SERUM REDOX MARKERS IN UNCOMPLICATED TYPE 2 DIABETES MELLITUS ACCOMPANIED WITH ABNORMAL IRON LEVELS

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SUMMARY

Objectives: This study aimed at evaluating the serum redox status in type 2 diabetes mellitus (T2DM) accompanied with an imbalance in iron concentrations.

Methods: Diabetic patients were grouped according to serum iron levels [normal (DNFe), low (DLFe), and high (DHFe)], and their clinical and redox parameters [total sulfhydryl groups (tSH), uric acid (UA), and total bilirubin (tBILI) as non-enzymatic antioxidants, and malondialdehyde (MDA) and advanced oxidation products of proteins (AOPP) as markers of oxidative stress] were determined.

Results: Glucose and HbA1c levels in the T2DM patients did not differ in function of serum iron. T2DM was associated with reduced tSH levels. In the diabetic patients, tSH, UA, and tBILI negatively correlated with MDA, as well as HbA1c with UA. Accordingly, AOPP and MDA were higher in the diabetic groups compared to the controls. The reduced antioxidant capacity was particularly pronounced in the DLFe group, which was further characterized by lower levels of UA and tBILI compared to the other groups. Subsequently, the level of MDA in the DLFe group was higher compared to the DNFe and DHFe groups. The positive correlation between serum iron levels and the antioxidants UA and tBILI, in conjunction with the negative correlation between serum iron levels and the markers of oxidative stress in the diabetic patients, corroborated the indication that comparatively higher level of oxidative stress is present when T2DM coexists with decreased iron levels.

Conclusions: T2DM-associated redox imbalance is characterized by a decrease in serum total sulfhydryl groups and low serum iron-associated reduction in uric acid and total bilirubin levels, accompanied by increased oxidative stress markers. The relatively noninvasive and simple determination of these parameters may be of considerable interest in monitoring the pathophysiological processes in T2DM patients, and may provide useful insights into the effects of potential therapeutic or nutritional interventions.

Key words: type 2 diabetes mellitus, serum iron, oxidative stress, total sulfhydryl groups, uric acid, total bilirubin

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INTRODUCTION

Type 2 diabetes mellitus (T2DM) is a progressive disease characterized by deterioration in β-cell function and insulin resistance. This leads to impaired blood glucose level control and increased risk of development of different complications. Hyperglycaemia has been associated with oxidative stress, a state of loss of balance between oxidants and antioxidants, caused by accumulation of reactive oxygen species (ROS) and cumulative damage to the complex biomolecules within the cells. Oxidative stress leads to significant chemical modifications of cellular lipids and proteins. The alterations of proteins include polypeptide fragmentation and modification of amino acid side chains leading to the formation of hydroxyl or carbonyl derivatives (1). Membrane lipid damage occurs via the process of peroxidation, generating various end products (aldehydes, hydroxy-alkenals, ketones, etc.), which can disturb normal cellular processes (2). Hyperglycaemia-associated oxidative stress is specifically attributed to the auto-oxidative glycosylation, formation of advanced glycation end products, as well as impairment of the antioxidant mechanisms (3).

Free iron is another potential contributor to oxidative stress. In its unbound form, the redox-active iron (Fe^{2+}) is a potent pro-oxidant, and has been shown to cause biomolecule oxidation by participating in the formation of harmful ROS via the Fenton and Haber-Weiss reactions (4). Both increased and decreased iron levels may lead to oxidative stress and cellular damage. In T2DM patients, free serum iron has been linked with uncontrolled diabetes, contributing to oxidative stress, which may affect the development of chronic diabetic complications (5). On the other hand, iron deficiency impairs the production of iron-containing

antioxidant enzymes, as well as the mitochondrial ATP production, leaning the balance towards pro-oxidative state in cells (6).

T2DM alters the availability of redox-active iron by its mobilization from iron stores or by disturbance of the protective mechanisms that restrict the release of free iron (7). This is accompanied by reduced serum total antioxidant level, particularly of its main determinant – the level of uric acid (UA), as well as by decreased levels of bilirubin, serum free thiols, glutathione, vitamin C, and vitamin E, which are also important contributors to the serum non-enzymatic antioxidant capacity (8–10). Hence, in the settings of T2DM, along with the impaired glucose control, the complex interrelations between serum antioxidants, circulating iron levels, and oxidative stress become abnormal (7).

