VIA MEDICA
RESEARCH PAPER
ISSN 2450–7458
e-ISSN 2450–8187

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Oxidative Stress and Inflammatory Leukocyte Markers in People with Type 2 Diabetes: A Single Center, Cross-Sectional Study

ABSTRACT

Objective: The aim of the study was to investigate whether oxidative stress resulting from enhanced free-radical formation, inflammation and higher total oxidation status plays a role in the initiation, progression and pathology of type 2 diabetes (T2D).

Materials and methods: A total of 207 participants were enrolled, which comprised of 157 T2D patients (experimental group), and 50 people without diabetes (control group). Any subject suffering from cardiovascular diseases, impaired renal or liver functions has been excluded. The activities of certain oxidase enzymes, total oxidative stress and inflammatory leukocyte markers were investigated and compared between the studied groups.

Results: Of the patients, 29.3% had HbA1c level < 7% and 28.0% had FPG level ≤ 7 mmol/L. The NADPH oxidase and myeloperoxidase, both of which are major contributors to reactive oxygen species (ROS) production, showed significantly higher activities in patients than in controls ($p < 0.0001$ and $p < 0.001$ respectively). Malondialdehyde (MDA), one of the end products of lipid peroxidation, was significantly higher in the patients ($p < 0.0001$). The total oxidative stress that estimates the overall oxidation status, was also significantly higher in the patients. The inflammatory leukocyte markers including total white blood cell count, percentage of neutrophils, and the neutrophil to lymphocyte ratio (NLR) were significantly higher in the patients ($p < 0.0001$). Further, the NADPH oxidase activity showed significant positive correlations with MDA, and NLR in T2D patients.

Conclusions: These findings suggest that excessive productions of ROS by the oxidase enzymes, NADPH oxidase and myeloperoxidase, cause increased oxidative stress which in turn led to cellular inflammatory responses in patients with T2D. (Clin Diabetol 2023; 12; 3: 156–163)

Keywords: type 2 diabetes, oxidative stress, NADPH oxidase, myeloperoxidase, inflammatory leukocyte markers

Introduction

Diabetes is one of the largest global health emergencies of the 21st century and the seventh leading cause of death worldwide, which has been about 1.5 million in 2019 [1]. Diabetes is a metabolic disorder of multiple etiology characterized by impaired action, secretion of insulin or both, resulting in hyperglycemia [2]. In diabetes, persistent hyperglycemia increases the levels of free radicals, especially ROS, due to glucose oxidation and non-enzymatic protein glycation [3]. The abnormally elevated levels of ROS and the simultaneous decrease of the antioxidants generate oxidative stress in patients with diabetes. The increase in oxidative

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Clinical Diabetology 2023, 12; 3: 156–163
DOI: 10.5603/DK.a2023.0017
Received: 30.01.2023 Accepted: 18.04.2023
Early publication date: 9.06.2023

stress leads to cellular injury, such as damage to DNA, proteins, and lipid membranes [3].

The nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (NOX) is a membrane-associated enzyme complex that uses NADPH as the electron donor to catalyze the one-electron reduction of oxygen [4]. NOX is viewed as the principal source of glucose--induced ROS formation in diabetes [5]. Myeloperoxidase (MPO) is a member of subfamily of peroxidases, which catalyzes the reaction between hydrogen peroxide and chloride ions to produce hypochlorous acid which is the most powerful bactericidal oxidant produced by neutrophils [6]. In a study, the plasma MPO activity was found significantly enhanced in T2D patients with and without symptoms of cardiovascular disease [7]. This observation indicated that elevated MPO activity in the blood could be an additional marker for oxidative stress and cardiovascular risk in T2D patients.

