The effects of acute hyperhomocysteinemia induced by DL-homocysteine or DL-homocysteine thiolactone on serum biochemical parameters, plasma antioxidant enzyme and cardiac acetylcholinesterase activities in the rat

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Abstract: The aim of this study was to assess the effects of DL-homocysteine (DL-Hcy) and DL-homocysteine thiolactone (DL-Hcy TLHC) on selected serum biochemical parameters, markers of oxidative stress and the activities of antioxidant enzymes (catalase (CAT), glutathione peroxidase (GPx), superoxide dismutase (SOD)) in the plasma, as well as on acetylcholinesterase (AChE) activity in the cardiac tissue homogenate in the rat. Male Wistar rats were divided into three groups as follows: control group (1 mL 0.9% NaCl, intraperitoneal (i.p.) injection), DL-Hcy group (8 mmol/kg body mass (b.m.), i.p.) or DL-Hcy TLHC group (8 mmol/kg b.m., i.p.). One hour after administration, the rats were euthanized, whole blood was collected for biochemical analysis, and the heart was excised. Following the i.p. administration of DL-Hcy and DL-Hcy TLHC, the activities of antioxidant enzymes were mostly significantly increased, while plasma malondialdehyde (MDA) was decreased. Administration of DL-Hcy and DL-Hcy TLHC significantly inhibited AChE activity in rat cardiac tissue. Our findings suggest that DL-Hcy and DL-Hcy TLHC exerted prooxidant effects; however, the decrease in MDA points to an inverse response to the increase in antioxidant enzyme activities. While both substances inhibited AChE activity in rat cardiac tissue, DL-Hcy TLHC induced stronger effects than DL-Hcy.

Key words: acetylcholinesterase; antioxidant enzymes; DL-homocysteine; DL-homocysteine thiolactone; rat

Abbreviations: ACh – acetylcholine; AChE – acetylcholinesterase; Alb – albumin; ALT – alanine aminotransferase; AMY – alfa amylase; AST – aspartate aminotransferase; CAT – catalase; CHD – coronary heart disease; Chol – cholesterol; CSE – cystathionine gamma lyase; Crea – creatinine; DL-PAG – DL-propargylglycine; ECL – electrochemiluminescence immunoassay system; eNOS – endothelial nitric oxide synthase; Fibr – fibrinogen; Gluc – glucose; GPx – glutathione peroxidase; GSH – glutathione; Hcy – homocysteine; HHcy – hyperhomocysteinemia; HDL – high density cholesterol; HO-1 – heme oxygenase-1; hsTnT – high sensitivity troponin T; LDH – lactate dehydrogenase; LDL – low density cholesterol; L-NAME – Nω-nitro-L-arginine methyl ester; MDA – malondialdehyde; SOD – superoxide dismutase; TGL – triglycerides; TP – total protein; Urca – uric acid; VWF Act – Von Willebrand factor activity; ZnPPR IX – zinc protoporphyrin IX

INTRODUCTION

Homocysteine (Hcy) is an endogenous sulfhydryl-containing amino acid that is an intermediate product in the normal biosynthesis of the amino acids methionine and cysteine [1]. Deficiency of the enzymes and/or vitamins that are involved in the homocysteine metabolic pathway leads to its increased levels in the plasma, referred to as hyperhomocysteinemia (HHcy) [2,3]. Data suggests that metabolic conversion of Hcy to the thioester Hcy-thiolactone contributes to Hcy pathobiology [4].

Literature findings indicate that there is a clear correlation between total serum Hcy and the incidence of cardiovascular disease and its complications, such as heart attacks and strokes, thus promoting Hcy as a new and independent risk factor for these diseases [5,6]. HHcy leads to endothelial dysfunction, which may further promote the proliferation of vascular smooth muscle cells [7] and increase collagen synthesis in vascular smooth muscle cells [8]. There is evidence that HHcy is associated with increased ROS formation, including the superoxide anion radical (O_2^-) and hydrogen peroxide (H_2O_2) [9], thereby leading to oxidative stress and cell damage [10]. The main antioxidant enzymes responsible for controlling oxygen free radicals are SOD, CAT, and glutathione-dependent enzymes, including GPx, GR and GST [10]. While it has been found that HHcy may impair the glutathione-related antioxidant defense system [11,12], data about its effect on SOD activity are still controversial.

