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Effect of carnosine treatment on oxidative stress in serum, apoB-containing lipoproteins fraction and erythrocytes of aged rats

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Abstract:
One of the mechanisms underlying the aging process is proposed to be oxidative damage by free radicals. Carnosine (β-alanyl-L-histidine) is a dipeptide with antioxidant properties. In this study, we investigated the effect of carnosine supplementation on oxidative stress in serum, apoB-containing lipoproteins (LDL + VLDL) and erythrocytes of young and aged rats. At the initiation of the study, young and aged rats were 5 and 22 months old, respectively. Carnosine (250 mg/kg, daily, *ip*) was administered for 1 month to young and aged rats. We found that serum malondialdehyde (MDA) and diene conjugate (DC) levels and endogenous DC and copper-induced MDA levels in the LDL + VLDL fraction increased in aged rats, but there was no change in plasma antioxidant activity. Endogenous DC and H_2O_2-induced MDA levels were also higher, but glutathione (GSH) levels were lower in erythrocytes of aged rats. Administration of carnosine for 1 month to aged rats resulted in decreased levels of MDA and DC in serum, the LDL + VLDL fraction and erythrocytes and increased levels of GSH in erythrocytes. Our findings indicate that *in vivo* carnosine treatment may be useful for the decrease in aged-induced oxidative stress in serum, the LDL + VLDL fraction and erythrocytes.

Key words:
carnosine, serum, apoB-containing lipoproteins, erythrocytes, aged rats

Abbreviations: AOA – antioxidant activity, DC – diene conjugate, FRAP – ferric reducing antioxidant power, GSH – glutathione, LDL – low-density lipoproteins, MDA – malondialdehyde, VLDL – very low-density lipoproteins

Introduction

Aging is a complex process that results in some physiological changes. One of the mechanisms underlying the aging process is the oxidative damage caused by free radicals [9]. Increases in oxidative stress parameters in plasma [15, 39], plasma lipoproteins [15, 28], erythrocytes [29, 40] and several tissues such as liver, heart and brain [23, 30, 31, 35, 39] have been observed in experimental animals. Increases in oxidative stress parameters have also been detected in humans with aging [5, 26, 38]. Oxidative stress plays an important role in aging, and supplementation of several antioxidants is reported to be beneficial in reversing the deleterious effects of free radicals on aging [9], although some controversial data are available [8, 17].

Carnosine (β-alanyl-L-histidine) is a dipeptide having strong antioxidant effects [2, 10, 20]. The antioxidant potential of carnosine was suggested to be dependent on its ability to inactivate reactive oxygen species, scavenge free radicals and chelate prooxidant metals [2, 10, 20]. Some investigators have reported that carnosine may have anti-aging actions [11, 19, 20]. Therefore, carnosine supplementation may be an effective agent to decrease prooxidant status in aging and age-related pathologies such as atherosclerosis and diabetes mellitus [20, 24]. Indeed, we recently reported that carnosine treatment decreased lipid peroxide levels in the liver, but not the heart and brain, of aged rats. Although there were no changes in non-enzymatic and enzymatic antioxidants in the heart and brain of carnosine-treated aged rats, vitamin E and superoxide dismutase activity increased in the liver of aged rats due to carnosine treatment [3].

In this study, we investigated how carnosine supplementation influenced oxidative stress in serum, apoB-containing lipoproteins and erythrocytes in young and aged animals.

Materials and Methods

Animals and treatments

Young and aged male Wistar rats were obtained from the Center for Experimental Medical Research Institute of Istanbul University. The animals were allowed free access to food and water and were kept in wire-bottomed stainless steel cages. Food intake was controlled periodically to avoid differences between the groups in the amount of food consumption. All experimental procedures used in this study met the guidelines of the Animal Care and Use Committee of the University of Istanbul.

At the initiation of the study, young and aged rats were 5 and 22 months old, respectively. Young and aged rats were divided into two subgroups as untreated and carnosine-treated rats. Carnosine and other chemicals were purchased from Sigma Aldrich Co. (USA). Carnosine (250 mg/kg, *ip*) was given daily for 1 month. At the end of this period, blood was collected following an overnight fasting period by cardiac puncture into dry tubes and ethylenediaminetetraacetic acid (EDTA)-containing tubes. EDTA-plasma and serum were obtained by centrifugation at 1500 × *g* for 10 min and stored at –80°C until they were analyzed.

