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The role of advanced oxidation protein products and total thiols in diabetic retinopathy

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PURPOSE. *This study aimed to investigate the role of protein peroxidation by detecting the serum levels of advanced oxidation protein products (AOPP), a novel marker for the degree of oxidative damage to proteins, and total thiol as a marker of antioxidant status in diabetic patients with or without diabetic retinopathy (DR) and to compare the results with those of control subjects.*

METHODS. *The study groups consisted of two separate subgroups: 1) 37 patients (14 male, 23 female) with noninsulin-dependent diabetes mellitus (NIDDM) showing diabetic retinopathy (DR) and 2) 20 patients with NIDDM and without any signs of DR (9 male, 11 female); 26 healthy non-diabetic control subjects (15 male, 11 female) were selected from the patients attending our department for refractive disorders. Venous blood samples of all participants were collected in the morning after an overnight fast, and serum samples stored at –70°C until assay for AOPP, and total thiol.*

RESULTS. *AOPP levels were significantly higher in diabetic patients with (210.9±73.0 µmol/L) or without DR (222.7±94.4 µmol/L) when compared to those of controls (152.4±72.04 µmol/L) (p=0.004). Even though the difference was not statistically significant (p=0.095), total thiol levels in cases with DR (278.7±139.1 µmol/L) were lower than those without DR (334.0±129.4 µmol/L) and controls (353.2±145.6 µmol/L). Correlation tests did not reveal any association between these parameters and age, sex, or duration of DM.*

CONCLUSIONS. *The present study suggests that increased protein oxidation may contribute to the pathogenesis of DR. (Eur J Ophthalmol 2008; 18: 792-8)*

KEY WORDS. *Advanced oxidation protein products, Diabetic retinopathy, Total thiols*

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INTRODUCTION

The role of oxidative stress in diabetes and microvascular diabetic complications including retinopathy has gradually gained profound interest by many investigators (1-4). Even though a great deal of research was carried out to clarify the exact role of oxidative stress and antioxidant status in diabetic retinopathy (DR), the pathogenesis of DR and its association with oxidative stress remain largely unresolved. To clarify this issue, various molecules such as advanced glycation end products (AGE), vascular endothelial growth factor (VEGF), mitochondrial superoxide dismutase (SOD), malondialdehyde, and glutathione peroxidase were investigated in patients with DR (1, 5-9). However, there are few studies in literature regarding the role of protein oxidation in the pathogenesis of DR (10-13). On the other hand, there is considerable evidence that the maintenance of protein redox status has a crucial importance for cell function, and thereby structural changes in proteins are considered to be among the molecular mechanisms leading to diabetic microvascular complications (10).

In the literature, advanced oxidation protein products (AOPP) and total thiol levels have not concomitantly been

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investigated in patients with DR, and there are restricted data about AOPP in DR. In this study, we aimed to investigate protein peroxidation by detecting the serum levels of AOPP, a novel marker providing information on the degree of oxidative damage to proteins, and total thiol as a marker of antioxidant status in diabetic patients with or without retinopathy and control subjects.

METHODS

The study groups consisted of 37 patients (14 male and 23 female, mean age: 59.7±9.6 years) with noninsulin-dependent diabetes mellitus (NIDDM) showing DR and 20 diabetic patients without DR (9 male and 11 female, mean age: 57.7±7.7). These two groups were well matched for duration of both diabetes and hypertension. Mean duration of DM in the two groups was 6 years. The diagnosis of DR was established upon compatible fundus findings. All patients underwent a detailed ophthalmologic examination including best-corrected visual acuity, slit-lamp examination, tonometry, and direct and indirect ophthalmoscopy. At the time of study entry, all patients were treated with oral antidiabetic drugs. All had adult-onset DM with no history of ketoacidosis. Chronic renal insufficiency, uncontrolled primary and secondary hypertension, previously documented myocardial infarction, angina pectoris, and cardiovascular disease or other life-threatening diseases such as cancer were the exclusion criteria from the study. All patients had similar fundus findings revealing background type of DR. At the time of the study, mean duration of diabetes was 6 years (range 3–11 years).

The study was performed in accordance with the Declaration of Helsinki and was approved by the ethics committee of Erciyes University Faculty of Medicine, Kayseri, Turkey. Informed consent was obtained from all subjects.

