Effects of 3-months carnosine supplementation on insulin resistance and glucose intolerance in overweight and obese sedentary individuals: Pilot Clinical Trial

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What is already known about the subject:

- carnosine supplementation prevents type 2 diabetes in rodents
- muscle carnosine content is cross-sectionally associated with type 2 diabetes
- carnosine supplementation increases exercise tolerance and has positive mental health effects in humans

What does this study add:

- this is the first evidence that carnosine supplementation has positive effects on glucose metabolism and specifically on glucose tolerance, insulin resistance and insulin secretion in humans

Abstract

Objectives: Carnosine is a naturally present dipeptide in humans ~~and over-the counter food additive~~. Evidence from animal studies support the role for carnosine ~~supplementation~~ in the prevention and treatment of diabetes and cardiovascular disease~~,~~ ~~yet there is limited human data~~. We ~~aimed to~~ investigated if carnosine supplementation in overweight or obese humans improves insulin sensitivity, glucose tolerance, blood pressure and lipid profile.

Methods: In a double blind randomized pilot trial in non-diabetic overweight and obese individuals (age 43±8y; BMI 31±4 kg/m^2), we randomly assigned 15 to 2g carnosine daily and 15 to placebo for 12 weeks. Insulin sensitivity by HOMA, blood pressure, plasma lipid profile, hsCRP, adiponectin, urinary carnosine levels and *in vivo* muscle carnosine deposition (^{1}H MRS) were measured. Effect of carnosine on glucose tolerance was examined in 12 individuals from carnosine (n=6) and placebo (n=6) groups with impaired glucose tolerance by oGTT.

Results: Carnosine concentrations increased in urine after supplementation ($p<0.05$ ~~for change between the treatment groups~~) and a tendency towards carnosine muscle accumulation was observed. An increase in fasting insulin and insulin resistance was hampered in individuals receiving carnosine compared to placebo and this remained significant after adjustment for age, sex and change in body weight ($p=0.02$, $p=0.04$, respectively). Glucose intolerance was improved by carnosine treatment in a subgroup of individuals with impaired glucose tolerance ($p<0.05$). There was no difference in body weight, resting energy expenditure, dietary fat preference or physical activity between the carnosine and placebo groups~~(p=0.1)~~.

Conclusions: These first pilot-human interventional metabolic data suggest that carnosine may improve glucose tolerance and impede insulin resistance development and thus could contribute to the prevention of type 2 diabetes.

Word Count Abstract: 269 words

Keywords: Carnosine, obesity, central adiposity, insulin resistance, impaired glucose tolerance, HOMA-IR, insulin secretion, inflammation

Introduction

Type 2 diabetes is a global health problem as it is associated with a substantial disease burden from both diabetes complications and cardiovascular diseases generating considerable associated health care costs (1). Obesity itself markedly increases the risk of developing type 2 diabetes (DM2). Obesity and diabetes dramatically increase the risk of heart disease and stroke, the two most prevalent forms of cardiovascular disease (CVD); and ~80% of those with both obesity and DM2 develop CVD (2). Importantly, obesity and type 2 diabetes is preventable. Currently available interventions are effective but often difficult to implement at a population level as they require intensive lifestyle modification over a long period of time and/or a profound change in the public health policies. This underpins the urgent need to identify and test other safe, low-cost strategies, with the potential to prevent the type 2 diabetes alone or in synergy with lifestyle changes.

Carnosine (β-alanyl-L-histidine) is a dipeptide present in mammalian tissues and is available as an over-the-counter food additive. In humans, beneficial effects of carnosine have been shown in clinical trials in exercise physiology, psychology, psychiatry and recently in heart failure (3-9). Importantly, carnosine supplementation was not associated with any significant side effects (3-5, 7-9). A significant body of animal evidence has suggested a role for carnosine supplementation in the prevention and treatment of type 2 diabetes and diabetes complications as well as cardiovascular risk factors (adverse lipid profile and hypertension) and disease (10-17). Cross-sectional studies in patients with type 2 diabetes have shown both increased and decreased muscle carnosine levels (18, 19). There are no human intervention studies in diabetes. There is only one clinical trial showing benefits of carnosine supplementation in patients with heart failure (9).

Suggested mechanisms of carnosine's action on the diabetes and cardiovascular risk factors include lowering chronic low-grade inflammation, oxidative stress, advanced

glycation and lipidoxidation endproducts (AGEs and ALEs, respectively) as well as chelating properties and effects on autonomic nervous system (6, 13, 14, 20-26).

Based on the animal data on protective effects of carnosine supplementation on diabetes and cardiovascular risk factors, we hypothesized that carnosine supplementation will improve insulin sensitivity (primary outcome), insulin secretion, glucose tolerance blood pressure and lipid profiles and reduce weight and central adiposity (secondary outcomes) in overweight and obese individuals. Overweight and obese individuals were targeted as the population with increased risk of type 2 diabetes and cardiovascular disease.

