



The Effects of Gasotransmitters Inhibition on Homocysteine Acutely Induced Changes in Oxidative Stress Markers in Rat Plasma

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ABSTRACT

Background: The importance of homocysteine (Hcy) is increasingly recognized in last few decades as an independent risk factor for atherosclerosis and thrombosis, but there is lack of data referring to influence of Hcy on plasma oxidative stress parameters as well as the role of gasotransmitters in these effects. Therefore, this study aim was to assess the role of gasotransmitter inhibitors in Hcy-induced effects on plasma oxidative stress in rats.

Material and Methods: Study involved 96 male *Wistar albino* rats divided into 8 groups: 1) Control group – saline (1ml 0.9 % NaCl i.p.); 2) DL-Hcy (8 mmol/kg i.p. DL homocysteine (DL-Hcy); 3) L-NAME (10 mg/kg i.p. N ω -Nitro-L-arginine methyl ester (L-NAME), inhibitor of NO production); 4) ZnPPR IX (30 μ mol/kg i.p. protoporphyrin IX zinc (ZnPPR IX), inhibitor of CO production); 5) DL-PAG (50 mg//kg i.p. DL-propargylglycine (DL-PAG), inhibitor of H₂S production); 6) DL-Hcy+L-NAME (8 mmol/kgi.p. DL-Hcy + 10 mg/kg i.p. L-NAME); 7) DL-Hcy+ZnPPR IX (8 mmol/kgi.p. DL-Hcy + 30 μ mol/kg i.p. Zn PPR IX), and 8) DL-Hcy+DL-PAG (8 mmol/kg i.p. DL-Hcy + 50 mg//kg i.p. DL-PAG). In all experimental groups, tested substances were administered in a single dose, intraperitoneally, 60 minutes before animals' euthanasia. In the collected blood samples malondialdehyde concentration, catalase, glutathione peroxidase and superoxide dismutase activity were measured.

Results: Applied substances induced rapid and strong increase of plasma antioxidant enzymatic activity probably as a compensatory response to its pro-oxidant influence.

Conclusion: The effects of Hcy on the activity of plasma antioxidant enzymes are in part mediated via interaction with gasotransmitters.

Keywords: gasotransmitters, homocysteine, oxidative stress markers, rat plasma.

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INTRODUCTION

Homocysteine (Hcy) is a S-containing amino acid and its plasma concentrations can be raised by various constitutive, genetic and lifestyle factors. Hyperhomocysteinemia is a modest independent predictor (risk factor or marker) of cardiovascular disease, stroke, neurodegeneration

and cancer.^{1,2} Some recent studies have shown that Hcy toxicity is mainly caused by oxidative stress via increasing inflammatory response³ and augmenting reactive oxygen species (ROS).⁴ Hyperhomocysteinemia is related with increased ROS formation, including the superox-

ide anion radical (O₂⁻) and hydrogen peroxide (H₂O₂),⁵ and in that way it leads to oxidative stress and cellular damage.⁶ Oxidative stress may influence the endogenous antioxidant system that includes glutathione-dependent enzymes (glutathione peroxidase (GPx), glutathione reductase (GR) and glutathione-S-transferase (GST)), superoxide dismutase (SOD), and catalase (CAT) antioxidant enzymes.^{6,7} There is a evidence that hyperhomocysteinemia may impair the glutathione-related antioxidant defense system,^{8,9} however data about its effect on SOD activity are still controversial. Wilcken et al. have revealed a positive correlation between SOD activity and Hcy levels in patients with homocystinuria,¹⁰ while other authors showed that hyperhomocysteinemia decreased erythrocyte SOD activity in patients with cardiovascular disease.^{9,11}

On the other hand, there are increasing number of assumptions that signaling gaseous molecules as nitric oxide (NO), carbon monoxide (CO) and hydrogen sulfide (H₂S)¹² may have an important role in effects of Hcy-thiolactone on the myocardial function and coronary circulation.¹³ This is very plausible having in mind that gasotransmitters have an important role in the regulation of inflammation, oxidative stress, modulation of mitochondria respiration and activation of antioxidant enzymes.¹⁴ With participation of NO, S-nitroso-Hcy inhibits hydrogen peroxide.¹⁵ H₂S also decreases level of Hcy in plasma¹⁶ and it is strong antioxidant.¹⁷ CO is the most stable gasotransmitter, and it is capable of exerting its effects during long time and distances.¹⁸

However, Hcy-thiolactone and gasotransmitters connection is still not fully understood. Thus, the aim of this research was to examine the effects of acute administration of DL-Hcy alone or in combination with specific inhibitors of different gasotransmitters, such as N ω -nitro-L-arginine methyl ester (L-NAME), Zinc protoporphyrin IX (ZnPPR IX) and DL-Propargyl Glycine (DL-PAG) on oxidative stress markers in the rat plasma – catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (GPx) activities and malondialdehyde (MDA) concentration.