Twenty-six healthy nondiabetic control subjects (15 male and 11 female, mean age: 55.5±11.1 years) were selected mainly from the patients attending our department for refractive disorders. All study subjects (controls and NIDDM patients) had normal renal and hepatic function without any evidence of acute infections or active inflammatory processes. None of the women in the study were on oral contraceptive agents or any hormones.

All blood samples were collected in the morning after an overnight fast, and serum samples stored at –70 ºC until assay for AOPP and total thiol. All chemicals used in this study were purchased from Sigma Chemical Co. (St. Louis, MO) and were of analytical grade or the highest grade available.

Determination of AOPP levels

Spectrophotometric determination of AOPP levels was performed by Witko-Sarsat's method (14). Samples were prepared in the following way: 200 µL of serum were diluted 1/5 in PBS, 10 µL of 1.16 M potassium iodide were then added to each tube, followed by 20 µL of acetic acid. The absorbance of the reaction mixture was immediately read at 340 nm, against a blank, containing 1000 µL of PBS, 10 µL of potassium iodide, and 20 µL of acetic acid. Chloramine T solution (0–100 µmol/L) was used as calibrator. The chloramine T absorbance at 340 nm was linear within range of 0–100 µmol/L. AOPP concentrations were expressed as micromoles per liter of chloramine T equivalents.

Determination of thiol levels

A spectrophotometric assay based on 2,2-dithiobisnitrobenzoic acid (DTNB or Elman's reagent) was used for thiol assay (15). An aliquot of serum was mixed with Tris-EDTA buffer; then DTNB was added. After 15-minute incubation at room temperature, the absorbance was measured at 405 nm. A reagent blank without sample and a sample blank with methanol instead of DTNB were prepared in a similar manner. GSH (50–100 µmol/L) solution was used as calibrator. Thiol levels were expressed as µmol/L.

Statistical analysis

Statistical evaluation was carried out with SPSS® software version 11.5 (SPSS Inc., Chicago, IL, USA). All results were expressed as mean±standard error of the mean. Categorical variables such as sex were compared by Chi-square or Fisher exact test (two tailed) between the study and control groups. Continuous variables such as AOPP and total thiol levels were compared by one-way analysis of variance (ANOVA) with Tukey test following the test of homogeneity of variances. Pearson's correlation and the point biserial correlation were performed to evaluate the correlation between variables. Statistical comparisons of the subgroups were made using nonparametric tests such as Mann-Whitney U and Kruskal Wallis tests.

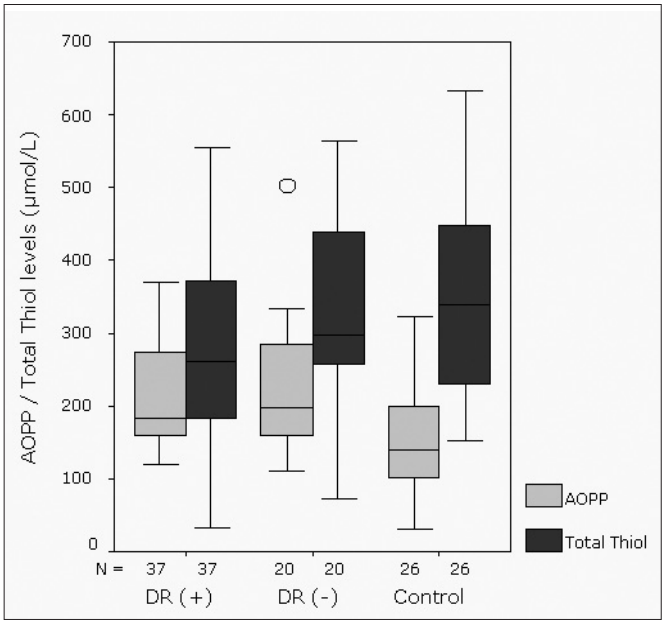

Fig. 1 - *Distributions of advanced oxidation protein products and total thiol levels in diabetic patients and controls.*

