Low Dose β-Carotene Supplementation Diminishes Oxidative Stress in Type 2 Diabetics and Healthy Individuals

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Abstract: Type 2 diabetes is a chronic, multifactorial disease, and oxidative stress one of the pathophysiological mechanisms associated with its appearance and development. The objective was to determine the effect of supplementation with β-carotene to type 2 diabetics and healthy individuals, on iron metabolism, oxidative balance, and antioxidant plasma capacity, using doses similar to the daily nutritional requirement. A total of 117 randomly selected non-smoker volunteers participated in the study. Type 2 diabetics (34) and healthy individuals (24), received 6 mg β-carotene for 45 d, and were compared to similar non-supplemented diabetic (33) and control (26) groups. Blood samples were taken at the beginning, end and 30 days after finishing supplementation, to determine hemoglobin, hematocrit unsaturated iron binding capacity, total iron binding capacity, transferrin saturation, ferritin, glycemia, glycosilated hemoglobin, cholesterol, triglycerides, HDL, LDL, oxidized LDL, copper, zinc, TBARS, FRAP, nitrites, GPx, SOD, folates, retinol and β-carotene. In supplemented diabetics, there was a significant diminution in copper concentrations (24.8%), and TBARS (9.5%), associated with a non significant increase in FRAP. Also, hemoglobin decreased and oxidized LDL augmented in supplemented diabetics. All the changes were still present one month after finishing the supplementation. In conclusion, supplementation with β-carotene had a positive effect on the oxidative balance of both, type 2 diabetics and healthy individuals, which makes the inclusion of β-carotene rich foods, part of the prevention and/or treatment strategies in type 2 diabetes.

Keywords: Type 2 diabetes, β-carotene, oxidative balance, copper, zinc, FRAP, TBARS.

INTRODUCTION

Type 2 diabetes mellitus is a chronic disease widely studied in recent years due to the alarming projections about its prevalence, indicating that approximately 360 million people will be diabetic by 2030, besides the increasing number of children and adolescents that are being diagnosed as diabetics [1-3]. As diabetes progresses life quality diminishes, due to its effect on organs such as eyes, kidneys, nerves, big blood vessels and heart.

This condition has multiple etiologies, being oxidative stress one of the proposed mechanisms, defined as an imbalance between prooxidant and antioxidant agents, which could be the result of an increase in oxidants production, a decrease in antioxidant defense or the simultaneous occurrence of both conditions, which is the case in diabetes mellitus [4].

In diabetes, several mechanisms have been implicated in the increased production of prooxidants that include the generation of Advanced Glycation End Products (AGE), the increase in the hexosamine pathway, as well as in the sorbitol pathway, among others [5]. On the other hand, there are reports about alterations in the generation and activity of antioxidants, either enzymatic or non enzymatic, associated to diabetes mellitus. Among non enzymatic antioxidants β-carotene, due to its multiple double bonds, posses the ability to interact with unpaired electrons from free radicals [6]. Studies about the role of β-carotene in diabetes have shown a decrease in serum concentrations of the carotenoid in diabetics, as well as a decreased risk of disease associated to both, high consumption and serum levels of β-carotene [7-9].

Regarding β-carotene supplementation in diabetes, there are no clear results due to methodological aspects such as combination with other antioxidants, duration of supplementation and the doses used that varied widely, being in most cases higher than the dietetic recommendations [10, 11].

The objective of this work was to determine the effect of the supplementation with β-carotene to type 2 diabetic patients and healthy individuals, on iron metabolism, oxidative balance, and antioxidant plasma capacity, to evaluate the effect of doses similar to the daily nutritional requirement on diabetics and a possible protective role on healthy individuals.

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METHODS AND MATERIALS

Subjects

A total of 117 non-smoker individuals participated in the study, 67 randomly selected type 2 diabetics from the Diabetes External Unit of Hospital Baudilio Lara in Venezuela, and 50 healthy individuals from social and cultural centers from the same city, with similar anthropometric characteristics than the diabetic group. The detailed protocol was explained to each individual, and if interested and after understanding the complete protocol, signed and informed consent. The protocol was registered in clinicaltrials.gov as IVIC-HUMNUT-001.

