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Antioxidant Status, Lipid Peroxidation and Protein Oxidation in Type 2 Diabetic Patients; Beneficial Effects of Supplementation with Carnosine: A Randomized, Double-Blind, Placebo-Controlled Trial

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Abstract

Background: Oxidative stress in the pathogenesis of diabetes is of great prominence and that Carnosine is a natural antioxidant.
Objectives: This study was aimed to investigate the effect of Carnosine supplementation on different oxidative stress biomarkers in patients with type 2 diabetes mellitus (T2DM).
Methods: In this randomized, double-blind placebo-controlled trial, 54 patients with T2DM were employed from Tabriz Sheikhorraees polyclinic and Imam-Reza Hospital endocrine center, and assigned to either the intervention group (n = 27) to receive two capsules of Carnosine 500 mg each or the control group (n = 27) to take two capsules of crystalline microcellulose for 12 weeks. Serum levels of fasting blood sugar (FBS), malondialdehyde (MDA), protein carbonyl (PC) and erythrocytes content of catalase (CAT), and superoxide dismutase (SOD) were assessed before and after the supplementation.
Results: A total of 44 patients completed the study. Anthropometric indices and energy intake did not show a significant change in both studied groups. Improved catalase level and decreased serum levels of fasting blood sugar, malondialdehyde and protein carbonyl occurred in the carnosine group compared to the placebo group after adjusting for baseline values and confounders ($P < 0.05$). Between-group differences were not significant for other variables at the end of the research. In the placebo group, none of the studied biomarkers were altered significantly.
Conclusions: Our findings divulge the beneficial effects of carnosine supplementation in improving the antioxidant status and attenuating some of the oxidative stress biomarkers in patients with T2DM making it a useful adjunct therapy.

Keywords: Antioxidant, Carnosine, Diabetes Mellitus, Type 2, Lipid Peroxidation, Protein Oxidation, Type 2

1. Background

Diabetes mellitus, as a metabolic disorder, is considered to be a major health problem in the world and can boost the overall risk of premature death (1). The World Health Organization (WHO) estimates that, by the year 2030, approximately 366 million people will suffer from diabetes (2). Type 2 diabetes mellitus (T2DM) accounts for 90% - 95% of all types of diabetes (3). Oxidative stress, by an imbalance between free radical formation and body's antioxidants, plays a major role in the pathogenesis of both type 1 and T2DM as well as the onset and progression of late diabetic complication. This occurs by the potential of oxidative stress to damage lipids, proteins, and DNA (4). Oxidative stress-induced complications include coronary artery disease, neuropathy, nephropathy, retinopathy (5), and stroke (6). In diabetes, rising of Reactive Oxygen Species (ROS) level is due to decrease in the destruction or an increase in the production of superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GSH-Px) antioxidants. The changes in the levels of these enzymes make the tissues susceptible to oxidative stress resulting in the progression and development of diabetic complications (7). Strategies to reduce oxidative stress in T2DM may exert favorable effects on the progression of diabetic microvascular and macrovascular complications of the disease.

Carnosine is a water-soluble, naturally occurring, dipeptide consisting of beta-alanyl-l-histidine (8), com-

mercially available as an over-the-counter "health food" supplement, which meets almost all requirements for an ideal antioxidant. It is synthesized and contained in the human muscle and nervous tissues, which is easily absorbed in the digestive tract, penetrates through the blood-brain barrier, and has high bioavailability and membrane-stabilizing action with powerful antioxidant, free radical scavenging, and neurotransmitter properties. Carnosine scavenges reactive oxygen radicals and impedes the oxidation of the cell membrane (8, 9). Suggested mechanisms of carnosine's action on diabetes and cardiovascular risk factors include attenuating oxidative stress, chronic low-grade inflammation, and lipid oxidation end-products as well as chelating properties and impacts on the autonomic nervous system (10, 11). In addition, carnosine improves wound healing, helps prevention of cataract, and inhibits diabetic nephropathy (12). The compound, at low concentrations, could improve glycemic control as well (13, 14). In physiological conditions, carnosine was found to limit, on the one hand, the oxidative damage, and on the other hand to improve the enzymatic and non-enzymatic antioxidant activity.