Methods

Study population

The trial took place from September until December 2013 and all the metabolic measurements were completed before the Christmas period. We performed a single center double-blind randomized parallel design clinical trial in 30 non-diabetic overweight and obese sedentary non-vegetarian individuals randomly assigned to receive 2g carnosine supplements daily (administered orally in 2 divided doses) *or* identical looking placebo (2g sucrose/day) for 12 weeks. Identical settings dose/study duration have been proved safe & effective earlier (7). All individuals underwent a study protocol employing anthropometric measurements and insulin resistance, lipid profile, blood pressure, daily free-living ambulatory activity, dietary fat preference, as well as assessment of plasma, urinary and muscle carnosine, plasma free fatty acids, carnosinase 1, hsCRP and adiponectin levels. All volunteers were recruited from the community via newspaper advertisement by the Institute of Experimental Endocrinology at Slovak Academy of Sciences.

The protocol was approved by the Ethics Committee of the University Hospital Bratislava, Comenius University Bratislava and it conforms to the ethical guidelines of the Helsinki declaration of 1964, as revised in 2000. All individuals signed a written informed consent prior study entry.

Methods

Participants eligible for the study underwent medical screening including history, physical examination and routine blood tests (including fasting plasma lipid levels, urinary and liver function tests, an oral glucose tolerance test (OGTT)). Prior to metabolic testing, participants were asked to abstain from strenuous exercise and caffeine for 3 days. All the metabolic testing, blood and urine collection and magnetic resonance studies were performed after a 12-h overnight fast and 12-h after carnosine ingestion.

Participants were non-diabetic, but 6 individuals in each treatment group had impaired glucose tolerance according to an OGTT, they were non-smokers and healthy according to a physical examination and routine blood analyses. No participant had clinical or laboratory signs of acute or chronic infection, or took any medication, food supplements or illicit drugs at the time of the study. Participants were asked to refrain from substantial changes in their lifestyle habits during the course of the study. As such, participants with weight change over >5kg over 12 weeks would be excluded from the study. Adherence to the study protocol was encouraged by regular phone calls and monthly follow-up anthropometric and blood pressure measurements.

<u>Anthropometric measurements</u>

Body weight and height were measured and used to calculate body mass index (BMI). Waist circumference was measured at the midpoint between the lower border of the rib cage and the iliac crest. Bioelectric impedance was used to evaluate total fat and to estimate lean body mass (Omron BF511, Omron Healthcare LTD., Japan),

measurements were performed in the morning between 8:00 and 9:00 a.m., individuals were in the fasted state, after the void.

Resting energy expenditure (REE) and metabolic substrate preference (RQ) were measured after an overnight fast with the aid of indirect calorimetry. Thirty minute measurement was initiated after the 30 minute bed rest at thermal comfort conditions with the Ergostik (Geratherm Respiratory, Germany).

Physical activity & dietary preference

Daily free-living ambulatory activity was assessed by accelerometers (Lifecorder Plus, Kenz, USA) during the three consecutive working days and accelerometer was used more than 12 hours a day.

Food Preference Questionnaire is a reliable and validated tool (27) to assesses determine the preference for High-Fat or Low-Fat foods (72 foods list). Participants rate each food hedonically on a 9-point Likert scale by rating how much they like each food, with 1 = dislike extremely, 5 =neither like nor dislike, and 9 = like extremely. Fat preference score was calculated as a ratio between High-Fat and Low-Fat score.

Metabolic studies

75-g OGTT was performed after a 12-h overnight fast and glucose tolerance status was determined (American diabetes association criteria 2006). Bloods were collected at 0, 30, 60, 90, 120 min for glucose and insulin concentrations. These measurements were used to calculate the Area Under the Curve (AUC) according to the trapezoid rule.

Blood pressure was measured on 2 different days in sitting position between 8-9 a.m. after 30 minutes of rest three times, separated by 5 minute using a Dinamap Compact (Johnson & Johnson Inc, UK). The mean of the three measurements taken was calculated and reported. Coefficient of variation for systolic and diastolic BP was 2.8 and 3.8% respectively.

Insulin sensitivity was estimated on the basis of fasting glucose and insulin measurements and calculated as HOMA-IR = fasting glucose concentration x fasting insulin

concentrations/22.5. Insulin secretion (β-cell function) was calculated as HOMA-β =(20 x fasting insulin concentration)/(fasting glucose concentrations-3.5)%.

