

## ORIGINAL ARTICLE

# The influence of vitamin B6 on cardiac oxidative stress, cardiometabolic and histological markers in monocrotaline-induced heart failure in wistar albino rats

✉ Jovana Jakovljevic Uzelac<sup>1</sup>, Dusan Todorovic<sup>1</sup>, Tatjana Djukic<sup>2</sup>, Slavica Mutavdzin<sup>1</sup>, Sanja Stankovic<sup>3</sup>, Milica Labudovic Borovic<sup>4</sup>, Jelena Rakocevic<sup>4</sup>, Natasa Milic<sup>5</sup>, Irina Nenadic<sup>6</sup>, Marko Djuric<sup>6</sup>, Ana Savic-Radojevic<sup>2</sup>

<sup>1</sup> University of Belgrade, Faculty of Medicine, Institute of Medical Physiology “Rihard Burian”, Belgrade, Serbia

<sup>2</sup> University of Belgrade, Faculty of Medicine, Institute of Medical and Clinical Biochemistry, Belgrade, Serbia

<sup>3</sup> Center for Medical Biochemistry, University Clinical Centre of Serbia, Belgrade, Serbia

<sup>4</sup> University of Belgrade, Faculty of Medicine, Institute of Histology and Embryology “Aleksandar Dj. Kostic”, Belgrade, Serbia

<sup>5</sup> University of Belgrade, Faculty of Medicine, Institute of Medical Statistics and Informatics, Belgrade, Serbia

<sup>6</sup> Clinic for Anesthesiology and Intensive Care, University Clinical Hospital Center “Dr Dragisa Misovic - Dedinje”, Belgrade, Serbia

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## Competing interests:

The authors have declared that no competing interests exist

## ✉ Correspondence to:

Jovana Jakovljevic Uzelac  
 Institute of Medical Physiology “Richard Burian”  
 26/II, Visegradska 26/II Street, 11000 Belgrade, Serbia  
 Email: jovanavjakovljevic@gmail.com

## Summary

**Introduction/Aim:** Heart failure (HF) induced by monocrotaline (MCT) is common in the pulmonary arterial vessels remodeling mechanisms with increased pulmonary resistance and oxidative stress markers. The purpose of this study was to validate the hypothesis that the treatment with vitamin B6 could affect HF by modulating cardiometabolic and oxidative stress biomarkers, and the structure of the rat heart.

**Material and Methods:** Male Wistar albino rats were divided into 3 groups: blank solution-exposed control (C physiological saline 1ml/kg 28 days ip., n=8), B6 (vitamin B6 7mg/kg/day 28 days ip., n=8), and MCT+B6 (MCT 50mg/kg once ip. plus vitamin B6 7mg/kg/day 28 days ip., n=8).

**Results:** The four-week vitamin B6 treatment significantly affected certain biochemical parameters. The activity of key antioxidant enzymes, superoxide dismutase (SOD) and glutathione peroxidase (GPX) did not change, whereas the total glutathione (GSH) was significantly decreased in the MCT+B6 group. This was followed by a slightly decreased level of the total glutathionylation observed in the MCT+B6 group. The parameters of protein oxidative damage (reactive carbonyl derivatives, thiol groups and nitrotyrosine) did not significantly change in the MCT+B6 group. An increasing trend in RV and LV wall thickness was observed in the MCT+B6 compared to the C group, as well as in Ki67 and PCNA positivity.

**Conclusion:** The four-week treatment with vitamin B6 significantly affected certain biomarkers. The activity of SOD and nitrotyrosine content did not change, while GPX activity, total glutathione and total glutathionylation level were decreased in the MCT+B6 group. We observed an increase in RV and LV wall thickness in the MCT+B6 group compared to the C group, as well as in Ki67 and PCNA positivity.

**Key words:** heart failure, monocrotaline, vitamin B6, oxidative stress, rats

## INTRODUCTION

Vitamin B6 in its active form, pyridoxal 5'-phosphate (PLP) is a cofactor of a variety of enzymes which are necessary for metabolism of amino acids, lipids and carbohydrates (1), but it also has an important role as co-factor in many inflammatory pathways (2). Vitamin B6 serves as a co-factor for two main enzymes in transsulfuration metabolic pathway of homocysteine, cystathionine  $\beta$ -synthase and cystathionine  $\gamma$ -lyase (3). This pathway is important for synthesis of hydrogen sulfide ( $H_2S$ ) and cysteine which is the key amino acid for the function of tripeptide glutathione (GSH) (4). GSH is an important intracellular antioxidant protecting cells from reactive oxygen species (ROS) and it also takes part in the regulation of inflammatory response by mediating in cytokine synthesis and the activation of transcription factors, like nuclear factor-kappa B (NF- $\kappa$ B) and hypoxia-inducible factor-1 $\alpha$  (HIF-1 $\alpha$ ) (5). In addition, the relevance of  $H_2S$  in blood pressure modulation, glucose uptake and metabolism of cardiomyocytes has emerged recently (6). Indeed, decreased  $H_2S$  levels have been found in patients with congestive heart failure (7). It has been proposed that beneficial effects of vitamin B6 on cardiovascular system are mediated by reducing inflammation and the degree of oxidative stress (8). Low vitamin B6 levels may interrupt the metabolism of homocysteine and lead to hyperhomocysteinemia which is a risk factor for coronary artery disease (9). A study on experimental animals by Mahfouz et al. (10) showed that a treatment with vitamin B6 and vitamin C achieved antioxidant effects in rats with experimentally developed hyperhomocysteinemia and the maintained ability of their aortas to produce prostacyclin at a normal rate. Deficiency of vitamin B6 is reflected in an increase of lipid peroxidation and a reduction of antioxidant defense systems, which can be in correlation with atherogenesis (11, 12). Vitamin B6 deficiency is really rare in developed countries, however low levels of vitamin B6 have been detected in patients suffering from alcoholism, diabetes, in women using oral contraceptives, and in smokers (13).