Several experiments carried out in animal models have shown that use of carnosine in metabolic syndrome was promising to impede oxidative stress and inflammation leading to the development of diabetes and cardiovascular diseases (15). Furthermore, anti-glycation (16) and anti-cross-linking (17) properties of carnosine have been shown, which are, in essence, reflections of its antioxidant effects, the ability to block the oxidation of biomolecules. Most of the studies exploring carnosine antioxidant potential were carried out on animals. Only one recent research investigated the effect of 12 weeks of carnosine supplementation on renal functional integrity and oxidative stress in type 1 pediatric diabetic patients with nephropathy, where carnosine supplementation was found to exert a significant improvement of oxidative stress, glycemic control, and renal function (18).

2. Objectives

To the best of our knowledge, no previous studies examined the antioxidant function of Carnosine in patients with T2DM. There is insufficient evidence to recommend its supplementation for those patients. Therefore, this study was undertaken to divulge the role of carnosine, as an adjuvant therapy, for T2DM patients and appraise the effects of carnosine supplementation on different biomarkers of oxidative stress in T2DM patients.

3. Methods

3.1. Patients

Patients with T2DM, using only oral agents for controlling hyperglycemia, aged 30 to 60 years old, and BMI < 40 Kg/m^2 were eligible to participate in the study. The exclusion criteria included patients with clinical or laboratory signs of acute or chronic infection, non-steroidal anti-inflammatory drug or food supplement use at the time of the study, using insulin, smoking, inflammatory, allergic and cardiovascular disease, hypo/hyperthyroidism, liver disease, and polycystic ovary syndrome. During the research, individuals were asked to remain on their routine habits of physical activity, dietary intake, as well as medication and were encouraged to keep a record of any signs of illness or deviations from the protocol. Considering α = 0.05, a power of 90%, and based on a 25% change in serum malondialdehyde (MDA) concentrations obtained from the study by Kim et al., (2011), the sample was determined with at least 22 cases in each group. The sample size was increased to 27 cases in each group for a possible drop out of 20%. A total of 100 individuals referred to two outpatient endocrinology clinics affiliated to the Tabriz University of Medical Sciences, Tabriz, Iran participated in a subsequent face-to-face screening session. Finally, 54 participants were randomly allocated to two control groups of carnosine (n = 27) and placebo (n = 27). After the 12-week intervention, 44 participants (22 participants in each group) completed the study. The patients were given a full explanation of the study procedure and signed a written informed consent before initiation of the clinical trial.

3.2. Experimental Design

This was a double-blind, randomized, parallel designed, clinical trial study (trial registration: IRCT2016011211689N2 at www.irct.ir). The protocol was approved by the research ethics committee of Tabriz University of Medical Sciences (TBZMED.REC 1394.854) and the trial took place from March to August 2016. The eligible participants (n = 54) were randomly allocated into the carnosine (n = 27) and the placebo groups (n = 27) based on the random block procedure consisting of four subjects per block, which matched participants to each block based on age, gender, physical activity level and type/dose of medication received, produced by random allocation software, version 1.0. A computer-generated random sequence was administered by an independent third party who was not involved with the clinical manipulation of the study and kept in a secure remote location until the data were collected and verified. Patients and those involved in enrolling patients, administering the interventions, and assessing results were blind to group

assignments. The participants in the intervention group received two capsules of L-Carnosine, each 500 mg (1 g/day, Myprotein UK and Ireland) after a meal for 12 consecutive weeks. The subjects in the placebo group consumed two capsules of the microcrystalline cellulose/day. In an earlier study, identical dose/study duration had been confirmed as safe and effective (19). Primary outcomes were changes in FBS, CAT, SOD, MDA, and protein carbonyl (PC) levels.

3.3. L-Carnosine Capsules and Placebo Preparation

Carnosine supplements contained L-Carnosine (Myprotein UK and Ireland), 500 mg capsules. The placebo capsules, which were identical to the carnosine supplements in color and size, were prepared by the School of Pharmacy, Tabriz University of Medical Sciences, Tabriz, Iran.

3.4. Anthropometric, Dietary and Physical Activity Assessments

Body weight was assessed to the nearest 0.1 kg using a Seca scale (Hamburg, Germany). Height was also measured to the nearest 0.5 cm using a mounted tape. Waist (WC) was measured with a tape measure to the nearest 0.1 cm. Dietary intake was assessed with three nonconsecutive day food records (two weekdays and one weekend) at baseline and at the end of the trial. Three-day average macronutrient and energy intakes of participants were evaluated by nutritionist 4 software (First databank Inc., Hearst Corp., San Bruno, CA). The international physical activity questionnaire (IPAQ) was used to assess the physical activity of participants. Validity and reliability of this tool have been evaluated previously (20). The physical activity of each patient was calculated considering the energy requirements defined as metabolic equivalent. All the patients were categorized into different activity levels (low, moderate, high).