Blood samples were drawn using standard phlebotomy technique and were taken in the morning at 8 a.m. after the 12-h overnight fast (12 to 14 hours after the last evening dose of carnosine (1 g), before and after 12-week carnosine supplementation. The tubes were centrifuged immediately (1600g, 10 mins, 4°C), and the serum stored at -80°C until analyses. During the oGTT, blood glucose was immediately measured with Super GL2 -SN1440 analyzer (Dr Muler Geratebau, GmbH, Germany). Serum glucose was later reanalyzed at the certified laboratory using glucose-hexokinase 3 kit (Siemens Health Care Diagnostics, USA). Insulin was determined with IRMA (Immunotech, France), total cholesterol, HDL-cholesterol and triglycerides with diagnostic kits from Roche (Germany). Friedewald formula was used to estimate LDL-Cholesterol & atherogenic index was calculated with the formula (Total cholesterol - HDL-cholesterol)/HDL-cholesterol (28). High sensitivity CRP (hsCRP) and adiponectin were measured by an immunoturbidimetric method (Randox, UK). Urine samples were taken at baseline, in the fasted state, centrifuged for 10 min at 4°C at 400xg and stored at -80ºC prior analysis.

Carnosine and carnosinase measurements

The blood for carnosine analyses was taken into precooled EDTA syringes. HPLC-grade water was prepared with a Milli-Q water purification system of Millipore (Italy). NFPA (nonafluoropentanoic acid), trichloroacetic acid, sulphosalycilic acid, formic acid, sodium phosphate dibasic and LC-grade and analytical-grade organic solvents were from Sigma-Aldrich (Milan, Italy). Carnosine (β-alanyl-L-histidine) and the internal standard (IS) H-Tyr-His-OH were a generous gift from Flamma S.p.A (Italy). The urinary carnosine was measured by using an internal standard and a triple quadrupole (TSQ quantum ultra, Thermo, Italy) in multiple reaction monitoring mode scan mode as mass analyzer. Calibration curves were set up for carnosine quantification. Blank matrices for calibration curves set-up were obtained by pooling urine from six volunteers aged from 24 to 28

years old and following a lacto-ovo-vegetarian diet for one day before the collection. Before pooling, the biological fluids were analyzed to ensure they did not contain the selected adduct above the 20 % of limit of quantitation. The internal standard, TH, was added at a final concentration of 35 µM for urine. Samples were then treated as the *ex vivo* samples, using the analytical procedure reported in the following paragraph. Three independent samples were prepared for each level of the calibration curve and each of them was analyzed in triplicate. The calibration curves were built by the least square linear regression analysis by plotting the ratios between the peak areas of the analyte and the IS against the analyte's nominal concentration. For LC-MS analysis the urine samples were treated as follows: aliquots of 150 µL were mixed with 150 µL of an aqueous solution of 4 % TCA (v/v) and the spiked with TH to reach a final concentration of 70 µM. The sample was then centrifuged at 14.000 g for 10 minutes by using a refrigerated centrifuge, Thermo Heraeus Megafuge (Thermo, Italy), in order to remove the particulate matter. All the urinary supernatant was collected and then an aliquot was placed in a plate well, ready to be injected in the chromatography system. Samples prepared as above described were than analysed by LC-ESI-MS in MRM as previously described (15).

Serum carnosinase activity was quantified by fluorometric determination of liberated histidine after carnosine addition. Briefly, the reaction was initiated by addition of 10mM carnosine (Flamma, Italy) to serum and stopped after 10 min incubation at 37°C by adding 600mM trichloroacetic acid (TCA). For controls, TCA was added before carnosine. After centrifugation (4500 rpm, 15 min), supernatant was added to a mixture of OPA (incomplete phtaldehyde with 0.2% 2-mercaptoethanol) and 4M sodium hydroxide and fluorescence was determined after 40 min (excitation: 360nm and emission: 465nm).

Serum carnosinase concentrations were determined by a sandwich ELISA (enzyme-linked immunosorbent assay) developed by Adelmann (29). High absorbent 96 well plates (Lab NUNC-Immuno Plate Maxi Sorp F96, Fisher Scientific GmbH) were incubated overnight with 100 µl of goat polyclonal anti-human CN-1 (10µg/ml) (R&D, Germany).

Afterwards, the plates were extensively washed (200µl Tween20 in 100ml PBS) and incubated with a blocking buffer (0.05 % W/V of dry milk powder) for 40 minutes on a shaker (350 rpm), followed by extensive washing. Next, 100 µl of sample (dilution: 1/300) and standard (recombinant human CN-1, R&D Systems; serial dilution) were added. The plates were placed on a shaker for 1 hour and subsequently extensively washed. Hereafter ATLAS anti-human CN-1 antibodies (Sigma PA) were added for 1 hour followed by extensive washing. Goat anti-rabbit IgG HRP (horseradish peroxidase) (Santa Cruz, USA) was added for 30 minutes. Again, extensive washing was performed. By adding deep-blue peroxidase (POD) (Roche diagnostics, Germany) a colour change was generated. This reaction was generally stopped after 10 minutes by addition of 50µl of 1M H_2SO_4. The plates were directly read at 450 nm. CN-1 protein concentrations were assessed in the linear part of the dilution curve.