This study was conducted according to the guidelines laid down in the Declaration of Helsinki and all procedures involving human subjects/patients were approved by the Bioethics Committee of Instituto Venezolano de Investigaciones Científicas.

Protocol

Diabetics and controls were further and randomly divided in 2 groups: supplemented and not supplemented. Volunteers from both supplemented groups, diabetics (34 individuals) and controls (24 individuals), received 6 mg of β-carotene as a soft gel capsule (GNC, Pennsylvania-USA), daily for 45 d. From non supplemented groups, 33 volunteers were diabetics and 26 were control individuals. Each participant was appointed and tested in 3 opportunities: at days 0, 45 and 75 that correspond to the beginning of the study (T0), the end of fortification period (T45), and 30 d after finishing the fortification period (T75).

In each visit, subjects were interviewed, physically examined and requested to complete a 24 h food consumption recall questionnaire. Also in each visit, 20 mL of blood were drawn and separated in 2 aliquots, one of them with 10% EDTA as anticoagulant to determine hemoglobin, hematocrit, glycosylated hemoglobin and erythrocyte folates. The rest of the sample was centrifuged to obtain serum, aliquoted and stored at -20°C, for the other determinations.

Hemoglobin and Hematocrit

The blood samples were processed within 2 h after extraction, for hemoglobin and hematocrit quantifications [12,13]. Normal ranges for hemoglobin and hematocrit were 130-180 g/L and 42-52% for men and 120-160 g/L and 37-48% for women, respectively.

Ferritin

Was measured by an ELISA with monoclonal antibodies against human ferritin. The sample analysis was made using a standard curve and run with quality controls of known concentrations. The inter and intrassay variations were 5 and 7 % respectively [14]. Normal values were 12-150 µg/L for women and 12-200 µg/L for men.

Serum Iron, Total Iron Binding Capacity (TIBC) and Unsaturated Iron Binding Capacity (UIBC)

Serum iron and TIBC were determined by the colorimetric method proposed by the International Committee of Standardization of Hematology. The UIBC was calculated from the results obtained for serum iron and TIBC. Values used to define normality for serum iron were 11.64-32.23 µmol/L (65-180 µg/dL) for men and 8.96-30.45 µmol/L (50–170 µg/dL) for women; for TIBC 42.98-80.6 µmol/L (240-450 µg/dL), for UIBC 14.33-71.64 µmol/L (80-400 µg/mL), and for transferrin saturation 20-50 % [15, 16].

Blood Chemistry

Glycemia, triglycerides, total cholesterol, LDL, and HDL were determined automatically in a Ciba Corning 550 Express autoanalizer, using classic enzymatic methods for the determination of these variables. Normal ranges used include: Glycemia 3.89-6.11 mmol/L (70-110 mg/dL), triglycerides < 1.69 mmol/L (150 mg/dL), cholesterol <5.17 mmol/L (200 mg/dL), LDL 2.59-3.34 mmol/L(100-129mg/dL) and HDL >1.03 mmol/L (40 mg /dL).

Glycosylated Hemoglobin

It was determined using a commercial kit (Bioscience, Caracas, Venezuela), based in the isolation of the hemoglobin glycosylated at the N-terminal end, using cation exchange chromatography. The normal range for this kits is 6 to 8,3 % [17].

Oxidized LDL

Were analyzed by a solid phase two-site enzyme immunoassay from Mercodia (Sweden), which contains 2 monoclonal antibodies directed against separated antigenic determinants on the oxidized apolipoprotein B molecule [18].

Tiobarbituric Acid Reactive Substances (TBARS)

Were detected by the method of Feix [19], based on the quantification of malondialdehyde present in the

sample, by reacting 2 molecules of thiobarbituric acid with 1 molecule of malondialdehyde, which produces an abduct that is detected at 535 mn. Malondialdehyde concentrations were calculated from a standard curve prepared from 1,1-3,3-tetramethoxypropane, and for comparison purposes, were also calculated using malondialdehyde molar extinction coefficient (1,56 x 10^5 $M^{-1}cm^{-1}$). Reference values 0.8 - 2.13 nmol/mL.

