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The role of protein oxidation in the development of diabetic microvascular complications

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ABSTRACT

OBJECTIVE: The role of protein oxidation in the development of diabetic microvascular complications was investigated.

METHODS: In total, 266 participants were split into five groups: Group 1; diabetes mellitus for at least 10 years without any complications, Group 2; diabetic nephropathy, Group 3; diabetic neuropathy, Group 4; diabetic retinopathy, and Group 5; control group. Thiol, disulfide, ferroxidase, and ischemia-modified albumin (IMA) levels were analyzed in the serum.

RESULTS: Native thiol, total thiol, and native thiol/total thiol were lower in Group 4 than Groups 1, 3, and 5 ($p<0.001$). However, disulfide/native thiol and disulfide/total thiol were higher in Group 4 than all other groups ($p<0.001$). IMA was higher in Groups 3 and 4 than all other groups ($p<0.001$). Ferroxidase was lower in Groups 3 and 4 than Group 2 ($p<0.001$).

CONCLUSION: Thiol-disulfide homeostasis impairment in favor of disulfide may have a function in the progress of diabetic retinopathy. Furthermore, the disruptions of IMA and ferroxidase levels involve in the development of diabetic retinopathy and neuropathy.

Keywords: Diabetes mellitus; microvascular complications; neuropathy; retinopathy; thiol-disulfide.

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Diabetes mellitus refers to a metabolic disease with entire or partial absence of insulin or resistance of insulin. The damages to the retina, kidney, peripheral, and autonomic nerves are caused by the structural and functional changes occurring in the microvascular compartments. The microvascular complications of diabetes mellitus include retinopathy, nephropathy, and neuropathy. The development of microvascular complications is related to hyperglycemia, hyperlipidemia, epigenetic dysregulation, and genetics [1]. However, the pathophysiology of microvascular complications has not yet been fully explained. Hyperglycemia serves a function in the formation of microvascular complications, however, it is not only the cause of tissue damage [2]. Oxidative stress in diabetic patients is known to increase. Oxidative stress plays an important role in the pathophysiology of microvascular complications [3–6].

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Thiols (-SH) are functional organic compounds of proteins. Amino acid and protein structures, such as albumin, cysteine, cysteinylglycine, glutathione, and homocysteine containing thiol groups, serve functions in the defense against oxidative stress [7, 8]. Reversible disulfide (-S-S-) bonds are produced by the interaction of thiols with oxidants [9].

The formation of ischemia-modified albumin (IMA) takes place by the changes occurring in the structure of albumin by free radicals. IMA is the only ischemia marker that is validated by the Food and Drug Administration [10]. It was observed that elevated IMA was linked to pathogenesis of type 2 diabetes mellitus [4, 11, 12].

Ceruloplasmin has ferroxidase enzyme activity. Iron (II) ions are converted iron (III) by ferroxidase enzyme, thus allowing them to bind to transferring. Therefore, it protects the body from the harmful effects of iron (II) ions [13].

Thiol/disulfide, IMA, and ferroxidase levels were examined to understand the function of protein oxidation in the physiopathology of diabetic microvascular complications in the present study.

MATERIALS AND METHODS

The present study was conducted between April 17, 2017, and January 31, 2018. While diabetic patients with diabetes for at least 10 years without any complication and diabetic nephropathy individuals were chosen from internal medicine outpatient clinic, diabetic retinopathy individuals were chosen from ophthalmology outpatient clinic, and diabetic neuropathy individuals were chosen from neurology outpatient clinic. A control group that included the individuals with similar demographic character who were examined to the internal medicine outpatient clinic for a routine check-up was formed. Written informed consent was taken from all subjects and the permission was received from the Clinical Research Ethics Committee of Erzincan Binali Yildirim University Faculty of Medicine (dated 11/04/2017 and numbered 4/09).

