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RESEARCH ARTICLE

Evaluation of Oxidative Protein Damage in Patients with Type 1 and 2 Diabetes Mellitus in Bangladesh

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ABSTRACT

Objective: Oxidative stress (OS) has been linked to the development and progression of diabetes mellitus (DM). Although maintaining the redox status of protein is crucial for proper cellular function, proteins are likely to be damaged by OS. Therefore, the present study aimed to evaluate oxidative protein damage (OPD) in patients with DM.

Materials and Methods: A total of 160 participants were recruited, of whom 16 were patients with type 1 DM (T1DM), 84 were patients with type 2 DM (T2DM), and 60 were healthy control subjects. The activities of NADPH oxidase (NOX) and myeloperoxidase (MPO); total oxidative stress (TOS), ferric reducing ability of plasma (FRAP), oxidative stress index (OSI); serum albumin; OPD markers-total thiols (T-SH), protein carbonyls (PCO), and advanced oxidation protein products (AOPP) were assessed.

Results: The activities of serum NOX and MPO were significantly higher in both DM groups compared to controls. Significantly higher TOS and OSI and lower FRAP values were observed in both DM groups than in controls ($p<0.001$, for all). In patients, the levels of albumin and T-SH were significantly lower, but PCO was significantly elevated, while AOPP was higher in T1DM and significantly elevated in T2DM compared to controls. Correlation analyses between these parameters linked hyperglycemia with enhanced NOX, MPO and AOPP, and decreased FRAP and T-SH in diabetic patients. Further, significant correlations of albumin with T-SH and AOPP suggested an association of OS with hypoalbuminemia.

Conclusion: These findings highlight that hyperglycemia induces enhanced OS and consequent protein damage in both T1DM and T2DM patients.

Keywords: Oxidative protein damage, Advanced oxidation protein products, Total thiols, Ferric reducing ability of plasma, Type 1 diabetes mellitus, Type 2 diabetes mellitus

INTRODUCTION

Diabetes is characterized by chronic hyperglycemia associated with damage, dysfunction, and failure of different organs, which consequently manifest various complications.[1] One of the major mechanisms underlying the onset of diabetes and latter diabetic complications is oxidative stress (OS), an imbalance between free radical generation and the antioxidant defense systems.[2] OS plays a pivotal role in the autoimmune destruction of pancreatic β-cells in type 1 diabetes mellitus (T1DM) and in type 2 diabetes mellitus (T2DM), which can cause insulin resistance.[3]

Hyperglycemia can augment the activity of reactive oxygen species (ROS)-generating enzymes such as nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (NOX) and myeloperoxidase (MPO).[4,5] The higher activity of NOX as a major source of ROS has been implicated in the pathophysiology of diabetic vascular disease and diabetic nephropathy.[6] The heme enzyme MPO utilizes hydrogen peroxide to oxidize chloride and generates oxidants, hypochlorous acids (HOCl). These oxidants can modify cellular macromolecules, leading to damage of tissues.[7] There is growing evidence that MPO is associated with insulin resistance and inflammation.[8] Analysis of MPO activity in diabetic patients provides a way to assess both OS and cardiovascular disease (CVD) risk considering its involvement in the pathophysiology of the latter.[9]

Higher levels of ROS cause peroxidation of lipids, amino acids, peptides, and proteins with the resultant production of hydroperoxides.[10] These hydroperoxides have been measured as total oxidative stress (TOS) marker in diabetes.[11] The ferric

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reducing ability of plasma (FRAP) manifests the total antioxidant capacity (TAC) based on the cumulative actions of total antioxidants in plasma to inhibit the oxidative effects of ROS. Furthermore, the oxidative stress index (OSI), a ratio of TOS to TAC, might more accurately index oxidant/antioxidant imbalance in diabetic patients.[12]

