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Original Article

Biomarkers of Oxidative Stress and Antioxidant Defense in Patients with Type 1 Diabetes Mellitus

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Abstract

Background: Oxidative stress has become the focus of interest in most biomedical disciplines and many types of clinical research. Increasing evidence from research on several diseases shows that oxidative stress is associated with the pathogenesis of diabetes and many other diseases. **Objectives:** We aimed to investigate the status of oxidative stress and antioxidant enzymes related parameters in type 1 diabetes mellitus (T1DM) patients. **Patients and Methods:** A total of 110 patients (70 patients newly diagnosed diabetic and 40 healthy) were studied by evaluating the level of lipid peroxidation (malondialdehyde [MDA]), nitric oxide (NO), fasting blood sugar (FBS), and glycated hemoglobin (HbA1c). Antioxidant enzymes, including superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx), were also evaluated. **Results:** The FBS and HbA1c levels were significantly higher in diabetic patients compared to those of healthy participants. Higher levels of MDA and NO were observed in the diabetic group compared to those in the healthy participants. A significant decrease was observed in serum SOD, CAT, and GPx activities in the serum of T1DM patients by 16.7%, 72.8%, and 15.3%, respectively ($P < 0.05$), as compared with their activities in the controls. On the basis of sex, both male and female patients showed a significant reduction in antioxidant levels as compared to their respective controls. **Conclusions:** These results indicated that oxidative status and antioxidant levels were affected in T1DM. The results suggested that the biomarkers such as the plasma levels of lipid peroxidation and antioxidants in early diagnosed diabetics can be used to monitor the developing complications of the diabetes.

Keywords: Antioxidants, lipid peroxidation, oxidative stress, type 1 diabetes mellitus

Introduction

Diabetes is a well-known health burden worldwide for its spread in both developed and developing countries. Diabetes is one of the major causes of disability and death in all countries of the world.[1] Type 1 diabetes mellitus (T1DM) causes approximately 5%–10% of all diagnosed cases of diabetes mellitus.

Free radicals recognized are short-lived, very unstable, and extremely reactive.[2] They are formed excessively in diabetes due to glucose oxidation and nonenzymatic protein glycation. Abnormally elevated free radical levels and the simultaneous decrease of antioxidant protective mechanisms can cause damage to cellular organelles and DNA, increased lipid peroxidation, and induction of insulin resistance.[3] Since diabetes is considered as a consequence of increased in the radicals formation, the autoimmune disorder is described during excessive and accumulation of free radicals, correlated with immune dysfunction and oxidative stress.[4]

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The leading antioxidant enzymes that are engaged in the neutralization of both reactive nitrogen species (RNS) and reactive oxygen species (ROS) are superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), and glutathione reductase (GRx).[5] Studies have demonstrated that SOD can be used to prevent the destructive consequences of hyperglycemia-induced ROS production. In diabetes, a notable increase in the CAT activity in lymphocytes was discovered in children with T1DM through all phases. Conversely, it has been reported that significant decreased CAT and significant increase

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in thiobarbituric acid reactive species concentration in T1DM patients with or without nephropathy compared with control.[6] Studies also indicated that GPx activity was lower in children with diabetes compared with healthy children (controls).[7,8] According to these results, it has been implied that lowered antioxidative defenses from the excessive production of lipid hydroperoxide and NOx overproduction are present in juvenile patients with T1DM.[9]

Patients and Methods

Study design

This study was conducted which recruited both males and females with TIDM in which they have not diabetic diagnosed before as well as nondiabetic participants. A cross-sectional study was conducted on 70 patients with T1DM (36 males and 34 females) aged 3–15 years who attended the Tripoli Medical Center (TMC), Tripoli, Libya. In addition, a control group composed of 40 nondiabetic individuals (20 males and 20 females) all within the same age range as the other patients who were not administered any medications. A detailed medical history was taken, and a physical examination was performed upon all participants. The Ethics committee of Biotechnology Research Center (Tripoli, Libya) approved the study (BEC-BTRC 03-2017). All patients provided a written informed consent before the start of the study procedures.

Selection criteria

Participants included in the current study were selected according to the following criteria: first, they were newly diagnosed with T1DM patient; second, they were free of any ailment which could affect the parameters under study; and third, they are not on any medication. Hemolytic anemia, hemoglobin variants, hepatic disease, and infectious diseases, such as tuberculosis and sarcoidosis, were excluded from the study. In addition, participants under treatment with drugs, such as chelating agents, ethambutol, D-penicillamine, were also excluded from the study.

