Acta Poloniae Pharmaceutica – Drug Research, Vol. 76 No. 1 pp. 147–157, 2019
DOI: 10.32383/appdr/94079

ISSN 0001-6837
Polish Pharmaceutical Society

THE EFFECTS OF SULFUR-CONTAINING COMPOUNDS ON REDOX STATUS IN HOMOCYSTEINE-TREATED RATS

TANJA S. SOBOT[1], VLADIMIR I. ZIVKOVIC[2*], IVAN M. SREJOVIC[2], JOVANA N. JEREMIC[3], TAMARA R. NIKOLIC TURNIC[3], NENAD D. PONORAC[1], ANICA M. PETKOVIC[3], VLADIMIR Lj. JAKOVLJEVIC[2,4] and DRAGAN M. DJURIC[5]

[1]Department of Physiology, Faculty of Medicine, University of Banja Luka, Save Mrkalja 14, Banja Luka 78000, Bosnia and Herzegovina
[2]Department of Physiology, Faculty of Medical Sciences, University of Kragujevac, Svetozara Markovica 69, Kragujevac 34000, Serbia
[3]Department of Pharmacy, Faculty of Medical Sciences, University of Kragujevac, Svetozara Markovica 69, Kragujevac 34000, Serbia
[4]Department of Human Pathology, I.M. Sechenov First Moscow State Medical University (Sechenov University), Trubetskaya st. 8, Moscow 119991, Russia
[5]Institute of Medical Physiology "Richard Burian", Faculty of Medicine, University of Belgrade, Visegradska 26, Belgrade 11000, Serbia

Abstract: There is growing interest in the activity of sulfur-containing compounds on redox balance in physiological and pathological conditions, considering that some of these compounds have not only antioxidative but also pro-oxidative activities. The aim of this study was to assess possible differences in the effects of various sulfur-containing compounds on redox balance of cardiovascular system in its physiological state and in the early onset of hyperhomocysteinemia. This experimental study divided *Wistar* albino rats into two groups: saline-treated (control) and DL-homocysteine-treated (experimental group). Rats from experimental group were subjected to subchronic subcutaneous administration of DL-homocysteine at dose of 0.45 ěmol/g body weight twice a day for 2 weeks. At the end of this period, rats were sacrificed, and blood samples were collected to be analyzed for homocysteine concentration and systemic oxidative stress. Isolated rat hearts were excised and attached to the Langendorff apparatus. To assess the effects of acute administration of L-methionine, L-cysteine, N-acetylcysteine, and sodium hydrogen sulfide, the hearts were perfused individually with each of the mentioned substances at the same single dose of 0.5 mmol/L for 5 min. In collected samples of coronary venous effluent oxidative stress biomarkers were determined using spectrophotometry. Total homocysteine level was significantly higher in the experimental group than in the control group, and the effects of applied sulfur-containing compounds were significantly different in experimental and control groups. DL-homocysteine induced considerable changes in the functioning of a cardiovascular system even before an increase in plasma homocysteine values, and action of sulfur-containing compounds varied depending on the presence of homocysteine.

Keywords: sulfur amino acids; L-methionine; L-cysteine; N-acetylcysteine; homocysteine; oxidative stress

Abbreviations: CPP, Coronary perfusion pressure; CF, Coronary flow; CAT, Catalase; GSH, Reduced glutathione; GSSG, Glutathione disulfide; DNA, Deoxyribonucleic acid; Hcy, Homocysteine; H_2O_2, Hydrogen peroxide; HPLC, High-performance liquid chromatography; Meth, Methionine; Cys, Cysteine; NAC, N-acetylcysteine; H_2S, Hydrogen sulfide; NaHS, Sodium hydrogen sulfide; NO_2-, Nitrites; NO, Nitric oxide; O_2, Superoxide anion radicals; OH, Hydroxyl radical; ROS, Reactive oxygen species; RNS, Reactive nitrogen species; RNOS, Reactive nitrogen oxygen species; RSS, Reactive sulfur species; SOD, Superoxide dismutase; SEM, Standard error mean; SD, Standard deviation; SAM, S-adenosylmethionine; SAH, S-adenosylhomocysteine; TBARS, Reactive thiobarbituric substances; VSMC, Vascular smooth muscle cells

INTRODUCTION

Oxidative stress is usually defined as an imbalance in the production and elimination of free radicals, where they accumulate due to either increased production or insufficient elimination. Some free radicals are commonly produced in various concentrations in different places in the cell, primarily in mitochondria, and these physiological concentrations are easily disintegrated by scavenger enzymes

* Corresponding author: e-mail: vladimirziv@gmail.com

and antioxidants but also have an important role in cell signaling (1). The oxidative stress induced by excessive accumulation of reactive oxygen species (ROS) and reactive nitrogen species (RNS), two of the most important types of free radicals, plays a primary role in numerous diseases and disorders, including cardiovascular diseases (2, 3). On the other hand, the significance of cardiovascular diseases and their prevention is reflected by the fact that these diseases are the most common cause of death in the modern world (4).

There is a growing interest in the activity of different sulfur-containing compounds on redox balance in physiological and pathological conditions, considering that some of these compounds can exert not only antioxidative but also pro-oxidative activities (5, 6). In addition to the ability of some sulfur-containing compounds to induce increased production of ROS and RNS, the third group of redox active molecules containing sulfur, usually termed as reactive sulfur species (RSS), have a similar harmful potential to oxidize organic compounds, causing peroxidation of membranes and DNA damage (7).

Homocysteine (Hcy) is a nonprotein sulfur-containing amino-acid byproduct in methionine metabolism and is connected with a variety of pathological disorders in increased concentrations (hyperhomocysteinemia, HHcy) (8, 9). Although previous studies have depicted the role of Hcy in various cardiovascular disorders and in changing cellular function and structure in the cardiovascular system, the mechanisms of these modifications have not been fully elucidated (10).

