BMI, body mass index; DBP, diastolic blood pressure; F, female; HDL, high-density lipoprotein; LDL, low-density lipoprotein; M, male; SBP, systolic blood pressure; TG, triglycerides.

fundus photographs and eliminating those with potential macular changes, only 47 healthy subjects (29 women and 18 men) were included in the final analysis. The characteristics of the study participants are provided in Table 1. Table 2 shows the anthropometric and vascular parameters determined by sex. Men exhibited higher SBP ($P = 0.018$) and DBP ($P = 0.01$) and lower levels of HDL cholesterol ($P = 0.002$) than women. However, MPOD and systemic oxidative markers were similar between the sexes (all $P > 0.05$).

A simple correlation model revealed that the level of MPOD correlated significantly and positively with both blood GSH ($r = 0.64$; $P < 0.001$) and t-GSH ($r = 0.63$; $P < 0.001$) levels but not with those of GSSG ($P > 0.05$). Age, systemic BP parameters, BMI, and plasma levels of cholesterol and TGs did not have any influence on either MPOD or blood GSH levels (all $P > 0.05$). A stepwise multiple regression analysis revealed MPOD levels to be independently, significantly, and positively correlated with blood GSH levels ($\beta = 0.64$; $P < 0.001$; Fig. 1).

DISCUSSION

At the retinal level, oxidative stress results in degeneration and death of the RPE and photoreceptors.[38] Because these retinal structures are not able to regenerate after such insult, protective mechanisms have developed to ensure a minimal local effect of free radicals.[39] Indeed, MP is present in the rod outer segments, and RPE and its specific spectral absorption and the presence of lutein and zeaxanthin have enabled it with strong, protective antioxidant properties. There are several methods for measuring the level of MP, including various subjective psychophysical and objective optical techniques.[39] One of these subjective methods is represented by the heterochromatic flicker photometry that involves the calculation of MPOD based on the luminance ratio of short wavelength blue light presented in the central retina (where is assumed to be partially absorbed by the MP) compared to that presented at a more peripheral retinal point, where MP levels are assumed to be minimal.[40] This method offers a good measure of the MPOD levels and is widely available in practice. By using this method, our analysis has shown for the first time an independent, significant, and positive relationship between MPOD and blood GSH levels. At this stage, more research is necessary to provide better knowledge of the exact mechanisms responsible; nevertheless, we can still propose a few hypotheses. It has been reported that the dietary intake of carotenoids had an influence not only on the level of MP[22,23] but also on the systemic circulating antioxidant markers.[24] In addition, exogenous supply of GSH protects the RPE against oxidative damage.[41] Although the individuals included in the present study did not receive either a special diet rich in carotenoids nor GSH or other antioxidant supplementation, the aforementioned results simply show that a relationship between local and systemic protective mechanisms—such as that found by our study—could exist. Consequently, although novel, our results should not be surprising.

Other mechanisms can also be speculated. Melatonin, a neurohormone that is secreted by retinas and the pineal gland, has an influence on the RPE and controls the amount of light reaching the photoreceptors; in addition, it also acts as potent antioxidant at both the ocular and systemic level and, in such capacity, it has been advocated to reduce the risk for pathologies associated with high oxidative damage, such as AMD[42] and cardiovascular disease.[43] In addition, melatonin also activates other antioxidant defenses including GSH peroxidase (GPx), an enzyme that uses GSH as a substrate to eliminate ROS.[44,45] Melatonin production could be affected by aging as the pupil diameter and the light absorption through the crystalline lens changes with age progression.[46–48] Consequently, aging contributes to an abnormal melatonin production and

TABLE 2. Measured Parameters for Men and Women

	Female	Male	*P*
Age, y (± SD)	49 ± 9	50 ± 8	>0.05
MPOD (± SD)	0.46 ± 0.18	0.47 ± 0.12	>0.05
TG, mmol/L (± SD)	1.16 ± 0.65	1.35 ± 0.58	>0.05
Total cholesterol, mmol/L (± SD)	4.52 ± 0.69	4.37 ± 1.00	>0.05
HDL cholesterol, mmol/L (± SD)	1.40 ± 0.29	1.11 ± 0.30	0.002
LDL cholesterol, mmol/L (± SD)	2.58 ± 0.75	2.65 ± 0.89	>0.05
BMI, kg/m^2 (± SD)	24.48 ± 4.06	26.37 ± 4.13	>0.05
SBP, mm Hg (± SD)	115 ± 12	124 ± 12	0.018
DBP, mm Hg (± SD)	70 ± 9	77 ± 8	0.010
HR, bpm (± SD)	72 ± 7	69 ± 8	>0.05
t-GSH, μmol/L (± SD)	1177 ± 564	1089 ± 416	>0.05
GSSG, μmol/L (± SD)	88 ± 44	78 ± 51	>0.05
GSH, μmol/L (± SD)	1002 ± 526	932 ± 383	>0.05
Redox index, GSH:GSSG (± SD)	11 ± 7	12 ± 6	>0.05

BMI, body mass index; DBP, diastolic blood pressure; GSH, reduced glutathione; GSSG, oxidized glutathione; HDL, high-density lipoprotein; HR, heart rate; LDL, low-density lipoprotein; MPOD, macular pigment optical density; SBP, systolic blood pressure; TG, triglycerides; t-GSH, total GSH.