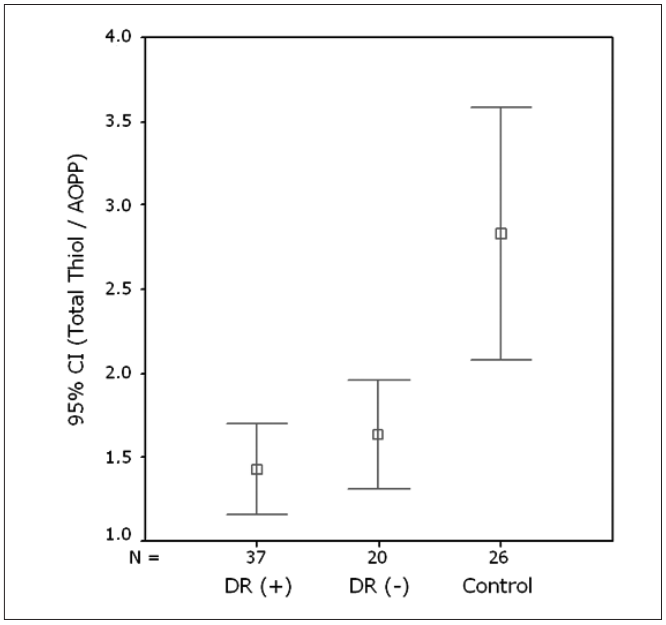

Fig. 2 - *Error bars of the total thiols/advanced oxidation protein products ratios in diabetic patients and controls.*

The binary logistic regression method was used to determine whether AOPP, total thiol, and their ratio were risk factors for the formation of DR. A p value less than 0.05 was considered statistically significant.

RESULTS

There were no statistically significant differences between the age and sex distribution of the groups (p=0.232 and p=0.296, respectively). No correlation was found when AOPP and total thiol levels were analyzed regarding their associations with the duration of DM.

Higher AOPP levels were found in diabetic patients both with DR (210.9±73.0 µmol/L) and without DR (222.7±94.4 µmol/L) when compared to those of controls (152.4±72.4 µmol/L) (p=0.004). Although it did not reach statistical significance, total thiol levels were found to be lower in diabetic patients especially with DR (278.4±139.1 µmol/L) than those without DR (334.0±129.4 µmol/L) and control subjects (353.2 ± 145.6 µmol/L) (p=0.095). The details of AOPP and total thiol levels in all subjects according to age, sex, and duration of DM subgroups are documented in Tables I and II. Distribution of these parameters in cases with and without DR and in healthy subjects is shown in Figure 1.

Total thiol/AOPP ratios were also investigated among the groups. Because the test of homogeneity of variances showed statistically significant difference (p=0.002), the logarithmic ratio values were figured out and used in one-way ANOVA test. According to this analysis, NIDDM patients with and without DR had much lower total thiol/AOPP ratios in comparison to healthy subjects (p=0.000) (post hoc Tukey test results: DR+ vs Control p=0.000, DR– vs Control p=0.032, and DR+ vs DR– p=0.347). Moreover, both increasing levels of AOPP (p=0.006; OR=1.012; 95% CI=1.003–1.021) and lower total thiol/AOPP ratio values (p=0.001; OR=0.330; 95% CI=0.168–0.648) were found to be important risk factors for the formation of DR on logistic regression analysis. The error bar graphic of these ratios is also shown in Figure 2.

When these parameters were separately compared in each group between female and male subjects, no statistically significant difference was found. Furthermore, the correlation between AOPP and total thiol levels were analyzed in each group; no statistically significant correlation was found between these two parameters in cases with DR (p=0.237 and r=0.199), cases without DR (p=0.284 and r=0.252), and controls (p=0.304 and r=0.210). The correlations between age and AOPP and total thiol levels were also analyzed and no statistically significant correla-

tion was found in cases with DR (age–AOPP; p=0.799, r=–0.043 and age–total thiol; p=0.845, r=–0.033), cases without DR (age–AOPP; p=0.977, r=0.007 and age–total thiol; p=0.260, r=–0.264), and controls (age–AOPP; p=0.705, r=0.078 and age–total thiol; p=0.675, r=0.086).

