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Original Article

Asymmetric dimethylarginine and homocysteine in exudative age-related macular degeneration

Alireza Javadzadeh[1], Amir Ghorbanihaghjo*[2], Sima Manzouri[3], Nadereh Rashtchizadeh[1]

[1] *Professor, Drug Applied Research Center, Tabriz University of Medical Sciences, Tabriz, Iran*
[2] *Professor, Biotechnology Research Center, Tabriz University of Medical Sciences, Tabriz, Iran*
[3] *General Practitioner, Drug Applied Research Center, Tabriz University of Medical Sciences, Tabriz, Iran*

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Abstract

Introduction: The oxidative stress has been proposed as an important case of exudative age-related macular degeneration (E-ARMD). The aim of the present study was to investigate homocysteine (Hcy), asymmetric dimethylarginine (ADMA) and oxidized low-density lipoprotein cholesterol (Ox-LDL-C) levels, the factors involved in oxidative stress, in the patients with E-ARMD.

Methods: In a cross-sectional study, 45 patients with E-ARMD were compared with 45 sex- and age-matched healthy controls. The levels of biochemical factors, Hcy, ADMA, and of Ox-LDL were estimated by standard methods in both study groups.

Results: The levels of Hcy (15.4 ± 7.2 vs. 10.7 ± 3.7 μM, P = 0.001), Ox-LDL (52.2 ± 13.8 vs. 37.8 ± 10.8 U/l, P = 0.001), and ADMA (0.84 ± 0.23 vs. 0.71 ± 0.26 μM, P = 0.012) were significantly higher in the patients with E-ARMD than those in the controls. In the patient group, there was a positive and significant correlation between serum Ox-LDL and Hcy concentrations (r = 0.719, P = 0.001), but no correlation was found between serum ADMA and Ox-LDL (r = 0.010, P = 0.900) and also between serum Hcy and ADMA levels (r = -0.070, P = 0.600).

Conclusion: The high levels of Hcy as an oxidant agent and ADMA as an endogenous nitric oxide synthase inhibitor can lead to increase Ox-LDL levels, and they may have important roles in oxidative stress, which can be a trigger in E-ARMD.

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Introduction

Age-related macular degeneration (ARMD) is the leading cause of blindness particularly in elderly people throughout the world.[1] It affects about 25% of people over 65, and the incidence is likely to rise as an outcome of increasing longevity.[2,3] ARMD is not painful, however, it affects the central vision and patients with ARMD may have blurred vision (early ARMD), or even a total loss of central vision (advanced ARMD), and they cannot see things in details.[4] The loss of central vision caused by ARMD can decrease quality of life by severely affecting basic daily tasks such as reading, driving, and facial recognition. In addition, approximately one-third of patients with ARMD suffer from depression.[1] The disease is complex and multifactorial; also several risk factors are associated with its development. Some recognized risk factors are age, white race, heredity, exposure to light, smoking, and

*** Corresponding Author:** Amir Ghorbanihaghjo, Email: ghorbaniamir@hotmail.com

inflammation.[1,2] There have been recent significant advances in the understanding of ARMD, but exact etiology and knowledge of the precise mechanisms responsible for its progression remains unclear.[1]

It is traditionally divided into three categories: early ARMD, characterized by the presence of pigmentary changes of the retinal pigment epithelium (RPE) and/or hard small drusen; intermediate ARMD, characterized by the presence of soft large drusen and/or geographic atrophy (GA) of the RPE with foveal sparing; and late ARMD, characterized by GA with foveal involvement and/or choroidal neovascularization (CNV).[5] ARMD may progress from the early form to the intermediate form and then to the advanced form, where two subtypes exist: the nonexudative (dry) type and the exudative (wet) type.[3] The nonexudative form is more common and accounts for most ARMD cases. The exudative ARMD (E-ARMD), however, is more debilitating and is responsible for more than 80% of the visual loss in such patients.[6] There is a general consensus that cumulative oxidative damage is responsible for aging, and may, therefore, play the important role in the pathogenesis of E-ARMD.[7]

