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# ZNAČAJ MARKERA OKSIDATIVNOG STRESA KOD KORONARNIH BOLESNIKA SA DIJABETESOM MELITUSOM TIPA 2

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*S obzirom na značaj oksidativnog stresa u aterosklerozi kao glavnom etiopatogenetskom mehanizmu nastanka koronarne bolesti (KB), cilj ovog rada bio je odrediti značaj pojedinih parametara oksidativnog stresa kod koronarnih bolesnika sa dijabetesom melitusom (DM) tipa 2. Analizirani su uzorci krvi pre terapije i određivana je aktivnost antioksidativnog enzima superoksid dizmutaze (SOD), enzima ksantin oksidaze (XO), intenzitet lipidne peroksidacije i produkt oksidativne modifikacije proteina (karbonilne grupe). Istraživanjem je obuhvaćeno 40 bolesnika sa dokazanim KB i DM tipa 2, 30 bolesnika sa KB i 20 ispitanika bez znakova za KB, DM tipa 2 i intoleranciju glikoze. Značajno veća aktivnost XO ($p < 0,01$) i intenzivnija lipidna modifikacija MDA ($p < 0,01$) su zapažene kao karakteristike kod bolesnika sa DM. Slično, značajno veća aktivnost XO ($p < 0,05$) i intenzivnija lipidna modifikacija MDA ($p < 0,01$) bile su karakteristike nalaza i kod bolesnika sa KB. Urađeni Backward Logistic regresioni model nije pokazao značajnu povezanost markera oksidativnog stresa sa pojavom KB kod ispitivanih bolesnika. Određivanje aktivnosti XO i koncentracije MDA kod bolesnika sa koronarnom bolešću, tako i kod bolesnika sa koronarnom bolešću i dijabetesom melitusom tipa 2, imaju važan djagnostički značaj.*

***Ključne reči:*** *oksidativni stres, koronarna bolest, dijabetes melitus tipa 2*

# SIGNIFICANCE OF OXIDATIVE STRESS MARKERS IN CORONARY PATIENTS WITH TYPE 2 DIABETES MELLITUS

*Considering the importance of oxidative stress in atherosclerosis as the primary etiopathogenetic mechanism of coronary artery disease (CAD), the aim of this study was to determine the significance of specific oxidative stress parameters in coronary patients with type 2 diabetes mellitus (DM). The blood samples were analyzed prior to therapy, and the activities of the antioxidant enzyme superoxide dismutase (SOD) and the enzyme xanthine oxidase (XO) were determined, as well as the intensity of lipid peroxidation and the protein oxidative modification product (for the carbonyl group). The study included 40 patients with confirmed CAD and type 2 DM, 30 patients with CAD and 20 control subjects without signs of CAD, type 2 DM or glucose intolerance. Significantly higher XO activity ($p < 0.01$) and more intense MDA lipid modification ($p < 0.01$) were observed as characteristics of patients with type 2 DM. Similarly, significantly higher XO activity ($p < 0.05$) and more intense MDA lipid modification ($p < 0.01$) were also characteristic findings in patients with CAD. The performed Backward Logistic regression model did not show a significant association of the oxidative stress markers with the occurrence of CAD in the examined patients. Measuring the XO activity and MDA concentration in patients with coronary artery disease, as well as patients with coronary artery disease and type 2 diabetes mellitus, has an important diagnostic significance.*

***Key words:*** *oxidative stress, coronary artery disease, type 2 diabetes mellitus*

## Introduction

Oxidative stress is a significant pathogenic mechanism in the development of endothelial dysfunction in diabetes mellitus (DM). It is defined as the state with elevated levels of oxygen free radicals that can result from the increased production of free radicals and/or the decrease of the antioxidant defense mechanism (1). The role of oxidative stress in the etiopathogenesis of DM is significant and it is connected to several molecular cascades in different metabolic pathways of glycolysis, hexosamine, the protein kinase C and polyol, and the advanced glycation end-product pathways (2). The conducted studies indicate a reduction of superoxide dismutase concentration in plasma or tissue, catalase, glutathione and ascorbic acid, both in clinical and experimental diabetes. Furthermore, diabetes is associated with increased formation of oxygen free radicals (3). Free radicals can impair endothelium-dependent vasodilation by inactivating nitric oxide (NO) (4).