DISCUSSION

In diabetes, persistent hyperglycemia is a well-known risk factor for the development and progression of retinopathy. Even though there is some evidence that hyperglycemia may disrupt natural antioxidant defense mechanisms (16, 17), the main pathogenic mechanisms linking hyperglycemia with DR include several interacting abnormal biochemical systems, such as increased advanced glycation end product (AGE) formation, protein kinase C (PKC) β activation, the polyol pathway, and oxidative stress (9, 18-22).

The possible sources of oxidative stress in diabetes may include auto-oxidation of glucose, shifts in redox balances, decreased tissue concentrations of low molecular weight antioxidants such as reduced glutathione (GSH) and vitamin E, and impaired activities of antioxidant defense enzymes such as superoxide dismutase (SOD) and catalase (23-26). Although the exact mechanism by which oxidative stress could contribute to the development of diabetic complications remains to be clarified, it is well known that consequences of chronic oxidative stress include damage to biological macromolecules such as DNA, lipids, proteins, and carbohydrates, disruption in cellular homeostasis, and generation of other ROS creating further damage resulting in many serious diabetic eye complications (27).

Indirect methods that measure the levels of secondary products of oxidative modified molecules became much more practical, since direct measurement of oxidative stress in vivo is very complex. For instance, one of these products, AOPP, a novel index of oxidative damage, was first described by Witko-Sarsat et al (14). AOPP, defined as dityrosine containing cross-linked protein products, are formed during oxidative stress by the action of chlorinated oxidants, mainly hypochlorous acid and chloramines produced by myeloperoxidase in activated neutrophils. Because of structural similarity between AOPP and AGE-proteins, AOPP exert similar biological activities as AGEs such as induction of proinflammatory cytokines and adhesive molecules. Moreover, it has been shown that AGEs and AOPP accumulate in biological systems and therefore take part in diabetic long-term complications by causing damage to biological membranes and endothelium (28). Besides, the relationship between AOPP and monocyte seems to be very important in various conditions. For instance, in one study by Witko-Sarsat et al, a close correlation was observed between AOPP and neopterin, which is one of the monocyte activation markers (29), and it may be suggested that AOPP is not only a marker of oxidative stress, but also acts as an inflammatory mediator.

Biochemical characterization of AOPP in plasma revealed that both the high- and low-molecular-weight AOPP peaks contain oxidized albumin in aggregate-forming or monomeric form (14). Moreover, since albumin is the most abundant plasma protein and a powerful extracellular antioxidant, AOPP measurement is a good reflection of excess free radical generation and the degree of protein oxidative damage. In the present study, we assessed AOPP for protein oxidation together with changes in the status of antioxidant defense system in diabetic patients with and without DR and compared them with those of healthy controls. Since thiol groups are important members of the antioxidant system as they have been shown to destroy ROS and other free radicals by enzymatic as well as non-enzymatic mechanisms, total thiol levels were also investigated (30). According to the results of the present study, all diabetic patients showed significantly higher AOPP levels. Total thiol levels were found to be lower in diabetic patients especially with DR than those without DR and control subjects, however, this did not reach statistical significance. This decrease in the level of total thiols in patients with DR may be described as a consumption of thiol in the face of oxidants giving rise to oxidative stress. Moreover, the total thiol/AOPP ratios were found to be significantly lower in diabetic patients with and without DR than those in controls. When the correlation of AOPP and total thiol levels with the duration of diabetes is analyzed, no statistically significant results were established. However, when the subgroup analysis shown in Table I was carefully evaluated, it was found that diabetic patients with DR who have been followed up for more than 5 years had much higher levels of AOPP, suggesting that AOPP elevation may be related to diabetic complications. However, as a result of these data obtained in this study, and as indicated by Cakatay (12), it is not easily possible to conclude whether increased protein oxidation and indirectly increased AOPP levels is a cause or only a secondary process in diabetes. Interestingly, increased AOPP

TABLE I - AOPP LEVELS (µmol/L) IN PATIENTS WITH DR, PATIENTS WITHOUT DR, AND CONTROLS ACCORDING TO AGE, SEX, AND DURATION OF DM SUBGROUPS