Ferric Reducing Ability of Plasma (FRAP)

Measured after 4 and 10 min incubation, was used to determine the ability of plasma to reduce iron from ferric to ferrous state, based on the formation of a triazine-Fe^{+3} complex, that when reduced to Fe^{+2}, generate a change in color that is measured at 593 nm. Reference values: 0.6-1.6 mmol/L [20].

Activities of the Enzymes Superoxide Dismutase (SOD) and Glutathione Peroxidase (GPx)

Determined by commercial kits (Cayman Chemicals, Pittsburg) following the recommended protocols [21, 22]. For superoxide dismutase activity in samples, a tetrazolium salt was used to detect superoxide radicals generated by xanthine oxidase and hypoxanthine. Glutathione peroxidase activity was measured indirectly by a coupled reaction with glutathione reductase. Reference values: SOD 0,5 -1,5 U/mL, GPx 50-300 nmol/min/mL

Serum Nitrites

They were determined as an indirect measure of the concentration of nitric oxide, since nitrites are the stable end products of its degradation. Nitrates were reduced to nitrites by activated cadmium. Then sulfanilamide and nitrites generate a chromophore that reacts with naftilethylenediamine, to generate a product visible at 540 nm. For comparison purposes, standard curves were prepared from potassium nitrate (KNO_3) and sodium nitrite ($NaNO_2$). The KNO_3 curve had the same activation and incubation steps than serum samples, to allow transformation of all nitrates into nitrites, while processed samples were also quantified directly through the standard curve of $NaNO_2$. Reference values : 15-100 µmol/L [23].

Serum and Erythrocyte Folates

Determined by an adaptation from the Centers for Diseases Control and Prevention (Atlanta, USA) of the methods of O'Broin & Kelleher in 1992, and Molloy & Scott in 1997 [24, 25].The method is based in the folate-dependent controlled growth of a Lactobacillus strain that is measured spectrophotometrically and quantified against a standard curve. Normal values: serum folate: >13.2 nmol/L (>6 ng/mL), erythrocite folate > 360 nmol/L (>159 ng/mL).

Serum Zinc and Copper

By flame atomic absorption spectrophotometry in an AAnalyst 200 Perkin Elmer equipment. Samples were diluted 1:10 in deionized water. Working conditions were: flux 10 mL/min, acetylene 200 psi, wavelength 219,9 nm for zinc and 324,75 nm for copper. Concentrations were calculated against standards curves from 0 to 1 ppm of each element [26]. Normal ranges: zinc 10.0 -16.8 µmol/L (0.66 - 1.10 µg/mL), copper 11.48 - 22.19 µmol/L (0.75 - 1.45 µg/mL).

β-Carotene

It was determined by HPLC, with a reverse fase C_{18} column. The mobile phase was acetonitrile-dichlorometane-methanol (85:10:5) with 0.05 % ammonium acetate, at 1 mL/min. Detection was performed in a PDA detector at 450 nm. Reference values 0.41 µmol/L (0.215 µg/mL) [27].

Vitamin A

It was determined by HPLC, with a reverse fase C_{18} column. The mobile phase was 100% methanol at 0.8 mL/min and detection at 322 nm. Reference values: > 0,698 µmol/L (20 µg/dL) [28].

The rationale of the determinations was to assess the hematological, pro-oxidant, antioxidant, micronutrient and vitamin status of individuals and how are affected by diabetes. After detecting differences due to diabetes, the tests were repeated to evaluate the role of β-carotene on those parameters and on the prevention, development or prognosis of the disease.

Statistical Analysis

Results were expressed as arithmetic mean ± standard deviation, except for ferritin that was presented as geometric mean. One way ANOVA was used for comparisons between different groups: unsupplemented controls (CS), supplemented controls (CB), unsupplemented diabetics (DS) and supplemented diabetics (DB) at different times of sample extractions: T0, T45 y T 75. Paired t-test was used for comparisons between samples in the same group during times of extraction. The significance level was $p \leq 0.05$. The statistical program used was SPSS version 13.0.