METHODS

Physiological Assay and Experimental Protocol

Male Wistar albino rats (n = 96, 12 in each experimental group, 10 weeks old, body weight (250 ± 30 g) have been used for investigation. Animals were housed in strictly controlled conditions (air temperature of 22±1°C, relative humidity of 50%, a cycle of brightness: darkness 12:12 hours, starting bright period at 8 AM), with free access to water and standard food. In all experimental groups, tested substances were administered in a single dose, intraperitoneally (i.p.), 60 minutes before euthanasia of animals. All animals were divided into 8 groups, and received: 1) Control group – saline (1 ml 0.9% NaCl i.p., pH 7.4); 2) DL-Hcy group (8 mmol/kg i.p. DL homocysteine); 3) L-NAME group (10 mg/kg i.p. L-NAME as inhibitor of NO production via inhibition of nitric oxide synthase); 4) ZnPPR IX group (30 μ mol/kg i.p. ZnPPR IX as inhibitor of CO production via inhibition of heme oxygenase-1); 5) DL-PAG group (50 mg//kg i.p. DL-PAG as inhibitor of H₂S production via inhibition of cystathionine gamma lyase); 6) DL-Hcy+L-NAME group (8 mmol/kg i.p. DL-Hcy + 10 mg/kg i.p. L-NAME); 7) DL-Hcy+ZnPPR IX group (8 mmol/kg i.p. DL-Hcy + 30 μ mol/kg i.p. ZnPPR IX), and DL-Hcy+DL-PAG group (8 mmol/kg i.p. DL-Hcy + 50 mg//kg i.p. DL-PAG).

All experimental procedures were done in accordance with prescribed legislation (EU Directive for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes 86/609/EES) and the principles of ethics.

Biochemical analyses

Sixty minutes after administration of tested substances, the rats were euthanized by decapitation. Blood was collected through a glass funnel and placed in test tubes coated in heparin. The blood samples were left at the room temperature for 15 min and afterwards centrifuged (15 min×3000 rpm). The obtained plasma was used for the analyses.

The evaluated parameters were determined in the control condition, and then in acute series of experiments.

Determination of Hcy

The plasma samples were analyzed using the electrochemiluminescence method (ECL- electrochemiluminescence immunoassay system,

ADVIA Centaur XP System, Siemens Healthcare GmbH, Erlangen, Germany); the range of reference values was $\text{Hcy} < 15 \mu\text{mol/l}$.

Determination of lipid peroxidation products (MDA)

MDA content in plasma was determined by using thiobarbituric assay.¹⁴ 500 μl of 25% HCl and 500 μl of 1% thiobarbituric acid (in 50 mM NaOH) was added in 500 μl of sample. The mixture was heated for 10 minutes on boiling water bath, and cooled to room temperature. Then 3 mL of n-butanol was added and shaken on a Vortex for 30 seconds. In order to successfully separate phases, samples are centrifugated (10 minutes at $2000 \times g$). Content of malondialdehyde was determined spectrophotometrically by measuring the absorbance of the organic phase (upper layer) at 532 nm. The blanks contain 50 mM NaOH instead of thiobarbituric acid, and they are prepared for each sample separately. The values of MDA content (nmol of MDA/ml plasma) was determined on the basis of absorbance values and molar absorption coefficient of the malondialdehyde-thiobarbituric acid complex.

Determination of CAT activity

CAT activity was measured by an essay that accompanies the degradation of H_2O_2 according to Beutler.¹⁹ 50 μl of a suspension of plasma was added to the quartz glass tubes at room temperature, consisting of 2.975 ml of 50 mM phosphate buffer solution in 0.4 mM EDTA. The enzyme reaction is initiated by adding 30 μl of 3% H_2O_2 . Reduction in absorbance due to enzymatic degradation of H_2O_2 (at 240 nm for 3-5 minutes) was monitored. CAT activity was expressed as U/ml of plasma. One unit (U) of enzyme activity was defined as 1 micromol of spent H_2O_2 /min.

