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Is the insulin necessary for the struggle against oxidative stress in diabetes mellitus type 2 – a pilot study

Da li je insulin neophodan za borbu protiv oksidativnog stresa u dijabetesu melitusu tip 2 – pilot studija

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Abstract

Background/Aim. Hyperglycaemia has a detrimental effect on the progress of micro/macrovascular complications in patients with diabetes mellitus type 2 (T2DM). Additionally, all known complications in T2DM are coupled with oxidative stress developed from different metabolic pathways. The aim of this study was to estimate the quality of glucoregulation and the degree of oxidative stress in T2DM patients depending on the applied therapeutic protocol and assess their correlation with clinical data and crucial biochemical parameters important for the development of diabetes complications. **Methods.** All included patients were divided into two groups: those treated with oral antidiabetic drugs (OAD) and those treated with oral antidiabetic drugs and insulin (OADINS). Thiobarbituric acid reactive substances (TBARS), total sulfhydryl groups (TSH), the activity of superoxide dismutase (SOD), total nitrites (NOx), vascular endothelial growth factor (VEGF), and activities of matrix metalloproteinase 9 (MMP9) were measured, together with lipid profile and routine biochemical parameters. All subjects were analyzed for demographic characteristics and detailed medical history as well as smoking habits and calculated for body mass index (BMI). **Results.** All patients were uniformly poor glucoregulated and dyslipidemic. SOD activity was decreased, and lipid peroxidation was increased in the OAD group compared to OADINS. Deficient glucoregulation in both the OAD and the OADINS groups did not associate with an oxidative state outcome. In both of these groups, the concentrations of VEGF and MMP9 were significantly higher than in controls. **Conclusion.** The better antioxidative outcome, expressed with a normalized concentration of TBARS, preserved TSH, and normalized SOD activity in T2DM patients treated with OADINS compared to those treated exclusively with OAD, suggests the need for more careful consideration of earlier insulin introduction into T2DM therapy in order to prevent the development of complications.

Key words:
diabetes mellitus, type 2; insulin; oxidative stress.

Apstrakt

Uvod/Cilj. Kod bolesnika sa dijabetesom melitusom tipa 2 (T2DM), hipergikemija podstiče progresiju mikro/makrovaskularnih komplikacija. Dodatno, sve poznate komplikacije u T2DM povezane su sa oksidativnim stresom koji nastaje različitim metaboličkim procesima. Cilj rada bio je da se proceni kvalitet glikoregulacije i stepen oksidativnog stresa kod bolesnika sa T2DM u zavisnosti od primenjenog terapijskog protokola i njihova povezanost sa kliničkim podacima i ključnim biohemijskim parametrima važnim za razvoj dijabetesnih komplikacija. **Metode.** Svi ispitivani bolesnici bili su podeljeni u dve grupe: grupa bolesnika lečenih samo oralnim antidijabetičnim lekovima (OAD) i grupa lečenih OAD i insulinom (OADINS). Praćeni su tiobarbiturna kiselina-reagujuće supstance (TBARS), totalni sulfhidrili (TSH), aktivnost superoksid dizmutaze (SOD), ukupni nitriti (NOx), vaskularni endotelni faktor rasta (VEGF), aktivnost matriksne metaloproteinaze 9 (MMP9), lipidni profil i rutinski biohemijski parametri. Svim ispitanicima su analizirane demografske karakteristike, detaljna medicinska istorija, pušačke navike, a izračunat im je i indeks telesne mase (BMI). **Rezultati.** Svi bolesnici su imali loše regulisanu glikoregulaciju i bili su dislipemični. Pokazana je smanjena aktivnost SOD i povećana lipidna peroksidacija u OAD grupi u poređenju sa OADINS grupom. Loša glikoregulacija u grupama OAD i OADINS nije bila povezana sa rezultatima oksidativnog stanja. I u OAD i u OADINS grupi, koncentracije VEGF i MMP9 bile su značajno više u odnosu na kontrole. **Zaključak.** Bolji

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antioksidativni odgovor registrovan kroz normalizovanu koncentraciju TBARS, očuvan TSH i SOD u granicama normalnih vrednosti kod T2DM bolesnika lečenih OADINS u odnosu na bolesnike lečene samo OAD, upućuju na potrebu za pažljivijim razmatranjem ranijeg uvođenja insulina u terapiju obolelih od T2DM, kako bi se sprečio razvoj komplikacija.

