Documenta Ophthalmologica **106:** 129–136, 2003.

Nitric oxide and lipid peroxidation are increased and associated with decreased antioxidant enzyme activities in patients with age-related macular degeneration

Cem Evereklioglu[1], Hamdi Er[2], Selim Doganay[2], Mustafa Cekmen[3], Yusuf Turkoz[2], Baris Otlu[2] & Elif Ozerol[2]

[1]*Department of Ophthalmology, Erciyes University;* [2]*Inönü University, Medical Faculty, Turgut Ozal Medical Center, Turkey;* [3]*Gaziantep University Medical Faculty, Research and Application Hospital, Turkey*

Accepted 23 December 2001

Key words: age-related macular degeneration, antioxidant enzymes, lipid peroxidation, nitric oxide

Abstract

Background: Nitric oxide (NO), hydroxyl radical ($OH^{\cdot}$), superoxide anion (O_2^-) and hydrogen peroxide (H_2O_2) are free-radicals released in oxidative stress. Superoxide dismutase (SOD), glutathione peroxidase (GSH*Px*) and catalase (CAT) are antioxidant enzymes, mediating defense against oxidative stress. Excess NO and/or defective antioxidants cause lipid peroxidation, cellular dysfunction and death. Age-related maculopathy (ARM) or degeneration (ARMD) is the leading cause of irreversible blindness in developed countries. The etiology is unclear and the molecular factors contributing this disease remain to be specified. *Aims*: This multicenter, double-blind, cross-sectional study aimed to investigate plasma NO and lipid peroxidation levels with relation to antioxidant enzyme activities in erythrocyte and plasma of patients with ARMD compared with healthy control subjects. *Methods*: NO, lipid peroxidation (measured as plasma malondialdehyde [MDA] levels) and the catalytic activity of SOD, GSH*Px* and CAT were measured in a group of 41 patients with maculopathy (19 men, 22 women; 67.12 ± 3.70 years) and compared with 25 age- and sex-matched healthy control subjects without maculopathy (12 men, 13 women; 68.04 ± 3.02 years). NO and MDA levels were measured in plasma, CAT in red blood cells (RBCs), and SOD and GSH*Px* in both plasma and RBCs. Color fundus photographs were used to assess the presence of maculopathy, and the patients were divided into two groups using clinical examination and grading of photographs; early-ARM (n = 22) and late-ARMD (n = 19). *Results*: All patients with maculopathy had significantly ($p < 0.001$) higher plasma NO levels over control subjects (mean ± SD, 48.58 ± 8.81 vs. 28.22 ± 3.39 μmol/l). Plasma MDA levels in patients and control subjects were 4.99 ± 1.00 and 2.16 ± 0.24 μmol/l, respectively, and the difference was significant ($p < 0.001$). On the other hand, SOD and GSH*Px* activities were significantly lower in both RBCs and plasma of patients with maculopathy than in control subjects (RBCs-SOD, 3509.30 ± 478.22 vs. 5033.30 ± 363.98 U/*g* Hb, $p < 0.001$; plasma-SOD, 560.95 ± 52.52 vs. 704.76 ± 24.59 U/*g* protein, $p < 0.001$; RBCs-GSH*Px*, 663.43 ± 41.74 vs. 748.80 ± 25.50 U/*g* Hb, $p < 0.001$; plasma-GSH*Px*, 98.26 ± 15.67 vs. 131.80 ± 8.73 U/*g* protein, $p < 0.001$). RBCs-CAT levels were not different between groups (131.68 ± 12.89 vs. 133.00 ± 13.29 k/*g* Hb, $p = 0.811$). Late-ARMD patients had significantly lower antioxidant enzyme levels and higher MDA levels when compared with early-ARM patients (for each, $p < 0.001$). In addition, plasma NO and MDA levels were negatively correlated with SOD and GSH*Px* activities. *Conclusions*: This study demonstrated for the first time that NO, the most abundant free-radical in the body, might be implicated in the pathophysiology of ARMD in association with decreased antioxidant enzymes and increased lipid peroxidation status.