Homocysteine (Hcy) is an intermediary amino acid normally formed during the conversion of methionine (Met) to cysteine in the body. It is rapidly auto-oxidized in serum, forming Hcy, mixed disulfides, and Hcy thiolactone. Potent reactive oxygen species (ROS), including superoxide anion and hydrogen peroxide, are produced during the auto-oxidation of Hcy.[8] The generation of these free radicals leads to the promotion of lipid peroxidation. This affects not only low-density lipoprotein (LDL) and other lipoproteins but also cellular lipids, including those in arterial walls[6] that can lead to induce the vascular dysfunction and atherosclerosis.[8,9] Although the cause-effect relationship is not clear, atherosclerosis may be a potential risk factor for the development of ARMD.[9]

Asymmetric dimethylarginine (ADMA), a naturally occurring L-arginine (Arg) analog, is a methylated amino acid derived from the proteolysis of proteins.[10] It is a potent endogenous competitive inhibitor of the endothelial nitric oxide (NO) synthase (NOS). Increased levels of ADMA reduce NO formation and are associated with endothelial dysfunction.[11] In addition, several studies have demonstrated that high ADMA level can induce oxidative stress in vascular tissues, and clinical evidence suggests that serum ADMA may be a novel cardiovascular risk factor. In order that increased level of ADMA can have the important role in the pathogenesis of atherosclerosis and E-ARMD.[10-14]

Dimethylarginine dimethylaminohydrolase (DDAH) is an enzyme that it degrades methylarginine, specifically ADMA. The activity of DDAH is impaired by oxidative stress, permitting ADMA to accumulate. A wide range of pathologic stimuli induces endothelial oxidative stress such as oxidized LDL (Ox-LDL) and hyperhomocysteinemia. Each of these insults attenuates DDAH activity in vitro and in vivo and leads to increase ADMA level.[15]

The aim of the present study was to evaluate Hcy, ADMA, and (Ox-LDL) levels, the factors involved in oxidative stress, in the patients with E-ARMD.

Methods

This cross-sectional study was performed from June 2008 to January 2009 in the department of Retina in Nikokari Eye Hospital, Tabriz University of Medical Sciences, Iran. The study groups were composed of 90 individuals ranging in age between 50 and 80 years, including 45 patients with newly diagnosed E-ARMD and 45 healthy age and sex matched controls.

All participants underwent a complete ophthalmic examination consisting of the best corrected visual acuity (BCVA) (using Snellen-Chart and calculated as the logarithm of the minimal angle of resolution, notated "LogMAR"), slit-lamp biomicroscopy, dilated fundoscopy (using a Slit-lamp, Haag-Streit, R 900; Haag-Streit AG, Switzerland with a super-field indirect lens), fundus photography, and fundus fluorescein angiography (ImageNet 2000; Topcon TRC

50IX; Topcon Corp., Japan) when necessary. Recruited patients in the study had either a classic form of CNV or a disciform scar. To limit the effects of interference factors in our results, we excluded other pathological ophthalmic conditions, such as trauma and angle-closure glaucoma. We also excluded those who were in an oxidative condition, such as smokers, subjects who were on antioxidant supplements, and subjects with systemic disorders such as diabetes, any inflammatory processes, and renal and/or liver dysfunction.

The Ethics Committee at the Tabriz University of Medical Sciences reviewed and approved the present study, in compliance with the declaration of Helsinki. Informed consent was obtained from all participants.

Venous blood samples of healthy controls and patients groups, before starting any medications were obtained in the morning following an overnight 8 hour fast and centrifuged at 2000 g for 15 minutes. The sera were separated immediately and then analyzed using enzymatic assays with an automated chemical analyzer (Abbott Analyzer; Abbott Laboratories, Abbot Park, IL, USA) for glucose, urea, creatinine, alanine aminotransferase, aspartate aminotransferase, total cholesterol, triglycerides, and high-density lipoprotein cholesterol. LDL-C levels were calculated using the Friedewald equation.[16] The remaining samples were stored at -70 °C pending analysis.