HF as one of the leading mortality causes worldwide, is accompanied with complex metabolic changes, including oxidative stress, inflammation, fibrosis, angiogenesis, and apoptosis. Valid HF experimental method (via pulmonary arterial hypertension-PAH) is achieved by MCT application. Obstruction of pulmonary blood vessels increased the afterload of the right ventricle leading to its malfunction and failure (14). Lung toxicity of MCT is caused by its effects on nitric oxide metabolism, antiapoptotic, and proliferative factors and membrane proteins (15). MCT affects the nuclear factor-E2-related factor (Nrf2) pathway leading to an increased caspase three activation, oxidative damage and inflammation (16). In an already failing heart, increased production of ROS could lead to ad-

verse myocardial remodeling accompanied by HF progression (17).

Taking all these effects into consideration, the aim of this study was to investigate if a treatment with vitamin B6 could modify biochemical, oxidative stress, histomorphometric and immunohistochemical parameters, as well as the damage of rat heart tissue in a model of the right ventricle failure developed by intraperitoneal monocrotaline application.

## MATERIAL AND METHODS

### Animal ethics report

We treated animals in compliance with the Guide for the Care and Use of Laboratory Animals (8th ed., National Academies Press) and the European Directive for the Welfare of Laboratory Animals (No. 2010/63/EU). Our research has been credited by the Ethical Council for the Welfare of Experimental Animals, Ministry of Agriculture, Forestry and Water Management, Veterinary Directorate, Republic of Serbia (No. 323-07-01339/2017-05/2).

### Experimental animals

Male Wistar albino rats were used in our study, with body mass around 160 g and age 25–30 days at the beginning of the experiment. The experimental animals were placed in transparent Plexiglas cages with a wood-chip floor, in pairs per each cage. Basic physiological needs, such as food and water, were accessible ad libitum. The ambient conditions such as 12 h light–dark cycle (the light period beginning at 7:30 a.m.), temperature ( $21 \pm 2$  °C), and humidity ( $55\% \pm 5\%$ ) were constant.

### Protocol of the experiment

A single intraperitoneal (ip.) application of MCT (50 mg/kg body mass) was used for inducing HF, and the induced HF animal model was verified earlier (18). The experimental period lasted for 28 days. The experimental animals were split into 3 groups: blank solution-exposed control (C physiological saline 1ml/kg 28 days ip., n=8), B6 (vitamin B6 7mg/kg/day 28 days ip., n=8), and MCT+B6 (MCT 50mg/kg once ip. plus vitamin B6 7mg/kg/day 28 days ip, n=8). The animals were euthanized after the experimental period. Heart tissue and blood samples of all animals were gathered for particular analysis (biochemical, histomorphometric and immunohistochemical).

### Biochemical analysis

Competitive immunoassay using direct, chemiluminescent technology on an ADVIA Centaur XP system (Siemens Healthcare Diagnostics, Tarrytown, New York,

USA) was used for serum homocysteine (Hcy) measuring. Glucose, urea (UREA) and creatinine (CREA) levels, the lipid profile parameters (total cholesterol (TC), high-density lipoprotein cholesterol (HDL-C), triglycerides (TG)), total proteins (TP), albumin (ALB), marker of liver function (aspartate aminotransferase (AST), alanine aminotransferase (ALT)),  $\alpha$ -amylase ( $\alpha$ -AMY), and alkaline phosphatase (ALP)) were determined by using spectrophotometry commercial kits (Siemens Healthcare Diagnostics Inc., Newark, New Jersey, USA) on an automatic analyzer (Dimension Xpand, Siemens). Friedewald's equation was used for the estimation of LDL-C level. Commercial test based on a particle enhanced turbidimetric immunoassay technique on a Dimension Xpand analyzer (Siemens) was used for measuring the serum C-reactive protein (CRP) concentration. The levels of cardiac troponin T (hs cTnT) were determined with a highly sensitive assay using the Roche Cobas e601 automated analyzer (Roche Diagnostics, Mannheim, Germany). The automated electrochemiluminescence immunoassay on a Cobas E601 analyzer (Roche Diagnostics) was used for measuring the concentration of interleukin (IL)-6. The concentration of fibrinogen was measured by the modified Clauss assay (Siemens Healthineers Headquarters, Erlangen, Germany) and the activity of von Willebrand factor (vWF) was determined by particle enhanced assay INNOVANCE® VWF Ac using a BCS XP analyzer (Siemens Healthineers Headquarters).

### Oxidative stress analysis

Uric acid (UA) serum levels were measured spectrophotometrically on an automatic biochemical analyzer (Dimension Xpand, Siemens) using commercial kits (Siemens Healthcare Diagnostics Ltd., Frimley, Camberley, UK). Cardiac tissue homogenate was used for measuring other oxidative stress parameters. The heart was washed with saline (0.9% NaCl) after isolation, and then dried on filter paper. Homogenization was carried out in 50 mmol/L RIPA buffer (radioimmunoprecipitation assay), pH 7.4, with protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO, USA), and it was centrifuged at 14,000 rpm for 30 min. Up to the moment of specific analyses, the cardiac tissue was frozen at  $-80^{\circ}\text{C}$ . The activity of superoxide dismutase (SOD) was measured spectrophotometrically (19), and the activity of glutathione peroxidase (GPX) was measured by the coupled assay procedure (20). One unit of enzyme activity is presented as millimoles of NADPH oxidized per minute, assuming  $6.22 \times 10^3 \cdot \text{L}^{-1} \cdot \text{mol}^{-1} \cdot \text{cm}^{-1}$  to be the molar extinction coefficient value of NADPH at 340 nm. The content of thiol (P-SH) groups was appointed according to the method of (21) and presented as micromoles per gram of protein. Total glutathione (GSH) was measured spectrophotometrically and presented as nanomoles per

milligram of protein (22). The content of reactive carbonyl derivatives (RCD) was measured according to the method of Levine et al. (23). The level of protein oxidation was supervised by determination of a classic carbonyl reagent, 2,4-dinitrophenylhydrazine. Spectrophotometric measurement of RCD content was presented as micromoles per gram of protein. The nitrotyrosine content in rat heart tissue was determined by an ELISA test. Standard curves (24) were used for the nitrotyrosine content determination and the results were presented as nanomoles per litre. Bicinchoninic Acid Protein Assay kit (BCA-1) (Sigma-Aldrich) was used for determination of protein concentration.

