

Effect of Beta-Carotene on Oxidative Stress and Expression of Cardiac Connexin 43

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Abstract

Background: Intervention studies have shown an increased mortality in patients who received beta-carotene. However, the mechanisms involved in this phenomenon are still unknown.

Objective: Evaluate the influence of beta-carotene on oxidative stress and the expression of connexin 43 in rat hearts.

Methods: Wistar rats, weighing approximately 100 g, were allocated in two groups: Control Group (n = 30), that received the diet routinely used in our laboratory, and Beta-Carotene Group (n = 28), which received beta-carotene (in crystal form, added and mixed to the diet) at a dose of 500 mg of beta-carotene/kg of diet. The animals received the treatment until they reached 200-250g, when they were sacrificed. Samples of blood, liver and heart were collected to perform Western blotting and immunohistochemistry for connexin 43; morphometric studies, dosages of beta-carotene by high-performance liquid chromatography as well as reduced glutathione, oxidized glutathione and lipids hydroperoxides were performed by biochemical analysis.

Results: Beta-carotene was detected only in the liver of Beta-Carotene Group animals ($288 \pm 94.7 \mu\text{g/kg}$). Levels of reduced/oxidized glutathione were higher in the liver and heart of Beta-Carotene Group animals (liver - Control Group: 42.60 ± 1.62 ; liver - Beta-Carotene Group: 57.40 ± 5.90 ; $p = 0.04$; heart: - Control Group: 117.40 ± 1.01 ; heart - Beta-Carotene Group: $121.81 \pm 1.32 \text{ nmol/mg protein}$; $p = 0.03$). The content of total connexin 43 was larger in Beta-Carotene Group.

Conclusion: Beta-carotene demonstrated a positive effect, characterized by the increase of intercellular communication and improvement of anti-oxidizing defense system. In this model, mechanism does not explain the increased mortality rate observed with the beta-carotene supplementation in clinical studies. (Arq Bras Cardiol. 2013;101(3):233-239)

Keywords: Beta Carotene / adverse effects; Oxidative Stress; Connexin 43; Rats; Ventricular Remodeling.

Introduction

Communicating junctions, or gap junctions, are specialized intercellular contact regions that connect the cytoplasm of adjacent cells, allowing electrical and metabolic coupling¹. Each communicating junction consists of proteins called Connexins (Cx). More than 20 coding genes of Cx were identified. In the heart, the three main isoforms correspond to Cx40, Cx43 and Cx45, being Cx43 more abundant in atrial and ventricular myocytes, and in distal parts of the conduction system²⁻⁴.

Reduction of intercellular communication via communicating junctions is related to cardiac arrhythmia^{5,6} and is present in several situations in which the heart is subjected to any form of aggression. In this case, changes

on communicating junctions were verified in ventricular remodeling situations, such as hypertension³, congestive heart failure⁷ and acute myocardium infarction⁸.

Beta-carotene is a carotenoid with pro-vitamin A activity, present in human diet, fruits and colored vegetables^{9,10}. It has a variety of functions, including antioxidant action¹¹ and increase of intercellular communication through communicating junctions in several types of tissues of the body^{9,12,13}. However, there are not known studies to evaluate the effects of beta-carotene in relation to Cx43 in the heart.

Intervention studies, designed to test the hypothesis that beta-carotene would protect humans against the development of cancer or cardiovascular diseases, showed increased cardiovascular death rate in groups supplemented with carotenoid^{9,14-16}. Additionally, studies performed in Unidade de Pesquisa Experimental (UNIPLEX) showed the supplementation of rat diet with beta-carotene induced ventricular remodeling, characterized by increased systolic diameter of the Left Ventricle (LV) and decreased ejection fractions and ventricular shortening¹⁷. Moreover, in a study that analyzed the effects of beta-carotene before infarction in normal and smoking rats, it was observed that it eliminated the paradoxical cigarette smoke-induced protective effect¹⁸.

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Therefore, in face of the negative effects produced by beta-carotene in the heart, the objective of this study is to evaluate the influence of beta-carotene on oxidative stress and Cx43 expression in rat hearts.

Methods

The experimental protocol was approved by Ethics Commission in Animal Testing of Faculdade de Medicina de Botucatu, and it complies with Ethical Principles of Animal Testing, adopted by the Brazilian College of Animal Testing.

