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Effects of Blood Orange Juice Intake on Antioxidant Bioavailability and on Different Markers Related to Oxidative Stress

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Orange juice is a source of antioxidants that might afford in vivo protection from oxidative stress. To test this hypothesis, we carried out a human intervention study with blood orange juice containing high amounts of vitamin C, anthocyanins, and carotenoids. Sixteen healthy female volunteers were enrolled in a crossover study and were given 600 mL/day of blood orange juice or a diet without juice for 21 days. Before and after each intervention period, plasma vitamin C, cyanidin-3-glucoside, and carotenoids were quantified. Furthermore, plasma antioxidant capacity, malondialdehyde concentration in plasma, 11-dehydrotromboxane B_2 urinary excretion, and lymphocyte DNA damage were evaluated as biomarkers of oxidative stress. Blood orange juice consumption determined a significant increase in plasma vitamin C, cyanidin-3-glucoside, β-cryptoxanthin, and zeaxanthin. Also, lymphocyte DNA resistance to oxidative stress was improved whereas no effect was observed on the other markers that we analyzed. In turn, these results suggest that blood orange juice is a bioavailable source of antioxidants, which might moderately improve the antioxidant defense system; however, the long-term effects of its consumption are to be further investigated.

KEYWORDS: Blood orange juice; antioxidants; DNA damage; anthocyanins; cardiovascular disease; Mediterranean diet; cancer

INTRODUCTION

Extensive literature suggests that population groups whose dietary habits include large quantities of plant foods, including citrus fruits, have a reduced risk to develop chronic diseases, possibly due to the cancer preventive and cardioprotective properties of many antioxidant compounds present in these foods (*1*).

Citrus fruits are rich in bitter limonoids and vitamins such as ascorbic acid, carotenoids, folates, polyphenols, and flavonoids. The traditional Mediterranean diet includes the consumption of fresh fruit in place of sweet desserts; intake of blood oranges in Southern Europe, namely, Sicily, is quantitatively relevant, also because of the increasing availability of freshly squeezed and pasteurized blood orange juice. Furthermore, frequent campaigns promote orange consumption because of its putative protective and "anticancer" properties.

Few studies exist on the potential role of blond oranges and their juices (*2*−*4*); even fewer are those performed with blood orange varieties. Blood oranges are abundant in pigmented anthocyanins (e.g., cyanidin 3-glucoside)—which are responsible for their red color—and also contain high amounts of vitamin C and carotenoids. In vitro studies demonstrated the high antioxidant capacity of these orange juices, due to their phenolic (anthocyanins) rather than vitamin C contents (*5*).

No data exist on the in vivo effect of blood orange juice consumption, while very few human studies have been carried out on other anthocyanin-rich foods such as bilberries or blueberries (*6*, *7*). Anthocyanins have been proposed to exert beneficial effects on the vascular system, on the eyes, and on platelet aggregation (*8*, *9*).

Blood orange juice also contains carotenoids such as β-cryptoxanthin. This compound has important biological functions: the presence in its structure of a nonhydroxylated ionone ring is fundamental for its conversion to vitamin A, but like other

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carotenoids, it also can exert antioxidant activities per se, as demonstrated by several studies (*10*, *11*).

We supplemented human volunteers with blood orange juice rich in anthocyanins, vitamin C, and carotenoids, and we evaluated its effects on plasma antioxidant concentrations and on DNA damage and lipid peroxidation, which have been hypothesized to be involved in the etiology of cancer and cardiovascular disease (*12*).

MATERIALS AND METHODS

Subjects. This study conforms to the principles outlined in the Declaration of Helsinki and was approved by the local ethics committee. Sixteen healthy female volunteers (age, 20−27 years; body mass index, 16.0−23.3 kg/m^2) were recruited and selected after the administration of a questionnaire concerning their dietary habits and lifestyle. Women following vegetarian diets and women habitually consuming dietary supplements or drugs were excluded from the study. The groups of subjects were homogeneous for the amount of fruit and vegetables consumed weekly, as assessed by an interview conducted by a certified dietician.

