ORIGINAL ARTICLE
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UDC: 577.112.386:616.1-092.9
https://doi.org/10.2298/VSP171213055M

# The effects of acutely and subchronically applied DL-methionine on plasma oxidative stress markers and activity of acetylcholinesterase in rat cardiac tissue

## Efekti akutno i subhronično primenjenog DL-metionina na markere oksidativnog stresa u plazmi i aktivnost acetilholinesteraze u tkivu srca pacova

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**Abstract**

**Background/Aim.** Chronically induced hypermethioninemia leads to hyperhomocysteinemia which causes oxidative stress, atherogenesis, neurodegeneration and cancer. However, little is known about the acute and subchronic effects of DL-methionine (Met). The aim of study was to assess the effects of acutely and subchronically applied Met on oxidative stress parameters in rat plasma [enzymes: catalase (CAT), glutathione peroxidise (GPx), superoxide dismutase (SOD) and index of lipid peroxidation, malondialdehyde (MDA)], and acetylcholinesterase (AChE) activity in rat cardiac tissue. **Methods.** The enzymes activities, as well as MDA concentration were evaluated following acute (n = 8) and subchronic (n = 10) application of Met [i.p. 0.8 mmoL/kg body weight (b.w.) in a single dose in the acute overload or daily during three weeks in the subchronic overload]. The same was done in the control groups following application of physiological solution [i.p. 1 mL 0.9% NaCl (n = 8) in the acute overload and 0.1–0.2 mL 0.9% NaCl, daily during three weeks (n =10) in the subchronic overload]. Tested parameters were evaluated 60 minutes after application in acute experiments and after three weeks of treatment in subchronic experiments. **Results.** There were no difference in homocysteine values between the groups treated with Met for three weeks and the control group. Met administration significantly increased the activity of CAT and GPx after 1 h compared to the control group ($p = 0.008$ for both enzymes), whereas the activity of SOD and MDA concentrations were unchanged. Subchronically applied Met did not affect activity of antioxidant enzymes and MDA level. AChE activity did not show any change in rat cardiac tissue after 1 h, but it was significantly decreased after the subchronic treatment ($p = 0.041$). **Conclusion.** Results of present research indicate that Met differently affects estimated parameters during acute and subchronic application. In the acute treatment Met mobilizes the most part of antioxidant enzymes while during the subchronic treatment these changes seems to be lost. On the contrary, the acute Met overload was not sufficient to influence on the AChE activity, while longer duration of Met loading diminished function of the enzyme. These findings point out that methionine can interfere with antioxidant defense system and cholinergic control of the heart function.

**Key words:**
**oxidative stress; methionine; homocysteine; rats; plasma; enzymes; cholinesterases.**

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**Apstrakt**

**Uvod/Cilj.** Hronično indukovana hipermetioninemija dovodi do hiperhomocisteinemije koja izaziva oksidativni stress, aterogenezu, neurodegeneraciju i karcinome. Međutim, malo se zna o efektima akutne i subhronične primene DL-metionina (Met). Cilj ovog istraživanja bila je procena efekata akutno i subhronično primenjenog Met na parametre oksidativnog stresa u plazmi pacova [enzime: katalaza (CAT), glutation peroksidaza (GPx), superoksid dismutaza (SOD) i indeks lipidne peroksidacije, malondialdehid (MDA)] i na aktivnost acetilholinesteraze (AChE) u tkivu srca pacova. **Metode.** Aktivnosti enzima, kao i koncentracija MDA mereni su nakon akutne (n = 8) i subhronične (n = 10) primene Met (i.p. 0,8 mmoL/kg u jednoj dozi u akutnom eksperimentu ili svakodnevno tokom tri nedelje u subhroničnom eksperimentu). Isti način tretmana je bio primenjen i u kontrolnoj grupi, ali su životinje bile tretirane fiziološkim rastvorom [i.p. 1 mL 0,9% NaCl (n = 8) u akutnom i 0,1–0,2 mL 0,9% NaCl svakodnevno tokom tri nedelje (n = 10), u subhroničnom eksperimentu]. Testirani parametri su mereni 60 min nakon aplikacije supstanci u akutnim eksperimentima i nakon tri nedelje tretmana u subhroničnim eksperimentima. **Rezultati.** Nije bilo razlike u vrednostima homocisteina između grupe tretirane Met tokom tri nedelje i kontrolne grupe. Primena Met značajno je povećala aktivnost CAT i GPx nakon 1h u poređenju sa kontrolnom grupom ($p$ = 0,008 za oba enzima), dok je aktivnost SOD i koncentracija MDA bila nepromenjena. Subhronično primenjen Met nije uticao na aktivnost antioksidativnih enzima, ni na koncentraciju MDA u plazmi. Aktivnost AChE u srčanom tkivu pacova nije se menjala nakon 1 h, ali je bila značajno smanjena nakon subhroničnog tretmana ($p$ = 0,041). **Zaključak.** Rezultati istraživanja pokazuju da Met različito utiče na ispitivane parametre tokom akutne i hronične primene. Posle akutne primene Met mobiliše veći deo antioksidativnih enzima, dok se tokom subhroničnog tretmana ove promene gube. Nasuprot tome, akutna primena Met ne utiče na aktivnost AChE, dok duže trajanje metioninskog opterećenja smanjuje funkciju ovog enzima. Ovi nalazi ukazuju na to da metionin može da interferira sa antioksidativnim sistemom zaštite i holinergičkom kontrolom funkcije srca.

