Antihyperlipidemic potential of dietary supplementation with carnosine in high-fat diet-fed rats

A. RAŠKOVIĆ1, N. MARTIĆ1, D. ZAKLAN2, N. DUBORIJAKOVAČEVIĆ3, M. VUJČIĆ4, B. ANDREJIĆ-VIŠNJIC4, I. ČAPO4, R. MIJOVIĆ5, M. KRGA6, N. PAVLOVIĆ2, D. PRODANOVIĆ1, P. ARSENOVIĆ6, O. HORVAT1

1Department of Pharmacology, Toxicology and Clinical Pharmacology, Faculty of Medicine, University of Novi Sad, Novi Sad, Serbia
2Department of Pharmacy, Faculty of Medicine, University of Novi Sad, Novi Sad, Serbia
3Department of Pharmacology and Clinical Pharmacology, Faculty of Medicine, University of Montenegro, Podgorica, Montenegro
4Department of Histology and Embryology, Faculty of Medicine, University of Novi Sad, Novi Sad, Serbia
5Department of Pathophysiology and Laboratory Medicine, Faculty of Medicine, University of Novi Sad, Novi Sad, Serbia
6Faculty of Medicine, University of Novi Sad, Novi Sad, Serbia

Abstract. – OBJECTIVE: The aim of this study was to investigate the hypolipidemic effects of carnosine and a commercial carnosine supplement on lipid status, liver and kidney function, and inflammation associated with dyslipidemia in rats with high-fat diet-induced hyperlipidemia.

MATERIALS AND METHODS: The study was conducted on adult male Wistar rats, divided into control and experimental groups. Animals were kept in standard laboratory conditions and according to groups were treated with saline, carnosine, carnosine dietary supplement, simvastatin, and their combinations. All substances were prepared fresh every day and used by oral gavage.

RESULTS: Treatment with a carnosine-based supplement significantly improved total and LDL cholesterol levels in serum, especially in the combination with simvastatin as a conventional drug in dyslipidemia treatment. The effect of carnosine on the metabolism of triglycerides was not as evident as in the case of cholesterol. Nevertheless, the values of the atherogenic index showed that the combinations of carnosine and carnosine supplement with simvastatin were the most effective in lowering this comprehensive lipid index. Dietary carnosine supplementation resulted also in anti-inflammatory effects, as demonstrated by immunohistochemical analyses. Besides, the good safety profile of carnosine in terms of its effect on liver and kidney functions was also confirmed.

CONCLUSIONS: The use of carnosine supplements in preventing and/or treatment of metabolic disorders requires further investigations into the mechanisms of action and potential interactions with conventional therapy. Key Words: Carnosine, Antihyperlipidemic potential, High-fat diet, Supplementation, Rats.

Introduction

Metabolic syndrome (MetS) is a global public health problem with a significant prevalence, as the silent epidemic no longer knows economic or socio-cultural boundaries. MetS represent a spectrum of metabolic disorders, including insulin resistance, hyperinsulinemia, dysglycemia, dyslipidemia, and hypertension. It is identified as a major cardiovascular risk factor, along with cigarette smoking, unbalanced dietary patterns, and a sedentary lifestyle.

Hyperlipidemia is considered to be one of the major risk factors in developing cardiovascular events, including myocardial infarction and stroke. It is characterized by imbalanced blood cholesterol levels, including low-density lipoprotein cholesterol (LDL-C) and high-density lipoprotein cholesterol (HDL-C). Other forms are hypertriglyceridemia and mixed hyperlipidemia with both cholesterol and triglyceride levels elevated. Thus, adequate management of lipid metabolism and hyperlipidemia has an essen-

Corresponding Author: Aleksandar Rašković, MD; e-mail: aleksandar.raskovic@mf.uns.ac.rs
tial part in primary and secondary preventive strategies for cardiovascular diseases (CVDs). The reduction of LDL-C plasmatic concentrations represents the most relevant curative measure. Lifestyle changes remain imperative in the management of underlying risk factors but are difficult to sustain. The Diabetes Prevention Program (DPP) trial defines the first-line measures to include a target goal of 7% weight loss, a low-calorie, low-fat diet, and 150 minutes of moderate physical activity per week.