Albumin is a powerful extracellular antioxidant which contributes more than 70% to the free radical-trapping activity of blood plasma.[13] The conformation of albumin is altered in OS, allowing its thiol groups to be oxidized and decreased in number. The assessment of plasma total thiol (T-SH) levels reliably indicates excessive free radical generation in the body.[14] Protein carbonyls (PCO) are formed when proteins undergo oxidative damage when ROS react with the side chains of certain amino acid residues in proteins including lysine, arginine, proline, and threonine.[15] A novel protein oxidation marker is advanced oxidation protein products (AOPP), which correspond to highly oxidized proteins, especially albumin, and are formed from reactions of MPO-derived chlorinated oxidants and proteins. They include oxidatively modified protein aggregates by disulfide bridges and/or tyrosine cross-linking.[16]

The maintenance of the protein redox state is crucial for cell function. Increased aggregation, fragmentation, distortion of secondary and tertiary structures, susceptibility to proteolysis, and reduction in normal function might result from changes in protein conformations brought on by ROS attack.[17] Therefore, this study was designed to evaluate the oxidative damage to proteins by the analysis of albumin, T-SH, PCO and AOPP markers together with the investigation of NOX and MPO activities and levels of TOS, FRAP and OSI in diabetic patients and compare these findings with a non-diabetic control group.

MATERIALS AND METHODS

Study Design

This study was approved by the Ethical Review Committee of the Faculty of Biological Sciences, University of Dhaka, Bangladesh (Ref. No. 108 /Biol. Sci. /2020-2021), and the Diabetic Association of Bangladesh. The study was conducted from January 2021 to May 2022. All the participants gave their informed consent before being enrolled in the study.

A total of 160 subjects were enrolled, of whom 100 were diabetics, comprising 16 T1DM and 84 T2DM patients; the remaining 60 were non-diabetic controls. The patients were randomly approached from the outpatient department of the Bangladesh Institute of Research and Rehabilitation in Diabetes, Endocrine and Metabolic Disorders (BIRDEM) General Hospital. They were previously diagnosed with diabetes and expert physicians distinguished T1DM and T2DM according to the criteria of the American Diabetes Association.[18] The control group was randomly selected from the local community. The study included participants aged 20-50 years and excluded those having CVD and/or nephropathy. Participants suffering from any condition known to cause OS were excluded from the study to avoid false positive results.

Data and Sample Collection

The general health information of the participants including age, gender, height, weight, blood pressure, fasting plasma glucose (FPG), glycosylated hemoglobin HbA1c (%), disease duration, family history of diabetes, smoking status, hypertension, diabetic complications, and medications were recorded. Blood samples were collected, and the plasma and serum were separated, collected in small aliquots, and stored at $-20^{\circ}C$ until analyzed.

Assay Procedures

Serum NOX activity was measured using the method established by Reusch and Burger, as detailed previously.[19,20] Serum MPO activity was determined by Bradley et al.'s method[21]. The detailed procedure has been described elsewhere.[22] A modified free oxygen radical test was used to assess the level of plasma TOS and the result was expressed as mmol/L of H_2O_2 equivalents.[11] The total antioxidant capacity (TAC) was determined by the FRAP assay, as detailed in a recent study.[23,24] The OSI was determined by the ratio of TOS to TAC [12], and expressed in arbitrary units (AU), according to the following formula: OSI (AU) = TOS / TAC.

Determination of Oxidative Protein Damage

The serum albumin level was estimated using the bromocresol green (BCG) method, as detailed previously.[25,26] The level of T-SH in plasma was measured by Hu's method.[14] The plasma PCO content was quantified by the 2,4-dinitrophenyldrazine method described by Levine et al.[27] Serum AOPP levels were determined using the method of Witko-Sarsat et al.[16], with slight modifications. In brief, 300 μL diluted serum (1/10 in phosphate buffer saline) or chloramine T trihydrate (Merck) standard was mixed with 150 μL of acetic acid and 75 μL potassium iodide. The absorbance of the reaction mixture was read after 2 minutes at 340 nm. AOPP concentrations were expressed as μM chloramine T equivalents.

