Red blood cell antioxidant enzymes in age-related macular degeneration

Monica A De La Paz, Jian Zhang, Irwin Fridovich

Abstract

Aims/background—Oxidative damage has been proposed to be involved in the pathogenesis of age-related macular degeneration (ARMD). The purpose of this study was to evaluate whether red blood cell antioxidant enzyme activity correlates with severity of aging maculopathy in affected individuals.

Methods—Blood samples were obtained from 54 patients with varying severity of aging maculopathy and 12 similarly aged individuals with normal ophthalmoscopic examination. Macular findings were graded according to a modification of the method described for the Age-Related Eye Disease Study. (AREDS). The activities of superoxide dismutase, catalase, glucose-6-phosphate dehydrogenase, glutathione peroxidase, and glutathione reductase were measured in red blood cells. Haemoglobin content of whole blood was measured, and enzyme activity was determined per mg haemoglobin.

Results—Multiple regression analysis and ordinal logistic regression analysis were performed to determine whether antioxidant enzyme activity was associated with severity of ARMD. No significant association between disease severity of ARMD and antioxidant enzyme activity was identified for any of the enzymes.

Conclusion—These results do not provide evidence for a relation between oxidative stress, as measured by antioxidant enzyme activity in red blood cells, and disease severity in ARMD.

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Age-related macular degeneration (ARMD) is the most common cause of blindness in individuals over 55 years of age in the USA.1 It is expected to become of greater public health concern as the proportion of elderly people in the population increases over the next century.

The exact etiology of ARMD is not known. Oxidative damage has been implicated in the pathogenesis of the disease, but the precise role of the damaging effects of reactive oxygen species in development and progression of the disorder remains to be fully elucidated. A haematogenous basis for development of ARMD, which involves oxidative damage, has been proposed by Gottsch et al,5 who suggest that development of ARMD is caused by chronic light exposure with direct choriocapillaris damage. Further evidence for involvement of blood factors is suggested by the finding that a high plasma antioxidant index has a protective effect in ARMD.6 In addition, increased dietary consumption of foods high in carotenoids, which are known to protect against singlet oxygen mediated oxidative damage, has been implicated in protecting against development of severe ARMD.7 The exact role of blood antioxidant substances in disease development and progression remains to be fully elucidated.

Enzymes including superoxide dismutase, catalase, glucose-6-phosphate dehydrogenase, glutathione peroxidase, and glutathione reductase are involved in protecting against oxidative damage. Previous reports suggest that blood levels of antioxidant enzymes may be lower in individuals with ARMD than in controls. Low glutathione peroxidase,8,9 glutathione reductase,8 and superoxide dismutase9 activities in red blood cells of ARMD patients compared with age-matched controls have been identified.

In this report, red blood cell antioxidant enzyme activity in patients with ARMD is further evaluated.

Patients and methods

Over a 6 month period, 54 subjects were referred to one of the authors (MAD) for evaluation of ARMD and recruited into the study at the time of their clinic visit. Twelve similarly aged subjects without ARMD were recruited into the study for comparison. Exclusion criteria included presence of a visually compromising eye disease such as glaucoma, visually significant cataract, and other retinal disease including those resembling ARMD, such as myopic degeneration and ocular histoplasmosis. Use of antioxidant vitamin supplementation was not an exclusion criterion. A clinical evaluation questionnaire was completed for each patient, and included medical history, medication use, antioxidant vitamin supplementation, and smoking history.

All subjects underwent the same ophthalmic examination. Measurements of best corrected visual acuity, slit-lamp examination, and applanation tonometry were performed for each subject. Non-contact lens biomicroscopy was performed on each subject (by MAD). Fundus photography was performed for most subjects, and fluorescein angiography was performed when clinically indicated. Severity of aging maculopathy for each eye was evaluated according to a modification of the eligibility scale used in the ongoing multicentre clinical trial of the Age-Related Eye Disease Study (AREDS).10 In this grading system, we define five grades (Table 1). Twelve patients were classified as grade 1. Grade 1 patients are...
Table 1  Modification of the grading system used in the Age-Related Eye Disease Study (AREDS)  

| Stage 1 | No drusen or small non-extensive drusen, without pigment abnormalities |
| Stage 2 | Extensive small drusen or non-extensive intermediate drusen, or pigment abnormalities associated with age-related macular degeneration (ARMD) |
| Stage 3 | Extensive intermediate drusen or any large drusen |
| Stage 4 | Geographic atrophy, with or without involvement of the centre of the macula |
| Stage 5 | Exudative ARMD, including retinal pigment epithelial (RPE) detachments or signs of a choroidal or subretinal membrane (subretinal or sub-RPE haemorrhage or fibrosis, disciform scar, or photoocoagulation scars consistent with treatment of ARMD) |

normals who may or may not have a few small hard drusen in the macula. The presence of a few small hard drusen is considered a normal aging variant. Increasing grades represent increasing severity of macular findings, with grades 4 and 5 representing classic fundus features of ARMD (geographic atrophy of the retinal pigment epithelium and exudative maculopathy, respectively).

