Introduction

Diabetes-associated oxidative stress is a consequence of both increased production of free radicals and reduced capacity for antioxidative defense. Prolonged hyperglycemia is the major factor in the pathogenesis of atherosclerosis in diabetes which is the cause of 80% of total mortality in diabetic patients, while more than 75% of total hospitalizations due to diabetic complications are attributable to cardiovascular diseases. Hyperglycemia induces a large number of alterations in the vascular tissue that potentially accelerate the atherosclerotic processes. There are three major mechanisms that encompass most of the pathological alterations observed in the diabetic vasculature: 1) nonenzymatic glycosylation of proteins and lipids which can interfere with their normal function by disrupting molecular conformation, alter enzymatic activity, reduce degradative capacity and interfere with receptor recognition; 2) oxidative stress and 3) protein kinase C activation with subsequent alteration in growth factor expression (1, 2).

One of the most important mechanisms responsible for accelerated atherosclerosis in diabetes is the nonenzymatic reaction between glucose and proteins or lipoproteins in arterial walls, leading to for-
mation of advanced glycosylation end products (AGEs). Once formed, AGE protein adducts are stable and irreversible and continuously accumulate with aging and accelerated rate of diabetes on the long-lived vessel wall proteins. The degree of nonenzymatic glycation is determined mainly by glucose concentration and time of exposure (3). AGEs can accelerate the atherosclerotic process by diverse mechanisms, which can be classified as non-receptor dependent and receptor mediated.

The most studied example of a non-receptor dependent mechanism is the interference of normal physiology of the low-density lipoprotein particle. The glycosylation process occurs both on the apoprotein B and phospholipids components of LDL, leading to functional alteration of LDL clearance and increased susceptibility to oxidative modifications (4–7). LDL-oxidation following AGE-LDL formation occurs in direct proportion to glucose concentration and it is considered a critical step in the atherosclerotic process (8).

The presence of AGE receptor (RAGE) has been demonstrated in all cells relevant to the atherosclerotic process including monocyte-derived macrophages, endothelial cells and smooth muscle cells (9). AGE interaction with RAGE on endothelial cells results in introduction of oxidative stress and consequently of transcription factor NF-kB and Vascular Cell Athesion Molecule-1 (VCAM-1) (10) resulting in reduced barrier function with increased permeability of endothelial cell monolayers (11). This reaction can initiate events in atherogenesis as well as monocyte-macrophage interaction of RAGE and AGE resulting in the production of mediators such as interleukin-1 (IL-1), tumor necrosis factor-α (TNF-α), platelet-derived growth factor (PDGF) and insulin growth factor-1 (IGF-1) (12–14). In smooth muscle cells, binding of AGE-modified proteins with RAGE is associated with increased cellular proliferation (15).

High glucose concentration activates protein kinase C (PKC) by increasing the formation of diacylglycerol (DAG) which is the major endogenous cellular co-factor of PKC activation (16). The PKC system is involved in the transcription of several growth factors and in signal transduction as response to growth factors (17,18). PKC activation increases the expression of transforming growth factor-β (TGF-β), which is one of the most important growth factors regulating extracellular matrix production (19). Increased expression of TGF-β is thought to lead to the thickening of capillary basement membrane, one of early structural abnormalities observed in almost all tissues in diabetes.

Hyperglycemia can increase oxidative stress through several pathways. The main mechanism is hyperglycemia-induced intracellular reactive oxygen species, produced by the proton electrochemical gradient generated by mitochondrial electron transport chain and resulting in increased production of superoxide (2). The other mechanism involves the transition metal-catalysed auto-oxidation of free glucose yielding superoxide anion and hydrogen peroxide (20). The third mechanism involves the transition metal-catalysed auto-oxidation of protein-bound Amadori products, which yields superoxide and hydroxyl radicals and highly reactive dicarbonyl compounds (21).

There is also evidence that hyperglycemia may compromise natural antioxidant defense. Under normal circumstances free radicals are rapidly eliminated by antioxidants such as reduced glutathione, vitamin C and vitamin E (22, 23). Reduced glutathione content, as well as reduced vitamin E, have been reported in diabetic patients (24). Plasma and tissue levels of vitamin C are 40–50% lower in diabetic patients compared with non-diabetic subjects. Nonenzymatic glycation of enzymes, especially intra- and extracellular SOD, compromise their catalytic activity.

