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

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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[1]. 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[2,3].

Hyperlipidemia is considered to be one of the major risk factors in developing cardiovascular events, including myocardial infarction and stroke[4,5]. 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[4]. Thus, adequate management of lipid metabolism and hyperlipidemia has an essen-

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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[6]. 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[7,8].

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[9,10]. *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[4,6]. Numerous studies[5,6,9] 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[11]. 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 are 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[4,9,10]. Mentioned effects seem to be the major reason for patients to discontinue the therapy, as non-adherence to statin treatment is a common issue[6,12-14].

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[15-17]. 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[17,18]. 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[2,19].

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[20,21]. 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[22]. 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[20,23]. 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[20,21,24]. 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 (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 end of the experiment. At the end of the experiment, all animals were anesthetized with urethane and sacrificed by cardio punction 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

Table I. Effects of carnosine supplementation on the body weight change of animals.

		Initial body weight [g]	Terminal body weight [g]	Difference, Δ [g]
Group 1	ConS	185.0 ± 12.2	301.5 ± 9.9	116.5
Group 2	ConHLP	208.2 ± 41.7	394.0 ± 59.9	185.8
Group 3	Car	219.1 ± 21.9	334.7 ± 18.6	115.6
Group 4	Car.Supp	234.6 ± 24.1	344.3 ± 27.0	109.7
Group 5	Sim	227.3 ± 55.2	336.2 ± 55.6	108.9
Group 6	Sim.Car	240.7 ± 39.9	366.1 ± 59.3	125.4
Group 7	Sim.Car.Supp	259.2 ± 50.8	365.8 ± 57.1	106.6

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

		Triglycerides [mmol/L]	Total cholesterol [mmol/L]	LDL [mmol/L]	HDL [mmol/L]	LDL coefficient	Atherogenic index
Group 1	ConS	0.94 ± 0.12	1.64 ± 0.21	0.52 ± 0.13	0.70 ± 0.07	2.32 ± 0.15	0.72 ± 0.13
Group 2	ConHLP	1.08 ± 0.27	5.42 ± 1.45*	4.39 ± 1.41*	0.56 ± 0.03*	9.85 ± 3.03*	7.91 ± 2.86*
Group 3	Car	1.07 ± 0.33	4.21 ± 0.58*	3.20 ± 0.63*	0.52 ± 0.08*	8.28 ± 2.03*	6.36 ± 2.01*
Group 4	Car.Supp	1.25 ± 0.32	4.10 ± 0.86* #	2.99 ± 0.85* #	0.52 ± 0.06*	8.02 ± 1.98*	5.94 ± 1.86*
Group 5	Sim	0.98 ± 0.29	3.47 ± 0.83* #	2.44 ± 0.92* #	0.60 ± 0.05	5.91 ± 1.98* #	4.14 ± 1.96* #
Group 6	Sim.Car	1.09 ± 0.50	3.25 ± 0.46* #	2.11 ± 0.67* #	0.65 ± 0.10	5.23 ± 1.66#	3.46 ± 1.69#
Group 7	Sim.Car.Supp	1.26 ± 0.34	3.05 ± 0.39* #	1.89 ± 0.30* #	0.59 ± 0.08	5.19 ± 0.83#	3.23 ± 0.70#

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

it as well, but without statistical significance (p=0.41) (Table II).

