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ORIGINAL ARTICLE

Evaluation of antioxidants and antioxidant capacity among Bangladeshi patients with Type 2 diabetes mellitus

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ABSTRACT

Diabetes mellitus (DM) is one of the leading causes of mortality and reduced life expectancy both worldwide and in Bangladesh. This cross-sectional study investigated the prevalence of Type 2 DM (T2DM) among Bangladeshi adult, and evaluated the status of antioxidant defense biomarkers and total antioxidant capacity (TAC) against an apparently healthy non-diabetic control group.A total of 158 participants, aged 20-50 years, were enrolled of whom 100 were T2DM patients and 58 were non-diabetic controls. Certain antioxidant biomarkers and the ferric-reducing ability of plasma (FRAP) were assayed by the standard procedures. In this study, the age at the first diagnosis of T2DM was 20-30 years in 24% of the patients. The fasting plasma glucose (FPG) and family history of diabetes were significantly higher in patients. The antioxidants - reduced glutathione, ascorbic acid and total thiol were significantly lower in patients (7.36±2.89 μM, 0.49±0.21 mg/dL, and 323.7±132.7 μM, respectively) than controls (10.233.13 μM, 0.66±0.20 mg/dL and 472.7±61.5 μM, respectively). The FRAP, a measure of TAC, was found to be significantly lower in patients. The activities of paraoxonase-1 (410.9±47.08 U/L) and superoxide dismutase (3.12±2.40 U/mL) were significantly lower while glutathione peroxidase (97.93±27.38 U/mL) and catalase (65.97±33.39 U/mL) were significantly higher than in controls. A significant negative correlation was found between FPG and FRAP in patients. A high prevalence of T2DM was found among young people. The overall findings suggested lower antioxidants enabled high levels of oxidative stress leading to the development of T2DM in young people.

Keywords : Ascorbic acid, FRAP, Paraoxonase1, Reduced glutathione, T2DM

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INTRODUCTION

Diabetes mellitus (DM) is a metabolic disorder characterized by impaired action, secretion of insulin or both, resulting in hyperglycemia. The appropriate secretion of insulin from pancreatic beta cells is critically important for energy homeostasis. Pancreatic beta cells secrete insulin in response to rising blood glucose by increasing oxidative metabolism, leading to increased ATP production in mitochondria [1,2] and maintain a normal glucose level in the blood. But, inadequate production of insulin by pancreatic beta cells, or the cells of the body not responding properly to insulin[3], which are insufficient to effectively lower plasma glucose concentrations, leading to high levels of blood glucose (hyperglycemia).

Oxidative stress (OS) may contribute to the pathogenesis of DM through impairment of insulin action, injury to pancreatic beta cells, increased lipid peroxidation and vascular endothelial damage. In diabetes, persistent hyperglycemia increases the levels of free radicals, especially reactive oxygen species (ROS), due to glucose oxidation and nonenzymatic protein glycation. The abnormally elevated levels of ROS and the simultaneous decrease of the antioxidants generate OS in diabetic patients. The increase in OS in pancreatic beta cells can ultimately result in immune dysfunction in type 1 diabetes mellitus (T1DM) and induction of insulin resistance and impaired insulin secretion in type 2 diabetes mellitus (T2DM)[4].

It has been found that chronic hyperglycemia and subsequent overproduction of ROS impair pancreatic beta cell function and increase insulin resistance which leads to deterioration of T2DM[5]. In this situation, the circulatory antioxidants such as reduced glutathione (GSH), ascorbic acid, ferric-reducing ability of plasma (FRAP) may reduce drastically. The concentration of GSH as well as the [GSH]/[glutathione disulfide, GSSG] ratio are markers of OS and of cell redox homeostasis. GSH plays the role of an antioxidant as a scavenger of electrophilic and oxidant species either in a direct way or through enzymatic catalysis[6]. Glutathione peroxidase (GPx) is a selenium-containing antioxidant enzyme that effectively reduces hydrogen peroxide (H_2O_2) and lipid peroxides to water and lipid alcohols, respectively, and in turn oxidizes glutathione to GSSG[7].