At the time of the examination, 5 ml of venous blood was drawn into a tube containing ethylenediamine tetra-acetic acid (EDTA). The tube was inverted several times to ensure thorough mixing. Processing of blood was performed by a single technician unaware of the clinical examination results. The blood sample was refrigerated immediately until the time of performance of enzyme assays. Assays were performed within 24 hours from the time the blood was drawn. Haemoglobin concentration in mg/ml was determined based on the cyanmethaemoglobin method (Sigma kit).  

The blood sample was centrifuged at 3000 g for 10 minutes to separate plasma and buffy coat from red blood cells. The plasma and buffy coat were carefully removed from the red blood cells using a glass pipette. Then 3 ml of deionised water were added to the red blood cells to induce haemolysis. The tube was gently vortexed. The sample was then centrifuged at 13500 g for 5 minutes to separate the haemolytate from the pellet of red blood cell membranes.

Assays of superoxide dismutase, catalase, glucose-6-phosphate dehydrogenase, glutathione peroxidase, and glutathione reductase were performed immediately using the red blood cell haemolysate. Spectrophotometric determination of enzyme activity was carried out using a Shimadzu UV-260 spectrophotometer, 3 ml quartz cuvettes, path length 10 mm. All assays were performed at 25°C. Unless otherwise specified, all reagents were obtained from Sigma Chemical Company (St Louis, MO, USA).

Superoxide dismutase activity was determined based on the kinetic assay of McCord and Fridovich, 12 which is based on the inhibition of cytochrome c reduction by xanthine oxidase in the presence of substrate. The reaction mixture consisted of $2 \times 10^{-5}$ M cytochrome c, 100 μM xanthine in 0-05 M phosphate buffer, pH 7-8, containing 1 $\times 10^{-4}$ M EDTA. The increase in absorbance at 550 nm was measured after addition of xanthine oxidase once measurement of baseline absorbance had been noted to verify stability. The assay was based on the inhibition of cytochrome c reduction by xanthine oxidase in the presence of 10 μl of haemolysate.

Definition of enzyme activity unit was as described by McCord and Fridovich, 12 and activity was expressed as units per mg haemoglobin.

Catalase assay was based on the method of Beers and Sizer, 13 in which decrease in ultraviolet absorption of hydrogen peroxide at 230 nm is measured spectrophotometrically as a function of time. The reaction mixture consisted of 9-20 mM hydrogen peroxide in 0-05 M phosphate buffer, pH 7-0. The decrease in absorbance over 4 minutes was followed after the addition of 20 μl of a 1:2000 dilution of haemolysate. Enzyme activity was measured as μmol hydrogen peroxide consumed per mg haemoglobin per minute.

Glucose-6-phosphate dehydrogenase activity was determined based on the method described by Kirkman, 14 in which the rate of generation of reduced nicotinamide adenine dinucleotide phosphate (NADPH) was measured as an increase in absorbance at 340 nm. The reaction mixture consisted of a final concentration of 0-06 mM glucose-6-phosphate, 0-20 mM nicotinamide adenine dinucleotide phosphate (NADP+), and 0-10 M magnesium chloride in 0-10 M TRIS-HCl, pH 8-0. Increase in absorbance over 4 minutes was measured after addition of 100 μl of a 1:40 dilution of haemolysate. Enzyme activity was expressed as μmol NADPH generated per mg haemoglobin per minute.

Glutathione peroxidase activity was measured based on the procedure described by Paglia and Valentine, 15 in which the oxidation of glutathione and NADPH is coupled in the presence of glutathione reductase and hydrogen peroxide. The reaction mixture consisted of 0-28 mM NADPH, 1 unit of glutathione reductase in pH 7-2, 5-74 mM sodium azide, 5-0 mM glutathione, 0-1 mM desferrioxamine, and 10 μl of a 1:4 dilution of haemolysate in 0-05 M phosphate buffer, pH 7-0 containing 0-1 mM EDTA. After incubation for 3 minutes, the rate of decrease in absorbance at 340 nm was measured over 3 minutes following addition of 0-073 mM hydrogen peroxide. The spontaneous reaction rate was measured in the absence of haemolysate and subtracted from the final rate. Enzyme activity was expressed as μmol NADPH consumed per mg haemoglobin per minute.