Ključne reči:
dijabetes melitus, tip 2; insulin; stres, oksidativni.

Introduction

Diabetes mellitus (DM) type 2 (T2DM) is a foremost public health problem and one of the health challenges of the XXI century. It includes a heterogeneous group of metabolic dysfunctions characterized by impaired insulin secretion, increased glycemia, and variable degrees of insulin resistance. It precedes by a period of abnormal glucose homeostasis classified as impaired fasting glucose or impaired glucose tolerance [1, 2].

In patients with T2DM, hyperglycaemia has a detrimental effect on the progress of micro/macrovascular complications. Glycaemia variability is positively associated with the development of diabetic retinopathy, neuropathy, and nephropathy, whose manifestations are related to atherosclerosis [3]. All of them, including diabetic neuropathy, are particularly emphasized in the appearance of oxidative stress, which promotes the functional and structural changes of the tissue and organs [4]. Besides endothelial dysfunction and inflammation, oxidative stress participates in all known complications during DM [5].

Oxidative modifications of lipids and proteins have been identified in vascular lesions, confirming the role of oxidative stress in atherogenesis. Underlying mechanisms of provoked oxidative stress during DM include the hexosamine pathway, pathway of polyols, activation of protein kinase C, and expansion of advanced glycation end-products [6]. Published data revealed that intermittent hyperglycaemia rather than chronic hyperglycaemia amplifies the production of reactive oxygen species (ROS) [7]. The complications can be suppressed and slowed by adequate therapeutic regulation of glucose and lipids levels. Therefore, regular monitoring of long-term glucose level control through the concentration of the haemoglobin fraction A1c (HbA1c) represents a gold standard in assessing the quality of the therapy [8].

The concentration of substances that react with thiobarbituric acid (TBA) may realize a significant task as a lipid peroxidation indicator as well as the content of total sulfhydryl groups (TSH), which are indicators of the oxidative protein damage [9]. These parameters are also useful in assessing antidiabetic therapy.

Enzyme activities of superoxide dismutase (SOD) and matrix metalloproteinase 9 (MMP9) in plasma represent the reflection of tissue events during the disease-systemic redox milieu and disturbed tissue structure [10]. The proinflammatory actions of MMP9 in DM have already been documented [11]. MMP9 also influences tissue availability to bound vascular endothelial growth factor (VEGF), which is a prominent factor in the development of diabetic complications [12]. VEGF in the systemic circulation is an indicator of endothelial activity whose integrity and functions are of great importance during therapy of DM [13]. The predictive values of these parameters are particularly significant from the aspect of their correlation with other proatherogenic parameters as well as HbA1c concentration.

This study intended to estimate the quality of glucoregulation and the degree of oxidative stress in T2DM patients depending on the applied therapeutic protocol, with the aim to assess their correlation with clinical data and crucial biochemical parameters important for the development of diabetes complications.

Methods

Patients and clinical protocol

This prospective study was conducted in agreement with ethical principles confirmed in the Helsinki Declaration and the approval from the Ethics Committee of the Military Medical Academy (MMA) from April 11, 2017. The clinical part of the study was carried out at the Clinic of Endocrinology of the MMA, while the laboratory measurements were achieved at the Institute for Medical Research and Central Chemical Laboratory of the MMA. All the participants were aware of the purpose of the investigation, after which they authorized the information approval.