Statistical Analyses

Statistical analysis and graphical presentation of the data were performed using GraphPad Prism (version 8.0.1, GraphPad Software, USA). Statistical significance of differences between the values of the continuous variables in the three groups (T1DM, T2DM and controls) was assessed by one-way ANOVA (Analysis of Variance) with post-hoc Games-Howell's multiple comparisons test. For each parameter, the mean ± SD

values were computed. The chi squared test was used to compare categorical variables. Spearman's correlation was conducted to evaluate the correlation between variables. The results were considered significant when p value was <0.05.

RESULTS

Baseline Characteristics of the Studied Subjects

A comparison of the baseline characteristics of the studied subjects has been presented in Table 1. It was found that both the T1DM and T2DM patients had significantly higher FPG levels compared to the controls (p<0.05 and p<0.001, respectively), FPG among the three groups was significantly different (p<0.001, one-way ANOVA), while the level between T1DM and T2DM patients did not differ significantly. The mean disease duration of the T1DM and T2DM patients were 12.8 ± 9.4 and 4.8 ± 3.7 years, respectively.

Statistical analyses of the values in the three groups showed family history of diabetes was significantly higher among the diabetics (p<0.01, Table 1), but a pairwise analysis revealed that compared to the controls, the family history of diabetes was significantly associated with the development of diabetes in T2DM patients only (χ^2 = 12.5, p<0.001). On the other hand, there was no significant relationship between hypertension or smoking with the risk of developing diabetes. Among all patients, the number of diabetics taking oral hypoglycemic drug, insulin injection, or both were 24, 37, and 33, respectively, and 8 had retinopathy, 9 had neuropathy, and 11 had both complications.

Comparison of NOX, MPO, TOS, FRAP and OSI

It was found that the mean activities of the oxidase enzymes NOX and MPO were significantly higher in both T1DM and T2DM patients compared to control subjects (NOX: p<0.01 and p<0.001, respectively; MPO: p<0.001, both), while the activities between the T1DM and T2DM patients did not differ significantly (Table 2). Similarly, the mean TOS values in both T1DM and T2DM patients were significantly elevated compared to controls (p<0.001, both) (Table 2), and no significant difference was found between the DM groups. The mean FRAP value in the control subjects was 1221.1 ± 305.0 μmol/L, and in the T1DM and T2DM patients were 723.6 ± 150.9 μmol/L and 789.6 ± 142.1 μmol/L, respectively, which were significantly lower (p<0.001, both) (Figure 1). The mean OSI values were significantly elevated in both types of DM patients compared to the controls (p<0.001, both) (Table 2). The OSI values between the DM groups were not significantly different.

Evaluation of Oxidative Protein Damage

The mean serum albumin levels in the T1DM and T2DM patients were 41.0 ± 10.0 g/L and 45.0 ± 6.0 g/L, respectively,

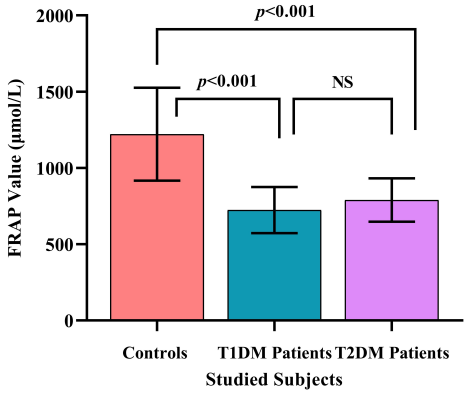

Figure 1. Comparison of the ferric reducing ability of plasma (FRAP) values between the studied subjects. Both types of diabetic patients had significantly lower FRAP values compared to the controls. There was no significant difference between the FRAP values of T1DM and T2DM patients.

which were significantly lower than 51.0 ± 7.0 g/L in the controls (p<0.01 and p<0.001, respectively). The albumin levels in T1DM and T2DM patients were not significantly different. The mean T-SH concentration in the control subjects was 472.7 ± 61.5 μM, and the corresponding values in the T1DM and T2DM patients were 301.1 ± 139.2 μM and 323.6 ± 132.7 μM, respectively, which were significantly lower (p<0.001, both).