Subgroups	Diabetes with DR (n = 37)	Diabetes without DR (n = 20)	Controls (n = 26)
<50 y	238.5±84.0	215.4±66.8	139.1±58.3
	220.3	226.3	139.5
51–60 y	187.4±61.9	200.1±73.9	171.1±70.1
	169.3	176.4	150.1
>60 y	217.5±75.2	255.9±129.2	154.5±89.4
	193.2	244.9	128.8
*p values**	*0.231*	*0.696*	*0.613*
Female	224.9±73.4	233.3±111.3	137.0±69.5
	205.1	201.9	123.5
Male	187.9±68.7	209.7±73.1	163.7±74.8
	172.1	193.2	146.7
p values†	*0.079*	*0.676*	*0.312*
DM 0–5 y	195.8±70.5	224.8±70.6	—
	170.0	201.9	
DM >6 y	228.7±74.1	222.5±122.2	—
	205.1	177.1	
p values†	*0.091*	*0.569*	—

As the numbers of the subgroups were believed to be inadequate, nonparametric tests were used in this analysis. All values given as mean±SD (above) and median (below).

*p values of Kruskal-Wallis H test.

†p values of Mann-Whitney U test.

AOPP = Advanced oxidation protein products; DR = Diabetic retinopathy

TABLE II - TOTAL THIOL LEVELS (µmol/L) IN PATIENTS WITH DR, PATIENTS WITHOUT DR, AND CONTROLS ACCORDING TO AGE, SEX, AND DURATION OF DM SUBGROUPS

Subgroups	Diabetes with DR (n = 37)	Diabetes without DR (n = 20)	Controls (n = 26)
<50 y	232.9±115.3	356.1±104.3	367.2±180.8
	246.3	311.0	363.8
51–60 y	303.4±150.9	390.8±142.5	317.8±77.1
	320.9	421.5	315.3
>60 y	278.7±141.6	248.5±82.4	360.4±147.4
	324.6	270.3	320.9
*p values**	*0.699*	*0.170*	*0.913*
Female	285.7±133.2	324.8±99.0	316.1±122.5
	272.4	283.5	279.8
Male	267.3±152.8	345.4±165.0	380.3±158.9
	233.2	311.0	380.6
p values†	*0.684*	*0.621*	*0.312*
DM 0–5 y	271.4±140.0	356.3±156.5	—
	246.3	317.1	
DM >5 y	287.3±141.9	306.8±87.4	—
	320.9	273.2	
p values†	*0.807*	*0.425*	—

As the numbers of the subgroups were believed to be inadequate, nonparametric tests were used in this analysis. All values given as mean±SD (above) and median (below).

*p values of Kruskal-Wallis H test.

†p values of Mann-Whitney U test.

DR = Diabetic retinopathy

levels were not only found in diabetic patients with DR but were also found in diabetic patients without any signs of diabetic complications. The similar results were also pointed out by some study groups (12, 31), and it has been suggested that the increased protein oxidation may not be due to the complications, but rather contribute to their progression. In the same study by Cakatay, it was also emphasized that poor glycemic control was another important factor in generation of increased protein oxidation in type 2 diabetic patients clinically free of complications (12). In another study by Piwowar et al, plasma levels of TRAP, as a marker of antioxidative defense, and CO, SH, NH_2 groups, and AOPP, as markers of oxidative protein damage, were investigated in 94 diabetic patients and 36 healthy controls (13). It was concluded that AOPP was found to show the most expressive rise in plasma of diabetic patients and thereby AOPP seems to be considered as a useful marker to estimate the degree of oxidative protein damage in diabetic patients (13). Another study by Martin-Gallan et al aimed at ascertaining the role of indicative parameters of lipoperoxidation, protein oxidation, and changes in antioxidant defense system status in 26 young diabetic patients with recently diagnosed microangiopathy, 28 diabetic patients without complications, and 40 healthy age-matched controls; their results showed that the increased protein oxidative damage and reduced antioxidative defenses were greater in young diabetic patients only with microvascular complications than in those without them (11).

In conclusion, increased protein oxidation might be an important early event in the pathogenesis of diabetic retinopathy. Moreover, total thiol/AOPP ratio seems to be an important prognostic parameter during follow-up for DR. Furthermore, inhibition of AOPP formation or oxidative stress generation might be considered as a potential target for therapeutic intervention in sight-threatening DR.

The authors have no financial or proprietary interest in any product, method, material, or concept used or discussed in this study.

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