Serum levels of ADMA, Hcy, and Ox-LDL were measured once after collecting of all samples.

Serum ADMA concentrations were determined using a Human ADMA enzyme-linked immunosorbent assay (ELISA) Kit from EIAab and USCNLIFE (Wuhan EIAab Science Co., Ltd). The microtiter plate provided in this kit has been pre-coated with an antibody specific to ADMA. Standards or samples are then added to the appropriate microtiter plate wells with a biotin-conjugated polyclonal antibody preparation specific for ADMA. Next, Avidin conjugated to horseradish peroxidase (HRP) is added to each microplate well and incubated. Then a 3,3′,5,5′-tetramethylbenzidine substrate solution is added to each well. Only those wells that contain ADMA, biotin-conjugated antibody, and enzyme-conjugated Avidin will exhibit a change in color. The enzyme-substrate reaction is terminated by the addition of a sulphuric acid solution, and the color change is measured spectrophotometrically at a wavelength of 450 ± 2 nm. The concentration of ADMA in the samples is then determined by comparing the OD of the samples to the standard curve.

Serum Hcy concentrations were determined using a commercially available enzyme-linked immunoassay (Axis-Shield, Axis Biochemical ASA, Distributed by IBL, Hamburg, Germany). With this method, total free and protein bound circulating Hcy moieties are reduced to free Hcy with dithiothreitol. The Hcy was then converted to S-adenosyl-L-homocysteine (SAH) by using SAH hydrolase and excess adenosine. After the addition of an anti-SAH antibody, in the next stage, a secondary rabbit anti-mouse antibody was labeled with the enzyme HRP. The peroxidase activity was measured spectrophotometrically after the addition of the substrate. The absorbance was inversely related to the concentration of Hcy in the sample. Serum Ox-LDL levels were measured using an ELISA kit (Mercodia, Uppsala, Sweden).

Statistical analyses were performed using SPSS software for Windows (version 18, SPSS Inc., Chicago, IL, USA).

The Kolmogorov–Smirnov test was used to evaluate the distributions, and results were expressed as means ± standard deviation (SD). The independent t-test was used to assess the significance of the differences between the two groups. $P < 0.050$ was considered statistically significant.

Results

About 45 patients with E-ARMD (27 women and 18 men) with a mean age of 71 ± 7 years were compared with 45 matched healthy controls (27 women and 18 men) with a mean age of 69 ± 5 years. Table 1 summarizes the clinical characteristics and laboratory findings from the patients with E-ARMD and

from the controls. The total serum Hcy concentration was significantly higher (71%) among the patients with E-ARMD than among the controls (15.4 ± 7.2 vs. 10.7 ± 3.7 µM, P = 0.001). The total serum Ox-LDL level in the patients with E-ARMD was 40.5% higher than the controls (52.2 ± 13.8 vs. 37.8 ± 10.8 U/l, P = 0.001).

The serum levels of ADMA were significantly higher, almost 14.5%, in the patients with E-ARMD than in the controls (0.84 ± 0.23 vs. 0.71 ± 0.26 µM, P = 0.012) (Figure 1). In the patient group, no correlation was found between serum Hcy and ADMA levels (r = -0.074, P = 0.630). There was a positive significant correlation between serum Ox-LDL and Hcy concentrations (r = 0.719, P = 0.001) (Figure 2). However, no correlation was found between serum ADMA and Ox-LDL levels (r = 0.010, P = 0.900) in the patients with E-ARMD.