Diabetes affects the free radical formation through the glucose-dependent and glucose-independent mechanism. Glucose auto-oxidation is known as a mechanism for free radical generation. Recent studies have shown that cellular glucose oxidation leads to the formation of excessively reactive types of oxygen radicals in the mitochondria cytosol (5). These free radicals can lead to an increase of lipid peroxidation, including lipoprotein oxidation. Numerous molecules in the arterial wall may get modified through the spontaneous process of glycation that occurs under hyperglycemic conditions, which is followed by the process of oxidation in the process of glycoxidation. This process of advanced glycation end-product (AGE) formation, which rests on the key elements (proteins, lipids and nucleic acids), is a process that affects every tissue and cell group, either as normal aging of an organism or one accelerated by the presence of diabetes (6).

The presence of glucose or phosphorlipids significantly accelerates the glycoxidation reactions. This is observed in accelerated atherosclerosis in diabetic patients with dyslipidemia. It was shown that, in diabetes, there is an increase in oxidative modification of LDL due to the presence of small and dense LDL particles (7). Increased lipid peroxidation induces excessive oxidative stress in diabetes (8) and probably reduces antioxidant protection. Oxidative stress can influence the expression of numerous genes in vascular cells, which accelerate atherosclerosis. This includes the genes for signal molecules such as the protein kinase C, nuclear factor-B and the extracellular signal-regulated kinases (9, 10).

In hyperglycemic states, increases in superoxide dismutase and catalase activity are more effective than cyclooxygenase inhibitors in restoring impaired acetylcholine-induced vasodilation, suggesting that the oxygen radicals produced by prostanoid synthesis, rather than prostanoid itself, are responsible for endothelial dysfunction (11). Also, the literature data indicate that antioxidants restore the endothelium-dependent vasodilation, while superoxide dismutase has little or no effect, highlighting the important role of hydroxyl radicals in inducing endothelial dysfunction (12–14).

Considering the importance of oxidative stress in atherosclerosis as the main etiopathogenetic mechanism of coronary artery disease, the aim of this study was to determine the significance of specific oxidative stress parameters in coronary patients with type 2 diabetes mellitus.

## Material and methods

The study included 90 participants, of whom 70 were patients with coronary artery disease treated at the Military Hospital Niš, and 20 were healthy subjects.

Patients with valvular heart diseases, a cerebrovascular disease, an implanted pacemaker, chronic liver or kidney diseases, malignant diseases, or other conditions that could affect the modification of characteristics of coronary artery disease, were excluded from the study.

Based on the presence of diabetes and signs of glucose intolerance, all the participants were divided into three groups:

Group I: 40 patients with confirmed coronary artery disease and type 2 diabetes mellitus (CAD and type 2 DM).

Group II: 30 patients with coronary artery disease (CAD). In this group, the patients had no diagnosed diabetes and no signs of glucose intolerance, which was confirmed by an OGTT test.

Group III: 20 subjects without signs of coronary artery disease, diabetes or glucose intolerance.

A detailed medical history was taken from all the patients, followed by blood sampling from the cubital vein prior to the initiation of therapy.

Methods for assessing oxidative stress

The superoxide dismutase (SOD) activity was determined from whole blood with an addition of EDTA, with hemolysate prepared on ice. The SOD concentration was measured photometrically (λ = 505 nm) using a commercial Randox kit. The superoxide anion radical, which is generated by the xanthine/xanthine oxidase system, reacts with the INT electron acceptor, forming a red formazan color. The SOD activity

was determined by the degree of inhibition of this reaction and calculated using the standard curve, while the values were expressed per gram of hemoglobin (U/g Hb).

The activity of xanthine oxidase (XO), a potent oxidative enzymatic system, was measured from plasma using the spectrophotometric method of Kizaki and Sakurada from 1977 (21). The method is based on the oxidation of xanthine to uric acid under the action of this enzyme. The extinction was measured at a wavelength of 292 nm and the difference in the UV absorbtion spectrum between xanthine and uric acid was calculated.