In addition to healthy lifestyle interventions, therapeutic modalities could be necessary. Among lipid-lowering drugs, statins have become the most extensively used, with proven effects in CVD prevention in various clinical settings. Via inhibition of 3-hydroxy-3-methyl-glutaryl-coenzyme A (HMG-CoA) reductase, statins cause the reduction of hepatic synthesis of cholesterol, intracellular cholesterol concentrations, and elevation of LDL receptors expression on hepatic cells’ surface. Numerous studies have shown the efficacy of statins in both primary and secondary cardiovascular prevention. On the other hand, the discussion about the legitimacy of statins is ongoing since conflicting data emerges and the interpretation of existing clinical trials differs. Statins are usually well-tolerated and safe, albeit statin-associated side effects can be challenging to assess and manage. Numerous associated adverse effects include gastrointestinal events, liver dysfunction, new-onset diabetes mellitus, neurological disorders, and respiratory infections, most frequent being statin-associated muscle symptoms (SAMS). The prevalence varies between statin classes, but 7-29% of statin-treated patients report SAMS, such as weakness, inflammation, and myalgia. Myopathy and rhabdomyolysis are less common and serious adverse effects, albeit combinations with various drug classes, such as CYP3A4 inhibitors, can increase the risk. Mentioned effects seem to be the major reason for patients to discontinue the therapy, as non-adherence to statin treatment is a common issue.

Recently, a growing enthusiasm opened a new market for nutraceuticals and functional foods with the potential to modify the plasma lipid profile and reduce the burden of CVD. The European Society of Cardiology and the European Society of Atherosclerosis has classified nutraceutical supplementation for the clinical management of dyslipidemia as a pre-pharmacological intervention. Yet, existing clinical evidence in this field is incomplete, and reliable safety data are still lacking. Thus, there is a major need to test the efficacy through well-designed experiments and clinical studies, before introducing potential new agents in the treatment. Nutraceuticals and functional food might complement lipid-lowering therapy with statin or non-statin agents but cannot replace pharmacotherapy.

Carnosine is an endogenous dipeptide, synthesized from β-alanine and L-histidine by carnosine synthase, abundantly present in excitable tissues. It is estimated that over 99% of the carnosine present in an organism is in skeletal muscle, with high concentrations in the brain, heart, and gastrointestinal tissue of humans. This dipeptide is easily absorbed in the gastrointestinal tract, crosses the blood-brain barrier, and does not accumulate in the organism of healthy individuals, due to the activity of hydrolytic enzymes, present in serum or tissues, named carnosinases. Although there is still little certainty about its biochemical role, pleiotropic physiological functions of carnosine include pH-buffering, metal-ion chelation and homeostasis, direct and indirect antioxidant capacity, protection against lipid peroxidation and protein oxidation, and glycation. A great effort to utilize carnosine in the treatment of a plethora of diseases is being made. Considerable results and potential benefits have been reported in the fields of neurological disorders, malignant diseases, wound healing, as well as cardiometabolic diseases, including obesity and atherosclerosis. While the pleiotropic effects of carnosine are challenging, further research activity is necessary to assess and evaluate its therapeutic capacity.

The objective of the present study was to investigate the hypolipidemic effects of carnosine and a commercial carnosine supplement on lipid status, liver and kidney function, and inflammation associated with dyslipidemia in rats exposed to cholesterol-fortified food.

**Materials and Methods**

**Materials**

Active ingredient L-carnosine was a gift from CarnoMed, (Novi Sad, Serbia). A commercial dietary supplement of carnosine [Karnozin extra®, CarnoMed, Serbia, (batch No. C66S110719)]
that contains L-carnosine (125 mg), coenzyme Q10 (20 mg), L-carnitine (20 mg), vitamin E (20 mg), and standardized grape seed extracts (20 mg) and north blueberry seed extracts (20 mg) was used in our work as well. Standard pellet food for laboratory rats was enriched with cholesterol and cholic acid: 2% of cholesterol (Acros Organics, Geel, Belgium) and 0.5% of cholic acid (Acros Organics, Italy) were added to 1 kilogram of granules. All chemicals and commercial kits used for biochemical and immunohistochemical analyses were used as received without any further modifications.

**Experimental Design and Animal Treatment**

The study was conducted on adult male Wistar rats, divided into control and experimental groups. At the beginning of the experiment, the rats weighed 180-280 grams. The animals were brought from the Military Medical Academy in Belgrade, Serbia. During the entire experiment, the animals were kept in standard laboratory conditions, which include staying in laboratory cages with a day and night rhythm of 12 hours, a temperature between 22-25° C, and a humidity of 30-40%. Animal care and all experimental procedures were carried out by EU Directive 2010/63/EU care for laboratory animals and the Law of Animal Welfare of the Republic of Serbia (OG RS 41/09). The study was approved by the Ethics Commission for the Protection of Laboratory Animal Welfare of the University of Novi Sad (Novi Sad, Serbia; No. 01-107/6-1) and the Ministry of Agriculture and Environmental Protection of the Republic of Serbia (Belgrade, Serbia; No. 323-07-04785/2019-05).