Bearing in mind all the abovementioned facts, the aim of this investigation was to assess the effects of Hcy on cardiovascular redox state during the onset of HHcy, as well as the effects of various sulfur-containing compounds on ROS balance.

EXPERIMENTAL

Animals

All research procedures in this investigation were carried out in accordance with the European Directive No. 86/609/EEC on the protection of animals used for laboratory purposes and principles of good laboratory practice and approved by the ethical committee of the Faculty of Medical Sciences, University of Kragujevac, Serbia.

The present study was carried out using 80 adult male Wistar albino rats (aged 16 weeks; body weight, 300-400 g). The animals were housed under standard controlled environmental conditions, with a temperature of 23 ± 1°C and a 12/12 h light/dark cycle. Food and water were provided *ad libitum*. All animals were obtained from the Military Medical Academy (Belgrade, Serbia).

The animals were divided into two groups: control (healthy, saline-treated rats) and experimental (DL-homocysteine-treated rats). Rats from the experimental group were subjected to the subchronic administration of a subcutaneous injection of DL-homocysteine (DL-Hcy) at a dose of 0.45 μmol/g body weight twice a day (every day at the same time) for 2 weeks. Rats from the control group were subjected to the administration of a subcutaneous injection of saline twice a day for 2 weeks to achieve the same injection-induced stress effect on rats.

Each group was divided into 4 subgroups (10 animals each), depending on the acute treatment with sulfur-containing compounds (in the same single dose of 0.5 mmol/L).

Control (saline-treated) group:

1. L-Meth subgroup (acute administration of L-methionine);
2. L-Cys subgroup (acute administration of L-cysteine);
3. NAC subgroup (acute administration of N-acetylcysteine);
4. NaHS subgroup (acute administration of inorganic sodium hydrogen sulfide).

Experimental (DL-Hcy-treated rats) group:

5. DL-Hcy + L-Meth subgroup (acute administration of L-methionine);
6. DL-Hcy + L-Cys subgroup (acute administration of L-cysteine);
7. DL-Hcy + NAC subgroup (acute administration of N-acetylcysteine);
8. DL-Hcy + NaHS subgroup (acute administration of inorganic sodium hydrogen sulfide).

Doses of the above substances were chosen empirically in accordance with previously published data (11-13).

Experimental protocol (sample preparation and biochemical analyses)

After subchronic administration of DL-Hcy or saline, rats from both groups (experimental and control) were exposed to short ketamine/xylazine-induced narcosis and sacrificed by decapitation. For analysis of the Hcy concentration and systemic oxidative stress, blood was collected into two different types of vacutainer tubes. The first type was a plain tube for serum preparation to measure Hcy concentration; the second contained EDTA as an anticoagulant for the assay of pro-oxidative markers in plasma and antioxidant markers in erythrocyte lysate.

Total serum Hcy concentrations were measured with high-performance liquid chromatography (HPLC) procedure with reverse-phase separation and fluorescence detection, as described previously (14).

Furthermore, for examining the effect of subchronic DL-Hcy administration on redox status, we used the blood samples to compare values of prooxidative and antioxidative markers between the control and experimental (DL-Hcy-treated) groups. Collected blood samples were centrifuged to separate plasma and erythrocytes. In the plasma samples, the following markers of oxidative stress were measured spectrophotometrically: index of lipid peroxidation (measured as TBARS-thiobarbituric acid reactive substances), nitrites (NO_2-), superoxide anion radical (O_2-), and hydrogen peroxide (H_2O_2). In the lysate of erythrocytes, we determined the activity of the enzymatic defense system by evaluating catalase (CAT) and superoxide dismutase (SOD) concentration, as well as the activity of non-enzymatic antioxidants such as reduced glutathione (GSH).

Immediately after venous blood sampling, quick thoracotomy was performed, followed by rapid cardiac arrest via superfusion with ice-cold isotonic saline; then, the hearts were promptly excised and attached to the Langendorff apparatus (Experimetria Ltd, 1062 Budapest, Hungary) via aortic cannulation. The hearts were retrogradely perfused under a constant perfusion pressure (CPP) of 70 cm H_2O with complex Krebs–Henseleit solution containing the following compounds (in mmol/L): NaCl, 118; KCl, 4.7; $CaCl_2 \cdot 2H_2O$, 2.5; $MgSO_4 \cdot 7H_2O$, 1.7; $NaHCO_3$, 25; KH_2PO_4, 1.2; glucose, 11; pyruvate, 2; equilibrated with 95% O_2 plus 5% CO_2 at 37°C (pH 7.4). After a stabilization period (the same values of coronary flow in three consecutive measurements, approximately lasting for 25 min), a sample of coronary venous effluent was collected (control value, C), and the experimental protocol was initiated. To assess the effects of acute administration of L-Meth, L-Cys, NAC, and NaHS, the hearts were perfused individually with each of the mentioned substances at the same single dose of 0.5 mmol/L for 5 min. During the last minute of treatment, a sample of coronary venous effluent was collected (effect value, E). After the treatment with experimental substances, the washout period of heart perfusion followed, lasting for 10 min. At the end of this period, a sample of coronary venous effluent was collected (washout value, W). In the collected samples of coronary venous effluent (control, effect, and washout), the same oxidative stress biomarkers as in plasma were determined: index of lipid peroxidation (TBARS), nitrite (NO_2-), superoxide anion radicals (O_2-), and hydrogen peroxide (H_2O_2).

All biochemical parameters were determined spectrophotometrically using Shimadzu UV 1800 spectrophotometer (Japan).

Determination of TBARS

The degree of lipid peroxidation in the plasma and coronary venous effluent was estimated by measuring TBARS. Each sample was incubated with 1% thiobarbituric acid in 0.05 M NaOH at 100°C for 15 min and then measured at 530 nm. In the plasma samples, distilled water solution with 1% thiobarbituric acid in 0.05 NaOH served as a blank probe. In the coronary venous effluent samples, Krebs–Henseleit solution was used as a blank probe (15).

