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Research Article

Thiol and Glutathione Homeostasis Parameters as Plasma Biomarkers of Oxidative Stress in Age-Related Macular Degeneration

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ABSTRACT

Purpose: To determine extracellular thiol homeostasis and intracellular glutathione homeostasis as a plasma biomarker for oxidative stress and to compare these parameters in non-exudative/exudative AMD patients and healthy individuals.

Method: 30 non-exudative AMD, 28 exudative AMD, and 36 age-matched healthy control subjects enrolled to the study. Extracellular total thiol, native thiol, disulphide amounts and intracellular oxidized/reduced glutathione levels of subjects were determined, and disulphide/thiol and oxidized/reduced glutathione percent ratios were calculated.

Results: In comparison with the control group both non-exudative and exudative AMD patients had higher plasma disulfide levels (20.5(4.8) vs. 4(3.1), p<0.001 and 22.5(7.5) vs. 15.4(3.1), p<0.001; respectively) and higher disulphide/thiol (6.64(2.57) vs. 5.4(1.9), p=0,002 and 7.05(3.14) vs. 5.4(1.9), p<0.001; respectively), in addition to higher oxidized glutathione levels (64.6(40.8) vs. 27.3(21.9), p=0.015 and 73.9(44.1) vs. 27.3(21.9), p=0.002; respectively) and oxidized/reduced glutathione ratio(6.48(8.35) vs. 3.14(3.31), p=0,034 and 10.21(10.28) vs. 3.14(3.31), p=0,003; respectively). Although there was no significant difference between groups in term of total thiol (361.5(61.6), 355.1(87.7) and 340.9(72.4), respectively, p=0,585); native thiol (318.8(62.4), 307.1(73.7) and 299.3(79.2), respectively, p=0,382); total reduced glutathione (986.3(282.1), 871.5(271.6) and 881.8(290.9), respectively, p=0.344) and native reduced glutathione (873.4(367.6), 723.7(379.0) and 797.2(307.5), respectively, p=0,113). However, there was no significant difference between non-exudative and exudative AMD groups in terms of both extracellular thiol homeostasis and intracellular glutathione homeostasis.

Conclusion: Greater extent of both extracellular disulphide and intracellular oxidized glutathione production occurred in AMD patients compared to age-matched healthy controls indicates the role of increased oxidative stress in AMD development. Further studies are needed to confirm the pathophysiologic role of homeostasis in these buffer systems in AMD.

Keywords: Age-related macular degeneration; Extracellular thiol homeostasis; Intracellular glutathione homeostasis; Oxidative stress; Plasma biomarker

INTRODUCTION

Age-related Macular Degeneration (AMD) is the leading cause of progressive and irreversible vision loss in the aging population in developed countries and the third most common cause of adult blindness worldwide. It is anticipated that global AMD prevalence will reach 196 million in 2020 and 288 million in 2040 [1]. Approximately 90% of patients with AMD have non-exudative form, characterized by the loss of Retinal Pigment Epithelium (RPE) and photoreceptors. The rest 10% of the AMD patient population has the exudative form of the disease, which manifests as abnormal growth of blood vessels into the retina from the choriocapillaris, a fenestrated blood vessel network outside the eye [2].

The impact of AMD on an individual's quality of life is high. Often asymptomatic in the early stages, in some patients AMD ultimately leads to loss of central vision deterioration and interferes with daily living activities, with profound effects on the quality of life of the elderly [3]. For example, Brown et al. [4] found

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that the decrease in quality of life from early AMD is similar to a person with symptomatic HIV, and with advanced AMD, to one with metastatic prostate cancer having poorly controlled pain [4]. With vision loss, a person with AMD is less active [5], and is at higher risk of depression [6,7] or anxiety [8] than unaffected elderly people.

AMD pathogenesis is multifactorial, and oxidative stress appears to play a major role in this pathogenesis. Several demographic and environmental risk factors for AMD, such as aging, smoking, and light exposure, have been linked to increased production of reactive Oxygen Species (OS) and thus cumulative cellular oxidative injury [9]. Supplementation with antioxidants (vitamin C, vitamin E, and βcarotene) and zinc was shown to slow AMD progression in the Age-Related Eye Disease Study (AREDS), a multicenter, randomized clinical trial, and high dietary intake of antioxidants (particularly carotenoids) has been correlated with lower AMD prevalence and incidence [10,11].