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

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

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

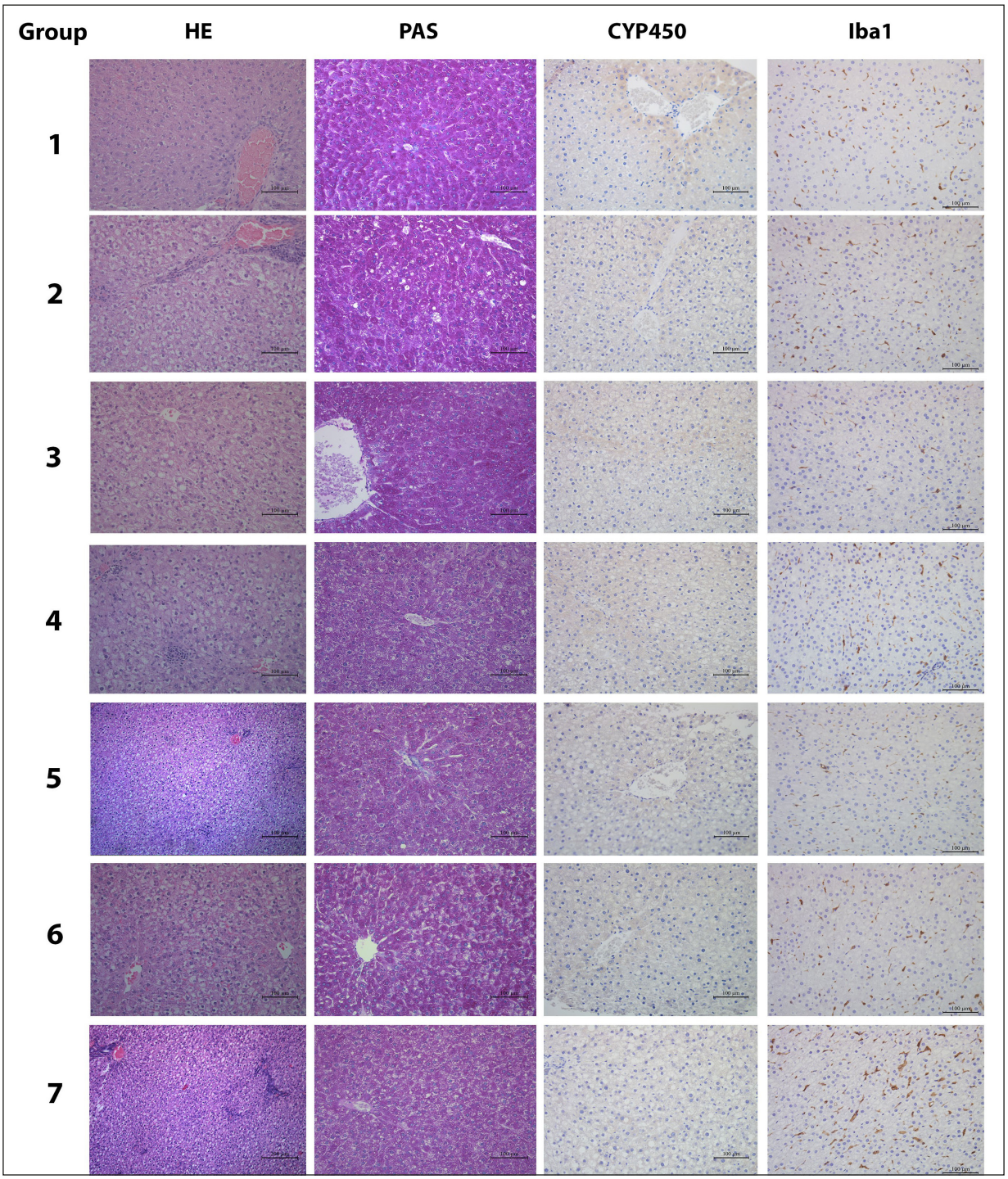

Figure 1. Histological examination of liver structure (20×).

(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)

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.

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

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

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

Discussion

A plethora of *in vitro* studies[20-22] 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[21-23,25] 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[25]. 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[28]. 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[29]. However, it was shown that while carnosine appears to have a consistent cross-species hypotriglyceridemic action, this is not true for cholesterol levels. In the same study, carnosine supplementation did not reduce the plaque area in the brachiocephalic artery and aortic sinus, when compared to the non-supplemented mice matched for glycemic status, despite its triglyceride-lowering effects. It was suggested that carnosine can modulate oxidation and glycation reactions by acting as a scavenger of both radicals and reactive aldehydes, such as methylglyoxal, and the lipid-derived aldehydes, malondialdehyde and 4-hydroxynonenal, and thus prevent pro-atherosclerotic aldehyde-mediated modification of low-density lipoproteins[29]. In another study[30], it was demonstrated in apoE-null mice that a readily bioavailable analog of carnosine, octyl-D-carnosine, prevents LDL oxidation and forms stable covalent conjugates with aldehydes generated during LDL oxidation, despite the fact it did not affect animals body weight or plasma cholesterol levels.