Manifestation of oxidative activities of the free radicals can be obtained by measuring their oxidative yields in biological systems [8]. Therefore, lipid peroxides are the most useful biomarkers of oxidative stress. Malondialdehyde (MDA) is one of the end products of lipid peroxidation and eventually increases in the plasma under oxidative condition [9]. The total oxidative stress (TOS) is usually used to estimate the overall oxidation state of the body. Consequently, higher levels of ROS cause peroxidation of lipids, amino acids, peptides, and proteins with the resultant production of hydroperoxides [10]. Previous studies reported increased oxidative stress in both T1D [11] and T2D patients [12] by measuring the levels of hydroperoxides. It has been shown that elevated white blood cell (WBC) count, even within the normal range, is associated with both macro- and microvascular complications in T2D [13]. The neutrophil to lymphocyte ratio (NLR) also reflect the presence of oxidative stress [14].

Therefore, the objectives of this study were to assess certain oxidative stress markers such as NOX and MPO, oxidative stress indicators MDA and TOS, and cellular inflammatory biomarkers in patients with diabetes compared to a control group. It was hoped that the findings would improve understanding of the complex pathophysiology of oxidative stress and inflammation in T2D.

Materials and methods

Study design

This study was conducted at the Immunology, Noncommunicable Diseases and Environmental Toxicology Laboratory of the Department of Biochemistry and Molecular Biology, University of Dhaka, Bangladesh. The study was approved by the Ethical Review Committee of the Faculty of Biological Sciences, University of Dhaka, Bangladesh. Each individual was informed about the objectives and significance of the study. Only the full consenting volunteers were enrolled. The study was conducted from January 2021 to April 2022.

Study population

A total of 207 participants were enrolled in this cross-sectional study which comprised 157 patients, aged 20 to 50 years and diagnosed with T2D (inclusion criteria) who attended at the Bangladesh Institute of Research and Rehabilitation in Diabetes, Endocrine and Metabolic Disorders (BIRDEM) outpatient department in the BIRDEM General Hospital. Exclusion criteria included those suffering from cardiovascular diseases (CVD), peripheral vascular disease, impaired renal and liver functions, and other chronic inflammatory conditions. A total of 50 subjects, from the employees of different offices of the local community, having the similar socioeconomic background as the patients, aged 20 to 50 years and not having diabetes (inclusion criteria) were enrolled as the control group. Exclusion criteria included those suffering from CVD, peripheral vascular disease or any other diseases known to develop oxidative stress.

The patients (experimental group) were previously diagnosed with diabetes by examining fasting plasma glucose, random plasma glucose, oral glucose tolerance test and HbA1c level. Expert physicians ascertained T2D by measuring fasting serum C-peptide level, autoantibody test for insulin and glutamic acid decarboxylase and observing clinical manifestations.

Sample collection

About 10 mL of 12-hour overnight fasting peripheral venous blood was collected from each participant, 5 mL taken in a lavender capped tube containing ethylenediaminetetraacetic acid (EDTA) for plasma collection, and the rest in a glass tube for serum collection. Serum and plasma were separated and stored in small aliquots at –20°C until analyzed.

Measurements performed

The NADPH oxidase (NOX) activity was measured according to the method established by Reusch and Burger [15], as detailed previously [16]. Briefly, a cocktail (450 μL) was prepared with phosphate buffer, nano-pure water and EDTA (4:4:1). Then 25 μL of serum and 25 μL of NADPH were added. Immediately, the absorbance was taken at 340 nm for 5 minutes.

Serum myeloperoxidase (MPO) activity was determined by the method of Bradley et al. [17]. The detailed procedure has been described elsewhere [18]. A mixture of 2.9 mL of O-dianisidine solution and 100 μL of H_2O_2

was added to 100 μL of test serum to start the reaction, and the absorbance was taken at 460 nm.

