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ORIGINAL CONTRIBUTION

Effect of the consumption of a fruit and vegetable soup with high in vitro carotenoid bioaccessibility on serum carotenoid concentrations and markers of oxidative stress in young men

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Abstract
Aim To evaluate the effect of the daily intake of a fruit & vegetable soup with high in vitro bioaccessibility of carotenoids on β-carotene and lycopene serum concentrations.
Methods Fourteen healthy young men (24 ± 1 years) received 300 mL/day of a carrot, tomato, and broccoli soup, containing 3.9 mg β-carotene and 4 mg lycopene, for 4 weeks followed by a 4-week washout period. The serum carotenoid response and oxidative markers were analyzed after 3 and 4 weeks of soup consumption and after a 4-week washout.
Results The in vitro bioaccessibility of β-carotene and lycopene was 55 and 43%, respectively, in the soup. Serum β-carotene concentrations were significantly higher than baseline (0.33 ± 0.05 μmol/L) after 3 weeks (0.69 ± 0.06 μmol/L) and 4 weeks (0.78 ± 0.10 μmol/L) of soup consumption ($P < 0.001$). Serum lycopene was also significantly higher compared with baseline levels (0.26 ± 0.08–0.56 ± 0.04 μmol/L and 0.60 ± 0.04 μmol/L, after 3 and 4 weeks, respectively) ($P < 0.001$). Although the highest concentration of both carotenoids was found after 4 weeks, the levels were not statistically different from the levels at 3 weeks. A 4-week washout significantly decreased serum carotenoid concentrations, although only β-carotene returned to baseline. Glutathione peroxidase (GPx) increased significantly after soup supplementation compared with baseline, while superoxide dismutase was significantly lower only after 3 weeks. Glutathione reductase, lipid, protein, and DNA oxidative markers remained unchanged.
Conclusions The soup contributed to increasing the concentration of each carotenoid by more than 100% after 3 and 4 weeks of consumption, the maximum increase being observed after 4 weeks. Oxidative markers did not show any variation except for GPx. Serum lycopene half-life was longer than that of β-carotene, which may be important for studies evaluating both carotenoids.

Keywords β-carotene · Lycopene · Oxidative stress · Glutathione peroxidase

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Introduction

Several studies have pointed to an association between the adequate dietary intake of fruit and vegetables and the decreased risk of chronic diseases such as cardiovascular disease, obesity, and some types of cancer [1–4]. The World Health Organization suggests that increasing individual fruit and vegetable consumption to more than 600 g per day could reduce the total worldwide disease burden (measured as number of cases) by 1.8% [5]. The World Cancer Research Fund (WCRF)/American Institute for

Cancer Research (AICR) [6] recommends consuming between 400 and 600 g/day of fruit and vegetables. However, consuming these amounts of fruit and vegetables may be difficult in many countries. Data from the last European Nutrition and Health Report (2009) [7] reported that only four countries (Poland, Germany, Italy, and Austria) met the recommendation of 400 g/day, while the populations of northern European countries (Denmark, Estonia, Finland, Latvia, Lithuania, Norway, and Sweden) consumed an average of 269 g/day of fruit and vegetable.

Epidemiological studies have shown that dietary components such as carotenoids are associated with the reduced risk of the chronic diseases mentioned above [8–10]. Thus, the development of new plant food products with an increased bioavailability of carotenoids might contribute to the healthy effects associated with a high fruit and vegetable diet. However, serum carotenoid responses after the intake of fruit and vegetable products or carotenoid supplements have been reported to depend on several factors, such as the food matrix, the amount of carotenoids, the length of supplementation, and the washout period used in human trials, which makes it difficult to extract firm conclusions [11, 12]. Horvitz et al. [13] reported that 5 mg/day of lycopene supplementation for 10 days increased serum lycopene by 28, 35, or 45% above baseline values, depending on the food matrix used (red carrots muffin, tomato paste plus white carrots muffin, and tomato paste muffin, respectively). In addition, postprandial studies have shown that there is a wide interindividual response to carotenoids when using isolated carotenoids administered with a single meal [14, 15]. A coefficient of variation of 61% has been reported for β-carotene concentrations in chylomicrons after a single meal consumption of 120 mg of β-carotene [14]. Due to this high variability, it would probably be of interest to identify the carotenoid response in serum or in chylomicrons and to identify potential health benefits of any new carotenoid-rich plant food product.