While some studies showed that HHcy decreased erythrocyte SOD activity in patients with cardiovascular disease (CVD) [12,13], others have established a positive relationship between SOD activity and Hcy levels in patients with homocystinuria [14]. Acetylcholinesterase (AChE) is the serum protease that terminates neurotransmission at cholinergic synapses by splitting the acetylcholine into choline and acetate, and it is predominantly distributed in different brain regions [15]. Interestingly, elevated homocysteine levels increase AChE activity in the central nervous system [15,16], while homocysteine thiolactone slowly and irreversibly inhibits it [17]. Recent investigations have proved the presence of AChE activity in the heart [18].

Taking into account that the molecular mechanisms of Hcy-induced cardiac effects are still poorly understood, this study aimed to examine the effect of DL-homocysteine and its thiolactone metabolite on serum biochemical parameters, markers of oxidative stress, the activities of antioxidant enzymes and cardiac AChE activity in the rat.

MATERIALS AND METHODS

Chemicals

All chemicals were purchased from the Sigma Chemical Co. (USA).

Experimental animals

Male Wistar albino rats (n=36, 12 in each experimental group; 10 weeks old, body mass 250±30 g) were used. The rats were housed under strictly controlled conditions of air temperature (22±1°C), relative humidity (50%) and a 12 h light/dark cycle (with the light period commencing at 9 am), with free access to water and standard food. All experimental procedures were performed in accordance with the EU Directive for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes 86/609/EES.

Experimental protocol

To produce acute HHcy, DL-homocysteine (DL-Hcy) and DL-homocysteine thiolactone (DL-Hcy TLHC) were administered i.p. in a single dose. Rats were divided into three groups as follows: control group (1 mL 0.9% NaCl, intraperitoneal (i.p.) injection), DL-Hcy group (8 mmol/kg b.m., i.p.) or DL-Hcy TLHC group (8 mmol/kg b.m., i.p.). One hour after administration, the rats were euthanized, whole blood was collected for biochemical analysis, and the heart was excised. Saline and both Hcy compounds were administered in the same volume (1 mL). Sixty min after application, the animals were killed using a rat guillotine, blood samples were collected, and the heart was removed. After collection, the samples were kept at room temperature for 15 min, centrifuged for 15 min at 2000 x g and analyzed.

Serum biochemical parameters

The following serum biochemical parameters: Alb, ALT, AMY, AST, Chol, C, Fibr, Gluc, HDL, Hcy, hsT-nT, LDH, LDL, TGL, TP, Urca, Urea, VWF Act, were analyzed using spectrophotometry and commercial kits (Siemens Healthcare Diagnostics Ltd. Frimley, Camberley, UK) on an automatic biochemical analyzer (Dimension Xpand, Siemens). An immunoturbidimetric commercial assay (Siemens Healthcare GmbH, Marburg, Germany) was used for the determination of fibrinogen and von Willebrand factor activity on a BCS XP coagulation analyzer (Siemens Healthcare GmbH, Marburg, Germany). Serum homocysteine was measured by an automated electrochemiluminescence immunoassay system ADVIA Centaur XP system (Siemens Healthcare GmbH, Erlangen, Germany).

Determination of lipid peroxidation products

The MDA content in plasma was determined by the TBARS assay according to the standard procedure [19]. The values of MDA content (nmol of MDA/mL plasma) were determined on the basis of absorbance values and molar absorption coefficient of the malondialdehyde-thiobarbituric acid complex.

Determination of CAT activity

CAT activity was estimated by measuring the degradation of H_2O_2 [20]. The rate of decomposition of H_2O_2 by CAT is measured spectrometrically at 230 nm. CAT activity was expressed as U/mL of plasma, with 1 U of enzyme activity defined as 1 µL of spent H_2O_2/min.

Determination of SOD

SOD activity was measured according to the method of Misra and Fridovich [21], with 1 U of SOD defined as the amount of enzyme that inhibits the rate of the epinephrine oxidation by 50%; enzyme activity was expressed as U/mL of plasma.