Malondialdehyde (MDA) is one of the final products of lipid peroxidation which can be measured as thiobarbituric acid reactive substances. MDA reacts with thiobarbituric acid (TBA) to form a pink colored complex that was assayed according to the method of Yagi [19], as described previously [20]. Briefly, freshly prepared TBA reagent (2 mL) was added to 1.0 mL of sample (100 μL plasma + 900 μL saline) and 30 μL of 50 mM butylated hydroxy toluene was added. The mixture was incubated for 15 minutes in a boiling water bath and then centrifuged after cooling. The supernatant was collected to measure the absorbance at 535 nm and the amount of MDA present in the plasma samples was determined using a standard graph consisting of 0–16 μmol/L of standard MDA and the value was expressed as MDA equivalent in nmol/mL. The method could accurately detect 0.5 to 15 nmol/mL of MDA.

A colorimetric free oxygen radical test was used to assess the level of total oxidative stress (TOS), by the method of Saha et al. [21]. Briefly, a volume of 50 μL plasma was diluted 2 times by phosphate buffer and mixed with 900 μL of acetate buffer. Then 25 μL of the freshly prepared chromogen (N,N-dimethyl-p-phenylenediamine sulphate, Sigma-Aldrich) solution was added and the absorbance was recorded at 505 nm for 3 minutes. The minimum detection limit of the assay is 1.22 mmol of H_2O_2/L.

The total and differential white blood cells (WBCs) were counted from fresh blood samples using the standard procedures, also detailed previously [18]. The neutrophil to lymphocyte ratio (NLR) was calculated from the differential WBC count.

Statistical methods used for data analysis

Statistical analyses were carried out using the GraphPad PRISM (version 8.0.1 for Windows, GraphPad Software, California, USA). The statistical methods used were independent samples t-test for comparison of the continuous variables, and chi-square test for categorical variables between the T2D patients and control subjects, and also the correlation analyses and descriptive statistical analyses. The mean ± SD values were calculated for each parameter. The results were considered significant when the value of p was < 0.05. Graphical presentations were done using the GraphPad PRISM and Microsoft Excel (version 2019 for Windows, Microsoft Corporation, USA).

Results

The baseline health characteristics including gender, age, body mass index, fasting plasma glucose (FPG), systolic blood pressure (SBP), diastolic blood pressure (DBP), smoking status, family history of diabetes, previous history of hypertension, glycosylated hemoglobin, diabetes duration, age at first diagnosis of T2D, diabetes complications, and type of medications of the studied subjects have been recorded in questionnaire forms and the values have been compared (Tab. 1).

The mean serum NOX activity in T2D patients was 11.3 ± 3.2 U/L, and the activity ranged from 5.1 to 19.3 U/L, which was significantly higher than 6.3 ± ± 1.8 U/L (activity range: 2.6–10.9 U/L) found in the control subjects (Fig. 1A).

The mean MPO activity of the controls was 45.8 ± 12.7 U/L, and the activity ranged from 27.4 to 79.6 U/L, while the corresponding value of the patients was 64.5 ± 11.3 U/L, with the activity ranging from 34.3 to 89.2 U/L, which was significantly higher ($p < 0.001$).

The total oxidative stress in plasma of the control subjects had a mean value equivalent to 11.5 ± ± 2.4 mmol of H_2O_2/L (range: 6.9–18.5 mmol of H_2O_2/L) and that of the patients was 14.8 ± 3.4 mmol of H_2O_2/L (range: 6.9–25.2 mmol/L), which was significantly higher ($p < 0.0001$).

The mean MDA concentration in T2D patients was 6.2 ± 1.0 nmol/mL (values ranging from 4.0 to 9.7 nmol/mL) and that in the control subjects was 3.4 ± 0.7 nmol/mL (values extending from 2.6 to 5.5 nmol/mL), implying that the patients had significantly higher plasma MDA concentrations ($p < 0.0001$) than the controls (Fig. 1B).

The mean total WBC count in the control subjects was 8.0 ± 1.6 × 10^6 cells/mL while the corresponding value in T2D patients was 10.9 ± 3.5 × 10^6 cells/mL, which was significantly higher ($p < 0.0001$) (Fig. 2). Evaluation of the differential counts of leukocytes between the studied groups showed significantly higher neutrophils, eosinophils, and monocytes but significantly lower lymphocytes in the peripheral blood of T2D patients compared to the control subjects. These results have been presented in Table 1.