Determination of nitrites (NO_2-)

Nitric oxide (NO) decomposes rapidly to form stable metabolite nitrite (NO_2-) or nitrate products. The nitrite level (NO_2-) was determined as an index of NO production with Griess reagent (16).

To detect nitrite in plasma, 0.1 mL of 3 N perchloride acid, 0.4 mL of 20 mmol/L ethylenediaminetetraacetic acid (EDTA), and 0.2 mL of plasma were placed on ice for 15 min and then centrifuged for 15 min at 6000 rpm. After pouring off the supernatant, 220 µL of K_2CO_3 was added. NO_2- was measured at 550 nm, and distilled water was used as a blank probe.

To detect nitrite in the coronary venous effluent, a total of 0.5 mL of perfusate was precipitated with 200 µL of 30% sulfosalicylic acid, vortexed for 30 min, and centrifuged at 3000 × g. Equal volumes of the supernatant and Griess reagent, containing 1% sulfanilamide in 5% phosphoric acid/0.1% naphthalene ethylenediamine dihydrochloride, were added and incubated for 10 min in the dark and measured at 543 nm. The nitrite levels were calculated using sodium nitrite as the standard (16).

Determination of superoxide anion radical (O_2-)

The concentration of the superoxide anion radical (O_2-) was measured after the reaction of nitro blue tetrazolium in TRIS buffer with the plasma or coronary effluent samples at 530 nm. Krebs-Henseleit solution was used as a blank probe (17).

Determination of hydrogen peroxide (H_2O_2)

The measurement of hydrogen peroxide (H_2O_2) was based on the oxidation of phenol red by hydro-

gen peroxide, in a reaction catalyzed by horseradish peroxidase (HRPO) (15). For each sample, 200 µL of plasma or coronary effluent was precipitated with 800 µL of freshly prepared phenol red solution, followed by the addition of 10 µL of (1 : 20) HRPO (made ex tempore). For the blank, an adequate volume of Krebs–Henseleit solution was used. The level of H_2O_2 was measured at 610 nm (18).

Determination of antioxidant enzymes (CAT, SOD)

Isolated erythrocytes were washed three times with 3 volumes of ice-cold 0.9 mmol/l NaCl. Haemolysates containing approximately 50 g of Hb/L (prepared according to McCord and Fridovich (19)) were used for the determination of CAT activity. CAT activity and the levels of reduced glutathione (GSH) were determined according to Beutler (20). Lysates were diluted with distilled water (1 : 7 v/v) and treated with chloroform-ethanol (0.6 : 1 v/v) to remove hemoglobin (21). Then, 50 µL of CAT buffer, 100 µL of samples, and 1 mL of 10 mmol/L H_2O_2 were added to the samples. Detection was performed at 360 nm, and distilled water was used as a blank probe. SOD activity was determined by the epinephrine method of Misra and Fridovich (22). Briefly, 100 µL of lysate and 1 ml of carbonate buffer were mixed, and then 100 µL of epinephrine was added. Detection was performed at 470 nm.

Determination of GSH

The level of reduced glutathione (GSH) in plasma was determined spectrophotometrically, based on GSH oxidation via 5,5-dithiobis-6,2-nitrobenzoic acid. GSH extract was obtained by combining 0.1 mL of 0.1% EDTA, 400 µL of plasma, and 750 µL of precipitation solution (containing 1.67 g of metaphosphoric acid, 0.2 g of EDTA, 30 g of NaCl, and filled with distilled water until 100 mL;

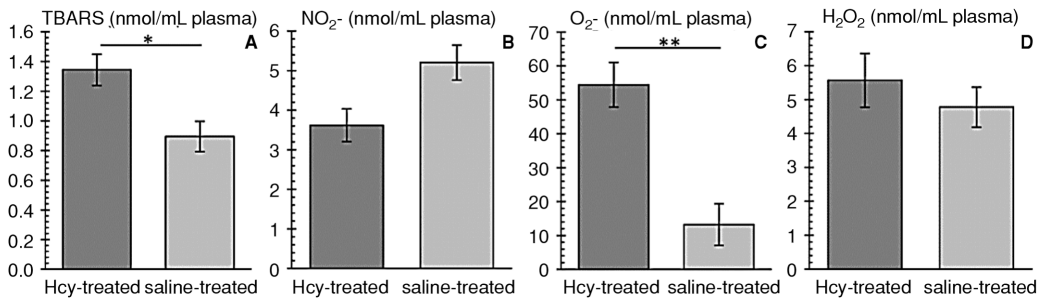

Figure 1. The values of oxidative stress biomarkers (TBARS, NO_2-, O_2-, H_2O_2) in the plasma of saline- and DL-Hcy-treated rats. Data are expressed as the means ± SE; *p < 0.05, **p < 0.01

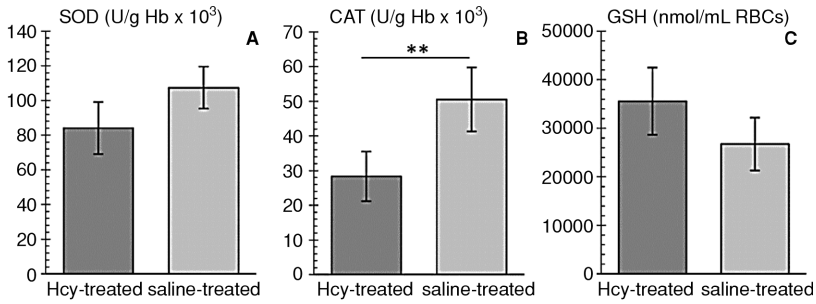

Figure 2. The values of enzymatic (SOD, CAT) and non-enzymatic (GSH) antioxidants in erythrocytes lysate of saline- and DL-Hcy-treated rats. Data are expressed as the means ± SE; *p < 0.05, **p < 0.01

Table 1. Differences in serum concentrations of homocysteine in saline- and DL-Hcy-treated rats. Data are expressed as the means ± SE.