Introduction

Age-related maculopathy (early-ARM) [1] or degeneration (late-ARMD) [2] is the leading cause of blindness in elderly population in the developed world. Although smoking [3], oxidative stress [4], atherosclerosis [5], genetic factors [6], and fibrinogen [7] have been implicated in this disease, data are still

scarce and the etiopathogenesis of this disorder remains to be specified. Macula is highly susceptible to oxidative stress because of its high polyunsaturated fatty acid content, its high O_2 consumption, and its exposure to visible light. Therefore, this area is prone to lipid peroxidation [8]. For a long time, therapeutic nihilism has been the rule among physicians confronted with such patients. It is, therefore, urgent to determine the factor(s) that may lead to this disease.

Oxidative stress is a well-known phenomenon in the body, which is thought to play an important role in various disease processes and syndromes [9,10]. Reactive oxygen species (ROS) consist of superoxide anion (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radical ($OH^{\cdot}$), and increased free-radical production leads to oxidative stress with the formation of self-propagating lipid peroxidation [11]. Primary defense against oxidative stress includes the antioxidant enzymes superoxide dismutase (SOD, EC1.15.1.1), glutathione peroxidase (GSH*Px*, EC1.11.1.9) and catalase (CAT, EC1.11.1.6). SOD catalyzes first the dismutation of O_2^- to H_2O_2. Then, GSH*Px* and CAT independently convert H_2O_2 to H_2O [12]. When, however, excess free-radicals or deficient antioxidant defense system are present, the increased H_2O_2 reacts with O_2^-, producing $OH^{\cdot}$, which is one of the most active ROS. On the other hand, nitric oxide (NO) is one of the most abundant free-radicals in the body and excess NO production causes mitochondrial respiratory enzyme inhibition [13]. Although the formed O_2^- is scavenged by superoxide dismutase (SOD), NO is the only known molecule that can be produced high enough to out-compete SOD for O_2^-[13]. If NO production is increased, short-lived NO rapidly reacts with O_2^- to form a potent and powerful long-lived oxidant free-radical, peroxynitrite ($ONOO^-$) [14]. These oxidative radicals interact with nucleic acids, proteins and lipids causing cellular dysfunction and even death.

Since NO is a well known free-radical in the body and has a close relation with oxidative stress and antioxidant enzymes, the present study investigated for the first time plasma NO levels in patients with ARMD. The interrelation between NO, lipid peroxidation, and antioxidant enzyme status was also determined by measuring MDA in plasma, CAT in erythrocytes, and SOD and GSH*Px* in both plasma and erythrocytes of the subjects.

Patients and methods

Patients

In this multicenter study, we obtained informed written consent from all patients and control subjects. Patients with maculopathy were selected from the routine clinical services and retinal divisions at research hospitals between 1999 and 2001. The Ethics Board of the university approved the study. A total of 41 patients with maculopathy (mean $\pm$ SD, 67.12 $\pm$ 3.70 years, 19 men, 22 women) and 25 age- and sex-matched healthy control subjects without maculopathy (68.04 $\pm$ 3.02 years, 12 men, 13 women) included in this cross-sectional investigation. Detailed family history of ARMD, smoking status, past medical history, and dietary intake were elicited by questionnaire in both groups. The subjects in both groups had to fulfill the following criteria: age over 60 years, stable weight, BMI within normal range (20–25), sedentary lifestyle, and normal caloric intake. Exclusion criteria were smokers, diabetes, cardiovascular disease, neurological disorder, malignant or chronic inflammatory diseases, eating disorders, and medication with lipid-lowering drugs or agents affecting metabolism [15]. Standard blood screening parameters (white and red blood cell count, platelet count, sodium, potassium, creatinine, aspartate, and alanine aminotransferase) and routine urine analyzes had to be within normal limit.

Definitions and grading of early and late maculopathy

Fundus photography and fluorescein angiography were performed in all subjects with maculopathy. The grading of the individual lesions was performed according to the Wisconsin Age-Related Maculopathy Grading System, developed by Klein et al. [16] and incorporated in an international classification system [2]. A grid centered on the fovea of stereo-color fundus photographs with a radius of 3000 μm was used. All photographs were graded by two experienced graders. The patients with maculopathy were divided into two groups; early-ARM and late-ARMD. Late- (advanced) ARMD included neovascular macular degeneration, subretinal fibrous scar, and geographic atrophy with confluent patches of RPE atrophy at least in one eye. Early-ARM was defined as either the presence of soft indistinct or reticular drusen, or the presence of both soft distinct drusen and retinal pigmentary abnormalities in the absence of

late-ARMD in either eye. If both eyes were graded, participants were categorized according to the findings in the field of the worse eye. Finally, 22 patients were classified as having early-ARM and the remaining 19 as late-ARMD.