We determined that carnosine supplementation is safe for use, considering its effects on hepatic and renal functions. Induction of hyperlipidemia slightly increased activities of AST and ALT in serum, and carnosine commercial supplement in combination with simvastatin managed to significantly reduce AST levels. Nephroprotective effects were demonstrated too, as manifested through the reduction of creatinine serum levels. Similarly, in a study of Fatih Aydın et al[31] 4-week carnosine treatment decreased serum lipids, creatinine, and urea levels in high-fat diet fed streptozotocin-induced diabetic rats. On the other hand, it did not alter glucose and HbA1c levels[31]. In obese Zucker rats, both L- and D-CAR exerted nephroprotective effects, as demonstrated by both urinary parameters and electron microscopy examinations of renal tissue[27]. Studies[32-34] on the nephroprotective activity of carnosine in rat models in which kidney damage was caused by nephrotoxic agents such as gentamicin, ifosfamide, nickel, were also performed, and they demonstrated that carnosine in pretreated animals may normalize urea and creatinine levels and reduce kidney damage.

The mechanism by which carnosine supplementation impacts the metabolic parameters in animal models has not been well understood yet. It was suggested that carnosine can serve as a histamine precursor and act *via* the H3 receptor to control the autonomic nervous system for regulation of blood glucose, as well as induce lipoprotein lipase activity and serve as a free radical scavenger to reduce lipid parameters[35]. However, 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 epiobesigenic genes[39].

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[40]. 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 Iba-1[41]. 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[42]. 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[41].

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[43-45]. 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[46].

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[15]. 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[15].

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.

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Authors' Contributions
AR, OH, NS and MK contributed to conception and design of the study. NM, DZ, DP and PA performed the experiments. DZ, NM and PA contributed to sample preparation. RM performed the biochemical analysis. BAV and IC performed the histology assessment. 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 sections of the manuscript. AR and NDK revised 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 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).