Table 1: Anthropometric Characteristics of Participants

	CS	CB	DS	DB
Age (years)	52 ± 9	56 ± 6	55 ± 12	58 ± 10
BMI (Kg/m^2)	25.4 ± 2.9	25.1 ± 3.7	24.7 ± 2.8	25.3 ± 3.3
Gender				
Male	10	7	11	9
Female	16	17	22	25
Total	26	24	33	34

CS= control group without supplementation CB= control group supplemented, DS= diabetic group without supplementation, DB= diabetic group supplemented BMI= Body mass index.

RESULTS

The 4 study groups were comparable in age and body mass index (BMI) and in most cases this parameter was close or at the upper limit of normality, resulting that all groups, controls and diabetics, were classified as overweight (Table **1**). There was also a higher, but similar, proportion of females in all groups.

The evaluation of hematological parameters indicated that all groups had similar values of hemoglobin at the beginning of the study that did not change during the study, except for the group of supplemented diabetics that showed a statistically significant decrease in hemoglobin concentrations after supplementation with β-carotene (T45) and one month after the supplementation period ended (T75) (Table **2**).

Glycemia was between normal ranges for both control groups while it was around 8.33 mmol/L (150 mg/dL) for diabetic groups, which indicates that although above normal ranges, individuals were compensated or controlled. The same evidence for controlled diabetics was obtained with glycosylated hemoglobin, showing that both diabetic groups were around 9%, being the normal range for the kit used 6 -

Table 2: Changes in Hemoglobin, Hematocrit, Blood Glucose, Glycosylated Hemoglobin and Lipids in Diabetics and Controls, Supplemented or Not with Low Doses of β-Carotene and Evaluated Before (T0), After (T45) and 30 Days After the End of Supplementation (T75)[1]

	Hemoglobin (g%)	Hematocrit (%)	Blood Glucose (mmol/L)	Glycosylated Hemoglobin (%)	Triglycerides (mmol/L)	Cholesterol (mmol/L)	HDL (mmol/L)	LDL (mmol/L)	LDL Oxidized (U/L)
Unsupplemented Controls (CS)									
T0	13.2 ± 1.6	41.4 ± 3.4	4.59 ± 0.77	7.6 ± 0.7	2.33 ± 2.07	5.51 ± 1.38	1.25 ± 0.27	3.05 ± 1.26	47.1 ± 19.5
T45	13.6 ± 1.4	41.8 ± 3.3	4.87 ± 0.54	7.2 ± 0.8	2.30 ± 1.89	5.74 ± 1.38	1.17 ± 0.26	3.65 ± 1.42	45.2 ± 22.1
T75	13.5 ± 1.3	41.5 ± 2.7	4.54 ± 0.69		2.09 ± 1.99	5.44 ± 1.34	1.03 ± 0.43	3.74 ± 1.56	45.4 ± 16.9
Supplemented Controls (CB)									
T0	13.5 ± 1.3	41.9 ± 3.1	4.47 ± 0.84	7.6 ± 0.8	1.92 ± 1.17	4.89 ± 1.56	1.31 ± 0.35	2.85 ± 1.10	50.5 ± 19.5
T45	13.6 ± 1.3	40.5 ± 3.0	4.44 ± 0.96	7.6 ± 0.8	1.85 ± 1.26	5.42 ± 1.24	1.15 ± 0.52	3.35 ± 1.03	50.3 ± 15.9
T75	13.2 ± 1.4	41.5 ± 3.5	4.39 ± 0.96		2.11 ± 1.63	5.39 ± 1.44	1.05 ± 0.41	3.58 ± 1.17	49.4 ± 18.04
Unsupplemented Diabetics (DS)									
T0	13.6 ± 1.7	40.8 ± 5.6	9.23 ± 5.10[a]	9.4 ± 2.0[b]	1.72 ± 1.28	4.84 ± 1.26	1.18 ± 0.35	2.74 ± 0.99	45.1 ± 20.5
T45	13.6 ± 2.1	41.6 ± 5.7	7.94 ± 4.32[a]	9.5 ± 2.0[b]	1.61 ± 1.13	4.85 ± 1.41	1.16 ± 0.33	2.94 ± 1.25	43.3 ± 18.8
T75	13.3 ± 2.1	40.2 ± 5.1	9.00 ± 4.87[a]		1.89 ± 1.02	5.06 ± 1.33	1.27 ± 0.41	2.87 ± 1.16	48.8 ± 17.7
Supplemented Diabetics (DB)									
T0	13.2 ± 1.1	39.3 ± 3.3	7.72 ± 3.57[a]	9 ± 1.6[b]	1.96 ± 0.79	5.27 ± 0.84	1.13 ± 0.45	3.13 ± 0.70	47.9 ± 14.9
T45	12.7 ± 1.2*	38.7 ± 3.3	7.53 ± 3.23[a]	8.8 ± 1.6[b]	2.15 ± 0.97	5.05 ± 1.16	1.16 ± 0.32	2.96 ± 1.07	55.3 ± 18.1*
T75	12.4 ± 1.2*	38.7 ± 3.4	7.61 ± 3.01[a]		2.26 ± 1.02	5.61 ± 1.23	1.13 ± 0.39	3.40 ± 1.18	57.5 ± 18.9*

