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THE INFLUENCE OF TYPE AND DURATION OF CARDIOVASCULAR COMPLICATIONS ON ANTIOXIDATIVE PARAMETER VALUES IN TYPE 2 DIABETIC PATIENTS

UTICAJ VRSTE I DUŽINE TRAJANJA KARDIOVASKULARNIH KOMPLIKACIJA NA VREDNOST ANTIOKSIDATIVNIH PARAMETARA U TIPU 2 DIABETES MELLITUSA

Emina Čolak[1], Vesna Dimitrijević-Srećković[2], Predrag B. Đorđević[2], Sanja Stanković[1], Nada Majkić-Singh[1], Katarina Lalić[2], Nebojša Lalić[2]

[1]Institute of Medical Biochemistry, Clinical Center of Serbia, Belgrade, Serbia
[2]Institute of Endocrinology, Diabetes and Metabolic Disorders, Clinical Center of Serbia, Belgrade, Serbia

Summary: It is well established that type 2 diabetes mellitus is associated with highly increased risk of coronary heart disease, cardiovascular disease (CVD), and total mortality. CVD is the leading cause of death of people with diabetes. The aim of our study was to test the effect of type and duration of cardiovascular complications on antioxidant parameter values: superoxide dismutase (SOD), glutathione peroxidase (GPx), glutathione reductase (GR) and total antioxidant status in patients with type 2 diabetes mellitus and manifested cardiovascular complications. Out of 100 subjects included in the study, 69 subjects were type 2 diabetic patients with cardiovascular complications and 31 age-matched controls. Statistical data processing revealed significantly lower antioxidant defense ($p<0.001$) in patients with type 2 diabetes and cardiovascular complications manifested as coronary artery disease (CAD), hypertension (HTA) and myocardial infarction experienced in the previous 8 years (AMI). The type 2 diabetics with longer history of diabetes and coronary artery disease had higher fasting glucose values, higher GR activity, but lower TAS and SOD activity ($p<0.05$). Fasting glucose levels were in negative correlation with SOD and GPx activities in the subgroups of diabetics with severe cardiovascular complications (CAD+AMI, CAD+AMI+ HTA) ($p<0.05$).

Keywords: oxidative stress, antioxidant defense, type 2 diabetes mellitus, cardiovascular complications

Address for correspondence:
Emina Čolak, M.D.
Institute of Medical Biochemistry
Clinical Center of Serbia
11000 Belgrade
Višegradska 26
Tel.: +381 11 361 56 31
e-mail: cholakaa@yubc.net

Kratak sadržaj: Poznato je od ranije da pacijenti sa tipom 2 diabetes mellitusa imaju povećan rizik za nastanak koronarne bolesti srca, kardiovaskularnih bolesti i mortaliteta. Kardiovaskularne bolesti predstavljaju vodeći uzrok smrti u pacijenata s tipom 2 dijabetesa. Cilj ovog rada bio je da se ispita uticaj vrste i dužine trajanja kardiovaskularnih komplikacija na vrednost antioksidativnih parametara: superoksid dismutaze (SOD), glutation peroksidaze (GPx), glutation reduktaze (GR) i ukupnog antioksidantnog statusa (TAS) pacijenata sa tipom 2 diabetes mellitusa i sa izraženim kardiovaskularnim komplikacijama. Ispitano je 100 ispitanika, među njima 69 obolelih od dijabetesa tipa 2 sa prisutnim kardiovaskularnim komplikacijama i 31 zdrav ispitanik. Statističkom obradom podataka dobijeno je da pacijenti s tipom 2 dijabetesa i sa izraženim kardiovaskularnim komplikacijama u vidu koronarne bolesti srca (KB), hipertenzije (HTA) i preležanog akutnog infarkta miokarda (AIM) u poslednjih 8 godina, imaju značajno niže vrednosti antioksidativnih parametara ($p<0,001$) u odnosu na zdrave ispitanike. Dijabetičari tipa 2 koji imaju duže vremena dijabetes i koronarnu bolest imaju i veće vrednosti glukoze, višu aktivnost GR, ali i niže koncentracije TAS-a i niže aktivnosti SOD-a ($p<0,05$). Jutarnja koncentracija glukoze negativno korelira sa aktivnostima SOD-a i GPx-a u podgrupama dijabetičara sa ozbiljnijim kardiovaskularnim komplikacijama (KB+AIM; KB+HTA+AIM) ($p<0,05$).