Determination of GPx activity

GPx activity was estimated essentially as described [22] and expressed as ΔA/min/mL of plasma.

Determination of AChE activity

AChE activity was determined in cardiac tissue homogenates. Heart tissue was homogenized (20 mg of tissue per 1 mL of phosphate buffer (pH 8.0, 0.1 M)) and centrifuged for 15 min at 10000 x g. The specific activity of AChE in the heart homogenate was determined *in vitro* by the method of Ellman [23].

Statistics

Statistical analysis of experimental data included the following basic descriptive statistics using the mean value (X)±standard error mean (SEM). Since the sample data do not have a normal distribution, in order to determine statistically significant differences between the groups, the Kruskal-Wallis H test (the nonparametric alternative to the one-way ANOVA) was used, as well as multiple comparisons test and Pearson's chi-square test. $P < 0.05$ was considered to be significant. A database analysis of the results was performed using the software package SPSS 22th (SPSS Inc., Chicago, IL, USA).

RESULTS

Plasma biochemical parameters

The plasma values of biochemical parameters are expressed in standard units and are presented in Table 1. It is important to note that the concentrations of Hcy in both experimental groups were >65 mmol/L. The glucose values were higher in DL-Hcy TLHC in comparison to both the control ($p < 0.05$) and DL-Hcy groups ($p < 0.05$). Total protein values were lower in the DL-Hcy group ($p < 0.01$) when compared to the control and DL-Hcy TLHC groups ($p < 0.05$). Alb, ALT and Fibr values were lower in both experimental groups as compared to the control ($p < 0.01$), while the DL-Hcy TLHC group had higher values of Alb than the DL-Hcy group ($p < 0.05$). Chol and HDL values were higher in the DL-Hcy TLHC group in comparison to the control and DL-Hcy group ($p < 0.01$, $p < 0.05$). LDL values were higher in the DL TLHC group when compared to the control ($p < 0.01$). Compared to the control group, serum AMY values were higher in both experimental groups ($p < 0.01$) groups while the HDL values were higher in both DL-Hcy groups ($p < 0.05$).

Table 1. Values of measured biochemical parameters in the plasma expressed in standard units (x±SEM).

Parameter/ Groups	Control	DL-Hcy	DL-Hcy TLHC
(X±SE)			
Gluc (mmol/L)	6.07±0.21	5.66±0.97	8.44±0.75 [b*, c*]
Urea (mmol/ L)	10.6±0.89	9.83±0.61	10.03±1.81
Crea (µmol/ L)	48.28±1.34	48.20±3.79	45.00±2.34
Urca (µmol/ L)	86.38±8.01	189.78±50.37	147.26±61.34
TP (g/L)	65.63±1.35	55.00±1.77 [a**]	63.80±3.84 [c*]
Alb (g/L)	41.98±0.59	31.00±0.69 [a**]	35.20±1.96 [b**,c*]
Chol (mmol/L)	1.39±0.65	1.61±0.10	2.03±0.19 [b**,c*]
HDL (mmol/L)	1.12±0.48	1.19±0.06	1.52±0.12 [b**,c*]
LDL (mmol/L)	0.08±0.01	0.19±0.05	0.30±0.05 [b**]
TGL (mmol/L)	0.59±0.05	0.52±0.08	0.46±0.09
AST (IU/L)	312.38±22.61	306.00±34.70	232.80±27.03
ALT (IU/L)	122.63±8.67	62.67±7.62 [a**]	68.00±10.57 [b**]
AMY (IU/L)	1281.50±83.50	2462.17±232.00 [a**]	2398.20±304.51 [b**]
hsTnT (ng/L)	25.80±6.20	40.88±7.14	19.75±7.36
LDH (IU/L)	3887.13±358.51	5655.00±605.73 [a*]	3789.25±625.50 [c*]
Fibr (g/L)	2.60±0.26	1.06±0.04 [a**]	1.07±0.07 [b**]
VWF Act (%)	214.25±30.45	236.17±36.13	162.00±24.28

Statistical comparisons are marked as follows: a – control vs DL-Hcy $p<0.05$; b – control vs DL-Hcy TLHC; c – DL-Hcy vs DL-Hcy TLHC. P values less than 0.05 were considered to be significant (*$p<0.05$; **$p<0.01$).