Muscle carnosine measurements with ^{1}H-MRS

All measurements were performed on a 7 T whole-body MR system (Magnetom, Siemens Healthcare, Erlangen, Germany) equipped with a 28-channel ^{1}H knee RF coil (QED, Mayfield Village, OH). The local ethics committee approved the protocol, and written, informed consent was obtained from all volunteers. All volunteers were examined in supine position, with the widest part of the right calf placed in the middle of the RF coil in the magnet isocenter as described in (Kukurova et al., 2015).

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Statistical Analysis:

Statistical analyses were performed using SAS Jump Statistics Software (USA). Results are given as mean±SD (unless indicated otherwise). Paired t-tests were used for assessing the difference between baseline and follow-up variables. Unpaired t-tests and general linear models were used to assess the differences between the intervention effects (delta follow-up-baseline) between the carnosine and placebo treatment groups and to examine the

relationships after adjusting for covariates (age, sex and change in body weight; HOMA-β was additionally adjusted for HOMA-IR). Statistical significance was assumed when $p<0.05$.

Sample size calculation:

Insulin sensitivity (measured by HOMA) was considered as primary outcome for this pilot study. There are currently no studies published assessing the impact of carnosine on insulin resistance in humans. For this study, we estimated the need for a sample size of 14 participants in each treatment arm (carnosine and placebo) to detect a clinically significant 40% change in insulin sensitivity based on HOMA data from similar overweight or obese individuals in our laboratory (mean 2.8, SD 1.0) with a type I error of 0.05 (two-tail) and a type II error of 0.20 (power = 80%).

Results

The anthropometric, metabolic, biochemical and behavioural characteristics of the 26 non-diabetic overweight and obese sedentary non-vegetarian individuals (5F/21M, age 43±8y; BMI 31 ± 4 kg/m^2) are summarized in Table 1. None of the baseline characteristics were different between the treatment groups. The treatment with carnosine was well tolerated and no side effects were reported throughout the study. There was one drop-out from the study (did not attend follow-up measurements) and 3 participants from the placebo group were excluded from the final analysis due to substantial weight loss of ≥4kg during the course of the study. Table 2 presents characteristics of the study population subdivided to groups of individuals with normal and impaired fasting glucose.

Effect of carnosine supplementation on carnosine and carnosinase 1 ~~levels~~

There was an increase in urinary carnosine levels in carnosine group ($p=0.046$) and no change in the placebo ($p=0.4$) with significant overall difference (1.5 vs. 60.4 nmoles/ml,

p=0.04). Baseline pre-treatment concentration of carnosine in castrocnemius muscle was 12.9+/-4.9 mM, range (5.8-20.6 mM). Post-treatment levels were 17.2+/-7.2 mM with the range (9.2-36.2 mM). Values corresponding to participants with normal and impaired glucose tolerance are shown in table 2. It is important to note, that gastrocnemius muscle carnosine content was negatively associated with 2h insulinemia (oGTT; R=-0.640, P=0046, n=10) in a subgroup of individuals with impaired glucose tolerance. There was no difference in serum carnosinase activity or carnosinase content between the carnosine and placebo treatment groups (both p=0.6).

Effect of carnosine supplementation on anthropometric variables and resting energy expenditure

There was no difference in the intervention-induced change in body weight (p=0.3), waist circumference (p=0.1), Waist-to-hip ratio (p=0.8), BMI (p=0.5), % of body fat (p=0.8), resting energy expenditure (p=0.5), respiratory quotient – metabolic substrate preference (p=0.4) or daily free-living ambulatory activity (steps/hour) (p=0.3) between the two groups.

Effect of carnosine supplementation on insulin sensitivity and secretion

There was an increase in HOMA-IR in both groups during the 12-week period (both $p<0.05$) but this increase was smaller in the carnosine group (mean difference in HOMA-IR; 1.14 vs. 0.3 mmol/l*mU/l for placebo and carnosine, respectively, Fig. 1, Table 1). The difference was significant after adjustment for age, sex and change in body weight (p=0.038, Table 3). There was a significant increase in insulin secretion in the placebo group (HOMA-β = 36 %, p=0.02) but not in the carnosine group (HOMA-β = 3 %, p=0.7) with overall difference of 33 % (p=0.04, Fig. 1, Table 1). This difference remained significant after adjustment for age, sex and change in body weight (p=0.009) and additional adjustment for change in insulin sensitivity (p=0.02, Table 3).

There was also an increase in fasting insulin concentrations in the placebo group (4.3 mU/l, p=0.01) and in the carnosine group (1.1 mU/l, p=0.03). The difference was significant before (3.2 mU/l, p=0.049) and after adjustment for age, sex and change in body weight (p=0.01). There was no difference in fasting and 2-hour glucose (both p=0.8) and 2-hour insulin concentration (p=0.6) between the two groups.

Carnosnine and glucose tolerance

This analysis of the dynamic response to the glucose challenge during the oGTT clearly showed that in individuals with impaired glucose tolerance carnosine normalized 2h glucose levels and showed a tendency to lower 2h insulinemia (Figure 2). Carnosine intervention, however, had no effect on the glycemic curve in individuals with normal glucose tolerance (Figure 2).