Investigation of PCO showed significantly higher values in both T1DM and T2DM patients compared to controls (p<0.01, both), and the mean values were 2.2 ± 0.5, 2.1 ± 0.8 and 1.6 ± 0.8 nmol/mg, respectively (Figure 2). The mean AOPP levels in the controls, T1DM, and T2DM patients were 441.6 ± 215.7, 605.7 ± 235.6, and 624.3 ± 233.1 μM chloramine T equivalents, respectively (Figure 3). Statistical analyses revealed that the T2DM patients had significantly elevated AOPP levels compared to the controls (p<0.001) but the level in T1DM patients was not significantly elevated (p=0.053).

Correlations Between Different Parameters

Spearman correlation analyses between different parameters of the pooled DM patients and control subjects have been presented in Table 3. There was a significant negative correlation between AOPP and albumin (p=0.006) (Figure 4a) and a significant positive correlation between OSI and FPG (p=0.004) (Figure 4b) in DM patients, which were not found in controls. In DM patients, NOX and MPO each showed significant positive correlations with FPG (p=0.003 and p=0.049, respectively), and a significant negative correlation was found between FRAP and FPG (p<0.001). No such significant correlations were observed in controls. A significant positive correlation was found between TOS and OSI in both DM patients and controls (p<0.001, both). The patients showed significant positive correlations of T-SH with albumin (p=0.001), and NOX with OSI (p=0.027). Further, AOPP significantly correlated with NOX in controls

Table 1. Comparison of baseline characteristics of the control subjects, T1DM and T2DM patients.

Variables	Controls (N=60)	T1DM (N=16)	T2DM (N=84)	Statistics (*p*-value)
Gender (M/F) (%)	80/20	94/6	90/10	0.128*
Age (years)	38.5 ± 6.0	34.4 ± 8.0	38.7 ± 4.8	0.075≠
FPG (mmol/L)	5.2 ± 0.3	10.1 ± 6.3	10.3 ± 4.5	< 0.001≠
HbA1c (%)	-	11.7 ± 2.0	8.7 ± 2.8	NA
BMI (kg/m²)	25.5 ± 3.7	23.2 ± 2.9	24.5 ± 3.0	0.061≠
SBP (mmHg)	128.7 ± 12.0	130.0 ± 16.0	132.0 ± 19.5	0.512≠
DBP (mmHg)	88.2 ± 10.4	94.3 ± 19.7	92.6 ± 11.0	0.217≠
Family history of DM: positive/negative (%)	34/66	50/50	65/35	< 0.01*
Hypertension (%)	40	40	44	0.945*
Smoking status: current/ex-smokers/ non-smokers (%)	18/2/80	25/13/62	17/11/72	0.336*

T1DM, type 1 diabetes mellitus; T2DM, type 2 diabetes mellitus; M, male; F, female; FPG, fasting plasma glucose; HbA1c, glycosylated hemoglobin; NA, not applicable; BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; *, *p*-value of chi-square test; ≠, *p*-value of one-way ANOVA (analysis of variance).

Table 2. Comparison of NOX and MPO activities, and TOS and OSI values between the control subjects, T1DM and T2DM patients.