We used Wistar male rats, weighing approximately 100 g. The animals were allocated, randomly, in two experimental groups: Control Group (C), consisting of 30 animals, that received the diet routinely used in Unidade de Pesquisa Experimental (UNIPEX); and Beta-Carotene Group (BC), consisting of 28 animals which received beta-carotene (in crystal form, added and mixed to the diet) at a dose of 500mg of beta-carotene/kg diet. The animals received the treatment until they reached 200-250 g (between 4 and 5 weeks).

After the end of the treatment, the animals were sacrificed with an excessive dose of anesthetic (100 mg/kg of pentobarbital sodium) and, subsequently, blood, liver and heart samples were collected for performing the experimental protocol.

Morphometric study

After euthanizing the animals, their hearts were removed and dissected; atria and ventricles were separated and weighed. The weights of LV and Right Ventricle (RV) were adjusted according to final body weight of the rat and used as ventricular hypertrophy index.

Heart tissue samples were fixed in formalin solution at 10%, according to method previously described¹⁹. After fixation, the tissue was placed in paraffin, subsequently obtaining sections measuring 4 μ m. Histological sections were stained in microscope slides with Haematoxylin-Eosin (HE) solution for analyzing areas of the myocytes cross-section, using Leica DM LS microscope coupled to video camera, which sends digital images to computer with the image analysis program Image-Pro Plus (Media Cybernetics, Silver Spring, Maryland, USA). We measured 50-70 cells per analyzed ventricle²⁰. Myocytes selected were cross-sectioned and had rounded shape and visible core in the center of the cell; they were located in the subendocardial layer of LV muscular wall. This procedure aimed at standardizing to the fullest extent the set of myocytes from different groups. Mean sectional areas obtained for each group were used as an indicator of cell size.

For evaluating LV myocardium interstitium, slides with coronary histological sections of 5 μ m, stained by the Picrosirius red technique and specific for visualization of collagen. Reading was performed using Leica DM LS microscope coupled to video camera, which sends digital images to computer with image analysis program Image-Pro Plus (Media Cybernetics, Silver Spring, Maryland, USA). We analyzed 30-40 fields per ventricle²⁰, using a 40X objective. Fields chosen were distant from the perivascular region.

Western blotting for Cx43

Western blotting experiments were performed in compliance with Rolim et al²¹, with a few modifications²². LV samples were homogenized using the device Polytron (Ika Ultra-Turrax® T25 Basic, Wilmington, USA) with hypotonic lysis buffer (50 mM potassium phosphate; 7.0 pH; 0.3M sucrose; 0.5mM DTT; 1mM EDTA; 8.0 pH; 0.3 mM PMSF; 10 mM NaF and phosphatase inhibitor) and centrifuged (Eppendorf 5804R, Hamburg, Germany) at 12,000rpm, for 20 minutes, at 4°C. Protein concentration was analyzed through Bradford method, using BSA Protein Assay Standard (Bio-Rad, Hercules, CA, USA) curves as standard, with 595nm absorbance reading.

Electrophoretic run was performed in biphasic stacking gel (240mM Tris-HCL; 6.8 pH; 30% polyacrylamide; APS and Temed) and running gel (240mM Tris-HCL; 8.8 pH; 30% polyacrylamide; APS and Temed), at 10% concentration at 120V (PowerPac HC 3.0, Bio-Rad, Hercules, CA, USA) for 2 hours and 10 minutes. Transfer to nitrocellulose membrane performed in Mini Trans-Blot (Bio-Rad, Hercules, CA, USA) system, using a transfer buffer (25mM Tris; 192mM glycine; 20% methanol and 0.1% SDS). After transfer, the membranes were incubated in blocking solution at 0.5% of skimmed milk powder, dissolved in buffer 7.4 pH TBS (20mM HCl Tris; 137mM NaCl and detergent 0.1% 20 Tween) for 2 hours, in ambient temperature. After this period, the membrane was rinsed three times in basal solution (Tris 1M; 2.8 pH; 5M NaCl and Tween 20) and incubated with primary antibodies diluted in blocking solution, under constant agitation for 12 hours. After incubation with primary antibody Mouse Monoclonal to GJA1 (Abcam) – for total Cx43, at a concentration of 1:250, and Mouse Monoclonal Anti-Connexin-43 (Zymed) – for non-phosphorylated Cx43, at a concentration of 3 μ g/mL, the membrane was rinsed in basal solution and incubated with secondary antibody (1:10,000) IgG anti-mouse HRP (Sigma), in blocking solution, for 2 hours under ambient temperature and constant agitation.