Forty T2DM patients diagnosed according to the criteria of the World Health Organization were included in the study. Patients were divided into two groups based on medical therapy for T2DM: those treated with oral antidiabetic drugs (OAD group; n = 20) and those treated with OAD and insulin (OADINS group; n = 20). Baseline insulin substitution in the OADINS group was applied with the whole average daily amount of 0.5 units *per* kilogram of body weight (U/kg bw). The control group of 20 healthy volunteers consisted of 13 males and 7 females, with an average age of 52.6 ± 7.4 years and an HbA_{1c} level of 5.21 ± 0.43%.

Demographic characteristics and detailed medical history, as well as smoking habits, were analyzed. Smokers were considered those respondents who have regularly smoked more than five cigarettes a day for the past six months. Data about comorbidities, such as cardiovascular events, nephropathy, retinopathy, polyneuropathy, and microangiopathy, have been analyzed as well.

The nephropathy was evaluated based on the estimated glomerular filtration rate (eGFR), dividing the T2DM patients into those with eGFR below 60 mL/min/1.73 m² (kidney disease) and those with eGFR above 60 mL/min/1.73 m² (normal) [14].

The degree of retinopathy was assessed based on ophthalmologist examinations, which were performed with ophthalmoscopy and fluorescence angiography. Based on the ophthalmic examination, T2DM patients were classified as subjects with no diabetic retinopathy or those with nonproliferative diabetic retinopathy.

The neuropathy was assessed by an experienced neurologist based on diabetic neuropathic symptoms and electrophysiological analysis of nerve conduction. Cardiovascular events included diagnosed coronary arterial disease, stroke, or peripheral arterial disease [14].

All subjects were anthropometrically measured for body weight (kg) and height (cm) and then calculated for body mass index (BMI).

Biochemical parameters

The concentrations of fasting plasma glucose, HbA1c, creatinine, urea, cholesterol, low-density lipoprotein (LDL), high-density lipoproteins (HDL), and triglycerides were determined from the blood by standard laboratory methods on the apparatus Siemens ADVIA 1800 Chemistry system in the Central Chemical Laboratory MMA.

The plasma concentration of TSH was measured spectrophotometrically at 412 nm in phosphate buffer (0.2 moL + 2 mmoL EDTA, pH 9) using 5,5-dithiobis-(2-nitrobenzoic acid) (DTNB, 0.01 M, Sigma). The results are expressed as μmol/L [15].

Lipid peroxidation in the plasma was measured as the production of substances assayed in the reaction with thiobarbituric acid, as described by Girotti et al. [16]. Reactions were performed with TBA reagent [15% trichloracetic acid + 0.375% TBA (both supplied by Merck, Darmstadt, Germany)] in boiling water for 15 min. TBA acid reactive substances (TBARS) were measured spectrophotometrically at 533 nm. The results are expressed as μmol/L.

Total SOD activity in plasma was measured spectrophotometrically at 480 nm as a percentage of inhibition of epinephrine autooxidation in an alkaline medium. After adding 10 mM epinephrine (Sigma, St. Luis, USA), the analysis was performed kinetically, for 10 min, in the sodium carbonate buffer (50 mM, pH 10.2; Serva, Feinbiochemica, Heidelberg, New York) containing 0.1 mM ethylenediaminetetraacetic acid (Sigma, St. Luis, USA). The activity of SOD is expressed in international units (U/mL). An international unit is defined as an enzyme activity that inhibits 50% of epinephrine autooxidation [17].

Nitrite/nitrate concentration (NOx) was assayed spectrophotometrically at 492 nm, using the colorimetric method of Griess [18].

VEGF in plasma was measured with commercial Quantikine Immunoassay VEGF (R&D System) with a minimum detection level of 9.0 pg/mL. The results are expressed as pg/mL.

MMP9 in plasma was measured with commercially available Elisa kits (Quantikine Elisa Human MMP-9 (R&D System). The results are expressed as ng/mL.