Ključne reči: oksidativni stres, antioksidantna zaštita, tip 2 diabetes mellitusa, kardiovaskularne komplikacije

Introduction

It is well established that type 2 diabetes mellitus is associated with highly increased risk of coronary heart disease (CHD), cardiovascular disease (CVD), and total mortality. CVD is the leading cause of death

of people with diabetes. Previous studies have reported that patients with CVD and type 2 diabetes have two- to fourfold higher risk of mortality compared to patients without diabetes (1, 2). Hyperglycemia and oxidative stress are the major factors in the pathogenesis of atherosclerosis in diabetes. Hyperglycemia associated with diabetes can lead to modification of macromolecules, resulting in the formation of advanced glycation end products (AGE) (3). Once generated, the AGE product is stable and irreversible, and it constantly accumulates in long-lived proteins in the blood vessel walls. This accumulation rises with aging and the rapid development of diabetes. AGE products accelerate atherosclerotic processes via several mechanisms, which are classified as non-receptor dependent and receptor-mediated mechanisms (4).

Glycosylation processes are, to a large extent, present on the apoprotein B (5) and phospholipid components of the LDL particle (6), where they cause functional changes of LDL clearance and higher susceptibility of LDL to oxidative modification. Takeover of these modified LDLs is completed by non-specific scavenger receptors, which are found on the surface of human macrophages; it causes the production of foam cells and initiates atherosclerosis.

Binding of AGE to specific receptors on the surface of endothelial cells, monocytes-macrophages and smooth muscle cells gives rise to the induction of oxidative stress, by increasing the expression of nuclear factor (NF-κ B) and adhesive molecule VCAM-1 synthesis (7), resulting in increased permeability of the endothelial cell monolayer. The increased chemotaxis (8), followed by infiltration of mononuclears through the intact endothelium cell monolayer (9), and the increased synthesis of proinflammatory reaction mediators such as: interleukin-1 (IL-1), tumor necrosis factor-α (TNF-α), platelet-derived growth factor (PDGF) and insulin-like growth factor-1 (IGF-1) (10) in the monocytes-macrophages, accelerate the atherosclerotic processes (11).

The antioxidant system (ADS) of the organism plays a crucial role in reducing the increased levels of free radicals generated by impaired glucose metabolism. ADS involves some enzymatic components such as: superoxide dismutase (SOD, EC 1. 15.1.1), catalase (CAT, EC 1.11.1.6), glutathione peroxidase (GPx, EC 1.11.1.9), glutathione reductase (GR, EC 1.6.4.2), glutathione-S-transferase (GST, EC 2.5.1.18), but also some low molecular weight non-enzymatic components, e.g. reduced form of glutathione (GSH), uric acid, albumin, bilirubin, vitamin E and vitamin C (12–14).

Previous studies have reported that the antioxidant defense system of diabetic patients with cardiovascular complications (15) is seriously altered. The aim of our study was to analyze the effect of type and duration of cardiovascular complications on antioxidant parameter values in patients with type 2 diabetes mellitus and manifested cardiovascular complications.

Material and Methods

Out of 100 subjects included in the study, 69 subjects (32 females and 37 males) were type 2 diabetic patients, aged 57.9 ± 8.7 years (from 39 to 78 years), treated at the Institute of Endocrinology, Diabetes and Metabolic Disorders, Clinical Center of Serbia, and 31 were age-matched healthy subjects (8 males and 25 females), recruited from hospital staff, who were without any acute or chronic diseases, and did not take any nutritional supplements containing substances with antioxidative effect.