The groups were formed as the following:

Group 1: Individuals with diabetes for minimum 10 years with no complications (n=21)

Group 2: Individuals with nephropathy (n=22)

Group 3: Individuals with neuropathy (n=69)

Group 4: Individuals with retinopathy (n=126)

Group 5: Healthy individuals (n=50)

Highlight key points

- Thiol-disulfide homeostasis deteriorates in favor of disulfide in diabetic retinopathy.
- IMA and ferroxidase levels changes in diabetic retinopathy and neuropathy.
- Protein oxidation may have role in the progress of diabetic microvascular complications.
- Increased glucose levels are possible to be responsible for increased protein oxidation.

Definitions

The patients were diagnosed as the following:

Diabetes Mellitus and Diabetic Nephropathy

The diagnosis of diabetes mellitus and diabetic nephropathy was made in line with the standards that were specified by the American Diabetes Association [14] and the Renal Pathology Society [15], respectively.

Diabetic Neuropathy

Diabetic sensorimotor polyneuropathy was diagnosed based on the Toronto Expert Panel on Diabetic Neuropathy which involves the symptoms, clinical, and electroneuromyography (Medelec Synergy, England) findings [16].

Diabetic Retinopathy

Diabetic retinopathy was diagnosed according to the Modified Klein Classification (Modified Early Treatment Diabetic Retinopathy Study scales). It was classified according to the presence or absence of abnormal new vessels as non-proliferative retinopathy and proliferative retinopathy [17].

Exclusion Criteria

Other than diabetes, the patients who had any heart, liver, pancreas, or hematological disease, the patients who had acute or chronic infection, hyperlipidemia, and hyperuricemia were not protected in the present study. Furthermore, the subjects with acute diabetic complications such as ketoacidosis, hyperosmolar non-ketotic diabetic coma, and lactic acidosis or macrovascular diabetic complications were excluded from this study.

Sample Collection

Samples of blood were obtained to the gel separated biochemistry tube between 08:00 and 10:00 in the morning.

Table 1. Comparison of clinical demographic characteristics of the groups

Variable	Groups					p	Multiple comparisons
	DM (1) Mean±SD	Nephropathy (2) Mean±SD	Neuropathy (3) Mean±SD	Retinopathy (4) Mean±SD	Control (5) Mean±SD		Group (p)
Age (years)	55.9±12	60.7±9.8	64.7±9.7	58.±8.9	54.7±13.3	<0.001	3–5 (0.001) 3–4 (<0.001)
Height (cm)	161.8±13	158.9±11.4	163.0±7.6	163.0±8.0	167.1±11.0	0.14	
Weight (kg)	80.6±14	80.3±18.0	85.5±13.6	80.5±14.8	83.8±20.3	0.35	
BMI (kg/m2)	31.2±6.8	31.7±6.2	32.2±5.1	30.3±5.8	29.9±5.9	0.207	
Diabetes duration (years)	12.8±2.6	11.5±5.2	10.2±7.9	11.8±7.0		0.458	
Insulin treatment duration (years)	6.6±5.6	5.6±4.7	7.5±8.1	6.8±6.2		0.801	

SD: Standard deviation; BMI: Body mass index; DM: Diabetes mellitus.

After the clotting, serum was acquired through centrifugation at 3000 g for 15 min. Serum was separated into sample separation tubes with the aim of measuring thiol-disulfide, IMA, and ferroxidase, and then, it was kept at 80°C below zero until the study was conducted.

Biochemical Measurements

Serum creatinine, urea, and fasting glucose were analyzed by spectrophotometric method on Olympus AU 2700 (Beckman Coulter Corporation, Tokyo, Japan). HbA1C was analyzed using the Tosoh G 8 instrument (Tosoh Corporation, Tokyo, Japan) by high-performance liquid chromatography method. Thiol, disulfide, IMA, and ferroxidase tests were analyzed in the Cobas 501 (Roche, Mannheim, Germany). The native thiol (-SH) and total thiol (-SH + -SS) were analyzed and disulfide (-S-S), disulfide/native thiol, disulfide/total thiol, and native thiol/total thiol values were obtained by calculation. "Modified Ellman method" of Erel et al. [8] was used for total and native thiol measurement. IMA was detected with a quick colorimetric method adapted by Bar-Or et al. [18] The method defined by Neselioglu et al. [13] was used to analyze ferroxidase activity.