Sample collection

This was a double-blind study. Therefore, both the physician taking the blood and the analyser were blinded to the group of the subject. Antecubital whole-blood samples were drawn from a peripheral vein using a 25-gauge needle, avoiding hemolysis, into evacuated heparinized plain tubes in the morning hours (08.00–10.00) after an overnight fast and 30 min of supine rest. None of the patients and control subjects received any topical or systemic medication at least 2 weeks prior to blood collection. The samples were centrifuged at 3000 × *g* for 10 min at 4°C, and the harvested plasma was collected and kept at −70°C until the time of analysis. The erythrocytes were subsequently washed twice with two volumes of 0.9% sodium chloride solution. Following this, the erythrocytes were hemolyzed with a two-fold volumes of ice-cold distilled water. After centrifugation (5000 × *g*, 10 min, 4°C) the supernatant was frozen at −70°C until the time of analysis. The plasma was used to measure MDA as the end product of lipid peroxidation, total nitrite concentrations as an indicator of recent NO production, and the catalytic activities of antioxidant enzymes (SOD, GSH*Px*). The supernatant was used to measure red blood cells (RBCs)-SOD, RBCs-GSH*Px* and RBCs-CAT levels. Hemoglobin content of whole blood was measured, and enzyme activity was determined per mg hemoglobin.

Total nitrite analysis for nitric oxide by Griess reaction

To study the biological role of NO in a particular process requires measuring its concentrations. NO is a labile compound, has a brief half-life and is rapidly converted to the stable end-products nitrite (NO_2^-) and nitrate (NO_3^-) in typical oxygenated aqueous solutions. Because an excellent and sensitive colorimetric reagent (the Griess reagent) exists for the determination of NO_2^-, it is common practice to use enzymatic or chemical reduction to convert all NO_3^- to NO_2^- by nitrate reductase prior to spectrophotometric quantitation of total NO_2^-, and measure total nitrite (NO_2^- + NO_3^-) as an indicator of recent NO production in biological samples as described before [17,18]. In addition to providing all necessary components in a microtiter format, it employs affinity purified *Zea mays* nitrate reductase and NADH, thereby circumventing some of the potential problems reported for NO_2^- measurement using nicotinamide adenine dinucleotide phosphate (NADPH)-dependent nitrate reductases.

In short, samples (250 μl) was incubated at room temperature with 250 μl of substrate buffer (imidazole 0.1 mol/l, NADPH 210 μmo/L, flavineadenine dinucleotide 3.8 μmol/l: pH 7.6) in the presence of nitrate-reductase (*Aspergillus niger*, Sigma) for 45 min to convert NO_3^- to NO_2^-. Excess reduced NADPH, which interferes with the chemical detection of NO_2^-, was oxidized by continuation of the incubation of 5 μg (1 μl) of LDH (Sigma), 0.2 mmol/l (120 μl) pyruvate (Sigma) and 79 μl of water. Total nitrite was then analyzed by reacting the samples with Griess reagent (1% sulfanilamide, 0.1% *N*-(1-naphthyl)-ethylenediamine dihydrocholoride in 5% H_3PO_4 spectroquant: Merck, Darmstadt, Germany). Reacted samples were treated with 500 ml of trichloroacetic acid (20%), centrifuged for 15 min at 8000 × *g* and the absorbance at 548 nm was compared with that of $NaNO_2$ standard (0–100 μmol/l). Total nitrite/nitrate levels were expressed as μmol/l. This method can be used to accurately measure as little as 1 μmol of NO_2^- (final concentration in the assay). Very little sample is required (5 – 85 μl for most samples).