Study Design. This study followed a crossover design. Volunteers were randomly divided into two groups. The first one (group A, eight subjects) was assigned a standardized diet devoid of orange juice for 21 days. After a period of washout (WO, 21 days), the blood orange juice under study (600 mL/day for 21 days) was added to their diet. The second group (group B, eight subjects) was assigned the opposite sequence: a standardized diet with added orange juice (21 days), WO (21 days), and a standardized diet devoid of orange juice (21 days).

Throughout the study, subjects had to follow a standardized diet in which the intake of foods rich in vitamin C, anthocyanins, and carotenoids was controlled. We gave subjects a list of foods not allowed or of limited intake. Volunteers were asked to avoid red vegetables and fruits, with particular attention being paid to citrus fruits, tomato and tomato products, berries and their derivatives, red wine, fruit juices, eggplant, red onion, red cabbage, plums, and red grapes. With this type of diet, food sources of cyanidin 3-glucoside were virtually excluded, while we estimated that the carotenoid intake was lower than 10 mg/day. Vitamin C consumption approached the current RDA (about 60 mg/day). During the WO period, subjects followed their own unrestricted diet, which was scheduled in order to avoid potential carryover effects.

At each time point, i.e., before and after the standardized diet with and without juice, blood samples (a total of four samples) were drawn from fasting volunteers into evacuated tubes containing Li^+ heparin as the anticoagulant. Additional blood samples were drawn from fingertips into heparinized microtubes. Plasma was obtained by centrifugation at 3500*g* for 15 min and was stored at −80 °C. Twenty-four hour urine was gathered on the day of blood collection. Its volume was measured, and aliquots were stored at −80 °C.

Characteristics of the Blood Orange Juice. The blood orange juice that we used (Oranfrizer, Scordia Catania, Italy) was obtained by squeezing and was pasteurized and portioned in 600 mL containers, which were immediately frozen and delivered to our laboratory. After the containers were thawed, the juices were kept refrigerated for no more than 2 days.

The juice contained 75.2 mg/100 mL vitamin C, as determined by high-performance liquid chromatography (HPLC) analysis performed according to the method of Mannino et al. (*13*). The carotenoid composition was analyzed as previously described; furthermore, an aliquot of the sample was subjected to saponification in order to evaluate carotenoids eventually present in the esterified form (*14*). In turn, 100 mL of the juice contained about 67 μg of β-cryptoxanthin, 20 μg of lutein, 18 μg of zeaxanthin, 17 μg of lycopene, 10 μg of β-carotene, and 8 μg of α-carotene. By calculating carotenoids before and after saponification, we found that 46 μg of the total β-cryptoxanthin was present as esters.

The concentration of cyanidin-3-glucoside, the most abundant anthocyanin present in the juice, was determined by HPLC. Identification of the compounds was obtained by means of mass spectrometry (Micromass, Beverly, MA; **Figure 1**). Furthermore, small amounts of cyanidin-3-glucoside-6″-malonyl were detected and quantified by the cyanidin-3-glucoside calibration curve. The chromatographic system consisted of an Alliance model 2695 (Waters) equipped with a model 2996 (Waters) photodiode array detector. A C_{18} Symmetry column (4.6 mm × 250 mm, Waters) was used for the separation, which was performed by means of a gradient elution (eluent A, formic acid 5% in water; eluent B, acetonitrile) at a flow rate of 0.8 mL/min. The gradient was as follows: from 10 to 40% B in 20 min and then from 40 to 60% B in 10 min. Chromatographic data were acquired by a Millennium 4.0 workstation (Waters) in the 200−600 nm range and were integrated at 520 nm. In addition to cyanidin-3-glucoside, small amounts of cyanidin-3-glucoside-6″-malonyl were detected and quantified based on the cyanidin-3-glucoside calibration curve. The concentration of cyanidin-3-glucoside in the juice was 3.5 mg/100 mL, while that of cyanidin-3-glucoside-6″-malonyl was 1.2 mg/100 mL. In turn, each of the 600 mL blood orange juice portions provided approximately 450 mg of vitamin C, 21 mg of cyanidin-3-glucoside, 0.4 mg of β-cryptoxanthin, 0.12 mg of lutein, 0.11 mg of zeaxanthin, 0.1 mg of lycopene, 0.06 mg of β-carotene, and 0.05 mg of α-carotene.