Index of lipid peroxidation (MDA)

Administration of DL-Hcy ($p<0.01$) and DL-Hcy TLHC ($p<0.05$) induced a statistically significant decrease in MDA as compared to the control group (Fig. 1A).

CAT, GPx and SOD activities

Administration of DL-Hcy TLHC induced a statistically significant increase in CAT activity in the plasma as compared to the control group ($p<0.01$). Furthermore, there was significant increase of CAT activity after administration DL-Hcy TLHC when compared to the administration of DL-Hcy ($p<0.05$) (Fig. 1B).

The same trend in GPx activity was observed after the administration of DL-Hcy and DL-Hcy TLHC. Administration of DL-Hcy TLHC induced a statistically significant increase in GPx activity as compared to the control ($p<0.01$). There was highly significant increase in GPx activity after DL-Hcy TLHC administration when compared to DL-Hcy ($p<0.01$) (Fig. 1C).

After DL-Hcy administration, SOD activity was statistically increased ($p<0.01$). DL-Hcy TLHC administration decreased SOD activity without statistical significance. A highly significant increase in SOD activity was noted after DL-Hcy administration in comparison to DL-Hcy TLHC administration ($p<0.01$) (Fig. 1D).
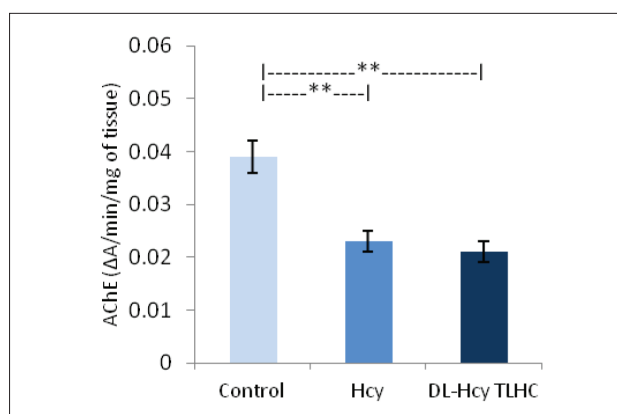
AChE activity

AChE activity was significantly decreased after the administration of DL-Hcy ($p<0.01$) and DL-Hcy TLHC ($p<0.01$) as compared to the control (Fig. 2).

DISCUSSION

Our first objective was to investigate the influence of acute HHcy on different serum biochemical parameters in order to assess the systemic response to an DL-Hcy load. Our results showed that an acute rise in DL-Hcy and its thiolactone metabolite did not affect the concentrations of most routinely examined biochemical markers (Table 1), the only exception being an increase in total cholesterol concentrations and its HDL and LDL fractions in rats treated with DL-Hcy TLHC. The data referring to the effect of increased Hcy on the lipid profile are debatable. A previous study of rats that received DL-Hcy TLHC for 30 days (1 g/kg body weight/day) showed elevated serum cho-
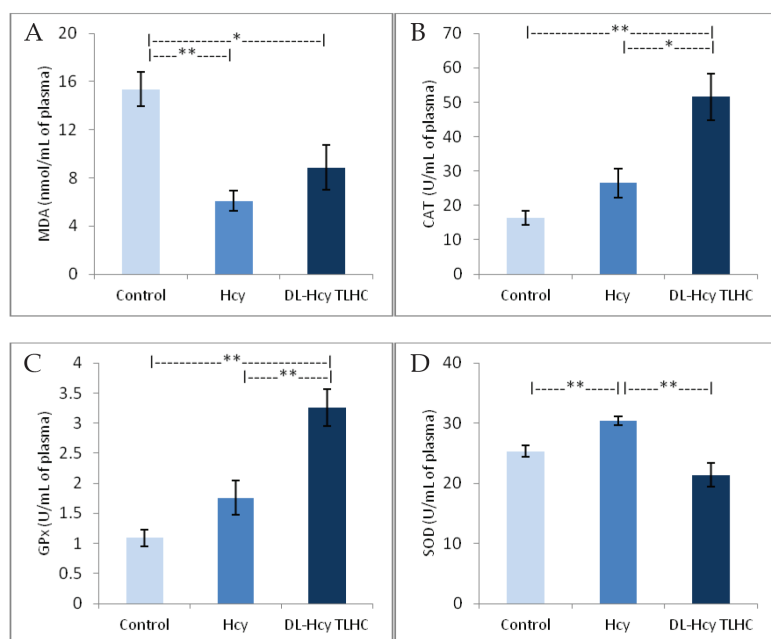
Fig. 1. The effects of DL-Hcy and DL-Hcy TLHC on selected markers of oxidative stress in rat plasma. **A** – MDA concentration; **B** – CAT activity; **C** – GPx activity; **D** – SOD activity. P values less than 0.05 were considered to be significant (*p<0.05; **p<0.01).