Plasma MDA analysis for lipid peroxidation

Plasma MDA level, referred as thiobarbituric acid-reactive substance (TBARS), was measured according to the method described by Wasowicz et al. [19]. In brief, 50 ml of plasma or an adequate volume of MDA working standard solution were introduced into 10-ml glass tubes containing 1 ml of distilled water. After addition of 1 ml of the solution containing 29 mmol/l TBARS in acetic acid and mixing, the samples were placed in a water bath and heated for 1 h at 95–100°C. After the samples were cooled, 25 μl of 5 mol/l HCI was added, and the reaction mixture was extracted by agitation for 5 min with 3.5 ml of *n*-butanol. Butanol phase was separated by centrifugation at 1500 × *g* for 10 min. The butanol extract was measured with a spectrofluorometer (Hitachi, F-4010 fluorescence spectrophotometer) at wavelengths of 525 nm for excitation. The calibration curve was prepared with

Table 1. Plasma nitric oxide, lipid peroxidation, and primary antioxidant enzyme activities in plasma and erythrocytes of patients with age-related maculopathy, age-related macular degeneration, and healthy control subjects

	Controls (n = 25 (12 M, 13 F)) Mean ± SD	All patients (n = 41 (19 M, 22 F)) Mean ± SD	Early-ARM (n = 22 (10 M, 12 F)) Mean ± SD	Late-ARMD (n = 19 (9 M, 10 F)) Mean ± SD
Age years	68.04 ± 3.02	67.12 ± 3.70	64.50 ± 2.40	70.15 ± 2.38
Plasma-NO (μmol/l)	28.22 ±3.39	48.58 ± 8.81*	41.75± 4.44	56.50 ± 5.13‡
Plasma-MDA (μmol/l)	2.16 ±0.24	4.99 ± 1.00*	4.11 ± 0.22	6.02 ± 0.31‡
RBCs-SOD (U/g Hb)	5033.30 ± 363.98	3509.30 ± 478.22†	3825.49 ± 401.39	3143.19 ± 237.86§
Plasma-SOD (U/g protein)	704.76 ± 24.59	560.95 ± 52.52 †	602.72 ± 26.69	512.57 ± 26.39§
RBCs-GSH*Px* (U/g Hb)	748.80 ± 25.50	663.43 ± 41.74†	695.50 ± 27.30	626.31 ± 17.05§
Plasma-GSH*Px* (U/g protein)	131.80 ± 8.73	98.26 ± 15.67 †	109.86 ± 9.36	84.84 ± 9.44§
RBCs-CAT (k/g Hb)	133.00 ± 13.29	131.68 ± 12.89||	128.68 ± 13.08||	135.15 ± 12.07||

ARM, age-related maculopathy; ARMD, age-related macular degeneration; M, male subjects; F, female subjects; SD, standard deviation; NO, nitric oxide; MDA, malondialdehyde; RBCs, red blood cells; SOD, superoxide dismutase; GSH*Px*, glutathione peroxidase; CAT, catalase.
*Significantly higher than control subjects by Mann-Whitney U-test (for each, $p < 0.001$).
†Significantly lower than control subjects by Mann–Whitney U-test (for each, $p < 0.001$).
‡Significantly higher than ARM patients by Mann–Whitney U-test (for each, $p < 0.001$).
§Significantly lower than ARM patients by Mann–Whitney U-test (for each, $p < 0.001$).
||Statistically not different when compared with control subjects ($p > 0.05$).

MDA standards of 0–10 μmol/l. Intra- and inter-assay CVs were 3.5 and 6%, respectively.

Plasma and RBCs-SOD analysis

SOD activities in plasma and supernatant were measured according to the method of Sun et al. [20] by determining the inhibition of nitroblue tetrazolium (NBT) reduction with xanthine–xanthine oxidase used as a O_2^- generator. One unit of SOD is defined as the amount of protein or hemoglobin that inhibits the rate of NBT reduction by 50%. Results were defined as units per gram protein or hemoglobin (U/*g* protein or U/*g* Hb).

Plasma and RBCs-GSHPx analysis

GSH*Px* activity in plasma and supernatant was measured according to the method of Paglia and Valentine [21]. Enzyme activity was determined from the oxidation of reduced NADPH in the presence of H_2O_2 used as substrate. The decrease in concentration of NADPH was monitored and recorded at 340 nm in a mixture containing reducted glutathione and glutathione reductase (pH 7.8, 25°C). Enzyme units were defined as the number of micromoles of NADPH oxidized per minute. Results were defined as units per gram protein or hemoglobin (U/*g* protein or U/*g* Hb).

RBCs-CAT analysis

CAT activity in supernatant was determined according to the method of Aebi [22] by monitoring the initial rate of disappearance of H_2O_2 (Initial concentration 10 mm) at 240 nm (e = 0.041 $mmol^{-1}$ 1 cm^{-1}) in a cuvette containing 10.5 mm H_2O_2 in 1 ml of 50 mm phosphate buffer (pH 7, 25°C), in a spectrophotometer. Results were reported as constant rate per second per gram hemoglobin (k/*g* Hb). Protein concentrations in plasma samples were measured according to Lowry et al. [23].