The mean neutrophil to lymphocyte ratio (NLR) in the control group was 1.5 ± 0.2 and that in the patient group was 2.3 ± 0.7, implying that the NLR in the patients was significantly higher ($p < 0.0001$) than the control group (Fig. 3A).

In T2D patients, serum NOX activities showed significant positive correlations with NLR (Fig. 3B), and MDA, with the Spearman correlation coefficients rho of 0.27 and 0.24, respectively. The fasting plasma glucose, a diagnostic parameter of diabetes, showed positive correlations with NOX ($\rho = 0.16$) and MPO ($\rho = 0.26$).

Table 1. Comparison of the Demographic and Baseline Characteristics, Type of Medications, and Differential WBC Counts of the Studied Groups

Variables	Control subjects (n = 50)	T2D patients (n = 157)	P-value
Gender (M/F)	41/9	133/24	0.6
Age [years]	38.6 ± 5.9	39.3 ± 4.1	0.4
BMI [kg/m^2]	25.5 ± 3.7	24.6 ± 2.6	0.1
FPG [mmol/L]	5.2 ± 0.3	9.8 ± 3.9	< 0.0001
SBP [mmHg]	128.4 ± 12.1	129.8 ± 16.9	0.5
DBP [mmHg]	88.1 ± 10.4	90.4 ± 9.7	0.1
Family history of diabetes [%]	34	70.1	< 0.0001
Nonsmoker/current smoker/ex-smoker [%]	80/18/2	70.7/21.0/8.3	0.1
Previous history of hypertension [%]	40.0	47.8	0.2
Glycosylated hemoglobin, HbA1c [%]	ND	8.5 ± 2.0	NA
Diabetes duration [years]	NA	5.2 ± 3.8	NA
Age at first diagnosis of T2D [years]	NA	34.5 ± 4.9	NA
Diabetes complications in T2D patients [%]	NA	None (67), retinopathy (15.3), neuropathy (10), retinopathy and neuropathy (7.7)	NA
Type of medications [%]	NA	OHD (metformin, sitagliptin, linagliptin)/insulin and OHD: 47.1/52.9	NA
Differential WBC counts [%], n = 50 for control subjects; n = 135 for T2D patients			
Neutrophil [%]	56.9 ± 3.9	63.3 ± 5.6	< 0.0001
Lymphocyte [%]	37.4 ± 3.3	29.4 ± 5.5	< 0.0001
Monocyte [%]	3.7 ± 1.1	4.5 ± 1.4	< 0.0001
Eosinophil [%]	1.2 ± 0.6	1.9 ± 0.8	< 0.0001
Basophil [%]	0.9 ± 0.5	0.9 ± 0.5	0.9

BMI — body mass index; DBP — diastolic blood pressure; F — female; FPG — fasting plasma glucose; HbA1c — glycosylated hemoglobin M — male; NA — not applicable; ND — not done; OHD — oral hypoglycemic drug; SBP — systolic blood pressure; T2D — type 2 diabetes; WBC — white blood cell