3.5. Laboratory Analysis

Before and after 12-week intervention, blood samples were drawn using the standard phlebotomy technique in the morning after a 12-hours overnight fasting. Blood samples were centrifuged immediately (1,600 g, 10 minutes) and the serum stored at -70°C until analysis. Serum glucose was measured in the certified laboratory using a glucose-hexokinase kit (Siemens Health Care Diagnostics, USA, Tarrytown, NY) (21). Serum MDA levels were measured through a reaction with thiobarbituric acid (TBA) as a TBA-reactive substance, as described previously (22). Summarily, serum samples were mixed with 3 mL of 1.0% phosphoric acid and 1.0 mL of 0.67% TBA, afterward heated in a boiling water bath for 45 minutes. After samples cooled, 3 mL of n-butanol was added, then, the mixture was centrifuged at 3000 rpm for 10 minutes to separate into two layers. TBA-reactive substance content of the n-butanol layer was determined by spectrophotometry at 532 nm. All the reagents applied in this assay were obtained from Merck (Darmstadt Germany). For SOD and CAT determination, erythrocyte lysates were used. Briefly, blood was collected in tubes containing EDTA and centrifuged (1500 g) for 15 minutes at 4°C. The sediment containing erythrocyte was suspended in normal saline and was recentrifuged. Sediment red cells were added to ice-cold distilled water and mixed thoroughly. SOD activity in red blood cells was measured using a commercially available SOD kit (Randox Co, Germany) at 505 nm by the spectrophotometer, according to the manufacturers' instructions. SOD activity that could trigger inhibition of 50% superoxide, produced by reaction nitroblue-tetrazolium, was defined as 1 unit (U). The ultimate concentration of total SOD was calculated from a semi-logarithmic, a standard curve of standard samples vs. absorbance. The ultimate concentration of total SOD was calculated from a semi-logarithmic standard curve of standard samples vs. absorbance. CAT activity was evaluated using the Aebi method (23). CAT activity of red cells was determined by monitoring the decline in absorbance at 240 nm in the presence of 10 mM of hydrogen peroxide at 25°C. One unit of CAT activity was described as the decomposition of 1 M of hydrogen peroxide min^{-1} at 25°C. The serum carbonyl group was measured by determining the PC residues using the dinitrophenylhydrazine (DNPH). According to the procedure supplied by Cayman's protein carbonyl colorimetric assay kit (Cayman Chemical, Ann Arbor, USA), the amount of protein-hydro zone product was evaluated via spectrophotometry at the wavelength of 360 nm.

3.6. Statistical Analyses

Statistical analyses were performed using SPSS Statistics Software, ver. 21 (IBM Corp, Armonk, New York, USA). All analyses were performed on the Intention-To-Treat principle (24). Normality of variables distribution was determined using the Kolmogorov-Smirnove test. Normally, distributed variables were represented as means $\pm$ standard deviation, while non-normally distributed variables were reported as median (25th and 75th percentiles), respectively. The changes between variables before and after the supplementation were assessed by paired t-test and as for non-parametric data, Wilcoxon signed-rank test was applied. Analysis of covariance (ANCOVA) was employed to draw any differences between the two groups at the end of the research, adjusting for baseline values and covariates (duration of diabetes, changes in BMI, and calorie intake). Statistical significance was assumed when $P < 0.05$.

4. Results

A total of 54 T2D patients were recruited in the present clinical trial. However, there were five dropouts in the intervention group and five dropouts in the control group. Therefore, 44 participants completed the study (Figure 1). There were no missing values in the measured main outcome variables. No symptom or adverse effects during and after carnosine supplementation were reported by the patients. Baseline characteristics of the patients are presented in Table 1. No significant differences were found between the two groups in the means of gender, age, energy intake, weight, BMI, diabetes duration, oral agent consumption, systolic and diastolic blood pressure, as well as physical activity level ($P > 0.05$).