Statistical analysis

Data were presented as mean ± standard deviation (SD) or percentage (%). Parametric variables between groups were analyzed by independent *t*-test and one-way ANOVA with the Tukey *post hoc* test. Nonparametric data were analyzed by the χ^2 test. Spearman's bivariate testing was applied to establish the correlations between biochemical parameters, expressing them through correlation coefficients (ρ). All statistical tests were performed using the statistical software package GraphPadPrism, version 5.03. Statistical significance was set at $p < 0.05$.

Results

Baseline clinical characteristics of T2DM patients divided into two equal groups according to antidiabetic therapy are presented in Table 1. The study groups were consistent in terms of

Table 1

Baseline clinical characteristics of the study population

Characteristics	OAD (n = 20)	OADINS (n = 20)
Age (years), mean ± SD	60.6 ± 11.1	61.9 ± 12.3
Male gender (%)	65	75
DM duration (years), mean ± SD	13.8 ± 7.8	15.2 ± 10.3
Smokers (%)	25	25
Ex-smokers (%)	15	20
Medical history (%)		
hypertension	90	90
nephropathy	85	70
retinopathy	35	30
neuropathy	35	65*
dyslipidaemia	65	65
cardiovascular event	20	25
Additional therapy (%)		
ACE inhibitors	65	65
Ca- antagonists	50	40
beta-blockers	30	40
ATR 2 antagonists	10	10
aspirin	50	45
statins	45	55
BMI (kg/m^2), mean ± SD	29.35 ± 5.13	26.5 ± 4.4*

DM – diabetes mellitus; OAD – oral antidiabetic drugs; OADINS – OAD and insulin; ACE – angiotensin-converting enzyme; Ca – calcium; ATR – angiotensin receptor; BMI – body mass index; SD – standard deviation.

***$p < 0.05$ – statistical significance compared to the OAD group.**

age, DM duration, smoking habits, applied medications (except insulin), and most of the comorbidities, except neuropathy (Table 1). Namely, in the OADINS group, a significantly higher percentage of neuropathy was recorded compared to the OAD group ($p < 0.05$). Furthermore, it was found that patients of the OAD group had a higher BMI ($p < 0.05$).

Compared to the healthy controls, biochemical parameters of both OAD and OADINS subjects showed significantly increased values of HbA1c ($p < 0.001$), cholesterol ($p < 0.001$), MMP9 ($p < 0.001$), and VEGF ($p < 0.001$). On the other hand, plasma HDL level was reduced ($p < 0.001$). No significant difference was noted between study groups in terms of the above-mentioned parameters (Table 2).

OAD respondents showed significant oxidative protein damage compared to control individuals, evaluated by plasma concentrations of TSH ($p < 0.05$). Simultaneously, plasma TSH levels of OADINS subjects were close to the control values (Table 2).

As we already emphasized, SOD is an antioxidant enzyme that plays an important role in protecting against damage that produces ROS. Therefore, the measurement of SOD activity was of particular importance in our investigation. We found significantly decreased SOD activity in the OAD group compared to the OADINS group ($p < 0.001$). At the same time, there was no difference between OADINS subjects and controls (Figure 1A). Another relevant parameter of oxidative damage is the degree of lipid peroxidation. An increased degree of lipid peroxidation, estimated by plasma concentration of TBARS, was detected in the OAD group compared to the controls and the OADINS group (Figure 1B).