### Determination of the total S-glutathionylation

Heart tissues were homogenized in radioimmunoprecipitation assay buffer supplemented with protease cocktail inhibitor and N-ethylmaleimide for measuring the total S-glutathionylation levels. Identical amounts of proteins (40 g) from the purified homogenate were separated by sodium dodecyl sulfate – polyacrylamide gel electrophoresis on Criterion™ TGX precast gels (4%–15%) (Bio-Rad, USA) under non-reducing conditions. The transfer of proteins is implemented to a nitrocellulose membrane. After that, immunodetection was determined by using a primary mouse monoclonal anti-glutathione antibody (Abcam, Cambridge, UK) followed by incubation with goat anti-mouse antibody (Dako, Glostrup, Denmark). Chemiluminescent detection system (kit ECL Western blotting analysis system, GE Healthcare, Amersham) was used for visualization performing. ImageLab software (Bio-Rad) was used for densitometric analysis.

### Histomorphometric and immunohistochemical analysis

Heart tissue was regularly oriented and transversely cut in 3 mm sections. The treated tissues were then set up in 4% neutral buffered formaldehyde, and fixed by the immersion procedure for 24 h. In the next step, it was inserted in paraffin, cut in 5 mm serial sections until the complete wall thickness of heart appeared. After all, the tissue was stained with hematoxylin and eosin. Ten measurements per heart were made from the endocardium to epicardium of the left ventricle (LV), right ventricle (RV) free wall thickness, and interventricular septum (IVS) thickness. The average dimension of a cardiomyocytes was calculated by measuring the diameter of 100 cardiomyocytes (at the nucleus level) of the LV free wall. Olympus BX 41 microscope was used for analyzing all slides. All slides were photographed with an Olympus C-5060 wide zoom digital camera and Olympus DP-soft Image Analyzer program. All the values were presented in micrometres. All tissue specimens were immunohistochemically stained using anti-Ki67 (RM-9106-S0 Ki67 clone SP6,

dilution ratio 1:200, ThermoScientific) and anti-PCNA (RB-9055-P0 PCNA rabbit polyclonal 1:200, ThermoScientific). After dewaxing and rehydration, a heat-induced antigen retrieval procedure using Tris-EDTA at pH 9.0 for 30 min was performed on all tissue sections. After that, sections were washed with TBS and incubated with the primary antibody diluted at an adequate ratio. The commercial UltraVision/DAB staining kit (ThermoScientific LabVision TL-060-HD) was used for treating the sections. Immunoreactions were subsequently developed by using DAB. The sections were counterstained with Mayer's hematoxylin. Negative controls were performed on myocardial tissue sections using the same methodology but with the omission of primary antibody. Olympus BX 41 microscope was used for analyzing all the slides, which were photographed with an Olympus C-5060 wide zoom digital camera and Olympus DP-soft Image Analyzer program. The results were analyzed on 100 counted cardiomyocytes by determining the number of cells expressing a percentage of Ki67 and PCNA positivity and were expressed as percentages.

### Statistical analysis

Obtained data were expressed as means  $\pm$  SD. Discrete variables were presented as frequencies and percentages. ANOVA with post hoc analysis as well as a non-parametric variant of the Kruskal-Wallis test and Mann-Whitney test were used to compare the values between the groups, depending on the data distribution. The value of  $p < 0.05$  was considered to be statistically significant.

## RESULTS

In the experimental model of monocrotaline-induced HF, the effects of four-week vitamin B6 treatment were assessed on biochemical, oxidative stress, histomorphometric and immunohistochemical parameters. Rats were divided according to the protocol regimen into 3 groups: control group (C), vitamin B6 treatment (B6), and MCT-induced HF group on vitamin B6 treatment (MCT+B6).

### Biochemical analysis

Levels of Hcy did not differ significantly between MCT+B6, B6 and the control group ( $7.68 \pm 2.26$  vs.  $7.21 \pm 1.05$  vs.  $10.19 \pm 3.87$   $\mu\text{mol/L}$ ). However, in the group of rats treated only with vitamin B6 in comparison with the control group a beneficial effect for blood glucose and HDL concentrations was noticed. On the other hand, glucose concentrations were significantly increased in MCT+B6 group compared to B6 group ( $7.33 \pm 0.78$  vs.  $5.88 \pm 0.31$   $\text{mmol/L}$ ). The levels of total cholesterol and its HDL fraction were increased in the sera of rats that

were treated with vitamin B6 after MCT-induced heart injury (CHL =  $2.18 \pm 0.48$   $\text{mmol/L}$ ; HLD =  $1.47 \pm 0.29$   $\text{mmol/L}$ ), compared to the control group (CHL =  $1.45 \pm 0.23$   $\text{mmol/L}$ ; HDL =  $0.64 \pm 0.09$   $\text{mmol/L}$ ). The levels of triglycerides did not differ significantly between groups. In B6 group, higher activity of AST, ALT and ALP were determined in comparison to the control group ( $p < 0.05$ ), but there were no changes in the activity of AST, ALT and ALP between MCT+B6 and C groups. Levels of TP and ALB had decreasing trend in the MCT+B6 group compared to the C group. The concentration of uric acid was not significantly changed comparing the groups. The activity of  $\alpha$ -AMY in the sera of rats was lower in the C group compared to the MCT + B6 group ( $3097.4 \pm 386.83$  U/L vs.  $3460.00 \pm 80.41$  U/L).

In all tested groups, in each sample, CRP was under the limit of detection (2 mg/L). We got similar results for the concentration of IL 6 (in all tested groups 100% of samples were under 1.5 pg/mL).

Fibrinogen values were under 1.8 g/L in 75% MCT+B6 group samples. The treatment with vitamin B6 caused a decrease in von Willebrand factor (vWF) concentration in rats with MCT-induced HF ( $36.87 \pm 26.01$  %).

