RESEARCH ARTICLE

Investigation of oxidative stress markers and antioxidant enzymes activity in newly diagnosed type 2 diabetes patients and healthy subjects, association with IL-6 level

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Abstract

Objective Oxidative stress and inflammation play an important role in the pathogenesis of diabetes and its complication. In this study, we aimed to evaluate and compare oxidative stress markers and antioxidant enzymes activity, as well as Interleukin 6 (IL-6) level in newly diagnosed type 2 diabetes mellitus (T2DM) patients and healthy subjects.

Material and methods 30 newly diagnosed type 2 diabetes patients and 30 healthy subjects (age and sex matched) were recruited in this study. After anthropometric parameters measurement, blood sample were collected from all participant. Serum and plasma were isolated. Biochemical parameters were evaluated in serum. Plasma was used to measure malondialdehyde (MDA), total oxidant status (TOS), total antioxidant capacity (TAC) levels, as well as superoxide dismutase (SOD) and glutathione peroxidase (GPx) activity. Also, IL-6 level was investigated in plasma using ELISA method.

Results MDA and TOS levels were significantly higher in T2DM patients than the control group ($p < 0.05$). However, TAC and SOD were significantly lower in T2DM as compared with healthy subjects ($p < 0.05$). Also, IL-6 level was higher in T2DM in comparison to healthy subjects ($p = 0.004$). Furthermore, there was a significant positive correlation between IL-6 with MDA ($p = 0.031$, r = 0.482) and TOS ($p < 0.001$, r = 0.744). In addition, a negative correlation was observed between IL-6 and SOD activity ($p = 0.002$, r = −0.660).

Conclusion Reducing oxidative stress and inflammation could be effective in improvement of T2DM.

Keywords Type 2 diabetes mellitus (T2DM) · Malondialdehyde (MDA) · Total oxidant status (TOS) · Total antioxidant capacity (TAC) · Superoxide dismutase (SOD) and glutathione peroxidase (GPx) · Interleukin 6 (IL-6)

Introduction

Type 2 diabetes mellitus (T2DM) is an endocrine disorder which is associated with impaired insulin function and secretion. The prevalence of T2DM is increasing worldwide, such that it has become a global concern. Hyperglycemia is the main characteristic of T2DM [1–3]. Hyperglycemia leads to an increase in reactive oxygen species (ROS) formation and inflammation [4].

ROS plays a critical role in T2DM and its complications. Hydroxyl radical, nitric oxide, peroxynitrite, superoxide, and hydrogen peroxide are among the most important ROSs [5]. Antioxidants protect the cells against oxidative stress by eliminating ROS. The antioxidant system involves enzymatic and non-enzymatic activities. The most important antioxidant enzymes include superoxide dismutase (SOD), glutathione peroxidase (GPx), and catalase (CAT). At the first line, SOD degrades superoxide radicals by converting these anions into hydrogen peroxide. Then, hydrogen peroxide is removed by GPx and CAT through oxidation of glutathione (GSH) and converting hydrogen peroxide into water and oxygen, respectively. On the other

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hand, non-enzymatic antioxidants include vitamins (A, C and E), glutathione, flavonoids, α-lipoic acid, coenzyme Q10, and thioredoxin [6, 7].

The imbalance between ROS production and antioxidant defense system plays an important role in the pathogenesis of T2DM and its complications [8]. Low concentrations of ROS have a physiological role such as proliferation and differentiation, while high levels of ROS damage the cells and is considered to be an important contributor to many diseases such as T2DM [9]. Increased ROS during T2DM promotes insulin resistance through various mechanisms including beta cell apoptosis and impaired insulin signaling pathway [7].

Furthermore, inflammation is another factor contributing to T2DM. IL-6 is one of the inflammatory cytokines involved in the pathogenesis of T2DM [10].

There are contradictory results over oxidative stress and inflammation levels in diabetic patients. In this study, we aimed to evaluate and compare oxidative stress markers including TAC (total antioxidant capacity), TOS (total oxidant status) and MDA (Malondialdehyde) as well as the activitiy of antioxidant enzymes including SOD and GPx, along with IL-6 level in plasma of newly diagnosed type 2 diabetes patients and healthy subjects.