Immunodetection was performed through chemiluminescence method according to manufacturer's instructions (Enhancer Chemi-Luminescence, Amersham Biosciences, NJ-USA).

The normalization antibody we used corresponded to GAPDH, Mouse IgG1 (ABR, Affinity BioReagents, Golden, CO, USA) (1:10,000).

Quantitative analysis of blots we obtained were performed using the program Scion Image (Scion Corporation, Frederick, Maryland, USA), in free software, available at the website <http://www.scioncorp.com>

Immunohistochemistry for Cx43

Immunohistochemistry experiments were performed in compliance with Saffitz et al²³, with a few modifications. Fragments of the left ventricular myocardium, successively fixed in formaldehyde at 10%^o for 24 hours, were immersed in paraffin and, subsequently, histological sections of 3 μ m were made. Then, the sections were kept incubated at 60°C, during 24 hours. Deparaffinization was performed in successive immersions in xylene, alcohol 100^o, alcohol 95^o and alcohol

70°, followed by hydration in distilled water. Histological sections were placed in citric acid solution at 0.01M, 6.0 pH and subjected to microwave environment for 10 minutes, for antigen recovery. After 20 minutes of cooling process, slides were rinsed in 7.2 pH sodium phosphate buffer (PBS) and incubated for 30 minutes in bovine serum albumin (BSA) solution at 3% (BSA solution in PBS), for blocking unspecific reactions. Subsequently, they were incubated overnight at 4°C in humid chamber with primary antibody (Rabbit Polyclonal Anti-Connexin 43; Abcam), diluted at 1:100.

After this period, the slides were rinsed in PBS, and sections were incubated with secondary antibody (Texas Red anti-rabbit IgG; Vector), diluted at 1:50, for 1 hour, in ambient temperature. Then, slides were rinsed in PBS and covered with mounting medium, composed by buffered glycerin and microslide. For each battery of immunohistochemical reactions performed (ten slides), the negative control corresponded to one LV slide, in which the primary antibody was omitted.

Reading was performed through a microscope embedded with an epifluorescence unit (Carl-Zeiss, Inc. North America) coupled to a video camera (AxioPlan 4.1; Carl-Zeiss Inc.) using 40x augmentation. We used the green filter (WG), with 550 nm excitation wavelength and 650nm emission.

Beta-carotene dosages

Beta-carotene concentrations, in serum, were quantified according to Yeum et al²⁴ and, in liver and heart, they were dosed according to Paiva et al²⁵. The methodology used was high-performance liquid chromatography (HPLC), with chromatograph Alliance 2695 (Waters) coupled to detector PDA 2996 (Waters). Analytical column corresponded to C₃₀ (Waters –YMC carotenoid: 4.6 x 150mm; 3.0µm). Beta-carotene was quantified by determining the sample/internal standard peak area. Values obtained were corrected according with losses during samples extraction and handling for recovery of internal standard.

Study of oxidative stress

The study of oxidative stress was performed in accordance with Azevedo et al²⁶. Samples of approximately 200 mg of tissue (LV or liver) were homogenized with 5mL of 0.1M sodium phosphate buffer, 7.0 pH, under ice, in homogenizer. The homogenized samples were centrifuged (10,000 rpm) for 15 minutes, in refrigerated centrifuge at -4°C. Supernatants obtained were used to determine the concentration of Reduced Glutathione (GSH), Oxidized Glutathione (GSSG) and lipids hydroperoxides. Spectrophotometric readings were obtained via ELISA (Biotek Instruments, INC) reader, and in spectrophotometry Pharmacia Biotech (England).

Definition of lipids hydroperoxides

Lipids hydroperoxides were determined by Fe⁺² oxidation (ferrous ammonium sulphate). Fe⁺³ reacts with orange xylenol, forming a colored compound. Readings were performed with a 560nm wavelength.

Definition of GSH and GSSG

GSH (non-protein groups of SH) concentration was determined in buffer tris-HCl, 8.9 pH, and 5.5'-dithiobis (2-nitrobenzoic acid), (DTNB) after previous precipitation with trichloroacetic acid (TCA) 50%. As standard, we used GSH 1 mM²⁷. GSSG concentration was determined by the difference between total GSH and reduced GSH, being the stoichiometric ratio (2GSH:GSSG)²⁸.

Statistical Analysis

Results of each variable, for each experimental group, were tested for normality and variance equality. Since in all comparisons these conditions were met, Student *t* test was deployed for analyzing the studied variables.

Results

In serum and heart, beta-carotene presence was not detected. In liver, only BC Group animals showed detectable concentrations of this carotenoid (288 ± 94.7 µg/kg).