Troponin T increased after vitamin B6 treatment ( $190.71 \pm 75.36$  ng/L) compared to the control group ( $57.00 \pm 87.68$  ng/L). The levels of UREA and CREA were significantly higher in the MCT+B6 group in comparison with the B6 group ( $22.77 \pm 8.56$   $\text{mmol/L}$  vs.  $12.30 \pm 2.18$   $\text{mmol/L}$ ,  $55.71 \pm 5.09$   $\mu\text{mol/L}$  vs.  $38.10 \pm 2.92$   $\mu\text{mol/L}$ , respectively). The results are presented in **Table 1**.

### Oxidative stress analysis

The key antioxidant enzymes, SOD and GPX, the main non-enzymatic antioxidant, GSH, as well as the parameters of oxidative damage of proteins (thiol, carbonyl groups and nitrotyrosine), were evaluated in all tested groups. Following B6 treatment, the activity of SOD did not change in the MCT + B6 group compared to C group ( $495.00 \pm 96.42$  U/ml vs.  $480.60 \pm 53.43$  U/ml) (**Fig. 1 A**), while GPX activity (**Fig. 1 B**) was decreased in both B6 and MCT + B6 group in comparison with the C group ( $197.27 \pm 29.90$  U/ml vs.  $317.60 \pm 151.48$  U/ml), but without reaching statistical significance. The total GSH concentrations were decreased in rats treated with B6 compared to the control group ( $33.23 \pm 3.06$  nmol/mg of proteins vs.  $70.62 \pm 11.44$  nmol/mg of proteins) which is more potentiated in the MCT+B6 group (**Fig. 1 C**). The intraperitoneal B6 application with MCT-induced HF very slightly decreased the content of thiol groups in comparison with C group ( $211.71 \pm 38.96$   $\mu\text{mol/g}$  of proteins vs.  $308.45 \pm 39.24$   $\mu\text{mol/g}$  of proteins) (**Fig. 2 A**). The content of RCD showed an increasing trend in comparison with the control group ( $26.99 \pm 13.68$   $\mu\text{mol/g}$  of proteins vs.  $18.22 \pm 5.40$   $\mu\text{mol/g}$  of proteins) (**Fig. 2**

**Table 1.** Certain biochemical parameters in the serum of the experimental animals

Groups/ parametres	C	B6	MCT+B6
°Hcy (µmol/L)	10.19 ± 3.87	7.21 ± 1.05	7.68 ± 2.26
°GLUC (mmol/L)	7.17 ± 0.58	5.88 ± 0.31 #	7.33 ± 0.78 <sup>^</sup>
°CHL (mmol/L)	1.45 ± 0.23	1.35 ± 0.13	2.18 ± 0.48 <sup>^</sup>
°HDL (mmol/L)	0.64 ± 0.09	1.12 ± 0.11 #	1.47 ± 0.29 <sup>^</sup>
°TGL (mmol/L)	0.77 ± 0.17	0.91 ± 0.21	1.18 ± 0.30
°AST (U/L)	222.80 ± 49.56	331.00 ± 36.26 #	190.29 ± 30.32 <sup>^</sup>
°ALT (U/L)	69.50 ± 8.77	121.40 ± 13.33 #	61.14 ± 11.91 <sup>^</sup>
°ALP (U/L)	347.80 ± 70.38	540.40 ± 119.90 #	346.00 ± 80.41 <sup>^</sup>
°TP (g/L)	60.60 ± 1.96	58.20 ± 2.97	52.43 ± 2.82 <sup>^</sup>
°ALB (g/L)	30.40 ± 1.58	34.30 ± 1.34 #	22.43 ± 1.62
°UA (µmol/L)	77.20 ± 14.11	62.25 ± 11.68	73.14 ± 20.05
°α-AMY (U/L)	3097.4 ± 386.83	3856.90 ± 952.10	3460.00 ± 80.41 <sup>^</sup>
¥IL6 < 1.5 (pg/mL)	8 (100%)	8 (100%)	8 (100%)
¥CRP < 2 (mg/L)	8 (100%)	8 (100%)	8 (100%)
¥Fibrinogen < 1.8 (g/L)	8 (100%)	3 (37.5%)	6 (75%)
¥D Dimer < 0.5 (mg/L)	2 (25%)	6 (75%)	7 (87.5%)
¥vWF Ac (%)	15.40 ± 0.89	130.52 ± 31.34 #	36.87 ± 26.01 <sup>^</sup>
hsTnT (ng/L)	57.00 ± 87.68	12.55 ± 3.70	190.71 ± 75.36 <sup>^</sup>
°UREA (mmol/L)	9.90 ± 0.54	12.30 ± 2.18	22.77 ± 8.56 <sup>^</sup>
°CREA (µmol/L)	29.00 ± 1.76	38.10 ± 2.92	55.71 ± 5.09 <sup>^</sup>

C - saline 1ml/kg/day i.p., B6 - vitamin B6 7mg/kg/day, MCT - 50mg/kg once ip. + vitamin B6 7mg/kg/day  
 Hcy-homocysteine, GLUC-glucose, CHL-total cholesterol, HDL-high density lipoprotein, TGL-triglycerides, AST-aspartate-aminotransferase, ALT- alanine - aminotransferase, ALP-alkaline phosphatase, TP-total proteins, ALB-albumin, UA-uric acid, α-AMY- α-amylase, IL6-interleukin 6, CRP-C- reactive protein, vWF-vonWillebrand Factor, hsTnT-troponin T, UREA-urea, CREA-creatinine;  
 °- results are presented as x ± SD; ¥ - results are presented as n (%)  
 # - P < 0.05 versus C group, ^ - P < 0.05 versus B6 group.

**B)**, and the nitrotyrosine content did not change in the MCT+B6 group compared to the C group (5.13 ± 0.35 nmol/L vs. 5.23 ± 0.82 nmol/L) (**Fig. 2 C**).