Material and methods

Subjects

In this study 30 newly diagnosed diabetic patients and 30 healthy subjects aged between 40 to 60 years (age and sex matched) participated. This study was approved by the Ethics Committee of Hamadan University of Medical Sciences (IR.UMSHA.REC.1396.837). Diagnosis of diabetes was performed based on American Diabetes Association criteria. According to these criteria, subjects with FBS ≥ 126 mg/dl grouped in the diabetic patients and subjects with FBS < 100 mg/dl were used as healthy control. People with types 1 diabetes, gestational diabetes, inflammatory diseases, cardiovascular disease, cancer, subjects who smoked were excluded from the study. It is worth noting that T2DM subjects were not using any medicine to control blood sugar. Also, all the patients and normal healthy subjects did not take any vitamin or antioxidant supplements. Informed consent was received from all participants. Height (±0.1 cm) and weight (±0.1 kg) were measured and body mass index (BMI) (weight/height^2) was calculated. Also systolic blood pressure (SBP) and diastolic blood pressure (DBP) were recorded.

Biochemical measurements

After 12 h fasting, 20 cc blood samples were taken in plain and EDTA-containing tubes. Serum and plasma were isolated by centrifugation at 2400 rpm for 15 min. Serum were used for evaluation of biochemical parameters including FBS, triglyceride (TG), total cholesterol (TC), high-density lipoprotein cholesterol (HDL-C), low-density lipoprotein cholesterol (LDL-C), aspartate aminotransferase (AST), alanine aminotransferase (ALT), Urea and Creatinine using colorimetric methods (Pars Azmoon, Tehran, Iran) on a BIOLIS24i Premium autoanalyzer (Tokyo Boeki Machinery Ltd., Japan). Insulin levels in serum were determined using enzyme-linked immunosorbent assay (ELISA) kit (MonobindInc.,USA). Homeostatic model assessment of insulin resistance (HOMA-IR) index was obtained using the following formula: serum insulin (μIU/ml) × FPG (mg/dl)/405. HbA1c was measured using HPLC method by Tosoh G8 instrument (South San Francisco, CA).

Lipid peroxidation assay (MDA)

Plasma was used to measure MDA, TOS, TAC levels, as well as SOD and GPx activity. Oxidative degradation of

Table 1 Anthropometric indices of the study population

Variable	Healthy subjects ($n = 30$)	T2DM patients ($n = 30$)	p value
Female/male	15/15	15/15	n.s.
Age (year)	44.97 ± 5.18	47.16 ± 5.94	n.s.
Height (cm)	173.28 ± 8.64	172.54 ± 9.45	n.s.
Weight (kg)	73.63 ± 7.17	77.48 ± 7.39	n.s.
BMI (kg/m2)	24.52 ± 2.28	26.02 ± 2.23	0.016
SBP (mmHg)	114.32 ± 5.53	127.64 ± 4.11	n.s.
DBP (mmHg)	79.04 ± 3.25	82.32 ± 3.63	n.s.

Data are expressed as Means ± SD. *BMI* body mass index, *SBP* systolic blood pressure, *DBP* diastolic blood pressure

Table 2 Biochemical parameters of the study population

Variable	Healthy subjects (n = 30)	T2DM patients (n = 30)	p value
FBS (mg/dl)	89.31 ± 6.24	184.76 ± 56.72	<0.001
Insulin (µIU/ml)	4.64 ± 1.29	13.51 ± 5.08	<0.001
HOMA-IR	1.02 ± 0.26	6.16 ± 3.85	<0.001
HbA1c (%)	4.57 ± 1.39	9.38 ± 3.21	<0.001
TG (mg/dl)	149.53 ± 39.22	284.62 ± 84.29	<0.001
TC (mg/dl)	159.27 ± 27.4	198.21 ± 41.2	<0.001
LDL-C (mg/dl)	87.42 ± 16.47	102.35 ± 23.46	n.s.
HDL-C (mg/dl)	46.68 ± 9.95	45.3 ± 7.28	n.s.
Urea (mg/dl)	26.29 ± 5.2	28.1 ± 5.72	n.s.
Creatinine (mg/dl)	1.05 ± 0.2	1.12 ± 0.53	n.s.
AST (U/L)	28.29 ± 5.37	28.94 ± 6.47	n.s.
ALT (U/L)	21.47 ± 6.4	25.88 ± 7.39	n.s.