Glutathione reductase activity was based on a modification of the procedure for glutathione peroxidase, in which oxidation of NADPH is measured as a decrease in absorption at 340 nm over time. The reaction mixture included 5-0 mM oxidised glutathione and 0-28 mM NADPH in 0-05 M phosphate buffer, pH 7-0, containing 0-10 mM EDTA. The reaction was initiated by the addition of 10 μl of a 1:4 dilution of haemolysate, and decrease in absorbance at 340 nm was followed over a period of 3-4 minutes. Spontaneous breakdown of NADPH was measured before addition of haemolysate, and subtracted from the final rate. Enzyme activity was expressed as in the glutathione peroxidase assay.

All enzyme assays were performed in duplicate. Activities were calculated to account for
the dilutions, and a mean specific enzyme activity was used in the statistical analyses. The χ² test was used to assess any potential relationship between variables, such as smoking history and disease severity. The likelihood ratio χ² test was used to evaluate the relation between disease stage of the two eyes of a participant to evaluate the appropriateness of using highest stage of the two eyes in the analysis. Multiple linear regression analysis was then performed to analyse the relation between antioxidant enzyme activity and ARMD stage. This statistical technique was chosen because it takes into account the potential effect of additional variables (covariates) on the response variable (enzyme activity). For example, in the analysis of the effect of ARMD stage on enzyme activity, covariates such as smoking history, antioxidant supplement use, and cardiovascular disease history are also included because these factors may also have a potential impact on enzyme activity. Results were also analysed using ordinal regression analysis, which was used to evaluate the predictive value of antioxidant enzyme activity of disease severity of a participant and to confirm results using multiple linear regression.

**Results**

Profiles of study participants are shown in Table 2. There was a positive association between the stage of ARMD between the two eyes of a participant (χ²; p=0.001). Because of the similarity of stage of ARMD between the two eyes, the highest stage of the two eyes of each participant was used in the analyses because it was believed to be a representative indicator of severity of macular findings.

The duplicate enzyme activities showed good reproducibility, and a mean of the activities was used in the statistical analyses. Multiple linear regression analysis modelled with age as the response variable with covariates indicated by sex, smoking history, cardiovascular disease, and ARMD stage demonstrated a tendency for older individuals to have more severe ARMD (p=0.01, 𝑟² 0.17). There was no significant effect of age on antioxidant enzyme activity for any of the enzymes.

Although the number of smokers and users of supplemental antioxidant vitamins was small, there was a tendency for smokers to have higher stages of ARMD (likelihood ratio χ², p=0.027). Smoking and antioxidant vitamin use were not found to be associated with higher antioxidant enzyme activity in these small subsets.

The distribution of mean antioxidant enzyme activity by stage of ARMD is shown in Table 3. Figures 1 to 5 show scatter plots of ARMD stage versus red blood cell enzyme activity for each of the enzymes evaluated. Multiple linear regression modelled with enzyme activity as the response variable with covariates of sex, smoking history, cardiovascular disease, antioxidant vitamin use, and disease severity was performed to evaluate whether there was an association between red

### Table 2 Patient characteristics based on stage of age-related macular degeneration in the more severely affected eye

<table>
<thead>
<tr>
<th></th>
<th>Stage 1</th>
<th>Stage 2</th>
<th>Stage 3</th>
<th>Stage 4</th>
<th>Stage 5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number</td>
<td>12</td>
<td>5</td>
<td>13</td>
<td>9</td>
<td>27</td>
</tr>
<tr>
<td>Mean age (years)</td>
<td>62.5</td>
<td>65.4</td>
<td>70.0</td>
<td>77.2</td>
<td>72.5</td>
</tr>
<tr>
<td>M:F</td>
<td>6:6</td>
<td>2:3</td>
<td>4:9</td>
<td>1:8</td>
<td>4:23</td>
</tr>
<tr>
<td>Smoking history</td>
<td>4</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td>10</td>
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<tr>
<td>Cardiovascular disease</td>
<td>3</td>
<td>2</td>
<td>3</td>
<td>6</td>
<td>8</td>
</tr>
<tr>
<td>Antioxidant vitamin use</td>
<td>2</td>
<td>0</td>
<td>1</td>
<td>2</td>
<td>2</td>
</tr>
</tbody>
</table>