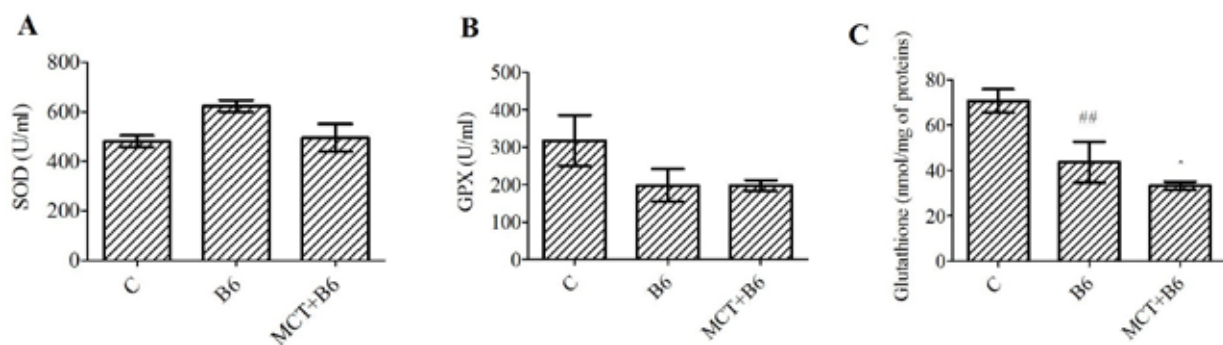
**Total S-glutathionylation measurements**

The total protein glutathionylation level was assessed by Western blot in 3 sample groups (C, B6 and MCT+B6). Monoclonal anti-glutathione antibody was used to investigate the total protein glutathionylation. **Figure 3A** showed bands reacting with anti-glutathione antibody in 3 investigated sample groups (C, B6 and MCT+B6). The

total glutathionylation level decreased in the MCT+B6 group in comparison with the C group but without statistical significance (**Fig. 3 B**). Densitometric analysis also showed decreased protein glutathionylation levels in the MCT+B6 group compared to the B6 group with borderline significance (p = 0.057).

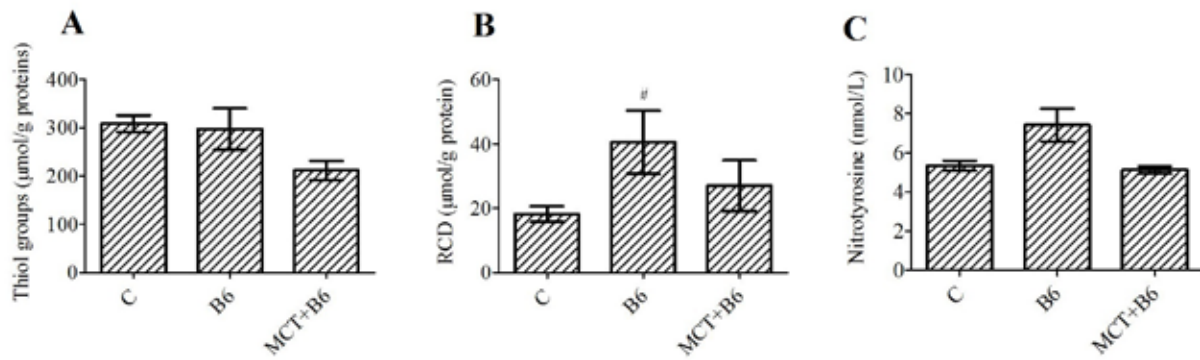
**Histomorphometric and immunohistochemical analysis**

The average dimension of cardiomyocytes of the LV free wall was significantly lower in the B6 group compared



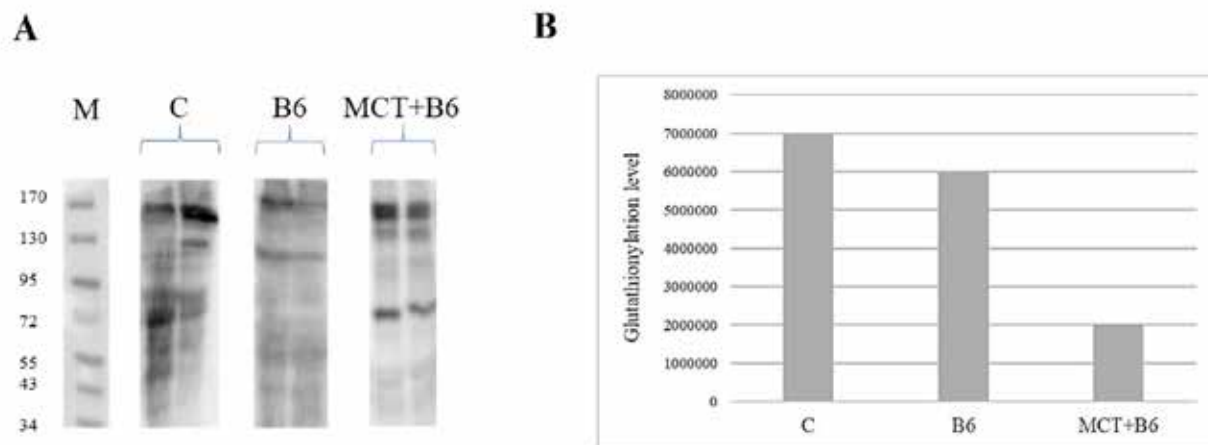
C - saline 1ml/kg/day ip., B6 - vitamin B6 7mg/kg/day, MCT - 50mg/kg once ip. + vitamin B6 7mg/kg/day  
 ## - P < 0.01 versus C group; ^ - P < 0.05 versus B6 group

**Figure 1.** A. Superoxide dismutase (SOD) activity in rat cardiac tissue; B. Glutathione peroxidase (GPX) activity in rat cardiac tissue; C. Glutathione content in rat cardiac tissue



C - saline 1ml/kg/day ip., B6 - vitamin B6 7mg/kg/day, MCT - 50mg/kg once ip. + vitamin B6 7mg/kg/day  
# – P < 0.01 versus C group