Data are expressed as Means ± SD. FBS: fasting blood glucose; HOMA-IR: Homeostasis Model Assessment-Insulin Resistance, TG: triglycerides; TC: total cholesterol; LDL-C: Low density lipoprotein cholesterol; HDL-C: High density lipoprotein cholesterol; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase

lipids in presence of free radicals was named lipid peroxidation. MDA as one of the most important end products of lipid peroxidation was measured according to the previous study [11]. 1,1,3,3- tetraethoxypropane was considered as standard.

Total oxidant status (TOS)

The ability of samples to change the ferrous ion (Fe III) to ferric ion (Fe II) was determined by measurement of total oxidant status (TOS). The reaction between the ferric ion
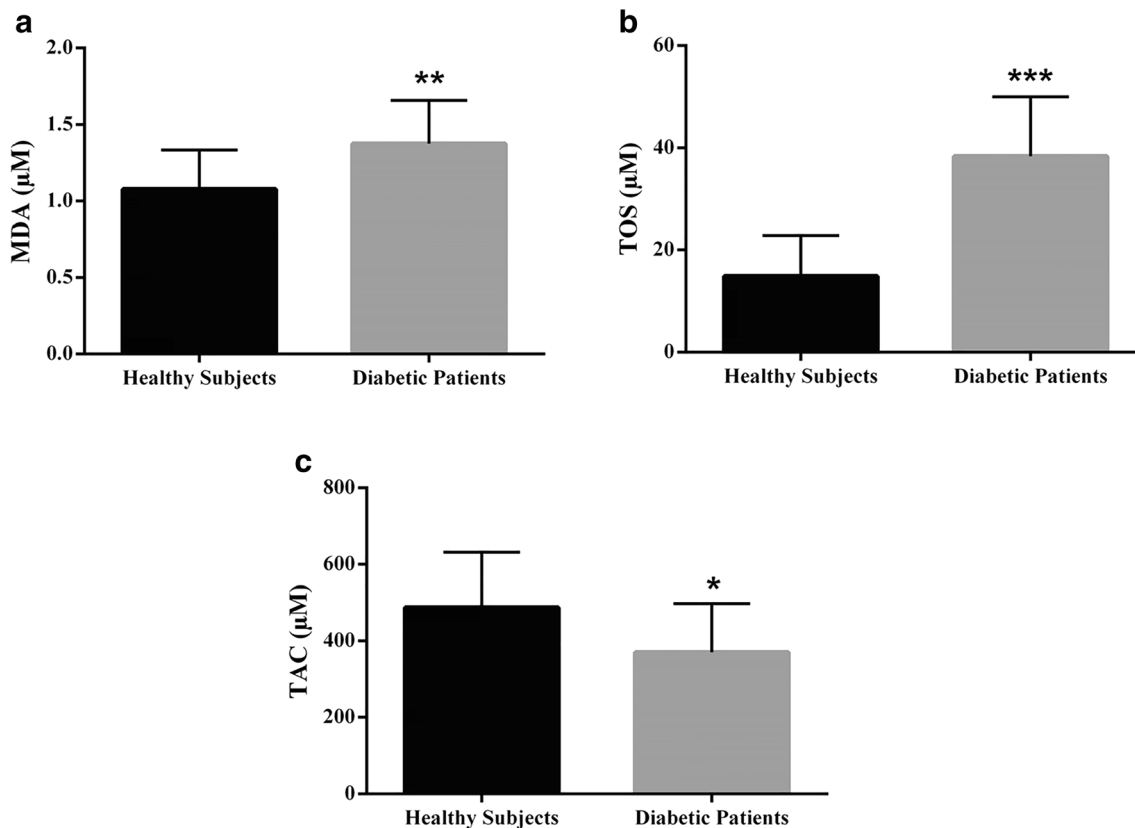