Catalase also reduces H_2O_2 by converting it into molecular oxygen (O_2) and water. Superoxide dismutase (SOD) catalyzes the dismutation of superoxide anion free radi- cal ($O_2^{\bullet-}$) into O_2 and H_2O_2, which reduces the cellular damage through decreasing $O_2^{\bullet-}$levels. Ascorbic acid is a potent reducing agent and scavenger of free radicals in bio- logical systems[8]. It is involved in the first line of antioxidant defense, protecting membrane lipids, and proteins from oxidative damage. Paraoxonase-1 (PON-1) is an enzymatic antiox- idant that prevents or reduces cardiovascular complications in patients with T2DM through different mechanisms such as reducing plasma oxidized low-density lipoprotein (ox-LDL) levels, reducing macrophage ability to uptake ox-LDL, promoting macrophage cholesterol efflux, reducing foam cell formation, and inhibiting monocyte chemotactic protein 1[9].

Thiols, compounds containing sulfhydryl groups (-SH), are one of the members of the antioxidant system as they have been revealed to devastate the ROS and other free radicals

by enzymatic and nonenzymatic mechanisms[10]. The FRAP is a measure of the presence of total antioxidants in plasma[11]. The measurement of this simple and robust method is based on the ability of antioxidants in the sample to inhibit the oxidative effects of reactive species generated in plasma.

In recent years, Bangladesh has seen a rapid transition due to industrialization, climate change and urbanization towards sedentary lifestyle, and because of high intake of carbohydrate-rich diets, the prevalence of diabetes and OS may be on the rise among young people, which has not been adequately addressed. Therefore, the present study was designed to investigate the prevalence of T2DM in young adults and evaluate enzymatic and nonenzymatic antioxidants and FRAP compared to a healthy group.

MATERIALS AND METHODS

Study place and subjects

This study was conducted at the Immunology, Non-communicable Diseases and Environmental Toxicology Laboratory of the Department of Biochemistry and Molecular Biology, University of Dhaka, Bangladesh. The study design has been presented in Supplementary Figure 1. A total of 158 subjects were enrolled comprising 100 patients diagnosed with T2DM who attended the Bangladesh Institute of Research and Rehabilitation in Diabetes, Endocrine and Metabolic Disorders (BIRDEM) outpatient department in the BIRDEM General Hospital. A group of 58 subjects, who were employees of local offices, were enrolled as the control group.

Inclusion and exclusion criteria

The inclusion criteria of all enrolled subjects were age 20 to 50 years, and for the patient group to have T2DM while the control group not to have T2DM. This study excluded participants above 50 years of age in order to avoid older age-related complications. The diagnosis of T2DM was done by physicians by examining fasting plasma glucose (FPG), random plasma glucose (RPG), fasting serum C-peptide and glycosylated hemoglobin (HbA1c) levels. The exclusion criteria of the patient group were cardiovascular diseases (CVD), infections, impaired renal and liver functions and any other chronic inflammatory conditions, and for the control group was no known history of CVD or any other inflammatory diseases.

Sample collection

This study was conducted by following the Declaration of Helsinki. The study protocol was reviewed and approved by the institutional Ethical Review Committee, and the experiments were carried out from February 2021 to April 2022. Before data collection, each individual was informed about the objectives and significance of the study. Only the full

consenting volunteers were enrolled. About 10 mL of venous blood was drawn from each subject, 5 mL was collected in a lavender capped tube containing EDTA for plasma collection and the rest was taken in a glass tube for serum collection. The serum and plasma were separated, collected in small aliquots and stored at -20°C until analyzed.

Measurement of nonenzymatic antioxidants

The serum GSH level was measured using 5-5'-di-thiobis [2-nitrobenzoic acid] (DTNB) according to the method described by Ellman[12]. A spectrophotometric assay based on DTNB or Ellman's reagent was used to measure the level of total thiol in plasma[13]. DTNB reacts with a free sulfhydryl group to yield a mixed disulfide and 2-nitro-5-thiobenzoic acid (TNB). The TNB has an intense yellow color that can be measured spectrophotometrically at 412 nm. Serum ascorbic acid levels were measured using di-nitro phenyl hydrazine, according to the method of Lowry et al.[14].

Measurement of antioxidant capacity

The total antioxidant capacity (TAC) was measured by the FRAP assay[15], as detailed in a recent study[16]. Briefly, the ferric ion (Fe^{3+}) when comes in contact with an antioxidant at an acidic pH, is reduced to a ferrous ion (Fe^{2+}) which can form a blue colored complex with 2,4,6-tripyridyl-S-triazine. The FRAP values were obtained by comparing the absorbance change at 593 nm.