All animals had body weight between 200 and 250 g, and there was no statistically relevant difference between C and BC Groups (Table 1). Collagen levels were lower in rats supplemented with BC (Table 1).

No significant difference was detected between C and BC Groups regarding location and staining intensity (quantity) for Cx43 (Figure 1 and Table 1). However, Cx43 total content (phosphorylated + non-phosphorylated) was larger in BC Group, when compared to C Group (Figure 2).

GSH/GSSG concentrations were higher in liver and heart of BC Group compared to C Group. As for other variables, no statistic difference was noticed between the experimental groups (Table 2).

Discussion

This study was performed to analyze the effects of beta-carotene on intercellular communication and oxidative stress in rats' heart. This study found carotenoid increases Cx43 expression, as well as heart defense antioxidant.

As for the effects of beta-carotene antioxidants, antioxidant vitamins have been studied due to their capacity of preventing chronic diseases, however, using these compounds for cardiovascular diseases remains controversial. Discrepancies in results obtained can be related to individual characteristics or administered antioxidant doses^{29,30}.

Beta-carotene antioxidant activity is well characterized in literature. In several studies, with animals and humans, it offers protection against oxidative damages¹⁰. Also after heart injuries, using antioxidants has a positive effect^{31,32}. Since the oxidative stress is involved in etiology and progression of several heart chronic diseases, antioxidants, such as beta-carotene, are considered important strategies in the prevention of these diseases^{30,33}. In this study, beta-carotene showed an antioxidant activity, characterized by the increased GSH/GSSG relation in heart and liver of rats, thus protecting the total antioxidant system.

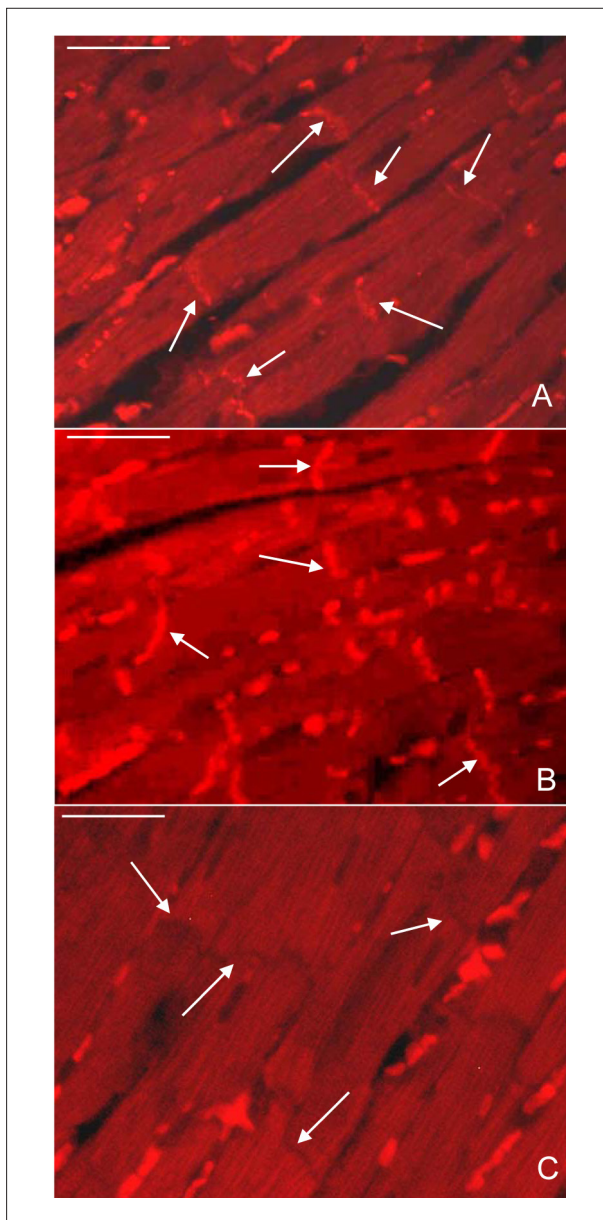


Figure 1 - Immunohistochemistry for connexin 43 in left ventricular myocardium. (A) Control; (B) supplemented with beta-carotene; (C): negative control.

Another beta-carotene effect, in this study, corresponded to decreased collagen levels in heart tissue. In cases of aggression to the heart, it is known the participation of free radicals in ventricular remodeling process³³⁻³⁵, as well as prevention or attenuation of this process, when antioxidants are used^{32,36}.