Fig 1 Oxidative stress markers (**a**) MDA, (**b**) TOS and (**c**) TAC in plasma of diabetic patients compared to healthy subjects. MDA and TOS were higher in T2DM patients than healthy subjects. TAC was lower in T2DM patients compared to healthy subjects. Data are expressed as mean ± SD. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$
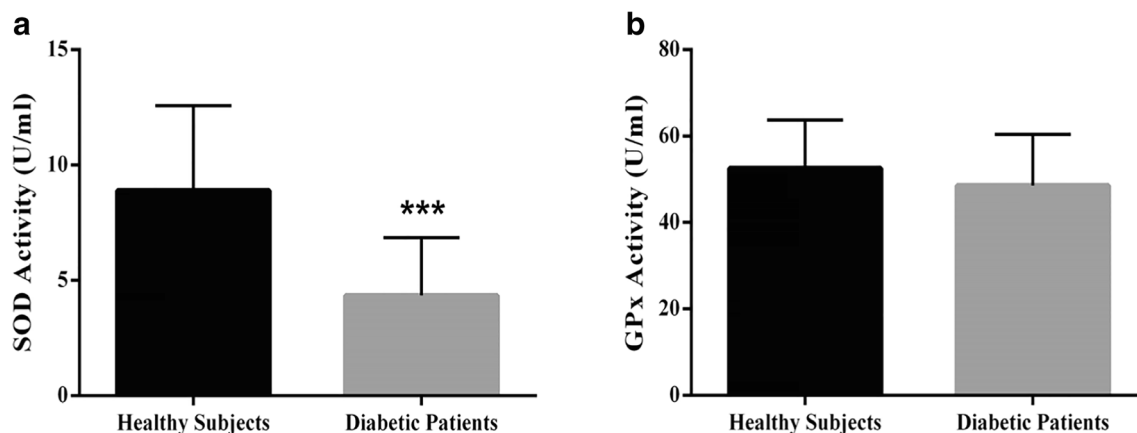
Fig 2 Enzyme activity of (A) SOD (U/ml) and (B) GPx (U/ml) in plasma of diabetic patients compared to healthy subjects. SOD and GPx activity were lower in T2DM patients compared to healthy subjects. Data are expressed as mean ± SD. ***$p < 0.001$

and xylenol orange formed a colored complex [12]. The assay was calibrated with H_2O_2.

Total antioxidant capacity (TAC)

The potential of samples to reduce ferric ion to ferrous ion represents total antioxidant capacity (TAC). The reaction between ferrous ion and 2,4,6-Tris(2-pyridyl)-s-triazine (TPTZ) forms a blue colored complex with maximum absorption at 565 nm [13].

SOD assay

SOD activity was determined by a commercial kit according to the manufacturer's instructions (SOD activity kiazist; Iran). The method is based on the ability of Mn-SOD to inhibit the conversion of resazurin to resorufin accompanied by reducing superoxide radicals produced by the xanthine/xanthine oxidase system.

GPx assay

GPx activity was measured using a commercial kit according to the manufacturer's instructions (GPx activity kiazist; Iran). The method is based on the reduction of hydrogen peroxide to water accompanied by oxidation of glutathione.

IL-6 level determination

Plasma IL-6 level was determined using ELISA kit according to the manufacturer's instructions (Diaclone Research, Besancon, France).

Statistical analysis

All data were analyzed by IBM SPSS Statistics 16.0 (IBM, USA). Normality of the variables was evaluated using a Kolmogorov–Smirnov test. Analyze of data were performed using the Mann–Whitney U test and unpaired student's t test for non-parametric and parametric data, respectively. Pearson's and Spearman's correlation tests were carried out to evaluated correlations between variables. $p < 0.05$ was considered as significant.

Results

The anthropometric indices of the subjects are represented in Table 1. There were no significant differences in age, height, weight, SBP and DBP between two studied groups ($P > 0.05$). BMI was significantly higher in T2DM compared to healthy control ($p = 0.016$).

As shown in Table 2 biochemical parameters including FBS, insulin, HOMA-IR, HbA1c, TG and TC were significantly higher in patients with T2DM as compared with non-diabetic subjects ($p < 0.001$). No significant difference in LDL-C, HDL-C, Urea, Creatinin, ALT and AST were observed between two groups ($P > 0.05$).
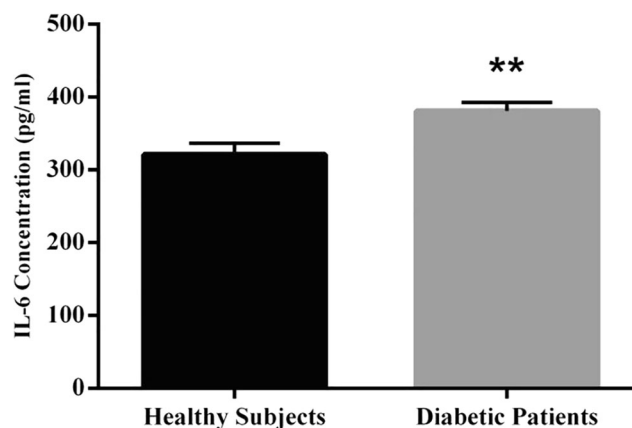