To reduce the influence of human variability in trials, some authors have used crossover designs in which the same subjects consumed different types of product and included a washout period between the different products. The length of the washout period used ranged between 1 and 10 weeks [16, 17], which makes it difficult to decide the best design to be used, and in some studies no measurements were made to verify that the selected washout period was long enough to allow serum carotenoid levels to return to baseline values [18]. Due to the many factors that influence the half-life of carotenoids in serum, this information would be very important for designing human studies aiming at evaluating carotenoid bioavailability in food products.

In the present study, the changes in serum carotenoid concentrations, and potential associated antioxidant effects, were assessed in subjects consuming a fruit and vegetable soup with a high in vitro bioaccessibility of β-carotene and lycopene for 3 and 4 weeks. We also evaluated whether a 4-week washout period was sufficient time to allow carotenoid levels to return to their baseline values for future crossover studies with similar food products.

Materials and methods

Subjects

Fourteen healthy young men were recruited in the University of Murcia, Spain. The study was conducted during May–July 2009. The inclusion criteria were: age 30 ± 10 years old (mean ± SD); fruit and vegetable consumption ≤4 portions/day, not taking vitamins, minerals, or other types of supplement during the previous 2 months; non-smoking; to be normolipidemic; and with body mass index (BMI) within the normal range according to the Spanish Society for the Study of Obesity (18.5–24.9 kg/m^2) [19]. Normal biochemical and hematological profile: serum cholesterol < 6.8 mmol/L, serum triglyceride < 2.3 mmol/L with a low-density lipoprotein (LDL)/high-density lipoprotein (HDL) ratio < 5.0. The exclusion criteria were: diagnosed diseases such as allergies, cancer, diabetes, obesity, mental diseases, gastro-intestinal or renal diseases, as well as intake of drugs related to these pathologies, alcohol consumption > 30 g/day, vegetarian diet, and subjects with deficiencies in serum vitamin B_{12}. The consumption of fruit and vegetables of the participants was evaluated by a validated food frequency questionnaire referring to the last year [20], while portion size was estimated by showing the subjects a picture of different fruits and vegetables [21].

The age of the participants (mean ± SEM) was 24.0 ± 0.7 years old. BMI and body fat percentage (means ± SEM) were within the normal range for men 22.3 ± 1.1 kg/m^2 and 14.7 ± 1.4%, respectively [19, 22], with no risk of cardiovascular disease according to waist/hip ratio (mean ± SEM) 0.88 ± 0.01 [23]. The consumption of fruit and vegetable of the participants before the supplementation period was (mean ± SEM) 2.4 ± 0.2 portions/day. None of the participants consumed 0 or 4 portions/day of fruit and vegetables, while five subjects consumed 1 portion, four subjects 2 portions, and five subjects 3 portions/day.

The sample size was chosen to have an α error of 0.05 and a β error of 0.2 for an estimated difference of 20% between groups for the mean value of lycopene, according to the variance in HPLC serum lycopene analyses in adult

men reported by Van den Berg et al. [24]. Subjects were recommended not to change their dietary habits during the study, especially as regards their consumption of a provided list of foods with high β-carotene, lycopene, and folate contents. The study was performed in accordance with The Helsinki Declaration of Human Studies and approved by the Ethical Committee of the University of Murcia. All participants signed an informed consent document.

Study design

Subjects consumed 300 mL/day of a fruit and vegetable soup for 4 weeks, followed by a 4-week washout period. Baseline blood and urine samples were collected twice (days 1 and 0), and the mean value of these measurements was taken as baseline value in this study. We also collected one blood sample on day 21 of the study (3 weeks of supplementation) and on day 28 (4 weeks of supplementation). Finally, we collected blood and urine samples 4 weeks after the end of soup consumption (day 56), as a washout sample.