Blood sample collection

Blood samples were collected into commercial tubes after overnight fasting for the analysis of laboratory parameters. Venous blood samples (5 ml) were obtained from the capital vein of each participant using sterile disposable plastic syringes. Specimens were collected at the same standardized time to minimize any effect of diurnal variation. The blood samples in the vacutainer tubes were left to clot and the serum was separated by centrifugation. The clear, nonhemolyzed supernatant sera were separated using clean, dry disposable plastic syringes. Samples were stored at −80°C and used within 1 month for the analysis of malondialdehyde (MDA), nitric oxide (NO), glutathione (GSH), and measurement of antioxidant enzymes.

Determination of biochemical parameters

Various biochemical parameters in blood were measured according to the standard methods including glucose, creatinine, total protein, alanine aminotransaminase, aspartate aminotransaminase, triglycerides (TG), total cholesterol, high-density lipoprotein (HDL), and low-density lipoprotein (LDL) cholesterol. All parameters measured according to the standard protocols in both T1DM and nondiabetic controls using premodular analytics by using a kit with Cobas Integra® 400 plus in the Biochemistry Laboratory, at the TMC, Tripoli, Libya.

Oxidative stress parameters

Thiobarbituric acid-reacting substances content, a measure of lipid peroxidation was determined spectrophotometrically in serum according to the method of Zhang *et al*.,[10] while NO in serum was determined according to the Griess method with some modification.[11]

The CAT activity was assayed using the spectrophotometric method, based on the ability of hydrogen peroxide to form a stable stained complex with molybdenum salts.[12] GPx activity was determined according to the method of Hafeman *et al*.[13] The estimation of SOD activity was performed using a SOD Assay kit-WST (Sigma Aldrich, USA) according to the manufacturers' protocol (Dojindo, Gaithersburg, MD, USA). Xanthine-xanthine oxidase system was used to generate a superoxide flux, and nitroblue tetrazolium was used as an indicator of superoxide production. Reduced GSH was determined using Ellman's reagent. Formations of color product monitored at 412 nm after oxidation of GSH by 5,5′-dithio bis-2-nitrobenzoic acid.[14]

Statistical analysis

Results were expressed as a mean ± standard deviation. The statistical significance was assessed using the analysis of variance. $P < 0.05$ was considered statistically significant.

Results

Characteristics of study subjects

Table 1 shows the clinical characteristics of the participants in the current study. The groups (diabetic patients and healthy control participants) were similar regarding sex and age. The study involved 70 patients with T1DM which are newly diagnosed. The ages in the control group are comparable to those in the overall group of type 1 diabetic cases studied. There was no gross obesity in the population studied, as can be seen from their body mass index [Table 1].

The demographic evaluation, fasting blood sugar (FBS), and glycated hemoglobin (HbA1c) levels are shown in Table 1. The participants were classified according to their sex. There were 36 male and 34 female represented 32.73% and 30.90% of the total patients' group, respectively. There were 20 males representing 22.22% of the control group and 20 females representing 22.22% of the same group. A significant increase in FBS was found in diabetic patients (173.09 ± 17.80 mg/dl) as compared to control group (68.42 ± 2.01 mg/dl) ($P < 0.05$). Furthermore, HbA1c is very significantly increased by 74.7% ($P < 0.0001$) in diabetic participants compared to

Table 1: The demographic, metabolic profiles of type 1 diabetes mellitus patients and healthy controls

Variable groups	Variables	Patients	Controls	Statistical significance
Demographic characteristics	Gender (male/female)	34/36	20/20	-
	Age (years)	10.9±4.0	10.8±4.0	NS
	Body mass index (kg/m^2)	19.1±4.1	13.4±15.4	P<0.0001
Glycemic control	Fasting plasma glucose (mg/dl)	173±18	68±2	P<0.0001
	HbA1c (%)	8.8±2.0	5.1±0.2	P<0.0001
Plasma lipids	Total Cholesterol (mg/dl)	158±27	147±6	NS (0.075%)[b]
	Triglycerides (mg/dl)	89±8	83±52	NS (7.58%)[b]
	LDL Cholesterol (mg/dl)	84±23	70±3	P<0.05 (19.8%)[b]
	HDL Cholesterol (mg/dl)	57±16	59±6	NS (−3.7%)[b]
Oxidant/antioxidant parameters	Catalase (U/mg protein)	0.49±0.42	1.80±0.94	−72.8%[b]
	SOD (U/mg protein)	11.05±2.48	13.26±3.47	P<0.05 (−16.7%)[b]
	GPx (U/mg protein)	6.59±1.96	7.78±1.13	P<0.05 (−15.29%)[b]
	MDA (nmol/mg protein)	0.37±0.10	0.19±0.08	P<0.0001, (94.73%)[b]
	NO (nmol/mg protein)	3.98±1.22	0.89±0.27	P<0.0001 (347.1%)[b]
	Glutathione (μg/mg protein)	0.017±0.004	0.023±0.006	P<0.0001 (26.08%)[b]