**Ključne reči:**
**stres, oksidativni; metionin; homocistein; pacovi; plazma; enzimi; holinesteraze.**

## Introduction

Methionine (Met) is an essential sulfur-containing amino acid. It is the first amino acid that is embedded during the process of protein synthesis. It is considered that its primary role is initiation of translation rather than inclusion in the protein structure because it is usually removed from proteins during their synthesis [1]. Activation of Met involves its conversion to S-adenosylmethionine (SAM), which is a methyl group donor in the methylation process. SAM, *via* S-adenosylhomocysteine translates into homocysteine (Hcy). It is normally metabolized *via* two biochemical pathways – remethylation and transsulfuration. Remethylation converts Hcy back to Met, in the presence of betaine or *via* Met synthase in the presence of folic acid and vitamin B12. Transsulfuration converts Hcy to cysteine and glutathione (GSH) in the presence of vitamin B6. GSH is the main product in fighting against oxidative stress [2]. Transsulfuration is regulated by the balance between prooxidants that favorize it and antioxidants that inhibit it [3].

It is believed that excess of Met in tissues is responsible for aging and a reduced life span. Prolonged Met overload can cause increased levels of hydroperoxide, LDL cholesterol, lipid peroxidation, oxidative stress in the liver and plasma Hcy level, which is angiotoxic, causes endothelial dysfunction, hypertension, and it is an important factor in the development of atherosclerosis [4]. Experimental hyperhomocysteinemia caused by long-term oral administration of Met shows the greatest reduction of vasodilatation after 8 h when Hcy level reaches maximum, and the value of Met normalizes [5]. This demonstrates that Hcy is responsible for endothelial dysfunction through the induction of oxidative stress or increased content of an endogenous inhibitor of NO synthesis, asymmetric dimethylarginine (ADMA), product of NO methylation [5, 6]. Another study has shown that, with an unchanged content of Met, and in the absence of B12, significantly elevated levels of Hcy does not cause endothelial lesions in rats with elevated LDL. It has also been shown that the administration of Met in the absence of B12 increases Hcy level to a lesser extent, but it causes significantly greater endothelial lesions [7]. This might mean that Hcy *per se* is not the culprit, and it does not induce oxidative stress itself but the excess of Met inhibits methylation of Hcy and redirects it to NO, forming ADMA. This is supported by the fact that ADMA elevation is observed only in homocysteinemia caused by Met, but not in chronic homocysteinemia [6].

Some authors suggest that cysteine is the one that causes oxidative stress, and has a higher vascular toxicity than Hcy [8]. Others assume that Hcy masks harmful effects of other substances, such as S-adenosylhomocysteine, formed from excess of Hcy, which inhibits methyltransferase and methylenetetrahydrofolate reductase with subsequent deleterious effects [9].

Despite numerous experiments that indicate possible toxicity of Met, primarily through Hcy, severe hyperhomocysteinemia occurs only after application of Met in a dose of 100 mg/kg during one week, which is seven times more than the necessary daily intake of sulfur-containing amino acids [10, 11]. Met is a precursor of glutathione, endogenous antioxidant, so physiological concentrations of Met are required for detoxication in the liver. One study showed that L-Met alone increased the reduction state of glutathione, as well as the total content of this tripeptide [12]. In investigations performed on thioredoxin and glutathione reductase knockout mouses, it has been shown that Met is an alternative fuel for the redox processes in the hepatocytes [13]. Other authors have demonstrated that Met applied during 3.5 days has protective effects against oxidative stress induced by polymyxin B in

rat kidney tubular cells [14]. Met may also directly neutralize reactive oxygen species (ROS) *via* sulfhydryl group [15].

In addition, there are three main enzymes that fight oxidative stress: catalase (CAT), glutathione peroxidase (GPx) and superoxide dismutase (SOD). They together reduce superoxide radicals and hydrogen peroxide to water [16].

Acetylcholinesterase (AChE) is a serine hydrolase that cleaves and inactivates the neurotransmitter acetylcholine (Ach) [17–19]. Although this enzyme is mostly present in the brain and sceletal muscle, it has a significant role in cholinergic system of the heart.[20]

It has been proven that excess of Ach or inadequate functioning of AChE causes hyperactivity of excitable tissues, muscle weakness and acute subjunctional necrosis of muscle fibers, as a consequence of increased levels of $Ca^{2+}$ and generation of ROS [21].

However, connection between AChE activity and oxidative stress is poorly investigated and still unclear. These informations have been accumulated in recent years and interaction between prooxidant and antioxidant moleculs and this enzyme seems to exist [22, 23]. In a very recent study it has been shown that increased ROS production during sepsis can reduce AChE activity in the diaphragms of rats [23].

As Met is an important factor in antioxidant defense, we aimed to examine the effects of acutely and subchronically applied Met on plasma oxidative stress markers and AChE activity in rat cardiac tissue.

## Methods

### *Physiological assay and experimental protocol*

Adult male Wistar albino rats, body weight 250 ± 50 g for acute experiments (n = 16) and around 140 ± 20 g (at the start) for subchronic experiments (n = 20) were used. Rats intended for subchronic experiments were three weeks younger in order to have the same age and approximately the same weight after the three weeks treatment with methionine as the animals from the acute series of experiments, on the day of sacrificing. Animals were raised in strictly controlled conditions (air temperature of 22 ± 1°C, relative humidity 50%, a cycle of brightness: darkness = 12 : 12 h, starting bright period at 8AM), with free access to water and standard food. For acute experiments, the animals were divided into two groups: the control group [0.9% NaCl i.p., pH 7.4; 1 mL/kg i.p.) (n = 8)]; Met group [0.8 mmoL/kg i.p. DL-Met) (n = 8)]. For subchronic experiments, the animals were also divided into two groups, which were given the substance according to the following scheme: the control group [0.9% NaCl, pH 7.4; 0.1–0.2 mL/day i.p., for 3 weeks (n = 10)] and Met group [0.8 mmoL/kg/day i.p. DL-Met, for 3 weeks (n = 10)]. Acute and subchronic experimental protocols were chosen according to literature data [24, 25].