Statistical Analysis

Statistical analyses were carried out with SPSS 20. The normality analyses were checked by the Kolmogorov–Smirnov test. The analysis of nominal data was performed with the Pearson's Chi-square test or Fisher's exact test. The data were compared with one-way analysis of variance. While Games-Howell multiple comparison test was applied, when the homogeneity of variance was not provided, Hochberg's GT2 test and Tukey test were performed when the homogeneity of variance was provided, and the relationships between the variables were determined using the Pearson correlation test. It was considered significant at $p<0.05$.

RESULTS

While 94 (35.3%) of a total of 266 individuals were male, 172 (64.7%) of them were female. No difference was found in terms of height, weight, body mass index, the duration of insulin treatment, and the duration of diabetes mellitus between the groups. The mean age was higher in Group 3 than Groups 4 and 5 (Table 1). All the participants included in the diabetic retinopathy group have non-proliferative retinopathy.

Glucose and HbA1C were higher in Groups 2, 3, and 4 than Group 5 ($p<0.001$). It was determined that urea and creatinine values were not different between the groups (Table 2).

Native thiol level, total thiol level, and native thiol/total thiol ratio were lower in Group 4 than Groups 1, 3, and 5 ($p<0.001$). Disulfide/native thiol and disulfide/total thiol rations were higher in Group 4 than all other groups, moreover, the level of disulfide was higher than Groups 3 and 5 ($p<0.001$). Ischemia-modified albumin level was higher in Groups 3 and 4 than Groups 1, 2, and

TABLE 2. Comparison of biochemical parameters of the groups

Variable	Groups					p	Multiple comparisons
	DM (1) Mean±SD	Nephropathy (2) Mean±SD	Neuropathy (3) Mean±SD	Retinopathy (4) Mean±SD	Control (5) Mean±SD		Group (p)
Glucose (mg/dL)	151.7±71.7	205.9±79.2	188.0±96.9	174.6±70.7	99.2±13.3	<0.001	5–2 (<0.001) 5–3 (<0.001) 5–4 (<0.001)
HbA1C (%)	7.5±2.4	9.9±1.6	8.7±2.6	8.1±1.7	5.5±0.7	<0.001	5–2 (<0.001) 5–3 (0.001) 5–4 (0.007)
Creatinine (mg/dL)	0.8±0.1	1.0±0.4	0.9±0.4	0.8±0.3	0.9±0.3	0.158	
Urea (mg/dL)	33.0±13.7	45.6±24.6	35.4±16.7	38.4±15.6	38.3±23.6	0.162	

SD: Standard deviation; DM: Diabetes mellitus; HbA1C: Hemoglobin A1C.

5 (p<0.001). The ferroxidase level was lower in Groups 3 and 4 than Group 2 (p<0.001) (Table 3).

There was a positive correlation between glucose, HbA1C, and IMA (p=0.003, r=0.188 vs. p=0.044, r=0.136, respectively) when the correlation analyses were carried out between the oxidative stress biomarkers and, demographic and biochemical parameters (Table 4).

DISCUSSION

Diabetic retinopathy, nephropathy, and neuropathy are the main reasons of blindness, end-stage renal disease, and some neuropathies. Hyperglycemia is the most significant reason, responsible for the production of microvascular complications in diabetes mellitus. There are a variety of possible pathways suggested to understand how hyperglycemia contributes to complications of diabetes mellitus. The enhance in oxidative stress, advanced glycation end-products formation, and polyol pathway are some of them [19]. Thiol compounds are a component of the antioxidant system. The oxidation of thiols is considered to be the early marker of protein oxidation [20]. In the present study, thiol/disulfide, IMA levels, and ferroxidase activity were analyzed by evaluating microvascular complications together in diabetes mellitus.