Parameters	Controls	T1DM	T2DM	*p*-value (ANOVA Post-hoc)	
				Control vs. T1DM	Control vs. T2DM
NOX (U/L)	6.3 ± 1.8	9.5 ± 2.8	11.3 ± 3.2	<0.01	<0.001
MPO (U/L)	45.8 ± 12.7	58.6 ± 10.2	64.5 ± 11.3	<0.001	<0.001
TOS (mmol/L)	11.4 ± 2.3	16.3 ± 3.7	15.0 ± 4.0	<0.001	<0.001
OSI (AU)	10.1 ± 3.6	24.0 ± 9.2	20.6 ± 7.0	<0.001	<0.001

T1DM, type 1 diabetes mellitus; T2DM, type 2 diabetes mellitus; NOX, NADPH oxidase; MPO, myeloperoxidase; TOS, total oxidative stress; OSI, oxidative stress index; ANOVA, analysis of variance; U/L, unit per liter; mmol/L, millimole per liter; AU, arbitrary units.

(p=0.007), but this correlation was not significant in DM patients.

DISCUSSION

This study investigated OPD in patients with diabetes mellitus by assessing activities of NOX and MPO, and levels of oxidative stress (OS) along with OPD markers, and compared the findings with a control group. The study enrolled younger patients of 20 to 50 years to avoid additional OS caused by older age-related diabetic complications. Both the T1DM and T2DM patients had significantly higher FPG levels compared to the controls. The family history of diabetes among first-degree relatives was found to be associated with the incident risk of T2DM in patients, which was in line with a previous study.[28] No such significant association was found in T1DM patients. The reason could be the small number of T1DM patients in this study, and also the incident risk of T2DM was more associated with family history than T1DM was.[18] Therefore, this finding suggests that family history could be used as an important tool for identifying the people at risk of developing diabetes.

This study demonstrated significantly higher NOX activities in both DM patient groups compared to the controls, and a significant positive association was observed between FPG and NOX in diabetic patients. These findings support a previous study showing hyperglycemia-induced higher NOX activity in T2DM patients and a positive correlation between glucose levels and p22phox expression, a critical component of NOX activation.[4] The current findings of significantly higher MPO activities in both types of DM patients were consistent with previous findings in T1DM and T2DM patients.[5,29] The latter study further reported enhanced levels of ROS including HOCl in the cultured peripheral blood mononuclear cells of T2DM

Table 3. Spearman correlation analysis between different parameters in diabetic patients and control subjects.

Correlation of parameters	Spearman correlation coefficient, ρ	*p*-value
Diabetic Patients		
FPG-NOX	0.428	0.003
FPG-MPO	0.236	0.049
FPG-OSI	0.325	0.004
FPG-FRAP	-0.693	<0.001
Duration of DM-FRAP	-0.324	0.003
TOS-OSI	0.766	<0.001
FRAP-OSI	-0.580	<0.001
NOX-OSI	0.378	0.027
T-SH-Albumin	0.377	0.001
AOPP-Albumin	-0.327	0.006
AOPP-NOX	0.181	0.229
Control subjects		
AOPP-NOX	0.379	0.007
TOS-OSI	0.508	<0.001
FRAP-OSI	-0.728	<0.001

DM, diabetes mellitus; FPG, fasting plasma glucose; NOX, NADPH oxidase; MPO, myeloperoxidase; TOS, total oxidative stress; FRAP, ferric reducing ability of plasma; OSI, oxidative stress index; T-SH, total thiol; AOPP, advanced oxidation protein products.

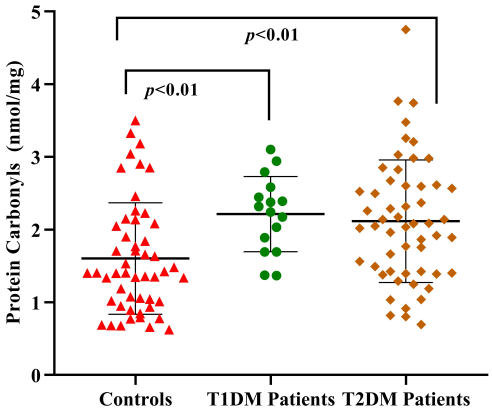

Figure 2. Comparisons of the protein carbonyls (PCO) levels in the studied subjects. Both types of diabetic patients had significantly higher PCO levels compared to the controls. There was no significant difference between the PCO levels of T1DM and T2DM patients.