Values are expressed as mean±SD for n participants. [b]Values in parentheses represent percentage change with respect to control, LDL: Low-density lipoprotein, HDL: High-density lipoprotein, NS: Nonsignificant, SOD: Superoxide dismutase, GPx: Glutathione peroxidase, MDA: Malondialdehyde, NO: Nitric oxide radical, SD: Standard deviation

the control group. Among 65% of diabetes participants had a very high HbA1c level among them 55.22% have HbA1c more than 8%.

Lipid profile assessment

Considering lipid profile, although there was an increase in TG levels in type 1 diabetic participants (89.3 ± 8.4 mg/dl) compared to control group (82.53 ± 52.08 mg/dl), no significant difference between the two groups was observed. Similar results were obtained regarding HDL cholesterol [Table 2]. However, LDL cholesterol was found to be significantly increased (84.29 ± 22.71 mg/dl) (P < 0.05) compared with healthy control participants. Furthermore, cholesterol level in diabetic patients (158.06 ± 26.58 mg/dl) was higher compared with its levels in healthy participants (147.05 ± 5.51 mg/dl) [Table 2]. In addition, the HDL/LDL cholesterol ratio did not show significant differences.

Oxidants and antioxidants levels

Table 1 shows serum MDA and NO levels, parameters of oxidative stress as well as serum antioxidants including GSH, CAT, GPx, and SOD. The level of MDA and NO was found to be significantly elevated almost 2-fold and 4.5-fold, respectively, in the blood of type 1 diabetic participants (0.37 ± 0.10, 3.98 ± 1.22 nmol/mg protein, respectively) than the respective control group (0.19 ± 0.08, 0.89 ± 0.27 nmol/mg protein, respectively) (P < 0.0001). A significant decrease was observed in serum SOD activity in the serum of type 1 patients (11.05 ± 2.49 U/ml; by −16.66%) as compared to healthy participants (13.26 ± 3.47 U/mg protein) (P < 0.05). A highly significant decrease in the serum CAT activity in patient with diabetic participants (0.49 ± 0.42 U/mg protein by −72.77%) as compared to control group (1.80 ± 0.94 U/protein) (P < 0.001) was observed. The activity of GPx was found to be lower in patients by 15.29% than the control group [Table 1].

When the comparison was made on the basis of sex, a significant increase in FBG and HbA1c compared to their respective controls was obtained [Table 2]. In diabetic females, the levels of cholesterol and LDL were markedly higher than their levels in healthy females [Table 2]. The concentrations of both MDA and NO were very significantly decreased (P < 0.0001) in the type I DM participants than their respective control groups [Figure 1]. A significant decrease was found in GSH level in diabetic males (0.018 ± 0.006 μg/mg protein) and diabetic females (0.016 ± 0.004 μg/mg protein) (P < 0.0001) as compared with their respective controls (0.02 ± 0.004 μg/mg protein; 0.024 ± 0.007 μg/mg protein, respectively) [Figure 2]. Moreover, there was a marked drop in the activity of CAT and GPx levels in the type I DM compared to their respective controls (P < 0.0001); however, there were no significant differences in the activity of SOD was found between the males and females with type I DM compared with their controls (P > 0.05) [Figure 3].

The demographic evaluations of the diabetic male and female participants are shown in Table 1. Considering the lipid profile, there was no significant difference between diabetic male and the diabetic female groups [Table 2]. Furthermore, HbA1c was higher in both diabetic males and females; however, GSH was higher in diabetic females only. The activities of SOD and GPx did not exhibit any significant difference (P > 0.05) between diabetic males and females [Table 2]. Furthermore, the level of MDA and NO was slightly higher (though not significantly) in diabetic males compared with diabetic females [Figure 1].