All experimental procedures were done in concordance with Directive of the European Parliament and of the Council (2010/63/EU) and approved by the Ethical Committee of the Faculty of Medicine, University of Belgrade.

### *Tissue and biochemical analyses*

Sixty minutes after administration of tested substances, the rats were anesthetized with ketamine (10 mg/kg) and xylazine (5 mg/kg) and euthanized by decapitation. After sacrificing of rats, venous blood samples were collected for biochemical analyses and hearts were isolated for determination of AChE activity in samples of cardiac tissue homogenate.

In samples of venous blood following biochemical parameteres were measured in the plasma: homocysteine, malondialdehyde (MDA), and activities of enzymes such as catalase (CAT), superoxide dismutase (SOD) and glutathione peroxidase (GPx). These parameters were determined in the control condition, and then in series of experiments.

For determination of AChE activity in samples of cardiac tissue homogenate whole hearts were isolated, rinsed in a phosphate buffer pH 8.0, and homogenized in cold phosphate buffer (pH 8.0). The final tissue concentration was 20 mg tissue per mL of the buffer [26].

### *Determination of plasma homocysteine level*

For this process, blood was collected through a glass funnel and placed in appropriate vacutainers coated by heparin. After the collection, the plasma samples remained at room temperature for 15 minutes and then were centrifuged (15 min × 3000 rpm) and analyzed. At the beginning of and after the experiment, the 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 Hcy < 15 μmol/L. Samples for determination of oxidative stress parameters were frozen (−80°C) until measurement. All measurements were performed on ice.

### *Determination of catalase activity*

Catalase activity was measured by an assay that follows the degradation of $H_2O_2$ [27]. Suspension of plasma (50 μL) was added to the quartz glass cuvette at room temperature, containing 2.975 mL of 50 mM phosphate buffer solution in 0.4 mM EDTA. The enzyme reaction was initiated by adding 30 μL of 3% $H_2O_2$. Reduction in the value of the absorbance due to enzymatic degradation of $H_2O_2$ at 240 nm for 3–5 minutes was monitored. Catalase activity was expressed as U/mL of plasma. One unit (U) of enzyme activity was defined as 1 μmoL of spent $H_2O_2$/min.

### *Determination of glutathione peroxidase activity*

To measure the glutathione peroxidase activity reaction cocktail was prepared as follows: 8.9 mL of phosphate buffer, pH 7 (50 mM $NaH_2PO_4$ + 0.40 mM EDTA), 50 μL of 200 mM reduced glutathione (GSH), 1 mg of β-NADPH, and 100 μL of 100 units/mL glutathione reductase from baker's yeast (*Saccharomyces cerevisiae*). The reaction cocktail (3 mL) and plasma sample (0.3 mL) were added in a quartz

glass cuvette (room temperature). Cuvette was placed in a spectrophotometer, and the enzymatic reaction was started by adding 50 μL of 0.042% $H_2O_2$ ($A_{340}$ = 0.52–0.56). The decline in the value of absorbance (λ = 340 nm) for 15 seconds during 4–5 minutes was monitored. GPx activity was expressed as ΔA/min/mL of plasma [28].

*Determination of superoxide dismutase activity*

Superoxide dismutase activity was measured according to the method of Misra and Fridovich [29]. Plasma sample (10–30 μL) was added to 3 mL of 0.5 M EDTA-sodium carbonate buffer, pH 10.2. The enzymatic reaction was started by the addition of 100 μL of adrenaline (30 mM in 0.1 M HCl) and the activity was measured at 480 nm during 4 minutes. One unit (U) was defined as the amount of enzyme that inhibits the rate of the oxidation of adrenaline by 50%. The enzyme activity was expressed as U/mL of plasma.

*Determination of malondialdehyde*

For the determination of MDA in plasma sample, thiobarbituric assay was used [30]. In 500 μL of plasma sample, 500 μL of 25% HCl and 500 μL of 1% thiobarbituric acid in 50 mM NaOH were added. The mixture was placed in a boiling water bath for 10 minutes, and then cooled to room temperature. N-butanol (3 mL) for extraction was added and shaken in vortex for 30 seconds. For the successful separation of the phases, centrifugation for 10 minutes at 2000 × g in a Sorvall centrifuge was necessary. Content of MDA was determined spectrophotometrically by measuring the absorbance of the organic phase (upper layer) at 532 nm. The blank probes contained 50 mM NaOH instead of thiobarbituric acid, and were prepared for each sample separately. The value of MDA content was expressed as nmol MDA/mL of plasma, and it was based on the measured values of absorbance and molar absorption coefficient of the complex malondialdehyde-thiobarbituric acid.

*Determination of acetylcholinesterase activity in cardiac tissue*

The specific activity of AChE in the cardiac tissue was measured *in vitro* by the Ellman method [31]. The method is based on the reaction of a color reagent 5,5'-dithio-bis-(2-nitrobenzoic acid) – DTNB, with the product of hydrolysis of the thiocholine substrate, acetiltioholine iodide (AChI), thiocholine, to give a yellow-colored compound, 5-thio-2- nitrobenzoate, whose intensity is proportional to the activity of AChE. An appropriate amount of a homogenate of the tested tissue (40 μL of the heart homogenate in 580 μL of phosphate buffer pH 8.0) was preincubated for 10 minutes at a temperature of 37°C. After preincubation, 20 μL of color reagent DTNB and 10 μL of AChI substrate were added. The change in absorbance at 412 nm was measured spectrophotometrically (Gilford Instrument, Model 250) for 3 minutes. The blank probe contained all the components of the assay for following AChE activity, except the tissue homogenate. The measurements were performed in duplicate. Specific enzyme activity of AChE in the heart was expressed as ΔA/min/mg of tissue.