Calculations and statistics

Mann–Whitney *U*-test was used for statistical analysis and results were expressed as mean ± standard deviation (SD). Multiple regression analysis, ordinal logistic regression analysis and Spearman's correlation coefficient analysis were performed as indicated to determine whether NO was correlated with lipid peroxidation and antioxidant enzymes or whether measured parameters were associated with severity of maculopathy. *P* values below 0.05 were considered to be significant. Statistical analysis was performed with Statistical Package for the Social Sciences for Windows (SPSS Inc., version 8.0, Chicago, IL, USA).

Results

The age and sex ratio were not substantially different (for each, $p > 0.05$) between all patients with maculopathy and control subjects. Clinical and photographic grading was available in all subjects included in the present study. Two patients with corneal opacity, two patients with cataract and one patient with poor dilation were not included in the present study for clinical ungradability. BMI was similar between patients (BMI: 22.79 ± 0.72) and control subjects (22.54 ± 0.69). The mean plasma total NO_2^- levels were significantly ($p < 0.001$) higher in patients with maculopathy over control subjects (48.58 ± 8.81 vs. 28.22 ± 3.39 μmol/l) and well-correlated with disease chronicity ($r = 0.99$, $p < 0.001$). Plasma MDA levels in patients and control subjects were 4.99 ± 1.00 and 2.16 ± 0.24 μmol/l, respectively, and the difference was significant ($p < 0.001$). Plasma NO levels were also positively correlated with plasma MDA concentrations ($r = 0.87$, $p < 0.01$). SOD and GSH*Px* activities were significantly lower in both RBCs and plasma of patients with maculopathy than in control subjects (RBCs-SOD, 3509.30 ± 478.22 vs. 5033.30 ± 363.98 U/*g* Hb, $p < 0.001$; plasma-SOD, 560.95 ± 52.52 vs. 704.76 ± 24.59 U/*g* protein, $p < 0.001$; RBCs-GSH*Px*, 663.43 ± 41.74 vs. 748.80 ± 25.50 U/*g* Hb, $p < 0.001$; plasma-GSH*Px*, 98.26 ± 15.67 vs. 131.80 ± 8.73 U/*g* protein, $p < 0.001$). On the other hand, RBCs-CAT levels were not different between groups (131.68 ± 12.89 vs. 133.00 ± 13.29 k/*g* Hb, $p = 0.811$). In addition, NO levels were negatively correlated with plasma-SOD ($r = -0.85$, $p < 0.01$), plasma-GSH*Px* ($r = -0.91$, $p < 0.01$), RBCs-SOD ($r = -0.88$, $p < 0.01$), RBCs-GSH*Px* ($r = -0.79$, $p < 0.01$). Furthermore, late-ARMD patients had significantly lower antioxidant enzymes with higher NO and MDA levels when compared with early-ARM patients or control subjects (Table 1).

Discussion

Diseases associated with aging appear to have a common denominator; oxidative damage [8,11]. ARMD, the leading cause of visual loss among people over 60 years old in the United States, Europe and the other developed world, is accompanied by degeneration of the RPE cells [1,2]. As the number of individuals with ARMD increases, the economic and social consequences of this blinding disease rise. Although some risk factors have been determined [3,15], the ultimate etiology remains unknown, treatment options are limited and no proven preventive measures exist to halt development or progression of this disease. The specific insults that trigger and perpetuate ARMD are unknown, however, it may be the result of repetitive oxidative injuries [11,15]. The clinicopathological basis for development of ARMD is divided into solar radiation effects, repeated photic insult, photochemical damage, lipofuscin accumulation, drusen development, choroidal neovascularization, scarification, and RPE detachment [24,25].