Interrelationships between oxidants and antioxidants between studied variables

To analyze the influence of oxidative stress parameters on antioxidants status, the Pearson correlation was performed. The analysis revealed a significant negative correlation between HbA1c and SOD in the diabetic patients (r = −0.339,

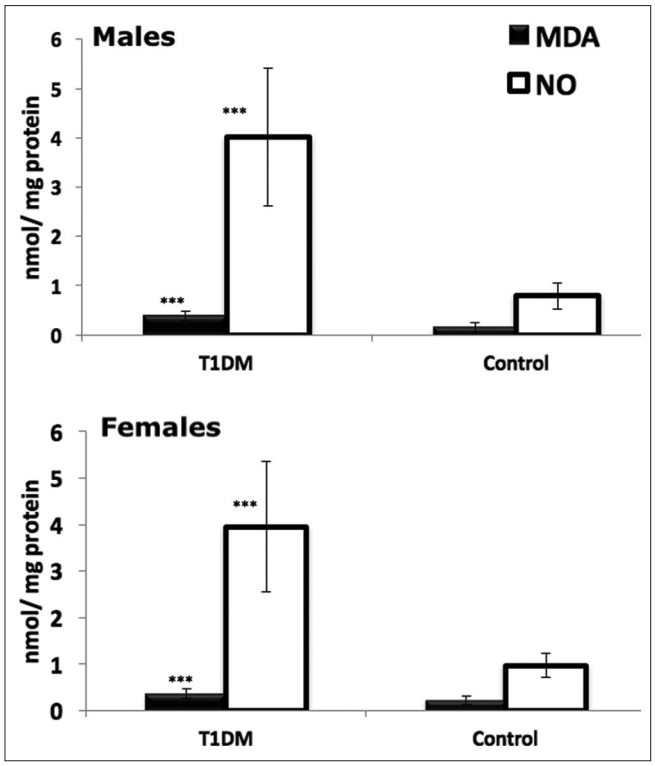

Figure 1: Malondialdehyde and nitric oxide levels in control and diabetic participants on the bases of sex. ***$P < 0.0001$ considered statistically significant as compared to control. Values are represented as mean ± standard deviation. T1DM: Insulin-dependent (Type-1) diabetes mellitus

Table 2: Glycated hemoglobin, total cholesterol, high-density lipoprotein cholesterol, low-density lipoprotein cholesterol, triglycerides in diabetic patients and control, and control participants presented in males and females separately

Parameters	Male ($n=36$)		Female ($n=34$)	
	Diabetic	Control	Diabetic	Control
HbA1c	9.3±2.2***	5.1±0.3	8.5±1.8***	5.1±0.2
Total Cholesterol	156±28 (NS)	148±6	161±26*	147±5
LDL cholesterol	84±24 (NS)	71±4	85±22*	70±3
Triglycerides	84±50 (NS)	88±8	81±55 (NS)	91±9
HDL cholesterol	54±16 (NS)	59±8	59±16 (NS)	59±5

Values are represented as mean±SD. *$P<0.05$; ***$P<0.0001$ considered statistically significant as compared to control. HbA1c: Glycated hemoglobin, LDL: Low-density lipoprotein, HDL: High-density lipoprotein, NS: Nonsignificant, SD: Standard deviation

$P < 0.01$). A significant positive correlation was also observed between TAG and MDA ($r = 0.203$, $P < 0.05$) and NO ($r = 0.311$, $P < 0.01$) in all the diabetics. In addition, a significant positive correlation was detected between HDL and GPx ($r = 0.296$, $P < 0.01$) as well as a significant negative correlation between HDL and NO ($r = -0.344$, $P < 0.01$) in type 1 diabetes participants. Moreover, a significant positive correlation was found between LDL and NO (0.263, $P < 0.05$). Furthermore, a negative correlation was observed between