*Chemicals*

All used substances were purchased from Sigma Aldrich (Germany). Substances used in the experiment were *pro* analysis quality.

*Statistical analysis*

Statistical significance of differences in the activity of the enzymes CAT, GPx, SOD, AChE and concentration of MDA between groups was analyzed by Student's *t*-test for independent samples. Statistical data were analyzed by a computer program "R". Values are presented as mean ± standard error of the mean (SEM). $P < 0.05$ was considered statistically significant.

## Results

*Determination of total plasma homocysteine level*

The homocysteine values in the subchronically methionine treated group were non-significantly different in relation to those in the control group (9.51 ± 0.59 μmol/L vs. 9.98 ± 0.65 μmol/L, respectively).

*Plasma catalase activity*

Acutely applied Met induced significant increase in CAT activity (78.37 ± 7.79 U/mL) compared to the control group (47.85 ± 4.78 U/mL). Enzyme activity was not changed after 3 weeks of Met administration (163.53 ± 21.33 U/mL) in comparison to the control (132.22 ± 10.37 U/mL) (Figure 1).

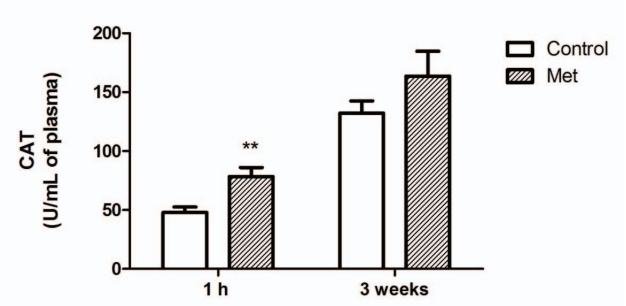


**Fig. 1 – The effects of methionine (Met) on catalase (CAT) activity after acute (1 h) and subchronic (3 weeks) application in the rat plasma. Values are presented as mean ± SEM. **$p < 0.01$ compared to the control group.**

*Plasma glutathione peroxidase activity*

The specific activity of GPx in the plasma of rats given physiological solution was 3.21 ± 0.33 U/mL, whereas in the

experimental group was 5.23 ± 0.510 U/mL suggesting that DL-Met increase the activity of this enzyme after 1 h. However, GPx activity remained unchanged after 3 weeks of Met application (10.90 ± 1.41 U/mL vs. 8.81 ± 0.69 U/mL in the control group) (Figure 2).

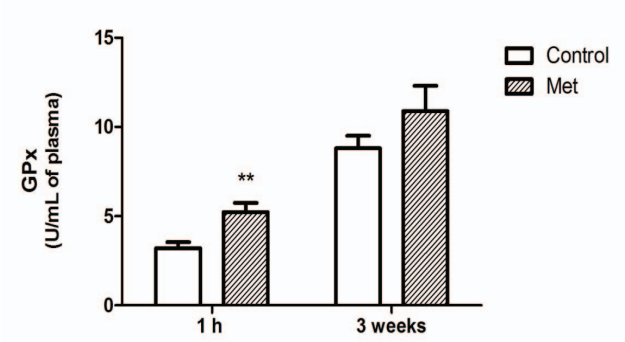


**Fig. 2 – The effects of methionine (Met) on glutathione peroxidase (GPx) activity after acute (1 h) and subchronic (3 weeks) application in the rat plasma. Values are presented as mean ± SEM. **$p < 0.01$ compared to the control group.**

*Plasma superoxide dismutase activity*

There was no statistically significant changes in SOD activity in the experimental group (23.47 ± 0.77 U/mL) compared to the control one (24.01 ± 0.84 U/mL) 1 h after Met administration. Also, there was no statistically significant difference in SOD activity between groups in the subchronic experiment. SOD activity was 29.92 ± 0.44 U/mL in the Met treated group and 30.09 ± 0.82 U/mL in the control group (Figure 3).

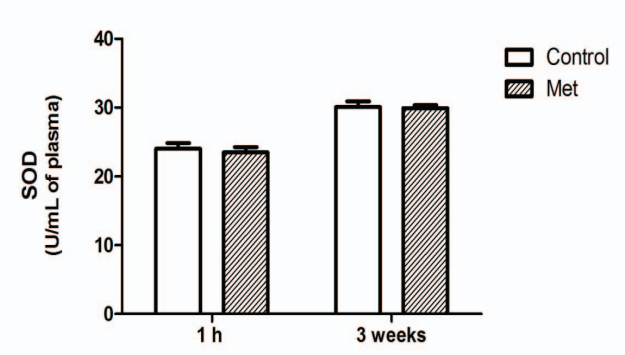


**Fig. 3 – The effects of methionine (Met) on superoxide dismutase (SOD) activity after acute (1 h) and subchronic (3 weeks) application in the rat plasma. Values are presented as mean ± SEM.**

*Plasma malondialdehyde concentration*

MDA concentration was not changed either after acutely or subchronically application of Met in relation to the control. After 1 h it was 4.77 ± 0.80 nmol/mL in the experimental group and 4.87 ± 0.43 nmol/mL in the control one, whereas after 3 weeks it was 15.03 ± 1.39 nmol/mL in the experimental group and 12.56 ± 1.38 nmol/mL in the control group (Figure 4).