Assay of enzymaticantioxidants

The paraoxonase-1 (PON-1) activity was determined by the spectrophotometric method using p-nitrophenyl acetate [2]. The plasma GPx activity was measured according to the method described by Flohé and Günzler [17]. The catalase activity was assayed using the method described by Goth[18] with a slight modification. A sample of 50 μL serum was incubated with 0.25 mL of 65 μmol H_2O_2 in 1 mL of 60 mM phosphate buffer at 37°C. SOD activity was assayed by the method of Marklund and Marklund[19], as detailed in a recent publication[20]. Briefly, 50 μL of serum was added to 2.85 mL of Tris-EDTA (Sigma-Aldrich) and 100 μL of pyrogallol (Merck).

Statistical analysis

Statistical analyses were carried out using GraphPad PRISM (version 8.0.1 for Windows, GraphPad Software, California, USA). The normality assessment of continuous variables was made by descriptive statistical analyses and presented as mean and standard deviation. For comparison between T2DM patients and control subjects, the independent samples t-test was used for continuous variables, and the chi-square test for nominal (categorical) variables (shown in Table 1). The relationship between variables in a group was analyzed by

the Pearson correlation test. Also, data were analyzed to determine the median (interquartile range) for the parameters (shown in Figure 4). The results were considered significant when the value of p was <0.05. Graphical presentations were done using both GraphPad PRISM and Microsoft Excel (version 2019 for Windows, Microsoft Corporation, USA).

Table 1: Demographic and baseline characteristics of control subjects and T2DM patients

Variables	Control Subjects (n=58)	T2DM Patients (n=100)	p-value
Gender (M/F), n	49/9	90/10	0.304*
Age (years)	38.54 ± 5.96	39.49 ± 4.77	0.298**
BMI (kg/m^2)	25.55 ± 3.67	24.57 ± 3.10	0.118**
FPG (mmol/L)	5.25 ± 0.33	9.76 ± 4.10	<0.0001**
SBP (mmHg)	128.70 ± 11.99	131.23 ± 15.16	0.309**
DBP (mmHg)	88.16 ± 10.45	92.54 ± 10.96	0.021**
Family history of DM, n (%)	17 (29)	69 (69)	<0.0001*
Nonsmoker/Current smoker /Ex-smoker (%)	79/19/2	72/25/3	0.581*

*: chi-square test; **: *t*-test.
BMI: Body mass index; DBP: Diastolic blood pressure; F: Female; FPG: Fasting plasma glucose; M: Male; n: number; SBP: Systolic blood pressure.

RESULTS

Demographic and baseline characteristics

The demographic and baseline characteristics including gender, age, body mass index (BMI), fasting plasma glucose, systolic blood pressure, diastolic blood pressure, smoking status, and family history of diabetes of the studied subjects have been recorded in questionnaire forms and the values have been compared in Table 1. There was a family history of diabetes in 69% of the patients. Their mean ± SD duration of DM was 5.26 ± 3.96 years, and the lowest age for the first diagnosis of DM was 20 years. The age at the first diagnosis of T2DM was 20-30 years in 24%, >30 to 40 years in 63%, and >40 to 50 years in 13% of the patients. The mean HbA1c level of the patients was 8.16 ± 2.26% which varied from 5.5 to 12.9%. Complications of diabetes were also recorded which showed 65% of the patients had no complications, 18% had retinopathy, 9% had neuropathy and 8% had both retinopathy and neuropathy. Of the patients, 49% were taking oral hypoglycemic drugs (OHD), while 51% were on OHD and insulin shots.

Evaluation of nonenzymatic antioxidants

The mean ± SD serum GSH concentration in T2DM patients was 7.36 ± 2.89 μM and that in the control subjects was 10.23 ± 3.13 μM. Statistical analysis showed the serum GSH concentration of the patients was significantly lower compared to the controls (Figure 1). The mean ± SD plasma protein total thiol level in the control subjects was 472.7 ± 61.5 μM and the corresponding value in T2DM patients was 323.7 ± 132.7 μM, which was significantly lower (Figure 2).