To evaluate the influence of carnosine on hyperlipoproteinemia in rats, a total of 56 animals were randomly divided into 7 groups, each containing 8 individuals. The groups were divided as shown below:

- **ConS** – standard pellet food + saline 1 mL/kg p.o.
- **ConHLP** – pellet food enriched with cholesterol + saline 1 mL/kg p.o.
- **Car** – pellet food enriched with cholesterol + carnosine 175 mg/kg p.o.
- **Car. Supp** – pellet food enriched with cholesterol + carnosine supplement 315 mg/kg p.o. (equivalent to 175 mg/kg of pure carnosine).
- **Sim** – pellet food enriched with cholesterol + simvastatin 3.5 mg/kg p.o.
- **Sim. Car** – pellet food enriched with cholesterol + simvastatin 3.5 mg/kg p.o. + carnosine 175 mg/kg p.o.
- **Sim.Car. Supp** – pellet food enriched with cholesterol + simvastatin 3.5 mg/kg p.o. + carnosine supplement 315 mg/kg p.o. (equivalent to 175 mg/kg of pure carnosine).

The study lasted for 4 weeks, and simvastatin, carnosine, and dietary supplement of carnosine were freshly dispersed in saline every day, 30 minutes before treatment. All animals were subjected to measurement of body weight immediately before and after the experiment. At the end of the experiment, all animals were anesthetized with urethane and sacrificed by cardiac puncture after which blood and tissue samples were collected for further analysis.

**Lipid Status and Serum Biochemical Parameters Determination**

Triglycerides and total, LDL, and HDL cholesterol levels were determined in serum using commercially available kits based on the well-established spectrophotometric methods, according to the manuals supplied. LDL coefficient and atherogenic index were further calculated for each animal.

To examine the safety of carnosine itself, a dietary supplement of carnosine and their combinations with simvastatin, serum enzyme activities as indicators of liver function were examined: aspartate aminotransferase (AST), alanine aminotransferase (ALT) and alkaline phosphatase (ALP), while the renal function was tested by indicators of renal function: urea, creatinine, and uric acid serum concentrations. Inflammatory factor C-reactive protein (CRP) examination as well as leptin testing were also conducted. All analyses were performed in triplicate for every sample on an Abbot Alinity analyzer (Chicago, IL, USA) using commercial kits.

**Histopathology and Immunohistochemistry Assessment**

The histological assessment was performed by light microscopy by two researchers on a blind study. The liver was sampled from each animal and histological analysis was performed on a small piece of tissue. Samples were fixed for 24 h in Bouin’s solution. Afterward, samples were dehydrated in a different concentration of isopropyl alcohol and embedded in paraffin blocks. For each animal, four successive 5 µm thick tissue sections were taken, using a rotation microtome (Sakura Finetek USA, Inc., Torrance, CA, USA).
Two sections were colored with the routine hematoxylin and eosin (H&E) and Periodic Acid Schiff (PAS) method. The two remaining pieces were exposed to the immunohistochemical procedure of staining: CYP2E1 (1:200, CSB-PA006425EA01H4, Flarebio, College Park, MD, USA) and Iba-1 [1:8000, AB178847, (Abcam, Cambridge, UK)] antibodies were applied by the manufacturer’s directions. All histological examinations were performed by Olympus BX-43 light microscope (Olympus, Tokyo, Japan) with an attached video camera Olympus DP 73 (Olympus, Tokyo, Japan). The free available image software Image J [version 1.51h, National Institute of Health, (Bethesda, MD, USA)] was used for additional morphometric analyses of CYP2E1 and Iba-1-stained slides. CYP2E1 antibody distinguishes cells with cytochrome P450 2E1 isoenzyme activity. The section of liver tissue with CYP2E1 positivity was determined using Image J software on 5 high-power field photographs for each slide of tissue. Iba-1 staining distinct the liver macrophages. Based on 5 HPF (high-power field, 40x magnification) photography, the average ratio of Iba-1 positive cells (Iba1+) was measured.

**Statistical Analysis**

Data are expressed as mean±standard deviation (SD). Paired two-tailed Student’s t-test was used for body weight comparison. The intergroup variation between various groups was measured by one-way analysis of variance (ANOVA) followed by Tukey’s multiple comparison test. Results were considered statistically significant when p<0.05. Data were analyzed using Origin 2018 software (OriginLab Corp, Northampton, MA, USA).

### Results

**Effects of Carnosine Supplementation on Body Weight and the Lipid Status**

In our study, there was a statistically significant increase in body weight within all experimental groups over a period of 4 weeks (Table I). Supplementation with carnosine preparations prevented the increase in the body weight of rats on a high-fat diet, both alone and in combination with simvastatin (Table I).