[1]Results represent mean ± standard deviation. * Statistically significant difference compared to T0. [a,b]Statistically significant difference compared to controls. < p 0.05.

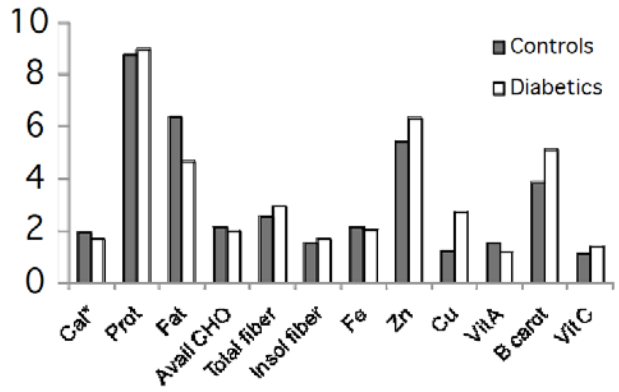

Figure 1: Comparison of daily nutrient consumption between diabetic and control groups, obtained from 24h- food consumption recall questionnaires.

* Cal = calories (Kcal x 1000), Prot = proteins (g x 10), Fat = fats (g x 10), Avail CHO = Available carbohydrates (g x 100), Total fiber = Total dietary fiber (g x 10), Insol fiber = Insoluble dietetic fiber (g x 10), Fe = Iron (mg x 10), Zn = Zinc (mg), Cu = Cooper (mg), Vit A = vitamin A (Retinol equivalents x 1000), β-carot= β-carotene (equivalents x 1000), Vit C = ascorbate (mg x 100), g = grams.

8.3%, and values above 10% indicate decompensation (Table **2**).

Triglycerides, cholesterol, HDL and LDL values showed no statistically significant differences between groups, although diabetic groups presented consistently lower mean values of these parameters when compared to controls. Oxidized LDL showed a statistically significant increase only in supplemented diabetics at the end of β-carotene administration period (T45) and 1 month later (T75) (Table **2**).

The 24h food recall questionnaires showed a higher consumption of calories, fat and carbohydrates in control groups compared to diabetics. On the other hand, zinc, copper, β-carotene, vitamin C, total and soluble fiber consumptions were higher in diabetic than in control groups (Figure **1**).

Regarding the parameters that measure iron metabolism, ferritin concentrations, serum iron, TIBC, UIBC and transferrin saturation did not change significantly in any of the groups studied during the protocol. However, serum iron and TIBC tended to be lower in groups receiving β-carotene, whether diabetics or not (data not shown).