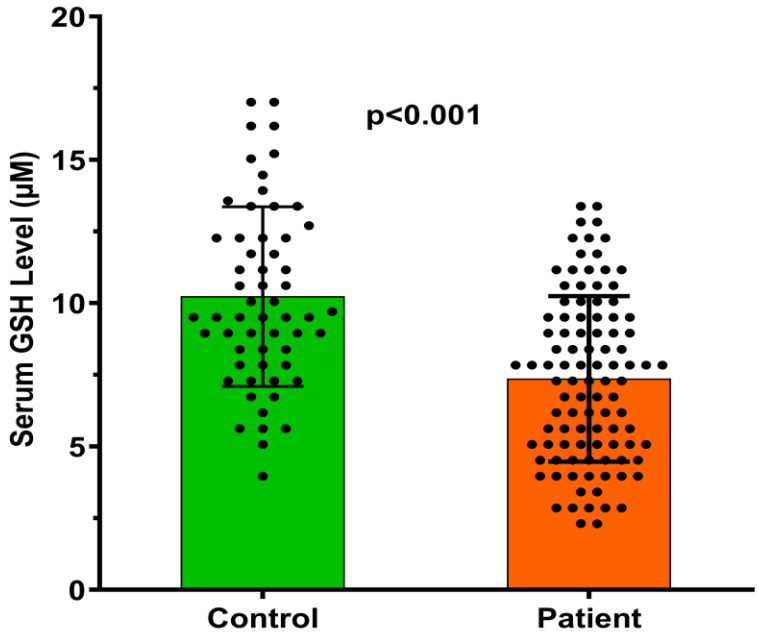

Figure 1 Serum reduced glutathione (GSH) levels in T2DM patients and control subjects

The mean serum ascorbic acid concentration in the control subjects was found to be 0.66 ± 0.20 mg/dL with values ranging from 0.36 to 1.26 mg/dL, and that of the patient group was 0.49 ± 0.21 mg/dL which varied from 0.09 mg/dL to 1.01 mg/dL (Figure 3), implying that the patients had significantly lower serum ascorbic acid concentration ($p<0.001$).

Similarly, the mean FRAP value in the control subjects was 1221.1 ± 305 μmol/L and that in the T2DM patients was 789.6 ± 142.1 μmol/L, which was significantly lower ($p<0.0001$). Further analysis of the data in different age groups showed the mean ± SD values of FRAP in T2DM patients of 20-30 years was 738.2 ± 196.4, >30 to 40 years was 822.2 ± 122.0, and >40 to 50 years was 754.5 ± 152.1 μmol/L, which were not significantly different (Tukey's multiple comparison test).

Assessment of enzymatic antioxidants

The mean serum PON-1 activity in the control subjects was 501.90 ± 39.92 U/L and the value in T2DM patients was 410.9 ± 47.08 U/L, which was significantly lower (Figure 4). The mean plasma GPx activity of the control subjects was 36.65 ± 14.45 U/mL and that of