The lipid peroxidation intensity was determined by measuring the concentration of malondialdehyde (MDA) in serum, one of the end products of lipid peroxidation, according to the method of Stroev and Makarov from 1989 (22). The precipitation was performed with trichloroacetic acid (TCA), followed by a reaction with an aqueous solution of thiobarbituric acid (TBA). The chromogen absorption was measured at 532 nm, while the plasma MDA levels were expressed in μmol/l.

The protein oxidative modification products (of the carbonyl group) were determined by a colorimetric reaction with 2,4-dinitrophenylhydrazine (2,4-DNPH) and TCA (23). Determining carbonyl groups in amino acid residues is an important indicator of protein oxidative modification. The concentration of carbonyl groups was expressed in μmol/g of plasma protein.

All the patients underwent ECG, exercise stress testing and echocardiographic examination.

## Results

The demographic characteristics of the formed groups are shown in Table 1.

Among the participants, 49 (55%) were female and 41 (45%) male. The mean age was 58.8 ± 6.49 years, with no statistically significant difference between the sexes.

The biochemical indicators of oxidative stress in relation to the presence of CAD are shown in Table 2.

Significantly higher XO activity ($p < 0.05$) and more intense MDA lipid modification ($p < 0.01$) were observed as characteristics in patients with CAD.

The association of CAD with the oxidative stress levels is shown in Table 3.

The Backward Logistic regression model did not show a significant association between CAD and the oxidative stress degree in the examined patients.

The biochemical indicators of oxidative stress in CAD patients in relation to the presence of DM are shown in Table 4.

Significantly higher XO activity ($p < 0.01$) and more intense MDA lipid modification ($p < 0.01$) were observed as characteristics in patients with DM.

**Table 1.** Demographic characteristics of the formed groups

| | Total | | Female | | Male | |
|---|---|---|---|---|---|---|
| | n | (%) | n | (%) | n | (%) |
| I | 40 | 44.5 | 23 | 25.5 | 17 | 19.0 |
| II | 30 | 33.5 | 14 | 15.5 | 16 | 18.0 |
| III | 20 | 22.0 | 12 | 14.0 | 8 | 8.0 |
| **Total** | **90** | **100.0** | **49** | **55.0** | **41** | **45.0** |

**Table 2.** Biochemical indicators of oxidative stress in relation to the presence of CAD

| | With CAD | Without CAD | Total |
|---|---|---|---|
| **SOD** | 2252.5 ± 425.3 | 2122.2 ± 137.4 | **2223.1 ± 320** |
| **XO** | 13.24 ± 8.1* | 9.49 ± 4.91 | **12.4 ± 7.2** |
| **Carbonyl groups** | 1.14 ± 0.92 | 1.18 ± 0.3 | **1.15 ± 0.9** |
| **MDA** | 14.91 ± 4.9** | 9.67 ± 4.9 | **13.74 ± 4.9** |

* $p < 0.05$; ** $p < 0.01$.

**Table 3.** Association of CAD with the level of oxidative stress

| | B | Wald | df | p | Exp (B) | 95.0% C.I. | |
|---|---|---|---|---|---|---|---|
| | | | | | | Lower | Upper |
| **SOD** | 0.03 | 0.096 | 1 | 0.757 | 1.1 | 0.9 | 1.4 |
| **XOD** | -0.02 | 0.002 | 1 | 0.865 | 0.9 | 0.8 | 1.19 |
| **Carbonyl groups** | 0.154 | 0.103 | 1 | 0.748 | 1.3 | 0.45 | 1.9 |
| **MDA** | -0.017 | 0.177 | 1 | 0.674 | 0.8 | 0.9 | 1.2 |

**Table 4.** Biochemical indicators of oxidative stress in CAD according to the presence of DM

| | CD + T2DM | CD without DM | Total |
|---|---|---|---|
| **SOD** | 2400.74 ± 356.77 | 2056 ± 505.8 | **2252.5 ± 425.3** |
| **XO** | 15.01 ± 4.11** | 10.88 ± 9.63 | **13.24 ± 8.1** |
| **Carbonyl groups** | 1.05 ± 0.96 | 1.27 ± 0.88 | **1.14 ± 0.92** |
| **MDA** | 16.47 ± 5.84** | 12.84 ± 3.25 | **14.91 ± 4.9** |

* $p < 0.05$; ** $p < 0.01$.

