

Oxidant/antioxidant status in Type-2 diabetes mellitus patients with metabolic syndrome

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Background: The concurrence of metabolic syndrome (MS) and diabetes mellitus (DM) is increasing worldwide. The long-term complications of these chronic diseases are a threat to patients' well-being. Oxidative stress is involved in the pathogenesis of several diseases. To understand the basic pathophysiological mechanisms of Type-2 DM (T2DM) and its related complications, we aimed to investigate the oxidant/antioxidant status and Na⁺-K⁺ ATPase activity in T2DM with MS. **Materials and Methods:** A population of ninety individuals including fifty patients diagnosed with T2DM and MS, but without overt diabetes complications, and forty individuals without T2DM or MS as control group participated in this study. Plasma malondialdehyde (MDA), catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (GPx) activities, total antioxidant capacity (TAC), and Na⁺-K⁺ ATPase activity were assessed by standard laboratory methods. **Results:** Plasma MDA in patients group was statistically significantly higher than that of controls ($P \leq 0.05$). Whereas, Na⁺-K⁺ ATPase activity was statistically significantly lower in patient group ($P \leq 0.05$). TAC, CAT, SOD, and GPx enzyme activities were not statistically significantly different between two groups ($P > 0.05$). Results from the patient group showed positive correlations between CAT activity and triglyceride and positive correlations between GPx activity and weight, body mass index (BMI), and waist circumference. In addition, there was a positive correlation between MDA results with high-density lipoprotein-cholesterol (HDL-C) and total cholesterol and a negative correlation with TAC, BMI, and weight ($P \leq 0.05$) in controls. **Conclusion:** Because T2DM patients were without any vascular complications, antioxidant defense results may reflect the lack of progression of diabetes complications in these patients. These results emphasize the need for initial and continued assessment of cardiovascular disease risks in diabetic individuals. Implementation of timely interventions may improve the management of diabetes and prevent the progression of diabetes complications.

Key words: Diabetes mellitus Type 2, metabolic syndrome, oxidative stress, sodium-potassium-exchanging ATPase

How to cite this article: Najafi A, Pourfarzam M, Zadhoush F. Oxidant/antioxidant status in Type-2 diabetes mellitus patients with metabolic syndrome. J Res Med Sci 2021;26:6.

INTRODUCTION

Type 2 diabetes mellitus (T2DM) refers to metabolic dysregulations recognized by chronic hyperglycemia along with carbohydrate, lipid, and protein metabolism disorders. According to International Diabetes Federation reports, the global number of people with diabetes would increase to 592 million people by 2035. It has been estimated that the prevalence of diabetes in Iran would increase from 8.4% of the total national population in 2013 to 12.3% in 2035.^[1] Metabolic syndrome (MS) is described by adverse cardiometabolic risk factors which increase the risk of developing T2DM

and cardiovascular disease.^[2] Recent findings suggest an emerging high prevalence of MS among the Iranian middleaged population, which is significantly higher than those reported for the general population of Iran.^[3]

A possible role of oxidative stress in concurrent T2DM and MS has been investigated. In fact, progression of insulin resistance (IR), β -cell dysfunction, mitochondrial dysfunction, and diabetes complications are among disorders that have been related to oxidative stress.^[4] Oxidative stress is defined as an imbalance between oxidant species production, such as reactive oxygen species (ROS) and reactive nitrogen species and enzymatic and nonenzymatic antioxidant system

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Quick Response Code: 	Website: www.jmsjournal.net
	DOI: 10.4103/jrms.JRMS_249_20

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Submitted: 09-Apr-2020; **Revised:** 26-May-2020; **Accepted:** 18-Aug-2020; **Published:** 28-Jan-2021

protection capability. Oxidative stress causes damage to tissues and cells by molecular alteration of cell components. Association of raised oxidative stress with cardiovascular, metabolic, inflammatory, and neurodegenerative disorders is well established.^[5]

Antioxidant defense systems include enzymatic and nonenzymatic antioxidants. Non-enzymatic ROS scavengers include glutathione; Vitamins A, C, and E; and several antioxidants present in the diet. Enzymatic antioxidant defense systems include superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx). In addition, malondialdehyde (MDA) is an end product of lipid peroxidation, which is a marker of oxidative stress.^[6]