Table 2 illustrates changes in biochemical parameters in both study groups after carnosine supplementation. In the baseline, no significant difference was seen between two studied groups in terms of FBS, CAT, SOD, MDA, and PC ($P > 0.05$). Mean serum levels of FBS were significantly attenuated ($P < 0.05$) in the carnosine group compared with its baseline concentration. At the end of the trial, a significant increase in serum levels of CAT ($P < 0.05$) happened in the carnosine group compared to the baseline values. Results of ANCOVA showed a statistically significant difference between the two groups in serum levels of CAT ($P < 0.05$), at the end of the study, adjusted for duration of diabetes changes in energy intake, BMI, and baseline values. Mean serum in the MDA level declined ($P < 0.05$) in the carnosine group compared to its baseline values. At the end of the survey, a significant decrease was observed in the carnosine group compared with the placebo group ($P < 0.05$). Mean plot for MDA changes is illustrated in Figure 2. After 12 weeks of supplementation, PC levels in the intervention group were decreased ($P < 0.05$) compared to the baseline values. In addition, at the end of the research a significant decrease ($P < 0.05$) was detected in changes of PC between the 2 study groups. However, no significant difference in changes of SOD between the two studied groups was detected at the end of the research ($P > 0.05$).

5. Discussion

In the present intervention study, we demonstrated that a 12-week carnosine supplementation led to a significant attenuation of FBS and oxidative stress biomarkers. Several studies have reported a hypoglycemic and protective effect of carnosine in diabetic rats. Reports from diabetic rodents suggest that supplementation of carnosine in a dose-dependent manner reduced glucose, HbA1c, and markers of oxidative stress (25-27). Furthermore, carnosine supplementation in db/db mice is known to delay development of T2DM (25). Similar to the above-mentioned studies, our results showed that 12 weeks of supplementation with carnosine led to a significant reduction in serum glucose levels possibly through its ability to act as a histamine precursor.

Oxidative stress is usually accompanied by alterations in the activities of the antioxidant enzyme in T2D patients (28). It is shown that subjects without known CAT gene mutations exhibit down-regulation of blood CATs (29). Further, some authors have reported a decrease in SOD levels in the blood of diabetic patients (30, 31). Inconsistent with previous reports (32), the current study revealed that supplementation of carnosine resulted in increased levels of CAT. Moreover, SOD levels did not significantly change with the intervention. The finding is in agreement with the results of Slowinska-Lisowska et al., who investigated the influence of carnosine supplementation (4 g/d for 14 days) on elite kayakers and canoeists (19). In contrast, Zhang et al., showed that carnosine attenuated early brain injury through its antioxidative effects in a rat experimental subarachnoid hemorrhage model (33). The discrepancy between various studies in terms of SOD may be justified by varying doses of carnosine used for supplementation, different study models, and different physiological conditions.

Evidence indicates that the level of MDA, a marker of oxidative stress and lipid peroxidation, is elevated in diabetic patients (34). Aydın et al., (35), demonstrated that administration of carnosine (250 mg/kg/day; i.p.; 5 days per week) for two months in D-galactose- treated rats, as an animal model for brain aging, diminished brain oxidative stress and apoptosis through a reduction in MDA, PC levels. In a recent study conducted on pediatric diabetic patients with nephropathy, 12 weeks supplementation of 1 gr carnosine resulted in a significant reduction of MDA (18). In accordance with previous experiments, our results similarly demonstrated that carnosine significantly attenuated oxidative damage of lipids by reducing serum levels of MDA.

Proteins are likely the most instant agent for striking oxidative damage in cells. PC content is absolutely the most general indicator and the most frequently used biomarker for assessing oxidation of proteins (36). Elevated PC content has been demonstrated in different cells and plasma of the diabetic patients (37). Carnosine is not only able to prohibit carbonylation of proteins, however, it also reacts directly with PC groups, producing the protein-carbonyl-carnosine adducts or “carbonylated” proteins, thus, hampering cross-linking to other unmodified protein (38). Carnosine supplementation for 24 weeks in Zucker rats was found to reduce carbonylation of proteins in the kidney and to boost collagen solubility as an index of

Table 1. Baseline Characteristics of the Study Participants (N = 22)[a]