Table 2

Biochemical parameters in the study population and controls

Biochemical parameters	Control (n = 20)	OAD (n = 20)	OADINS (n = 20)
HbA1c (%)	5.51 ± 0.73	8.68 ± 1.71***	8.37 ± 1.90***
Cholesterol (mmol/L)	3.08 ± 0.60	4.87 ± 1.39***	4.89 ± 0.89***
Triglycerides (mmol/L)	1.81 ± 0.33	2.01 ± 0.75	1.84 ± 0.98
LDL (mmol/L)	2.09 ± 0.46	2.90 ± 1.17	2.78 ± 0.73
HDL (mmol/L)	1.96 ± 0.50	1.12 ± 0.24***	1.24 ± 0.42***
Urea (mmol/L)	5.86 ± 1.08	6.37 ± 3.02	6.98 ± 2.33
Creatinine (mmol/L)	69.20 ± 9.83	82.70 ± 39.82	83.4 ± 27.6
TSH (μmol/L)	0.489 ± 0.043	0.40 ± 0.14*	0.45 ± 0.08
NOx (μmol/L)	6.58 ± 1.49	9.66 ± 4.01	7.88 ± 4.18
MMP9 (ng/mL)	42.96 ± 3.23	74.37 ± 9.82***	78.42 ± 10.75***
VEGF (pg/mL)	24.25 ± 4.52	56.04 ± 11.85***	63.43 ± 12.70***

OAD – oral antidiabetic drugs; OADINS – OAD and insulin; HbA1c – haemoglobin A1c; LDL – low-density lipoprotein; HDL – high-density lipoprotein; TSH – total sulfhydryl groups; NOx – nitrite and nitrate; MMP9 – matrix metalloproteinase 9; VEGF – vascular endothelial growth factor.

Data are shown as mean ± standard deviation. The statistical differences between groups were performed by one-way ANOVA with the Tukey *post hoc* test.

***$p < 0.05$; ***$p < 0.001$ – statistical significance compared to the control group.**

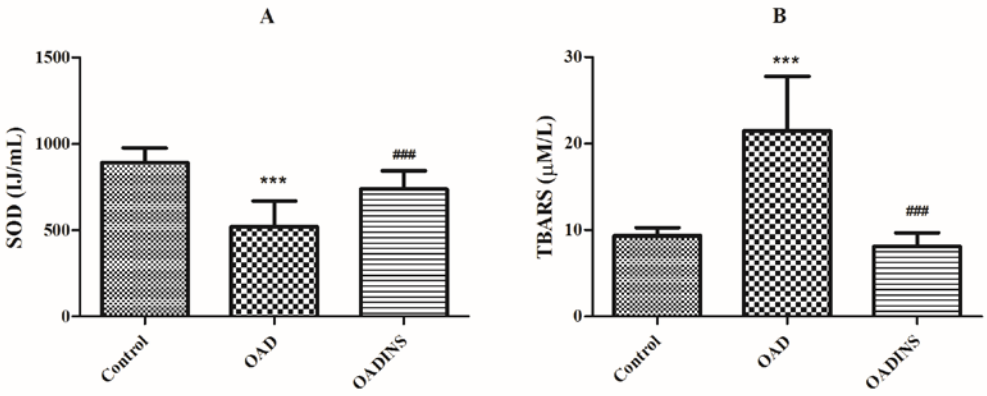

Fig. 1 – A) Superoxide dismutase (SOD) activity and B) thiobarbituric acid reactive species (TBARS) in plasma of the control and type 2 diabetes mellitus (T2DM) patients. Bars in the graph represent the mean ± standard deviation from 20 subjects per group. OAD – oral antidiabetic drugs; OADINS – OAD and insulin.

***$p < 0.001$, statistical significance compared to the control group; ###$p < 0.001$, statistical significance compared to the OAD group.**

Table 3

Spearman's correlation analysis between HbA_{1c} and biochemical parameters in OAD and OADINS groups