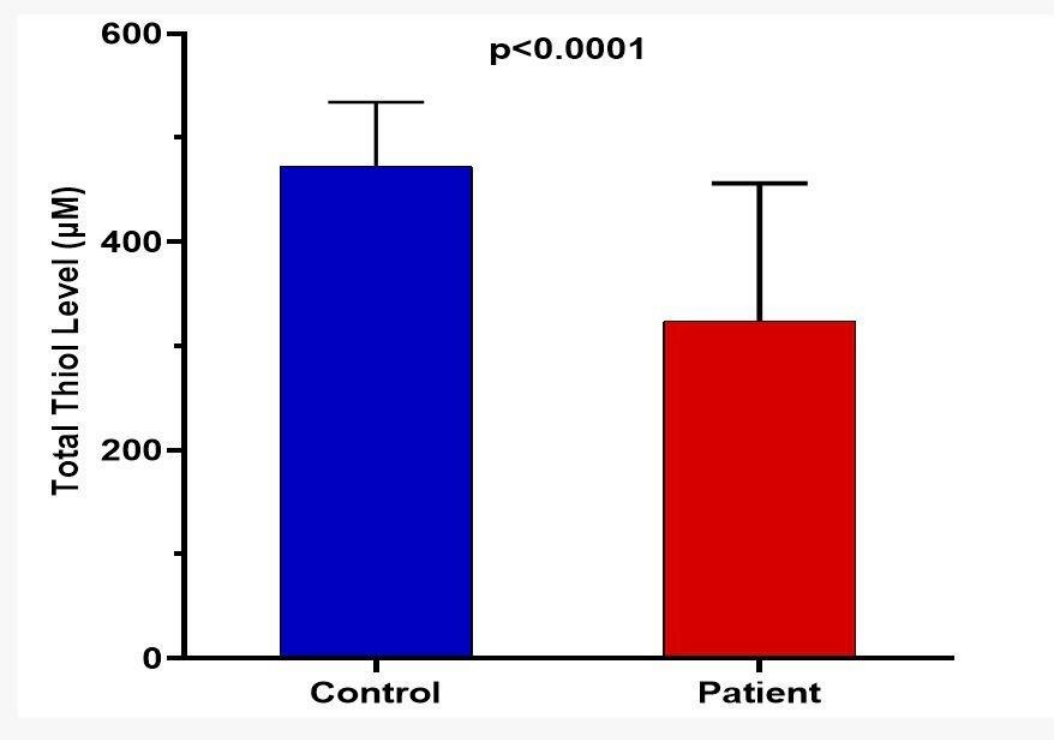

Figure 2 Comparison of the mean total plasma protein thiol levels studiedsubjects.

the patients was 97.93 ± 27.38 U/mL which was significantly higher ($p<0.0001$).

The mean serum catalase activity of the control group was 55.02 ± 21.97 U/mL and the corresponding value in the patient group was 65.97 ± 33.39 U/mL which was significantly higher ($p<0.05$). The mean serum SOD activity of the control subjects was 4.09 ± 1.97 U/mL and that in the patients was 3.12 ± 2.40 U/mL which was significantly lower ($p<0.05$).

Correlation of diabetic markers and TAC

In T2DM patients, a highly significant negative correlation was found between the fasting plasma glucose (FPG) level and total antioxidant capacity (TAC), measured by the FRAP assay (Figure 3). Also, there was a significant negative correlation between the duration of DM (years) and FRAP value ($r = -0.244$, $p=0.0034$).

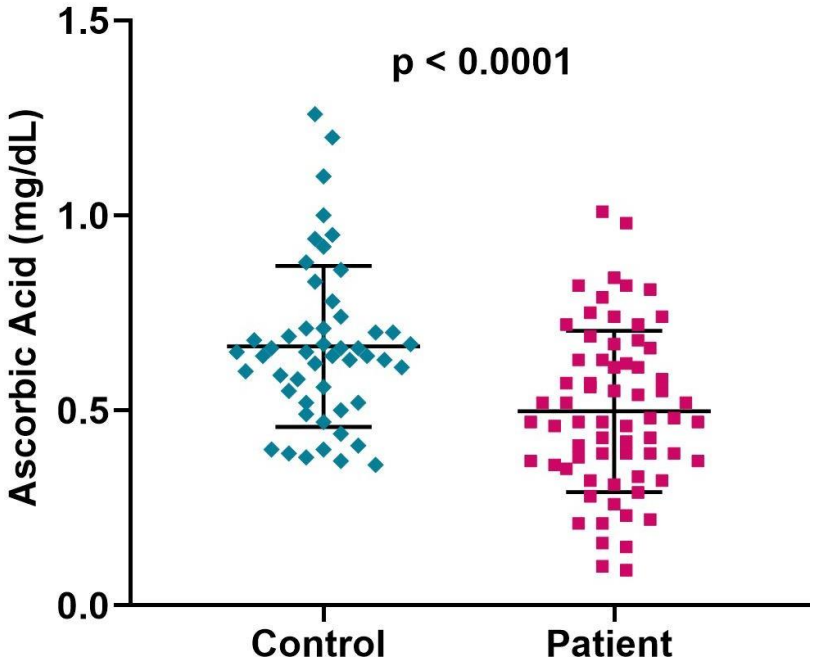

Figure 3 Comparison of serum ascorbic acid concentration between the control subjects and T2DM patients. Ascorbic acid concentration wassig- nificantly lower in the T2DM patients than in the control subjects ($p<0.0001$).