Glucose easily binds non-enzymatically to the amino groups of proteins after proteins have been exposed to elevated levels of glucose for a long time. Autoxidation of glycosylated proteins causes of the generation of free radicals [21]. Gulpamuk et al. [4] reported that native thiol and total thiol levels decreased, however, disulfide, disulfide/native thiol, disulfide/total thiol, and IMA levels enhanced in diabetic individuals with retinopathy compared to diabetic individuals without retinopathy. In other studies, it was shown that the level of IMA increased in diabetic retinopathy, however, thiol groups decreased, therefore, there was an augmentation in oxidative stress in these subjects [12, 22–24]. In this study, it was found an enhancement in IMA level and thiol/disulfide homeostasis in the group with retinopathy in favor of disulfide, which is compatible with the results of the above-mentioned studies. Thus, thiol/disulfide balance and protein oxidation have a significant function in the formation of diabetic retinopathy. However, it should be taken into consideration that all the patients in retinopathy group have non-proliferative retinopathy in the present study. Because, it is possible that proliferative or non-proliferative retinopathy has the potential to change the values mentioned for protein oxidation. Diabetic neuropathy is also associated with oxidative stress [25]. It was reported by Vural et al. [6] that thiol-disulfide homeostasis was disrupted in favor of disulfide in polyneuropathy cases contracted to diabetic and healthy individuals. In the present study, the lower ferroxidase activity in the neuropathy group than the nephropathy group and higher IMA level than the nephropathy and control group indicate that diabetic neuropathy patients are subjected to more oxidative stress than nephropathy and healthy individuals. Furthermore, higher level of thiol and lower level

Table 3. Comparison of thiol-disulfide, IMA, and ferroxidase parameters of the groups

Variable	Groups					p	Multiple comparisons
	DM (1) Mean±SD	Nephropathy (2) Mean±SD	Neuropathy (3) Mean±SD	Retinopathy (4) Mean±SD	Control (5) Mean±SD		Group (p)
Ferroxidase (µmol/L)	641.1±180	693.3±114.4	543.2±140.8	554.6±138.2	588.8±138.2	<0.001	2–3 (<0.001) 2–4 (<0.001)
IMA (IU/ml)	0.5±0.1	0.8±0.4	1.2±0.2	1.1±0.3	0.8±0.3	<0.001	1–3 (<0.001) 1–4 (<0.001) 2–3 (<0.001) 2–4 (0.001) 5–3 (<0.001) 5–4 (<0.001)
Native_thiol (µmol/L)	395.1±55.9	365.6±99.8	379.6±71.5	324.9±70.7	376.9±76.8	<0.001	1–4 (0.001) 3–4 (<0.001) 4–5 (0.001)
Disulfide/native thiol (%)	5.2±3.7	6.1±5.2	4.0±2.5	9.0±5.1	4.8±2.6	<0.001	1–4 (0.002) 2–4 (<0.001) 3–4 (<0.001) 4–5 (<0.001)
Disulfide/total thiol (%)	4.5±2.9	5.1±3.7	3.6±2.1	7.3±3.5	4.3±2.1	<0.001	1–4 (0.001) 2–4 (0.009) 3–4 (<0.001) 4–5 (<0.001)
Native thiol/total thiol (%)	90.8±5.8	89.7±7.4	92.7±4.2	85.2±7.0	91.3±4.2	<0.001	1–4 (0.001) 3–4 (<0.001) 4–5 (<0.001)
Disulfide (µmol/L)	20.7±14.5	19.7±11.7	14.7±8.9	27.6±13.5	17.7±9.5	<0.001	4–3 (<0.001) 4–5 (<0.001)
Total thiol (µmol/L)	433.9±67.5	405.0±102.1	409.1±75.1	380.2±72.2	412.4±82.1	0.015	4–1 (0.005) 4–3 (0.018) 4–5 (0.018)

SD: Standard deviation; DM: Diabetes mellitus; IMA: Ischemia-modified albumin.

of disulfide in the neuropathy group than the retinopathy group revealed that patients with diabetic neuropathy had less oxidative stress than patients with diabetic retinopathy. Ergin et al. [3] reported that thiol/disulfide balance was impaired in type 2 diabetes mellitus cases. Furthermore, they determined disulfide level higher in the group with diabetic complications than the group with no complications. Hence, they indicated the impaired thiol/disulfide balance in the progress of diabetic complications. Nevertheless, in their study, all individuals with diabetic microvascular complications were gathered in a single group and a small number of individuals were included. However, in this study, individuals with diabetic microvascular complications were separately grouped in accordance with the type of complication.