Table 1. Clinical data of E-ARMD (exudative age related macular degeneration) patients and controls

Parameters	Controls	Patients	P*
	Mean ± SD	Mean ± SD	
Age (year)	69 ± 5	71 ± 7	0.110
BCVA	0.10 ± 0.1	1.18 ± 0.36	0.001
BMI (kg/m²)	27.3 ± 5.6	25.3 ± 3.8	0.110
SBP (mmHg)	124.4 ± 7.2	129.8 ± 20.0	0.120
DBP (mmHg)	82.5 ± 8.1	77.5 ± 10.0	0.110
Glucose (mg/dl)	85.2 ± 14.9	88.0 ± 13.0	0.580
Urea (mg/dl)	34.3 ± 6.1	32.3 ± 7.2	0.160
Creatinine (mg/dl)	1.08 ± 0.2	1.0 ± 0.2	0.140
Triglycerides (mg/dl)	153.7 ± 49.5	144.5 ± 63.8	0.450
Cholesterol (mg/dl)	181.1 ± 36.3	204.0 ± 39.5	0.005
HDL (mg/dl)	44.2 ± 6.3	43.1 ± 5.0	0.300
LDL (mg/dl)	105.1 ± 35.3	131.6 ± 40.2	0.001
Ox-LDL (U/l)	37.8 ±10.8	52.2 ± 13.8	0.001
Homocysteine (µM)	10.7 ± 3.7	15.4 ± 7.2	0.001
Number/Sex	45/(18 M, 27 F)	45/(18 M, 27 F)	NS

M: Male; F: Female; BCVA: Best corrected visual acuity; BMI: Body mass index; SBP: Systolic blood pressure; DBP: Diastolic blood pressure; HDL: High-density lipoprotein; LDL: Low-density lipoprotein; Ox-LDL: Oxidized LDL cholesterol; NS: Non-significant; SD: Standard deviation

*Independent-sample t-test

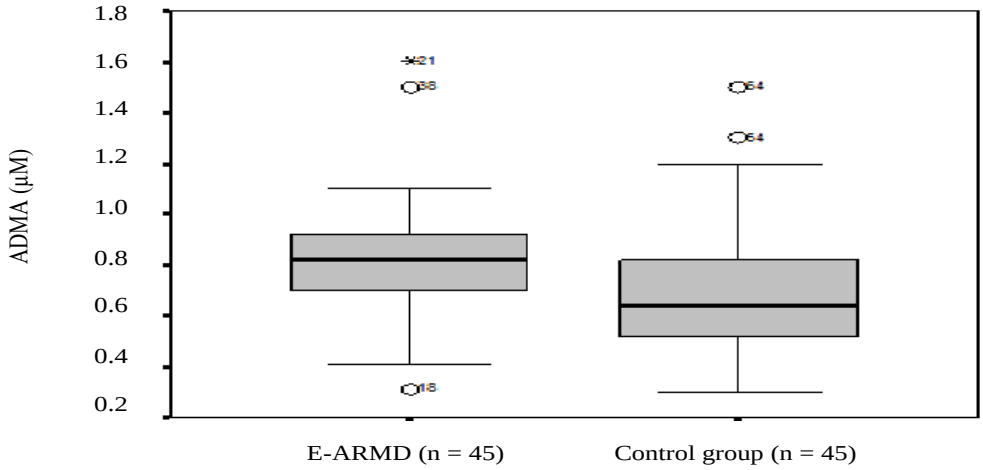

Figure 1. Comparison of ADMA (asymmetric dimethylarginine) concentrations between the control and E-ARMD (exudative age-related macular degeneration) groups (P = 0.012)
ADMA: Asymmetric dimethylarginine; E-ARMD: Exudative age-related macular degeneration