Among morphological changes observed during the remodeling process, is interstitial fibrosis³⁷. Studies have shown that oxidative stress induces collagen synthesis, producing, consequently, fibrosis in heart tissue^{34,36}. Among mechanism, it has been observed that free radicals induce the expression of TGFβ₁, growth factor that increases the collagen synthesis, in addition to suppressing its degradation in heart tissue³⁶.

This study is in compliance with such concept, since the decrease of oxidative stress was associated with a decreased collagen quantity. Thus, we may suggest the decreased collagen levels, in this study, occurred due to an improvement in the antioxidant system, related to the diet supplemented with beta-carotene^{10,12}.

Regarding the increased expression of Cx43 in heart tissue, induced by beta-carotene in this study, this was a very relevant finding, once stimulating intercellular communication using carotenoids is frequently documented in the literature of other tissues, being considered one of the mechanisms that prevent cancer^{9,10,13}. Moreover, it has been demonstrated that beta-carotene increases the expression of Cx43 in several tissues, such as human and mouse fibroblasts^{38,39}, adenocarcinoma cell lines in human lung¹³ and rat liver cells⁴⁰. In relation to beta-carotene effect on carcinogenesis, carotenoids, with the increased expression of Cx43, restore intercellular communication, an effect that is correlated to growth inhibition of chemically transformed cells⁹.

In this study, the effect of increased expression of Cx43 induced by beta-carotene occurred in non-damaged hearts. This finding is in compliance with other results published in the literature, in which it is observed the increased expression of Cx43 due to this carotenoid in several types of tissues of the body^{9,38}. Thus, in this experimental model, the supplementation of diet with beta-carotene does not behave as aggression, since, in damaged hearts, there is a decreased expression of Cx43^{3,7,8}. Additionally, this change does not explain the increased adverse events observed in intervention studies with beta-carotene.

Mechanisms through which beta-carotene increases the expression of Cx43 are yet not completely known. However, it is

Table 1 - Morphometric data in Control and Beta-Carotene Groups

	Control n = 23	Beta-carotene n = 25	P value
Body weight	237 ± 3.8	237 ± 4.0	0.95
Myocyte area (µm ²)*	190.10 ± 10.3	185.10 ± 8.0	0.76
Collagen (%)*	4.34 ± 0.55	2.65 ± 0.25	0.02
LV/BW (g)	2.21 ± 0.03	2.16 ± 0.03	0.37
Cx43 ⁺ Location	3.63 ± 0.12	3.68 ± 0.11	0.80
Cx43 ⁺ Quantity	2.32 ± 0.19	2.55 ± 0.27	0.51

Data expressed in mean ± standard error. * 5 animals in each experimental group; # 10 animals in each experimental group. LV: left ventricle; BW: body weight; Cx: connexin. Student t test.