**Figure 2.** A. The amount of thiol (P-SH) group in rat cardiac tissue; B. The content of reactive carbonyl derivatives (RCD) in rat cardiac tissue; C. The content of nitrotyrosine in rat cardiac tissue



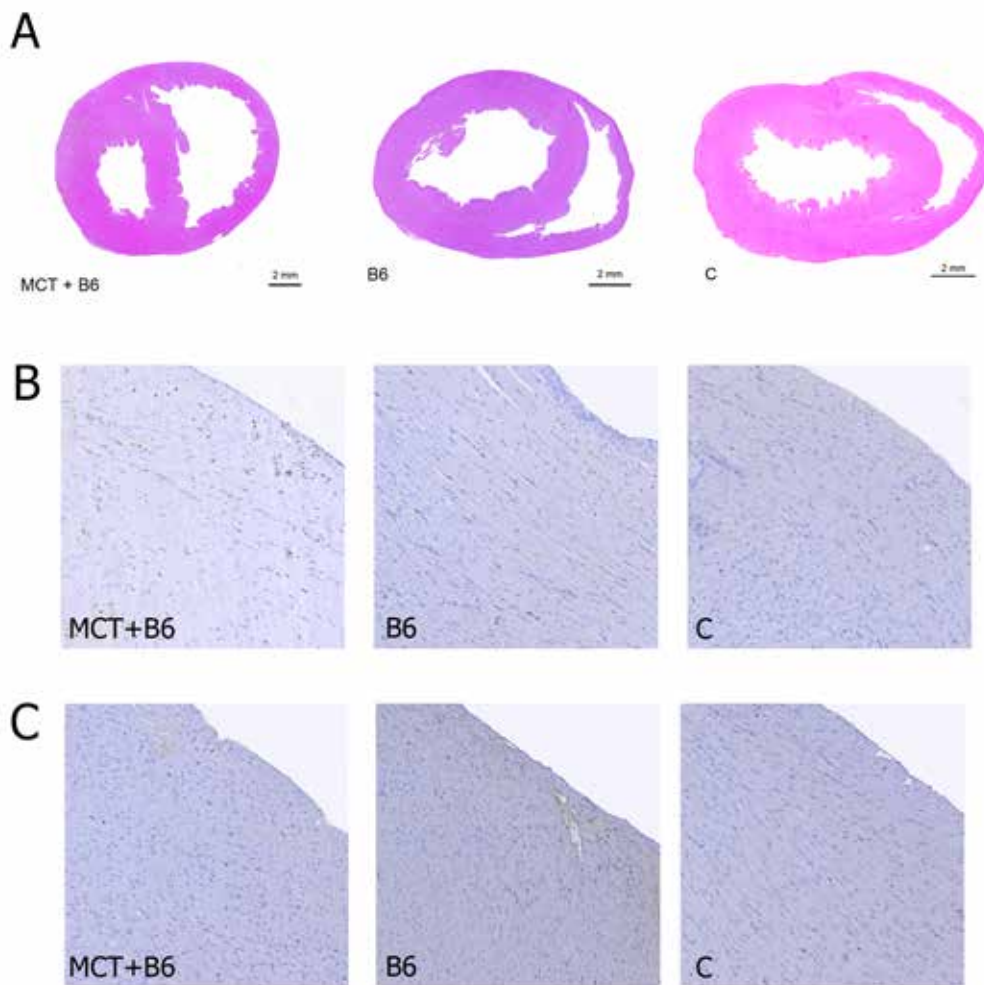
C - saline 1ml/kg/day ip., B6 - vitamin B6 7mg/kg/day, MCT - 50mg/kg once ip. + vitamin B6 7mg/kg/day

**Figure 3.** A. The level of total protein glutathionylation by Western blot; B. Densitometric analysis of obtained blots: ImageLab software (Bio-Rad, USA)

**Table 2.** Histomorphometric and immunohistochemical parameters of rat heart

Parameters	C	B6	MCT+B6
LV wall thickness (µm ± SD)	2363.81 ± 346.21	1943.37 ± 96.36	2259.30 ± 382.00
RV wall thickness (µm ± SD)	835.48 ± 144.63	707.58 ± 100.63	1000.71 ± 292.25
IVS thickness (µm ± SD)	2112.61±296.05	1762.42 ± 79.28	2194.91 ± 410.28
Dimension of cardiomyocyte of the LV free wall (µm ± SD)	22.50 ± 1.90	17.62 ± 1.43 #	25.21 ± 2.83 ^
Ki67 (RV) (%±SD)	1.25± 1.26	1.60 ± 0.89	17.50 ± 10.60 #
Ki67 (LV) (%±SD)	1.00 ± 0.82	1.00 ± 0.71	5.00 ± 4.24 #
PCNA(RV) (%±SD)	0.20 ± 0.45	0.40 ± 0.55	6.3 ± 1.41 #
PCNA(LV) (%±SD)	0.20 ± 0.45	0.20 ± 0.45	6.6 ± 0.78 #

C - saline 1ml/kg/day ip., B6 - vitamin B6 7mg/kg/day, MCT - 50mg/kg once ip. + vitamin B6 7mg/kg/day  
LV – left ventricle; RV – right ventricle; IVS – interventricular septum; SD – standard deviation  
# – P < 0.05 versus C group, ^ – P < 0.05 versus B6 group



C - saline 1ml/kg/day ip., B6 - vitamin B6 7mg/kg/day, MCT - 50mg/kg once ip. + vitamin B6 7mg/kg/day