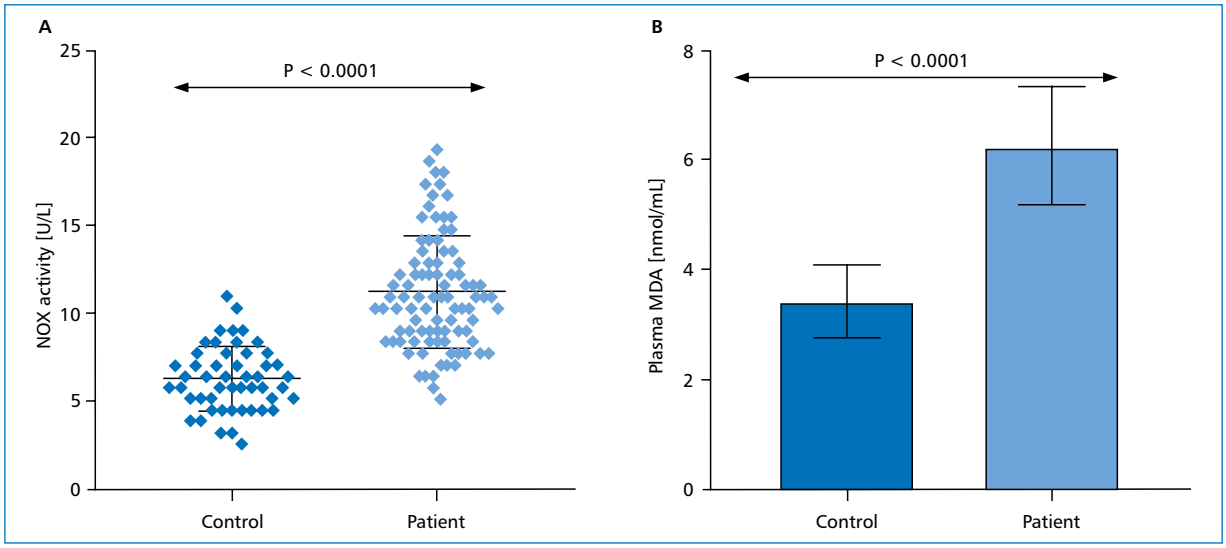

Figure 1. Comparison of **A**. Serum NOX Activity between the T2D Patients and Control Subjects and **B**. Plasma MDA Concentrations between the T2D Patients and Control Subjects
NOX — nicotinamide adenine dinucleotide phosphate oxidase; T2D — type 2 diabetes

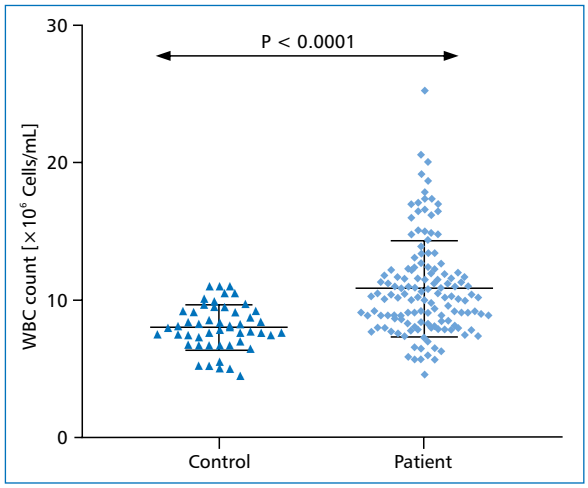

Figure 2. Comparison of the Total WBC Count between the Control Subjects and T2D Patients
T2D — type 2 diabetes; WBC — white blood cells

Discussion

The focus of the present study was to evaluate different biomarkers of oxidative stress and inflammation in T2D patients compared to a control group in order to investigate whether these conditions play a role in the development of T2D. Previous research showed that CVD, infectious diseases and other inflammatory conditions also contribute to the development of oxidative stress [22]. Therefore, to avoid false positive results, the present study excluded T2D patients with CVD, diabetic nephropathy or any other chronic inflammatory diseases. Smoking also causes oxidative stress and there were some smokers among the studied groups; our preliminary data showed no significant difference in NOX, MDA and NLR values between smokers and non-smokers. This study did not find smoking to be a risk factor for T2D.

Analyses of the baseline characteristics showed the family history of diabetes was a strong risk factor for the development of type 2 diabetes, as observed previously [23]. Although the patients enrolled in this study were not age-matched with the controls, the mean age of the studied groups did not vary significantly. Also, the body mass index (BMI), SBP and DBP did not vary significantly between the patients and controls. It should be noted that the T2D patients were taking many anti-diabetic drugs that might have an effect on their blood pressure regulation as one study suggested that some of the anti-diabetic medications might prove as important agents for the control of hypertension in individuals with diabetes [24].