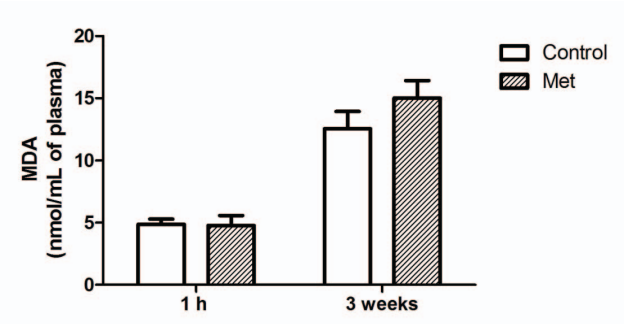


**Fig. 4 – The effects of methionine (Met) on malondialdehyde (MDA) concentration after acute (1 h) and subchronic (3 weeks) application in the rat plasma. Values are presented as mean ± SEM.**

*Cardiac tissue homognate acetylcholinesterase acitivity*

The acute application of Met did not induce change in AChE activity in the rat cardiac tissue homogenate (0.043 ± 0.001 ΔA/min/mg of tissue) vs. control (0.049 ± 0.002 ΔA/min/mg of tissue). However, subchronically applied, Met caused the significant decrease of the enzyme activity in the cardiac tissue homogenates of treated group (0.046 ± 0.004 ΔA/min/mg of tissue) in comparison to the control group (0.057 ± 0.002 ΔA/min/mg of tissue) (Figure 5).

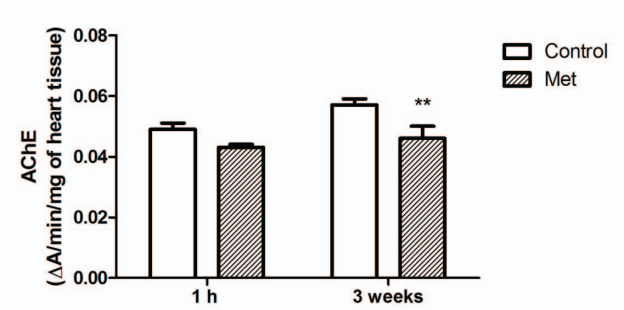


**Fig. 5 – The effects of methionine (Met) on acetylcholinesterase (AChE) activity after acute (1 h) and subchronic (3 weeks) application in the cardiac tissue of rats. Values are presented as mean ± SEM. **$p < 0.01$ compared to the control group.**

*Correlation analysis*

A positive correlation was noticed between following values/groups: CAT and SOD values in the control group from the acute experiment (Table 1); Hcy values from the acute experiment and GPx and MDA values from the subchronic experiment in the control group; CAT values in subchronically treated Met group and SOD values in the control group from subchronic experiment; GPx values and MDA values in the control group from the subchronic experiment; SOD values in the control group and CAT values in the subchronically treated Met group in the subchronic experiment (Table 2); CAT values in the control group from the acute experiment and CAT values in the subchronically Met treated group from the subchronic experiment; CAT values in the control group from the acute experiment and SOD values in the control group from the subchronic experiment;

CAT values in the acutely treated Met group and CAT values in the control group from the subchronic experiment; SOD values in the control group from the acute experiment and CAT values in the subchronically Met treated group from the subchronic experiment; SOD values in the control groups from the acute and subchronic experiment (Table 3).

**Table 1**
**Correlation matrix between hemocysteine (Hcy) and parameters of oxidative stress in acutely methionine treated rats**