**Figure 4.** A. Representative histological slides of rat's hearts in experimental groups; B. Representative immunohistochemical slides of rat hearts in experimental groups (Ki67 proliferation marker); C. Representative immunohistochemical slides of rat hearts in experimental groups (PCNA proliferation marker)

to the C group ( $17.62 \pm 1.43 \mu\text{m}$  vs.  $22.50 \pm 1.90 \mu\text{m}$ ,  $p < 0.05$ ). There was an increasing trend in RV wall thickness in the MCT+B6 group compared to the C group ( $1000.71 \pm 292.25 \mu\text{m}$  vs.  $835.48 \pm 144.63 \mu\text{m}$ ). IVS thickness did not change in the MCT+B6 group compared to the control group ( $2194.91 \pm 410.28 \mu\text{m}$  vs.  $2112.61 \pm 296.05 \mu\text{m}$ ). The same trend was observed in LV wall thickness. Ki67 positivity in the RV wall was increased in MCT+B6 group compared to the C group ( $17.50 \pm 10.60\%$  vs.  $1.25 \pm 1.26\%$ ). A similar pattern of Ki67 positivity was recorded in the LV wall between MCT+B6 and C groups ( $5.00 \pm 4.24\%$  vs.  $1.00 \pm 0.82\%$ ). PCNA positivity in the RV wall was increased in MCT+B6 group compared to the C group ( $6.3 \pm 1.41\%$  vs.  $0.20 \pm 0.45\%$ ), and the same increase was observed in the LV wall ( $6.6 \pm 0.78\%$  vs.  $0.20 \pm 0.45\%$ ). The results are presented in **Table 2** and **Figure 4 (A, B, C)**.

## DISCUSSION

In this study, we found some beneficial effects of the four-week treatment with vitamin B6 on histomorphometric

and immunohistochemical parameters in monocrotaline-induced right heart failure. Indeed, an increasing trend in RV and LV wall thickness, as well as, in Ki67 and PCNA positivity in rats with heart failure treated with B6 vitamin compared to control rats was determined. Interestingly, based on a significant decrease in total glutathione concentrations, unchanged SOD and GPX activity, as well as on the increasing trend of glutathionylation obtained in the control group treated only with vitamin B6, we hypothesized a potential involvement of vitamin B6 in redox signaling and regulation.

It has been well established that the experimental model of heart failure (HF), induced by intraperitoneal injection of the macrocyclic alkaloid monocrotaline (MCT), causes changes in various cardio-metabolic and oxidative stress parameters (REF). In our research, we reviewed retrieved information regarding potential effective impacts of vitamin B6 and determined the effects of four-week vitamin B6 treatment on cardiometabolic and oxidative stress biomarkers, but also the impact on histomorphometric and immunohistochemical changes in MCT-induced HF. Namely, the activity of key antiox-

idant enzymes, SOD and GPX, as well as, parameters of oxidative damage to proteins, such as thiol groups, reactive carbonyl derivatives and nitrotyrosine did not change in the MCT+B6 group compared to the control group. However, a significant decrease was determined only for the total GSH concentrations, followed by a decreasing trend in protein glutathionylation levels in rats with heart failure treated with vitamin B6. Interestingly, in the control group treated only with vitamin B6 a significant decrease in total glutathione concentrations, and an increasing trend of protein glutathionylation in comparison with the control group, clearly implies its potential involvement in redox regulation. Namely, the important role of protein S-glutathionylation in redox regulation of different signaling pathways has emerged recently. Moreover, it has been shown that perturbations in protein glutathionylation status might be involved in the pathogenesis of atherosclerosis and cardiac hypertrophy, underlying the possible molecular mechanism of redox disbalance in etiology of cardiovascular diseases (25). Until now, antioxidant effect of vitamin B6 was based mainly on its effect on inhibiting superoxide radical generation and reducing lipid peroxidation (26). Recently, it has been shown that a beneficial effect of vitamin B6 supplementation on the function of aortic endothelium in old rats is mediated by stimulation of H<sub>2</sub>S biosynthesis, reduction of oxidative/nitrosative stress and lipid peroxidation along with an increase in constitutive NO synthesis (27). In contrast, vitamin B6 deficiency increases lipid peroxidation and reduces antioxidant defenses, and it also shows a connection with atherogenesis (28). It has been observed that vitamin B6 is important as a significant and detached risk factor for cardiovascular diseases (29). Some case-control studies have shown that vitamin B6 deficiency is obviously associated with higher cardiovascular risk (9), even though some clinical trials evidenced ineffectiveness of vitamin B6 supplementation on recurrence of cardiovascular events (30).

The experimentally MCT-induced HF have effect primarily on pulmonary vessels without an effect on systemic vessels and right heart hypertrophy (31). In our study, we observed an increasing trend in RV wall thickness in the MCT+B6 group compared to the C group. Moreover, Ki67 and PCNA positivity, both in the RV and LV wall, was increased in the MCT+B6 group compared to the control group. Those histomorphometric and immunohistochemical changes were accompanied with a significant increase in troponin T and impaired renal function. Although most of the investigated parameters of oxidative stress was not significantly changed in the heart failure rats in comparison with the healthy ones, a significant decrease in glutathione concentrations might contribute to disturbances in redox sensitive signaling pathways involved in the pathogenesis of RV hypertrophy. Moreover, some studies have demonstrated a higher RV susceptibility to oxidative stress damage in compari-

son with the LV (32). In our previous study, we found that supplementation with folic acid reduced cardiomyocyte proliferation in the RV wall even if it did not significantly reduce hypertrophy of the RV wall (33).

In our study, we observed no change in Hcy plasma level in all investigated groups. Vitamin B deficiency (primarily folate, vitamin B6 and B12) could result in hyperhomocysteinemia, via the obstruction of Hcy detoxification, leads to oxidative imbalance and overbalance in ROS production. ROS have induced the DNA base damage, DNA strand breaks, and accelerated telomere shortening (34). Some studies describing the relationship between Hcy and telomere shortening are in conflict. A certain number of these reported an inverse association between telomere length and Hcy (35), and others did not (36). The high level of plasma Hcy promotes chronic systemic inflammation through the oxidative stress induction in different types of tissue and a subsequent intracellular and extracellular damage (37, 38). That connection between Hcy and chronic inflammation, is not a one-way road. Steele et al. (39) have shown, through *in vitro* experiments, a concentration-dependent fashion. Pro-inflammatory cytokines (interleukin-1 $\beta$  (IL-1 $\beta$ ) and TNF-alpha) have modified the cells' redox state and increased extracellular Hcy concentration. Furthermore, Ulvik et al. (40) have shown that systemic inflammation increased the catabolism of vitamin B6 and cellular uptake, and resulted in reduced B6 plasma concentrations. Moreover, vitamin B6 was also a strong predictor of death. In the crude model, subjects with the highest vitamin B6 concentrations had a 59% lower risk to die during follow-up. It has been shown that subjects with the highest age-corrected leukocyte telomere length (LTL) had a higher median concentration of vitamin B6 and, at the same time, a lower plasma Hcy concentration.