Biochemical parameters	OAD ρ	OADINS ρ
Cholesterol (mmol/L)	0.1429	0.2982
Triglycerides (mmol/L)	0.2475	0.2331
LDL (mmol/L)	0.2406	0.1645
HDL (mmol/L)	-0.3469	-0.00565
Urea (mmol/L)	-0.4170	-0.1597
Creatinine (mmol/L)	-0.0572	0.0407
TSH (μmol/L)	0.2851	-0.0659
TBARS (μmol/L)	-0.0376	0.0783
SOD (U/mL)	-0.1414	0.6052**
NOx (μmol/L)	0.0959	-0.0026
MMP9 (ng/mL)	-0.3143	-0.1292
VEGF (pg/mL)	0.0947	-0.1943

OAD – oral antidiabetic drugs; OADINS – OAD and insulin; HbA1c – haemoglobin A1c; LDL – low-density lipoprotein; HDL – high-density lipoprotein; TSH – total sulfhydryl groups; TBARS – thiobarbituric acid reactive species; SOD – superoxide dismutase; NOx – nitrite and nitrate; MMP9 – matrix metalloproteinase 9; VEGF – vascular endothelial growth factor; ρ – coefficient of correlatin.
****$p < 0.01$ – statistical significance compared to the OAD group.**

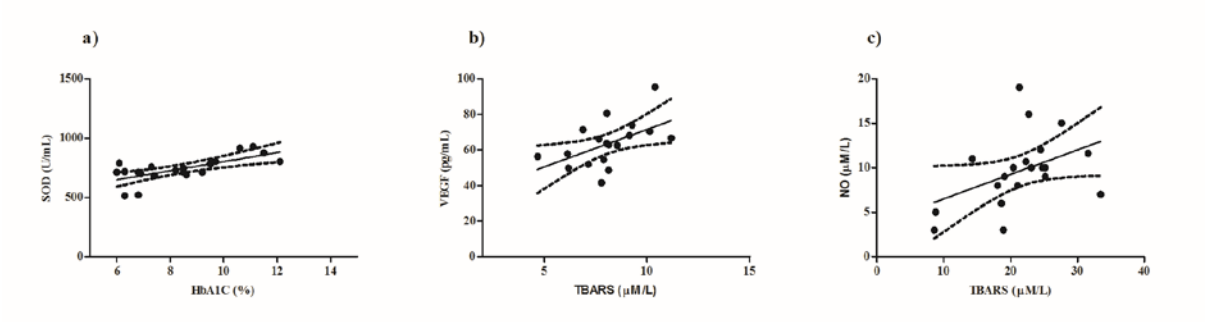

Fig. 2 – Correlation analysis between: a) haemoglobin A1c (HbA1c) (%) and superoxide dismutase (SOD) activity (U/mL) ($\rho = 0.6052$; $p < 0.01$), and b) thiobarbituric acid reactive species (TBARS) (μmol/L) and vascular endothelial growth factor (VEGF) (pg/mL) ($\rho = 0.5356$; $p < 0.05$) in patients treated with oral antidiabetic drugs (OAD) and insulin (OADINS); c) TBARS and total nitrite and nitrate (NOx) ($\rho = 0.4700$; $p < 0.05$) in patients treated only with oral antidiabetic drugs (OAD).

Performing Spearman's correlation analysis among HbA1c (%) and biochemical parameters (Table 3), we found a significant positive correlation between HbA1c and SOD activity in the OADINS group (Figure 2a). In the same group, there was also a positive correlation between TBARS and VEGF (Figure 2b). In the OAD group, a positive correlation was found between TBARS and total nitrite and nitrate (NOx) (Figure 2c).

Discussion

Hyperglycaemia stimulates ROS production from different cellular sources. The electron transport chain in mitochondria is initially a superoxide producer, which leads to the activation of protein kinase C and the formation of advanced glycation end products [19]. ROS-induced mechanisms in the development of T2DM complications are complex. Besides activating protein kinase C, ROS has also been shown to inhibit insulin signal transduction by nuclear kappa factor B, confirming further the association between T2DM and oxidative stress development [20].