To mimic more closely the dietary behavior of consumers, soup consumption was included in the normal daily diet, and no specific time of consumption or accompanying meal was established. The soup contained the same proportions of carrot, tomato, and broccoli (20% of each) with 5% olive oil. The tomatoes, broccoli, and carrots were heat-treated by blanching at >85 °C followed by high pressure homogenization (HPH) at 100 bar. The soup, which contained 3.9 mg of β-carotene, 4 mg of lycopene, and 52.2 μg of folate, was produced by Unilever Discover R&D within the European project "Healthy Structuring" (Proposal/Contract No: 023115) from the EU Sixth Framework Programme, to encourage the development of fruit and vegetable food products with a high bioavailability of carotenoids and folate. Many different soup prototypes were elaborated by Unilever, and the soup with the highest carotenoid content and in vitro bioaccessibility was used for this study. The soup is described as having high in vitro carotenoid bioaccessibility since this parameter was found to be much higher than that reported by other authors for raw and cooked carrots, tomato, and broccoli [12, 25, 26].

In vitro digestion procedure

The in vitro carotenoid bioaccessibility of the soup was estimated by measuring the percentage (in relation to the initial content of the soup) of carotenoids transferred to the micellar phase, using a static in vitro model as previously described [27]. Duplicate analyses were performed on fresh material, blanketed with nitrogen and in subdued light. To simulate the initial part of the gastric phase, the pH was adjusted to 4 with HCL (1 M) and a pepsin solution was added before incubating the samples for 30 min at 37 °C, followed by 30 min at pH 2 (adjusted with HCL 1 M). To mimic small intestinal digestion, the pH was raised to 6.9 by the addition of $NaHCO_3$ (1 M) and the samples were incubated for 120 min at 37 °C after adding pancreatine and bile extract solutions. After incubation, samples were centrifuged for 15 min at 4 °C (5,000 *g*) before the supernatants were vacuum filtrated into amber E-flasks to obtain a supernatant phase, which was used to measure the amount of nutrients released from the food matrix. To isolate the micellar fractions, aliquots of the supernatants were microfiltered (0.22 μm) and used to estimate of the efficiency of micellarization. The fraction of carotenoids transferred to the micellar phase was considered to reflect the amount of bioaccessible carotenoids.

Anthropometric measurements

Weight was measured to the nearest 0.1 kg in subjects wearing light clothes and barefooted, while total body fat (%) was assessed by electric impedance, using for both measurements a portable device (model TBF-300, Tanita Corporation, Japan). Height was estimated to the nearest 1 cm using a Harpenten digital stadiometer (range, 0.7–2.05 m; Holtain Ltd, Pembrokshire, UK), with the subject upright and the head in the Frankfurt plane. Waist circumference (at the level of the iliac crest) and hip circumference (the greatest perimeter at the level of gluteus) were also measured.

Dietary assessment

During the first week of the study, subjects completed a 7-day dietary record to evaluate energy and nutrient intake. The energy and nutrient contents were estimated by the software "GRUNUMUR" [28], using Spanish food composition tables [29, 30].

Analytical determinations

A total of 10 mL of fasting blood samples were collected from each subject by venepuncture from the antecubital vein: 3 mL was placed in EDTA tubes for hematological measurements, while 7 mL was placed in tubes without any anticoagulant and centrifuged at 1,500 *g* for 10 min at 4 °C for serum separation. Serum aliquots were prepared and stored at −80 °C until analysis. A first urine sample in the morning was collected and frozen at −20 °C until analysis.

Serum folate concentrations were analyzed by HPLC [31, 32]. Serum glucose, triglycerides, total cholesterol, high- and low-density lipoprotein cholesterol (HDL-C and

LDL-C), urea, creatinine, uric acid, serum albumin, glutamic-oxaloacetic transaminase (GOT), and glutamic-pyruvic transaminase (GPT) were analyzed by commercial kit in an autoanalyzer (Cobas 711, Roche Diagnostic, Mannheim, Germany). Total homocysteine in serum was analyzed by chemioluminiscence using a commercial kit (Boehringer, Mannheim, Germany).

The serum activity of the antioxidant enzymes, glutathione peroxidase (GPx), superoxide dismutase (SOD), and glutathione reductase (GR), was analyzed by spectrophotometry, using commercial kits (Randox, Crumlin, UK).

As markers of lipid peroxidation, we analyzed serum oxidized LDL particles by ELISA (Randox, Crumlin, UK) and 15-isoprostanes F_2 in urine using a commercially available enzyme-linked immunoassay kit (Oxford Biomedical Research, Inc. Oxford, MI, USA).