There is a consensus that the state of T2DM and the abnormal serum levels of iron could independently cause increased oxidative stress, which may lead to increased morbidity and death. However, there are a limited number of studies on patients that simultaneously exhibit both types of disorders. Thus, the aim of this study was to evaluate serum redox markers in T2DM accompanied with an imbalance in serum iron concentrations. The serum levels of malondialdehyde (MDA) and the advanced oxidation products of proteins (AOPP) were measured as markers of oxidative stress, while the concentration of total sulfhydryl groups (tSH), uric acid, and total bilirubin (tBILI) were determined as serum non-enzymatic antioxidants.

MATERIALS AND METHODS

Patients and Study Design

This study included 128 subjects from the Republic of Macedonia, of mixed ethnicity, living in the Polog region. The inclusion criteria were: subjects of either gender, aged between 30 and 66 years, healthy (for the control group) or diagnosed with type 2 diabetes mellitus according to the WHO criteria (11), and taking oral antihyperglycaemic agent – Metformin (prescribed by their physicians). The exclusion criteria were: acute illness, cardiovascular disorders (hypertension, ischaemic heart disease), asthma, alcoholics, smokers, secondary endocrine disorders, autoimmune disorders, anaemia (due to causes other than iron deficiency), hereditary hemochromatosis, dysmetabolic iron overload syndrome, and the presence of other diabetic complications. Subjects taking steroids, antioxidants, vitamins, or other supplements on chronic basis were also excluded.

Informed consent was obtained from all subjects, as well as medical history, anthropometric measurements (to calculate the body mass index – BMI), and treatment details. For grouping purposes, the serum iron level was defined as low (< 7.3 μmol/L), normal (7.3–29.9 μmol/L), and high (> 29.9 μmol/L). Henceforth, the patients with T2DM were grouped according to serum iron levels: diabetic, with normal serum iron level (DNFe); diabetic, with low serum iron level (DLFe); diabetic, with high serum iron level (DHFe).

Haematological and Biochemical Parameters

The samples were collected at the PHI Health Centre – Gostivar, during the year 2021. Blood samples were drawn after 12-hour overnight fasting. Blood serum was obtained after centrifugation at 1,500 g for 15 minutes in a refrigerated centrifuge, aliquoted, and stored at −80 °C until further analysis.

An automated haematological counter Beckman Coulter LH 500 was used to determine the red blood cell count (RBC), concentration of haemoglobin (Hb), haematocrit value (HCT), and the mean corpuscular volume of erythrocytes (MCV) in each sample. The fully automated analyser Beckman Coulter AU480 was used to determine the standard biochemical parameters in serum: fasting blood glucose (Glu), glycated haemoglobin (HbA1c), total iron, total bilirubin, uric acid, and total protein.

The total sulfhydryl group content in serum was measured using the Ellman's reagent 5,5-dithio-bis-(2-nitrobenzoic acid) – DTNB. The reagent is reduced by thiols and gives a yellow derivative that absorbs at 412 nm (12). The reaction mixture contained sample, Tris-buffer, pH 8.2, and DTNB, and colour was developed after 15 minutes. A molar extinction coefficient of 13,100 $M^{-1}cm^{-1}$ was used in the calculation of tSH, and the results were expressed per g protein.

The concentration of MDA in blood serum was determined by the method described by Yagi (13), based on the formation of coloured product in the reaction between MDA and thiobarbituric acid (TBA). Solutions of 50% TCA and 1.3% TBA (dissolved in 0.3% NaOH) were added to a sample. After 20-minute incubation period at a temperature of 90–95 °C in a water bath, the samples were instantly cooled on ice, followed by centrifugation at 4,000 g for 10 minutes. The absorbance of the supernatant was measured at a wavelength of 535 nm; 1,1,3,3-tetraethoxypropane was used as standard.