As we have already pointed out, HbA1c is the gold standard for monitoring the therapeutic effects in diabetics. Uniformly elevated HbA1c in both OAD and OADINS groups indicate poor glycaemic control regardless of the applied therapy (Table 2). The results of HbA1c follow previously published reports about the frequent occurrence of poor glucose control, which is associated with earlier and more severe complications in T2DM [6, 21].

Deficient glucose control in both OAD and OADINS groups did not associate with an antioxidative outcome. Compared to the OAD group, we found significantly lower lipid peroxidation and elevated SOD in the OADINS group, which indicates a higher antioxidant potential despite persistent poor glucose control (Figure 1). Additionally, a positive correlation between HbA1c and SOD is registered in

the OADINS group (Figure 2a), which is diverse from the published results of Verma et al. [22], who found a negative correlation between SOD and HbA1c in T2DM patients. In the OADINS group, despite the increase in HbA1c, lipid peroxidation reached the control level, and an increase in SOD activity might have been one of the reasons. Elevated SOD could target superoxide, eliminating their destructive effects on the cell membranes and improving antioxidative properties.

The only difference in treatment protocol between the two study groups was insulin, so the question arises about the potentially positive effect of insulin itself on antioxidant status. In the experimental model of DM induced by injection of streptozotocin, it has been proven that insulin may manifest antioxidative potential through the nuclear factor erythroid 2-related factor 2 (Nrf2) signaling pathway, which counterbalances endogenous antioxidants against oxidants [23].

In T2DM patients in our study, hypertension, nephropathy, retinopathy, and dyslipidaemia were revealed equally in both OAD and OADINS subjects (Table 1). However, neuropathy was more common in the OADINS group, suggesting this factor was perhaps significant for the introduction of insulin in the therapy. This was later confirmed by an analysis of medical reports. In the pathogenesis of diabetic neuropathy, oxidative stress is persistently active and supported by both inflammation and compromised neural conduction velocity [24]. Thus, the increased antioxidant potential in the OADINS group could have a positive effect on slowing the progression of neuropathy which requires further investigation.

Calculating BMI gives us data on overweight subjects in both OAD and OADINS groups, but BMI was remarkably higher in the OAD group (Table 1). Obesity is a promoting factor for metabolic disturbances, insulin resistance, and the development of metabolic syndrome during T2DM [25, 26]. Together with smoking, being overweight accelerates the development of complications in blood vessels. Several cohort studies with T2DM patients realized that HbA1c and smoking were the strongest predictors regarding the risk of the outcomes and death [27]. It is well documented that compounds from cigarette smoke have the potential to stimulate endothelial NADPH oxidase and promote mitochondrial oxidative stress. Besides the effects on promoting vascular resistance, compounds from cigarette smoke also stimulate the oxidation of LDL, which potentiates its pro-oxidative activity [28]. Oxidized LDL is more effective in activating NADPH oxidase, which stimulates ROS generation [29]. In both OAD and OADINS groups, smokers and ex-smokers were almost half of the subjects (Table 1). We did not obtain differences in LDL and triglyceride levels between the OAD and OADINS group, nor compared to controls, which could be due to the therapeutic usage of antilipemic drugs (Table 2). However, despite the uniform use of antilipemics (Table 1), cholesterol level was elevated in both the OAD and OADINS group compared to controls, while HDL concentrations were reduced (Table 2). In addition, there is a trend of a positive correlation between HbA1c and cholesterol, triglycerides and LDL levels, and a negative correlation between HbA1c and HDL ones (Table 3), confirming that faulty glycaemia control is associated with dysfunctional lipid metabolism. By inhibiting 3-hydroxy-3-methylglutaryl-coenzyme A reductase, statins reduce levels of total cholesterol, LDL, and triacylglycerols and concurrently increase HDL level in plasma. Statins are also known for their pro-oxidative potential as they induce metabolic transformation involving CYP450 enzymes, known to potentiate oxidative stress [30]. Assuming the nonsignificant difference in the use of statins in both study groups, this factor could not be the reason for a different antioxidant outcome.