Effect of carnosine supplementation on cardiovascular risk factors.

Neither systolic nor diastolic blood pressure was different between the groups or modulated by intervention. There was no difference in other parameters such as fasting lipid profile, atherogenic index, plasma free fatty acids or dietary fat preference (all p>0.5, Table 1 & 2).

Effect of carnosine supplementation on plasma inflammatory markers and adipocytokines

There was no difference in intervention-induced change in plasma hsCRP and adiponectinemia (both p=0.7, Table 1).

Discussion

In the presented ~~novel~~ human pilot intervention study, we showed that a 12-week carnosine supplementation resulted in increased concentrations of carnosine in the urine, a tendency to accumulate carnosine in gastrocnemius muscle, differences in insulin sensitivity and insulin secretion compared to placebo and normalisation of glucose intolerance in a subgroup of individuals with impaired glucose tolerance. There were no significant intervention-induced changes in anthropometric and cardiovascular risk factors in this pilot study. Importantly and as in previous trials (3-5, 23), there were no side effects reported from carnosine supplementation in the trial.

We have previously shown in rodents that carnosine supplementation resulted in a decrease in body weight, which was due to decreased food intake (15). Consistent with this, carnosine was previously suggested to regulate appetite in rodents (14). In addition, effects of carnosine on lipolysis have also been described (14). We have recently reported that muscle carnosine content was cross-sectionally related to percentage body fat (bioimpedance) and subcutaneous adipose tissue content (magnetic resonance) as well as to resting energy expenditure (de Courten, Plos one, 2015, in press). However, there are no human data to this date to support a role of carnosine supplementation in the regulation of body weight. Our pilot study did not show changes in body weight, percentage of body fat, energy expenditure or respiratory quotient in response to carnosine supplementation. However, our data are consistent with the data from a 3-month intervention study in patients with schizophrenia receiving 2g carnosine daily that revealed no changes in BMI (5), it is therefore plausible to think that carnosine does not affect body weight in humans. However, larger studies employing gold standard measures of total or central adiposity such dual X-ray absorptiometry and magnetic resonance imaging are necessary to solve this issue.

We showed that after carnosine supplementation, change in insulin levels, insulin sensitivity and secretion were smaller in the carnosine group compared to placebo, suggesting carnosine's ability to hamper insulin resistance and associated fasting

hyperinsulinemia. These changes were independent of changes in anthropometric parameters of obesity. Moreover, we clearly showed that the 3 month of carnosine treatment normalized 2h glucose levels and showed a tendency to lower 2h insulinemia in patients with impaired glucose tolerance. A growing body of evidence from animal studies indicates a protective role of carnosine supplementation in diabetes (12-17). Specifically, data from diabetic rodents indicate that supplementation of carnosine reduced fasting plasma glucose, insulin resistance, increased insulin secretion and **β**-cell mass as well as reduced markers of chronic inflammation and advanced glycation (12-15). Importantly, in db/db mice carnosine supplementation was able to delay the development of type 2 diabetes (12) suggesting that carnosine supplementation may prevent type 2 diabetes. Larger adequately powered human trials with gold standard measures of insulin sensitivity and secretion are needed to determine the potential of carnosine supplementation in prevention of obesity related insulin resistance and type 2 diabetes.

Mechanisms by which carnosine may decrease risk for type 2 diabetes include anti-inflammatory, anti-AGEs, anti-ALEs, reactive carbonyls species trapping activity and anti-oxidant effects in addition to the specific effects on the autonomic nervous system (13, 14, 20-22, 30). We and others have shown that these processes lead to insulin resistance in humans through NFKB and JNK pathways or directly effecting insulin signalling (31, 32). In addition, carnosine has been shown (i) to inhibit pathways important for insulin signalling such as Akt/mTOR/p70S6K pathway, (ii) to inhibit matrix metalloproteinases (MMP-2 & MMP-9) via urokinase plasminogen activator (uPA) - uPA receptor as well as (iii) to suppress transforming growth factor beta (TGFβ) production via inhibition of ALK5 pathways (17, 33-35). In this pilot trial, we did not see any changes in pro- and anti-inflammatory biomarkers such as plasma hsCRP and adiponectin suggesting that in humans the observed effect on insulin sensitivity and secretion are not primarily driven by changes in inflammation. Another possibility is that our obese but relatively healthy study

population did not have significantly increased biomarkers to start with and therefore no change was observed. Moreover, we measured only 2 plasma markers of inflammation.

Carnosine has been shown to improve lipid metabolism (13, 15, 36) and reduce blood pressure (11, 15, 37) in animal models. Specifically, carnosine has been shown to reduce lipid peroxidation (10), atherogenic ApoB containing lipoproteins (oxidized LDL and VLDL) (38), triglycerides and extracellular lipid in the plaque in rodents (35). The anti-hypertensive effect of carnosine has been described in various mammalian species and has been attributed to direct vasorelaxing effects of carnosine (11, 15), which is dose dependent (37). Suggested mechanisms of anti-hypertensive effects of carnosine have been mediated *via* histamine/histidine pathway (39), nitric oxide/cGMP mechanism (37) and the direct effects on autonomic nervous system (11, 14).