Furthermore, the augmentation of glucose and HbA1C values in the retinopathy and the neuropathy groups revealed that glucose regulation of these patients was poor. Hence, higher glucose levels may cause enhanced oxidative stress. In addition, the positive correlation between glucose and HbA1C with IMA suggests that higher serum glucose increases the IMA formation

Table 4. Correlation table of the oxidative stress markers with demographic and biochemical parameters

Variable	Ferroxidase	IMA	Native thiol	Total thiol	Disulfide	Disulfide/ Native thiol	Disulfide/ Total thiol	Native thiol/ Total thiol
Age								
r	–0.028	0.072	–0.089	–0.099	–0.043	–0.014	–0.017	0.170
p	0.653	–0.246	0.154	0.114	0.491	0.823	0.780	0.780
BMI								
r	–0.035	–0.042	0.077	0.095	0.055	0.020	0.016	–0.016
p	0.605	0.536	0.250	0.158	0.411	0.771	0.815	0.815
HbA1C								
r	–0.013	0.136	–0.060	–0.060	0.002	0.018	0.017	–0.017
p	0.848	0.044[a]	0.378	0.381	0.972	0.789	0.803	0.803
Glucose								
r	0.052	0.188	–0.036	–0.005	0.094	0.063	0.073	–0.073
p	0.414	0.003[a]	0.568	0.935	0.138	0.320	0.248	0.248

BMI: Body mass index; HbA1C: Hemoglobin A1C; IMA: Ischemia-modified albumin; a: P<0.05.

by altering albumin form. Indeed, in our another study, thiols reduced, however, disulfides increased after the glucose intake in pregnant who has an impairment as a result of 50 g glucose challenge test. Nevertheless, no difference was found between thiol/disulfide values before and after the glucose challenge test in pregnant with normal test results. Furthermore, it was found a positive correlation between glucose and disulfide level and a negative correlation between glucose and native thiol level. Hence, the impairment of thiol/disulfide balance in favor of disulfide was observed at higher glucose values [26].

Diabetic nephropathy, which leads to serious morbidity and mortality, is also linked with disturbing thiol/disulfide balance [5]. In the previous studies, it was reported that the IMA level was high in individuals with diabetic nephropathy [11, 27]. In the present study, patients with nephropathy were in the early stages which the kidney functions did not reduce yet, so oxidative stress did not increase in this group. In fact, it was demonstrated in a previous study that the deterioration of thiol/disulfide balance was linked with the degree of nephropathy [5]. The lower ferroxidase activity in the patients with neuropathy and retinopathy indicates that these patients are exposed to higher oxidative stress or there was an impairment in the antioxidant defense systems.

A particularly limited number of patients, the fact that oxidative stress markers were only carried out at once, and some patients under diabetes treatment were the main limitations of this study. Nevertheless, the measurement of some oxidative stress markers in diabetic microvascular complications using newly developed methods is important. In the future, prospective studies can be planned by following the progress of diabetic microvascular complications in larger patient groups.

Conclusions

Oxidative stress has a major position in the physiopathology of diabetic microvascular complications. Especially, patients with retinopathy and neuropathy are subjected to more oxidative stress and have the poorer blood glucose regulation. Therefore, this study stressed once again the importance of controlling blood glucose in the prevention of microvascular diabetic complications.

Ethics Committee Approval: The Erzincan Binali Yildirim University Faculty of Medicine Clinical Research Ethics Committee granted approval for this study (date: 11.04.2017, number: 4/09).

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Authorship Contributions: Concept – CM; Design – CM; Supervision – CM, TAC; Fundings – CM, TAC; Materials – CM, GS, YK, AE, AO; Data collection and/or processing – CM, GS, GG, OE, YA; Analysis and/or interpretation – CM, GS, GG, OE, YA; Literature review – CM, GS; Writing – CM, GS; Critical review – CM, GS, TAC, YK, AE, AO, YA, GG, OE.

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