The determinations in serum/plasma

The degree of endogenous lipid peroxidation in serum was assessed by two different methods. First, serum diene conjugate (DC) formation was determined spectrophotometrically at 234 nm [12]. For this assay, serum lipids were extracted with a chloroform/methanol (2:1, v/v) mixture. The extracted lipids were redissolved in cyclohexane, and the amounts of hydroperoxides were calculated using a molar extinction coefficient of 2.52×10^{-4} M^{-1} cm^{-1}. Second, malondialdehyde (MDA) levels were determined using thiobarbituric acid according to the method of Buege and Aust [12]. Plasma antioxidant activity (AOA) was evaluated using the ferric reducing antioxidant power (FRAP) assay [6]. The FRAP assay uses antioxidants as reductants in a redox-linked colorimetric method.

The determinations in apoB-100-containing lipoproteins (LDL + VLDL)

ApoB-100-containing lipoproteins (low-density plus very low-density lipoproteins; LDL + VLDL) were precipitated from EDTA-plasma by a dextran sulfate and $MgCl_2$ solution, pH 7.0. The pellet was suspended in 0.9% NaCl and reprecipitated by adding precipitation reagent, vortexing and centrifuging in order to remove EDTA from the non-HDL fraction. The pellet was redissolved with phosphate buffered saline (0.68 M NaCl, 10 mM NaH_2PO_4, pH 7.0) to obtain the LDL + VLDL fraction [41], and protein levels were determined in this fraction [36]. Lipids were extracted from the LDL + VLDL samples with chloroform-methanol (2:1, v/v), dried under nitrogen, then redissolved in cyclohexane, and analyzed spectrophotometrically at 234 nm to measure endogenous DC levels [1]. Absorbance units were converted to molar units using the molar extinction coefficient 2.95×10^4 $M^{-1}cm^{-1}$. Values were expressed as nmol DC/mg LDL + VLDL protein. To determine Cu-induced MDA levels, the LDL + VLDL fraction (200 g protein) was incubated with copper sulfate (final copper concentration, 50 M) at 37°C for 3 h. MDA produced during this period was estimated by taking the difference in levels from 0 h [41]. Values were expressed as nmol MDA/mg LDL + VLDL protein.

Determinations in erythrocytes

Lipids from erythrocytes were extracted with isopropanol-chloroform (11:7, v/v) by the method of Rose and Oklander [34]. DC formation was also determined in lipid extracts from erythrocytes. Extracted lipids were evaporated and dissolved in cyclohexane, and DCs were measured at 234 nm [12]. Erythrocyte susceptibility to lipid peroxidation was determined according to the method of Stocks and Dormandy [37]. The final composition of the incubation mixture was 5 mM H_2O_2, 2 mM sodium azide, and the erythrocyte suspension in phosphate buffered saline (30 mg hemoglobin (Hb)/ml incubation mixture). Lipid peroxidation was assayed by measurement of MDA production during a 2 h incubation period at 37°C. Values were expressed as nmol MDA/g Hb. Hb concentrations in erythrocyte suspensions and whole blood were measured with Drabkin's reagent. Glutathione levels in erythrocytes were determined according to Beutler et al. [7].

Statistical analysis

The results are expressed as the mean ± SD. Experimental groups were compared using the Kruskal-Wallis variance analysis test. When significant effects were found, *post-hoc* analysis using the Mann-Whitney U test was performed, and $p < 0.05$ was considered to be statistically significant.