The present study revealed that the patients exhibited significantly higher NOX activity compared to the controls. It has been reported that hyperglycemia is the main factor which induces oxidative stress mainly by activation of NOX in patients with diabetes [25]. Therefore, increased NOX activity was clearly associated with the underlying pathogenesis of T2D in patients.

One of the important findings of the present study was increase in lipid peroxidation, which was reflected by the significantly higher plasma MDA levels in T2D patients. This result supported previous studies

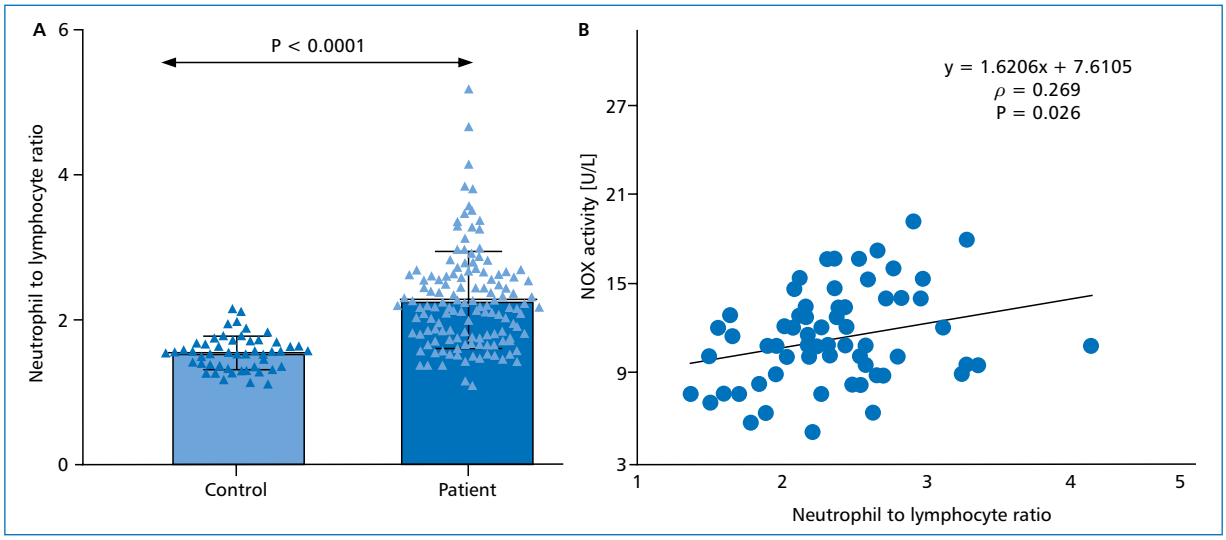

Figure 3. A. Comparison of the Neutrophil to Lymphocyte Ratios between the T2D Patients and Control Subjects; **B.** A Significant Positive Correlation of Neutrophil to Lymphocyte Ratios with NOX Activities in T2D Patients
NOX — nicotinamide adenine dinucleotide phosphate oxidase; T2D — type 2 diabetes

showing that hyperglycemia increased lipid peroxidation from overproduction of free radicals in diabetes [9, 26]. Increased level of MDA in diabetes suggested that peroxidative injury might be involved in the development of diabetic complications [27].

The present study found significantly higher total oxidative stress (TOS) in patients with diabetes which was consistent with a previous study [21]. The higher TOS values in patients with diabetes indicated increased level of hydroperoxides (ROOH) generation. Myeloperoxidase produced by circulating neutrophils plays an important role in both processes of inflammation and oxidative stress [28]. The current study found significantly higher MPO activities in T2D patients, as observed previously [29], suggesting that it might contribute to the enhanced oxidative stress through the generation of ROS.