Determination of SOD activity

The activity of total SOD was measured according to the method of Misra and Fridovich.²⁰ 10-30 μl of plasma was added in 3 ml of a 0.5 M EDTA-sodium carbonate buffer (pH 10.2). The enzymatic reaction started by adding 100 μl of epinephrine (30 mM in 0.1 M HCl). SOD activity was measured at absorbance of 480 nm during 4 minutes. One unit (U) of SOD was defined as the amount of enzyme that inhibits the rate of the epinephrine oxidation for 50%. The enzyme activity was expressed as U/ml of plasma.

Determination of GPx activity

Procedure for measuring of GPx activity begins with preparation of reaction cocktail which con-

sists of 8.9 ml of phosphate buffer, 50 μl of 200 mM reduced glutathione (GSH), 1 mg of β -NADPH, and 100 ml of 100 units/ml GSH-reductase from baker's yeast (*Saccharomyces cerevisiae*). pH value of reaction cocktail was adjusted to 7 (50 mM NaH_2PO_4 + 0.40 mM EDTA). 3 ml of a reaction cocktail and 0.3 ml of plasma sample was added in a quartz glass cuvette (room temperature). Cuvette was placed in a spectrophotometer, and 50 μl of 0.042% H_2O_2 was added in order to start enzymatic reaction ($A_{240} = 0.52$ to 0.56). The decline of absorbance ($\lambda = 340 \text{ nm}$) in intervals of 15 seconds during the 4-5 minutes was monitored. GPx activity was expressed as $\Delta A/\text{min}/\text{ml}$ of plasma.²¹

Chemicals used

All chemicals were of p.a. grade quality and were purchased from Sigma Aldrich (Germany).

Statistical analyses

The parameters of descriptive statistics were used. For testing statistical significance after testing normality of distribution, one-way analysis of variance (ANOVA), followed by Tukey's Post Hoc Test was used. Statistical calculation was done using SPSS computer program (SPSS Inc. Chicago, SAD). Values are presented as mean \pm SEM. $P < 0.05$ was considered statistically significant.

RESULTS

Determination of Hcy

In all plasma samples levels of measured Hcy were more than $65 \mu\text{mol/l}$, indicating moderate hyperhomocysteinemia (30-100 $\mu\text{mol/l}$), except in the Control group in which it was $10.4 \pm 0.6 \mu\text{mol/l}$.

Plasma MDA values

In all experimental groups: DL-Hcy ($6.09 \pm 0.85 \text{ nmol/ml}$ of plasma, Figure 1. a), L-NAME ($5.63 \pm 0.85 \text{ nmol/ml}$ of plasma, Figure 1. a), DL-PAG ($5.28 \pm 1.37 \text{ nmol/ml}$ of plasma, Figure 1. a), DL-Hcy+L-NAME ($4.10 \pm 0.74 \text{ nmol/ml}$ of plasma, Figure 1. b), DL-Hcy+ZnPPR IX ($4.28 \pm 0.59 \text{ nmol/ml}$ of plasma, Figure 1. b), DL-Hcy+DL-PAG ($9.90 \pm 0.94 \text{ nmol/ml}$ of plasma, Figure 1. b), except ZnPPR IX alone ($17.30 \pm 4.74 \text{ nmol/ml}$ of plasma, Figure 1. a), the administration of tested substances induced significant decrease in MDA values compared with control condition ($15.37 \pm 1.41 \text{ nmol/ml}$ of plasma) (Figure 1 a, b).

Plasma CAT activity

In relation to control group (16.40±2.11 U/ml of plasma, Figure 2. a, b), the administration of all tested substances induced significant increase in CAT activity: L-NAME (50.59±3.58 U/ml of plasma, Figure 2. a), ZnPPR IX (67.54±2.08 U/ml of plasma, Figure 2. a), DL- PAG (118.69±12.13 U/ml of plasma, Figure 2. a), DL-Hcy+L-NAME (127.87±19.17 U/ml of plasma, Figure 2. b), DL-Hcy+ZnPPR IX (181.19±16.64 U/ml of plasma, Figure 2. b), DL-Hcy+DL-PAG (109.58± 22.43 U/ml of plasma, Figure 2. b), however only in DL-Hcy group (26.49±4.22 U/ml of plasma, Figure 2. a) there was no significant change in this parameter.