For better analysis of the impact of type and duration of cardiovascular complications on antioxidative parameter values, each group was divided into five subgroups, covering a time period of 10 years, and including subjects aged 31–80 years, as follows: subgroup I (31–40 yrs.), subgroup II (41–50 yrs.), subgroup III (51–60 yrs.), subgroup IV (61–70 yrs.), and subgroup V (>70 years).

All type 2 diabetic patients had history of coronary events manifested as coronary artery disease, acute myocardial infarction in the previous 8 years and unregulated hypertension diagnosed according to the World Health Organization criteria. Mean duration of diabetes was 9.34 ± 9.65 years (1 to 43 years). All type 2 diabetic patients had coronary artery disease (CAD) with the duration of 6.39 ± 5.75 years (1 to 20 yrs.), out of whom 17 patients (24.6%) had CAD as only complication, while 32 patients (46.4%) had CAD with hypertension (CAD + HTA), with the mean duration of hypertension of 10.72 ± 9.7 years (1 to 33 yrs.). The rest of the patients (28.99%) had experienced acute myocardial infarction (AMI) in the 8 years before the time of testing, out of whom 7 patients (10.14%) had CAD and AMI, while 13 patients (18.84%) had all three types of complications (CAD + HTA + AMI).

Blood samples were drawn after 12–14 h of fasting. Antioxidant status was estimated by the activities of Cu, Zn erythrocyte superoxide dismutase (SOD), Se-dependent erythrocytes glutathione peroxidase (GPx), plasma glutathione reductase (GR), and total antioxidant status (TAS), using the commercial assays manufactured by Randox Laboratories, based on spectrophotometer determination methods. SOD was determined in a hemolysate prepared from blood collected in lithium-heparinized Vacutainer test tubes. The hemolysate preparation consisted of erythrocyte separation by centrifugation for 10 minutes at 3000 rpm, followed by washing four times with 3 mL of 154 mmol/L NaCl and centrifuged at 3000 rpm. After the last supernatant decantation, erythrocytes were lysed with 2 mL of cold deionized water and left for 15 minutes at 4 °C in order to complete the hemolysis. To obtain linear measurement, it was necessary to dilute the lysates 26 times with 10 mmol/L of pH 7.0 phosphate buffer. The SOD determination was performed using the Ransod test kit based on the method descri-

bed by Goldstein (16). For GPx determination, the whole blood was used, diluted with dilution solution (supplied with test reagents) and lysed with a double concentration of the Drabkin reagent. In this way, the blood was 41 times diluted with addition of equal amounts of indicated solutions, and then determined by using Ransel kits based on the method described by Paglia and Valentine (17). TAS and GR were determined in plasma obtained after 10-minute centrifugation of Li-heparinized blood at 3000 rpm, using the commercial kits developed by the same manufacturer, based on methods described by Miller (18) and Goldberg, respectively (19). Fasting glucose levels in serum as well as total cholesterol, triglycerides, HDL-cholesterol, creatinine, uric acid, total and direct bilirubin, albumin and total proteins were determined using the standard laboratory methods. LDL-cholesterol concentrations were calculated using the Friedewald formula.

For statistical evaluation, basic methods of descriptive statistics were used: mean values ($\bar{x}$) with dispersion measure (standard deviation). Statistical significance was calculated using the Student's t-test, Mann-Whitney U-test and one-way analysis of variance, as well as Spearman rank correlation test.

Results

The obtained values of antioxidative parameters along with patients' characteristics are presented in *Table I*.

The obtained values of all tested antioxidative parameters in type 2 diabetic patients were significantly lower in comparison to healthy subjects ($p<0.0001$).

Mean glucose values in the group of diabetic patients with cardiovascular complications were 8.66 ± 3.2 mmol/L (4.3 to 20.1 mmol/L), and they were significantly higher compared to healthy control subjects (5.8 ± 0.9 mmol/L) ($p<0.05$).