Effects of 2 weeks of treatment with DL-homocysteine on plasma homocysteine concentration	
Group	Values of total Hcy in plasma
Saline-treated	8.11 ± 1.28 μmol/L
DL-Homocysteine-treated	12.64 ± 1.53 μmol/L*

Statistical significance compared with previous value ($p < 0.05$, **$p < 0.01$)

the solution was stable for 3 weeks at +4°C). After mixing in the vortex machine and extracting on cold ice (15 min), the sample was centrifuged at 4000 rpm (10 min).

Detection was performed at 420 nm, and distilled water was used as a blank probe (20).

Drugs

All drugs used in this experimental protocol were provided by Sigma-Aldrich Chemie GmbH Eschenstr. 5, 82024 Taufkirchen, Germany.

Statistical analysis

The following basic descriptive statistics were determined for the experimental data: mean (x), standard deviation (SD) and standard error mean (SEM). To test the normality of the distribution parameters, the Kolmogorov–Smirnov test was used. To test the statistical significance of the results and to confirm the hypothesis, Student's t-test (parametric test) was used for dependent and independent variables. A database analysis of the results was performed using software package SPSS 20 (SPSS Inc., Chicago, IL, USA). A p-value = 0.05 was considered statistically significant.

RESULTS

Homocysteine concentration

The total serum homocysteine concentration of DL-Hcy-treated rats was significantly higher than that of saline-treated animals (12.64 vs 8.11 μmol/L) (Table 1).

Effects of DL-Hcy administration on systemic redox status

The values of TBARS and O_2- were significantly higher while the value of CAT was significantly lower in the experimental group (subchronic administration of DL-Hcy) than in the control group (Figs. 1, 2).

Effects of L-methionine on biomarkers of oxidative stress

The application of L-methionine induced a significant decrease in NO_2- levels and a significant increase in the production of O_2- in saline-treated rats (control group). These levels also held during the washout period (Figs. 3B and 3C). On the other hand, L-methionine did not affect any examined oxidative stress biomarker in DL-Hcy-treated rats (Fig. 4).

Effects of L-cysteine on biomarkers of oxidative stress

The application of L-cysteine in the isolated hearts of control rats induced an increase in O_2- production, while the values of H_2O_2 were significantly increased in the washout period compared with control values for that period (Figs. 3C and 3D). The administration of L-cysteine in the isolated hearts of DL-Hcy-treated rats induced a significant increase in NO_2- and O_2-, and the values of both mentioned parameters decreased significantly during the washout period similar to the control values (Figs. 4B and 4C).

Effects of N-acetylcysteine (NAC) on biomarkers of oxidative stress

In the control group, the acute administration of N-acetylcysteine induced a decrease in TBARS, and this reduction continued during the washout period, with a statistically significant difference between the values at the end of the washout period and the control values (Fig. 3A). Furthermore, in DL-Hcy-treated rats, the administration of NAC induced a statistically significant increase in NO_2- but did not change the values of any other oxidative stress biomarkers observed in this study (Fig. 4B).

Effects of inorganic sodium hydrogen sulfide (NaHS) on biomarkers of oxidative stress

The administration of NaHS induced a significant decrease in the control group and a significant