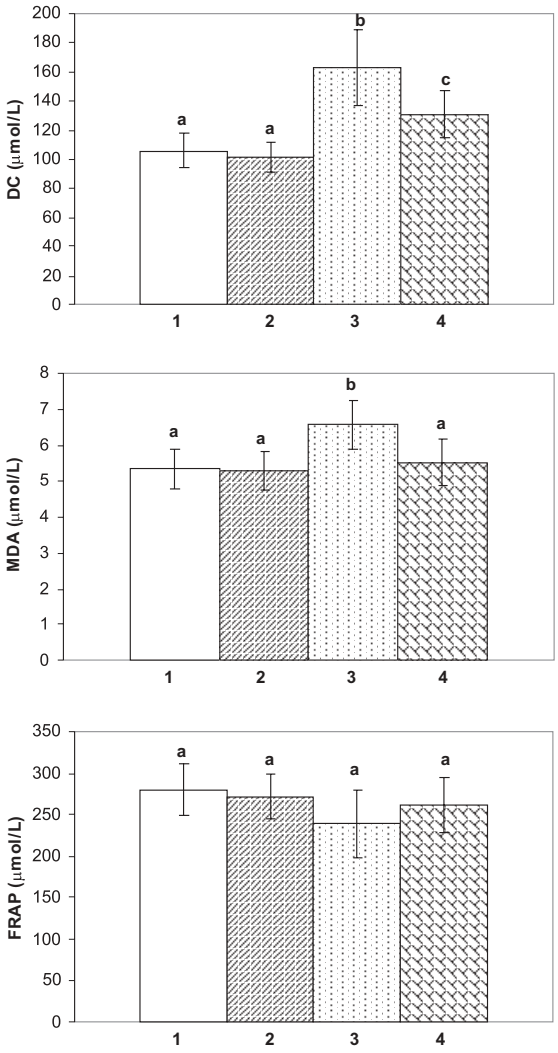

Fig. 1. Serum diene conjugate (DC) and malondialdehyde (MDA) levels and plasma ferric reducing antioxidant power (FRAP) values in young (1), carnosine-treated young (2), aged (3) and carnosine-treated aged (4) rats (the mean ± SD; n = 6 in groups 1 and 2; n = 8 in groups 3 and 4). Values with different superscripts (a-c) are significantly different by the Kruskal-Wallis test followed by the Mann-Whitney U test; $p < 0.05$

Results

When carnosine-treated and untreated groups were compared, the weight gain of rats during the experimental period was not significantly different in young and old rats (data not shown).

As shown in Figures 1 and 2, DC and MDA levels in the serum and endogenous DC and copper-induced MDA levels in the LDL + VLDL fraction were increased in aged rats as compared with young rats, but there was no change in plasma AOA. Endogenous DC and H_2O_2-induced MDA levels were also higher, but GSH levels were lower in erythrocytes of aged rats (Fig. 3). Administration of carnosine for 1 month to aged rats resulted in decreased levels of lipid peroxide in serum, the LDL + VLDL fraction and erythrocytes and increased levels of GSH in erythrocytes. However, there were no changes in these parameters due to carnosine treatment in young rats (Figs. 1–3).

Discussion

Oxidative stress can be assayed by measuring oxidized lipids, protein carbonyls, fragmented DNA and

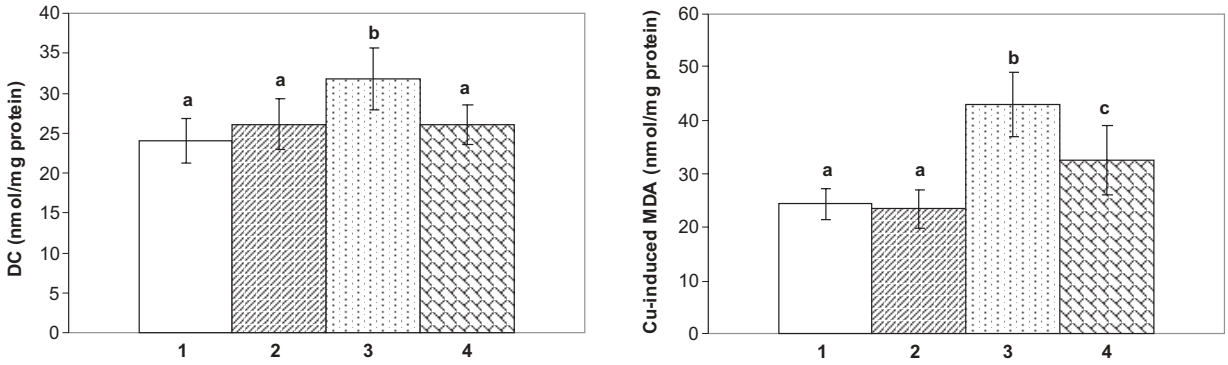

Fig. 2. Endogenous diene conjugate (DC) and copper-induced malondialdehyde (MDA) levels in the LDL + VLDL fraction of young (1), carnosine-treated young (2), aged (3) and carnosine-treated aged (4) rats (the mean ± SD; n = 6 in groups 1 and 2; n = 8 in groups 3 and 4). Values with different superscripts (a-c) are significantly different by the Kruskal-Wallis test followed by the Mann-Whitney U test; $p < 0.05$