Recent and first human study showed that carnosine supplementation (500 mg daily) for 6 months resulted in improved physical performance (6 min walk test and VO_2max) and improved quality of life – along with a trend to increase the end-diastolic volume (p=0.07) in individuals suffering stable chronic heart failure and severe left-ventricular dysfunction (9). In our study, there was no significant effect of carnosine on ~~change in~~ systolic or diastolic blood pressure or lipid parameters. It is possible that this lack of effect was due to the fact that we studied relatively young, healthy, normolipidemic and normotensive population of relatively small size. But, available evidence indicates that there is no effect of carnosine on lipids and blood pressure in humans. Large-scale ~~mechanistic~~ human trial is necessary to determine the potential of carnosine in prevention and treatment of dyslipidemia and hypertension as well as to study molecular mechanisms of carnosine-induced metabolic benefits in humans.

Limitations: (i) Firstly, we used~~,~~ HOMA-IR and HOMA-**β**, which are indirect measures of insulin sensitivity and secretion both calculated from the circulating levels of fasting glucose and insulin. Gold standard method to measure insulin sensitivity such as

euglycemic hyperinsulinemic clamp, would have to be employed in the future studies. (ii) In addition, in both treatment groups HOMA-IR and HOMA- **β** increased but the increase was significantly smaller in carnosine treatment group compared to placebo. This overall intervention-induced change in the study population could have been due to change in both diet and exercise when transitioning to winter months (trial took place between September and December). (iii) Study population contained only a small subpopulation of individuals with impaired glucose tolerance, where effect of carnosin on 2h glucose was observed. Larger studies with gold standard measures of body composition, insulin sensitivity and insulin secretion using a specific population of prediabetic individuals are essential to confirm our findings. Secondly, despite our population was overweight/obese and more insulin resistant compared to the general population, they had normal blood pressure, plasma lipids and inflammation markers. Findings might differ in hypertensive and dyslipidemic populations.

In conclusion, we have demonstrated that carnosine supplementation compared to placebo resulted in a relative conservation of insulin sensitivity and insulin secretion with no effect on CVD risk factors after 12 weeks of supplementation. 3-months carnosine supplementation normalized glucose intolerance in subgroup of individuals with impaired glucose tolerance. These findings need to be confirmed in larger studies in high-risk groups using gold standard measurements of insulin sensitivity and secretion. Further investigation is warranted to determine the mechanisms by which carnosine contributes to obesity and insulin resistance in humans.

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List of tables and figures

TABLE 1: Characteristics of the two intervention groups

Parameters	**Carnosine**			**Placebo**		
Gender (F/M)	3/12			4/7		
Age (yrs.)	42±7			43±10		
	Baseline	**Follow-up**	**Δ(FU-BL)**	**Baseline**	**Follow-up**	**Δ(FU-BL)**
Body weight (kg)	98.6±19.4	99.6±18.2	1.0±2.8	100.0±7.5	100.0±7.9	-0.03±1.9
BMI ($kg.m^{-2}$)	31.1±4.6	31.4±4.1	0.3±0.9	31.6±3.7	31.7±3.7	0.08±0.9
Waist circumference (cm)	101.5±12.9	101.1±12.4	-0.3±2.4	102.5±8.6	104.2±8.7	1.7±3.5
Body fat (%)	31.5±7.5	31.8±7.5	0.27±0.6	33.8±9.0	33.9±9.5	0.05±1.3
[a]**RQ**	0.79±0.09	0.83±0.08	0.05±0.09	0.83±0.09	0.83±0.07	0.00±0.11
RMR (kcal/24 hours)	2076±400	1924±295	-152±290	1996±362	1865±236	-131±279
Fasting glucose (mmol/L)	5.2±0.4	5.4 ±.0.5	0.2±0.4	5.2±0.4	5.3±0.5	0.1±0.4
2-hour glucose (mmol/L)	6.4±2.0	6.0 ±.0.4	-0.4±1.7	7.6±2.5	7.0±1.9	-0.61±1.9
Fating insulin (mU/L)	11.8±7.2	12.9±6.9	**1.1±1.8***	14.0±5.4	18.3±8.5	**4.3±4.6*#**
HOMA-IR (mmol/L*mU/L)	2.8±2.0	3.2±1.8	**0.3±0.6***	3.2±1.2	4.3±2.1	**1.14±1.3*#**
HOMA-β (%)	131±56	135±56	3±30	176±90	212±110	**36±43*#**
Triglycerides (mmol/L)	1.7±1.0	1.7±1.0	0.05±0.6	1.6±0.5	1.7±1.0	0.1±0.9
Total Cholesterol (mmol/L)	5.5±0.9	5.3±0.7	-0.1±0.6	5.4±0.9	5.2±0.7	-0.2±0.6
Systolic BP (mmHg)	121±12	115±14	-6±13	124±12	124±14	0±13
Diastolic BP (mmHg)	79±6	76±9	-3±7	83±8	81±7	-2±8
Plasma adiponectin (μg/ml)	6.1±3.1	6.4±3.2	0.3±1.0	5.5±2.0	5.7±2.3	0.2±0.6
Plasma CRP (mg/l)	3.2±2.5	2.5±2.3	-0.7±2.5	3.4±2.8	3.4±4.0	0.1±5.1
[b]**Fat preference score** (High-Fat/Low-Fat score)	0.96±0.14	0.98±0.12	0.02±0.17	0.88±0.15	0.95±0.13	0.08±0.08
[c]**Steps per hour**	614±257	637±294	23±172	533±181	430±133	-97±131