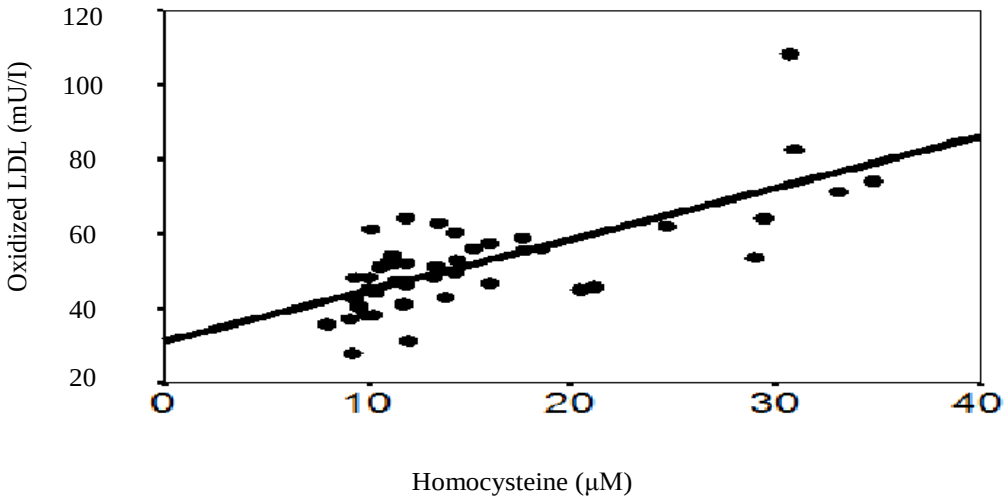

Figure 2. There is a direct linear correlation between the Ox-LDL (oxidized low-density lipoprotein) and homocysteine levels in the patient group (r = 0.719, P = 0.001).

Discussion

The main findings of the present study were that higher levels of Hcy and ADMA as oxidant agents and Ox-LDL as an oxidative product in the patients with E-ARMD, compared with controls. Furthermore in a study of Ugurlu et al.[17] significant increase in total oxidant status levels were observed in sera of ARMD patients compared to controls. Total antioxidant status, total thiol status and serum paraoxonase 1 (PON1) enzyme activities were significantly lower in ARMD patients relative to control groups. The results of these studies show that oxidative stress is the important factor for ARMD progression.[18]

It has been shown that ARMD affects the macula, the posterior aspect of the retina responsible for central visual acuity.[4] The retina is an ideal environment for the generation of ROS for several reasons. First, oxygen consumption by the retina is much greater than by any other tissue. Second, the retina is subject to high levels of cumulative irradiation. Third, photoreceptor outer segment membranes are rich in polyunsaturated fatty acids, which are readily oxidized and which can initiate a cytotoxic chain-reaction. Fourth, the neurosensory retina and the RPE contain an abundance of photosensitizers. Finally, the process of phagocytosis by the RPE is itself an oxidative stress and results in the generation of ROS.[7,19]

High ROS could lead to lipid peroxidation in retina, which initiates an inflammatory response. Mulero et al.[20] in their study found significant differences between ARMD patients and control group in baseline values of Hcy, CRP and fibrinogen, although they did not observe differences in levels of lipid profile. Their data support the role of chronic inflammation in the development of ARMD. The inflammation disrupts the RPE cell junctions and the integrity of the blood-retina barrier, both of which contribute to neovascular processes. Increased serum Ox-LDL as an oxidant agent may suggest the oxidative damage in retina or risk of ARMD more precisely than other serum factors.[21,22]

Xu et al.[23] have shown previously that a number of oxidized materials including nitrotyrosine, Ox-LDL and oxidized protein accumulate in the aging retina, which represent an endogenous threat to normal retina function and construction.

Increased serum Hcy levels in our E-ARMD group are confirmed by studies of Axer-Siegel et al.[24], and Coral et al.[8] that there were a significant, elevated Hcy levels in E-ARMD. Vine et al.[25] showed that elevated CRP, and Hcy levels are associated with ARMD and that they contribute to inflammation and atherosclerosis in the pathogenesis of ARMD. Moreover, Gopinath

et al.[26] in their cohort study showed that elevated serum Hcy, serum folate, and vitamin B-12 deficiencies predicted increased risk of incident ARMD, which suggests a potential role for vitamin B-12 and folate in reducing ARMD risk.