Fig. 2. The effects of DL-Hcy and DL-Hcy TLHC on AChE activity in rat heart. P<0.05 was considered to be significant (*p<0.05; **p<0.01).

lesterol and triglycerides [24], with which our results agree. Interaction between Hcy and lipids is poorly understood, especially with regard to the activity of the enzyme paraoxonase. Nevertheless, it has been proposed that DL-Hcy induces endoplasmic reticulum stress and activates sterol regulatory element-binding proteins (SREBPs), which could increase the expression of genes responsible for cholesterol/triglyceride biosynthesis [25].

The kidneys are important in the removal of Hcy, and they could be affected by elevated plasma DL-Hcy levels. Although a recent investigation on mice suggests that persistently increased levels of DL-Hcy induce extra-cellular matrix remodeling and renal fibrosis [26], the parameters of renal function we examined (Urea, Crea and Urca) were not changed following the acute increase in DL-Hcy.

The liver possesses the highest catabolic capacity for DL-Hcy, and increases in DL-Hcy or methionine levels can lead to liver oxidative/nitrosative stress [27]. Results linked with hepato-cellular damage (AST and ALT) showed that HHcy is not linked to hepatocel-lular damage; however, the observed lower levels of serum Alb and Fibr in both experimental groups indicate that there is an initial impairment of liver function. Disturbed fibrinogen levels may occur due to an established process of homocysteine vs proteins interactions. Recent studies have demonstrated that treatment with methionine for 8 weeks (100 mg/kg) led to liver dysfunction through increased ROS production [28].

The observed alterations in serum glucose and AMY concentrations in acute HHcy could be linked with pancreatic dysfunction. Also, the higher glucose concentration in the DL-Hcy TLHC group could be the result of insulin resistance. A previous study on rats has shown that HHcy was associated with a decrease in the glucose:insulin ratio and an increase in the insulin resistance index [29]. It was also found that a 2-week treatment with DL-Hcy decreased the cerebral expression of GLUT-1 and produced a concomitant increase in the percentage of damaged cerebral vessels in rats [30].

In addition, we observed increased levels of hsTnT (very close to statistical significance) and LDH, as indicators of cardiac and brain damage. The limited metabolic capacity for DL-Hcy in the cardiovascular system might be linked to injury of the cardiovascular system induced by Hcy.

Our study showed that both DL-Hcy and DL-Hcy TLHC decreased MDA, while there was no significant difference between these experimental groups and its metabolite. These results are not in agreement with the results of other studies where it was shown that acute HHcy increased lipid peroxidation in rats [11]. Additionally, it was shown [9] that chronic DL-Hcy administration also promoted oxidative damage by lipid peroxidation [31]. However, we did not note changes in the index of lipid peroxidation in the isolated rat heart treated with three isoforms of 10 μM homocysteine (DL-Hcy, DL-Hcy TLHC and L-Hcy TLHC) [32]. The discrepancy between our results and the mentioned investigations may be due to the acute application of substances and the resulting lack of time for achieving their negative effects on oxidative stress markers.