References

1) Bovolini A, Garcia J, Andrade MA, Duarte JA. Metabolic Syndrome Pathophysiology and Predisposing Factors. Int J Sports Med 2021; 42: 199-214.
2) Varghese JF, Patel R, Yadav UCS. Novel Insights in the Metabolic Syndrome-induced Oxidative Stress and Inflammation-mediated Atherosclerosis. Curr Cardiol Rev 2018; 14: 4-14.
3) Fahed G, Aoun L, Bou Zerdan M, Allam S, Bou Zerdan M, Bouferraa Y, Assi HI. Metabolic Syndrome: Updates on Pathophysiology and Management in 2021. Int J Mol Sci 2022; 23: 786.
4) Karr S. Epidemiology and management of hyperlipidemia. Am J Manag Care 2017; 23: S139-S148.
5) Ravnskov U, Alabdulgader A, de Lorgeril M, Diamond DM, Hama R, Hamazaki T, Hammarskjöld B, Harcombe Z, Kendrick M, Langsjoen P, McCully KS, Okuyama H, Sultan S, Sundberg R. The new European guidelines for prevention of cardiovascular disease are misleading. Expert Rev Clin Pharmacol 2020; 13: 1289-1294.
6) Volpe M, Volpe R, Gallo G, Presta V, Tocci G, Folco E, Peracino A, Tremoli E, Trimarco B. 2017 Position Paper of the Italian Society for Cardiovascular Prevention (SIPREC) for an Updated Clinical Management of Hypercholesterolemia and Cardiovascular Risk: Executive Document. High Blood Press Cardiovasc Prev 2017; 24: 313-329.
7) Dommermuth R, Ewing K. Metabolic Syndrome: Systems Thinking in Heart Disease. Prim Care 2018; 45: 109-129.
8) Saklayen MG. The Global Epidemic of the Metabolic Syndrome. Curr Hypertens Rep 2018; 20: 12.
9) Grundy SM, Stone NJ, Bailey AL, Beam C, Birtcher KK, Blumenthal RS, Braun LT, de Ferranti S, Faiella-Tommasino J, Forman DE, Goldberg R, Heidenreich PA, Hlatky MA, Jones DW, Lloyd-Jones D, Lopez-Pajares N, Ndumele CE, Orringer CE, Peralta CA, Saseen JJ, Smith SC, Jr., Sperling L, Virani SS, Yeboah J. 2018 AHA/ACC/AACVPR/AAPA/ABC/ACPM/ADA/AGS/APhA/ASPC/NLA/PCNA Guideline on the Management of Blood Cholesterol: A Report of the American College of Cardiology/American Heart Association Task Force on Clinical Practice Guidelines. Circulation 2019; 139: e1082-e1143.
10) Climent E, Benaiges D, Pedro-Botet J. Hydrophilic or Lipophilic Statins? Front Cardiovasc Med 2021; 8: 687585.
11) García-Fernández-Bravo I, Torres-Do-Rego A, López-Farré A, Galeano-Valle F, Demelo-Rodriguez P, Alvarez-Sala-Walther LA. Undertreatment or Overtreatment With Statins: Where Are We? Front Cardiovasc Med 2022; 9: 808712.
12) Nelson AJ, Puri R, Nissen SE. Statins in a Distorted Mirror of Media. Curr Atheroscler Rep 2020; 22: 37.
13) Mohammadkhani N, Gharbi S, Rajani HF, Farzaneh A, Mahjoob G, Hoseinsalari A, Korsching E. Statins: Complex outcomes but increasingly helpful treatment options for patients. Eur J Pharmacol 2019; 863: 172704.
14) Krähenbühl S, Pavik-Mezzour I, von Eckardstein A. Unmet Needs in LDL-C Lowering: When Statins Won't Do! Drugs 2016; 76: 1175-1190.

15) Cicero AFG, Colletti A, Bajraktari G, Descamps O, Djuric DM, Ezhov M, Fras Z, Katsiki N, Langlois M, Latkovskis G, Panagiotakos DB, Paragh G, Mikhailidis DP, Mitchenko O, Paulweber B, Pella D, Pitsavos C, Reiner Ž, Ray KK, Rizzo M, Sahebkar A, Serban MC, Sperling LS, Toth PP, Vinereanu D, Vrablík M, Wong ND, Banach M. Lipid-lowering nutraceuticals in clinical practice: position paper from an International Lipid Expert Panel. Nutr Rev 2017; 75: 731-767.

16) Sosnowska B, Penson P, Banach M. The role of nutraceuticals in the prevention of cardiovascular disease. Cardiovasc Diagn Ther 2017; 7: S21-S31.

17) Poli A, Visioli F. Pharmacology of Nutraceuticals with Lipid Lowering Properties. High Blood Press Cardiovasc Prev 2019; 26: 113-118.

18) Mach F, Baigent C, Catapano AL, Koskinas KC, Casula M, Badimon L, Chapman MJ, De Backer GG, Delgado V, Ference BA, Graham IM, Halliday A, Landmesser U, Mihaylova B, Pedersen TR, Riccardi G, Richter DJ, Sabatine MS, Taskinen MR, Tokgozoglu L, Wiklund O. 2019 ESC/EAS Guidelines for the management of dyslipidaemias: lipid modification to reduce cardiovascular risk. Eur Heart J 2020; 41: 111-188.

19) Banach M, Patti AM, Giglio RV, Cicero AFG, Atanasov AG, Bajraktari G, Bruckert E, Descamps O, Djuric DM, Ezhov M, Fras Z, von Haehling S, Katsiki N, Langlois M, Latkovskis G, Mancini GBJ, Mikhailidis DP, Mitchenko O, Moriarty PM, Muntner P, Nikolic D, Panagiotakos DB, Paragh G, Paulweber B, Pella D, Pitsavos C, Reiner Ž, Rosano GMC, Rosenson RS, Rysz J, Sahebkar A, Serban MC, Vinereanu D, Vrablík M, Watts GF, Wong ND, Rizzo M. The Role of Nutraceuticals in Statin Intolerant Patients. J Am Coll Cardiol 2018; 72: 96-118.