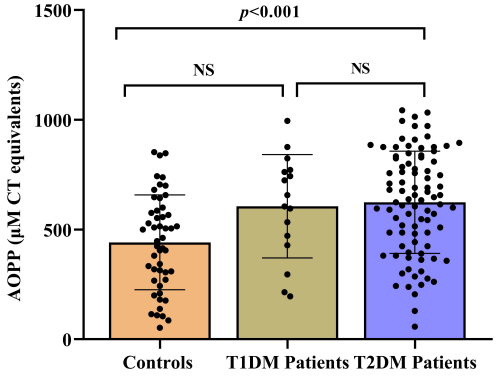

Figure 3. Comparisons of the advanced oxidation protein products (AOPP) levels in μM Chloramine T (CT) equivalents between the studied subjects. The T2DM patients had significantly higher AOPP levels than the controls.

patients in higher glucose condition.[5] Interestingly, the present study also found a significant positive correlation between FPG and MPO in diabetic patients. The enhanced activities of these two enzymes and their associations with FPG corroborate the involvement of hyperglycemia in the increased generation of ROS and higher OS in diabetes.

The present study demonstrated that both types of diabetic patients had significantly higher TOS levels than the controls, which was consistent with previous findings.[11,30] Elevated TOS levels provide pronounced evidence of oxidative damage in diabetic patients. This study further showed that the FRAP values were significantly lower in both types of diabetic patients, which confirmed existing studies reporting lower FRAP values in patients with DM.[31,32] Furthermore, there were significant negative correlations of FRAP with FPG and duration of diabetes. Similar findings were reported in other studies that validated the current observations.[32,33] These findings indicated that the exogenous pool of total antioxidants was depleted gradually by

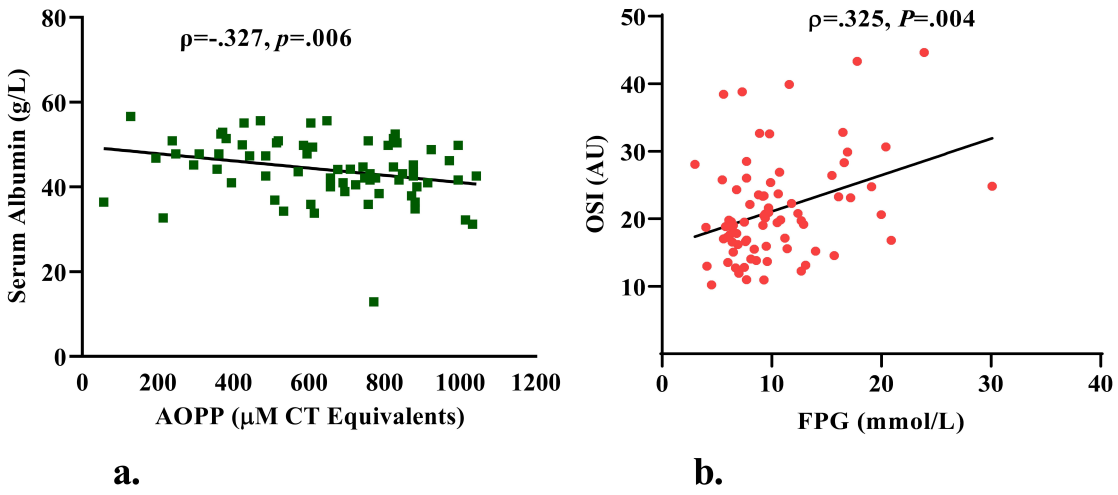

Figure 4. Spearman correlation analyses showed a significant negative correlation between advanced oxidation protein products (AOPP) and serum albumin **(a)**, and a significant positive correlation of oxidative stress index (OSI) with fasting plasma glucose (FPG) **(b)** in diabetic patients (T1DM + T2DM).

the increased oxidants, resulting from hyperglycemia, leading to disturbed redox balance in plasma and the progression of diabetes.