Oxidative stress is a general term that describes the steady-state level of oxidative damage in a cell, tissue, or organ caused by ROS, which the body produces as a natural product of normal oxygen metabolism [12]. Thiol molecules, which express the compounds containing the sulfur element, interact with all physiological oxidants and therefore play a key role as antioxidant buffer maintaining the oxidation/reduction (redox) balance inside the cell and the tissues [13]. Given that the dynamic thiol-disulphide homeostasis plays a critical role in apoptosis, regulation of enzyme activities, transcription, and cell signal transduction mechanisms, a defect in this homeostasis can cause a variety of diseases [14]. In many studies extracellular thiol levels had been measures to investigate the relationship between retinal diseases and oxidative damage [15-17]. However, evaluating the extracellular thiol-disulphide balance together with the oxidized and reduced forms of glutathione, which is the most important thiol source in the cell, will provide a better explanation of the total thiol balance.

The aim of this study was to evaluate the relationship between AMD and oxidative stress by measuring extracellular disulphide/thiol (SS/SH) levels and intracellular oxidized/reduced glutathione (GSSG/GSH) levels, and to compare these parameters in non-exudative/exudative AMD patients and healthy individuals.

MATERIALS AND METHODS

Study population

In this prospective, cross-sectional, and comparative study, we enrolled a total of 30 patients with non-exudative type AMD, 28 exudative type AMD patients and 36 healthy control subjects. All patients provided written informed consent before enrollment. All procedures conformed to the tenets of the Declaration of Helsinki. The study was approved by the Ethics Committee of the Bolu Abant Izzet Baysal University, Bolu, Turkey.

All subjects underwent complete ophthalmological examination including best-corrected Snellen visual acuity, slit-lamp biomicroscopy, applanation tonometry, dilated fundus examination, spectral domain optic coherence tomography, and fundus fluorescein angiography when necessary. A detailed ophthalmic and medical history was obtained from all subjects. Patients and healthy control subjects with a history of smoking, malignancy, coronary artery disease, rheumatic disease, cerebrovascular disease, liver, and kidney dysfunction, systemic inflammatory diseases, thyroid dysfunction, vitamin deficiencies, and other systemic disorders were excluded from the study due to the possibility of false-positive results in plasma thiol level analysis accompanying these disorders. Moreover, patients with significant refractive errors (>3 diopters of spherical equivalent refraction), intraocular pressure ≥ 21 mmHg, glaucoma, pseudoexfoliation, uveitis, retinal vascular diseases or anterior optic neuropathy, patients who had previous ocular surgery, and individuals taking vitamins or antioxidant supplements, were also excluded from the analysis.

Chemicals

All chemicals were purchased from Sigma-Aldrich Inc. (Taufkirchen, Germany) and Merck Co. (Darmstadt, Germany) and of analytical grade and type-1 reagent-grade deionized water was used.

Samples

Two blood samples, one to an EDTA-containing tube and one to a serum separating tube, from all individuals were obtained from median ulnar or basilica veins by an experienced phlebotomist. Samples in EDTA anticoagulant tubes were immediately centrifuged and washed thrice in 0.9% NaCl and lysed with deionized water. Then, 1-part 20% w/v TCA solution was mixed with 3-part erythrocyte lysate to precipitate proteins. Serum separating tubes allowed to clot for 15 minutes and centrifuged. Supernatant and serum samples were stored at -80°C. All supernatant and serum samples were thawed on the day of measurement and laboratory analysis were made.

SH/SS homeostasis parameters determination

Serum samples was used to measure SH/SS homeostasis parameters by the method described by Erel and Neşelioğlu [14]. Briefly, firstly native SH levels of sample were measured with 5,5′-dithiobis (2-nitrobenzoic acid) (DTNB, Ellman's reagent). Then, disulphides (SS) were reduced by 10 mM sodium borohydride (NaBH4), to form free functional thiol groups. Formaldehyde was used to the remnant unused sodium borohydride. After that, total SH levels including both reduced and native SHs were determined with the reaction of free functional SHs with DTNB. The amount of SS was determined by taking half of the difference between serum total and native SH levels. After the determination of SH (native thiol), total SH (total thiol), and SS amounts (disulphide), SS/SH (Disulphide/thiol), percent ratios were calculated.