Serum hydroperoxide levels were analyzed by the method described by Jiang et al. [33] and the thiobarbituric acid assay (TBARs) as described by Buege and Aust [34], using spectrophotometrical methods in both cases.

Protein oxidation was estimated by quantification of serum carbonyl groups and DNA oxidation by quantifying serum 8-hydroxy-2'-deoxyguanosine (8-OHdG), using commercial ELISA kits (Randox, Crumlin, UK).

Extraction of carotenoids from serum samples

An equal volume of ethanol containing 0.1% BHT was added to 200 μL of serum. The sample was mixed using a vortex for 10 s before adding 1 mL hexane containing 0.1% BHT. Samples were mixed again with the vortex and centrifuged (3,000 *g*). The carotenoid-containing hexane layer was transferred to a glass vial and the extraction procedure was repeated twice. The combined hexane layers were evaporated to dryness in a water bath at 35 °C under nitrogen and the residues were reconstituted with 100 μL of mobile phase consisting of Methanol/Methyl *tert*-butyl ether (50:50 v/v) and transferred to HPLC vials. Samples were protected from light throughout the extraction and analysis.

HPLC analysis of carotenoids

Carotenoids were quantified by HPLC (Waters 600) using a C_{30} column (dimension 4.6 mm × 25 cm and particle size 5 μm, YMC, Europe GMBH, Germany). To separate lycopene and *β*-carotene, a gradient starting with 70% methanol and 30% methyl *tert*-butyl ether for 17 min was followed by elution using 30% methanol (18 min) and 70% methanol (5 min). The flow rate was 1 mL/min and the injection volume 20 μL. Carotenoids were detected using a UV–visible photodiode array detector (Waters 996) operating at 450 nm.

External standards for lycopene and *β*-carotene were used for quantification. Two standard curves, one for lycopene and one for all-*trans*-*β*-carotene, of eight different concentrations were prepared.

Statistical analyses

The results are expressed as mean ± standard error mean (SEM) with 95% CI. The distribution of the variables was examined by Shapiro–Wilk test and Kolmogorov–Smirnov tests, and those not following a normal distribution (triglycerides, homocysteine, isoprostanes, and 8-hydroxy-2'-deoxyguanosine) were logarithmically transformed before analysis. A general lineal model of repeated measures was used to compare the basal values and those obtained at different sampling times, followed by a post hoc Bonferroni test. Differences were considered statistically significant for a P-value < 0.05. SPSS version 15.0 (Statistical Package for Social Sciences, SPSS Inc. Chicago, IL, USA) was used for the statistical analyses.

Results

Dietary assessment

During the soup consumption period, the dietary energy and protein intake of the participants were seen to be well balanced according to the Spanish recommendations for adults (Table 1). Nevertheless, the percentage of energy from lipids and carbohydrates did not follow healthy dietary recommendations, but rather, a normal western dietary pattern. The daily dietary intake of *β*-carotene, lycopene, and folate without considering the soup was 0.7, 4.3 mg, and 159 μg, respectively. The average daily intake of folate was lower than the recommended daily intake (RDI) even with the inclusion of the soup in the diet (Table 1).

In vitro bioaccessibility and serum response of carotenoids and folate

The relative in vitro bioaccessibility was calculated dividing the amount of carotenoids released from the food matrix and transferred to the micellar phase during digestion by the average carotene content of the soups. The proportion of bioaccessible carotenoids in the micellar fraction was 55% *β*-carotene and 43% lycopene. Serum concentrations of both *β*-carotene and lycopene increased significantly following the dietary incorporation of the fruit and vegetable soup, compared to baseline values (Table 2). After 3 weeks of soup consumption, both *β*-carotene and lycopene concentrations had increased by 114% compared with basal values, and after 4 weeks, serum *β*-carotene and

Table 1 Average daily dietary nutrient and phytochemical intake during the supplementation period, including the consumption of the soup

	Values	Reference values	References
Energy (MJ (kcal)/day)	10.4 ± 0.7 (2,484 ± 167)	11.3 (2,699)	[30]
Proteins (% of total energy)	14.9 ± 0.6	10–15	[30]
Lipids (% of total energy)	40.3 ± 1.4	30–35	[30]
Carbohydrates (% of total energy)	44.8 ± 1.2	50–65	[30]
β-carotene (mg/day)	4.6 ± 0.2	2.96†	[57]
Lycopene (mg/day)	8.3 ± 1.3	1.64†	[57]
Folate (μg/day)	211 ± 18	400	[30]