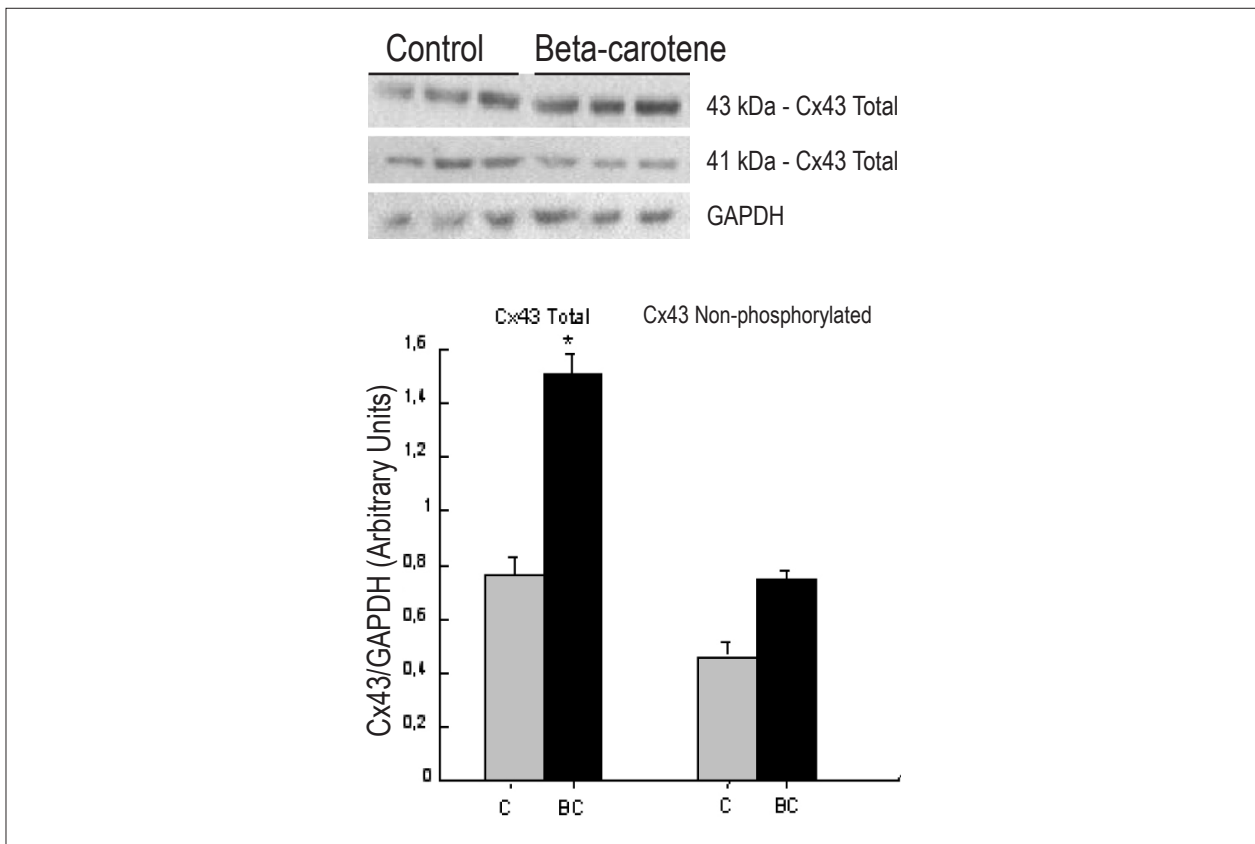


Figure 2 - Western blotting for connexin 43 in left ventricular myocardium. *P value = 0.02. C: control; BC: beta-carotene; Cx: connexin. Student t test.

Table 2 - Oxidative stress biomarkers in Control and Beta-Carotene Groups

		Control n = 5	Beta-carotene n = 5	P value
Serum (µmol/mL)	GSH	21.80 ± 0.14	22.36 ± 0.40	0.69
	GSSG	1.78 ± 0.02	1.78 ± 0.02	1.00
	GSH/GSSG	12.21 ± 0.2	12.13 ± 0.1	0.73
	HP	4.76 ± 0.11	4.92 ± 0.36	0.44
Liver (nmol/mg protein)	GSH	4.34 ± 0.22	4.83 ± 0.27	0.18
	GSSG	0.52 ± 0.05	0.54 ± 0.05	0.78
	GSH/GSSG	42.60 ± 1.62	57.40 ± 5.90	0.04
	HP	3.22 ± 0.12	3.06 ± 0.12	0.26
Heart (nmol/mg protein)	GSH	45.37 ± 2.44	49.41 ± 1.22	0.17
	GSSG	0.38 ± 0.02	0.40 ± 0.01	0.41
	GSH/GSSG	117.40 ± 1.01	121.81 ± 1.32	0.03
	HP	3.34 ± 0.31	2.52 ± 0.21	0.06

Data expressed in mean ± standard error. Student t test. GSH: reduced glutathione; GSSG: oxidized glutathione; GSH/GSSG: relation between reduced and oxidized glutathione; HP: lipids hydroperoxides.

possible to infer that this activity of carotenoids is not exclusively due to pro-vitamin A activity nor the antioxidant activity of these compounds. This affirmation is based on the fact that carotenoids without pro-vitamin activity also have this effect, and other antioxidants, such as alpha-tocopherol, do not¹⁰.

In conclusion, beta-carotene, in the experimental model used in this study, has a positive effect, characterized by the increased intercellular communication and antioxidant defense system.

Author contributions

Conception and design of the research: Novo R, Paiva SAR; Acquisition of data: Novo R, Azevedo PS, Paiva SAR; Analysis and interpretation of the data: Novo R, Azevedo PS, Minicucci MF, Zornoff LAM, Paiva SAR; Statistical analysis: Minicucci

MF; Writing of the manuscript: Novo R, Minicucci MF, Paiva SAR; Critical revision of the manuscript for intellectual content: Azevedo PS, Zornoff LAM, Paiva SAR.

Potential Conflict of Interest

No potential conflict of interest relevant to this article was reported.

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Study Association

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