Biochemical parameters of the lipid status in the serum of the treated rats are presented in Table II. Induction of hyperlipidemia was successful following a 4-week high-fat diet since a statistically significant increase in total and LDL cholesterol was observed (p<0.001). Triglycerides concentration was 15% increased in the serum of the animals on a high-fat diet compared to the healthy animals, but without statistical significance.

Pure carnosine and the dietary supplement based on carnosine efficiently reverted cholesterol levels toward the normal values, but they were still significantly higher than those of the saline-treated group. Simvastatin was more effective in cholesterol-lowering than carnosine and carnosine supplements. However, the hypocholesterolemia effect was the most pronounced in the group of simvastatin and carnosine supplement co-treatment (group 7).

The effect of carnosine, alone or in combination with simvastatin, on the metabolism of triglycerides, was not as evident as in the case of cholesterol. Nevertheless, the values of the atherogenic index showed that the combinations of carnosine and carnosine supplement with simvastatin were the most effective in lowering this comprehensive lipid index, although the dietary supplement based on carnosine alone decreased significantly.

<table>
<thead>
<tr>
<th>Group</th>
<th>Initial body weight [g]</th>
<th>Terminal body weight [g]</th>
<th>Difference, Δ [g]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group 1 ConS</td>
<td>185.0 ± 12.2</td>
<td>301.5 ± 9.9</td>
<td>116.5</td>
</tr>
<tr>
<td>Group 2 ConHLP</td>
<td>208.2 ± 41.7</td>
<td>394.0 ± 59.9</td>
<td>185.8</td>
</tr>
<tr>
<td>Group 3 Car</td>
<td>219.1 ± 21.9</td>
<td>334.7 ± 18.6</td>
<td>115.6</td>
</tr>
<tr>
<td>Group 4 Car.Supp</td>
<td>234.6 ± 24.1</td>
<td>344.3 ± 27.0</td>
<td>109.7</td>
</tr>
<tr>
<td>Group 5 Sim</td>
<td>227.3 ± 55.2</td>
<td>336.2 ± 55.6</td>
<td>108.9</td>
</tr>
<tr>
<td>Group 6 Sim.Car</td>
<td>240.7 ± 39.9</td>
<td>366.1 ± 59.3</td>
<td>125.4</td>
</tr>
<tr>
<td>Group 7 Sim.Car.Supp</td>
<td>259.2 ± 50.8</td>
<td>365.8 ± 57.1</td>
<td>106.6</td>
</tr>
</tbody>
</table>
Antihyperlipidemic potential of dietary supplementation with carnosine in high-fat diet-fed rats

Effects of Carnosine Supplementation on the Hepatic and Renal Functions

Biochemical parameters, related to hepatic and renal function, were determined in the serum of rats and the results are presented in Table III. The extent of hepatocellular damage was assessed by measuring the activities of AST, ALT, and ALP in serum. Induction of hyperlipidemia did not significantly change these serum parameters of liver function. The activities of AST and ALT were slightly increased in the group of animals on a high-fat diet in comparison to the negative control group. Simvastatin increased the serum activities of ALT in comparison to healthy rats, and this effect was particularly pronounced in combination with pure carnosine (ConS vs. Sim. Car, \(p = 0.06\)).

Concentrations of urea, creatinine, and uric acid, as indicators of renal function, were also determined in serum. In rats with hyperlipidemia (ConHLP), the serum concentration of urea was statistically significantly decreased in comparison to the rats treated with saline (\(p < 0.001\)). Although the supplementation with carnosine could not revert this serum parameter to normal values, the combination of carnosine and simvastatin managed to statistically significantly increase the concentration of urea in comparison to the animals of the ConHLP group (\(p < 0.001\)). None of the treatments significantly affected serum levels of uric acid (Table III).

Effects of Carnosine Supplementation on the Liver Histology

Liver histology is presented in Figure 1. It can be observed that the liver tissue of the control group (ConS) shows a usual, lobular structure with lobules of well-known hexagonal shape, centrally placed central veins with hepatocytes extended radially, arranged in the form of Remack’s beams. Hepatocytes show clear cell borders, the cytoplasm is homogeneous and plentiful, and one or two nuclei with a prominent nucleolus are present. The portal spaces contain rare connective tissue with elements of the portal triad.

Table II. Effects of carnosine supplementation on the serum lipid profile.