The concentration of AOPP in blood serum was determined by the method of Taylor et al., (14), based on the conversion of I^- to I_3^-. To each sample, 1.16 M KI solution was added, followed by glacial acetic acid 2 minutes later. The absorbance of the reaction mixture was measured after 10 minutes at a wavelength of 340 nm. AOPP concentration was calculated using the molar absorption coefficient of chloramine T.

Statistical Analysis

Data are presented as mean ± standard deviation. Considering the wide range of ages (30–66 years) of the subjects included in the study, the data were analysed by the two-way analysis of covariance (two-way ANCOVA), taking the "group in which the subjects are allocated" and the "gender" as factors, controlling for the "age" as covariate. In this way, the differences between the group means and the differences in function of the gender of the subjects were determined, as well as the interaction between these two factors. The Bonferroni correction was used for the multiple comparisons of group means. The correlation between the variables was tested using the Pearson's test. All analyses were performed using MedCalc Statistical Software version 14.8.1 (MedCalc Software bvba, Ostend, Belgium). P values less than 0.05 were considered statistically significant.

RESULTS

The anthropometric and clinical parameters of the subjects are shown in Table 1. The groups were age- and gender-matched.

The BMI did not differ significantly between the groups. Fasting glucose levels and HbA1c were significantly higher in the diabetic subjects compared to the controls (Table 2). However, there were no significant differences in the mean Glu or HbA1c levels between the diabetic groups. Iron deficiency (DLFe group) was characterized by lower RBC count, Hb, HCT, and MCV compared to the controls and/or to the other diabetic groups (Table 2). Expectedly, the haematological parameters Hb, HCT, and MCV were increased in function of serum iron level, i.e., the subjects in the DHFe group manifested elevated values of these parameters compared to the controls and to the DNFe group. Additionally, the RBC count, Hb, and HCT levels were significantly different in relation to gender (Table 2).

The serum redox status, represented by the levels of selected non-enzymatic antioxidants and the concentration of markers of oxidative stress are shown in Figure 1 and 2, respectively, and the significant correlations between these parameters are listed in Table 3. There was a T2DM-associated reduction in the levels of tSH, with the mean of the DLFe group having the lowest value, significantly lower compared to both the controls and the DNFe

Table 1. *Anthropometric and clinical parameters of the subjects*

Parameter	C	DNFe	DLFe	DHFe
Number of subjects	35	30	35	28
Gender, n (%)				
Males	17 (49)	16 (53)	17 (49)	15 (54)
Females	18 (51)	14 (47)	18 (51)	13 (46)
Age (years), mean (SD)	48.7 (9.3)	50.7 (8.9)	51.7 (8.6)	51.7 (8.9)
BMI (kg/m^2), mean (SD)	22.65 (1.62)	23.01 (2.47)	23.89 (1.92)	23.85 (2.03)
Treatment	–	+	+	+

C – healthy controls; DNFe – diabetic, normal serum iron; DLFe – diabetic, low serum iron; DHFe – diabetic, high serum iron; BMI – body mass index; treatment – oral antihyperglycaemic agent Metformin