Values are mean ± SD (*n* = 15 for the carnosine group and n = 11 for the placebo group).

Abbreviations: FU-follow-up, BL-baseline, CRP-high sensitivity C-reactive protein, BP-blood pressure, RQ-respiratory quotient, RMR-resting metabolic rate, HOMA-IR and HOMA-**β** - homeostatic model assessment of insulin resistance and insulin secretion, BP-blood pressure. None of the baseline characteristics in the control and placebo groups were significantly different between the treatment groups.
[a]RQ-respiratory quotient (VCO_2/VO_2) parameter of the fasting metabolic substrate preference, RQ=0.7 indicates 100% metabolic preference for fat, RQ=1.0 indicates 100% preference for carbohydrates.
[b]Data from the food preference questionnaire (27).
[c]Accelerometry data, accelerometer use >12h per day
(*) indicates significance within the intervention group and (#) shows significant differences between the intervention groups, both at $p<0.05$.

Table 2: Characteristics of the subgroups with normal and impaired glucose tolerance

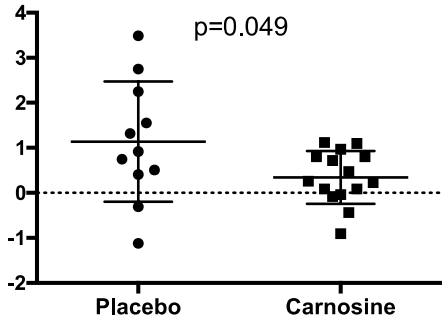
Parameters	**Carnosine**				**Placebo**			
	Normal glucose tolerance		**Impaired glucose tolerance**		**Normal glucose tolerance**		**Impaired glucose Tolerance**	
Gender (F/M)	**3/6**		**0/6**		**0/5**		**4/2**	
Age (yrs.)	43.5±7.1		40.5±6.8		43.8±10.2		45.3±9.4	
	Baseline	**Follow-up**	**Baseline**	**Follow-up**	**Baseline**	**Follow-up**	**Baseline**	**Follow-up**
Body weight (kg)	96.7±21.9	97.6±21.0	101.5±16.1	102.6±14.4	97.1±7.6	97.1±8.5	101.6±5.5	101.1±5.9
BMI ($kg.m^2$)	30.8±4.6^{a}	31.1±4.1^{a}	31.6±5.0^{ab}	31.9±4.4^{ab}	29.1±3.0^{a}	29.4±2.7^{a}	**35.5±3.6^{b}**	**35.4±3.5^{b}**
Waist circumference (cm)	99.1±14.7	99.5±14.3	103.0±11.4	103.5±9.6	101.2±11.3	102.6±10.7	105.3±5.6	107.3±6.4
Total body fat (%)	32.5±7.6^{a}	33.3±7.2^{a}	29.8±4.7^{a}	30.6±5.3^{a}	29.3±4.8^{a}	29.3±5.4^{a}	**43.0±10.1^{b}**	**42.4±9.9^{b}**
Visceral fat (%)	12.4±4.9	11.6±4.8	14.3±5.1	15.1±4.6	12.8±4.3	12.8±4.1	12.5±3.8	12.7±3.9
Fasting glucose (mmol/L)	5.19±0.44	5.23±0.28	5.26±0.25	5.58±0.62	5.23±0.17	5.41±0.40	5.25±0.43	5.27±0.61
2-hour glucose (mmol/L)	4.98±0.97^{a}	5.19±0.80^{a}	**8.60±0.81^{b}**	**7.15±0.99^{c}**	5.29±1.49^{a}	5.89±1.53^{ac}	**9.21±1.22^{b}**	**8.23±1.87^{bc}**
Fating insulin (mU/L)	11.0±9.0	12.2±8.8	12.5±3.8	13.5±3.9	12.1±6.4	17.2±11.5	18.2±2.6	18.8±5.8
2-hour insulin (mU/L)	67.3±67.6^{a}	62.9±53.3^{a}	**144.0±69.9^{b}**	76.1±61.8^{ab}	62.1±48.7^{a}	88.6±81.8^{ab}	**190.8±79.9^{b}**	**167.9±80.4^{b}**
HOMA-IR (mmol/L*mU/L)	2.74±2.53	2.91±2.27	2.94±0.98	3.43±1.21	2.81±1.47	4.22±2.84	4.21±0.51	4.42±1.53
Triglycerides (mmol/L)	1.33±0.55^{a}	1.37±0.68^{a}	**2.32±1.29^{b}**	2.15±1.32^{ab}	1.60±0.51^{ab}	1.80±0.69^{ab}	1.57±0.53^{ab}	1.26±0.34^{a}
Total Cholesterol (mmol/L)	5.26±0.87^{ab}	5.15±0.57^{ab}	5.75±0.96^{ab}	5.63±0.42^{ab}	**5.85±0.86^{a}**	5.56±0.39^{ab}	5.17±0.54^{ab}	**4.90±0.66^{b}**
HDL cholesterol (mmol/L)	1.37±0.47	1.35±0.42	1.06±0.15	1.09±0.20	1.18±0.21	1.21±0.20	1.32±0.24	1.30±0.24
Systolic BP (mmHg)	118±12^{ab}	120±17^{ab}	124±11^{ab}	**109±8^{b}**	**131±14^{a}**	**130±8^{a}**	**127±11^{a}**	**135±26^{a}**
Diastolic BP (mmHg)	81±6^{ab}	78±12^{ab}	**76±4^{b}**	**74±6^{b}**	**87±7^{a}**	83±2^{ab}	**87±11^{a}**	82±14^{ab}
Plasma hsCRP (mg/l)	3.26±3.2^{ab}	2.18±1.77^{a}	2.83±2.65^{ab}	3.00±2.91^{ab}	3.34±4.08^{ab}	1.24±0.89^{a}	**6.84±5.8^{bc}**	**9.52±6.89^{c}**
RQ	0.81±0.09	0.86±0.08	0.77±0.08	0.80±0.05	0.86±0.08	0.82±0.04	0.80±0.09	0.83±0.09
RMR (kcal/24 hours)	2008±456	1890±375	2178±309	1976±115	2113±297	1932±250	1898±408	1810±231
***Steps per hour**	657±298^{a}	659±294^{a}	516±93^{ab}	595±321^{ab}	**497±209^{b}**	**385±124^{b}**	557±176^{ab}	467±139^{ab}
Carnosine in *m. gastr.* (mM)	12.5±5.2	14.3±3.5	14.0±4.4	22.4±9.4	-	-	-	-
Fat preference score (High-Fat/Low-Fat score)	0.98±0.13^{ab}	0.98±0.12^{ab}	0.93±0.16^{ab}	0.93±0.12^{ab}	0.83±0.10^{b}	0.90±0.14^{ab}	0.91±0.18^{ab}	1.0±0.12^{a}