DISCUSSION

The purpose of the present study was to determine various enzymatic and non-enzymatic antioxidants in young T2DM patients and compare these parameters with a control group of similar age to determine the effect of excess ROS production in T2DM. Although some of these parameters have been investigated by previous research groups, this study evaluated a wide range of antioxidant biomarkers in T2DM patients and determined their correlations as possible pathogenic factors towards diabetic complications. In this study, the patients were not age-matched with the control subjects; however, the mean age of the control and patient groups did not vary significantly. The systolic blood pressure between the studied groups did not vary significantly but the diastolic blood pressure was significantly higher in patients. The HbA1c and FPG levels were higher in T2DM patients suggesting that they had poor glycemic status, and thus higher OS.

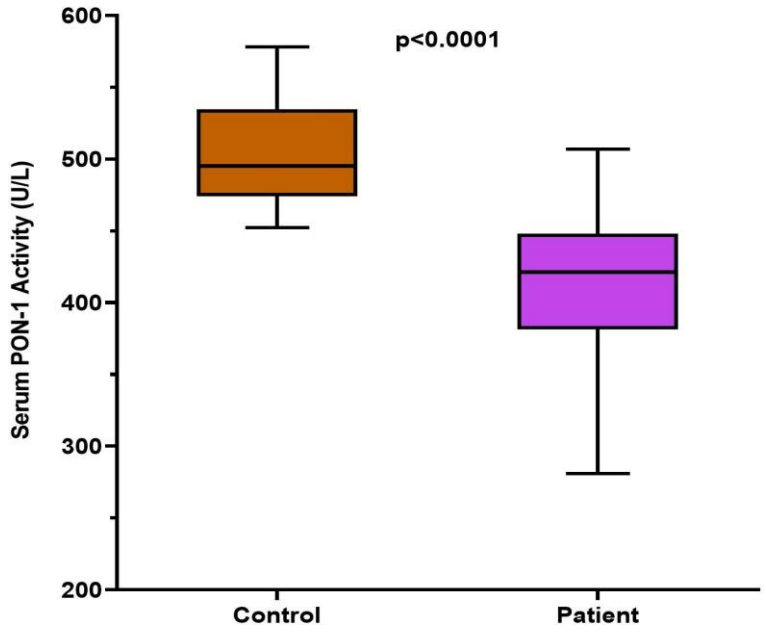

Figure 4 Box plot diagram comparing serum PON-1 activity between the T2DM patients and control subjects.The lower and upper sides of the box represent the lower and upper quartiles,the horizontal line in the box indicates the median value, and the whiskers belowand above the boxes represent the minimum and maximum values. The patients had significantly lower serum PON-1 activity than the control subjects($p<0.0001$).

This study enrolled participants aged between 20 and 50 years, to investigate the effect of T2DM in young people and to exclude older age-related complications. It was found for the first time that 24% of the patients were aged 20-30 years when they were first diagnosed with T2DM. The mean BMI of these patients was 23.78 that ranged from 17.7 to 33.6. The mean age at the first diagnosis of T2DM in the whole patient group was 33.8 ± 5.3 years. However, one study in Bangladesh reported higher prevalence of T2DM among adults aged ≥35 years which was linked to age and obesity[21]. Our study supports these findings partially that 63% of the patients were >30 to 40 years of age when they were first diagnosed to have T2DM, although they were not obese.

In the present study, the BMI of the patient group was not significantly different than the controls which corroborated a recent study on T2DM patients[22]. This finding suggests that in T2DM, the body cannot use insulin effectively and cannot transport glucose to the cells properly which builds up in the blood circulation while the body produces energy by burning fat and muscle at a rapid pace, leading to weight loss and eventually BMI is also reduced. However, one study suggested that Asians with lower BMI are at increased risk of developing T2DM which contributes significant burden of morbidity and mortality in developing countries like Bangladesh[23].