Mean duration of diabetes in this group of patients was 9.34 ± 9.65 yrs. The longest duration of diabetes was in the subgroup III of patients (51–60 yrs. old), 10.2 ± 11.5 years (34 patients), and the shortest duration was in subgroup II: 7.1 ± 6.6 yrs. (11 patients). The longest duration of coronary artery disease was in subgroup IV: 6.8 ± 6.1 yrs. and the shortest in the subgroup in which patients were 41–50 years of age (2 yrs.). The longest duration of hypertension was in the subgroup of patients older than 70 (19.5 ± 16.2 yrs.), and the shortest was in the subgroup II with mean duration of 7.5 ± 4.95 yrs. The highest incidence of acute myocardial infarction was in subgroup III (10 cases).

The mean values of tested parameters according to the patient's age are presented in *Figure 1*.

Significantly lower SOD activity was obtained in type 2 diabetic patients with cardiovascular complications in comparison to the healthy control group ($p<0.0001$). Significantly lower values were obtained in subgroups II ($t = 2.971$, $p<0.01$) and III ($t = 2.584$, $p<0.05$) compared to the same age group of the controls. No significant difference in SOD values was established between the tested age groups of diabetics with complications. GPx values were also significantly lower in diabetics with CVC compared to the control group. Significant difference was observed in the age subgroup II compared to the same age group of the controls ($t = 3.788$, $p<0.01$). TAS values were significantly higher in the controls compared to type 2 diabetic patients with cardiovascular complications, with significant difference in the age subgroups II and III between the two tested groups ($t = 1.987$, $p<0.05$). Glutathione reductase activity was also significantly lower in diabetics with CVC compared to the controls with significant difference in the subgroup II compared to the same age subgroup of the controls ($t = 3.008$, $p<0.05$). The lowest values of GR activities were observed in the youngest patients (subgroup I), and the highest values were in older patients (subgroups IV and V).

The patients with CAD and CAD with HTA had significantly lower TAS values compared to patients who had CAD and AMI ($p<0.05$) (*Table I*). The patients in CAD, AMI and CAD, AMI and HTA groups had significantly higher GR and lower glucose values compared to those patients who had CAD with or without HTA ($p<0.05$).

There was highly positive and significant correlation between glucose levels and duration of diabetes in the group of CAD+AMI+HTA ($r = 0.730$, $p = 0.005$), as well as between glucose concentration and duration of coronary artery disease ($r = 0.557$, $p<0.05$). In the group of diabetic patients with CAD and HTA, there was significant negative correlation between TAS concentration and duration of type 2 diabetes: $r = -0.393$, $p = 0.038$; and positive correlation between GR activity and duration of CAD ($r = 0.404$, $p<0.05$). There was also negative correlation between the duration of diabetes and SOD activity in the group of CAD and AMI ($r = -0.949$) at the border-line value of significance ($p = 0.051$).

In the subgroup of CAD+HTA there was significant positive correlation between SOD and GPx activities ($r = 0.42$, $p = 0.029$) and significant inverse correlation between TAS concentration and GR activity ($r = -0.392$, $p<0.05$). GPx values were in inverse correlation with glucose concentration values ($r = -0.382$, $p<0.05$). In the subgroup of diabetics with CAD and AMI but without HTA, highly significant and positive correlation was found between SOD and GPx values ($r = 0.925$, $p = 0.024$), and highly significant negative correlation between GPx activity and glucose concentration ($r = -0.860$, $p<0.05$). In the subgroup of CAD+AMI+HTA, significant negative correlation was

Table I Characteristics of type 2 diabetic patients with cardiovascular complications.