DNA oxidation products and assessing the activities or levels of antioxidants [18]. Tests used to evaluate the products of lipid peroxidation in serum include determinations of MDA, lipid hydroperoxides, DCs and F_2-isoprostanes [18]. Although several substances have interfering effects on this assay, the MDA assay is the most frequently used method because of its simplicity. In our previous studies, we detected that serum MDA levels correlated with serum DC levels [25], serum protein carbonyl content [26] and lymphocyte DNA damage [26]. Conjugated diene measurement is also often used to study peroxidation in pure lipids, lipoproteins and membrane fractions. Because conjugated dienes in serum may not only arise from oxidative stress but may also come from dietary sources, application of this method to plasma has some problems [18]. In our study, we determined endogenous serum MDA and DC levels as well as plasma FRAP values in rats. According to our results, serum MDA levels were found to be higher in aged rats. Similarly, serum DC levels increased in aged rats. Because carnosine-treated and untreated young and old rats were fed the same diet and no difference was observed in daily food consumption between carnosine-treated and untreated young rats or carnosine-treated and untreated old rats, differences observed in serum DC levels were likely not due to dietary factors. In addition, there was a tendency towards a decrease in plasma FRAP values in the aged rats, but this decrease was not statistically significant ($p = 0.242$). These results indicate that the prooxidant-antioxidant balance is disturbed in favor of prooxidation in the serum of old rats. Our results are accordance with those of other studies [15, 39].

Increased atherosclerosis is one of the major causes of morbidity and mortality during aging. There is increasing evidence that lipoprotein oxidation is an important primary event in atherogenesis [16, 27]. Indeed, LDL oxidation was found to be increased by using different parameters in aged rats [15, 28]. Because LDL and VLDL, apoB-containing lipoproteins, are atherogenic and both oxidized LDL and oxidized VLDL play a role in the development of atherosclerotic lesions [1, 41]. Therefore, in this study, endogenous DC levels and the copper-induced MDA levels of the LDL + VLDL fraction obtained from EDTA-plasma were determined in rats by using a dextran sulfate and $MgCl_2$ solution. According to our results, endogenous DC and copper-induced MDA levels were increased in the VLDL + LDL fraction of aged rats compared with young rats.

Erythrocytes are known to be sensitive to oxidative stress because their membranes are rich in polyunsaturated fatty acids; they are continuously exposed to high concentrations of oxygen, and they contain a powerful transition metal catalyst [13]. For these reasons, erythrocytes are an appropriate model for lipid peroxidation studies. The aging process is also reported to affect the prooxidant and antioxidant balance in erythrocytes [29, 40]. In this study, significant increases were observed in endogenous DC and H_2O_2-induced MDA levels in the erythrocytes of aged rats compared with young rats. In addition, erythrocyte GSH levels decreased in aged rats.