Free radicals and lipid peroxidation alter the cellular structure of membrane-bound proteins by changing phospholipids and fatty acid composition. In addition, chronic hyperglycemia may alter the glycosylation state of membrane-bound glycosylated proteins, resulting in decreased protein activity.^[7] One of the major membrane-bound enzymes which regulate the balance of electrolytes is Na⁺-K⁺ ATPase which is associated with insulin action. Alteration of this enzyme is thought to be linked to several complications of DM.^[8]

In this study, we aimed to provide further insights into the basic pathophysiological mechanisms of T2DM and its related complications by investigating the changes in oxidative status, erythrocyte membrane, and blood lipid profile in a group of Iranian patients with concurrent T2DM and MS, through determining antioxidant status and estimating erythrocyte Na⁺-K⁺ ATPase enzyme activity. This may be exploited to improve the management of consequences of these diseases.

MATERIALS AND METHODS

Study population

The study included fifty recently diagnosed T2DM patients with concurrence MS and forty sex- and age-matched normoglycemic apparently healthy controls. Diagnosis of diabetes was established based on the criteria proposed by the American Diabetes Association (fasting glucose ≥ 126 mg/dL or 2 h postprandial glucose ≥ 200 mg/dL, or if they were taking oral anti-diabetic medication but not insulin).^[9] Oral antidiabetic medications for T2DM patients were metformin or combined formulations of metformin/glibenclamide. MS was defined according to the NCEP Adult Treatment Panel III (ATP III). According to ATP III criteria, a patient is classified as having MS when three or more of the following risk determinants are present: hyperglycemia (fasting plasma glucose ≥ 100 mg/dL), hypertriglyceridemia (triglyceride ≥ 150 mg/dL), low high

density lipoprotein-cholesterol (HDL-C < 40 mg/dL for men and < 50 mg/dL for women), blood pressure elevation ($\geq 130/85$ mmHg), and increased waist circumference (WC ≥ 102 cm for men and ≥ 88 cm for women).^[10] Individuals taking medications known to influence the Na⁺-K⁺ ATPase enzyme activity, lipid-lowering agents, insulin, or smoking cigarettes or other tobacco products were excluded from the study.^[11] However, oral antidiabetic medications were acceptable. Written informed consent was obtained from each participant, and the study was approved by the Ethics Committee of Isfahan University of Medical Sciences.

Anthropometric measurements and blood pressure

The participants' weight, height, WC (at umbilicus) and hip circumference (HC) (at widest point), waist/hip circumference ratio (WHR), and body mass index (BMI) were measured according to standard conditions. The means of systolic and diastolic blood pressure (SBP and DBP), which determined three times by a single person at 2–5 min intervals, were considered as the blood pressure.

Blood collection and processing

After at least 10 h overnight fasting, venous blood samples were collected in heparinized, ethylenediaminetetraacetic acid (EDTA), and plain test tubes. The blood samples were centrifuged at $1500 \times g$ at 25°C for 10 min, and then serum and plasma were separated into plain test tubes. Serum samples were processed for fasting blood glucose (FBG), triglycerides (TG), total cholesterol (TC), low-density lipoprotein-cholesterol (LDL-C), HDL-C, fasting insulin (FINS), and high-sensitivity C-reactive protein (hs-CRP) measurement. EDTA blood samples were used for hemoglobin A1c (HbA1c) determination. Heparinized blood samples were used for the preparation of washed packed erythrocytes to measure membrane Na⁺-K⁺ ATPase activity and protein concentration.

Biochemical assays

FBG was measured by enzymatic colorimetric method using glucose oxidase test (Parsazmun, Alborz, Iran). HbA1c was analyzed by immunoturbidimetric latex method using kit (Parsazmun, Alborz, Iran), and hs-CRP was measured with a latex-enhanced immunoturbidimetric assay using an automated analyzer (Parsazmun, Alborz, Iran). Serum TC, TG, and HDL-C were determined by enzymatic methods using a Hitachi 902 automated analyzer (Parsazmun, Alborz, Iran). Serum LDL-C was calculated using Friedewald's formula. If serum TG concentration was > 400 mg/dL, LDL-C was determined directly by enzymatic method using the commercial kit (Parsazmun, Alborz, Iran). Serum FINS was measured using enzyme-linked immunosorbent assay kit according to manufacturer's instructions (Monobind, California, USA). IR was estimated using the HOMA-IR index according to the following formula: HOMA-IR =

Fasting serum insulin ($\mu\text{U/mL}$) \times fasting plasma glucose (mmol/L)/22.5.^[12]

Plasma malondialdehyde assay

Plasma MDA was assessed using a commercial assay kit (Kiazist, Hamedan, Iran). Briefly, reaction between MDA and thiobarbituric acid in 95°C produces a colored complex assessable at 540 nm. Plasma MDA concentration was calculated by reference to a standard curve. The results are reported as nmol/ml .