Variables	Carnosine	Placebo	P Value[b]
Gender, F/M	13/10	9/12	0.243
Age, y	43.0 ± 7.6	40.4 ± 5.1	0.188
Energy, kcal/d	2525.9 ± 588.6	2512.4 ± 869.6	0.950
Weight, kg	78.8 ± 16.4	77.6 ± 12.4	0.782
BMI, kg/m^2	43.0 ± 7.6	40.4 ± 5.1	0.188
Diabetes duration, y	4.5 ± 2.0	4.2 ± 2.2	0.549
Metformin, 500 mg, tablets/d	2.2 ± 0.6	2.1 ± 0.4	0.365
Glibenglamide, 5 mg, tablets/d	0.8 ± 0.3	0.7 ± 0.3	0.847
SBP, mmHg	11.7 ±1.3	11.8 ± 1.2	0.654
DBP, mmHg	7.8 ± 1.1	7.9 ± 0.8	0.721
Physical activity, N. %			
Low	18 (58.1)	13 (41.9)	0.325
Moderate	5 (38.5)	8 (61.5)	0.234
High	0 (0)	0 (0)	-

Abbreviations: BMI, Body Mass Index; DBP, Diastolic Blood Pressure; SBP, Systolic Blood Pressure.
[a]Values are reported as means ± SD unless otherwise indicated.
[b]P < 0.05 was considered significant. P values indicate the comparison between groups at baseline (independent-sample t-test).

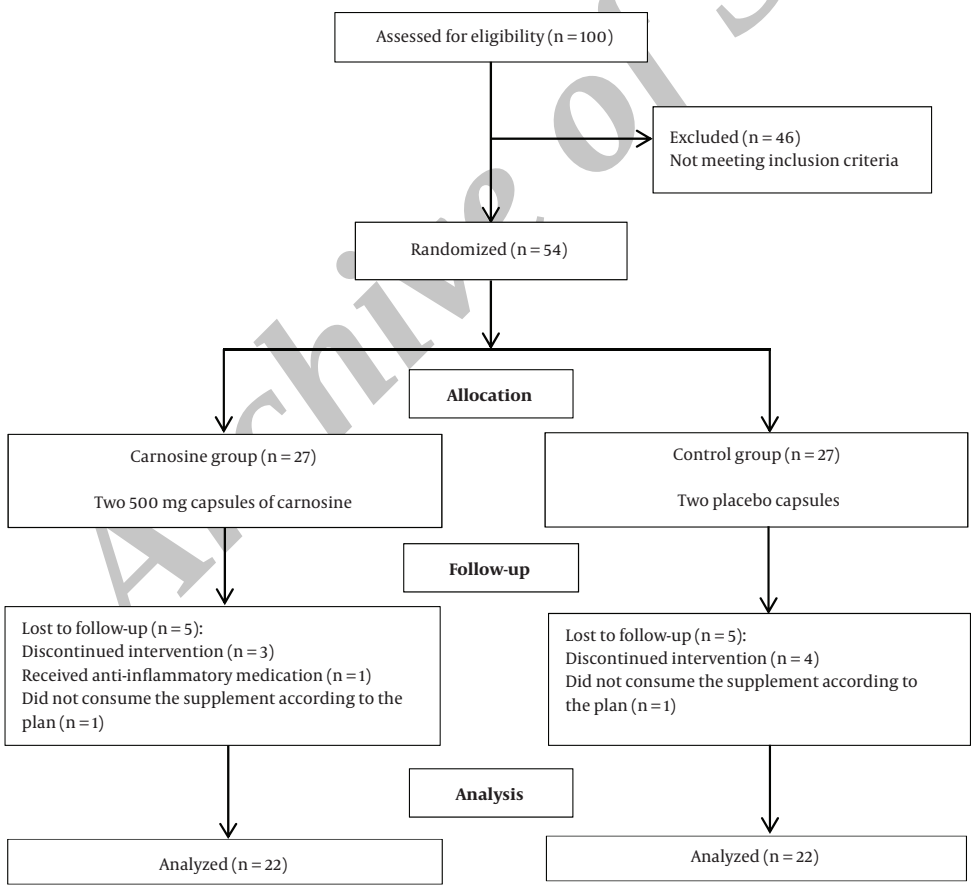

Figure 1. Flowchart of the study

Table 2. Changes in Biochemical Parameters in T2DM Patients After the Intervention (N = 22)[a]