hsCRP-high sensitivity C-reactive protein, BP-blood pressure, RQ-respiratory quotient, RMR-resting metabolic rate, HOMA-IR and HOMA-**β** - homeostatic model assessment of insulin resistance and insulin secretion. Values are mean ± SD, different letters (a,b,c) denote statistical significance at $p<0.05$.

Table 3: General linear models for HOMA-IR and HOMA- β

Change in HOMA-IR				
R^2=0.21	**Estimate**	**Standard error**	**t Ratio**	**p**
Intercept	1.02	1.12	0.91	0.4
Age (y)	0.002	0.03	0.1	0.9
Sex (M/F)	-0.02	0.26	-0.09	0.9
Change in body weight (kg)	0.1	0.08	1.26	0.2
Intervention group (CRN-PL)	-0.89	0.41	-2.21	0.038
Change in HOMA-beta				
R^2=0.45	**Estimate**	**Standard error**	**t Ratio**	**p**
Intercept	68.88	36.99	1.86	0.07
Age (y)	-1.01	0.83	-1.21	0.23
Sex (M/F)	-4.87	8.32	-0.59	0.56
Change in body weight (kg)	6.28	2.75	2.28	0.03
Change in HOMA- IR(mmol/l*mU/l)	6.93	7.07	0.98	0.33
Intervention group (CRN-PL)	-34.46	14.6	-2.35	0.03

Figure 1: Carnosine, HOMA IR & HOMA-β



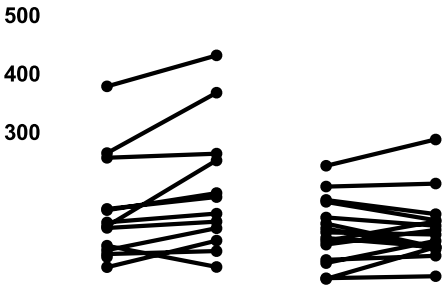


Figure 2: Effect of carnosine treatment on glucose metabolism in individuals with normal (A,B) and impaired (C,D) glucose tolerance.