Plasma total antioxidant capacity assay

Plasma total antioxidant capacity (TAC) was assessed by commercial kit (Kiazist, Hamedan, Iran). The assay is based on the reduction of Cu^{+2} by plasma antioxidants to Cu^{+1} in the presence of a chromogen reagent to produce a colored complex which is measured at 450 nm. Plasma TAC value was estimated by reference to Trolox standard curve. The results are reported as $\text{nmol of trolox equivalent/mL}$.

Plasma superoxide dismutase activity assay

Plasma SOD activity was estimated using a commercial kit (Kiazist, Hamedan, Iran). Briefly, resazurin reagent is used for the detection of superoxide radicals generated by xanthine oxidase by colorimetric evaluation at 570 nm. The results are reported as U/mL of SOD activity.

Plasma catalase activity assay

Plasma CAT activity was assessed using a commercial assay kit (Kiazist, Hamedan, Iran). Briefly, reaction of enzyme with methanol yields formaldehyde which reacts with purpald dye to produce a colored complex measurable at 570 nm. The results are reported as mU/mL of CAT activity.

Plasma glutathione peroxidase activity assay

The GPx activity was assessed using a commercial assay kit (ZellBio, Germany). Briefly, the enzyme converts glutathione to oxidized glutathione. The remaining glutathione then reduces 5,5-dithio bis (2-nitrobenzoic acid) or DTNB to generate a yellow color measured at 412 nm. The GPx activity (U/mL) of the samples is indirectly proportional to the color intensity.

Erythrocyte ghost preparation and $\text{Na}^+\text{-K}^+$ ATPase activity measurement

Erythrocyte packed cells were suspended and lysed by 10 volumes of ice-cold $5\text{ mM Tris}/0.1\text{ mM Na}_2\text{ EDTA}$, $\text{pH } 7.6$. After centrifugation at $20,000 \times g$ for 20 min at 4°C , the supernatant was discarded and the pellet was washed three times in $0.017\text{ M NaCl}/5\text{ mM Tris-HCl}$, ($\text{pH } 7.6$) and three times with 10 mM Tris-HCl ($\text{pH } 7.5$). Erythrocyte ghost was resuspended in 10 mM Tris-HCl buffer ($\text{pH } 7.5$). Erythrocyte membrane protein was measured according to

the modified method of Markwell *et al.* based on the reaction of the Folin–Ciocalteu reagent with protein by using bovine serum albumin as standard^[13] protein concentration. The erythrocyte membrane total ATPase activity was determined as it was described previously.^[14] A volume of $50\ \mu\text{l}$ erythrocyte membrane suspension was incubated with 5 mmol/L ATP , 25 mmol/L KCl , 75 mmol/L NaCl , 5 mmol/L MgCl_2 , 0.1 mmol/L EGTA , and $25\text{ mmol/L Tris-HCl}$ ($\text{pH } 7.5$) in a total volume of $500\ \mu\text{l}$ for 90 min at 37°C . The reaction was inhibited by adding trichloroacetic acid to a final concentration of 5% (w/v). After centrifugation for 20 min at $1500 \times g$, a supernatant aliquot was used to measure total inorganic phosphorus amount liberated by Fiske and Subbarow reaction.^[15] The assay was repeated in 1 mmol/L ouabain presence, an inhibitor of $\text{Na}^+\text{-K}^+$ ATPase activity. Total ATPase activity was reported as μmol of inorganic phosphorus liberated per milligram of membrane protein per hour. $\text{Na}^+\text{-K}^+$ ATPase activity subsequently determined by subtracting the ATPase activity in ouabain presence from the total $\text{Na}^+\text{-K}^+$ ATPase activity in ouabain absence.