| Parameters | Hcy-C | Hcy-M | CAT-C | CAT-M | GPx-C | GPx-M | MDA-C | MDA-M | SOD-C | SOD-M |
|---|---|---|---|---|---|---|---|---|---|---|
| Hcy-C | / | r = -0.34 | r = -0.60 | r = 0.60 | r = 0.24 | r = 0.24 | r = -0.45 | **r = -0.84** | r = -0.60 | r = 0.46 |
| | | *p* = 0.51 | *p* = 0.20 | *p* = 0.08 | *p* = 0.64 | *p* = 64 | *p* = 0.65 | ***p* = 0.04** | *p* = 0.20 | *p* = 0.36 |
| Hcy-M | r = -0.34 | / | r = -0.41 | r = -0.62 | r = -0.13 | r = 0.49 | r = -0.21 | r = 0.65 | r = -0.41 | r = -0.72 |
| | *p* = 0.51 | | *p* = 0.41 | *p* = 0.19 | *p* = 0.80 | *p* = 0.32 | *p* = 0.69 | *p* = 0.16 | *p* = 0.41 | *p* = 0.10 |
| CAT-C | r = -0.60 | r = -0.41 | / | r = -0.20 | r = 0.00 | r = -0.63 | r = 0.60 | r = 0.32 | **r = 1.00** | r = 0.2 |
| | *p* = 0.20 | *p* = 0.41 | | *p* = 0.70 | *p* = 1.00 | *p* = 0.18 | *p* = 0.21 | *p* = 0.54 | ***p* = 0.000** | *p* = 0.70 |
| CAT-M | r = 0.76 | r = -0.62 | r = -0.20 | / | r = 0.00 | r = 0.32 | r = -0.6 | r = -0.63 | r = -0.20 | r = 0.40 |
| | *p* = 0.08 | *p* = 0.19 | *p* = 0.70 | | *p* = 1.00 | *p* = 0.54 | *p* = 0.21 | *p* = 0.18 | *p* = 0.70 | *p* = 0.43 |
| GPx-C | r = 0.24 | r = -0.13 | r = 0.00 | r = 0.00 | / | r = -0.46 | r = 0.38 | r = -0.46 | r = 0.00 | r = 0.77 |
| | *p* = 0.64 | *p* = 0.80 | *p* = 1.00 | *p* = 1.00 | | *p* = 0.36 | *p* = 0.45 | *p* = 0.36 | *p* = 1.00 | *p* = 0.07 |
| GPx-M | r = 0.24 | r = 0.49 | r = -0.63 | r = 0.32 | r = -0.46 | / | **r = -0.95** | r = 0.25 | r = -0.63 | r = -0.63 |
| | *p* = 0.64 | *p* = 0.32 | *p* = 0.18 | *p* = 0.54 | *p* = 0.36 | | ***p* = 0.004** | *p* = 0.63 | *p* = 0.18 | *p* = 0.18 |
| MDA-C | r = -0.46 | r = -0.21 | r = 0.60 | r = 0.60 | r = 0.38 | **r = -0.95** | / | r = 0.00 | r = 0.60 | r = 0.40 |
| | *p* = 0.36 | *p* = 0.69 | *p* = 0.21 | *p* = 0.21 | *p* = 0.45 | ***p* = 0.004** | | *p* = 1.00 | *p* = 0.21 | *p* = 0.43 |
| MDA-M | **r = -0.84** | r = 0.65 | r = 0.32 | r = -0.63 | r = -0.46 | r = 0.25 | r = 0.00 | / | r = 0.32 | r = -0.79 |
| | ***p* = 0.04** | *p* = 0.16 | *p* = 0.54 | *p* = 0.18 | *p* = 0.36 | *p* = 0.63 | *p* = 1.00 | | *p* = 0.54 | *p* = 0.06 |
| SOD-C | r = -0.61 | r = -0.41 | r=-1.000 | r = -0.20 | r = 0.00 | r = -0.63 | r = 0.6 | r = 0.32 | / | r = 0.2 |
| | *p* = 0.20 | *p* = 0.41 | *p* = 0.00 | *p* = 0.70 | *p* = 1.00 | *p* = 0.18 | *p* = 0.21 | *p* = 0.54 | | *p* = 0.70 |
| SOD-M | r = 0.46 | r = -0.72 | r = 0.2 | r = 0.40 | r = 0.77 | r = -0.63 | r = 0.40 | r = -0.79 | r = 0.2 | / |
| | *p* = 0.36 | *p* = 0.10 | *p* = 0.70 | *p* = 0.43 | *p* = 0.07 | *p* = 0.18 | *p* = 0.43 | *p* = 0.06 | *p* = 0.70 | |

**C – control goup; M – methionine trated group.**
**CAT – catalase; GPx – glutathione peroxidase; MDA – malondialdehyde; SOD – superoxide dismutase.**
**Pearson correlation coefficient (r): low or no correlation $0 \leq r \leq 0.3$; moderate correlation $0.3 \leq r \leq 0.7$; strong correlation $0.7 \leq r < 1$ (- indicates negative correlation); *p* value less than 0.05 was considered as significant (statistically significant differences are bolded).**

**Table 2**
**Correlation matrix between homocysteine (Hcy) and parameters of oxidative stress in subchronically methionine treated rats**

| Parameters | CAT-C3 | CAT-M3 | GPx-C3 | GPx-M3 | MDA-C3 | MDA-M3 | SOD-C3 | SOD-M3 |
|---|---|---|---|---|---|---|---|---|
| Hcy-C | r = 0.72 | r = 0.36 | **r = 0.84** | r = 0.49 | **r = 0.54** | r = 0.34 | r = -0.36 | r = 0.00 |
| | p=0.10 | *p* = 0.48 | ***p* = 0.04** | *p* = 0.32 | **p=0.04** | *p* = 0.51 | *p* = 0.51 | *p* = 1.00 |
| Hcy-M | **r = -0.82** | r = -0.65 | r = -0.65 | r = 0.58 | r = -0.65 | **r = -0.93** | r = 0.65 | r = 0.16 |
| | ***p* = 0.047** | *p* = 0.16 | *p* = 0.16 | *p* = 0.22 | *p* = 0.16 | ***p* = 0.008** | *p* = 0.16 | *p* = 0.76 |
| CAT-C3 | / | r = 0.25 | r = -0.75 | r = -0.19 | r = 0.75 | r = 0.71 | r = 0.25 | r = 0.00 |
| | | *p* = 0.63 | *p* = 0.09 | *p* = 0.72 | *p* = 0.09 | *p* = 0.12 | *p* = 0.63 | *p* = 1.00 |
| CAT-M3 | r = 0.25 | / | r = 0.00 | **r = -0.96** | r = 0.00 | r = 0.71 | **r = 1.00** | r = 0.25 |
| | *p* = 0.63 | | *p* = 1.00 | ***p* = 0.003** | *p* = 1.00 | *p* = 0.12 | ***p* = 0.000** | *p* = 0.63 |
| GPx-C3 | r = -0.75 | r = 0.00 | / | r = 0.19 | r = **1.00** | r = 0.71 | r = 0.00 | r = -0.25 |
| | *p* = 0.09 | *p* = 1.00 | | *p* = 0.72 | ***p* = 0.000** | *p* = 0.12 | *p* = 1.00 | *p* = 0.63 |
| GPx-M3 | r = -0.19 | **r = -0.96** | r = 0.19 | / | r = -0.19 | r = -0.54 | r = **-0.96** | r = -0.19 |
| | *p* = 0.72 | ***p* = 0.003** | *p* = 0.72 | | *p* = 0.72 | *p* = 0.27 | ***p* = 0.003** | *p* = 0.72 |
| MDA-C3 | r = -0.75 | r = 0.00 | **r = 1.00** | r = 0.19 | / | r = 0.71 | r = 0.00 | r = -0.25 |
| | *p* = 0.09 | *p* = 1.00 | ***p* = 0.000** | *p* = 0.72 | | *p* = 0.12 | *p* = 1.00 | *p* = 0.63 |
| MDA-M3 | r = -0.71 | r = -0.71 | r = -0.71 | r = -0.54 | r = -0.71 | / | r = -0.71 | r = 0.00 |
| | *p* = 0.12 | *p* = 0.12 | *p* = 0.12 | *p* = 0.27 | *p* = 0.12 | | *p* = 0.12 | *p* = 1.00 |
| SOD-C3 | r = -0.25 | **r = 1.00** | r = 0.00 | **r = -0.96** | r = 0.00 | r = -0.71 | / | r = 0.25 |
| | *p* = 0.63 | ***p* = 0.000** | *p* = 1.00 | ***p* = 0.003** | *p* = 1.00 | *p* = 0.12 | | *p* = 0.63 |
| SOD-M3 | r = 0.00 | r = 0.25 | r = -0.25 | r = -0.19 | r = -0.25 | r = 0.00 | r = 0.25 | / |
| | *p* = 1.00 | *p* = 0.63 | *p* = 0.63 | *p* = 0.72 | *p* = 0.63 | *p* = 1.00 | *p* = 0.63 | |