Fig 3 IL-6 concentration in plasma of diabetic patients compared to healthy subjects. IL-6 level was higher in T2DM patients compared to healthy subjects. Data are expressed as mean ± SD. ** $p < 0.01$

Table 3 Correlation coefficients between variables

Variable	MDA (μM)		TOS (μM)		TAC (μM)		SOD (U/ml)		GPx (U/ml)		IL-6 (pg/ml)	
	r1	r2	r1	r2	r1	r2	r1	r2	r1	r2	r1	r2
BMI (kg/m2)	0.040	–	**0.367**	–	**−0.332**	–	−0.252	–	−0.129	–	**0.543**	–
SBP (mmHg)	0.081	−0.043	**0.424**	−0.038	−0.187	0.006	−0.141	−0.125	−0.115	0.233	0.319	0.024
DBP (mmHg)	0.109	−0.108	0.237	0.135	−0.156	−0.085	−0.123	−0/052	−0.084	0.036	0.121	0.271
FBS (mg/dl)	0.279	0.437	**0.330**	0.125	**−0.475**	−0.442	**−0.404**	−0.282	−0.169	−0.271	**0.649**	**0.635**
Insulin (μIU/ml)	0.220	0.324	**0.579**	0.468	−0.308	−0.198	**−0.465**	−0.308	−0.177	−0.293	**0.696**	**0.539**
HOMA-IR	0.194	0.309	**0.370**	0.165	**−0.404**	−0.363	**−0.384**	−0.190	−0.255	−0.425	**0.643**	**0.540**
HbA1c (%)	**0.396**	**0.649**	**0.396**	0.345	**−0.345**	−0.379	**−0.437**	−0.432	−0.068	0.005	**0.625**	**0.613**
TG (mg/dl)	**0.322**	0.346	**0.524**	0.543	−0.041	0.295	**−0350**	−0.450	−0.025	−0.031	**0.593**	**0.619**
TC (mg/dl)	−0.001	−0.018	**0.345**	0.491	−0.294	−0.088	−0.256	−0.089	−0.048	−0.086	0.376	0.245
LDL-C (mg/dl)	0.079	0.143	0.300	0.035	**−0.438**	−0.313	−0.079	0.260	−0.256	−0.397	0.086	−0.142
HDL-C (mg/dl)	−0.123	−0.150	−0.154	0.100	0.179	0.074	−0.127	0.018	0.225	0.223	−0.073	−0.40
Urea (mg/dl)	0.059	0.366	0.102	−0.153	**−0.314**	−0.173	0.124	−0.059	−0.112	−0.268	0.228	0.079
Creatinine (mg/dl)	−0.007	−0.044	−0.027	−0.256	−0.185	−0.153	0.033	0.086	−0.079	−0.335	−0.073	−0.005
AST (U/L)	0.052	−0.199	0.263	0.534	0.078	−0.081	−0.010	−0.127	−0.079	0.397	−0.143	−0.046
ALT (U/L)	0.070	−0.232	**0.390**	0.447	−0.083	−0.107	0.106	0.213	−0.096	0.070	0.061	−0.103

r1 unadjusted, r2 adjusted for BMI. All bolds are statistically significant (P value <0.05)

Results of oxidative stress marker showed that T2DM patients had significantly higher MDA ($p = 0.001$) and TOS ($p < 0.001$) levels than the healthy control subjects (Fig. 1a, b). However, TAC were significantly lower in T2DM in comparison with control groups ($p = 0.01$) (Fig. 1c). Also, SOD activity in patients with T2DM were significantly lower when compared to healthy individuals ($p < 0.001$) (Fig. 2a). GPx activity of diabetic patients were lower than control subject however is not significant ($p = 0.28$) (Fig. 2b).

Furthermore, IL-6 concentration was significantly higher in diabetic patients in comparison to healthy subjects ($p = 0.004$) (Fig. 3).

The relationship between the parameters was investigated before and after adjustment with BMI.

Correlation of parameters showed that MDA is positively correlated with HbA1c and TG ($p < 0.05$). There was a significant positive correlation between TOS with BMI, SBP, FBS, Insulin, HOMA-IR, HbA1c, TG, TC and ALT ($P < 0.05$). TAC inversely was associated with BMI, FBS, HOMA-IR, HbA1c, LDL-C and urea ($p < 0.05$). Significant negative correlation was observed between SOD with FBS, Insulin,

HOMA-IR, HbA1c and TG ($p < 0.05$). Also, IL-6 level was positively associated with FBS, Insulin, HOMA-IR, HbA1c and TG ($p < 0.05$) (Table 3).