The study of oxidative balance indicated no differences between groups for TBARS at the beginning of the supplementation. The administration of β-carotene produced a statistically significant

Table 3: Effect of Supplementation with β-Carotene in Diabetics and Control Subjects, on Some Indicators of Oxidative Stress, Evaluated Before (T0), After (T45) and 30 Days After the End of Supplementation (T75)[1]

	TBARS[2] (calculated by STD curve) (nmol/mL)	TBARS (Calculated by MEC) (nmol/mL)	FRAP (mmol/L)	Nitrites (µmol/L)
Unsupplemented Controls (CS)				
T0	1.17 ± 0.3	0.86 ± 0.22	0.532 ± 0.135	27.27 ± 16.28
T45	1.11 ± 0.24	0.81 ± 0.17	0.532 ± 0.135	20.68 ± 14.96
T75	1.1± 0.21	0.80± 0.14	0.511 ± 0.154	26.83 ± 15.71
Supplemented Controls (CB)				
T0	1.17 ± 0.33	0.84 ± 0.24	0.520 ± 0.112	21.30 ± 14.43
T45	1.11 ± 0.29	0.81 ± 0.21	0.546 ± 0.112	20.26 ± 12.23
T75	1.06 ± 0.27*	0.77 ± 0.20*	0.512 ± 0.132	24.38 ± 16.93
Unsupplemented Diabetics (DS)				
T0	1.16 ± 0.36	0.84 ± 0.25	0.512 ± 0.118	33.58 ± 19.8
T45	1.2 ± 0.35	0.88 ± 0.25	0.523 ± 0.126	31.18 ± 22.63
T75	1.25 ± 0.35	0.90 ± 0.25	0.522 ± 0.144	32.54 ± 19.25
Supplemented Diabetics (DB)				
T0	1.26 ± 0.31	0.91 ± 0.22	0.516 ± 0.120	35.01 ± 21.64
T45	1.15 ± 0.28*	0.83 ± 0.19*	0.536 ± 0.104	32.22 ± 29.50
T75	1.14 ± 0.26*	0.82 ± 0.19*	0.504 ± 0.164	34.65 ± 23.23

[1]Results represent mean ± standard deviation. * Statistically significant difference ($p < 0.05$) compared to T0. [2] TBARS = Thiobarbituric Acid Reactive Substances, MEC = Molar Extinction Coefficient, FRAP = Ferric Reducing Ability of Plasma.

reduction ($p<0.05$) in TBARS on both supplemented groups. In diabetics reduction was statistically significant at T45 and T75, and in supplemented control subjects only at T75 (Table **3**). Results for TBARS were obtained from a standard curve prepared with tetramethoxypropane, but also were calculated using the molar extinction (MEC) coefficient for malondialdehyde ($1{,}56 \times 10^5\ M^{-1}\ cm^{-1}$). Although concentration results obtained from the standard curve were higher than from MEC, differences were consistent and comparable in a reading to reading basis, concluding that either methodology could be used for this kind of study (Table **3**).

Along with the previous finding and also as a measure of oxidative balance status, both supplemented groups showed an increase in ferric reducing capacity (measured as FRAP), that increased during the fortification period and returned to normal values 1 month after it was finished (Table **3**). The enzymatic activities of SOD and GPx did not show changes associated to diabetes or as a result of the supplementation (data not shown).

The concentrations of nitrites were not statistically different between groups, although both diabetic groups had higher values of nitrites compared to controls at all times of sample extraction (Table **3**). The use of standard curves prepared from KNO_3 or $NaNO_2$ for calculation of nitrites concentration was similar for both methods and could be used indistinctly.

Copper concentrations were higher in diabetics than in controls at the beginning of the study, although differences were no statistically significant. After 45d supplementation with β-carotene, there was a significant ($p<0.05$) reduction in copper concentrations in diabetic individuals, that was still significant 1 month after finishing supplementation. Zinc concentrations did not change between the groups at any time point analyzed (Table **4**).

Serum and erythrocyte folates were higher in diabetics than in controls. The difference was statistically significant ($p<0.05$) when comparing supplemented diabetics vs controls at T0 (Table **4**).