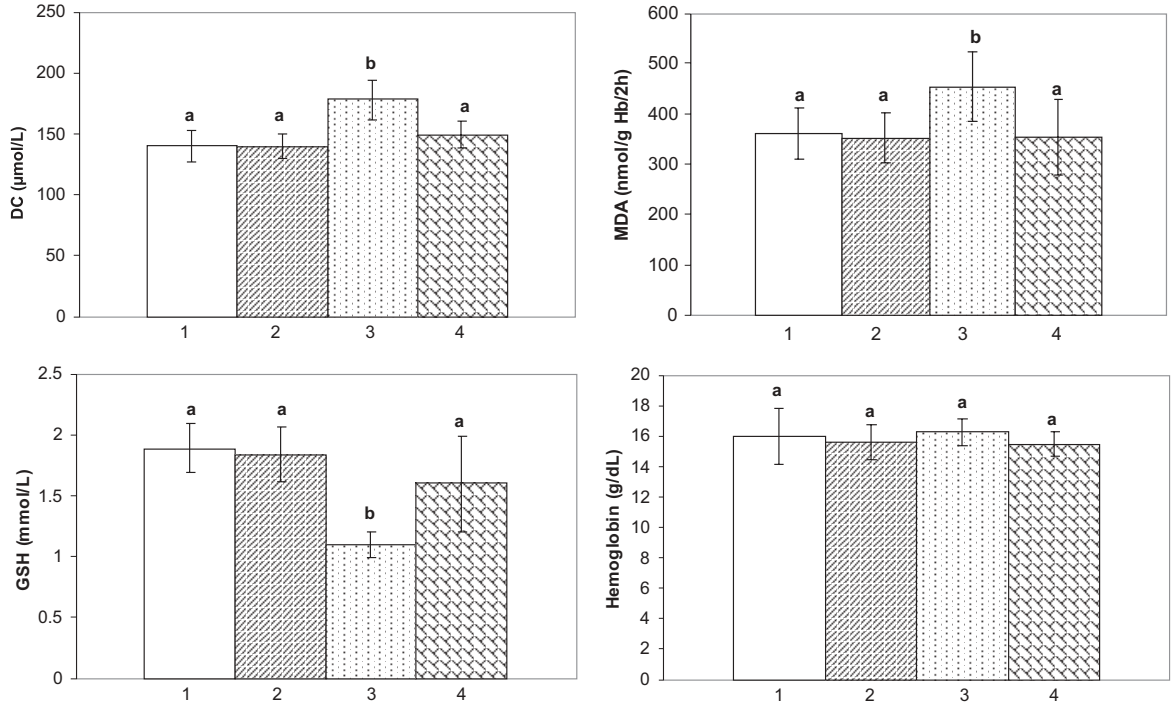

Fig. 3. Endogenous diene conjugate (DC), H_2O_2-induced malondialdehyde (MDA) and glutathione (GSH) levels in erythrocytes as well as blood hemoglobin levels from young (1), carnosine-treated young (2), aged (3) and carnosine-treated aged (4) rats (the mean ± SD; n = 6 in groups 1 and 2; n = 8 in groups 3 and 4). Values with different superscripts (a-b) are significantly different by the Kruskal-Wallis test followed by the Mann-Whitney U test; $p < 0.05$

There are no *in vivo* studies in the literature investigating the effect of carnosine treatment on age-induced oxidative stress in the LDL or LDL + VLDL fractions and erythrocytes . However, some investigators have reported that carnosine inhibits LDL oxidation [14, 24, 33] and protects erythrocytes from oxidative damage [4, 32] *in vitro.* In our study, rats were treated with carnosine for 4 weeks, and this treatment caused significant decreases in serum DC and MDA levels without any change in FRAP levels in aged rats. Similarly, carnosine treatment caused significant decreases in endogenous DC and copper-induced MDA levels in the LDL + VLDL fraction from aged rats. This treatment also caused significant decreases in endogenous DC and H_2O_2-induced MDA levels and significant increases in GSH levels in erythrocytes from aged rats.

Carnosine has strong antioxidant effects. It scavenges reactive oxygen species and chelates a number of metal ions including copper and zinc [2, 10, 20]. Carnosine inhibits peroxidation of membrane lipids, oxidative modifications of proteins and DNA fragmentation caused by reactive oxygen species [2, 10, 20]. The dipeptide also reacts with deleterious aldehydic products of lipid peroxidation and thereby suppresses their toxicity [2, 21, 22]. Carnosine can react with proteins bearing carbonyls to form protein-carbonyl-carnosine adducts [2, 19]. Therefore, free radical scavenging, reacting with aldehydes and detoxifying aldehyde-modified proteins and chelating with redox metal ions may altogether contribute to the observed protective effect of carnosine in aged rats. Increased GSH levels in erythrocytes of aged rats after carnosine treatment may be due to the reaction of carnosine with deleterious aldehyde, thus sparing GSH.

This study is the first *in vivo* study in the literature investigating the effect of carnosine on endogenous and induced lipid peroxidation in the LDL + VLDL fraction and erythrocytes of aged rats. Our findings indicate that carnosine may be useful *in vivo* for the decrease in age-induced oxidative stress in the LDL + VLDL fraction and erythrocytes.

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