Variables	Carnosine	Placebo	MD (CI 95%)
FBS, mg/dL			
Baseline	137.04 ± 36.10	135.28 ± 25.14	1.75 (-17.34 to 20.86)
After 12 weeks	127.17 ± 21.03	139.90 ± 31.39	-13.09[b] (-25.34 to -0.84)
MD (CI 95%)	-9.86[c] (-21.17 to 1.43)	4.61 (-4.09 to 13.33)	-
CAT, U/mL			
Baseline	67.19 ± 9.71	67.40 ± 11.15	-0.21 (-6.56 to 6.13)
After 12 weeks	71.71 ± 9.25	67.44 ± 12.31	4.52[b] (2.33 to 6.71)
MD (CI 95%)	4.52[c] (2.56 to 6.47)	0.03 (-0.95 to 1.01)	-
SOD, U/mL			
Baseline	49.62 ± 3.81	49.48 ± 5.77	0.14 (-2.81 to 3.09)
After 12 weeks	50.39 ± 3.07	49.71 ± 3.95	0.62 (-0.82 to 2.08)
MD (CI 95%)	0.77 (-0.53 to 2.07)	0.22 (-1.31 to 1.77)	-
MDA, nmol/mL			
Baseline	2.20 ± 0.55	2.13 ± 0.63	0.07 (-0.29 to 0.43)
After 12 weeks	1.68 ± 0.44	2.05 ± 0.63	-0.38[b] (-0.64 to -0.11)
MD (CI 95%)	-0.52[c] (-0.72 to -0.32)	-0.08 (-0.32 to 0.15)	-
PC, U/mL			
Baseline	62.98 ± 11.74	53.50 ± 13.38	9.47 (1.82 to 17.11)
After 12 weeks	49.68 ± 11.59	58.00 ± 9.51	-13.46[b] (-19.12 to -7.81)
MD (CI 95%)	-13.29[c] (-17.97 to -8.61)	4.49 (0.09 to 8.89)	-

Abbreviations: CAT, Catalase; CI, Confidence Interval; FBS, Fasting Blood Sugar; MDA, Malondialdehyde; PC, Protein Carbonyl; SOD, Superoxide Dismutase.
[a] Data were expressed as mean ± SD.
[b] Significant between groups mean difference (P < 0.05; ANCOVA adjusted for duration of diabetes, changes in energy intake, BMI and baseline values after 12 weeks).
[c] Significant within groups mean difference (P < 0.05; paired Student's t-test).

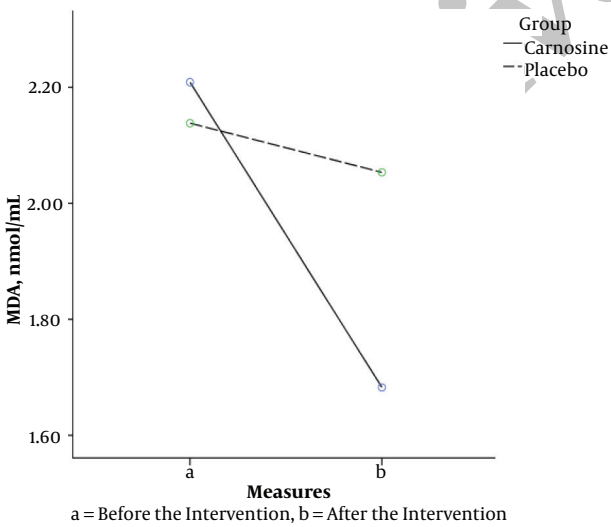

Figure 2. Mean plot for malondialdehyde (MDA)

the intensity of collagen crosslinking (26). Carnosine was also shown to act as an antioxidant and to exert beneficial effects on renal function, oxidation, and glycation products in the kidneys of high-fat diet/streptozotocin-induced diabetic rats as evidenced by attenuated PCs (39). Similar to the above-mentioned reports, our results demonstrated a remarkable fall in the oxidation of proteins mirrored by the reduced serum levels of PC in the carnosine group compared to controls.

Suggested mechanism of antioxidant potential of carnosine is to attenuate oxygen-free radical-mediated injury to cellular macromolecules either by scavenging hydroxyl radicals, or chelating divalent cations with its imidazole group (40). This study seems to be the first report regarding the effects of carnosine on oxidative stress status in patients with T2DM. In general, the findings of the current research should be interpreted with some restrictions. The most important limitation of our study was the relatively small sample size. In addition, we did not evaluate urine or muscle carnosine concentrations of patients due to technical difficulties. The strength of our research was the monitoring of patient's status by weekly telephone conversations. Additionally, carnosine appeared to be well tolerated by patients and anecdotal reports indicated high

acceptance of the supplements.

5.1. Conclusion

Our findings imply that carnosine improves the antioxidants status and attenuates lipid peroxidation and protein oxidation, resulting in a balanced prooxidant-antioxidant status.

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Footnote

Conflict of Interests: The authors declare that there is no conflict of interests.

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