<table>
<thead>
<tr>
<th>Group</th>
<th>Condition</th>
<th>Triglycerides [mmol/L]</th>
<th>Total cholesterol [mmol/L]</th>
<th>LDL [mmol/L]</th>
<th>HDL [mmol/L]</th>
<th>LDL Atherogenic coefficient index</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group 1</td>
<td>ConS</td>
<td>0.94 ± 0.12</td>
<td>1.64 ± 0.21</td>
<td>0.52 ± 0.13</td>
<td>0.70 ± 0.07</td>
<td>2.32 ± 0.15</td>
</tr>
<tr>
<td>Group 2</td>
<td>ConHLP</td>
<td>1.08 ± 0.27</td>
<td>5.42 ± 1.45*</td>
<td>4.39 ± 1.41*</td>
<td>0.56 ± 0.03*</td>
<td>9.85 ± 3.03*</td>
</tr>
<tr>
<td>Group 3</td>
<td>Car</td>
<td>1.07 ± 0.33</td>
<td>4.21 ± 0.58*</td>
<td>3.20 ± 0.63*</td>
<td>0.52 ± 0.08*</td>
<td>8.28 ± 2.03*</td>
</tr>
<tr>
<td>Group 4</td>
<td>Car.Supp</td>
<td>1.25 ± 0.32</td>
<td>4.10 ± 0.86*</td>
<td>2.99 ± 0.85**</td>
<td>0.52 ± 0.06*</td>
<td>8.02 ± 1.98*</td>
</tr>
<tr>
<td>Group 5</td>
<td>Sim</td>
<td>0.98 ± 0.29</td>
<td>3.47 ± 0.83*</td>
<td>2.44 ± 0.92*</td>
<td>0.60 ± 0.05</td>
<td>5.91 ± 1.98*</td>
</tr>
<tr>
<td>Group 6</td>
<td>Sim.Car</td>
<td>1.09 ± 0.50</td>
<td>3.25 ± 0.46*</td>
<td>2.11 ± 0.67**</td>
<td>0.65 ± 0.10</td>
<td>5.23 ± 1.66*</td>
</tr>
<tr>
<td>Group 7</td>
<td>Sim.Car.Supp</td>
<td>1.26 ± 0.34</td>
<td>3.05 ± 0.39*</td>
<td>1.89 ± 0.30*</td>
<td>0.59 ± 0.08</td>
<td>5.19 ± 0.83*</td>
</tr>
</tbody>
</table>

All values are expressed as mean ± SD. (*) Significantly different from ConS group; (#) Significantly different from ConHLP group; \(p < 0.05\).

Table III. Effects of carnosine supplementation on the serum biochemical parameters of the hepatic and renal function.

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Group 1</td>
<td>ConS</td>
<td>139.0 ± 19.6</td>
<td>30.9 ± 6.7</td>
<td>305.3 ± 74.4</td>
<td>7.9 ± 0.6</td>
<td>49.7 ± 2.0</td>
<td>70.7 ± 15.5</td>
</tr>
<tr>
<td>Group 2</td>
<td>ConHLP</td>
<td>170.1 ± 72.7</td>
<td>31.3 ± 10.8</td>
<td>240.9 ± 56.1</td>
<td>4.8 ± 0.5*</td>
<td>56.0 ± 5.3*</td>
<td>74.7 ± 8.3</td>
</tr>
<tr>
<td>Group 3</td>
<td>Car</td>
<td>126.6 ± 8.2</td>
<td>29.9 ± 3.9</td>
<td>244.7 ± 29.9</td>
<td>4.9 ± 0.6*</td>
<td>52.0 ± 1.5</td>
<td>81.1 ± 9.9</td>
</tr>
<tr>
<td>Group 4</td>
<td>Car.Supp</td>
<td>121.7 ± 13.9</td>
<td>30.7 ± 2.1</td>
<td>220.6 ± 23.3</td>
<td>5.0 ± 0.6*</td>
<td>52.5 ± 2.7</td>
<td>74.2 ± 18.2</td>
</tr>
<tr>
<td>Group 5</td>
<td>Sim</td>
<td>119.9 ± 21.4</td>
<td>34.4 ± 2.9</td>
<td>274.4 ± 77.5</td>
<td>5.2 ± 0.3*</td>
<td>49.6 ± 2.2*</td>
<td>64.1 ± 7.2</td>
</tr>
<tr>
<td>Group 6</td>
<td>Sim.Car</td>
<td>119.9 ± 17.1</td>
<td>41.3 ± 6.5</td>
<td>309.7 ± 67.0</td>
<td>6.1 ± 0.7*</td>
<td>47.1 ± 1.4*</td>
<td>65.2 ± 10.9</td>
</tr>
<tr>
<td>Group 7</td>
<td>Sim.Car.Supp</td>
<td>111.7 ± 22.3</td>
<td>33.7 ± 7.5</td>
<td>250.3 ± 49.9</td>
<td>5.2 ± 0.3*</td>
<td>47.3 ± 2.3*</td>
<td>72.4 ± 7.1</td>
</tr>
</tbody>
</table>

All values are expressed as mean ± SD. (*) Significantly different from ConS group; (#) Significantly different from ConHLP group; \(p < 0.05\).
(interlobular artery and vein and interlobular bile ducts) and rare lymphocytes. PAS staining shows a uniform, homogeneous distribution of glycogen in hepatocytes.