Values are means ± SEM, $n = 14$

† Reported values

lycopene were enhanced by 141 and 132%, respectively, compared with basal values. The subsequent 4-week washout period significantly decreased serum concentrations of both carotenoids compared with the levels observed after 4 weeks of soup consumption ($P = 0.001$). This decrease was particularly pronounced in the case of β-carotene, which returned to baseline values. Serum concentrations of folate increased after 4 weeks of soup consumption and decreased after the washout period, but not significantly in either case ($P = 0.120$).

Biochemical and antioxidant parameters

The biochemical parameters analyzed were within the normal ranges, with no significant differences of note during the trial (Table 3). As regards antioxidant enzymes, the effect was limited. Glutathione peroxidase (GPx) activity increased statistically after 3 and 4 weeks of soup consumption compared with basal values, but decreased significantly after the washout period ($P = 0.001$). In contrast, superoxide dismutase (SOD) tended to decrease with soup consumption and washout period with respect to baseline values, although the differences were only significant after 3 weeks of soup consumption ($P = 0.001$) (Table 4). Glutathione reductase (GR) enzyme activity was not modified during the study ($P = 0.212$). No significant effect of soup consumption on lipid, protein, or DNA oxidized metabolites was observed (Table 4). F_2-isoprostane levels were higher in the washout period than at other time points, although this result was not reflected in the other lipid oxidative metabolites analyzed such as oxidized LDL or TBARs.

Discussion

In the present study, the dietary β-carotene and lycopene provided in a fruit and vegetable soup designed to enhance the bioaccessibility of these compounds significantly increased serum carotenoid concentrations after 3 and 4 weeks of supplementation in healthy humans. Dragsted et al. [35] estimated that the inclusion in the diet of 600 g of fruit and vegetables provides about 18 mg of total carotenoids; in our experiment, subjects with a low intake of fruit and vegetables reached about 13 mg of carotenoids with the daily supplementation of soup in their diet. Thus, supplementation with the fruit and vegetables soup may contribute to increasing some of the compounds associated with the health benefits of 600 g/day fruit and vegetables proposed as a public health goal.

Literature data on carotenoid bioaccessibility from vegetable soups are currently very limited, most studies report data from evaluations of the in vitro bioaccessibility from single fruit and vegetables. The in vitro carotenoid bioaccessibility of our soup containing carrot, broccoli, and tomato was 55% for β-carotene and 43% for lycopene, which was markedly higher than the levels reported in other studies (0.1–20%) using raw or cooked single fruit and vegetables [12, 26]. Granado-Lorencio reported high levels (about 80%) of β-carotene and lycopene

Table 2 Serum concentrations of β-carotene and lycopene at baseline, after 3 and 4 weeks of soup consumption (wk 3 and wk 4, respectively), and after a 4-week washout period

	Baseline (μmol/L serum)	Wk 3 (μmol/L serum)	Wk 4 (μmol/L serum)	Washout (μmol/L serum)	*P*
β-carotene	0.33 ± 0.05^{b} (0.21, 0.44)	0.69 ± 0.06^{a} (0.57, 0.81)	0.78 ± 0.10^{a} (0.58, 0.97)	0.31 ± 0.04^{b} (0.23, 0.39)	0.001
Change		−0.36 ± 0.03 (−0.47, −0.26)	−0.45 ± 0.06 (−0.63, −0.27)	0.02 ± 0.03 (−0.06, 0.09)	
Lycopene	0.26 ± 0.03^{c} (0.22, 0.34)	0.56 ± 0.04^{ab} (0.48, 0.63)	0.60 ± 0.04^{a} (0.51, 0.68)	0.45 ± 0.03^{b} (0.38, 0.52)	0.001
Change		−0.30 ± 0.04 (−0.39, −0.16)	−0.34 ± 0.05 (−0.46, −0.17)	−0.19 ± 0.02 (−0.24, −0.09)	