## Discussion

The relationship between CAD risk and baseline measurements of oxidative stress parameters in plasma has been shown in prospective and retrospective studies. This association remains even after adjusting for traditional risk factors, indicating an independent role of oxidative stress in increasing the risk of coronary artery disease. It is known that in this multistep process, an important role is played by both increased oxidative stress and inflammation, which are classified as non-traditional risk factors for the development of cardiovascular disease (15).

The significance of oxidative stress in the development of endothelial dysfunction and CAD was investigated by comparing the oxidative stress markers between the groups of patients with CAD and the healthy control subjects (Table 2). Even in this case, significantly higher XO activity ($p < 0.05$) and MDA concentration ($p < 0.01$) were observed in CAD patients in comparison with the healthy subjects. The results of the conducted study show that there was no significant association between CAD and the oxidative stress degree in the observed patients (Table 3). In the patients with existing CAD, the lowest tertile of SOD values doubles the risk of subsequent coronary events in both men and women. This doubling of risk remains even after excluding other traditional risk factors. Besides, this risk is comparable to that associated with smoking and a positive family history of CAD (16).

Free radicals can modify the endothelial function through various mechanisms. These include the direct effects on the endothelium, such as the membrane lipid peroxidation, the activation of transcription factors (NF-kB) leading to downstream regulation of adhesion molecules for platelets and leukocytes, the reduction of quantity of the available NO, the increase of LDL oxidation and the platelet and monocyte activation (16).

The significance of oxidative stress as a risk factor for endothelial dysfunction and CAD in diabetes was assessed by comparing the values between the groups of patients with CAD and type 2 DM and the patients with CAD and without diabetes mellitus (Table 4). In the group of patients with type 2 DM, there was a significantly higher XO activity and MDA concentration ($p < 0.01$) compared to the patients without diabetes.

Hyperglycemic pseudohypoxia, glucose auto-oxidation and AGE formation are important determinants of increased oxidative stress in diabetes. Besides, hyperglycemia may weaken the endothelial antioxidant mechanisms by reducing the pentose-phosphate pathway activity and decreasing the quantity of available NADPH and glutathione (17). The endothelial cells are also, at least in culture, highly sensitive to free radicals and lipid peroxidation. Under normal conditions, endothelial cells can respond to high glucose levels by increasing the expression of antioxidant enzymes such as Cu/Zn-superoxide dismutase, catalase and glutathione peroxidase (18). This is in accordance with the findings that in conditions of chronic hyperglycemia, increased oxidative stress results more from enhanced pro-oxidant activity than from decreased antioxidant protection (16).

Paraoxonase 1 (PON1) is an anti-inflammatory enzyme on the HDL particle that protects against atherosclerosis. Its inverse

relationship with the CRP values is considered a significant prognostic factor for the development of atherosclerosis, because reduced values of PON1 are observed in patients with CAD, with the particularly low values found in patients with CAD and type 2 DM (19).

In diabetics, there is a pro-oxidative state that is associated with an increased risk of CAD. This leads to reduced paraoxonase activity due to the inhibition of paraoxonase 1 by its substrates, such as lipid peroxides, resulting in elevated oxidative stress, inflammation and the increase of CRP levels. Paraoxonase activity is associated with the surface of the HDL particles, which are significantly reduced under the condition of diabetes. In this manner, it is possible to explain the pathogenic aspects of increased lipid peroxidation, as measured by the increase of MDA levels, and low HDL-C concentration in diabetes, on the reduction of paraoxidase 1 activity (20).

Given the importance of this anti-inflammatory enzyme for CRP production, it is possible to explain the close association between diabetes, oxidative stress and inflammation in the development of endothelial dysfunction and atherosclerosis pathogenesis. It should also be noted that the PON1 activity is sex-dependent and higher in women, which can be another explanation for the significantly higher protection that women have among the general population for the development of coronary artery disease (21).

## Conclusion

The measurement of XO activity and MDA concentration as parameters of oxidative stress, both in patients with coronary artery disease and in those with coronary artery disease and type 2 diabetes mellitus, has a significant diagnostic value.

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