**C – control group and M – methionine treated group in the acute experiment; C3 – control group and M3 – methionine treated group in the subchronic experiment.**
**CAT – catalase; GPx – glutathione peroxidase; MDA – malondialdehyde; SOD – superoxide dismutase.**
**Pearson correlation coefficient (r): Low or no correlation $0 \leq r \leq 0.3$; moderate correlation $0.3 \leq r \leq 0.7$; strong correlation $0.7 \leq r < 1$ (- indicates negative correlation); *p* – value less than 0.05 was considered as significant (statistically significant differences are bolded).**

**Table 3**
**Correlation matrix for parameters of oxidative stress between acutely and subchonically methionine treated rats**

| Parameters | CAT-C3 | CAT-M3 | GPx-C3 | GPx-M3 | MDA-C3 | MDA-M3 | SOD-C3 | SOD-M3 |
|---|---|---|---|---|---|---|---|---|
| CAT-C | r = 0.00 | **r = 0.95** | r = -0.32 | **r = -0.97** | r = 0.32 | r = 0.45 | **r = 0.95** | r = 0.32 |
| | *p* = 1.00 | ***p* = 0.004** | *p* = 0.54 | ***p* = 0.002** | *p* = 0.54 | *p* = 0.37 | ***p* = 0.004** | *p* = 0.54 |
| CAT-M | **r = 0.95** | r = 0.00 | r = -0.63 | r = 0.00 | r = -0.63 | r = 0.45 | r = 0.00 | r = 0.00 |
| | ***p* = 0.004** | *p* = 1.00 | *p* = 0.18 | *p* = 1.00 | *p* = 0.18 | *p* = 0.37 | *p* = 1.00 | *p* = 1.00 |
| GPx-C | r = 0.15 | r = 0.15 | r = 0.46 | r = 0.10 | r = 0.46 | r = 0.43 | r = 0.15 | r = 0.15 |
| | *p* = 0.74 | *p* = 0.74 | *p* = 0.36 | *p* = 0.86 | *p* = 0.36 | *p* = 0.40 | *p* = 0.77 | *p* = 0.77 |
| GPx-M | r = 0.00 | r = -0.75 | r = 0.25 | r = 0.57 | r = 0.25 | r = -0.71 | r = -0.75 | r = 0.00 |
| | *p* = 1.00 | *p* = 0.09 | *p* = 0.63 | *p* = 0.23 | *p* = 0.63 | *p* = 0.12 | *p* = 0.09 | *p* = 1.00 |
| MDA-C | r = -0.32 | r = 0.63 | r = 0.00 | r = -0.48 | r = 0.00 | r = 0.45 | r = 0.63 | r = 0.00 |
| | *p* = 0.54 | *p* = 0.18 | *p* = 1.00 | *p* = 0.33 | *p* = 1.00 | *p* = 0.37 | *p* = 0.18 | *p* = 1.00 |
| MDA-M | r = -0.75 | r = 0.00 | r = -1,000 | r = -0.19 | r = -1,000 | r = -0.71 | r = 0.00 | r = 0.25 |
| | *p* = 0.09 | *p* = 1.00 | *p* = 0.00 | *p* = 0.72 | *p* = 0.00 | *p* = 0.12 | *p* = 1.00 | *p* = 0.63 |
| SOD-C | r = 0.00 | **r = 0.95** | r = -0.32 | r = -0.97 | r = -0.32 | r = 0.45 | **r = 0.95** | r = 0.32 |
| | *p* = 1.00 | ***p* = 0.004** | *p* = 0.54 | *p* = 0.002 | *p* = 0.54 | *p* = 0.37 | ***p* = 0.004** | *p* = 0.54 |
| SOD-M | r = 0.63 | r = 0.47 | r = 0.79 | r = -0.24 | r = 0.79 | r = 0.89 | r = 0.47 | r = 0.00 |
| | *p* = 0.18 | *p* = 0.34 | *p* = 0.06 | *p* = 0.64 | *p* = 0.06 | *p* = 0.02 | *p* = 0.34 | *p* = 1.00 |

**C – control group and M – methionine treated group in the acute experiment; C3 – control group and M3 – methionine treated group in the subchronic experiment.**
**CAT – catalase; GPx – glutathione peroxidase; MDA – malondialdehyde; SOD – superoxide dismutase.**
**Pearson correlation coefficient (r): Low or no correlation $0 \leq r \leq 0.3$; moderate correlation $0.3 \leq r \leq 0.7$; strong correlation $0.7 \leq r < 1$ (- indicates negative correlation); *p* – value less than 0.05 was considered as significant (statistically significant differences are bolded).**

## Discussion

Present investigation aimed to assess the influence of acute and subchronic Met treatment on plasma oxidative stress markers and AChE activity in rat cardiac tissue.