GSH/GSSG homeostasis parameters determination

GSH/GSSG homeostasis parameters were determined by the method described by Alısık et al. [18]. GSH amount of supernatant samples were evaluated using the Ellman method that was using 500 mM Tris solution (pH: 8.2). GSSG in the 600 µL supernatant samples were reduced with 150 reagents µL including 3.5 M NaBH4 and 1.5 M NaOH to form GSH. After that, 70 µL HCl solution was added to remove the excess unused the remnant NaBH4 in order to prevent extra-reduction of DTNB molecules and re-oxidation of GSH molecules. Total GSH levels (including native GSH contents and GSH from reduction of GSSG) were measured using the Ellman method as determination of GSH. GSH content was subtracted from the total GSH (GSH+GSSG) content and divided by two equals to the GSSG amount. Results were expressed as µmol/L. GSSG/GSH percent ratios were calculated.

Statistical analysis

Analysis were performed using the SPSS statistical software for Windows, version 25, released in 2012 (IBM, Armonk, NY, USA). The descriptive statistics are expressed as means ± standard deviations for variables with normal distributions, medians (interquartile range for non-normal distributions), and the number of cases and percentages (%) for nominal variables. The Kolmogorov-Smirnov distribution test was used to examine the normal distribution. Pearson Chi-square test and Fisher's Exact test were used for comparison of descriptive statistics, as well as qualitative data. Mann-Whitney U test was performed for comparison of non-normally distributed quantitative data of two groups; Student's t test was used for normal distributed data. Kruskal-Wallis test was performed for comparison among more than two groups of non-normally distributed quantitative data, and Mann-Whitney U test was performed to analyze the group causing the difference. For comparison among more than two groups of normally distributed quantitative data, ANOVA test was performed and the group causing the difference is defined with post hoc Tukey test. The results were evaluated at 95% confidence interval, $p<0.05$ significance level.

RESULTS

In this study, we enrolled a total number of 58 AMD patients (30 (51,7%) non-exudative, 28 (48,3%) exudative AMD), and 36 age-matched healthy control subjects. The mean age of the non-exudative AMD group, exudative AMD group and healthy controls were 64.9 ± 5.8 years, 63.5 ± 5.5 years and 62.5 ± 4.3 years, respectively (p=0,176). there were 4 males and 26 females in non-exudative AMD group, 7 males and 21 females in exudative AMD group and 6 males and 30 females in healthy control group. There was no statistically significant difference between groups in term of gender (p=0,494). Demographic data is shown in Table 1.

Although there was no significant difference between groups in term of total thiol (361.5(61.6), 355.1(87.7) and 340.9(72.4), respectively, p=0,585) and native thiol (318.8(62.4), 307.1(73.7) and 299.3(79.2), respectively, p=0,382), in compared to healthy subjects both non-exudative and exudative AMD patients had higher plasma disulfide levels (20.5(4.8) vs. 15.4(3.1), $p<0.001$ and 22.5(7.5) vs. 15.4(3.1), $p<0.001$; respectively) and higher SS/SH (6.64(2.57) vs. 5.4(1.9), p=0,002 and 7.05(3.14) vs. 5.4(1.9), $p<0.001$; respectively). Although there was no significant difference between groups in term of total GSH (986.3(282.1), 871.5(271.6) and 881.8(290.9), respectively, p=0.344) and native GSH (873.4(367.6), 723.7(379.0) and 797.2(307.5), respectively, p=0,113), in compared to healthy subjects both non-exudative and exudative AMD patients had higher GSSG levels (64.6(40.8) vs. 27.3(21.9), p=0.015 and 73.9(44.1) vs. 27.3(21.9), p=0.002; respectively) and GSSG/GSH ratio(6.48(8.35) vs. 3.14(3.31), p=0,034 and 10.21(10.28) vs. 3.14(3.31), p=0,003; respectively). Extracellular SH/SS and intracellular GSH/GSSG homeostasis parameters of each group are shown in Table 2. However, there was no significant difference between non-exudative and exudative AMD groups in terms of both extracellular thiol and intracellular glutathione balance. Pairwise comparisons are shown in Table 3.