Parameters	CAD	CAD + HTA	CAD+AMI	CAD+AMI +HTA	Total DM patients	Control group
Number of subjects	17	32	7	13	69	31
Sex (M/F)	10/7	16/16	3/4	8/5	37/32	8/25
Age (yrs.)	60.8 ± 6.7	59.5 ± 7.11	58 ± 7.55	6.3 ± 6.1	57.9 ± 8.7	41.6 ± 11.4
Duration of DM (yrs.)	5.8 ± 4.9	11.5 ± 11.7 ♣	4.0 ± 6.7	6.3 ± 6.1	9.34 ± 9.6	–
Duration of CAD (yrs.)	4.2 ± 3.4	5.8 ± 5.7	3.8 ± 4.3	5.7 ± 5.3	6.39 ± 5.76	–
Duration of hypertension (yrs.)	–	6.78 ± 6.67	–	7.3 ± 6.46	10.7 ± 9.7	–
SOD (U/gHb)	792 ± 107	799 ± 113	842 ± 53	805 ± 120	806 ± 103.6 •	969.4 ± 104.8
GPx (U/gHb)	23.8 ± 5.3	23.1 ± 4.0	25.9 ± 4.5 ♦	23.1 ± 5.8	23.6 ± 4.6 •	29.1 ± 3.5
GR (U/L)	52.4 ± 10.5*	55.4 ± 10.3	60.4 ± 8.5	57.8 ± 6.0 ♥	55.1 ± 9.5 •	62.5 ± 7.9
TAS (mmol/L)	1.11 ± 0.15*	1.17 ± 0.19	1.34 ± 0.1 ♦	1.18 ± 0.25	1.18 ± 0.18 •	1.35 ± 0.23
Glucose (mmol/L)	9.1 ± 2.3*	8.9 ± 3.8	7.3 ± 2.1	7.5 ± 1.8 ♥	8.6 ± 3.16 •	5.04 ± 0.8
Total bilirubin (μmol/L)	11.9 ± 4.3	15.5 ± 7.5	15.1 ± 6.7	17.8 ± 10.6	14.83 ± 6.8	12.5 ± 3.8
Direct bilirubin (μmol/L)	3.4 ± 1.2	5.9 ± 3.1	6.06 ± 2.6	5.85 ± 2.5	5.4 ± 2.8	4.69 ± 1.3
Total protein (g/L)	69.9 ± 5.2	69.3 ± 5.7	72.3 ± 5.9	66.8 ± 9.1	69.6 ± 6.1 •	73.4 ± 4.6
Albumin (g/L)	47.2 ± 6.7	43.4 ± 5.3	47.3 ± 5.6	41.3 ± 4.3	43.5 ± 4.9	45.3 ± 4.9
Acidum uricum (μmol/L)	295.9 ± 84.2	351.6 ± 90.8	271.2 ± 152.2 ♦	311.3 ± 128.3	327 ± 101 •	250.6 ± 88.7
Haptoglobin (g/L)	1.48 ± 0.73	1.06 ± 0.67	2.69 ± 3.35 ♦	2.72 ± 2.84 ♥	1.38 ± 1.27 •	0.74 ± 0.2
Total cholesterol (mmol/L)	6.04 ± 1.28	6.33 ± 1.23	5.95 ± 0.48	5.05 ± 0.79	6.07 ± 1.26	5.76 ± 0.9
Triglycerides (mmol/L)	2.78 ± 1.66	2.42 ± 1.38	2.33 ± 1.09	3.01 ± 2.1	2.58 ± 1.6 •	1.60 ± 0.66
HDL-cholesterol (mmol/L)	1.13 ± 0.34	1.21 ± 0.29	1.36 ± 0.37	0.88 ± 0.24	1.15 ± 1.58	1.21 ± 0.19
LDL-cholesterol (mmol/L)	3.32 ± 1.03	4.11 ± 1.29	3.45 ± 0.77	3.12 ± 0.59	4.01 ± 1.2	3.84 ± 0.94

Significance of difference:
* $p<0.05$ = difference between CAD and CAD+AMI
♦ $p<0.05$ = difference between CAD + AMI and CAD + HTA
♥ $p<0.05$ = difference between CAD + AMI + HTA and CAD
♣ $p<0.05$ = difference between CAD + HTA and CAD
• $p<0.05$ = difference between CG and all type 2 diabetics with CVC
Legend:
CAD – coronary artery disease
CAD + HTA – coronary artery disease with hypertension
CAD + AMI – coronary artery disease and acute myocardial infarction
CAD + AMI + HTA – coronary artery disease, acute myocardial infarction and hypertension

obtained between SOD activity and fasting glucose levels ($r = -0.590$, $p<0.05$).