The current study showed significantly higher OSI values in both types of DM patients, which suggests a greater intensity of OS. Accordingly, OSI was positively associated with TOS, and FPG, and negatively with FRAP. The significant increase of OSI in diabetic patients and its association with FPG were in accordance with previous findings.[12] The association of OSI with NOX and FPG highlighted OSI as a promising marker to evaluate oxidant/antioxidant imbalance and further indicated linkage of hyperglycemia with OS. The current findings of significantly lower levels of serum albumin in both types of DM patients corroborated a similar observation in diabetic patients.[34] Previous studies reported an association of hypoalbuminemia with an increased risk of ketosis in patients with T2DM.[35]

The present study found significantly elevated PCO and AOPP, and significantly lower T-SH levels in T2DM patients. The T1DM patients demonstrated significantly higher PCO, but the AOPP levels were slightly elevated without any significant difference from controls, while the T-SH levels were significantly lower. Overall, these findings are in accordance with those reported by previous investigators.[31,36–38] Taken together, the present study explored the effects of hyperglycemia-induced ROS generating enzymes/oxidants and evaluated subsequent deleterious damage to proteins in both types of diabetic patients.

The most important findings of the present study include significant positive and negative correlations of albumin in diabetic patients with two OPD markers, T-SH and AOPP, respectively. The most compelling explanation of these correlations could be that extremely oxidative conditions modify albumin through irreversible oxidations of its cysteine-34 residue and hence lessen thiol levels; AOPP are known to be oxidized albumin products in aggregate or monomeric forms[16], therefore, oxidative modifications of albumin may lead to underestimation of total albumin concentrations by the conventional bromocresol green assay method, which may be the reason for inverse correlation with AOPP. The interpretation that protein oxidation might interfere with albumin measurement was supported by previous studies.[39]

The present study showed a noteworthy finding in a significant positive correlation between AOPP and NOX in control subjects. Growing evidence demonstrated that AOPP might be not only a marker of oxidant-mediated protein damage, but also a potential inducer of oxidative stress and inflammation by activating neutrophils, monocytes, and T lymphocytes.[40] A previous study reported that AOPP mediates the activation of NOX to induce ROS generation in human endothelial cells.[41] These studies indirectly supported the correlation of AOPP with NOX.

Finally, there were at least two potential limitations to this investigation. The first limitation concerned the small sample size, particularly since the number of T1DM patients was extremely low. Due to the lower prevalence of T1DM, stringent inclusion criteria and the need to perform the analysis on fresh samples, it was difficult to collect a large number of samples. The second limitation was that the analysis was performed on patients, the majority of whom were on some form of antidiabetic treatment, which might play a role in subduing some of their oxidative stress and represent a partial result. Despite these limitations, the significant findings of this study on oxidative protein damage in patients with T1DM and T2DM could

be used as a reference for future studies with larger sample size, considering the significance of the measured parameters, and also taking into account the use of anti-diabetic treatment.

CONCLUSION

The present study showed increased activities of oxidative enzymes NOX and MPO and decreased FRAP levels, reflecting disturbances between ROS and antioxidants in patients with diabetes mellitus. Increased levels of TOS and OPD markers indicate damage of cellular macromolecules in diabetic patients and draw attention to future intervention studies concerning the development of secondary diabetic complications. The correlations of parameters with FPG observed in this study showed hyperglycemia triggering both the excessive burden of oxidants and the declined FRAP levels in diabetic patients. This study sheds light on the concerted mechanism connecting the activity of oxidative enzymes and oxidant mediated damage of proteins. To the best of our knowledge, no previous study focused simultaneously on the activity of oxidative enzymes and OPD markers in both T1DM and T2DM patients. Finally, this study showed an interesting association of a novel OPD marker, AOPP, with NOX.

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