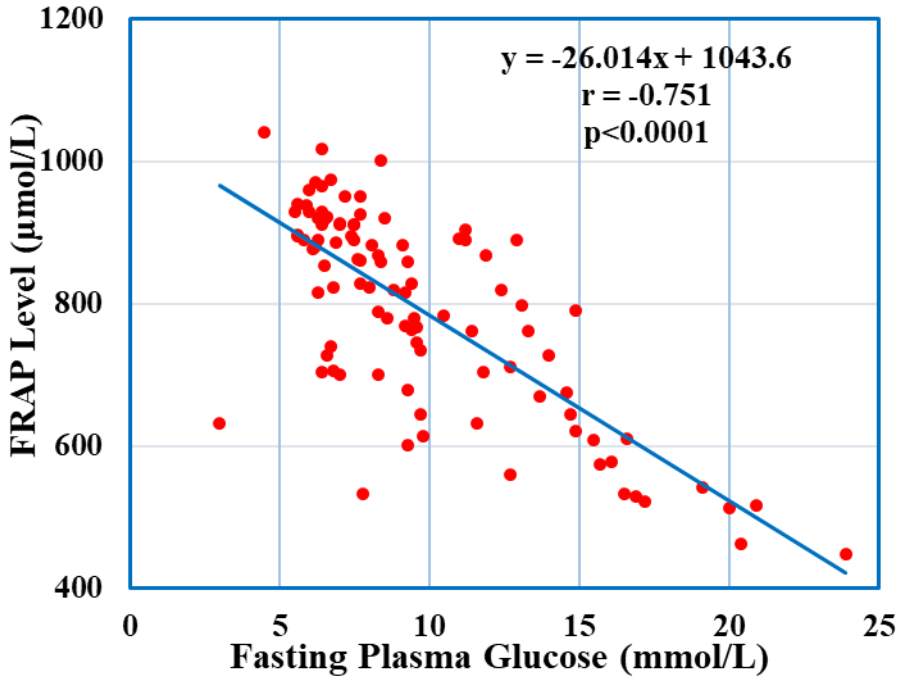

Figure 5 Scatter plot showing a significant negative correlation between FPG level and FRAP value in the T2DM patients, where r = -0.751 ($p<0.0001$).

The selection criterion of this study excluded those suffering from CVD, infections, impaired kidney and liver functions to avoid the effects of additional OS on the findings. The mean duration of DM of the patients was 5.26 ± 3.96 years which explains why 49% of the patients were treated with oral hypoglycemic drugs only as they had no severe diabetic complications. The remaining 51% were treated with both oral hypoglycemic drugs and insulin. In general, oral hypoglycemic drugs are suggested during the initial stage of T2DM and depending on the duration of DM with a poor glycemic control status and beta cell dysfunction, insulin therapy is added with oral hypoglycemic drugs. In this study, the family history of diabetes was found to be significantly higher in the patient group which was consistent with a previous finding [24]. Although smoking increases OS and aggravates the micro- and macro-vascular complications[25], this study did not find a significant association of tobacco smoking and T2DM.

In this study, the serum GSH concentrations in T2DM patients were significantly lower which was consistent with a similar study[26]. This finding suggested that OS could consume some naturally occurring local antioxidants such as GSH which might manifest decreased levels in the serum of patients[27]. Also, the levels of total thiols were significantly lower in

the patient group which was consistent with a previous study[28]. The reduction in the level of total thiols indicates free radical mediated oxidation of protein thiol groups, as essentially all of them are associated with proteins.

Ascorbic acid, another potent antioxidant, plays a protective role in detoxifying oxygen radicals and therefore, prevents cellular damage from OS. In this study, the serum ascorbic acid (vitamin C) concentrations in T2DM patients were significantly lower and the finding was consistent with a similar study[29]. A possible explanation for the low vitamin C levels in diabetics could be linked to increased ascorbic acid oxidation or impaired regeneration from its oxidized state[30].

In agreement with a previous report[31], the present study found the FRAP value to be significantly lower in T2DM patients compared to the control subjects, which suggested that the depletion of exogenous pools of total antioxidants in the face of heightened OS could be due to elevated levels of blood glucose thereafter leading to disturbed redox balance and the progression of T2DM. Hence, this progression may be associated with the gradual loss of total antioxidant capacity.

In this study, the activity of the antioxidant enzyme PON-1 was significantly lower in T2DM patients which was in accordance with another study[2], suggesting that elevation of blood glucose caused glycosylation of the enzymes with their structural and functional properties altered resulting in decreased PON-1 activity. The present study found significantly higher GPx activity in T2DM patients which corroborated a previous study suggesting an adaptive response against excess lipid peroxidation and accumulation of hydrogen peroxide in a hyperglycemic state[32]. The catalase activity in T2DM patients was also significantly higher than the controls. A similar result was found in another study where the antioxidant capacity of catalase was found to be higher[33], suggesting a compensatory response to the increased oxidative stress in T2DM patients.