VEGF is an important participant in vascular tissue remodeling during T2DM [31]. In both OAD and OADINS groups, the concentration of VEGF was significantly higher than in controls but with no difference between them (Table 2) and with no correlation with statin use. These results were contrary to expectations given the published data on suppressed angiogenesis and decreased VEGF in T2DM patients using statins [32]. A significant positive correlation between VEGF and TBARS in the OADINS group (Figure 2b) draws attention to the possible indirect relationship of elevated VEGF with the degree of lipid peroxidation in the OADINS group. In the same group, elevated HbA1c level positively correlated with the elevation of SOD activity, indicating a higher degree of antioxidative defence (SOD) in more compromised glycaemia control (Figure 2a).

Tissue changes in both the OAD and OADINS group are also supported by homogenously elevated MMP9 activity compared to controls. Uniformly increased MMP9 activity in both the OAD and OADINS group decreases the possibility of its direct influence on the better antioxidant output in the OADINS group.

We have already mentioned that statins have pro-oxidative potential. About half of the participants in our study were taking statins. Furthermore, the additional therapy of our subjects included other drugs that are known to affect oxidative status – angiotensin-converting enzyme (ACE) inhibitors, beta-blockers, and calcium channel antagonists.

Angiotensin II manifests the potential to activate NADPH oxidase and induce ROS production in the mitochondria *via* respiratory chain complexes I and III and a protein kinase C-dependent pathway, improving endothelial function and insulin resistance during T2DM. The increased levels of angiotensin II in diabetic patients may additionally reduce dihydrofolate reductase expression and decrease tetrahydrobiopterin (BH4) recycling from dihydrobiopterin (BH2), thus indirectly influencing endothelial nitric oxide synthase [33, 34]. Hence, ACE inhibitors act as antioxidants. Beta-blockers are also established antioxidants through several different mechanisms, while calcium channel antagonists promote superoxide scavenging, thus increasing the antioxidant capacity of the vessel endothelium [35-37]. Equal frequencies of applied beta-blockers, ACE inhibitors, and calcium channel antagonists in both study groups (Table 1) eliminate them as prominent factors of better antioxidant outcomes in the OADINS group.

In T2DM, glucose intolerance impairs endothelial nitric oxide (NO) synthase (eNOS) activity directly through improved oxidative stress [38]. An excessive amount of superox-

ide anion, which is an inducer of oxidative stress in mitochondria, rapidly induces NO to convert it to highly reactive peroxynitrite. Peroxynitrite expresses prooxidative potential to oxidize lipids, induce cellular injury, and modulate arterial contraction. It also depresses NO bioavailability and additional impairment of endothelium-mediated vasodilation. The enhanced ROS production and consecutive peroxynitrite in T2DM promote vascular inflammation, DNA damage, and vascular aging [39]. Unchanged concentrations of NOx in both OAD and OADINS patients compared to controls and each other indicate a reduced potential for peroxynitrite production. It also directs to higher bioavailability of NO to improve vascular response during T2DM. Mutually, compared with the OAD group, elevated SOD (which removes superoxide), depressed TBARS, and unchanged NOx in the OADINS group suggest a better antioxidant capacity and suppressed nitrosative stress. Since better antioxidant outcomes were not achieved in the OAD group, these quote a significant effect of insulin given in the OADINS group.

Conclusion

The therapeutic approach in T2DM is directional at preserving endothelial function. Obtained results indicate the improvement of antioxidative defence in T2DM patients due to supplemented insulin therapy. Normalized SOD activity and regulated lipid peroxidation upgrade a redox status, which is a promising outcome for restraint of complications in T2DM. The better antioxidative outcome expressed with a normalized concentration of TBARS and SOD activity in T2DM patients treated with insulin besides OAD supports the need for more careful consideration to upstart earlier with insulin in T2DM therapy.

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