Values are means ± SEM (95% CI), $n = 14$. A general lineal model for repeated measures followed by the Bonferroni post hoc test was used. The *P*-value was based on the Fisher *F*-test. Values not sharing the same superscript are significantly different

Table 3 Biochemical parameters in men before (baseline), after 3 and 4 weeks of soup consumption (wk 3 and wk 4), and after the washout period

	Baseline	Wk 3	Wk 4	Washout	*P*
Glucose (mmol/L)	4.95 ± 0.10^{a} (4.71, 5.15)	4.83 ± 0.10^{ab} (4.58, 5.04)	4.79 ± 0.12^{a} (4.55, 5.04)	4.64 ± 0.10^{b} (4.40, 4.83)	**0.009**
Total cholesterol (mmol/L)	4.48 ± 0.21^{a} (3.95, 4.84)	4.48 ± 0.22^{a} (3.94, 4.87)	4.23 ± 0.21^{ab} (3.79, 4.65)	4.15 ± 0.22^{b} (3.60, 4.56)	**0.029**
HDL-cholesterol (mmol/L)	1.42 ± 0.11 (1.18, 1.66)	1.44 ± 0.11 (1.18, 1.69)	1.47 ± 0.12 (1.22, 1.72)	1.49 ± 0.11 (1.25, 1.72)	0.613
LDL-cholesterol (mmol/L)	2.54 ± 0.16^{ab} (2.12, 2.75)	2.58 ± 0.14^{a} (2.22, 2.80)	2.31 ± 0.12^{ab} (2.05, 2.56)	2.17 ± 0.15^{b} (1.81, 2.46)	**0.002**
Triglycerides (mmol/L)	0.85 ± 0.27 (0.53, 1.07)	0.75 ± 0.30 (0.44, 1.05)	0.73 ± 0.29 (0.45, 1.00)	0.81 ± 0.29 (0.52, 0.96)	0.547
Homocysteine (μmol/L)	10.7 ± 1.2 (8.1, 13.3)	11.9 ± 2.1 (7.5, 16.3)	10.4 ± 1.4 (7.5, 13.3)	13.1 ± 2 .6 (7.8, 18.5)	0.078

Values are means ± SEM (95% CI), $n = 14$. *HDL* High-density lipoprotein, *LDL* Low-density lipoprotein. A general lineal model for repeated measures followed by the Bonferroni post hoc test was used. The *P*-value was based on the Fisher *F*-test. Significant differences are shown in bold. Values not sharing the same superscript are significantly different

Table 4 Serum markers of oxidative stress in men before (baseline), after 3 and 4 weeks of soup consumption (wk 3 and wk 4), and after the washout period

Biomarker	Baseline	Wk 3	Wk 4	Washout	*P*
Antioxidant enzymes					
GPx (U/g Hb)	16.4 ± 0.8^{c} (14.7, 17.9)	28.3 ± 0.8^{a} (26.6, 30.1)	29.5 ± 1.1^{a} (27.3, 31.4)	23.0 ± 1.1^{b} (20.0, 24.7)	**0.001**
SOD (U/g Hb)	1,393 ± 93^{a} (1,158, 1,522)	544 ± 59^{b} (420, 669)	827 ± 65^{ab} (645, 982)	839 ± 33^{ab} (774, 948)	**0.001**
GR (U/L)	55.0 ± 2.0 (51.0, 59.1)	57.4 ± 4.8 (47.4, 67.5)	51.9 ± 2.6 (46.5, 57.4)	52.5 ± 2.1 (48.1, 56.8)	0.212
Lipid oxidation					
Oxidized LDL (U/L)	59.6 ± 4.1 (51.0, 68.2)	57.9 ± 3.4 (50.7, 65.0)	55.0 ± 3.1 (48.5, 61.5)	62.9 ± 4.5 (53.5, 72.3)	0.122
F_2-Isoprostanes (pmol/mmol creatinine)	0.56 ± 0.07^{b} (0.43, 0.70)	0.58 ± 0.08^{ab} (0.41, 0.76)	0.52 ± 0.06^{b} (0.39, 0.64)	0.82 ± 0.05^{a} (0.70, 0.93)	**0.001**
Hydroperoxides (μmol/L)	1.87 ± 0.08 (1.69, 2.04)	1.91 ± 0.10 (1.69, 2.12)	1.90 ± 0.10 (1.70, 2.10)	2.13 ± 0.12 (1.88, 2.37)	0.078
TBARS (μmol/L)	3.17 ± 0.17 (2.77, 3.55)	3.17 ± 0.17 (2.79, 3.54)	3.17 ± 0.17 (2.78, 3.54)	3.02 ± 0.41 (2.16, 3.88)	0.757
Protein oxidation					
Carbonyl (nmol/mg protein)	0.28 ± 0.02 (0.19, 0.36)	0.28 ± 0.02 (0.19, 0.36)	0.25 ± 0.01 (0.20, 0.28)	–	0.112
DNA oxidation					
8 OHdG (mol/L)	78.8 ± 9.4 (51.9, 100.3)	78.8 ± 13.6 (48.0, 68.8)	78.3 ± 10.1 (32.5, 116.9)	–	0.741