The aim of this study was to test the parameters of antioxidative defense: SOD, GSH-Px, GR and TAS in type 2 diabetic patients with and without cardiovascular complications in order to discover the effect of hyperglycemia on the extent of disorders of antioxidative parameters.

### Material and Methods

A total of 159 subjects: 71 males and 88 females aged 32 to 90 years, were included in the study. They were divided into three groups: 69 of them, aged 57.9 ± 8.7 years (37 males and 32 females), were type 2 diabetics with cardiovascular complications (DM + CVC), 48 of them were type 2 diabetics without complications (DM) (25 males and 23 females), aged 58.1 ± 10 years, while the control group consisted of 42 age-matched healthy subjects (9 males and 33 females). Type 2 diabetic patients were tested angiographically in order to establish the presence and type of complications. All of type 2 diabetic patients with cardiovascular complications had coronary artery disease (CAD) as primary complication, 32 (46.4%) of them had CAD and hypertension (CAD + HTA), 7 (10.1%) patients had CAD and acute myocardial infarction (CAD + AMI), while 13 (18.8%) had all three types of complications (CAD + HTA + AMI).

Determination of antioxidant parameters: SOD, GSH-Px, GR and TAS, was performed using commercial tests manufactured by Randox Laboratories, UK, based on spectrophotometer determination methods. SOD was determined in a hemolysate preparation of hyperglycemic subjects, aged 58.1 ± 10 years, while the control group consisted of 42 age-matched healthy subjects (9 males and 33 females). Type 2 diabetic patients were tested angiographically in order to establish the presence and type of complications. All of type 2 diabetic patients with cardiovascular complications had coronary artery disease (CAD) as primary complication, 32 (46.4%) of them had CAD and hypertension (CAD + HTA), 7 (10.1%) patients had CAD and acute myocardial infarction (CAD + AMI), while 13 (18.8%) had all three types of complications (CAD + HTA + AMI).
lowed by washing four times with 3 mL of 154 mmol/L NaCl and centrifuged at 3 000 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 finish hemolysis. To obtain a linear measurement, it was necessary to dilute the lysates 26 times with 10 mmol/L of pH 7.0 phosphate buffer. SOD determination was performed using Ransod test kit (Randox Laboratories Ltd, UK) based on the method described by Goldstein (26).

For GSH-Px determination, the whole blood was diluted with dilution solution (obtained through test-reagents) and lysed with a doubly concentrated Drabkin reagent. In this way, blood was diluted 41 times with addition of equal amounts of indicated solution, and then examined using Ransel kits based on the method described by Paglia and Valentine (27).

TAS and GR were determined in plasma obtained after the 10-minute centrifugation of Li-heparinized blood at 3 000 rpm using commercial kits developed by the same manufacturer, based on methods described by Miller (28) and Goldberg (29), respectively.

Fasting glucose levels were also determined in sera for all subjects using a standard method.

For statistical evaluation, basic methods of descriptive statistics were used: mean values with dispersion measure (standard deviation). Statistical significance was determined using 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 and glucose concentration are presented in Table I.

Statistical data processing revealed significantly lower SOD values in diabetics with cardiovascular complications compared to the control group (p<0.0001) and to diabetics without complications (p<0.001), as well as significantly lower GSH-Px values in both pathological groups in comparison to the control group (p<0.05). There was also a significant difference of GSH-Px values between diabetics with and without complications (p<0.001). Mean GR and TAS values of diabetics with complications were also significantly lower in comparison to the control group (p<0.0001 and p = 0.0002 respectively). Lower GR and TAS values were also obtained in diabetics without complications, but such difference was not statistically significant.

Diabetics with coronary disease as their complication had significantly lower values of TAS (t= 2.57, p<0.05), GR (t = 1.96, p<0.05) and glucose (t = 1.965, p<0.05) in relation to patients with coronary disease and experienced acute myocardial infarction as complications (Table II). Patients with coronary disease and AMI manifested significantly higher values of TAS in relation to DM patients having CD with HTA (t=1.965, p<0.05). Reviewing as a whole the diabetics with complications and interrelating the values of antioxidative parameters, significant positive correlation was found between SOD and GPx, where Spearman’s factor was r = 0.259 for p<0.05, while there was negative correlation between TAS and GPx and GR, but it was not statistically significant.