20) Boldyrev AA, Aldini G, Derave W. Physiology and pathophysiology of carnosine. Physiol Rev 2013; 93: 1803-1845.

21) Jukić I, Kolobarić N, Stupin A, Matić A, Kozina N, Mihaljević Z, Mihalj M, Šušnjara P, Stupin M, Ćurić Ž B, Selthofer-Relatić K, Kibel A, Lukinac A, Kolar L, Kralik G, Kralik Z, Széchenyi A, Jozanović M, Galović O, Medvidović-Kosanović M, Drenjančević I. Carnosine, Small but Mighty-Prospect of Use as Functional Ingredient for Functional Food Formulation. Antioxidants (Basel) 2021; 10: 1037.

22) Prokopieva VD, Yarygina EG, Bokhan NA, Ivanova SA. Use of Carnosine for Oxidative Stress Reduction in Different Pathologies. Oxid Med Cell Longev 2016; 2016: 2939087.

23) Wu G. Important roles of dietary taurine, creatine, carnosine, anserine and 4-hydroxyproline in human nutrition and health. Amino acids 2020; 52: 329-360.

24) Chmielewska K, Dzierzbicka K, Inkielewicz-Stępniak I, Przybyłowska M. Therapeutic Potential of Carnosine and Its Derivatives in the Treatment of Human Diseases. Chem Res Toxicol 2020; 33: 1561-1578.

25) Caruso G. Unveiling the Hidden Therapeutic Potential of Carnosine, a Molecule with a Multimodal Mechanism of Action: A Position Paper. Molecules 2022; 27: 3303.

26) Aldini G, de Courten B, Regazzoni L, Gilardoni E, Ferrario G, Baron G, Altomare A, D'Amato A, Vistoli G, Carini M. Understanding the antioxidant and carbonyl sequestering activity of carnosine: direct and indirect mechanisms. Free Radic Res 2021; 55: 321-330.

27) Aldini G, Orioli M, Rossoni G, Savi F, Braidotti P, Vistoli G, Yeum KJ, Negrisoli G, Carini M. The carbonyl scavenger carnosine ameliorates dyslipidaemia and renal function in Zucker obese rats. J Cell Mol Med 2011; 15: 1339-1354.

28) Kim MY, Kim EJ, Kim YN, Choi C, Lee BH. Effects of α-lipoic acid and L-carnosine supplementation on antioxidant activities and lipid profiles in rats. Nutr Res Pract 2011; 5: 421-428.

29) Brown BE, Kim CH, Torpy FR, Bursill CA, McRobb LS, Heather AK, Davies MJ, van Reyk DM. Supplementation with carnosine decreases plasma triglycerides and modulates atherosclerotic plaque composition in diabetic apo E(-/-) mice. Atherosclerosis 2014; 232: 403-409.

30) Barski OA, Xie Z, Baba SP, Sithu SD, Agarwal A, Cai J, Bhatnagar A, Srivastava S. Dietary carnosine prevents early atherosclerotic lesion formation in apolipoprotein E-null mice. Arterioscler Thromb Vasc Biol 2013; 33: 1162-1170.

31) Fatih Aydın A, Küçükgergin C, Bingül İ, Doğan-Ekici I, Doğru-Abbasoğlu S, Uysal M. Effect of Carnosine on Renal Function, Oxidation and Glycation Products in the Kidneys of High-Fat Diet/Streptozotocin-Induced Diabetic Rats. Exp Clin Endocrinol Diabetes 2017; 125: 282-289.

32) Soliman KM, Abdul-Hamid M, Othman AI. Effect of carnosine on gentamicin-induced nephrotoxicity. Med Sci Monit 2007; 13: 73-83.