Table 2. *Haematological and biochemical parameters of the subjects*

Parameter	C Mean (SD)	DNFe Mean (SD)	DLFe Mean (SD)	DHFe Mean (SD)	Groups compared	p-value
Serum iron (µmol/l)	17.03 (5.71)	17.81 (5.01)	5.48 (1.56)	33.92 (5.76)	C vs. DLFe C vs. DHFe DNFe vs. DLFe DNFe vs. DHFe DLFe vs. DHFe	< 0.0001 < 0.0001 < 0.0001 < 0.0001 < 0.0001
Glu (mmol/l)	5.68 (0.71)	11.13 (2.74)	10.57 (3.50)	9.59 (3.21)	C vs. DNFe C vs. DLFe C vs. DHFe	< 0.0001 < 0.0001 < 0.0001
HbA1c (%)	5.75 (0.67)	9.17 (2.04)	8.94 (2.06)	8.85 (1.94)	C vs. DNFe C vs. DLFe C vs. DHFe	< 0.0001 < 0.0001 < 0.0001
RBC (x10^6/µl) Males Females	4.85 (0.55) 5.13 (0.59) 4.62 (0.40)	5.14 (0.51) 5.34 (0.52) 4.89 (0.37)	4.51 (0.53) 4.95 (0.74) 4.44 (0.47)	5.23 (0.53) 5.36 (0.29) 4.99 (0.78)	DNFe vs. DLFe DHFe vs. DLFe Gender	< 0.0001 < 0.0001 < 0.001
Hb (g/l) Males Females	138.09 (19.59) 153.94 (10.37) 124.74 (14.96)	147.37 (13.46) 153.76 (11.80) 139.00 (10.89)	114.69 (15.09) 128.20 (15.74) 112.43 (14.00)	156.11 (13.30) 159.33 (7.72) 150.30 (18.96)	C vs. DLFe C vs. DHFe DNFe vs. DLFe DLFe vs. DHFe Gender	< 0.0001 < 0.0001 < 0.0001 < 0.001 < 0.001
HCT (l/l) Males Females	0.44 (0.06) 0.48 (0.05) 0.40 (0.06)	0.47 (0.04) 0.48 (0.04) 0.45 (0.03)	0.37 (0.05) 0.42 (0.05) 0.36 (0.04)	0.49 (0.03) 0.50 (0.02) 0.48 (0.05)	C vs. DLFe C vs. DHFe DNFe vs. DLFe DLFe vs. DHFe Gender	< 0.0001 < 0.0001 < 0.0001 < 0.0001 0.001
MCV (fl)	88.97 (5.56)	90.92 (4.83)	82.30 (7.94)	93.99 (2.81)	C vs. DLFe C vs. DHFe DNFe vs. DLFe DLFe vs. DHFe	< 0.0001 0.0098 0.0001 0.0001

C – healthy controls; DNFe – diabetic, normal serum iron; DLFe – diabetic, low serum iron; DHFe – diabetic, high serum iron; Glu – fasting blood glucose; HbA1c – glycated haemoglobin; RBC – erythrocytes; Hb – haemoglobin; HCT – haematocrit; MCV – mean corpuscular volume

group (Fig. 1). Additionally, the cases where T2DM was accompanied with low serum iron (DLFe group) also had the lowest concentration of the other two antioxidants – UA and tBILI (Fig. 1). Specifically, UA in the DLFe group was significantly reduced compared to the controls and to the other diabetic groups, while tBILI levels were higher in the DNFe and in the DHFe groups compared to the DLFe group.

The negative association between T2DM and the serum antioxidant status was supported by the negative correlation between UA and the glycaemic status (Glu and HbA1c) (Table 3). As expected, the reduced antioxidant levels in the serum of the diabetic subjects were associated with increased oxidative stress. Figure 2 shows that the concentration of AOPP was significantly higher in all diabetic groups compared to the controls. The rate of lipid peroxidation, reflected by MDA levels, was higher in the cases where T2DM was accompanied with abnormal serum iron (DLFe and DHFe groups) compared to both the DNFe group and the controls (Fig. 2). In support of this, MDA negatively correlated with the non-enzymatic antioxidants – tSH, UA, and tBILI in the diabetic subjects (Table 3). Furthermore, the positive correlation between the serum iron level and the antioxidants UA and tBILI, in conjunction with the negative correlation between the serum iron level and the markers of oxidative stress – MDA and AOPP in the diabetic subjects (Table 3), corroborated the indication that comparatively higher level of oxidative stress is present when T2DM coexists with low serum iron level.