Statistical analysis

Data were expressed as mean \pm standard deviation. IBM SPSS Statistics for Windows, Version 20.0 (Armonk, NY: IBM Corp.) was utilized for all statistical analyses. The normal variable distribution was checked by Kolmogorov–Smirnov test. Baseline characteristics of controls and cases were compared using independent sample *t*-tests for continuous variables and Mann–Whitney U-test for nonparametric variables. The equality of variances was calculated with the Levene’s test. The bivariate correlations between quantitative variables were determined by the Pearson’s correlation (for parametric data) and Spearman’s test (for nonparametric data). $P < 0.05$ were considered statistically significant.

RESULTS

Characteristics of the study participants

The patient group had statistically significantly higher weight, WC, HC, WHR, BMI, SBP, DBP, insulin, FBG, HbA1c, HOMA-IR, and TG and lower HDL-C levels in comparison to control group ($P < 0.05$). No statistically significant differences ($P > 0.05$) in the serum levels of hs-CRP, LDL-C, and TC were observed between the two groups of study [Table 1].

Systemic oxidative stress

Plasma MDA levels were statistically significantly elevated ($P < 0.05$) in patients. In contrast, we did not find any statistically significant differences ($P > 0.05$) in plasma CAT, SOD, and GPx activities and TAC value between the two groups [Table 2].

Table 1: Characteristics of the study participants

Parameter	Control (n=40)	T2DM with MS (n=50)	P
Age (year)	54.24±5.11	55.44±7.03	0.406
Weight (kg)	76.09±12.38	84.54±13.04	0.003
WC (cm)	95.22±10.5	105.52±9.91	0.000
HC (cm)	99.327±9.25	106.09±7.26	0.001
WHR	0.96±0.094	0.99±0.050	0.05
BMI (kg/m ²)	22.69±3.43	24.85±0.3.54	0.007
SBP (mmHg)	123.16±14.44	130.75±13.44	0.015
DBP (mmHg)	79.45±9.74	79.65±17.15	0.947
FBG (mg/dL)	96.66±10.62	157.02±42.05	0.000
A1c (%)	5.46±0.41	7.50±±1.59	0.000
HOMA-IR	2.67±1.30	5.46±3.74	0.000
TC (mg/dL)	192.16±30.37	193.18±44.92	0.904
TG (mg/dL)	126.19±31.16	203.82±85.29	0.000
HDL-C (mg/dL)	46.37±5.71	35.96±6.19	0.000
LDL-C (mg/dL)	117.5±24.57	114.57±33.61	0.653
FINS (U/L)	11.3±5.18	13.79±6.69	0.04
Hs-CRP (mg/L)	1.77±1.89	2.12±1.54	0.366

Data are expressed as mean±SD. Significant P values are shown in bold. BMI=Body mass index; DBP=Diastolic blood pressure; FBG=Fasting blood glucose; FINS=Fasting insulin; HbA1c=Hemoglobin A1c; HC=Hip circumference; HDL-C=High-density lipoprotein-cholesterol; HOMA-IR=Homeostasis Model Assessment of Insulin Resistance; hs-CRP=High-sensitivity C-reactive protein; LDL-C=Low-density lipoprotein-cholesterol; SBP=Systolic blood pressure; TC=Total cholesterol; TG=Triglyceride; WC=Waist circumference; WHR=Waist-hip ratio; SD=Standard deviation; T2DM=Type-2 diabetes mellitus; MS=Metabolic syndrome

Table 2: Oxidative stress markers and Na⁺/K⁺ATPase activity of the study participants (n=90)

Parameter	Controls (n=40)	T2DM with MS (n=50)	P
Na ⁺ -K ⁺ ATPase activity (μM P/mg protein/h)	0.64±0.21	0.52±0.24	0.019
MDA (nmol/mL)	345.32±52.54	379.09±81.40	0.023
TAC (nmol/mL)	1138.16±220.21	1084.39±214.04	0.267
CAT (mU/mL)	177.9±20.66	179.42±21.55	0.755
SOD (U/mL)	14.12±0.75	13.92±1.18	0.373
GPX (U/mL)	305.02±110.84	275.99±113.59	0.249

Data are expressed as mean±SD. Significant P values are shown in bold. MDA=Malondialdehyde; TAC=Total antioxidant capacity; CAT=Catalase; SOD=Superoxide dismutase; GPX=Glutathione peroxidase; SD=Standard deviation; T2DM=Type-2 diabetes mellitus; MS=Metabolic syndrome

SOD activity correlated positively with CAT in the control group ($P = 0.004$) and in all the individuals ($P = 0.046$). In addition, in patient group, TAC correlates positively with MDA levels ($P = 0.025$) (data are not shown).