Free-radicals (O_2^-, H_2O_2 and $OH^{\cdot}$), considered as the pathogenic agents of many diseases and of aging, are mainly derived from the metabolism of free acids and arachadonic acid, and from an univalent sequential reduction of molecular oxygen with beneficial (defense against microbial aggression by phagocytes) and detrimental effects at cellular and molecular levels [26]. The main target of oxidative injury is mitochondria, an organelle known to accumulate damages in postmitotic tissues during aging [27]. The burst of activated, oxygen-derived free-radical species is responsible for peroxidation of cell membranes, resulting in tissue edema, and protein and enzyme degradation. Lipid peroxidation in turn leads to the subsequent formation of free fatty acids and arachadonic acid [28]. A vicious circle ensues, whereby the metabolism of these molecules leads to further free-radical formation, and further oxidative damage. Defense systems of cells against this free-radical induced toxic lipid peroxidation consist of antioxidative free-radical scavenging molecules such as SOD, GSH*Px* and CAT [29]. These molecules block the initiation of free-radical chain reactions, and therefore, lipid peroxidation [11].

If SOD levels decrease concomitantly with GSH*Px*, then the first (O_2^-) and second step (H_2O_2) intermediate radicals accumulate. This oxygen free-radicals could undergo the Fenton's reaction, generating $OH^{\cdot}$, which may lead to lipid peroxidation in cells [4,8,11].

Important in this respect is the role of NO in oxidative stress, which is one of the most abundant free-radical in the body. Normally, the detrimental effect of NO is destroyed by O_2^- and stabilized by SOD [30]. The result is the elimination of both radicals. If excess amount of NO is produced, and/or SOD is decreased, then the short-lived NO combines with mildly reactive free-radical O_2^- to form a potent and long-lived toxic oxidant free-radical species, peroxynitrite anion ($ONOO^-$): $O_2^{\cdot -} + NO^{\cdot} \rightarrow ONOO^-$ [14]. A vicious circle ensues, whereby $ONOO^-$ breaks down to $OH^{\cdot}$ [31]. Formation of $ONOO^-$ and $OH^{\cdot}$ also mediate tissue injury. Therefore, decreased SOD and GSH*Px* activities paired with the increased NO levels reacting with O_2^- cause further antioxidant depletion and further oxidative damage with mitochondrial respiratory enzyme inhibition. This oxidation of cell membrane lipids leads to the further formation of lipid peroxidation, with subsequent formation of free fatty acids, followed in turn by further free-radical formation [32].

Although NO is one of the well-known free-radical in the body with its role in oxidative stress, its role in the etiopathogenesis of ARMD have not been previously investigated. Evidence is now emerging that NO is a mediator of physiological, and possibly pathological, processes in the retina [33]. This investigation was conducted to measure circulating levels of NO and the components of the primary free-radical defense system in patients with maculopathy. In addition, we also assessed the levels of plasma TBARS (MDA) as an estimate of lipid peroxidation. Using NO_2^-as an index of NO synthesis, we showed higher NO_2^-concentrations in the plasma of patients with ARMD when compared with healthy control subjects without ARMD. This was to be expected, since free-radical insult is well defined phenomenon in patients with ARMD and protection of the retina from light damage using a NO synthase inhibitor has been previously reported [34]. The concept that excessive NO insult with oxidative lipid peroxidation might be a factor contributing to the development of ARMD may be suggested by the higher levels of MDA concentrations. In addition, NO levels were positively correlated with disease chronicity as well as plasma MDA levels. Moreover, late-ARMD patients showed statistically significant higher NO levels when compared with early-ARM subjects. On the other hand, both plasma and erythrocyte activities of SOD and GSH*Px* were significantly decreased in ARMD patients when compared with control subjects without ARMD. Furthermore, a negative correlation could be established between maculopathy grading and plasma-SOD ($r = -0.89$, $p < 0.01$) or plasma-GSH*Px* levels ($r = -0.77$, $p < 0.01$). This suggests that the RBCs and plasma of ARMD patients have an unbalanced antioxidant system with a concomitant increase in free-radical NO. Therefore, it may be suggested that higher NO levels with concomitant lower antioxidant enzyme status results in exaggerated oxidative stress and, therefore, increased lipid peroxidation in ARMD patients.