Discussion

Based on the obtained results, it may be concluded that type 2 diabetic patients with cardiovascular complications had significantly lower enzymatic antioxidant defense and higher fasting glucose levels compared to healthy control subjects ($p<0.001$) (20). Significant impairment of SOD, GPx, GR and TAS values was more pronounced in younger diabetic patients (40–60 years of age) with CVC than in older ones compared to the corresponding age group of the control subjects, meaning that younger type 2 diabetic patients with CVC had more severe impairment of antioxidant defense parameters than older patients. Type 2 diabetics with longer duration of diabetes and coronary artery disease had higher fasting glucose values, higher GR activity, but lower TAS and SOD activity ($p<0.05$). Fasting glucose levels correlated negatively with SOD and GPx activities in the subgroups of diabetics with severe cardiovascular complications (CAD+AMI, CAD+AMI+ HTA), meaning that patients with higher glucose levels had lower SOD and GPx activities.

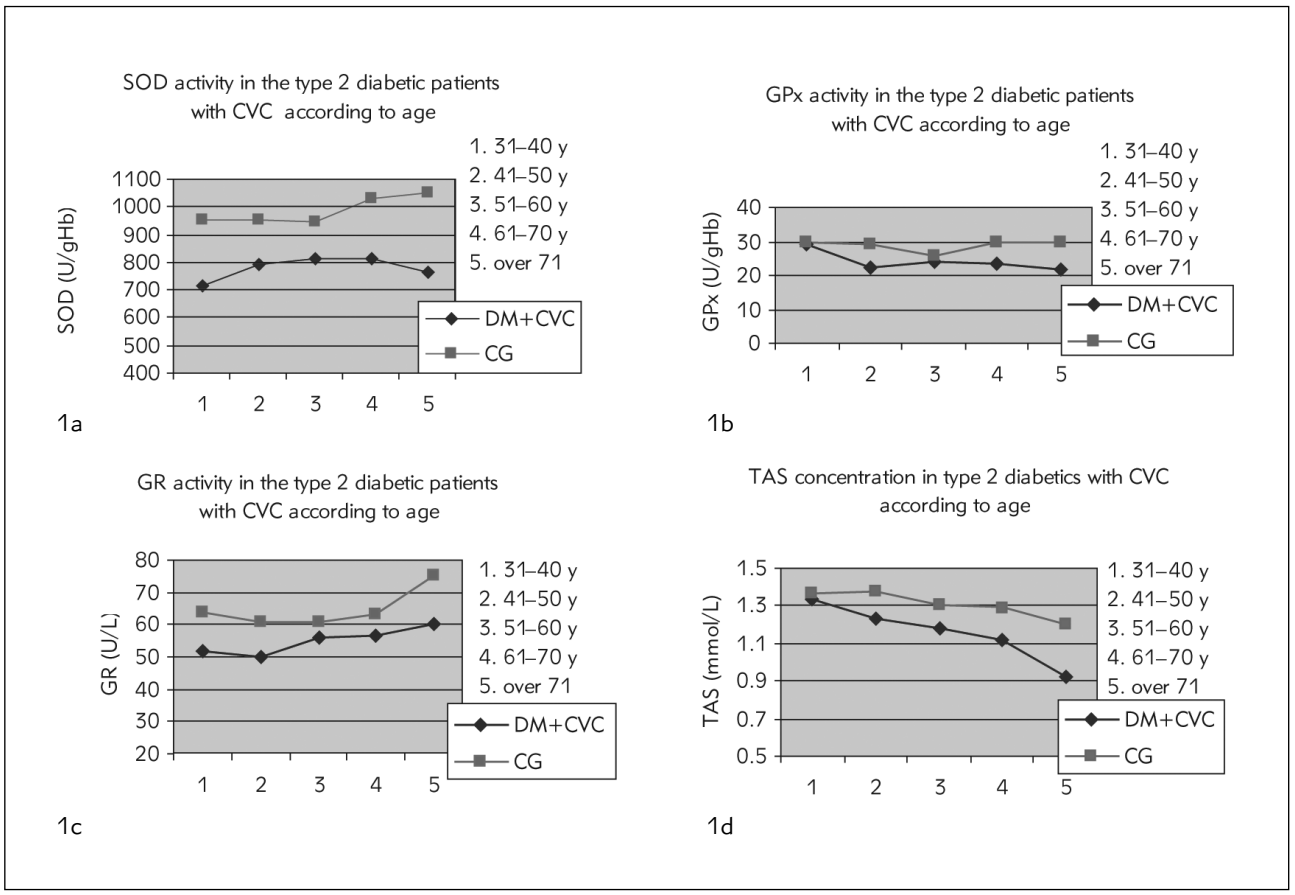

Figure 1 Mean values of SOD (a), GPx (b), GR (c), and TAS (d) in type 2 diabetic patients with cardiovascular complications (DM+CVC) and control group (CG) according to age decades.

Our study showed a positive relationship between fasting glucose levels and the duration of type 2 diabetes and the duration of cardiovascular complications, respectively ($p<0.05$). The study also showed negative correlation of SOD activity and TAS concentration with duration of type 2 diabetes in patients who had coronary artery disease, hypertension and experienced acute myocardial infarction in the past 8 years.

Similar results have been obtained by other authors having studied this subject. Kimura et al. (21) showed that the extracellular superoxide dismutase (EC-SOD) correlated positively with duration of type 2 diabetes, carotid artery intimal-media thickness and severity of nephropathy and retinopathy, which suggested that serum EC-SOD concentration levels might be a marker of vascular injury. Komosinska-Vassev (22) demonstrated the significant increase of SOD and decrease of GPx, GR and TAS values in type 2 diabetic patients with angiopathy. SOD correlated positively