The liver tissue of the ConHLP group shows a typical, lobulated structure and centrally located central veins. Hepatocytes show intact cell membranes. Pronounced perinuclear halo and hydropic degeneration are observed in some hepatocytes as an indicator of damage to the cell ultrastructure. In some of the tissue samples, smaller accumulations of inflammatory cells (lymphocytes)
Antihyperlipidemic potential of dietary supplementation with carnosine in high-fat diet-fed rats

...were present in the lobules and in the connective tissue of the portal spaces. The port spaces at the corners of the lobules contain thin to moderately abundant connective tissue containing elements of the port triad. PAS staining is less pronounced in the central parts of the lobules, indicating glycogen depletion in hepatocytes.

The use of carnosine itself and carnosine supplement (groups Car and Car.Supp) did not significantly affect the normalization of histological changes in the liver. Disturbance of the hepatocyte ultrastructure is evident, with the appearance of macrovesicular fat change in some animals. Fewer hepatocytes with signs of degeneration and perinuclear illumination were observed in the liver tissue of Sim, Sim.Car and Sim.Car.Supp groups. In animals treated with statins and any formulation of carnosine, there is no occurrence of inflammatory infiltration in the lobules and portal spaces (Figure 1).

**Effects of Carnosine Supplementation on the Biochemical and Immunohistochemical Inflammation Markers**

Table IV shows the serum levels of C-reactive protein (CRP) and leptin in control and experimental obese rats treated with carnosine. The serum levels of CRP, as an acute marker of inflammation, were low in all investigated groups of animals. Besides, neither the hyperlipidemia induction nor carnosine supplementation influenced the serum concentrations of leptin, as a proinflammatory adipokine.

The results of the immunohistochemical analysis are shown in Table V and in Figure 1. In our study, the number of hepatocytes with elevated CYP2E1 activity, estimated as the percentage of liver tissue expressing positive CYP2E1 staining, showed relative decline values in groups treated with carnosine (Car) and its commercial supplement (CarSupp). Their use in combination with a statin drug (Sim.Car and Sim.Car.Supp) neutralized CYP2E1 activity and these values were statistically significantly different compared to the control group. The percentage of Iba1 positive (Iba1+) cells in the liver were lower in animals treated with carnosine (Car) and commercial carnosine dietary supplement (Car.Supp). While simvastatin alone did not affect the Iba1+ cell population, its co-administration with carnosine significantly reduced the number of Iba1+ cells.

**Table IV. Effects of carnosine supplementation on the serum levels of CRP and leptin.**

<table>
<thead>
<tr>
<th></th>
<th>CRP [mg/L]</th>
<th>Leptin [ng/mL]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group 1 ConS</td>
<td>&lt; 1.0</td>
<td>0.0283 ± 0.0256</td>
</tr>
<tr>
<td>Group 2 ConHLP</td>
<td>&lt; 1.0</td>
<td>0.0288 ± 0.0247</td>
</tr>
<tr>
<td>Group 3 Car</td>
<td>&lt; 1.0</td>
<td>0.0225 ± 0.0104</td>
</tr>
<tr>
<td>Group 4 Car.Supp</td>
<td>&lt; 1.0</td>
<td>0.0280 ± 0.0175</td>
</tr>
<tr>
<td>Group 5 Sim</td>
<td>&lt; 1.0</td>
<td>0.0300 ± 0.0082</td>
</tr>
<tr>
<td>Group 6 Sim.Car</td>
<td>&lt; 1.0</td>
<td>0.0188 ± 0.0035</td>
</tr>
<tr>
<td>Group 7 Sim.Car.Supp</td>
<td>&lt; 1.0</td>
<td>0.0220 ± 0.0063</td>
</tr>
</tbody>
</table>

**Table V. Effects of carnosine supplementation on the percentage of CYP2E1 and Iba1 positive cells in the liver following the immunohistochemical staining.**

<table>
<thead>
<tr>
<th></th>
<th>CYP2E1 [%]</th>
<th>Iba1 [%]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group 1 ConS</td>
<td>10.0 ± 7.6</td>
<td>26.1 ± 2.9</td>
</tr>
<tr>
<td>Group 2 ConHLP</td>
<td>5.1 ± 3.6</td>
<td>26.5 ± 3.4</td>
</tr>
<tr>
<td>Group 3 Car</td>
<td>4.2 ± 2.2</td>
<td>12.81 ± 5.1</td>
</tr>
<tr>
<td>Group 4 Car.Supp</td>
<td>4.3 ± 1.8</td>
<td>17.79 ± 6.8</td>
</tr>
<tr>
<td>Group 5 Sim</td>
<td>3.1 ± 1.7</td>
<td>27.47 ± 9.0</td>
</tr>
<tr>
<td>Group 6 Sim.Car</td>
<td>3.1 ± 1.3</td>
<td>17.5 ± 8.2</td>
</tr>
<tr>
<td>Group 7 Sim.Car.Supp</td>
<td>3.6 ± 1.7</td>
<td>27.20 ± 5.9</td>
</tr>
</tbody>
</table>