Although certain risk factors have been identified, no interventions have been proven effective in prevention of ARMD. Laser photocoagulation has been known as an effective treatment, but its benefits are limited. Therefore, identifying the molecular defect of the disease is of high interest. The posterior region of the human retina is very susceptible to lipid peroxidation [35]. The photoreceptor–RPE complex contain high levels of lipofuscin and long-chain polyunsaturated fatty acids, where this complex is exposed to visible light and, therefore, susceptible to oxidative damage with its near-arterial oxygen level [36]. By other words, macula, a prime environment for the generation of ROS, is very prone to lipid peroxidation if antioxidants SOD and GSH*Px* activities are decreased. Although it has been demonstrated that retinal lipofuscin is a photoinucible generator of ROS, it is unclear how this may translate into cell damage in tissues highly susceptible to oxidative damage. Therefore, increased NO levels found in our ARMD patients might participate in this process by reacting with O_2^-, producing highly cytotoxic peroxinitrite with a consequent lipid peroxidation. It appears, therefore, that oxidative stress and the increased NO synthesis may be involved in the pathogenesis of early onset macular degeneration.

In conclusion, the present study demonstrated for the first time that plasma NO levels are increased in patients with ARMD and positively correlated with lipid peroxidation and negatively correlated with both SOD and GSH*Px* activities in plasma and RBCs. Our results are consistent with the epidemiological and experimental studies suggesting the involvement of lipid peroxidation in retinal diseases. Clinicians usually prescribe antioxidants to help in the management of ARMD. In spite of this, these processes inexorably

induce visual impairment and may progress towards blindness. Therefore, in addition to other pathogenic mechanisms not fully understood, it may be that NO, acting as cytotoxic oxidant when produced in excess, mediates in this chronic disorder. Thus, the possible detrimental value of NO on diseases with involvement of oxidative retinal tissue damage, such as ARMD, should be investigated further. Prospects for future research in the study of RPE require new initiatives that will probe more accurately into the localization of NO in the retina by detecting the tissue NO synthase levels in such patients. For this, surgical specimens of neovascular membranes from eyes with ARMD and the macular regions of whole donor eyes with neovascular ARMD should be studied further by sophisticated methods.

Acknowledgements

Authors have no financial or proprietary interest in any instrument or products used in this study.