This study found the SOD activity to be significantly lower in patients than in the control subjects, which supported the observation by Haddad et al.[34]. The decline in SOD activity in the patients might be due to hyperglycemia which activated various biochemical pathways such as glucose autooxidation, non-enzymatic glycosylation of proteins and activation of protein kinase C. These pathways in turn overproduce oxidants like superoxide and hydroxyl radicals as well as H_2O_2, or the increase of glycosylated SOD that leads to inactivation of enzyme or loss of its two cofactors Zn^{2+} and Cu^{2+}[35].

The present study showed a significant negative correlation between the fasting plasma glucose (FPG) level and FRAP value in T2DM patients. A similar study was found in the literature[31], which validated the current observation. A previous study suggested that high blood glucose levels could initiate the production of ROS, which were able to stimulate the decline of total antioxidants[36]. Interestingly, a similar trend of negative correlation was found between the FPG and FRAP values in the control subjects of this study, which was not significant. Further, a significant negative correlation was found between the duration

of diabetes mellitus (DM) and FRAP value in T2DM patients, which was consistent with a previous study[37]. Our finding suggested that the increase in the duration of DM with elevated blood glucose resulted in the generation of ROS and the reduction of total antioxidants, which may eventually lead to diabetic complications.

Finally, it should be mentioned that the major limitation of this study was a relatively small sample size which was probably inadequate to illustrate the full picture of a population. One of the reasons was having to collect blood samples from the outdoor patients attending the hospital for routine checkups following the safety measures of post COVID-19 pandemic. The other limitation being stringent inclusion criteria of the study participants, excluding CVD, infections, impaired liver and or kidney functions. Despite these limitations, this study revealed that 24% of the patients were 20-30 years of age at the time of first diagnosis of T2DM, and the current data showed patients aged 20-30 years had similar total antioxidant capacity as older adults. These findings can be used as references for future studies with larger sample size considering the significance of the present investigation for better management of T2DM in young people.

Conclusions

A high prevalence of T2DM was found among young people in Bangladeshi population. The family history of diabetes was recognized as the most important risk factor for the development of T2DM. This study sheds light on the interaction between ROS production by hyperglycemia induced oxidative stress and their neutralization by the antioxidants in T2DM. In patients, significantly lower SOD and Paraoxonase-1 activities, as well as GSH, ascorbic acid, total thiol and FRAP values indicated poor antioxidant condition in the circulation and a definite sign of OS while significantly higher catalase and glutathione peroxidase activities indicated the impelled mechanism to overtake OS by antioxidant power. Finally, a negative correlation between FPG level and FRAP value in the patients strongly represented the gradual progression of hyperglycemia linked with the loss of redox balance, leading to excessive ROS production in the circulation. These results suggested the development of greater oxidative burden even in younger adult T2DM patients.

Abbreviations:

ATP: adenosine triphosphate; BIRDEM: Bangladesh Institute of Research and Rehabilitation in Diabetes, Endocrine and Metabolic Disorders; BMI: body mass index; CVD: cardiovascular diseases; DM: diabetes mellitus; EDTA: ethylenediamine tetraacetic acid; FPG: fasting plasma glucose; FRAP: ferric reducing ability of plasma; GPx: glutathione peroxidase; GSH: reduced glutathione; HbA1c: glycosylated hemoglobin; OS: oxidative stress; PON-1: paraoxonase-1; ROS: reactive oxygen species; SOD: superoxide dismutase; TAC: total antioxidant capacity; T2DM: Type 2 diabetes mellitus.

DECLARATIONS

Authors' Contributions

Concept, design and supervision - LNI; Methodology, investigation and data collection, processing, analysis and interpretation - RS, TJ, ST, NIK; Literature search- RS, TJ, ST; Preparation of first draft - RS; Critical review – LNI, RS, TJ, ST, NIK.

Conflict of Interest

The authors declare that there is no conflict of interest.

Data availability

The data that support the findings of this study is available from the corresponding author, upon reasonable request.

Financial Disclosure

The authors did not receive any external funding for this study.

Ethics Committee Approval

The study was approved by the Ethical Review Committee of the Faculty of Biological Sciences, University of Dhaka (Ref. No. 108 /Biol. Sci. /2020-2021, Date: February 4, 2021). Each individual was informed about the objectives and significance of the study. Only the full consenting volunteers were enrolled.

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