Concentrations of serum β-carotene and retinol did not change between groups at any time of the study. There was a high variation in β-carotene concentrations which resulted in a high SD (mean 0.38 µmol/L, SD 0.42 µmol/L or 0,20 and 0,22 µg/mL,

Table 4: Effect of Supplementation with β-Carotene in Diabetics and Control Subjects, on Serum Zinc, Copper, Folates and Erythrocyte Folates, Evaluated Before (T0), After (T45) and 30 Days After the End of Supplementation (T75)[1]

	Zinc (µmol/L)	Copper (µmol/L)	Serum Folate (nmol/L)	Erythrocyte Folate (nmol/L)
Unsupplemented Controls (CS)				
T0	0.144 ± 0.03	0.148 ± 0.04	11.23 ± 8.0	214.2 ± 63.9
T45	0.145 ± 0.02	0.162 ± 0.04	12.69 ± 6.77	201.3 ± 75.2
T75	0.144 ± 0.02	0.158 ± 0.04	11.67 ± 6.53	187.7 ± 82.0
Supplemented Controls (CB)				
T0	0.144 ± 0.02	0.156 ± 0.03	13.43 ± 19.65	198.9 ± 93.14
T45	0.138 ± 0.04	0.168 ± 0.04	14.73 ± 20.56	240.1 ± 233.3
T75	0.138 ± 0.03	0.145 ± 0.03	12.22 ± 18.0	323.4 ± 295.8
Unsupplemented Diabetics (DS)				
T0	0.139 ± 0.03	0.173 ± 0.05	26.34 ± 21.83	291.5 ± 148.5
T45	0.138 ± 0.03	0.165 ± 0.04	25.36 ± 26.26	277.6 ± 143.0
T75	0.144 ± 0.03	0.171 ± 0.05	21.64 ± 20.42	248.1 ± 135.8
Supplemented Diabetics (DB)				
T0	0.138 ± 0.03	0.185 ± 0.04	37.39 ± 37.73[+]	429.7 ± 393.5
T45	0.139 ± 0.02	0.150 ± 0.03*	29.64 ± 27.44	389.4 ± 324.4
T75	0.136 ± 0.02	0.139 ± 0.04*	32.85± 29.93	386.9 ± 314.6

[1]Results represent mean ± standard deviation. * Statistically significant difference ($p < 0.05$) compared to T0. [+] Statistically significant difference ($p < 0.05$) compared to control group at T0.

respectively), unrelated to presence of diabetes or time of sampling (data not shown).

DISCUSSION

This study shows the effect of the supplementation with β-carotene on oxidative balance of diabetic and non diabetic individuals, highlighting in the experimental design, the inclusion of diabetic and non diabetic groups without supplementation and also that the doses administered were similar to the daily recommendation for β-carotene.

The study groups had similar anthropometric characteristics and according to BMI presented overweight, a common finding in age groups close to 50 years, which also explains the alterations found in the lipid profiles, similar in diabetics and controls [29], although some parameters such as cholesterol and triglycerides concentrations were more elevated in control groups probably due to a nutritional guidance in controlled diabetic individuals. This aspect was corroborated by the 24h recall questionnaires indicating that diabetics consumed less calories, fat and carbohydrates and more micronutrients and vitamins (as fruits and vegetables) than non diabetic controls.

The statistically significant increase in oxidized LDL found in diabetic individuals after supplementation was unexpected, and we were not able to find in the literature, experimental evidence of an interaction between β-carotene and LDL in diabetics that explains an increase in oxidized LDL (total LDL did not change) or at least in the conformational epitope quantified by the method used. This finding needs to be corroborated and deserves further investigation.

The decrease in hemoglobin concentrations in supplemented diabetics, associated to normal ferritin values, was another unexpected finding of this study, since it has been reported a positive effect of β-carotene on iron solubility, which results in increased iron absorption [30]. Although it is common to observe a decrease in hemoglobin concentrations in the diabetic nephropathy, Craig *et al.* reported that diminution in hemoglobin is a common finding with the development of diabetes even without nephropathy [31]. In agreement with these results, our data shows that hemoglobin values were lower as time of diabetes diagnosis increased, finding the lowest concentrations in subjects that have been diagnosed as diabetics for more than 10y (data not shown). Although the diminution in hemoglobin seems to be a consequence of diabetes, it remains to elucidate if β-carotene has an additional effect, probably affecting iron mobilization. The lower values found for serum iron and TIBC after supplementation may indicate such mobilization, but this has to be clarified.