Values are means ± SEM (95% CI), $n = 14$. *GPx* Glutathione peroxidase, *U* Units, *Hb* Hemoglobin, *SOD* Superoxide dismutase, *GR* Glutathione reductase, *LDL* Low-density lipoprotein, *TBARS* Thiobarbituric acid reactive substances, *8 OHdG* 8-hydroxy-deoxyguanosine. A general lineal model for repeated measures followed by the Bonferroni post hoc test was used. The *P*-value was based on the Fisher *F*-test. Significant differences are shown in bold. Values not sharing the same superscript are significantly different

bioaccessibility in a tomato paste, and approximately 75% β-carotene bioaccessibility in carrots, both of which are higher than those obtained in our study, but only about 17% β-carotene bioaccessibility was found for broccoli [25].

With the fruit and vegetable soup used in the present study, serum levels of both carotenoids increased by 114% at 3 weeks, while after 4 weeks of supplementation, this increase was 141% for β-carotene and 132% for lycopene. In addition, the observed serum values of carotenoids were within the range of previously reported values for Spanish subjects [36]. Dragsted et al. [35] observed that daily dietary intakes of 600 g of fruit and vegetables for similar time periods (16 or 24 days) significantly increased both serum β-carotene and lycopene concentrations compared

with the responses observed in a control group, with higher values observed on day 24 than on day 16 (about 50 and 60% change with respect to the basal values for β-carotene and lycopene, respectively, on day 16, and about 65 and 80% on day 24). In other studies, 3 weeks of supplementation with 9.3 mg of β-carotene from spinach products increased serum concentrations by 57% [37], while 8 mg lycopene consumption from tomato products increased serum lycopene by about 70% [38]. Although the carotenoid content of the soups in the present study was lower (4 mg of each carotenoid) than in the previously reported studies, we obtained higher serum levels of both carotenoids, which seems to be in agreement with the estimated high bioaccessibility of carotenoids in this food matrix. The inter-relationship of different carotenoids present in the food matrix also affects carotenoid absorption [11]. Lutein has been shown to have the ability to reduce but also enhance the plasma area under the curve (AUC) for β-carotene [39], thus, it is possible that the presence of lutein may have influenced the observed increase in serum response. However, the lutein content of our soup was not determined. Dietary lipids are known to be an important factor of carotenoid bioavailability in humans, and the presence of 5% olive oil in our soup (15 g/serving) might also have contributed to the observed increase in serum β-carotene and lycopene concentrations. The amount of fat used was in the range reported in other studies [38].

In crossover trials, differences in the length of washout periods have been reported [17, 40]. In the present study, the serum carotenoid levels obtained in the subjects after 4 weeks of soup consumption significantly decreased after a 4-week washout period, confirming that the carotenoid levels recorded during the study were mainly the result of soup consumption. A 4-week period for carotenoid washout has been considered as appropriate, as described in several reviews [41, 42], but, depending on the food matrix, and the amount of carotenoids, a longer washout might be necessary. The β-carotene concentrations returned to basal values after 4 weeks in our experiment which agrees with the observations made by Rock et al. [43]. In a crossover study, plasma β-carotene remained significantly higher than baseline after the subsequent period of placebo treatment for 26 days; however, these authors used a high dose of β-carotene (15 mg/day) provided in capsules [44]. Moreover, carotenoid bioavailability from supplements has been found to be higher than that from dietary sources [45, 46]. The half-life for β-carotene in serum has been estimated to be about 7–14 days [47, 48], thus, a washout period of 4 weeks seems quite reasonable.