### Table 3 Mean antioxidant enzyme activity in red blood cells of patients based on stage of age-related macular degeneration

<table>
<thead>
<tr>
<th></th>
<th>Stage 1</th>
<th>Stage 2</th>
<th>Stage 3</th>
<th>Stage 4</th>
<th>Stage 5</th>
<th>p Value</th>
<th>𝑟²</th>
</tr>
</thead>
<tbody>
<tr>
<td>SOD</td>
<td>4.248</td>
<td>3.620</td>
<td>3.625</td>
<td>2.623</td>
<td>3.961</td>
<td>0.67</td>
<td>0.08</td>
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<tr>
<td>CAT</td>
<td>174.0</td>
<td>221.5</td>
<td>227.6</td>
<td>150.1</td>
<td>216.6</td>
<td>0.43</td>
<td>0.12</td>
</tr>
<tr>
<td>G-6-PDH</td>
<td>0.0038</td>
<td>0.0037</td>
<td>0.0041</td>
<td>0.0038</td>
<td>0.0039</td>
<td>0.07</td>
<td>0.03</td>
</tr>
<tr>
<td>GSH-Px</td>
<td>0.0479</td>
<td>0.0429</td>
<td>0.0579</td>
<td>0.0538</td>
<td>0.0514</td>
<td>0.37</td>
<td>0.12</td>
</tr>
<tr>
<td>GSSG-Red</td>
<td>0.0073</td>
<td>0.0073</td>
<td>0.0082</td>
<td>0.0061</td>
<td>0.0088</td>
<td>0.60</td>
<td>0.09</td>
</tr>
</tbody>
</table>

SOD=superoxide dismutase, CAT=catalase, G-6-PDH=glucose-6-phosphate dehydrogenase, GSH-Px=glutathione peroxidase, GSSG-Red=glutathione reductase. Enzyme activities expressed as in Methods section. p Values and 𝑟² are from multiple regression analysis.

![Figure 1](http://bjo.bmj.com/)

**Figure 1** Plot of age-related macular degeneration (ARMD) grade versus superoxide dismutase (SOD) activity in units/mg haemoglobin. ARMD grade as defined in text (grade 1 is normal).

![Figure 2](http://bjo.bmj.com/)

**Figure 2** Plot of ARMD grade versus catalase (CAT) activity in pmol hydrogen peroxide consumed/mg haemoglobin per minute.
blood cell enzyme activity and disease severity. There was no significant association for any of the enzymes (Table 3). In addition, none of the covariables had a significant impact on antioxidant enzyme activity. Ordinal regression analysis was performed to evaluate the predictive value of antioxidant enzyme activity for each enzyme on severity of ARMD, and once again no significant association was found.

In a separate analysis, three new stages were defined by combining previously defined stages of disease: stage 1 remained as a single level (level 1); stages 2 and 3 were combined (level 2); and stages 4 and 5 were combined (level 3). Once again there was no significant association between severity of ARMD and antioxidant enzyme activity when stages were grouped into these new levels.

Discussion
Oxidative damage is a form of tissue injury which is initiated by reactive oxygen species known as free radicals. These reactive molecules can form during the course of normal aerobic metabolism or as the result of a particular insult such as light exposure. Complex defence mechanisms against such damage exist in tissues exposed to oxidative stress. However, when defences are inadequate, damage can result. The final consequence of oxidative damage includes loss of normal structural and functional integrity of cells.

Within the eye, these damaging reactions have been proposed to be involved in the pathogenesis of ARMD.2-4 The retina is considered to be especially at risk because photoreceptor membranes contain extremely high levels of polyunsaturated fatty acids, which are molecules particularly susceptible to these damaging reactions.16 In addition, the high metabolic rate and exposure to light may contribute to an underlying oxidative stress. A wide variety of protective mechanisms exists. Protective vitamins such as α tocopherol function to quench free radicals and thus prevent propagation of the damaging reactions. In addition, antioxidant enzymes such as superoxide dismutase catalyse reactions which prevent the damaging reactions induced by free radicals. Catalase and the glutathione enzyme system function to prevent the damaging effects of hydrogen peroxide. Glucose-6-phosphate dehydrogenase participates indirectly by catalysing the generation of reduced nicotinamide adenine dinucleotide phosphate, a necessary cofactor for the protective glutathione system.