DISCUSSION

To the best of our knowledge, the present study is the first investigation in the literature evaluating the association between AMD and intracellular GSH/GSSG homeostasis parameters besides extracellular SH/SS homeostasis parameters compared to healthy subjects to reflect the relation between oxidative stress imbalance and AMD. Higher plasma disulfide and GSSG levels and GSSG/GSH ratio and higher SS/SH ratio in both non-exudative and exudative AMD patients in compared to heathy subjects supported the hypothesis that oxidative stress plays role in AMD pathogenesis, although the exact mechanism of this interaction remains to be defined.

The RPE is a single layer of postmitotic pigmented cells located between the photoreceptors and the choriocapillaris. These cells have multiple functions involved in maintaining retinal health including photoreceptor phagocytosis, nutrient transport, and cytokine secretion. Disruption of RPE cell function is a key event in the pathogenesis of AMD [19]. Oxidative stress has long been considered a major influence on the RPE in AMD pathophysiology. It can be assumed that the retina, and in particular the macula, is disposed to suffer from oxidative stress, being exposed to high oxygen concentration through the choriocapillaris, with high oxygen consumption, while the external segments of the photoreceptors contain a high quantity of unsaturated fatty acids and are continually exposed to light [20]. Previous studies suggest that the pathologic mechanism involves mitochondrial dysfunction resulting from oxidative stress and subsequent damage to proteins, lipids, and mt DNA at RPE cells [6-8].

It has been shown that proteins from the external segment of photoreceptors modified as a result of lipid peroxidation are protected from lysosomal degradation and, transiting without modification to the sub-RPE space (apical-to-basolateral transcytosis), are forming sub-RPE deposits [21]. Handa et al. demonstrated that some advanced glycation end products, as a

Table 1: Demografic data.

	Non-Exudative AMD Group (n:30)	Exudative AMD Group (n:28)	Control Group (n:36)	p value
Age, years (Mean ± std)	64.9 ± 5.8	63.5 ± 5.5	62.5 ± 4.3	0,176
Gender				
Female	26	21	30	0,494
Male	4	7	6	

Table 2: Extracellular SH/SS and intracellular GSH/GSSG homeostasis parameters in study groups.

		Non-Exudative AMD Group (n:30)	Exudative AMD Group (n:28)	Control Group (n:36)	p value
		Median (Interquartile range)			
Extracellular	Total Thiol, µmol/L	361.5(61.6)	355.1(87.7)	340.9(72.4)	0.585
	Native Thiol, µmol/L	318.8(62.4)	307.1(73.7)	299.3(79.2)	0.382
	Disulfide, µmol/L	20.5(4.8)	22.5(7.5)	15.4(3.1)	<0.001
	SS/SH, %	6.64(2.57)	7.05(3.14)	5.4(1.9)	<0.001
Intracellular	Total GSH (GSH + GSSG), µmol/L	986.3(282.1)	871.5(271.6)	881.8(290.9)	0.344
	Native GSH, µmol/L	873.4(367.6)	723.7(379.0)	797.2(307.5)	0.113
	GSSG, µmol/L	64.6(40.8)	73.9(44.1)	27.3(21.9)	0.001
	GSSG/GSH, %	6.48(8.35)	10.21(10.28)	3.14(3.31)	0.002

Table 3: Pairwise comparisons of intracellular glutathione and extracellular thiol homeostasis.