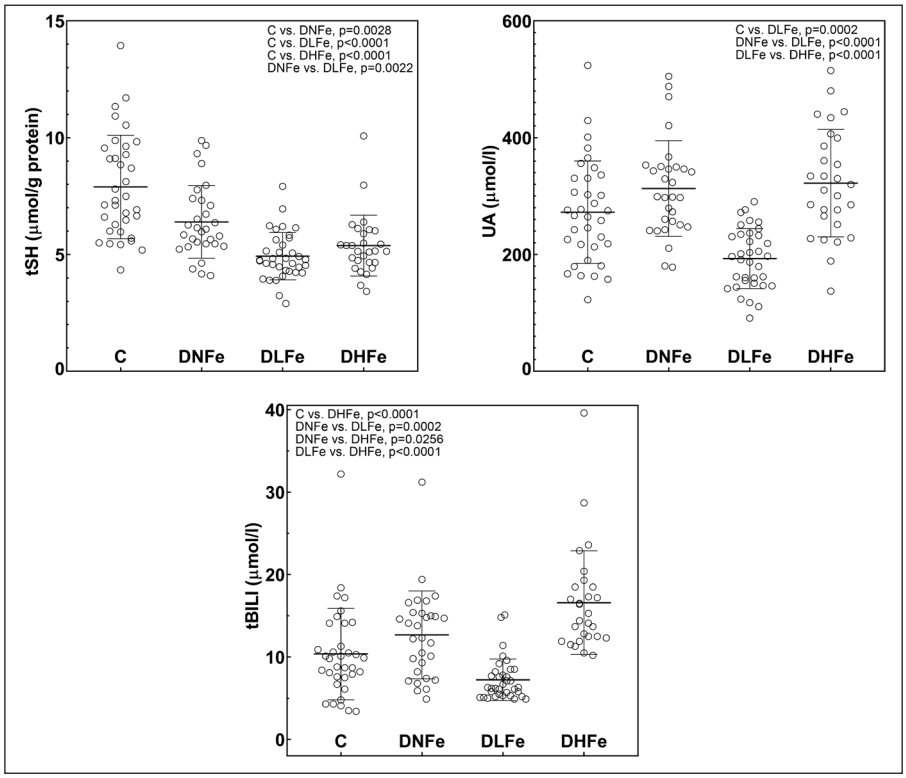

Fig. 1. *Levels of non-enzymatic serum antioxidants.*

C – healthy controls; DNFe – diabetic, normal serum iron; DLFe – diabetic, low serum iron; DHFe – diabetic, high serum iron; tSH – total sulfhydryl groups; UA – uric acid; tBILI – total bilirubin

The group comparisons with significant differences are indicated in the upper left or right corner of each graph.

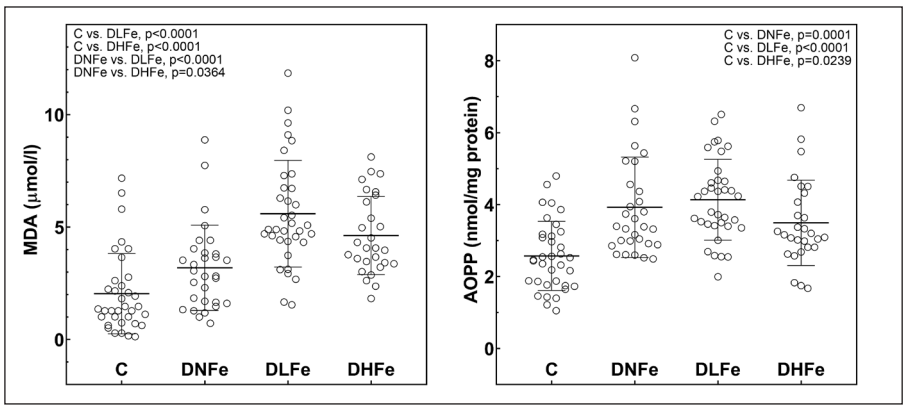

Fig. 2. *Serum markers of oxidative stress.*

C – healthy controls; DNFe – diabetic, normal serum iron; DLFe – diabetic, low serum iron; DHFe – diabetic, high serum iron; MDA – malondialdehyde; AOPP – advanced oxidation protein products

The group comparisons with significant differences are indicated in the upper left or right corner of each graph.

Table 3. *Correlations between different serum parameters – combined data from all diabetic patients (N = 93)*

Parameters	Correlation coefficient (r)	95% CI	p-value
HbA1c vs. UA	−0.2451	−0.4275 to −0.04355	0.0179
Glu vs. UA	−0.2377	−0.4211 to −0.03577	0.0217
MDA vs. tBILI	−0.2271	−0.4118 to −0.02454	0.0286
MDA vs. UA	−0.3711	−0.5344 to −0.1811	0.0002
MDA vs. tSH	−0.2205	−0.4060 to −0.01758	0.0337
MDA vs. serum iron	−0.2066	−0.3937 to −0.003010	0.0469
AOPP vs. serum iron	−0.2209	−0.4064 to −0.01805	0.0333
Serum iron vs. tBILI	0.6594	0.5264 to 0.7609	< 0.0001
Serum iron vs. UA	0.5490	0.3888 to 0.6770	< 0.0001