**Discussion**

A plethora of in vitro studies have shown the various protective activities of carnosine in multiple heterogeneous cell types, such as macrophages/microglia, myocytes, skeletal muscle myoblasts, podocytes, endothelial cells, pancreatic cells, etc. which strengthens the idea that carnosine has the potential to exert therapeutic effects in a broad spectrum of pathological conditions. Numerous studies have been published on the structure, role, function, and biological activities of carnosine under numerous experimental and clinical conditions. Nevertheless, advances need to be made in order to fully unveil the enormous therapeutic potential of this dipeptide, specifically in the context of in vivo studies, which are currently characterized by substantial heterogeneity regarding administration route, dosage, treatment duration, and animal model. In this research, we determined the influence of carnosine supplementation on lipid status, indicators of hepatic and renal function and dyslipidemia-related inflammation in high-fat diet-fed rats.

In our study, the anti-obesity effects and hypolipidemic activity of carnosine, especially in...
the form of commercial dietary supplement with other components and in the combination with simvastatin, was confirmed. In accordance with our results, Aldini et al.26 found that 24-week supplementation with both L- and D-carnosine greatly reduced obese-related diseases in non-diabetic, Zucker obese rats, by significantly restraining the development of obesity, dyslipidemia, hypertension and renal injury.27 Similarly, 6-week treatment of high-fat diet fed Sprague-Dawley rats with L-carnosine, alone or in combination with α-lipoic acid, improved serum lipid profiles by reducing LDL cholesterol serum levels and increasing HDL levels.31 On the other hand, carnosine supplementation had no effect upon the hypercholesterolemia in streptozotocin-induced diabetic mice, while it managed to significantly reduce plasma triglyceride levels.28 The mechanisms through which carnosine exerts its pharmacological effects are most likely multifactorial and involve several pathways. It has been demonstrated that carnosine is effective in reducing oxidative stress, protein carbonylation leading to advanced glycation end products (AGEs) and advanced lipoxidation end products (ALEs) and inflammation. The ability of carnosine to prevent the formation of these end products by detoxifying reactive carbonyl species (RCS) generated from lipid and sugar oxidation explains the protective effect of carnosine in atherosclerosis and diabetes complications.26 Besides, RCSs have a role in obesity-related metabolic disorders and novel compounds that mitigate the production or enhance the removal of RCSs represent the good therapeutic strategy for cardiovascular and metabolic diseases associated with obesity.36

Obesity is a chronic inflammatory disorder in which increased plasma levels of leptin and CRP have been linked to cardiovascular pathophysiological processes and increased cardiovascular risk.37 In our study, neither the hyperlipidemia induction nor carnosine supplementation influenced the serum levels of CRP and leptin. Although leptin is produced in proportion to body fat mass, factors other than adipose tissue mass may influence circulating leptin concentrations as well. Previously, it was shown that short-term high-fat diet (less than 4 weeks) is associated with lower circulating leptin concentrations than expected, which is in accordance with our results.38 Not only leptin concentration, but also
leptin methylation pattern, can be influenced by diet-induced obesity, which suggests that epigenetic mechanisms could be involved in obesity by regulating the expression of important epigenetic genes.42,

One of the consequences of hyperlipoproteinemia in tissues, and especially in the liver, is reflected in the initiation of inflammation and migration of inflammatory cells. Interleukin-6 (IL-6) and tumor necrosis factor (TNF-α) are involved in the progression of hepatotoxicity.46 The increased release of inflammatory factors could consequently lead to cytokine imbalance, immune dysfunction and even liver cell apoptosis. Thus, agents with anti-inflammatory activity may potentially improve hepatic inflammatory injury.