In this study, the WBC indices including neutrophil, monocyte and eosinophil counts were significantly higher but lymphocyte were significantly lower in T2D patients. In addition, the total WBC count was significantly higher in patients, which was consistent with a previous finding [30]. One study reported involvement of peripheral blood polymorphonuclear leukocytes in oxidative stress and inflammation in T2D patients, suggesting that the inflammatory reactions result in cell recruitment and ultimately in oxidative stress-induced endothelial dysfunction [31].

In a hyperglycemic condition, advanced glycation end-products could upregulate angiogenic and pro-inflammatory cytokine production in human monocyte/macrophages [32]. The present study found significantly higher NLR in T2D patients compared to control subjects. This finding was in agreement with Lou et al. [33], which observed that increased NLR was significantly associated with insulin resistance that plays a vital role in the pathogenesis of T2D.

It is believed that the release of inflammatory mediators is prompted by high glucose concentration and mediated by oxidative stress [34], although the interactions between these factors are not well studied. One of the critical findings of the current study is a significant positive correlation between NOX activity and NLR in T2D patients. NOX, a respiratory burst enzyme initially identified in neutrophils, is one of the principal sources of ROS production in hyperglycemic conditions. Therefore, increased activation of neutrophils due to oxidative stress and inflammation in T2D patients [31] could lead to the increased activation of NOX resulting excessive ROS production. This finding suggested that high NLR was associated with increased NOX activity. Further research may identify NLR as an inexpensive yet important biomarker to monitor inflammation in T2D patients along with the routine biochemical markers FPG and HbA1c.

Further, there was a significant positive correlation between NOX activity and MDA concentration which was consistent with a previous finding [35]. This observation implied that activation of NOX released ROS, which enhanced lipid peroxidation process, and consequently MDA level was increased. This study found direct correlations, although not significant, between activities of NOX/MPO with fasting plasma glucose supporting that in diabetes, hyperglycemia induces a vicious cycle of ROS generation which eventually leads to an increased oxidative stress, as suggested by Fakhruddin et al. [36].

It should be mentioned that there were certain limitations to this study. Firstly, the sample size was small and probably inadequate to illustrate the full picture of a population. Secondly, the patients were collected from the outpatient department of a single hospital. Lastly, although it was ensured that the control subjects did not suffer from any major chronic illness despite some of them had hypertension, it could not be checked, which might affect the results. Albeit these limitations, the noble finding of this study can be used as a stepping stone for undertaking future studies with larger sample size considering the significance of the present investigation, and the outcome can help the researchers and clinicians design better management and treatment strategies for this complex disease.

Conclusions

The present study highlighted the extent of oxidative stress due to excessive production of ROS by the oxidase enzymes NOX and MPO. The effect of this enhanced oxidative condition was measured as higher MDA and TOS concentrations in patients. The present study attempted to explain the role of oxidative stress-induced inflammation in T2D patients by the elevated values of neutrophils and NLR. MPO produced by activated neutrophils played important roles in both oxidative stress and inflammation in T2D. The correlations between oxidative stress and inflammatory markers NOX and NLR respectively, and oxidative stress markers NOX and MDA strongly supported the development of greater oxidative burden in T2D patients, which can lead to diabetes related complications. Therefore, the assessment of these oxidative stress and inflammatory markers in diabetes can provide benefits in terms of early diagnosis (of inflammatory conditions) and treatment interventions to prevent the progression into other complications.

Funding

This publication was prepared without any external source of funding.

Acknowledgements

The authors gratefully thank Professor Dr. M. Sawkat Hassan, Director, Laboratory Services, and Md. Nayeemul Islam Khan, Principal Scientific Officer, Clinical Biochemistry Laboratory, BIRDEM General Hospital, Dhaka for their kind cooperation in collecting patient samples.

Conflicts of interests

None declared.

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