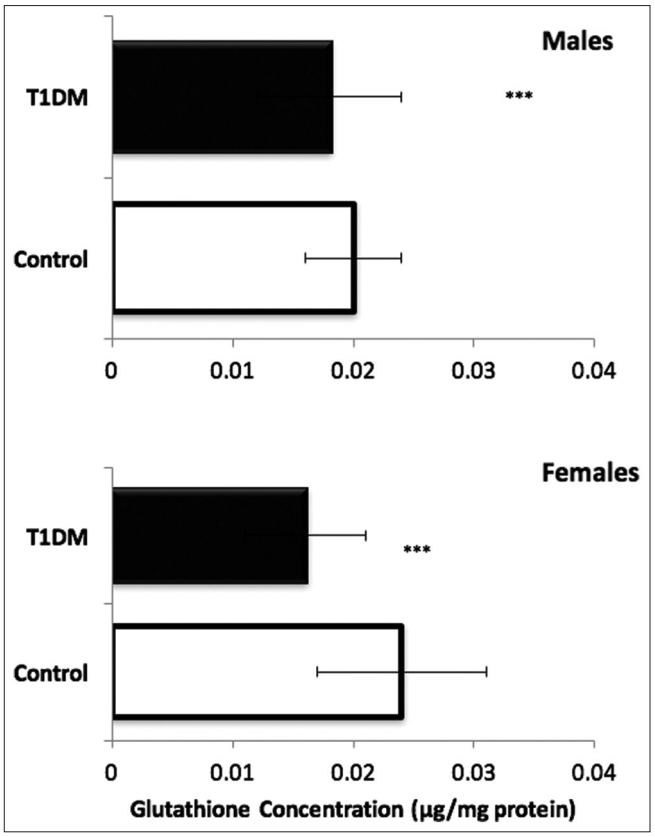

Figure 2: Level of glutathione in healthy participants and type 1 diabetic patients on the bases of sex. T1DM: insulin-dependent (Type-1) diabetes mellitus. ***$P < 0.0001$ considered statistically significant as compared to control. Values are represented as mean ± standard deviation

GPx with NO ($r = -0.355$, $P < 0.01$) and GSH ($r = -0.315$, $P < 0.05$). On the other hand, no correlation was found between lipid peroxidation (MDA) and nonenzymatic (GSH) or any of the other enzymes (SOD, GPx, and CAT) in the assessed antioxidants. No correlation was found between lipid profile parameters (TAG, cholesterol, LDL, and HDL) with regard to peroxidation parameters (MDA and NO) and antioxidants (SOD, CAT, and GSH) or HbA1c in females type 1 diabetes. The only positive correlation was found between GPx and NO ($r = -0.510$, $P < 0.01$) in female diabetics.

Regarding diabetic males, a statistically significant negative correlation was found between the levels of HbA1c with SOD (−0.440, $P < 0.05$) and MDA (0.402, $P < 0.01$). A significant positive correlation between TG and NO (0.486, $P < 0.01$) was also found in diabetic males. In addition, there was a significant positive correlation was found between LDL and NO (0.373, $P < 0.01$). A significant negative correlation was also obtained between HDL with NO (−0.403, $P < 0.01$) and positive correlation with GPx (0.396, $P < 0.01$).

Discussion

There are some contradictory results regarding the role of oxidative stress in the etiology of type I DM. Therefore, the present study was aimed to investigate whether oxidative stress

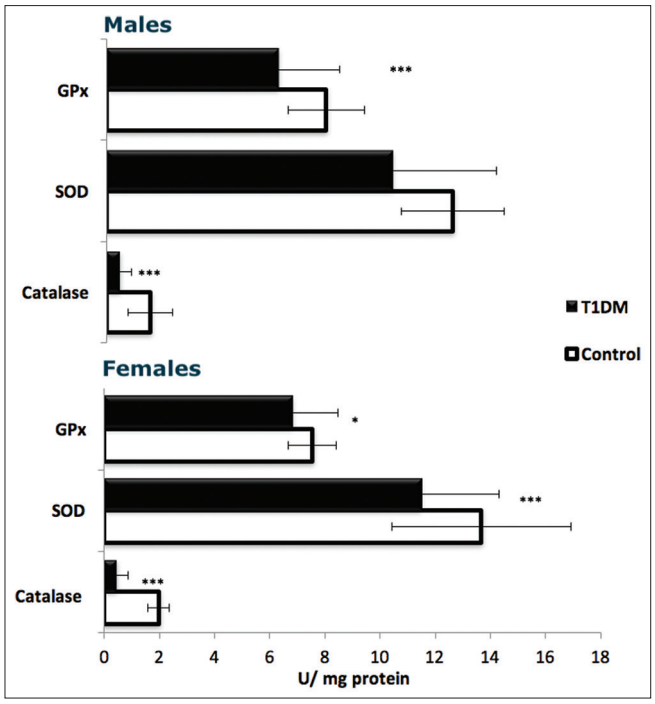

Figure 3: Antioxidant enzymes activities in healthy participants and type 1 diabetic patients on the bases of sex. Values are represented as mean ± standard deviation. *$P < 0.05$; ***$P < 0.0001$ considered statistically significant as compared to control. SOD: Superoxide dismutase, GPx: Glutathione peroxidase, MDA: Malondialdehyde, NO: Nitric oxide radical, T1DM, insulin-dependent (Type-1) diabetes mellitus

occurs early during the disease without diabetic complications and whether any difference exists between diabetic males and females in this regard. This study focuses on the Libyan patients for whom there are very little data.