33) Ommati MM, Farshad O, Ghanbarinejad V, Mohammadi HR, Khadijeh M, Negar A, Zahra M, Ilkhaninasab F, Moezi L, Heidari R. The Nephroprotective Role of Carnosine Against Ifosfamide-Induced Renal Injury and Electrolytes Imbalance is Mediated Via the Regulation of Mitochondrial Function and Alleviation of Oxidative Stress. Drug Res (Stuttg) 2020; 70: 49-56.

34) Hasanein P, Felegari Z. Chelating effects of carnosine in ameliorating nickel-induced nephrotoxicity in rats. Can J Physiol Pharmacol 2017; 95: 1426-1432.

35) Peng W, Mao P, Liu L, Chen K, Zhong Y, Xia W, Guo Q, Tan SC, Rahmani J, Kord Varkaneh H, He P. Effect of carnosine supplementation on lipid profile, fasting blood glucose, HbA1C and insulin resistance: A systematic review and meta-analysis of long-term randomized controlled trials. Complement Ther Med 2020; 48: 102241.

36) Anderson EJ, Vistoli G, Katunga LA, Funai K, Regazzoni L, Monroe TB, Gilardoni E, Cannizzaro L, Colzani M, De Maddis D, Rossoni G, Canevotti R,

Gagliardi S, Carini M, Aldini G. A carnosine analog mitigates metabolic disorders of obesity by reducing carbonyl stress. J Clin Invest 2018; 128: 5280-5293.

37) Hribal ML, Fiorentino TV, Sesti G. Role of C reactive protein (CRP) in leptin resistance. Curr Pharm Des 2014; 20: 609-615.

38) Ainslie DA, Proietto J, Fam BC, Thorburn AW. Short-term, high-fat diets lower circulating leptin concentrations in rats. Am J Clin Nutr 2000; 71: 438-442.

39) Milagro FI, Campión J, García-Díaz DF, Goyenechea E, Paternain L, Martínez JA. High fat diet-induced obesity modifies the methylation pattern of leptin promoter in rats. J Physiol Biochem 2009; 65: 1-9.

40) Ridker PM. Hyperlipidemia as an instigator of inflammation: inaugurating new approaches to vascular prevention. J Am Heart Assoc 2012; 1: 3-5.

41) Yamate J, Izawa T, Kuwamura M. Histopathological Analysis of Rat Hepatotoxicity Based on Macrophage Functions: in Particular, an Analysis for Thioacetamide-induced Hepatic Lesions. Food Saf (Tokyo) 2016; 4: 61-73.

42) Wijesundera KK, Juniantito V, Golbar HM, Fujisawa K, Tanaka M, Ichikawa C, Izawa T, Kuwamura M, Yamate J. Expressions of Iba1 and galectin-3 (Gal-3) in thioacetamide (TAA)-induced acute rat liver lesions. Exp Toxicol Pathol 2013; 65: 799-808.

43) Kessova I, Cederbaum AI. CYP2E1: biochemistry, toxicology, regulation and function in ethanol-induced liver injury. Curr Mol Med 2003; 3: 509-518.

44) Bardag-Gorce F, Li J, French BA, French SW. The effect of ethanol-induced CYP2E1 on proteasome activity: the role of 4-hydroxynonenal. Exp Mol Pathol 2005; 78: 109-115.

45) Xu J, Ma HY, Liang S, Sun M, Karin G, Koyama Y, Hu R, Quehenberger O, Davidson NO, Dennis EA, Kisseleva T, Brenner DA. The role of human cytochrome P450 2E1 in liver inflammation and fibrosis. Hepatol Commun 2017; 1: 1043-1057.

46) Liu WH, Liu TC, Yin MC. Beneficial effects of histidine and carnosine on ethanol-induced chronic liver injury. Food Chem Toxicol 2008; 46: 1503-1509.

47) Rivellese AA, Ciciola P, Costabile G, Vetrani C, Vitale M. The Possible Role of Nutraceuticals in the Prevention of Cardiovascular Disease. High Blood Press Cardiovasc Prev 2019; 26: 101-111.