Absence of increase in Hcy values in the group subchronically treated with Met may be consequence of duration of methionine loading and/or applied dose. In our previous study we showed that rats treated for 4 weeks with diets enriched in methionine (with or without deficiency in B vitamins) had increased Hcy levels especially in conditions of deficit in vitamin B complex [32]. Furthermore, some human studies pointed out that methionine overload can increase Hcy levels in only 33% of cases [33]. In that sense it is possible that time of exposure to methionine and its concentration were insufficient to cause elevation in Hcy levels in the present study.

In this study it was found that acutely applied Met increased activities of CAT and GPx, whereas did not significantly altered activity of SOD and MDA level in the rat plasma after 1 h.

The liver is particularly sensitive to prolonged administration of Met, and some authors claim that after chronic application, level of MDA in the liver is increased, which could be due to increased levels of iron [34, 35]. However, other experiments have shown that after 1 h MDA in the liver is significantly decreased [36]. The same authors have shown that after 1 h, CAT activity in the liver is increased *in vitro* and decreased *in vivo*, which means that there is a possibility that CAT is released from the liver and its activity could be increased in plasma. Previously may explain the increase in CAT activity in the plasma observed in our study. We obtained that SOD activity in the plasma was unchanged. This result is in agreement with the results of Costa et al. [36]. Some authors suggest that 2–3 h after the application of Met concentration of MDA in plasma is not changed and that is noticed only after 8 h, which corresponds to a maximum concentration of Hcy [37].

A lot of research has been done in order to investigate the origin and mechanism of Hcy toxicity, as well as the connection between Met and Hcy on one side and oxidative stress and vascular diseases on the other side.

In one study it is shown that Met has a protective effect in atherosclerotic lesions by increasing the activity of antioxidant enzymes in the heart up to 24 h from the application and then this effect begin to decrease [38]. It is also shown that Met increases GPx activity at the level of mRNA, while the activities of CAT and SOD are regulated by post-transcriptional or post-translational modification. GPx and CAT were significantly increased, and SOD was unchanged, which is consistent with our results. In that study, it was demonstrated that a significant reduction of MDA in the heart coincides with a maximum of GPx activity after 24 h.[38] In another study it was shown that chronic application of Met caused an increase of MDA level and GPx activity in the heart as a response to increased level of ROS (due to elevated Hcy), not as a direct effect of Met [39].

Tests conducted on the rat hippocampus showed no change in the level of MDA 1 h after giving Met [40], which is in accordance with the obtained values in the plasma in our study. On the other hand, the same study indicates an increase in MDA in hippocampus after 3 h [40], which may be the result of increased concentrations of iron, lipid content and low activity of antioxidants in basal conditions [41]. Other autors demonstrated that acutely given Met increased SOD and GPx activity 1h after application, but decreased CAT activity in the rat brain cortex [42]. In another experiment SOD activity in the rat brain remained unchanged, which is consistent with the results obtained in the heart in our study.

No data were found in the literature about the impact of Met on the AChE activity in the heart tissue. However, our recent published study investigated the effects of Hcy or Hcy-thiolactone on the plasma oxidative stress and AChE activity in the rat cardiac tissue 1 h after application [24]. Statistically significant reduction of the AChE activity was found, and the same was observed in this study after subchronic Met overload, contrary to the acute treatment that did not give any change. Although stress-induced increased sympathetic action is inevitable and can cause a weaker function of AChE, there is a significant activity of the enzyme in most of the sympathetic ganglions in the rat (including, among others, the heart). On this way, sympathetic preoccupation may also be associated with an increased activity of AChE [43].

Moreover, it is known that excess of AchE has protective effects in the heart only in conditions related to the oxidative stress such as inflammation, hypoxemia, ischemia [44–46]. Reduction of AChE in these cases could be a compensatory mechanism. Taking into consideration that subchronically given Met did not induce oxidative stress, but can induce increase of CRP level [47], we were not able to assume that reduction of the enzyme activity was a direct effect of Met. However, Met could also provoke oxidative stress in the heart *via* high level of AChE. Because of that, further studies are needed to differentiate whether this is consequence of elevated Met, Hcy or some other products levels obtained from Met cycle after 3 weeks. Besides, some authors claimed that maximum concentration of Met in the plasma was achieved after 15 min of application [4], and it would be also preferable to examine such effects.

## Conclusion

The present study showed that acutely applied Met has some beneficial effects; it protects from oxidative stress, through increasing activities of two antioxidant enzymes in the plasma – CAT and GPx. Nevertheless, after 3 weeks of the treatment these changes seems to be lost. On the other hand, given in both manners, acutely and subchronically, Met did not influence on lipid peroxidation process.

Acute Met overload was not sufficient to influence on activity of AchE in the rat heart, while longer duration of Met loading diminished function of the enzyme. These findings point out that Met can interfere with antioxidant defense system and cholinergic control of the heart function. More detailed examinations are needed to determine the effects of this amino acid and its possible therapeutic options.

## Acknowledgements

This work is supported by the grant No.175043 from the Ministry of Education, Science and Technological Development of the Republic of Serbia.

## Conflict of interest

None.

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Received on December 13, 2017.
Revised on February 27, 2018.
Accepted on March 26, 2018.
Online First April, 2018.