Current studies indicate ineffectiveness of vitamin B6 supplementation for the prevention of cardiovascular disease recurrence. Nevertheless, it remains open to determine whether vitamin B6 supplementation is effective for the primary CVD prevention. Given that vitamin B6 occurs through various physiological forms and in combination with other nutrients and other B vitamins which have a role in the complex vitamin B6 metabolism, the question arises whether supplementation with a single high-dose of vitamin B6 is effective as an adequate intake of this vitamin. More studies are needed to find the optimal, right dose and an adequate combination of vitamin B6 forms for maximizing the efficacy and minimizing detrimental effects, with respect to genetic susceptibility and environmental factors to properly estimate the role of B vitamins in the primary prevention of CVD.

## CONCLUSIONS

In this study, we observed that four-week treatment with vitamin B6 had some beneficial effects on certain



cardio-metabolic biomarkers in monocrotaline-induced right heart failure. A four-week treatment with vitamin B6 significantly affected certain biomarkers like glucose, HDL cholesterol, aminotransferase, alkaline phosphatase and amylase activities, vonWillebrand factor activity, cardiac troponin T, urea and creatinine. The activity of SOD did not change, while GPX activity decreased in the MCT+B6 group. The total glutathione values and the content of thiol groups had a decreasing trend in the MCT+B6 group. The content of reactive carbonyl derivatives showed an increasing trend, and the nitrotyrosine content did not change in the MCT+B6 group. The total glutathionylation level was decreased in the MCT+B6 group. We observed an increase in RV and LV wall thickness in the MCT+B6 group compared to the C group, as well as in Ki67 and PCNA positivity.

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### Conflict of interest statement

The authors declare that there are no conflict of interest associated with this work.

### Author Contributions

Conceptualization, A.S.R., J.J.U., T.D.; methodology, A.S.R., J.J.U., T.D., M.L.B., J.R., D.T.; software, D.T., I.N., M.D.; validation, A.S.R., T.D., S.S., M.L.B., J.R., N.M.; formal analysis, J.J.U., D.T., T.D., S.M., J.R., I.N., M.D., S.S., M.L.B., N.M., A.S.R.; research, J.J.U., D.T., T.D., J.R., I.N., M.D.; resources, A.S.R., S.S.; data curation, J.J.U., D.T., T.D., J.R., I.N., M.D.; writing-original draft preparation, J.J.U.; writing—review and editing, D.T., T.D., S.M., A.S.R.; visualization, J.J.U., D.T., T.D. All authors have read and agreed to the published version of the manuscript.

### Ethical approval

Animals were treated in accordance with the Guide for the Care and Use of Laboratory Animals (8th edition, National Academies Press) and the European Directive for welfare of laboratory animals No: 2010/63/EU. The study was approved by the Ethical Council for the Welfare of Experimental Animals, Ministry of Agriculture, Forestry and Water Management, Veterinary Directorate, Republic of Serbia (Number: 323-07-01339/2017-05/2).

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## UTICAJ VITAMINA B6 NA MARKERE OKSIDATIVNOG STRESA, KARDIOMETABOLIČKE I HISTOLOŠKE MARKERE U USLOVIMA MONOKROTALINOM INDUKOVANE SRČANE SLABOSTI KOD WISTAR ALBINO PACOVA

Jovana Jakovljević Uzelac<sup>1</sup>, Dušan Todorović<sup>1</sup>, Tatjana Đukić<sup>2</sup>, Slavica Mutavdžin<sup>1</sup>, Sanja Stanković<sup>3</sup>, Milica Labudović Borović<sup>4</sup>, Jelena Rakočević<sup>4</sup>, Nataša Milić<sup>5</sup>, Irina Nenadić<sup>6</sup>, Marko Djurić<sup>6</sup>, Ana Savić-Radojević<sup>2</sup>

### Sažetak

Srčana insuficijencija (HF) izazvana monocrotalinom (MCT) je dobro poznata po mehanizmima remodelovanja plućnih arterijskih krvnih sudova sa povećanom plućnom rezistencijom i povećanim markerima oksidativnog stresa. Svrha ove studije je bila da se potvrdi hipoteza da tretman vitaminom B6 može uticati na srčanu insuficijenciju modulacijom biomarkera kardiometaboličkog i oksidativnog stresa, i strukture srca pacova. Mužjaci *Wistar albino* pacova podeljeni su u 3 grupe: kontrola izložena praznom rastvoru (C fiziološki rastvor 1ml/kg 28 dana ip., n=8), B6 (vitamin B6 7mg/kg/dan 28 dana ip., n=8), i MCT+B6 (MCT 50 mg/kg jednom ip. plus vitamin B6 7 mg/kg/dan 28 dana ip., n=8). Četvoronedeljni tret-

man vitaminom B6 značajno je uticao na određene bi-hemijske parametre. Aktivnost ključnih antioksidativnih enzima, superoksid dismutaze (SOD) i glutacion peroksidaze (GPx) nije se promenila, dok je ukupni glutacion (GSH) značajno smanjen u grupi MCT+B6. Nakon toga je usledilo blago smanjenje nivoa ukupne glutacionilacije, uočeno u grupi MCT+B6. Parametri oksidativnog oštećenja proteina (reaktivni karbonilni derivati, tiol grupe i nitrotirozin) nisu se značajno promenili u grupi MCT+B6. Uočen je trend povećanja debljine zida desne (RV) i leve (LV) komore u grupi MCT+B6 u poređenju sa kontrolnom (C) grupom, kao i povećanje Ki67 i PCNA pozitivnosti.

**Ključne reči:** srčana insuficijencija, monocrotalin, vitamin B6, oksidativni stres, pacov

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