References

1. Klein R, Klein BE, Jensen SC, Meuer SM. The five-year incidence and progression of age-related maculopathy. Ophthalmology 1997; 104: 7–21.
2. Bird AC, Bressler NM, Bressler SB, et al. An international classification and grading system for age-related maculopathy and age-related macular degeneration: The International ARM Epidemiological Study Group. Surv Ophthalmol 1995; 39: 367–74.
3. Risk factors for neovascular age-related macular degeneration. The Eye Disease Case-Control Study Group. Arch Ophthalmol 1992; 110: 1701–8.
4. Christen WG Jr. Antioxidants and eye disease. Am J Med 1994; 97: 14–7.
5. Vingerling JR, Dielemans I, Bots ML, Hofman A, Grobbee DE, de Jong PT. Age-related macular degeneration is associated with atherosclerosis. The Rotterdam Study. Am J Epidemiol 1995; 142: 404–9.
6. Allikmets R, Shroyer NF, Singh N, et al. Mutation of the Stargardt disease gene (ABCR) in age-related macular degeneration. Science 1997; 277: 1805–7.
7. Smith W, Mitchell P, Leeder SR, Wang JJ. Plasma fibrinogen levels, other cardiovascular risk factors, and age-related maculopathy: the Blue Mountains Eye Study. Arch Ophthalmol 1998; 116: 583–7.
8. Gerster H. Review: antioxidant protection of the ageing macula. Age Ageing 1991; 20: 60–9.
9. Pastor MC, Sierra C, Dolade M, et al. Antioxidant enzymes and fatty acid status in erythrocytes of Down's syndrome patients. Clin Chem 1998; 44: 924–9.
10. Ben-Menachem E, Kyllerman M, Marklund S. Superoxide dismutase and glutathione peroxidase function in progressive myoclonus epilepsies. Epilepsy Res 2000; 40: 33–9.
11. Beckman KB, Ames BN. The free radical theory of aging matures. Physiol Rev 1998; 78: 547–81.
12. Chance B, Sies H, Boveris A. Hydroperoxide metabolism in mammalian organs. Physiol Rev 1979; 59: 527–605.
13. Sozmen EY, Kerry Z, Uysal F, et al. Antioxidant enzyme activities and total nitrite/nitrate levels in the collar model. Effect of nicardipine. Clin Chem Lab Med 2000; 38: 21–5.
14. Beckman JS, Beckman TW, Chen J, Marshall PA, Freeman BA. Apparent hydroxyl radical production by peroxynitrite: implications for endothelial cell injury from nitric oxide and superoxide. Proc Natl Acad Sci USA 1990; 87: 1620–4.
15. Hirvela H, Luukinen H, Laara E, Laatikainen L. Risk factors of age-related maculopathy in a population 70 years of age or older. Ophthalmology 1996; 103: 871–7.
16. Klein R, Davis M, Magli YL, Segal P, Klein BE, Hubbard L. The Wisconsin age-related maculopathy grading system. Ophthalmology 1991; 98: 1128–34.
17. Granger DL, Taintor RR, Boockvar KS, Hibbs JB Jr. Measurement of nitrate and nitrite in biological samples using nitrate reductase and Griess reaction. Methods Enzymol 1996; 268: 142–51.
18. Evereklioglu C, Turkoz Y, Er H, Inaloz HS, Ozbek E, Cekmen M. Increased serum nitric oxide levels in patients with Behçet's disease: is it a new activity marker? J Am Acad Dermatol 2002; 46: 50–4.
19. Wasowics W, Neve J, Peretz A. Optimized steps in flourometric determination of thiobarbituric acid-reactive substances in serum: importance of extraction pH and influence of sample preservation and storage. Clin Chem 1993; 39: 2522–6.
20. Sun Y, Oberley LW, Li Y. A simple method for clinical assay of superoxide dismutase. Clin Chem 1988; 34: 497–500.
21. Paglia DE, Valentine WN. Studies on the quantitative and qualitative characterization of erythrocyte glutathione peroxidase. J Lab Clin Med 1967; 70: 158–69.
22. Aebi H. Catalase in vitro. Methods Enzymol 1984; 105: 121–6.
23. Lowry OH, Rosenbrough NJ, Farr AL. Protein measurement with the Folin phenol reagent. J Biol Chem 1951; 193: 265–75.
24. Tso MO. Retinal photic injury in normal and scorbutic monkeys. Trans Am Ophthalmol Soc 1987; 85: 498–556.
25. Alexander LJ. Age-related macular degeneration: the current understanding of the status of clinicopathology, diagnosis and management. J Am Optom Assoc 1993; 64: 822–37.
26. Crastes de Paulet A. Free radicals and aging. Ann Biol Clin 1990; 48: 323–30.
27. Cai J, Nelson KC, Wu M, Sternberg P Jr, Jones DP. Oxidative damage and protection of the RPE. Prog Retin Eye Res 2000; 19: 205–21.
28. Halliwell B. Reactive oxygen species in living systems: source, biochemistry, and role in human disease. Am J Med 1991; 91: 14–22.
29. Guemouri L, Artur Y, Herbeth B, Jeandel C, Siest G. Biological variability of superoxide dismutase, glutathione peroxidase, and catalase in blood. Clin Chem 1991; 37: 1932–7.
30. Goldstein IM, Ostwald P, Roth S. Nitric oxide: a review of its role in retinal function and disease. Vision Res 1996; 36: 2979–94.
31. Muijsers RB, Folkerts G, Henricks PA, Sadeghi-Hashjin G, Nijkamp FP. Peroxynitrite: a two-faced metabolite of nitric oxide. Life Sci 1997; 60: 1833–45.
32. Beckman JS. The double-edged role of nitric oxide in brain function and superoxide-mediated injury. J Dev Physiol 1991; 15: 53–9.

33. Bredt DS, Hwang PM, Snyder SH. Localization of nitric oxide synthase indicating a neural role for nitric oxide. Nature 1990; 347: 768–70.
34. Goureau O, Jeanny JC, Becquet F, Hartmann MP, Courtois Y. Protection against light-induced degeneration by an inhibitor of NO synthase. Neuroreport 1993; 5: 233–6.
35. De La Paz M, Anderson RE. Region and age-dependent variation in susceptibility of the human retina to lipid peroxidation. Invest Ophthalmol Vis Sci 1992; 33: 3497–9.
36. Wassell J, Davies S, Bardsley W, Boulton M. The photoreactivity of the retinal age pigment lipofuscin. J Biol Chem 1999; 274: 23828–32.

Address for correspondence: C. Everekliogl u, Dept. of Ophthalmology, Erciyes University Medical Faculty, Kayseri, Turkey
Phone: +90-352-233 15 65; Fax: +90-422-341 06 19;
E-mail: everekliogl u@hotmail.com