Erythrocyte Na⁺-K⁺ ATPase activity

Statistically significant decrease ($P = 0.019$) in erythrocyte membrane Na⁺-K⁺ ATPase activity (20.3%) was observed in patients compared to control group [Table 2].

Relationships between individual characteristics, insulin resistance, oxidative stress markers, and Na⁺-K⁺ ATPase activity
Bivariate Pearson's correlation between the study participants' main characteristics is summarized in

Table 3. CAT activity correlated positively with TG levels in patients and all participants. GPx activity represented a positive correlation with weight, BMI, and WC in patient group and close to significance correlation with HDL-C and TC levels in all participants. MDA had positively correlated with HDL-C and TC and negatively correlated with WC and BMI in the control group. No correlation was observed between any of the oxidative stress markers and HOMA-IR. In addition, Na⁺-K⁺ ATPase activity did not indicate any association with IR and MS components and oxidative stress markers (data are not shown).

DISCUSSION

In the present study, plasma levels of MS components including SBP, FBG, WC, TG, and HDL-C level, in addition to HOMA-IR and BMI, showed significant differences between the study groups [Table 1]. Ruderman *et al.* showed the association of IR with some MS components, including FBG, obesity, dyslipidemia, and hypertension.^[6] Consistent with this study, we obtained significantly positive correlations between HOMA-IR and TG, WC, BMI, and hs-CRP and a significantly negative correlation with HDL-C in all participants. In addition, in patient group, there was a significant positive correlation between HOMA-IR and hs-CRP, which acts as an independent risk factor of cardiovascular disease.^[11]

Oxidative stress has been investigated in metabolic disorders in a number of studies. Evidences suggest that oxidative stress is associated with DM, both in pre-diabetes state and clinical phase. IR, β-cell dysfunction, and mitochondrial dysfunction are among complications linked with oxidative environment created in diabetes.^[4] Concurrent T2DM and MS has a relationship with the augmentation of oxidative stress. Hyperglycemia, hyperinsulinemia, and elevated free fatty acids can induce ROS production.^[17,18]

During diabetes state, levels of superoxide ion (O₂⁻) increase in several tissues because of hyperglycemia and hyperlipidemia. Indeed, O₂⁻ is known as one of the hallmarks of diabetes.^[19] O₂⁻ is also responsible for low bioavailability of nitric oxide. Oxidative stress has reported to intensify in MS cases.^[20] In addition, a considerably amplified number of neutrophils, lymphocytes, monocytes, and eosinophils, caused by MS, are observed in T2DM patients with MS.^[21] Activation of immune cells consequently may produce high level of ROS, which erode lipids, proteins, and nucleic acids.

In this study, plasma MDA, a lipid peroxidation marker, was significantly elevated in patient group compared to controls. Although there were negative correlations between MDA and BMI, and weight and positive correlation with

Table 3: Pearson's correlation coefficient between characteristics in all the participants

	Insulin	HOMA-IR	TG	LDL-C	HDL-C	WC	BMI	hs-CRP
FBG	0.261* (0.019)	0.707** (0.000)	0.437** (0.000)	0.087 (0.424)	-0.371** (0.000)	0.250* (0.023)	0.159 (0.151)	0.141 (0.22)
Insulin		0.812** (0.000)	0.197 (0.08)	-0.129 (0.25)	-0.332** (0.002)	0.359** (0.001)	0.335** (0.003)	0.256* (0.027)
HOMA-IR			0.329** (0.003)	-0.033 (0.769)	-0.361** (0.001)	0.287* (0.011)	0.244* (0.032)	0.295* (0.01)
TG				0.062 (0.568)	-0.393** (0.000)	0.165 (0.138)	0.128 (0.251)	0.111 (0.335)
LDL-C					0.461** (0.000)	-0.02 (0.86)	-0.034 (0.763)	0.138 (0.227)
HDL-C						-0.318** (0.003)	-0.235* (0.032)	-0.053 (0.648)
WC							0.874** (0.000)	0.188 (0.106)
BMI								0.221 (0.056)