In diabetics without complications, Spearman’s correlation coefficient revealed significant positive

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>DM + CVC</th>
<th>DM</th>
<th>CG</th>
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<tbody>
<tr>
<td>N</td>
<td>69</td>
<td>48</td>
<td>42</td>
</tr>
<tr>
<td>Sex (male/female)</td>
<td>37/32</td>
<td>25/23</td>
<td>9/33</td>
</tr>
<tr>
<td>Age (years)</td>
<td>57.9 ± 8.7</td>
<td>58.1 ± 10</td>
<td>51.6 ± 11.4</td>
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<td>Duration of DM (years)</td>
<td>9.34 ± 9.7</td>
<td>7.36 ± 7.5</td>
<td>–</td>
</tr>
<tr>
<td>SOD (U/gHb)</td>
<td>806.5 ± 103.6*</td>
<td>961.0 ± 92.9*</td>
<td>969.0 ± 104.8</td>
</tr>
<tr>
<td>GSH-Px (U/gHb)</td>
<td>23.6 ± 4.6*</td>
<td>27.2 ± 5.3*</td>
<td>29.1 ± 3.5</td>
</tr>
<tr>
<td>GR (U/L)</td>
<td>55.1 ± 9.5*</td>
<td>59.3 ± 8.8*</td>
<td>62.5 ± 8.0</td>
</tr>
<tr>
<td>TAS (mmol/L)</td>
<td>1.17 ± 0.19*</td>
<td>1.27 ± 0.21*</td>
<td>1.35 ± 0.23</td>
</tr>
<tr>
<td>Glucose (mmol/L)</td>
<td>8.6 ± 3.2*</td>
<td>9.2 ± 3.4*</td>
<td>5.0 ± 0.8</td>
</tr>
</tbody>
</table>

Significance of differences:
* p<0.05 – difference between pathological group and control group
▲ p<0.05 – difference between DM and DM + CVC
The effect of hyperglycemia on the values of antioxidative parameters

Correlation between SOD and glucose (r = 0.375, p<0.05), and between GPx and glucose (r = 0.384, p<0.05). If the diabetics with complications were observed as a heterogeneous group consisting of 4 subgroups, Spearman’s correlation coefficient disclosed important information: significant negative correlation was found between GPx and glucose (r = –0.382, p<0.05) in DM group with CD and HTA, and also in DM group with CD and AIM (r = –0.860, p<0.05); in DM group with all three types of complications highly significant negative correlation was found between SOD and glucose (r = –0.590, p<0.05).

These data suggest a direct correlation between glucose concentrations and the activities of studied enzymes: SOD and GPx, as well as a negative impact of glycosylation of proteins and proteinaceous enzymes involved in this process, which is manifested by reduced catalytic activity of the enzymes.

**Discussion**

On the basis of the obtained results, it may be concluded that the values of studied antioxidative parameters (SOD, GPx, GR, and TAS) were significantly lower in diabetics with cardiovascular complications both in relation to the controls (p<0.001) and to the diabetics without complications (p<0.05) (30, 31). Cardiovascular complications alter the antioxidative defense of diabetic patients in the way that TAS and GR values are significantly lower (p<0.05) in diabetics with coronary disease, regardless of the fact whether it is associated with hypertension or not, in relation to those diabetics who have experienced acute myocardial infarction in the last 8 years. It is important to highlight that, in the diabetic group without complications, the increase of glucose concentration is followed by higher activity of SOD and GPx, which means that in these patients hyperglycemia induces a positive response from the antioxidative defense system; on the contrary, in diabetics with cardiovascular complications, higher glucose concentration is associated with lower GPx activity in diabetic subgroups who had coronary artery disease with hypertension and AIM, respectively (p<0.05), as well as with lower SOD activity in diabetics with all three forms of complications (CAD+HTA+AIM). Such negative response of the antioxidative defense system may be related to the effect of protein glycosylation and the impact of oxidative stress on reduced catalytic SOD and GPx activity, all contributing to impaired total antioxidative defense of diabetics with cardiovascular complications.

Similar results have been obtained by other authors who had studied this issue.

Oda et al. (30) proved, by in vitro experiment, that incubation of Cu,Zn-SOD with increasing glucose concentration ranging from 10–100 mmol/L in a time period of 2–120 hours produces increased glycosylation of this enzyme and reduces its activity by 40%. The same authors confirmed this in vitro experiment by an in vivo one, where the activity of erythrocytic Cu,Zn-SOD in insulin-independent diabetics correlated negatively with glucose concentration, suggesting that hyperglycemia brings about the glycosylation and inactivity of this enzyme.