Glu – fasting blood glucose; HbA1c – glycated haemoglobin; MDA – malondialdehyde; AOPP – advanced oxidation protein products; tSH – total sulfhydryl groups; UA – uric acid; tBILI – total bilirubin

DISCUSSION

In this study, T2DM was associated with increased oxidative stress markers in the blood serum. This is in agreement with the findings from other studies (7, 10, 15). In T2DM patients, oxidative stress has been mostly related to the increased rate of lipid peroxidation and accumulation of lipid peroxidation products (10). The enzymatic and non-enzymatic antioxidant defences have a crucial function in scavenging ROS, which helps to limit lipid peroxidation. This study focused on the determination of the non-enzymatic antioxidants – tSH, UA, and tBILI in blood serum. It has been shown that T2DM subjects have reduced total antioxidant status compared to the controls, independently of glycaemic control, and low urate levels have been associated with enhanced oxidative stress and increased mortality risk (8). In the same manner, elevated bilirubin levels are associated with better disease control in T2DM subjects who are otherwise healthy (16). The free sulfhydryl moieties (tSH) are part of the thiols – cysteine-containing proteins as well as low-molecular weight compounds like glutathione, found in intra- and extracellular fluids. Because ROS oxidize thiols, the sulfhydryl groups act as ROS neutralizes and the circulating quantities of tSH may directly represent systemic redox state. According to Schillern et al. (9), high serum tSH are a reflection of a favourable redox status and may therefore positively associate with disease status. However, in patients with T2DM serum total thiol levels are usually lowered, which is a sign of an increase in oxidative stress (17). In this context, our results showed consistently lowered tSH levels in diabetic subjects irrespective of serum iron levels. Although UA and tBILI were negatively correlated with the degree of lipid peroxidation, their levels were mainly associated with serum iron levels in subjects with diabetes. Hence, in our study the increased serum MDA could be related to the generally lower levels of non-enzymatic antioxidants in the T2DM subjects, regardless of gender. Indeed, the indices of hyperglycaemia (Glu and HbA1c) negatively correlated with UA, while there was also negative correlation between MDA and tSH, UA, and tBILI, respectively. Of particular importance is the relation to tBILI, considering that the lipophilic bilirubin protects lipids from oxidation and aids the prevention of diabetic complications (18). These findings are significant because in T2DM patients, an increase in lipid peroxidation and a loss in antioxidant defences may arise before secondary complications develop (10). Protein oxidation may also be attributed to the decreased antioxidant capacity in the serum of T2DM subjects in the present study. The levels of AOPP, often formed in the reaction of oxidants with plasma proteins, were increased in the T2DM subjects. Protein structure changes caused by oxidants, such as amino acid side chain modifications, covalent cross-linking, and non-enzymatic cleavage, can result in a partial or complete loss of protein functioning (19). Our results have corroborated the findings from other studies in which AOPP levels positively correlated with HbA1c concentration and were significantly higher in T2DM subjects with or without diabetic complications in comparison to healthy controls (14, 20).

The necessity of adequate iron levels for good health and wellbeing is well understood. Iron is an important mineral for a number of molecules to keep their regular structures and functions, as well as for cells to live and proliferate. The body's iron status is mainly determined by dietary iron, which is not actively excreted, allowing a possibility of overload related to high dietary intake (21). Yet, due to insufficient content and limited bioavailability, normal iron requirements are frequently unmet by regular diet (22). Then it comes as no surprise that among the patients with T2DM both iron deficiency and iron overload can be prevalent, and in this study the total amount of circulating iron was used as a grouping parameter.