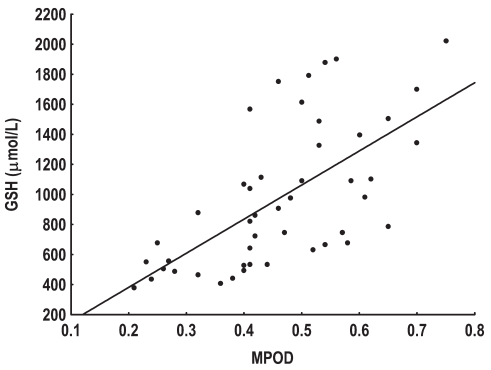

FIGURE 1. The correlation between the levels of macular pigment optical density and reduced glutathione in the study population ($P < 0.001$).

also decreases antioxidant defense, which in turn will result in accelerated aging processes with various effects throughout the human body, including the eye. Although measuring melatonin levels was not an aim in the present research, we could still hypothesize that a link between ocular and systemic antioxidant mechanisms could also be established indirectly via melatonin. This hypothesis should, however, be tested in various age groups. In addition, other mechanisms are most certainly involved and should be further researched using more complex analyses. The role of antioxidants proven to have a link to both macular pigment and circulating GSH levels should also be researched. Nevertheless, as previously emphasized, the positive correlation between the levels of MPOD and GSH seen in our study probably only reveal that, in healthy individuals, the antioxidant defense mechanisms present at various levels act in the same direction to protect the body against harmful effects of ROS. However—and maybe most importantly—our research points toward the necessity of studying various normal relationships between ocular and systemic protective mechanisms against diseases with multiple etiologies and complications, such as AMD. In this way, we could understand better results that are reported after various pathologic changes have already occurred. Reducing the risk for AMD is important, and strengthening natural bodily mechanisms that are at their best in healthy individuals seems to be one of the possible approaches.

Although some studies report sex differences in plasma GSH levels,[49] others did not confirm it in either plasma or blood GSH.[50] In agreement with later studies, we also could not find any difference between men and women with respect to blood GSH levels. It is possible that the various methods used for GSH assay are responsible for this lack of consistency in the results. There is no general agreement as to what method is best for analyzing circulating GSH. In the present study, we used a validated method for measuring blood levels of GSH and GSSG[30,33,34] that is known to minimize variability from more complicated sample preparation steps associated with methods measuring plasma GSH.[50] In addition, to avoid variations and GSH loss, we have paid particular attention to blood collection, initial processing, and storage. Blood samples were collected at the same time (between 9 AM and 10 AM) and processed in the same way and time interval from collection in all individuals. Moreover, incorporation of GSR in our assay confers it specificity to GSH.

Study Limitations

As previously emphasized, our research included only patients over 40 years of age that were carefully selected to exclude macular changes or other ocular pathologies as well as systemic chronic disease and various therapeutic interventions. We have also excluded smokers and individuals taking any antioxidant supplements. This careful selection has limited the number of individuals included in the final analysis. It is possible that the relationship between MPOD and circulating GSH level is different not only in various age groups but also in individuals with additional risk factors for AMD and/or vascular disease. Moreover, individuals having a diet either lacking or very rich in carotenoids or receiving various supplements as well as patients suffering from AMD with or without vascular complications may display very different results. More studies to include larger and more various populations to cover the many possible confounders that have been either missed or intentionally avoided in the present study are warranted.

In conclusion, our study has shown for the first time that in older, healthy, nonsmoking individuals, MPOD correlates with whole blood levels of GSH. Because assays for measuring systemic levels of antioxidant molecules are complex and need specialized laboratories, it is tempting to propose that a simple MPOD assessment could be used as surrogate indicator of the individual's systemic capacity of dealing with the damaging effect of free radicals and, consequently, of their risk for developing chronic ocular and/or systemic pathologies. Nevertheless, this assumption should be verified in larger studies, and the clinical significance of associations such as the one reported by our research should be carefully analyzed.

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