Plasma GPx activity

Levels of GPx were significantly increased by all of the applied substances: L-NAME (3.37±0.23 U/ml of plasma, Figure 3. a), ZnPPR IX (4.50±0.13 U/ml of plasma, Figure 3. a), DL-PAG (7.91±0.80 U/ml of plasma, Figure 3. a),

DL-Hcy+L-NAME (7.60±0.77 U/ml of plasma, Figure 3. b), DL-Hcy+ZnPPR IX (12.59±0.69 U/ml of plasma, Figure 3. b), DL-Hcy+DL-PAG (6.37±0.72 U/ml of plasma, Figure 3. b), except DL-Hcy alone (1.76±0.28 U/ml of plasma, Figure 3. a), compared with control condition (1.09±0.14 U/ml of plasma, Figure 3. a, b).

Plasma SOD activity

Administration of all tested substances: DL-Hcy (30.41±0.71 U/ml of plasma, Figure 4. a), L-NAME (31.07±0.40U/ml of plasma, Figure 4. a), DL-PAG (30.25±0.54 U/ml of plasma, Figure 4. a), DL-Hcy+L-NAME (32.98±0.94 U/ml of plasma, Figure 4. b), DL-Hcy+ZnPPR IX (33.36±0.43 U/ml of plasma, Figure 4. b), DL-Hcy+DL-PAG (32.66±1.20 U/ml of plasma, Figure 4. b) caused significant increase in SOD level compared with control condition (25.31±0.96 U/ml of plasma, Figure 4. a, b), except ZnPPR IX alone (26.46±2.65 U/ml of plasma, Figure 4. a).

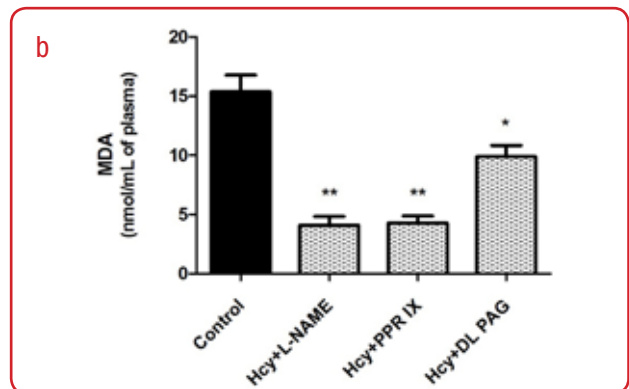
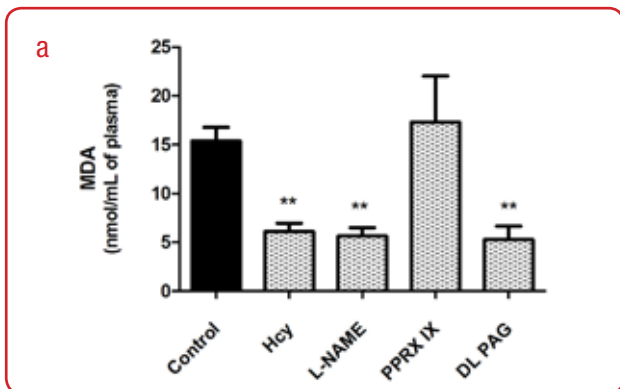


Figure 1. The effects of Hcy and gasotransmitters inhibition separately (a) and its combination (b) on MDA concentration in rat plasma. Values are presented as mean ± SEM. *P < 0.05; **P < 0.01 compared to control group.