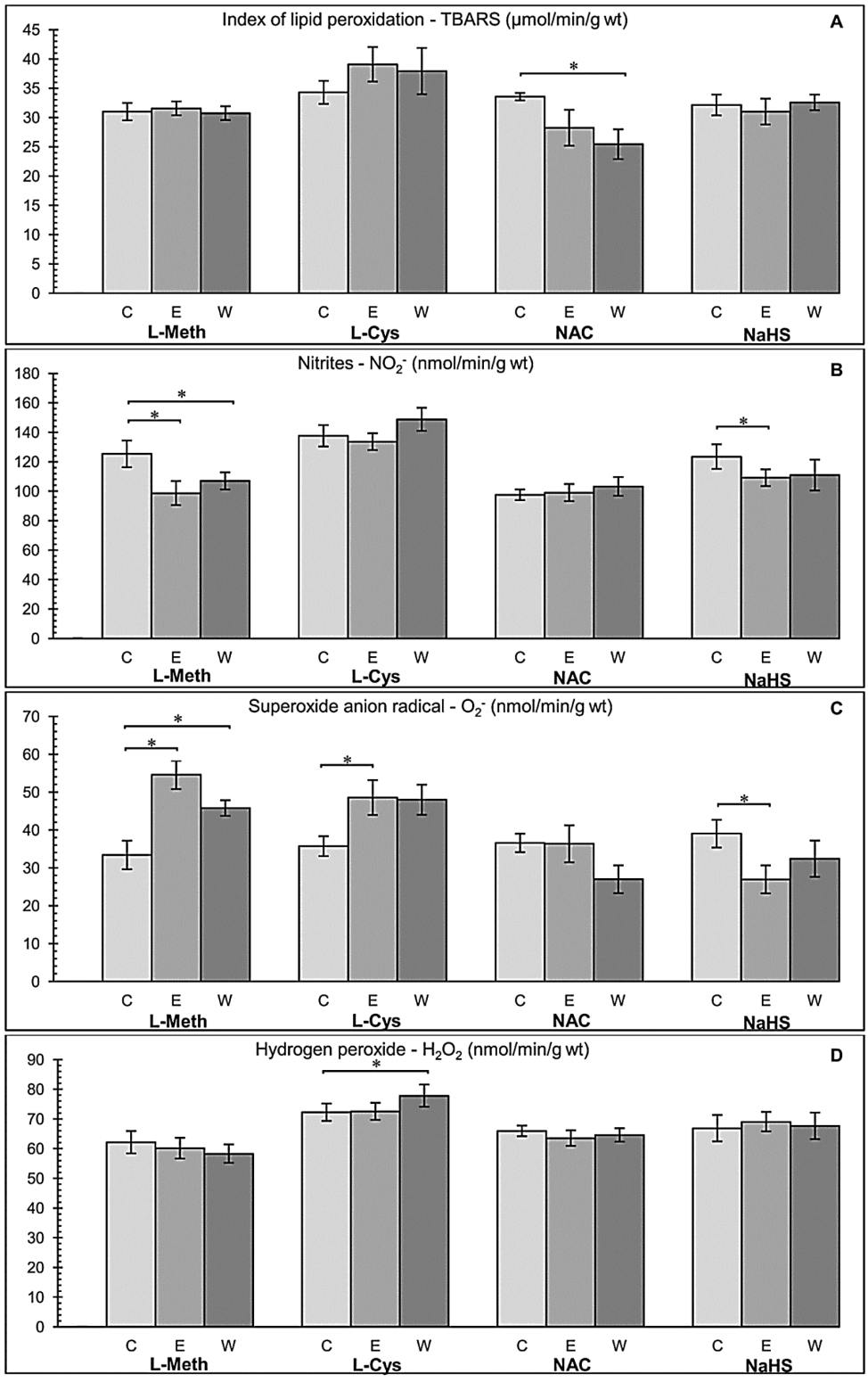

Figure 3. The values of oxidative stress biomarkers after acute administration of various sulfur-containing compounds (L-Meth, L-cysteine, NAC and NaHS) in saline-treated rats. The values were measured in 3 time periods (C, control; E, effect; W, washout). Data are expressed as the means ± SE; *$p < 0.05$, **$p < 0.01$

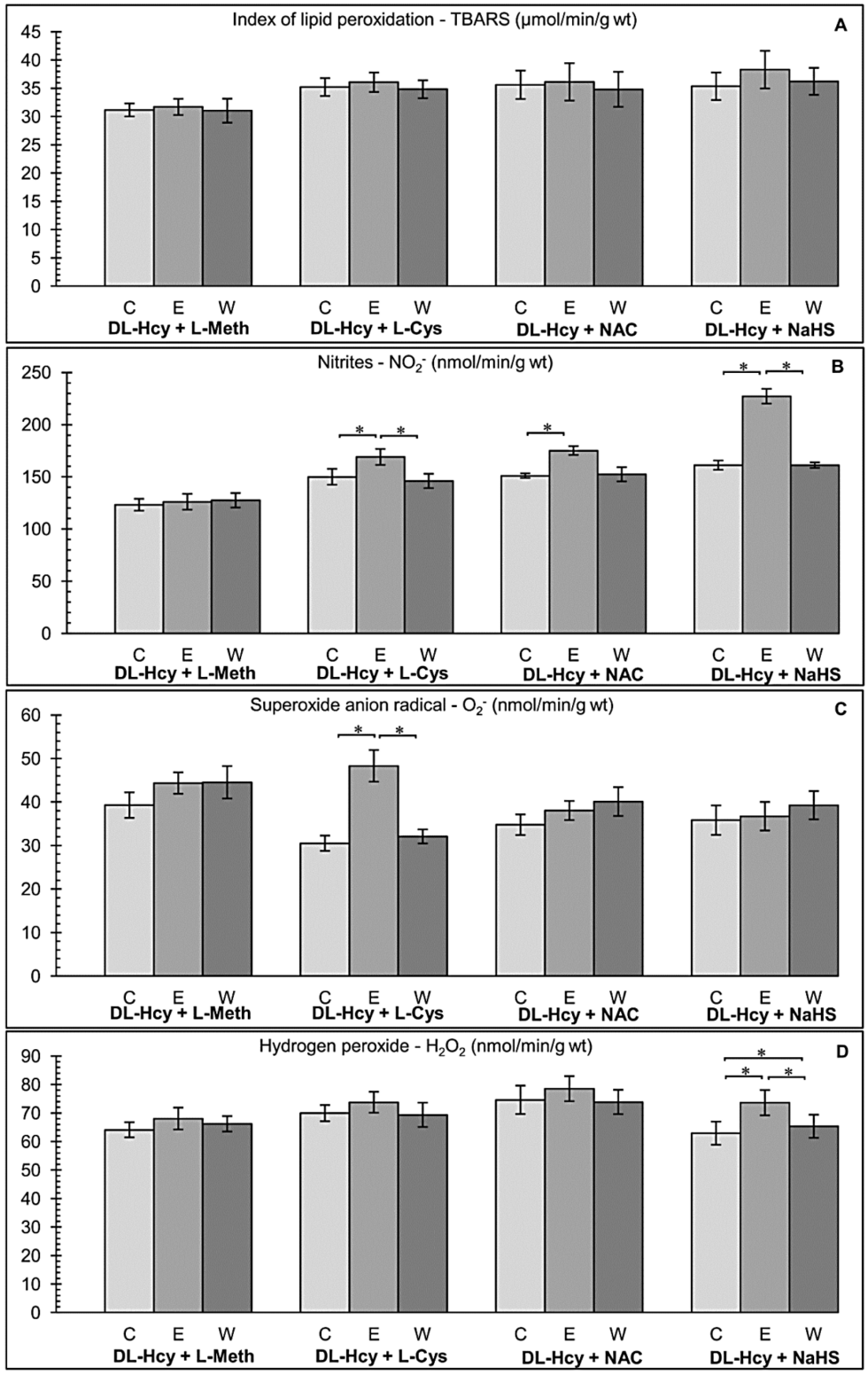

Figure 4. The values of oxidative stress biomarkers after acute administration of various sulfur-containing compounds (L-Meth, L-cysteine, NAC and NaHS) in DL-Hcy-treated rats. The values were measured in 3 time periods (C, control; E, effect; W, washout). Data are expressed as the means ± SE; *$p < 0.05$, **$p < 0.01$.