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>CAD</th>
<th>CAD+HTA</th>
<th>CAD+AMI</th>
<th>CAD+AMI+HTA</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>17</td>
<td>32</td>
<td>7</td>
<td>13</td>
</tr>
<tr>
<td>Sex (M/F)</td>
<td>10/7</td>
<td>16/16</td>
<td>3/4</td>
<td>8/5</td>
</tr>
<tr>
<td>SOD (U/gHb)</td>
<td>791.6 ± 106.6</td>
<td>799.4 ± 112.7</td>
<td>842.4 ± 53.4</td>
<td>805 ± 119.6</td>
</tr>
<tr>
<td>GSH-Px (U/gHb)</td>
<td>23.8 ± 5.3</td>
<td>23.1 ± 4.0</td>
<td>25.9 ± 4.5*</td>
<td>23.1 ± 5.8</td>
</tr>
<tr>
<td>GR (U/L)</td>
<td>52.4 ± 10.5*</td>
<td>55 ± 10.3</td>
<td>60.4 ± 8.5</td>
<td>57.8 ± 6.0 ▼</td>
</tr>
<tr>
<td>TAS (mmol/L)</td>
<td>1.15 ± 0.15*</td>
<td>1.17 ± 0.19</td>
<td>1.34 ± 0.1*</td>
<td>1.18 ± 0.25</td>
</tr>
<tr>
<td>Glucose (mmol/L)</td>
<td>9.1 ± 2.3*</td>
<td>8.9 ± 3.8</td>
<td>7.3 ± 2.1</td>
<td>7.5 ± 1.8 ▼</td>
</tr>
</tbody>
</table>

Significance of differences:
* p<0.05 difference between CAD and CAD + AMI
▼ p<0.05 difference between CAD + AMI + HTA and CAD
∧ p<0.05 difference between CAD + AMI + HTA and CAD
* p<0.05 difference between CAD + HTA and CAD
Kesavulu et al. (33) obtained low SOD and GPx values in type 2 diabetics with microvascular complications such as retinopathy and nephropathy which were associated with higher concentrations of lipid peroxide and HbA1C (>10%). Siemianowich et al. (34) obtained low values of GPx and catalase in children who were at high risk of coronary arterial disease because they had been born in families with positive history.

Valabhi et al. (35) proved that patients with type 1 DM and coronary arterial calcification had lower TAS value in relation to non-diabetics without calcification, which correlated negatively with diabetes duration, age, degree of calcification, cholesterol and creatinine concentrations, and arterial pressure level. Therefore, they suggest that this parameter be accepted as an independent predictive index of coronary arterial calcification.

Lapenna and assoc. (36) confirmed low GPx and GR values in carotid arterial plaque in 13 patients with marked atherosclerosis of carotid arteries. It is known that GPx and GR may be inactivated by free radicals, particularly hypochloric acid which results from myeloperoxidase action. GPx may also be inactivated by 4-hydroxinonenal, byproduct of lipid peroxidation, which originates from peroxidation of LDL in circulation and inactivates GPx in erythrocytes (36). Therefore, it is believed that these two enzymes are significant in conditions of oxidative stress in atherosclerotic lesions. GPx is an essential enzyme for the elimination of organic and inorganic peroxides, and it is a crucial intracellular antioxidative enzyme in mammals (38). Peroxides are cytotoxic to vascular cells, especially in the presence of transitory redox-active metals which are available in catalytic active form in human atherosclerotic plaque (38). The key role of GPx in vascular antioxidative defense is based on the fact that catalase is deficient while SOD is poorly effective in vascular cells (40). Accordingly, deficiency of GPx and redox glutathione cycle in atherosclerotic tissue may considerably weaken their antioxidative potential and therefore favor the pro-oxidative and atherosclerotic processes, even if there is a normal concentration of low-molecular «scavenging» antioxidants (41).

Determination of markers of antioxidative defense as very sensitive parameters not only contributes to a better understanding of the effect of oxidative stress on the development of diabetes and diabetic complications, but also opens new perspectives for the treatment of diabetic complications; in addition, it is particularly important in the prevention of atherosclerosis and diabetic micro- and macrovascular complications.

Acknowledgement: The present work was supported by the Ministry of Sciences and Environmental Protection of Serbia, on the basis of contact No. 145010.
References


Received: January 31, 2006
Accepted: February 25, 2006