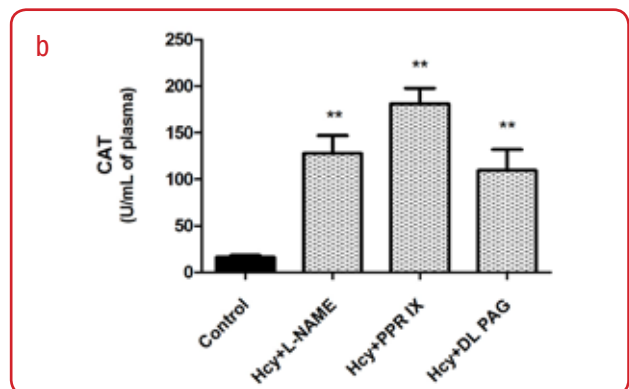
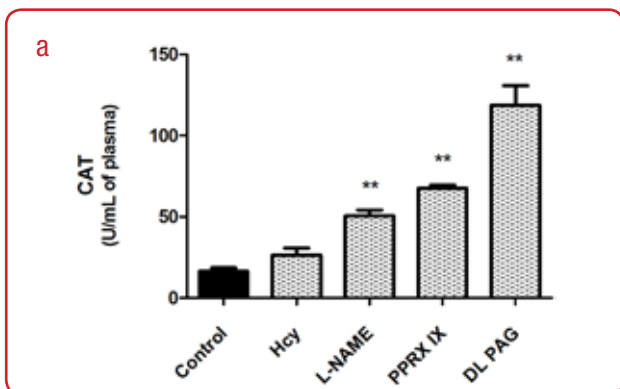


Figure 2. The effects of Hcy and gasotransmitters inhibition separately (a) and its combination (b) on CAT activity in rat plasma. Values are presented as mean ± SEM. **P < 0.01 compared to control group.

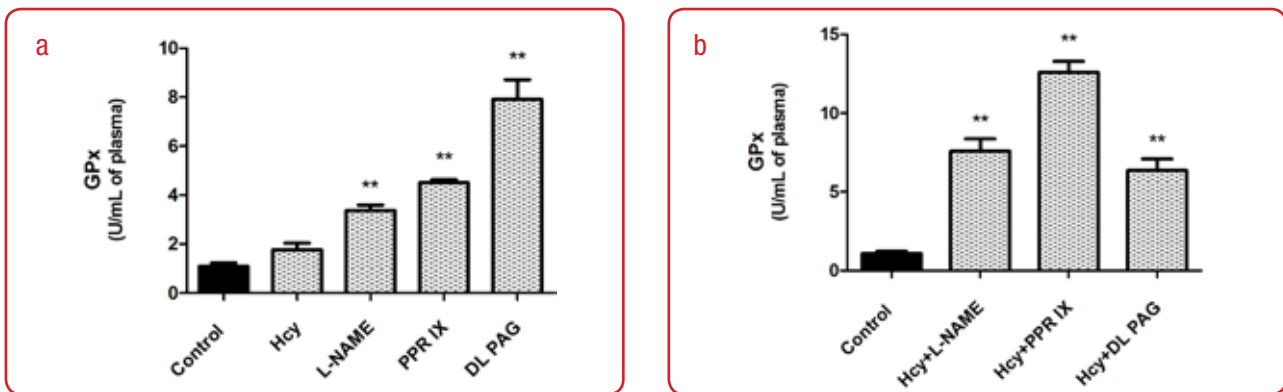


Figure 3. The effects of Hcy and gasotransmitters inhibition separately (a) and its combination (b) on GPx activity in rat plasma. Values are presented as mean \pm SEM. **P < 0.01 compared to control group.