Having in mind that GPx-1 may be a key target of the deleterious actions of DL-Hcy [33], it was important to attempt to reveal the complex relationship between HHcy and the activity of this enzyme. We showed that the basic form of DL-Hcy did not significantly affect GPx activity, while DL-Hcy TLHC induced an increase in the activity of this enzyme. One possible explanation for the rise in GPx activity could be the chemical reactivity of the toxic thiolactone metabolite, which possesses the ability to cause prominent activation of the glutathione antioxidant system [34]. In contrast, other investigations found that in acute HHcy, GPx activity was decreased in a similar experimental model (0.6 μmol DL-Hcy/g body weight) [11]. Nevertheless, the authors used a much lower concentration of DL-Hcy, which may be not have been enough to mobilize this part of the defense system.

On the other hand, we observed completely opposite results in the case of SOD activity. Namely, the application of DL-Hcy led to a significant increase in SOD, while DL-Hcy-TLHC did not change it. Literature data referring to the DL-Hcy-SOD relation are controversial. Previous studies in rats have shown that 3 weeks of DL-Hcy administration decreased SOD levels in the plasma [9,31]. However, although the time under HHcy was prolonged, its concentration was more than 1000 times lower, which may be the reason for the difference to our investigation. In addition, other investigations found that patients with significantly elevated DL-Hcy have elevated SOD levels [14]. Based on the abovementioned, it would

seem that concentration determines the direction of DL-Hcy toward SOD, i.e. in mild HHcy, SOD is decreased while in prominent HHcy the activity of this enzyme is strongly activated.

Unlike SOD, CAT values showed a different dynamic. Thus, only the thiolactone form induced a significant increase in plasma CAT activity. Recent investigations on animal models also observed increased CAT activity 15 min after acute DL-Hcy administration, suggesting that the oxidative status of rats was altered [11]. Taking into consideration the increased activities of all antioxidant enzymes, we suggested that DL-Hcy and DL-Hcy TLHC have prooxidant effects. However, the decrease in MDA levels during the same period could mean that the rise in antioxidant activity was inversed. In contrast, chronic administration of DL-Hcy can decrease CAT levels in the plasma, heart and aorta, which are accompanied by histological changes [9]. This finding indicates that, besides changed concentrations, the duration of HHcy can also contribute to the effects of DL-Hcy on antioxidative enzyme activity. The potential mechanisms of DL-Hcy-induced changes in antioxidant defense enzymes probably include different interactions of ROS and DL-Hcy [9].

As previously described [17], it has been assumed that the harmful effects of elevated homocysteine could in part be due to its actions on cholinesterases. In the available literature, there is almost no information concerning the effects of DL-Hcy on AChE activity in the heart. According to the presented results, both DL-Hcy and DL-Hcy TLHC decreased AChE activity in cardiac tissue homogenates (Fig. 2). However, the thiolactone form induced a more prominent decrease in AChE activity, revealing its more deleterious influence on cardiac tissue than Hcy itself. Investigations on human subjects also found that thiolactone slowly and irreversibly inhibited the activity of AChE in plasma [17]. Interestingly, in a recent paper [18] it was suggested that increased cholinergic tone in the heart caused by cholinesterase inhibitors has a positive effect on some cardiovascular disorders such as heart failure.

CONCLUSION

The results of the present study suggest that acute application of different forms of DL-Hcy can elicit

responses in different organs. Following acute i.p. administration of DL-Hcy or DL-Hcy TLHC, the activities of antioxidant enzymes in the plasma were significantly increased. Our findings demonstrate that these amino acids have prooxidant effects. In addition, the obtained results were dependent on the form of acutely applied DL-Hcy.

Author contributions: Duško Kornjača performed the experiments and the statistical analysis, interpreted the data, analyzed the results and prepared the manuscript; Vladimir Živković performed the statistical analysis, interpreted the data, analyzed the results and prepared the manuscript; Danijela Krstić contributed to the study design, performed the biochemical analysis and interpreted the data; Mirjana Čolović contributed to the study design, performed the biochemical analysis and interpreted the data; Marko Đurić performed the experiments; Sanja Stanković performed the biochemical analysis; Slavica Mutavdžin performed the experiments and the statistical analysis, provided the literature review and prepared the graphics; Vladimir Jakovljević contributed to the study design, performed the statistical analysis, interpreted the data, analyzed the results and prepared the manuscript; Dragan Đurić contributed to the study design, perform the experiments, performed the biochemical and statistical analysis, interpreted the data, analyzed the results and prepared the manuscript.

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