As CRP values did not indicate an inflammatory process, and the inflammatory infiltrate composed mainly of lymphocytes was not very pronounced, we tried to establish the presence of cells of the monocyte-macrophage system. The properties of macrophages are influenced by microenvironmental conditions. Recently, macrophages appearing in pathological lesions, are divided into classically activated macrophages (M1-macrophages) and alternatively activated macrophages (M2-macrophages). Generally, besides CD68, M1-macrophages express MHC class II and Iba1. Antibody to Iba1 is used usually to detect microglial cells, but recently Iba1 antigen has been assigned some roles in inflammation such as migration, proliferation, and signal transduction of macrophages.43 M1-macrophages, here detected by Iba1 staining, develop under the influence of IFN-γ at early stages, and become an effector that works in cell-mediated immunity as a combined response not only to IFN-γ itself, but also to TNF-β, IL-6 and IL-1β. Perhaps these inflammation markers would be more sensitive than CRP and leptin. In our study, the percentage of Iba1 positive (Iba1+) cells in the liver was lower in animals treated with carnosine dietary supplement in comparison to non-treated animals, indicating the anti-inflammatory activity of carnosine supplements.

In our study, the number of hepatocytes with elevated CYP2E1 activity, was reduced in rats treated with carnosine, especially in combination with simvastatin. CYP2E1 is a member of the P450 enzyme family that plays a vital role in alcohol, drug, toxin, lipid, and carcinogen metabolism. It is mainly expressed in hepatocytes, where it catalyzes the conversion of its substrates into more polar metabolites for secretion or use as substrates for other microsomal phase II enzymes. CYP2E1 also transfers active electrons from reduced nicotinamide adenine dinucleotide phosphate (NADPH) or reduced nicotinamide adenine dinucleotide to oxygen to produce reactive oxygen species (ROS). CYP2E1-induced toxic metabolites coupled with oxidative stress from ROS are proposed to be important mediators of liver injury by promoting an inflammatory and fibrogenic milieu to facilitate recruitment of leukocytes and activation of hepatic stellate cells (HSCs). CYP2E1 thus plays a critical role in oxidative stress and liver damage.44,45 In accordance with our results, CYP2E1 suppressive effect has been documented by Liu et al.46 in their study on carnosine and histidine prevention of alcohol-induced hepatotoxicity.

Given that the most pronounced results of the present study were obtained for the carnosine commercial supplement, it should be noted that additive or synergistic effects with other constituents of the investigated commercial dietary supplement are also possible. The results of meta-analyses showed a significant reduction of lipoprotein(a) levels, as well as serum CRP and TNF-α concentrations, following L-carnitine supplementation, which may also contribute to the hypolipidemic potential of the investigated formulation. Meta-analysis results demonstrated also that CoQ10 may significantly decrease total cholesterol and increase HDL cholesterol levels in patients with coronary artery disease, with no effect on LDL-cholesterol, triglycerides, and lipoprotein(a) levels.47 Several mechanisms of lipid-lowering action of vitamin E have been suggested, such as peroxisome proliferator-activated receptors (PPARs) activation, HMG-CoA reductase inhibition, and radical-scavenging efficacy. Although vitamin E does not seem to be an effective lipid-lowering agent, its supplementation might be associated with reduced risk of fatal myocardial infarction.

Conclusions

The overall data obtained by the present study demonstrated the hypolipidemic activity of carnosine, especially in the form of commercial dietary supplement with other components and in the combination with simvastatin as a conventional drug in dyslipidemia management in primary prevention of cardiovascular diseases. Dietary carnosine supplementation resulted also in anti-inflammatory effects. Besides, the good
safety profile of carnosine makes it an attractive adjunct to pharmacological management of metabolic syndrome. The use of carnosine supplements in preventing and/or treatment of metabolic disorders requires further investigations on the mechanisms of action and potential interactions with the conventional therapy.

**Conflict of Interest**
The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

**Informed Consent**
Not applicable.

**Funding**
This work was supported by the Provincial Secretariat for Higher Education and Scientific Research project No. 142-451-2574/2021-01/01.

**Authors’ Contributions**
AR, OH, NS and MK contributed to conception and design of the study. NM, DZ, DP and PA performed the experiments. DM, NM and PA contributed to sample preparation. RM performed the biochemical analysis. BAV, MV, RM, IC and PA wrote sections of the manuscript. AR and MV were involved in interpretation of the results. NM, DZ and NP wrote the first draft of the manuscript. BAV, MV, RM, IC and PA wrote and supervised the manuscript. AR provided the funding and supervised the project administration. All authors contributed to manuscript revision, read, and approved the submitted version.

**Availability of Data and Materials**
All generated data is available upon the reasonable request from the authors.

**Ethics Approval**
The study was conducted after obtaining the approval of the Ethics Commission for the Protection of Laboratory Animals of the University of Novi Sad (Novi Sad, Serbia; No. 01-107/6-1) and the Ministry of Agriculture and Environmental Protection of the Republic of Serbia (Belgrade, Serbia; No. 323-07-04785/2019-05).

**References**


Antihyperlipidemic potential of dietary supplementation with carnosine in high-fat diet-fed rats