Serum copper concentration was higher in diabetics compared to controls which is common in inflammatory diseases such as diabetes, which adds up to other mechanisms such as glycosilation and fragmentation of Zn-Cu SOD, which releases copper [32], highly reactive in the generation of free radicals [33]. For that reason, one of the most remarkable results from this study was the statistically significant diminution in copper concentrations of diabetics receiving β-carotene, that persisted for up to 1month after finishing the supplementation. It is possible, although it has to be demonstrated, that β-carotene mediates this effect by affecting copper solubility and mobilization, as has been reported for iron [30]. There are reports about low serum zinc concentrations in diabetics associated to an increase in urinary excretion. However, other studies indicate normal values especially when there is no kidney damage [34], as in this study.

The bibliography on folates and diabetes is scarce, although the connection is clear. Folates are part of the reactions responsible of $NADPH/NADP^+$ balance in cells as well as cofactors of enzymes like methionine synthase responsible of homocysteine detoxification. In fact, there is an inverse relationship between levels of folates and homocysteine, making folates an interesting tool for preventing or managing of vascular complications. In this study folates concentrations were higher in diabetics, supplemented or not, than in controls. This was the case for both, serum or erythrocyte folates, indicating that differences with controls was not the result of recent folates consumption, but to an adequate nutritional counseling [35] that resulted in a regular consumption of a folates richer diet in diabetics, which was corroborated by the 24h recall questionnaires.

The significant reduction of TBARS in both supplemented groups could be explained by the structural characteristics of β-carotene with multiple double bonds and associated to the plasmatic membrane, where it could reduce local lipid peroxidation and control oxidative stress. Furthermore, the fact that the effect was also obtained in supplemented controls, makes β-carotene a powerful tool not only for treatment but also for the prevention of pathologies in which oxidative damage or imbalance are part of the pathophysiologic mechanism.

Along with TBARS reduction, there was a tendency for increase of FRAP in supplemented groups that lasted during the supplementation period. This finding is interesting because indicates that β-carotene is being incorporated as part of the plasma reducing activity as well as antioxidant in membranes, but also because the effect, although not significant, was observable in control groups. It is probably necessary to increase β-carotene doses to evaluate the intensity and duration of the effect.

The evaluation of nitrites as an indirect measure of nitric oxide, SOD and GPx did not change as a result of β-carotene supplementation. This could be due to the low doses tested, which also explain that there was no change in serum β-carotene concentration in supplemented groups. As stated before, the doses used for supplementation were similar to the daily requirement and quite low compared to other studies [10]. Serum retinol was also measured to rule out a difference in conversion of β-carotene to retinol due to diabetes and/or supplementation [36], but there were no changes in serum retinol or β-carotene under our experimental conditions.

The fact that small doses of β-carotene produced favorable effects motivates to design further studies with different times and doses in order to improve the achieved effects, but also to issue nutritional recommendations to diabetics through daily consumption of β-carotene rich vegetables and fruits, that could improve their quality of life and make unnecessary the use of supplements. Moreover, the antioxidant effect described in supplemented controls makes possible to think in a preventive or protective effect against illnesses generated or propagated by oxidative imbalance.

In conclusion, supplementation with low doses of β-carotene produced a diminution in the generation of reactive oxygen species measured by TBARS, the increase in antioxidant defense measured by FRAP and the diminution of a pro-oxidant factor such as copper in controlled type 2 diabetics, that remained at least 1 month after finishing supplementation. The effect of different doses and periods of supplementation with β-carotene or other carotenoids should be evaluated to determine its impact on the evolution of the diabetes or other chronic diseases, as well as the preventive value of this treatment, that could be as simple as a change in food habits.

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