with plasma glucose concentration and glycated hemoglobin level, while negative correlation was found between GPx and glucose level or HbA1c, and TAS concentration and glucose or HbA1c level. The authors observed that blood glucose control and vascular complications had strong independent effects on prooxidant-antioxidant status, apart from blood glucose level and GR activity, as well as different mechanisms by which vascular complications and glucose control affected blood free radical indices and antioxidant status parameters in type 2 diabetic patients. Tosukhowong and assoc. (23) found significantly lower erythrocyte glutathione (GSH) and GPx activity in Thai patients with CAD, compared to control subjects, while TAS and vitamin E values were not significantly different between the tested groups. They obtained negative correlation between GSH and total cholesterol levels, GSH and LDL-cholesterol and between vitamin E and malondialdehyde levels as well. Ozdemir et al. (24) found significantly higher malondialdehyde levels and lower GSH levels and GPx activity in type 2 diabetic patients with microalbuminuria. These authors suggested that decreased antioxidant levels and increased lipid peroxidation and homocysteine levels observed in patients with microalbuminuria could contribute to vascular disease, which was particularly prevalent in type 2 diabetic patients with microalbuminuria. Nojiri et al. (25) demonstrated that TAS values, concentrations of retinol, albumin, total protein and HDL-cholesterol were significantly lower in CAD patients compared to control

subjects. TAS values correlated positively with uric acid and negatively with a number of diseased vessels. The study demonstrated the association of antioxidant parameters with the atherosclerosis progression, however, it failed to confirm antioxidants as independent risk factors of CAD events.

Determination of markers of antioxidative defense as very sensitive parameters contributes not only to a better understanding of the effect of oxidative stress on the development of diabetes and diabetic complications, but also on the prevention of atherosclerosis and micro- and macrovascular complications of diabetes.

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