It should be noted that we did not found any study about ADMA assessment on E-ARMD. Hcy, an amino acid that is found in the blood, is an independent and potentially modifiable risk factor for various forms of vascular disease also for E-ARMD. Its concentration normally increases with age. The adverse effects of Hcy on endothelial function may be mediated by the reduced production and bioavailability of NO as a result of oxidant stress with the formation of ROS and increasing lipid peroxidation.[6,8,9] In addition, it can impair production of the antioxidant glutathione peroxidase and more important by its metabolite, homocysteine thiolactone.[6,8] Thus, hyperhomocysteinemia can lead to increase Ox-LDL and oxidative stress that they also may result in increased ADMA level.[11,15,27,28]

High level of ADMA can also induce oxidative damage in vascular tissues results in endothelial dysfunction.[10,12] Endothelial function depends on the integrity of constitutive NOS and the availability and vascular signaling of its product, NO.[12]

Vallance et al.[29] reported a large increase in serum concentrations of ADMA in patients with CKD and end-stage renal disease and showed that ADMA inhibited NOS and that ADMA and cationic amino acid transferase. Selley[30] demonstrated that Hcy inhibits the activity of DDAH in cultured neuronal granule cells, causing the accumulation of ADMA and the inhibition of NO synthesis.

The observation that ADMA levels increase early in the development of atherosclerosis suggests that ADMA has the potential to be not only a marker, but a mediator of vascular lesions. Because the pathologic changes in E-ARMD are similar to those in atherosclerosis, and atherosclerosis may contribute to the pathogenesis of E-ARMD, choroidal vessel damage and neovascularization may be attributed to high levels of Hcy and ADMA in the study patients, which could improve oxidized lipoproteins in the macular area of eyes with E-ARMD.[15,25,31]

In the present study, we did not find any significant correlation between the high serum levels of ADMA and Hcy in the patient group that is in contrast with Selley[30] and Yoo and Lee.[32] It may be interpreted by Hcy status in our study patients, who had mild hyperhomocysteinemia; a state has been described in a study on the Iranian population by Ghaedi et al.[33] In that study, the Hcy means in cases and controls were 15.56 ± 6.77 and 11.51 ± 4.63 µM, respectively.

According to Stuhlinger et al.[34] findings, Hcy and its precursor L-Met increase ADMA elaboration by endothelial cells in a dose-dependent fashion at pathophysiologically relevant concentrations and this effect is associated with a dose-dependent impairment of the activity of endothelial DDAH. They suggested that in a cell-free system, Hcy directly inhibits the activity of DDAH and this effect of Hcy seems to be due to its attack on a critical sulfhydryl group of DDAH, therefore, Hcy may induces impairment of the NOS pathway. Then, they showed that the Hcy-induced increase in ADMA is associated with a reduction in DDAH activity and reduced NO elaboration by endothelial cells and aortic vessel segments. In addition, Ito et al.[35] found that when cultured endothelial cells are exposed to Ox-LDL cholesterol, ADMA accumulation in the medium is associated with a temporally related decline in DDAH activity.

Conclusion

The hyperhomocysteinemia, lipid peroxidation and high ADMA levels in E-ARMD patients as the results that were showed in this study, confirm that the generation ROS and oxidative stress may be one of the most important mechanisms in this context. This is the first study that we can show high level of ADMA as like as high Ox-LDL and hyperhomocysteinemia may be a prominent risk factor for E-ARMD. Although high Hcy and ADMA levels in our patient

group did not show significant correlation (may be because of mild hyperhomocysteinemia), Hcy could impair the NO pathway by increasing ADMA accumulation, because of inhibiting DDAH activity and induction of Ox-LDL production, then subsequently, reducing NO elaboration by endothelial cells. Our study had some limitations. We evaluated only a small number of subjects; thus, these findings may not be generalized to represent the entire E-ARMD patients. We found that the Ox-LDL level was associated with the serum Hcy concentration. However, further work is required in large case-control studies to determine the general applicability of our results, because of the multifactorial nature of E-ARMD disease.

Conflict of Interests

Authors have no conflict of interest.

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