A half-life of 11–14 days or 12–33 days has been described for serum lycopene by different authors [47, 48]. However, some studies [49, 50] have reported that serum lycopene decreases more rapidly than serum β-carotene. In our study, a 4-week washout returned serum β-carotene concentrations to basal values but not serum lycopene levels. This might be related with differences in the chemical structure of both carotenoids, which could determine higher lipophilicity on the part of lycopene in serum compared with β-carotene. In addition, the β-carotene functions as provitamin A and differences in the antioxidant activity of both carotenoids might also have contributed to the differences observed in their depletion rate [50]. A 4-week washout period was found to be sufficient for the serum lycopene concentrations to return to basal values after 4 weeks of 6 mg lycopene supplementation [40]. Nevertheless, as reported in another study, a 4-week washout period tended to increase serum lycopene compared with the values recorded in a 6-week intervention period of tomato juice consumption (12 mg lycopene) plus a controlled diet. The findings were explained by the fact that the self-selected diet contained more lycopene than the controlled diet [47]. The normal diet of the participants in our study contained almost the same amount of lycopene as provided by our soup, while the β-carotene content of the diet was low (0.7 mg/day). These findings also suggest that in populations consuming a diet containing higher levels of lycopene than other carotenoids, such as β-carotene [51], it is more difficult to control lycopene intake during the washout period in dietary intervention studies, underlining the need for longer washout period during crossover studies considering human lycopene responses.

Considering biochemical parameters, the differences observed were slight, in agreement with other studies [17, 24]. We found some differences in serum glucose, and total and LDL-cholesterol after the washout compared with the supplementation period, but no differences were observed for the levels measured during supplementation compared with the baseline levels for these parameters. As regards the effect of the soup on the antioxidant status of the subjects, GPx activity significantly increased after 3 and 4 weeks of soup consumption compared with the basal values, while this enzymatic activity decreased during the washout period. GPx catalyzes the degradation of peroxides with the concomitant oxidation of glutathione, which is immediately converted to its reduced form in the presence of GR and NADPH [37]. In our study, nevertheless, GR activity did not change. Dragsted et al. [35] also reported a significant increase in GPx activity after 25 days of a basic diet plus 600 g of fruit and vegetables compared with a control group, while they did not find significant effects in other antioxidant measurements. Nevertheless, not all authors report changes in GPx activity after carotenoid supplementation [52]. SOD catalyzes the dismutation of superoxide anion radicals into hydrogen peroxide [37].

The reason for the observed decrease in SOD activity after 3 weeks of soup supplementation is not clear. McGill et al. [53] also reported a decrease in human leukocyte SOD activity after β-carotene supplementation. They proposed that supplemental β-carotene may act as direct scavenger of reactive oxygen species, decreasing the body's need for certain antioxidant enzymes. Nevertheless, our data from 4 weeks of supplementation did not confirm this hypothesis. Moreover, some of the observed effects may be related with the presence of other carotenoids such as lutein, zeaxanthin, etc., or phenolic compounds in the olive oil, which also possess antioxidant properties [11, 54]. Thus, the effect of the intake of foods rich in carotenoids on the activity of the antioxidant enzymes remains unclear. Our results also suggest that a larger number of participants should be considered to determine the effect of carotenoids on the antioxidant status of subjects. Like other antioxidant compounds, the intake of β-carotene at pharmacological levels has been reported to act as pro-oxidant [55, 56], but no such effects have been reported for dietary intake.

In conclusion, an addition of a fruit and vegetable food product with enhanced carotenoid bioaccessibility to the diet may contribute to enhanced levels of bioavailable β-carotene and lycopene close to those achieved from a diet containing 600 g/day of fruit and vegetables. Both 3 and 4 weeks of soup consumption resulted in increases in serum carotenoid levels of more than 100%, the maximum increase being observed at 4 weeks. Oxidative markers did not show any variation except for GPx. A washout period of 4 weeks was sufficient to return serum β-carotene to basal values, but the serum lycopene concentrations still remained above the basal values after this period.

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Conflict of interest The authors declare that they have no conflict of interest.

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