Fernández-Real et al. (23) discovered a bi-directional link between iron and glucose metabolism, concluding that serum iron modulates glucose metabolism even when there is no major iron overload or deficiency. It is generally accepted that iron overload increases the risk of developing T2DM (24). However, once T2DM is established, according to the results of the present study, low serum iron, more so than high serum iron, was associated with unbalanced redox status of the serum in favour of the oxidants, promoting the state of oxidative stress. The increased oxidative stress could be attributed to increased production of ROS and/or decreased antioxidant defences. The results of this study showed that low serum iron affected the production of haemoglobin, but other iron-containing proteins like myoglobin, cytochromes, CAT, and peroxidase may be also affected (6, 7). Previous studies have shown that iron deficiency leads to decreased total antioxidant capacity, decreased antioxidant enzyme activity, and increased lipid peroxidation (15, 25). In patients with T2DM, the diabetes-related decrease in the antioxidant capacity

would be complemented by the iron deficiency-related reduction in enzymatic antioxidant activity, and this would cause increased utilization of non-enzymatic antioxidants. For example, vitamin C and E were found to be significantly depleted in patients with iron-deficiency anaemia compared to the control subjects (26). This concept would explain the decreased levels of the main determinants of serum antioxidant capacity – UA and tBILI, and the concomitantly increased level of MDA in the T2DM patients with low serum iron compared to the other diabetic groups in the present study. For instance, studies have shown that T2DM patients with iron deficiency have higher MDA and HbA1c, and lower UA compared to T2DM patients without iron deficiency (15, 27). In addition to the increased utilization of UA and tBILI as radical scavengers, other factors related to serum iron could also influence their levels. Serum iron was found to be independently correlated with bilirubin, and inflammation has been proposed as the basis for this relationship (28). This could be relevant to the low tBILI observed in diabetic subjects with low serum iron levels in our study, considering that T2DM is an inflammatory state (7). Moreover, increased production of uric acid is considered a protective response to increased iron availability (29). The observed positive correlation between UA and serum iron in our results might be related to this antioxidant response. Interestingly, although serum iron correlated negatively to both MDA and AOPP, the AOPP levels did not differ between the three diabetic groups in function of serum iron.

It is recognized that iron overload is not a prerequisite for iron to mediate either diabetes or its complications (30). That is, iron must be in its free, redox-active form to operate as a pro-oxidant agent. Although the serum iron correlated both to the markers of oxidative stress (negatively) and to the levels of the non-enzymatic antioxidants (positively), this study's results do not convey a message that high serum iron is beneficial per se to the serum redox status in T2DM subjects. It has been shown that iron supplementation may reverse the decline in antioxidant enzyme activity associated with iron deficiency, but iron therapy has also been shown to increase the generation of ROS (30). In the present study, the diabetic subjects with high serum iron had significantly higher markers of lipid peroxidation and protein oxidation, as well as reduced tSH compared to the controls. The same was also true for the diabetic subjects that were iron sufficient, suggesting that the main contributor to oxidative stress is the state of T2DM, with the availability of free iron being able to modulate the serum redox status to a certain degree. Considering the latter, the results showing no significant differences between the sexes with respect to markers of oxidative stress and levels of non-enzymatic antioxidants in our study might be related to the observed lack of gender-associated differences in serum iron levels.

The main limitation of the study is the small cohort from a single region. This limits the generalization of our data. The study lacks data on other serum antioxidants (such as vitamin C, vitamin E, etc.), other markers of oxidative stress (e.g. isoprostanes), markers of inflammation, as well as indices of iron metabolism (ferritin, transferrin, TIBC, etc.). However, the findings from the study highlight serum biomarkers, the evaluation of which may be useful for better phenotyping and follow-up of diabetic population. The study also emphasizes the importance of identifying individuals who are likely to require particular pharmaceutical therapies that target oxidative stress in addition to glucose control.

CONCLUSIONS

Our study supports the concept that oxidative stress mediates the relationship between serum iron abnormalities and the pathogenesis of T2DM. T2DM-associated redox imbalance is characterized by a decrease in serum total sulfhydryl groups and low serum iron-associated reduction in uric acid and total bilirubin levels. The lowered non-enzymatic antioxidant levels were accompanied by an increase in the concentration of lipid peroxidation and protein oxidation markers. The relatively noninvasive monitoring of these parameters may be of considerable interest in monitoring the pathophysiological processes in T2DM and may provide useful insights into the effects of potential therapeutic or nutritional interventions.

Conflicts of Interest

None declared

Adherence to Ethical Standards

All participants provided their written consent. The study was conducted in accordance with the Declaration of Helsinki.

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