In addition, as represented in Table 4 IL-6 level was positively correlated with MDA and TOS ($p < 0.05$). However, there was a significant negative correlation between IL-6 level and SOD activity ($p < 0.05$).

Discussion

This is the first study to examine oxidative stress and inflammation status in newly diagnosed type 2 diabetes patients and correlated these parameters with anthropometric and biochemical indices. Our study revealed that oxidative stress markers including MDA and TOS and IL-6 level increased in newly diagnosed T2DM subjects; these results were in agreement with previous reports in human and rat studies [3, 14, 15]. Hyperglycemia, the most important characteristic of diabetes, is the main reason of increased production of free radicals and inflammation. Another possibility is activation of

Table 4 Correlation coefficients between IL-6 concentration and oxidative stress markers

	MDA		TOS		TAC		SOD		GPx	
	r1	r2	r1	r2	r1	r2	r1	r2	r1	r2
IL-6 concentration	**0.482**	**0.564**	**0.744**	**0.620**	−0.410	−0.225	**−0.660**	**−0.601**	0.038	0.038

r1 unadjusted, r2 adjusted for BMI. All bolds are statistically significant (P value <0.05)

phagocytic cells during DM. Activated phagocytic cells enhance free radicals through promotion of inflammation [16].

Our study indicated that TAC and antioxidant enzymes including SOD and GPx activity were reduced in T2DM. Several studies confirm our results [2]. Hisalkar et al. (2012) reported lower SOD and GPx activity in diabetic patients compared with healthy subjects [5]. Also, we previously reported lower genes expression of Mn-SOD and CAT in obese children [9]. However, some studies have reported contradictory results [14]. Suziy et al. (2009) reported increased antioxidant enzymes in T2DM [16]. Meanwhile, some reports have indicated that antioxidant enzymes do not change in diabetes [17]. Differences in results could be due to the age, size of the population, usage of anti-diabetic drugs, and duration of the disease in different studies [7, 9, 16, 17]. There has been some reason for decreased antioxidant enzymes level in diabetes such as neutralization of high levels of oxidative stress by antioxidant enzymes, glycation, and inactivation of GPx due to hyperglycemia, decreased levels of Zn^{2+} and Cu^{2+} (cofactors of SOD), and reduction in GSH (substrate and a cofactor of GPX) concentration during diabetes [1].

High levels of oxidative stress markers in diabetes reduce the expression of antioxidant enzymes through oxidation of transcription factors. Meanwhile, reduced activity of antioxidant enzymes leads to elevation of ROS markers and lipid peroxidation [17].

Decreased SOD activity is accompanied by reduced GPx activity. GPx activity diminishes due to decreased production of H_2O_2 by SOD. Furthermore, increased superoxide radicals could inactivate GPx. However, in our study decreased GPx activity in T2DM subjects was not significant. Perhaps it is because of increased insulin levels in diabetic patients. Insulin enhances NADPH production by stimulating pentose phosphate pathway. NADPH is necessary for GSH formation. GSH is another substrate of GPx. Therefore, increased GSH may compensate H_2O_2 deficiency [3].

The positive correlation between oxidative stress markers and IL-6 levels with FBS and HbA1c suggests that control of blood glucose could relieve diabetes complications through reducing the oxidative stress and inflammation [3, 18].

Also, oxidative stress markers were correlated with TG, TC, and ALT. So, increased oxidative stress is associated with an increase in the risk of cardiovascular and hepatic diseases [19].

Our study revealed a positive and negative correlation between IL-6 level with oxidative stress markers and SOD activity, respectively. ROS increases the expression of inflammatory cytokines such as IL-6 by activating nuclear factor kappa B (NFκB) and activator protein-1 (AP-1). Further, macrophage infiltration into adipose tissue in diabetes stimulates the release of inflammatory cytokines with increasing the ROS production [18]. On the other hand, SOD mitigates inflammation through NFκB and AP-1 downregulation [20].

In conclusion, hyperglycemia leads to increased oxidative stress and inflammation in diabetic patients. So, hyperglycemic control may improve oxidative stress and inflammation, thereby further relieving diabetes complications.

Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

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