increase in NO_2- in DL-Hcy-treated rats (Figs. 3B and 4B). During the washout period in DL-Hcy rats, the value of NO_2- decreased significantly and reached values similar to those of the control group (Fig. 4B). Furthermore, the application of NaHS induced a significant reduction in O_2- production in control rats and a significant increase in H_2O_2 production in DL-Hcy-treated rats (Figs. 3C and 4D). The values of H_2O_2 in DL-Hcy-treated rats decreased significantly during the washout period, but at the end of this period, these values were significantly higher than the control group (Fig. 4D).

Comparisons between groups depending on the effects of treatment of DL-Hcy and the point of sampling the effluent (control, effect or washout)

To estimate the effect of DL-Hcy treatment and point of sample collection (control, effect and washout) on observed parameters, as well as the effect size, data were analyzed by two-way ANOVA (Table 2). Effect size is shown through the parameter partial eta squared (η_p^2). There was no statistically significant interaction between DL-Hcy treatment and point of sample collection on the value of any of the observed oxidative stress biomarkers regarding L-methionine application. On the other hand, NO_2- varied significantly depending on the DL-Hcy treatment, while O_2- varied significantly depending on the point of sample collection. In the case of L-cysteine application, there were statistically significant interactions between DL-Hcy treatment and point of sample collection on the values of NO_2- and O_2-. Furthermore, NO_2- varied significantly depending on the DL-Hcy treatment, and O_2- varied significantly depending on both DL-Hcy treatment and point of sample collection. Similarly, there were statistically significant interactions between DL-Hcy treatment and point of sample collection on values of NO_2- and O_2- regarding NAC administration. However, in the case of NAC administration, all examined oxidative stress biomarkers varied depending on the DL-Hcy treatment. Analysis of

Table 2. Comparisons between groups depending on the DL-Hcy treatment and the point of sample collection (control, effect and washout).

L-methionine	TBARS		NO_2-		O_2-		H_2O_2	
	Sig.	ηp^2	*Sig.*	ηp^2	*Sig.*	ηp^2	*Sig.*	ηp^2
DL-Hcy treatment	0.859	0.001	0.017	0.160	0.507	0.013	0.078	0.084
Sample collection time	0.867	0.009	0.289	0.072	0.001	0.334	0.860	0.009
DL-Hcy treatment and sample collection time interaction	0.998	0.001	0.132	0.115	0.069	0.150	0.596	0.031
L-cysteine	TBARS		NO_2-		O_2-		H_2O_2	
	Sig.	ηp^2	*Sig.*	ηp^2	*Sig.*	ηp^2	*Sig.*	ηp^2
DL-Hcy treatment	0.397	0.024	0.016	0.178	0.010	0.202	0.264	0.041
Sample collection time	0.521	0.043	0.584	0.035	0.001	0.497	0.740	0.020
DL-Hcy treatment and sample collection time interaction	0.648	0.029	0.040	0.193	0.031	0.207	0.371	0.064
N-acetylcysteine	TBARS		NO_2-		O_2-		H_2O_2	
	Sig.	ηp^2	*Sig.*	ηp^2	*Sig.*	ηp^2	*Sig.*	ηp^2
DL-Hcy treatment	0.005	0.230	0.001	0.860	0.001	0.298	0.001	0.358
Sample collection time	0.256	0.087	0.063	0.160	0.057	0.174	0.859	0.010
DL-Hcy treatment and sample collection time interaction	0.356	0.067	0.039	0.194	0.021	0.227	0.572	0.037
NaHS	TBARS		NO_2-		O_2-		H_2O_2	
	Sig.	ηp^2	*Sig.*	ηp^2	*Sig.*	ηp^2	*Sig.*	ηp^2
DL-Hcy treatment	0.059	0.121	0.001	0.830	0.006	0.228	0.867	0.001
Sample collection time	0.922	0.005	0.001	0.445	0.023	0.221	0.285	0.080
DL-Hcy treatment and sample collection time interaction	0.634	0.030	0.001	0.564	0.012	0.255	0.560	0.038

NaHS application showed statistically significant interactions between DL-Hcy treatment and point of sample collection on values of NO_2- and O_2-, and both mentioned parameters significantly varied depending on the DL-Hcy treatment and point of sample collection. Values of η_p^2 followed the *p* values in all mentioned parameters. Namely, η_p^2 values were always higher in cases where the p-value indicated statistical significance.

DISCUSSION AND CONCLUSION

The aim of this study was to assess the effect of acute, direct administration of various sulfur-containing compounds (L-meth, L-cysteine, NAC and NaHS) on oxidative stress biomarkers in saline- and DL-Hcy-treated rats, distinguish the early changes of redox balance in the myocardium induced by Hcy, and measure the effects of applied sulfur-containing compounds on these changes.

Based on the biochemical evaluation at the termination of treatment, we confirmed that subchronic (2 weeks long) administration of DL-Hcy at the dose of 0.45 μmol/g body weight twice a day led to a significant increase in plasma Hcy levels (Table 1) but did not induce hyperhomocysteinemia (HHcy). However, the values of plasma Hcy were lower than 15 μmol/L, which is defined as the limit value for HHcy (23). These results support those found by Timkova and colleagues who used the same experimental protocol and obtained similar values of Hcy (11).