		Group 1-Group 2	Group 1-Group 3	Group 2-Group 3	p value
		p value			
Intracellular	Total GSH (GSH + GSSG), µmol/L	0.191	0.882	0.213	0.585
	Native GSH, µmol/L	0.263	0.479	0.029	0.382
	GSSG, µmol/L	0.437	0.015	0.002	<0.001
	GSSG/GSH, %	0.401	0.034	0.003	<0.001
Extracellular	Total Thiol, µmol/L	0.446	0.152	0.635	0.344
	Native Thiol, µmol/L	0.297	0.56	0.688	0.113
	Disulfide, µmol/L	0.469	<0.001	<0.001	0.001
	SS/SH, %	0.319	0.002	<0.001	0.002

result of oxidation, can accumulate in Bruch's membrane and in the choroid with aging, and reported that these may damage the RPE–Bruch's membrane–choroid complex [22,23]. Oxidative stress has also been shown to damage especially mitochondrial DNA, and the defective mitochondrial DNA causes dysfunction and apoptosis of RPE [24,25]. Damages defined in these studies which were carried out with cell cultures or donor eyes have been presented as evidence for the relation between oxidative stress and AMD.

The role of plasma thiol molecules as physiological free radical scavengers was already described previously [26]. Previous studies indicated that total thiol levels were lower in patients with AMD compared to healthy subjects [27,28]. However, Brantley et al. [29] revealed that the difference in plasma SS levels between AMD patients and controls was no longer significant after adjusting for age alone (p=0.130), and a positive linear correlation was observed between SS levels and age (p<0.001), indicating that plasma SS levels may be more related to aging than AMD [29]. In our cohort, extracellular thiol or intracellular glutathione products alone were not sufficient biomarkers for identifying individuals at risk for AMD. However, in a recent study, Erel and Neselioglu described that total thiol levels might remain in a plateau level despite the significant variability in plasma oxidant concentrations in the human metabolism. The researchers used a novel automated assay for determination of plasma total, native, and disulfide levels, which was an easier method for the evaluation of thiol-disulfide balance in the human metabolism. Because total thiol content also involves static and structural bonds that do not have role in the oxidation-reduction reactions,

they proposed that SH/SS ratio and plasma disulfide levels might reflect the antioxidant capacity better than total thiol status [14]. In this study there were higher SS levels and higher SS/SH ratio levels in both type of AMD in compared to healthy subjects, although there was no significant difference in terms of total and native thiol levels between groups. This supports the conclusions of previous studies that rather than the total thiol concentration, the thiol/disulfide balance has a fundamental role in protection against oxidative stress.

Glutathione, apart from being an important antioxidant, is the main low-molecular-weight thiol-containing peptide with its cysteine residue present in most living cells. Its reduced and oxidized forms are indicators of the oxidation-antioxidation status [30]. Intracellular glutathione has two different forms, one of which is reduced glutathione (GSH) and the other being oxidized glutathione (GSSG). Glutathione is one of the most important antioxidants of the intracellular compartment [31]. It has various important metabolic functions such as detoxification, signal transduction, synthesis of DNA and protein, cell proliferation and apoptosis, mitochondrial function, neurodegeneration, and protection of cognitive function of the brain [32]. Therefore, it may play a role in the pathogenesis of many diseases such as neurological disorders, fibrosis of organs, cardiovascular disease, DM and liver diseases [33]. To the best of our knowledge this is the first study to evaluate relation with AMD and intracellular GSG parameters. In this study we found that there were higher GSSG levels and GSSG/GSH ratios in both type of AMD patients in compared to healthy subjects, although there was no significant difference in terms of total and native GSH levels between groups. This results, higher levels of oxidized glutathione in AMD patients, may support the role of oxidative stress in AMD pathophysiology.

Our study has also some limitations. First, our study is a cross sectional study with a limited number of patients. Second, we did not use other indices for oxidative stress in order to compare with SS/SH and GSSG/GSH ratios. Finally, apart from the sophisticated methods used in previous studies like high-performance liquid chromatography, fluorescence capillary electrophoresis, or bioluminescent systems, the clinical utility of this novel automated assay should be evaluated in large-scale studies.

CONCLUSION

In conclusion, a greater extent of SS and GSSG production occurred in AMD patients compared to age-matched healthy controls indicating the role of increased oxidative stress in AMD development. Therefore, SS/SH and GSSG/GSH ratios may have a potential to reflect the clinical disease status. Future large-scale studies are needed in order to evaluate the interaction between thiol/disulfide and GSH/GSSG metabolisms and AMD as well as the utility of these novel parameters in clinical practice.

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