Significant *P* values are shown in bold. **P*<0.05; ***P*<0.01. HOMA-IR=Homeostasis Model Assessment of Insulin Resistance; HDL-C=High-density lipoprotein-cholesterol; hs-CRP=High-sensitivity C-reactive protein; LDL-C=Low-density lipoprotein-cholesterol; BMI=Body mass index; TG=Triglyceride; WC=Waist circumference; FBG=Fasting blood glucose

HDL-C and TC in control group, such a correlation was not obvious among patient group or in all participants.

Furthermore, no significant difference was observed in TAC, SOD, CAT, and GPx activities between two groups. According to our study design, patients participated in the study were without diabetes complications. Because oxidative stress markers in T2DM patients with micro- and macrovascular complications show significant difference in comparison to those without complications,^[22] oxidative stress status potentially associates with complication occurrence.

Investigation of antioxidant enzymes' relationship with aging and diseases in human studies has resulted in inconsistent data. Although association of antioxidant enzymes polymorphism with T2DM suggested by meta-analyses, reduced or increased activities (or levels) of SOD, CAT and GPx have been reported in these diseases.^[6] In a previous study, Cardona *et al.*, despite a difference in GPx activity in serum sample of MS patients with controls, did not observe difference between SOD and CAT activity results.^[23] In a study performed in Iran, it was found that serum SOD activity significantly decreased in patients with MS, but not significantly different in persons with high body fat percentage and those with DM.^[24] In another study, serum SOD activity of MS cases was lower than that of control group, but GPx activity did not indicate any significant difference.^[25] While a study performed in Nigeria found SOD and GPx activities decreased and MDA elevated in MS Patients, CAT activity remained unchanged.^[26] In an obese and diabetic mouse model, activity and expression of CAT, SOD, and GPx were lower in the adipose tissue compared to control group. However, the results were unchanged in skeleton muscles and liver.^[27]

Compensatory upregulation of antioxidant enzymes has been suggested as a response to elevated oxidative

stress. Regarding T2DM patients, increased MDA levels were found simultaneously with higher CAT and/or SOD activities. Moreover, unchanged or reduced CAT and/or GPx and elevated SOD activities and lipid peroxidation markers were reported.^[6]

We found a significant reduction in erythrocyte membrane Na⁺-K⁺ ATPase activity in patient group compared to controls. Previously, Jain *et al.* showed that incubation of human *red blood cells* (RBCs) in higher glucose concentration leads to decreased Na⁺-K⁺ ATPase activity, increased lipid peroxidation, and protein glycosylation of RBC membranes *in vitro*.^[7] Lipid peroxidation products and ROS potentially cause protein oxidative damage and decrease the enzyme activity.^[11] Reduced Na⁺-K⁺ ATPase activity induced by diabetes may be involved in the pathogenesis of diabetes complications. Nonetheless, we did not find any correlation between MS component with Na⁺-K⁺ ATPase enzyme activity.

In the present study, no significant association was found between Na⁺-K⁺ ATPase activity and oxidative stress markers in the study groups. However, a statistically significant negative correlation was observed between MDA and TAC in the control group ($r = -0.361$, $P = 0.03$).

Finally, there are some limitations to our study. Participants should be assessed for natural antioxidant consumption supplied by their diet. Studies on Mediterranean-style diet intervention demonstrate effects on reducing oxidative stress and IR in MS patients.^[28] In addition, determination of Iranian dietary antioxidants intake could contribute in evaluating redox system more specifically. According to the signaling function of Na⁺-K⁺ ATPase, association of signaling components and the enzyme with redox system and metabolic disorders should be investigated.

CONCLUSION

Because the diabetic patients in our study were without any complications of DM, it seems that their antioxidant status did not have significant difference with the control group. Due to the potential association of complications of DM with undermining antioxidant protection, these results highlight the early diagnostic importance of DM and MS.

Acknowledgments

The authors thank all of the patients who participated in this study.

Financial support and sponsorship

This study was supported by grants from Isfahan University of Medical Sciences (grant no. 397683).

Conflicts of interest

There are no conflicts of interest.

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