In the first part of our study, we compared the systemic redox status of healthy rats and rats treated with DL-Hcy to examine the effects of the subchronic DL-Hcy administration. Our data showed that 2 weeks of subcutaneous DL-Hcy administration at a dose of 0.45 μmol/g body weight twice a day induced an increase in TBARS levels and ROS, such as superoxide anion radicals (O_2-) and hydrogen peroxide (H_2O_2), but reduced NO_2- levels (Fig. 1). Our results are consistent with the results obtained by Kolling and coauthors. They showed that chronic DL-Hcy application (twice a day, 0.3-0.6 μmol/g body weight, 22 days) increased TBARS and decreased NO in the hearts of young Wistar albino rats (24). TBARS values were also compatible with the previously mentioned investigation by Timkova and colleagues (23). Decreased values of NO_2- reflect decreased NO bioavailability and disturbed endothelial function. Decreased NO production during HHcy is a consequence of the decreased activity of constitutive isoforms of nitric oxide synthase (NOS), endothelial and neuronal, and the increased activity of inducible NOS, uncoupling of endothelial NOS, and the consequent increase of O_2- production, which reacts with NO and results in the production of one of the most toxic forms of ROS, peroxynitrite ($ONOO^-$) (25). Reactive oxygen species, such as O_2- and H_2O_2, are also generated during the oxidation and auto-oxidation of thiol groups in Hcy (26). These ROS are considered to be one of the primary causes for the endothelial cytotoxicity of DL-Hcy. Chang and coauthors showed stimulation of ROS generation by 0.5 and 1.0 mmol/L Hcy in rat vascular smooth muscle cells (VSMC) (27). The same study also demonstrated that CAT activity in VSMC was inhibited by Hcy, which is consistent with the results of our research. (Fig. 2). Our results for antioxidative enzymes activity are also similar to those of a previously mentioned study by Kolling and coauthors (24). The decrease of antioxidative enzyme activity may be another mechanism responsible for the oxidative stress injury induced by homocysteine.

In the next part of our study, we explored the effects of acute, direct administration of sulfur-containing amino acids (L-Meth, L-Cys, and NAC) or NaHS on redox status by measuring the levels of some oxidative stress parameters (TBARS, NO_2-, O_2- and H_2O_2) in the coronary venous effluent of saline- and DL-Hcy-treated rats.

Our data showed that acute application of L-Meth induced a decrease in NO_2- and an increase in O_2- in saline-treated rats, while the acute application of L-Meth did not affect the observed oxidative stress parameters in DL-Hcy-treated rats (Figs. 3B, 3C and 4). Soares and coworkers investigated the effects of acute Meth exposure (0.4 g/kg, subcutaneous injection) on oxidative stress parameters in the cerebral cortex of young rats. Their results showed a significant increase in ROS (3 h after) and SOD (1 h after), while CAT was reduced at 1 h and 3 h after Meth administration (28). Costa and coauthors also demonstrated that acute treatment with Meth alters lipid peroxidation, carbonyl content, ROS levels, and SOD and CAT activities in the liver of young rats (29). The difference in the effects of L-Meth between the control and DL-Hcy-treated groups may be the consequence of altered intracellular Hcy and L-Meth metabolic pathways.

The direct application of L-Cys induced an increase in the measured ROS values (during application: O_2-; during washout period: H_2O_2) in saline-treated rats and a significant increase in NO_2- and O_2- in DL Hcy-treated rats (Figs. 3C, 3D, 4B, and 4C). A previous study by Hogg proposed that the increased level of Cys induces thiol-dependent

oxidative stress due to this higher auto-oxidative potential and that Hcy reduces cystine to cysteine (30). On the other hand, some data suggests L-Cys has protective effects, indicating the double-edged sword of compounds containing thiol groups in terms of their antioxidant/pro-oxidant properties. Shackebaei and other authors showed the protective effects of L-Cys in the heart when preconditioned with 0.5 mmol/L L-Cys before long-term ischemia and suggested several potential mechanisms. However, considering the pathophysiological concept of preconditioning, it is possible that a mild increase in free radical production increases cell survival after ischemia and reperfusion (12, 13). Differences in the effects of L-Cys among the abovementioned studies may be the consequence of different experimental protocols, suggesting that L-Cys may exert various effects depending on the mechanism of administration or that our perception of its effects depends on the time of the measurement and the measured biomarkers.

The administration of NAC resulted in a significant decrease of TBARS in saline-treated rats and in a significant increase in NO_2- in DL-Hcy-treated rats (Figs. 3A and 4B). NAC is an antioxidant, although its exact mechanisms of action are still unclear. Literature data suggest its sulfhydryl group sparing potentials are responsible for its cell-protecting properties. NAC is a scavenger of free radicals, binds to transitions metals, and restores intracellular GSH levels (31). An increase in NO_2- levels in DL-Hcy-treated rats suggest an increase in NO production, which may be the part of the protective mechanism of NAC. However, other authors also demonstrated that NAC can increase NO production and restore endothelial dysfunction induced by Hcy (32).

The acute administration of NaHS induced a significant decrease in NO_2- and O_2- in the saline-treated group and a significant increase in NO_2- and H_2O_2 in DL-Hcy-treated rats (Figure 3B, 3C, 4B and 4D). Zhang and coworkers induced cardiac dysfunction with a single dose of isoprenaline (ISO) and concluded that the application of NaHS can mitigate ISO-induced changes in the myocardium by decreasing ISO-induced ROS production (33). Namely, NaHS decreases the activity of NADPH oxidase, resulting in a reduction in ROS formation and oxidative stress. The results of all these studies are consistent with those on the antioxidative effects of NaHS of saline-treated rats of this study, except that NaHS did not exhibit an antioxidative effect in Hcy-treated rats. On the other hand, Jiang and colleagues found that NaHS at a dose of 1 mmol/L induced a decrease in oxygen consumption in hiPSC-derived cortical neurons, primary human fibroblasts, and COS-7 monkey kidney cells (34). These authors proposed two ways to explain the NaHS-increased oxidative stress observed: sulfide from NaHS can react directly with oxygen to form reactive oxygen species, and sulfide can serve as a substrate for complex II of the mitochondrial electron transport chain. Based on these results and results from our investigation, we concluded that NaHS may have a protective or harmful effect depending on the mode of administration and the administered dose.