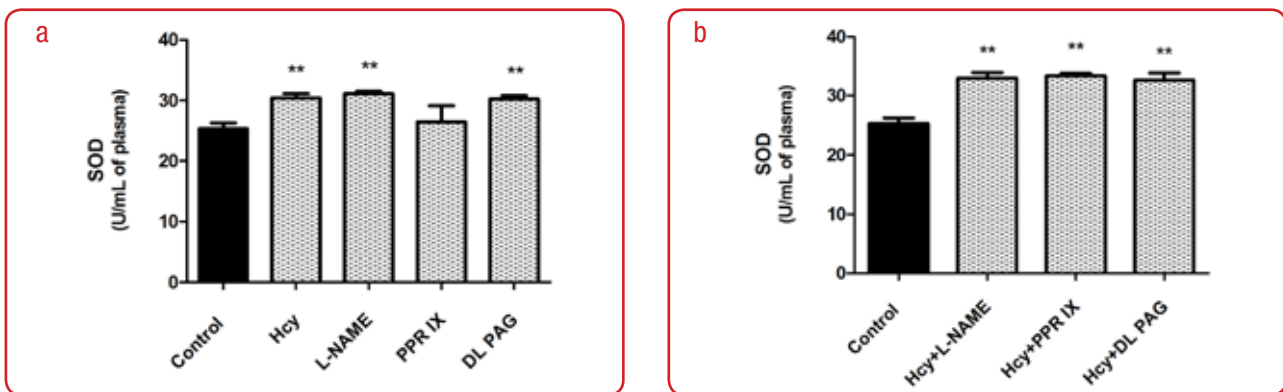


Figure 4. The effects of Hcy and gasotransmitters inhibition separately (a) and its combination (b) on SOD activity in rat plasma. Values are presented as mean \pm SEM. **P < 0.01 compared to control group.

DISCUSSION

In present investigation DL-Hcy caused decrease of MDA level in the plasma, indicating that acutely applied Hcy does not increase lipid peroxidation in plasma of rats. Similar results were observed in case of NO or H₂S inhibition, or during synergistic application of each inhibitor with Hcy. Therefore, we can assume that (except CO) gaseous molecules could induce plasma lipid peroxidation, while in presence of Hcy positive effect of their blockage seems to be even more obvious. Decreased level of MDA in all groups may be consequence of increased activities of antioxidant enzymes in same groups during the same time. We previously noted that thiolactone form of Hcy alone or in combination with gasotransmitter inhibitors non-significantly decreased level of lipid peroxidation measured in coronary venous effluent.¹³

In the second part of our research, we focused on the effects of Hcy and different gasotransmitters production inhibition and on antioxidative

enzyme system such as GPx, CAT, SOD. The obtained results have shown that acute intraperitoneal administration of DL-Hcy induced acute increase of antioxidative enzymatic activity, which correlates with decreased values of MDA. These findings probably mean that DL-Hcy induce strong pro-oxidant effects taking into consideration rapid and significant increased activities of antioxidant enzymes (CAT, GPx, SOD) in plasma following intraperitoneal administration of this substance in rat, and probably as an adaptive response to its pro-oxidant effects. This is in accordance with other study that also showed increase of CAT and SOD activity after acute application.¹¹ However, decrease of plasma MDA level during the same period lead to the conclusion that there was an inverse response to the raise of antioxidant enzymes.

On the other hand, chronic application of Hcy induce drop in antioxidant enzymatic activity,²² probably due to increased time of exposure. In-

terestingly, in our investigation we have noticed reduced levels of MDA in almost all groups, and significantly increased levels of GPx in the same groups. It seems logic, considering that GPx by reducing H₂O₂ level, inhibit lipid peroxidation directly or indirectly by mediation of lipid peroxides.²³

During inhibition of gasotransmitter production, it has been observed increased activity of plasma antioxidant enzymes, indicating that NO, CO or H₂S could react with these enzymes in plasma. However, during inhibition of gaseous signaling molecules production, Hcy con-

tinues to potentiate raise in activity of SOD, CAT and GPx. These results showed no changes in dynamics of measured plasma antioxidants in presence or in absence of gasotransmitter synthesis alone, suggesting that the influences of Hcy are not quite clear. At the end, it is important to emphasize that determination of each gasotransmitter effect was assessed indirectly by their inhibition of production, which could be limitation of this study also. Addition of any data on mRNA and also cellular data would provide more evidence to the notion that enzyme involved in oxidative stress are induced or suppressed.

CONCLUSION

Gasotransmitters inhibitors increased significantly antioxidant enzymes activities and that increase was higher when they were administered in combination with Hcy. This indirectly indicates that gasotransmitters have an important role in oxidative stress protection.

CONFLICT OF INTERESTS

The authors declare no conflict of interest.

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AUTHORSHIP STATEMENT

Marko Đjurić: performing of experimental procedures, statistical analysis, interpretation of data, preparation of the manuscript; Sanja Kostić: statistical analysis, interpretation of data, preparation of the manuscript; Dragana Lončar-Stojiljković: interpretation of data, analysis of results, preparation of the manuscript; Mirjana Čolović: biochemical analysis; Danijela Krstić: biochemical analysis, interpretation of data; Predrag Stevanović: interpretation of data, analysis of results; Dragan Djuric: study design, performing of experimental procedures, statistical analysis, interpretation of data, analysis of results, preparation of the manuscript.

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