The protective effect of antioxidant substances in ARMD is suggested by the finding that a high blood antioxidant index (composed of blood levels of vitamins E, C, selenium, and carotenoids) is associated with a decreased risk for development of neovascular ARMD.6 These results imply that oxidative damage is involved in development of neovascular ARMD. However, there is no conclusive evidence that an increased susceptibility to oxidative stress exists in patients with the disease. Concerning treatment options, further studies (such as the National Eye Institute
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sponsored Age-Related Eye Disease Study) are necessary before it can be concluded that supplemental antioxidant substances are of benefit in ARMD.

It has been proposed that patients with ARMD have lower levels of particular antioxidant enzymes compared with age-matched controls, and that this deficiency may be related to development of disease. The finding of lower levels of superoxide dismutase and glutathione peroxidase in small series of patients compared with controls led one group to propose that red blood cells can be used as a biological index for studying progress of disease in ARMD. The patient numbers in these studies were smaller than in our study. In this report, we investigated further whether an association between ARMD severity and particular major antioxidant enzyme levels in red blood cells exists. Such an association, if present, would imply that a particular blood antioxidant enzyme level may be a marker or risk factor for disease progression.

To evaluate this possible association, we needed to define a grading system of increasing levels of severity of aging macular changes. It is known that ARMD is a heterogeneous disease. We chose a grading system consisting of five subgroups of increasingly severe macular findings of aging individuals. Stage 1 includes participants with either a normal macular examination or few small hard drusen. A few small hard drusen in the macula of elderly individuals is a common finding and a normal aging variant, and not a definite risk factor for progression to exudative disease. The presence of pigment changes and large drusen of the macula (stages 2 and 3, respectively) represent minor changes of aging maculopathy which may cause minor vision loss. These changes are possible risk factors for exudative disease and more severe vision loss. Stages 4 and 5 (geographic atrophy of the retinal pigment epithelium and exudative maculopathy, respectively) are the classic manifestations of ARMD which are commonly associated with severe vision loss.

Using this grading system in our analysis, we found no evidence for an association between severity of aging maculopathy and antioxidant enzyme activity. One possible reason for the lack of identified association is that our grading system may not adequately represent a stratification of disease severity. However, even when stages were combined with comparison of more severe (stages 4 and 5) to less severe (stages 3 and 2) and normal (stage 1), the lack of association persisted. Although our results are limited by small sample size and variability of sample size subgroups with different ARMD severity, there is clearly no trend for an association between severity of ARMD and antioxidant enzyme activity for any of the enzymes evaluated. The lack of an effect of age on enzyme activity was supported by results of other investigators.

It is possible that a much larger sample size may show a trend for one or more enzyme activities to change with ARMD severity, but such a change would more than likely be so small that it would not be a practical clinical tool to screen for susceptibility to ARMD or study disease progression. In addition, it is possible that antioxidant enzyme activity correlates with presence of a particular subset of patients with ARMD. A larger sample size for each stage would be of use in analysing this possibility further. Finally, there may be an interaction of oxidative stress with other aetiological factors, such as a possible susceptibility gene. A more detailed knowledge of aetiological factors may provide further insight into the exact role of oxidative stress in ARMD.

The techniques for assay which we used differed from those of other investigators. However, we do not believe that our results were limited by experimental technique in measurement of enzyme activity. The enzyme activities which we measured tended to be higher for most of the enzymes. One investigator, who found differences in glutathione reductase and peroxidase in patients with ARMD, stored plasma samples at −70°C after venepuncture. We found that freezing at −70°C resulted in a decline in enzyme activity over a few days, which may account for the increased activities which we measured. The time interval between phlebotomy and clinical assay may contribute to variability of results. However, this interval was kept at no more than 24 hours. In our experience, no significant decline in enzyme activity occurs in this interval (unpublished results). In addition, the assay results at the time of patient examination were performed in duplicate, and showed good reproducibility.

Using similar assay techniques, we have previously identified a significant effect of age on the difference between the macular and peripheral superoxide dismutase and glutathione peroxidase specific activity of human cadaver retina. There was a significant decline in peripheral superoxide dismutase activity in the periphery with age, and the difference between glutathione peroxidase activity between the macula and periphery increased with age. Further research is in progress to evaluate the basis of this difference, which may in part be the result of differential cell loss with age between the macula and periphery.

Clearly, additional research is needed to further evaluate the role of oxidative stress in ARMD. We conclude from these results that antioxidant enzyme activities evaluated in the red blood cells of individuals with ARMD are not useful correlates of disease severity. There is no evidence for an underlying systemic oxidative stress as measured by antioxidant enzyme activities in red blood cells of patients with ARMD. Further research is needed to understand the role of oxidative damage in this important disorder of aging.

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