Furthermore, based on our obtained results, we concluded that homocysteine induces considerable changes in the function of the cardiovascular system even before the increase in plasma homocysteine values. Homocysteine affects redox balance, increases ROS production, decreases antioxidative defenses and acts as an overall pro-oxidant. Because the effects of the investigated sulfur-containing compounds varied considerably depending on the action of homocysteine, some of them may yield completely opposite effects depending on whether they act in the presence of homocysteine.

Conflict of interest

All authors of the present paper disclose that they have no actual or potential conflicts of interest, including any financial, personal, or other relationships with people or organizations.

Acknowledgments

This project was supported by the Ministry of Science and Technical Development of the Republic of Serbia (Grant no. 175043) and the Junior Project 04/2011 of the Faculty of Medical Sciences at University of Kragujevac, Serbia.

REFERENCES

1. Pizzino G., Irrera N., Cucinotta M., Pallio G., Mannino F. et al.: Oxid. Med. Cell. Longev. 2017, 8416763 (2017).
2. Ooi B.K., Goh B.H., Yap W.H.: Int. J. Mol. Sci. 18, E2336 (2017).
3. Adameova A., Xu Y.J., Duhamel T.A., Tappia P.S., Shan L. et al.: Curr. Pharm. Des. 15, 3094 (2009).
4. Prabhakaran D., Anand S., Watkins D., Gaziano T., Wu Y. et al.: Lancet 391, 1224 (2018).
5. Xiao Y., Su X., Huang W., Zhang J., Peng C. et al.: Int. J. Biochem. Cell. Biol. 67, 158 (2015).

6. Chung H.S., Wang S.B., Venkatraman V., Murray C.I., Van Eyk J.E.: Circ. Res. 112, 382 (2013).
7. Schöneich C.: Antioxid. Redox. Signal. 26, 388 (2017).
8. Martínez Y., Li X., Liu G., Bin P., Yan W. et al.: Amino. Acids. 49, 2091 (2017).
9. Škovierová H., Vidomanová E., Mahmood S., Sopková J., Drgová A. et al.: Int. J. Mol. Sci. 17, E1733 (2016).
10. Steed M.M., Tyagi S.C.: Antioxid. Redox. Signal. 15, 1927 (2011).
11. Timkova V., Tatarkova Z., Lehotsky J., Racay P., Dobrota D. et al.: Mol. Cell. Biochem. 411, 261 (2016).
12. Shackebaei D., King N., Shukla B., Suleiman M.S.: Mol. Cell. Biochem. 277, 27 (2005).
13. Elsey D.J., Fowkes R.C., Baxter G.F.: J. Cardiovasc. Pharmacol. Ther. 15, 53 (2010).
14. Ulbink J.B., Vermak W.J.H., Bissbort S.: J. Chromatogr. B. Biomed. Appl. 565, 441 (1991).
15. Ohkawa H., Ohishi N., Yagi K.: Anal. Biochem. 95, 351 (1979).
16. Green L.C., Wagner D.A., Glogowski J., Skipper P.L., Wishnok J.S. et al. Anal. Biochem. 126, 131 (1982).
17. Auclair C., Voisin E.: in: Handbook of Methods for oxygen radical research, Greenvvald R.A. Ed., p. 123, CRC Press Une, Boca Raton 1985.
18. Pick E., Keisari Y.: J. Immunol. Methods. 38, 161 (1980).
19. McCord J.M., Fridovich I.: J. Biol. Chem. 244, 6056 (1969).
20. Beutler E.: in: Manual of biochemical Methods, Beutler E. Ed. p. 105, Grune and Stratton, New York 1982.
21. Tsuchihashi M.: Biochem. Z. 140, 65 (1923).
22. Misra H.P., Fridovich I.: J. Biol. Chem. 247, 3170 (1972).
23. Cesari M., Rossi G.P., Sticchi D., Pessina A.C.: Nutr. Metab. Cardiovasc. Dis. 15, 140 (2005).
24. Kolling J., Scherer E.B., Cunha A.A., Cunha M.J., Wyse A.T.: Cardiovasc. Toxicol. 11, 67 (2010).
25. Pushpakumar S., Kundu S., Sen U.: Curr. Med. Chem. 21, 3662 (2014).
26. Ramakrishnan S., Sulochana K.N., Lakshmi S., Selvi R., Angayarkanni N.: Indian. J. Biochem. Biophys. 43, 275 (2006).
27. Chang L., Xu J., Zhao J., Pang Y., Tang C. et al.: Acta. Pharmacol. Sin. 25, 341 (2004).
28. Soares M.S.P., Viau C.M., Saffi J., Costa M.Z., da Silva T.M. et al.: Metab. Brain. Dis. 32, 1693 (2017).
29. Costa M.Z., Silva T.M., Flores N.P., Schmitz F., Scherer E.B.S. et al.: Mol. Cell. Biochem. 384, 21 (2013).
30. Hogg N.: Free. Radic. Biol. Med. 27, 28 (1999).
31. Samuni Y., Goldstein S., Dean O.M., Berk M.: Biochim. Biophys. Acta. 1830, 4117 (2013).
32. Liu Y.H., You Y., Song T., Wu S.J., Liu L.Y.: J. Cardiovasc. Pharmacol. 50, 155 (2007).
33. Zhang Z., Jin S., Teng X., Duan X., Chen Y. et al.: Nitric. Oxide. 67, 10 (2017).
34. Jiang J., Chan A., Ali S., Saha A., Haushalter K.J. et al.: Sci. Rep. 6, 20831 (2016).

Received: 20. 06. 2018