Clinical and experimental studies suggest that oxidative stress plays a major role in the pathogenesis of both type 1 and type 2 diabetes mellitus. Free radicals are formed excessively in diabetes due to glucose oxidation and nonenzymatic protein glycation.[3,15]

Abnormally elevated free radical levels and the simultaneous decrease of antioxidant protective mechanisms which lead to increase in the lipid peroxidation.[3] Lipid peroxidation products in diabetic patients are of special interest because their hyperlipidemia is considered to be a significant risk factor for the development of vascular complications.[16,17] Our results have found that MDA levels, a lipid peroxidation product which is the most important marker of oxidative stress, were increased significantly in male and female diabetic patients [Figure 1]. This obviously shows that regardless of the sex, diabetic patients were exposed to elevated oxidative stress through lipid peroxidation which was in parallel with previous results.[18-20]

Generation of NO and its effects are enhanced in diabetes mellitus[21] and in early stages of development of the diabetic complications.[22,23] NO is a highly unstable molecule and is rapidly converted to nitrite and nitrate. Present results showed significant higher NO end products in the serum of diabetic patients as compared with healthy participants [Figure 2]. This may be due to the overproduction of NO associated with higher oxidative stress status in diabetic patients.[24]

As a result of the progressive increases in lipid peroxidation and NO production levels in serum of the early stages of diabetic participants, a decline in antioxidant defense system which can lead to the damage of cellular organelles and enzymes and therefore can encourage the development of complications in diabetes mellitus patients. Antioxidant enzyme-dependent defenses play a crucial role in scavenging or preventing free radicals produced under oxidative stress.[25]

Antioxidant enzymes that are involved in the neutralization of free radical (RNS and ROS) including SOD, CAT, GPx, and GRx,[5] also they can be destroyed by free radicals.[26]

SOD catalyzes the depletion of superoxide $O_2^{-\bullet}$ to O_2 and to the less reactive species hydrogen peroxide H_2O_2 by reduction. The previous study demonstrated that SOD can be used to prevent the destructive consequences of hyperglycemia-induced ROS production in diabetes. While CAT plays a role in the conversion of hydrogen peroxide to oxygen and water and, therefore, protects cells from the damaging effect of hydrogen peroxide that is present intracellularly. GPx is the primary antioxidant enzyme involved with preventing the aggregation of intracellular hydrogen peroxide; it converts hydrogen peroxide (H_2O_2) to water.[27]

Various studies have reported that both increase[28,29] and decrease in enzyme activities.[30-32] The progressive glycation of enzymatic proteins leads to decline in activities of antioxidant enzymatic systems in diabetes. In diabetics, about 50% SOD is glycated which causes the enzyme low activity.[33,34] Besides SOD, GPx, and CAT showed decrease[35-37] or no change in diabetic patients.[38] The present results support these findings where patients with T1DM had significantly lower activity of antioxidant enzymes, especially SOD, as compared to control participants. Therefore, diabetic patients had an early impairment of plasma antioxidant capacity and increased oxidative stress.

GSH is oxidized by lipid peroxides under the influence of GPx yielding GSH disulfide and back to back into GSH by GRx, indicating that GSH may serve a purpose in detoxifying lipid peroxides.[39] Diabetes induces alterations in the activity of both enzymes which make the cells prone to oxidative stress and hence cell injury. Our results revealed that the level of GSH and the activity of GPx were significantly decreased in the sera of T1DM patients, indicating decreased scavenging capacity of GSH-dependent antioxidant defensive system against elevated lipid peroxidation processes and NO production in these patients.

Conclusions

The oxidative status is clearly affected the endogenous antioxidant status in the newly diagnosed diabetic patients

with T1DM. The plasma levels of lipid peroxidation and NO measurements can be considered as early biomarkers of oxidative damage, which can be used to monitor the developing complications of diabetes.

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Authors' contributions

RA conceived and designed the experiments. NA, OA, and SE performed the experimental works. RA, NA, and NAw analyzed and interpreted the data. RA drafted the paper. NAw revised the manuscript. All authors revised the paper and approved its final version.

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Conflicts of interest

There are no conflicts of interest.

Compliance with ethical principles

The study was approved by the Ethics Committee at the Biotechnology Research Center, Tripoli, Libya (BEC-BTRC 03-2017). Parents of all patients provided written informed consent before starting of the study procedures.

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