Determination of Vitamin C in Plasma. The extraction was performed in triplicate on 100 μL of plasma to which 100 μL of a 0.54 mmol/L Na_2EDTA in 10% metaphosphoric acid (MPA) solution had been added. After the mixtures were vortexed for 1 min, samples were centrifuged at 800*g* for 2 min and 50 μL of the supernatant was diluted with 950 μL of 0.54 mmol/L Na_2EDTA in 5% MPA solution. The samples were then stored at −80 °C for not more than 2 months. Prior to HPLC injection, samples were further centrifuged to remove the last particles. The HPLC system consisted of a model 2695 system pump (Waters) connected to a Coulochem II equipped with a model 5011 cell (ESA, Chelmsford, MA) and a Millenium Work station (Waters). An Aminex Fast Acid column (100 mm × 7.8 mm id; Bio-Rad Labs., Hercules, CA) was used. Samples were eluted (0.8 mL/min) with a mobile phase of 2 mmol/L sulfuric acid. Chromatograms were generated under the following conditions: guard cell at +800 mV, control cell at 0 mV, and analytical cell at +500 mV. The sample injection volume was 50 μL. Vitamin C standards (Sigma Chemical) in MPA buffer in the range of 0.1−1.0 μg/mL were prepared daily for plasma analysis. The detection limit was 0.05 μg/mL, and the CV was 4.3%.

Determination of Carotenoids in Plasma. Carotenoid extraction was performed in duplicate on 100 μL of plasma previously separated from blood samples (by centrifugation at 1000*g* for 10 min). The plasma samples were added to 100 μL of ethanol containing echinenone as the internal standard (F. Hoffmann-La Roche & Co., Basel, Switzerland). After the samples were vortexed, 200 μL of hexane was added and used for carotenoid separation. The supernatants (150 μL), obtained after centrifuging samples at 1000*g* for 5 min, were evaporated under N_2 and dissolved in 100 μL of eluent for HPLC analysis.

Carotenoid HPLC analysis was performed as previously described (*14*) by using a 5 μm Vydac 201 TP 54 C_{18} column (250 mm × 4.6 mm, i.d.) fitted with a C_{18} guard column and biocompatible frits. The eluent consisted of methanol:THF (95:5), and the flow rate was 1 mL/min. Visible detection was achieved at 445 nm (UV−vis detector Varian 2010). Recovery of the internal standard was between 90 and 100%. The carotenoid standards that we used were purchased from Sigma Chemical (Milan, Italy), except for lutein, zeaxanthin, and β-cryptoxanthin (F. Hoffmann-La Roche & Co.). Coefficients of variation were lower than 6% for all carotenoids, with the exception of lycopene (∼10%). Detection limits were as follows: 0.04 μg/mL for lutein, 0.07 μg/mL for zeaxanthin, 0.02 μg/mL for β-cryptoxantin, 0.06 μg/mL for α-carotene, 0.05 μg/mL for β-carotene, and 0.04 μg/mL for lycopene.

Determination of Cyanidin-3-glucoside in Plasma. Plasma samples (1 mL) were diluted with 1 mL of water, and the resulting solutions were then loaded onto HLB Oasys 300 mg SPE cartridges (Waters, Milford, MA) preactivated with methanol (2 mL) and then washed with water (4 mL). The SPE cartridge was then sequentially washed with 2 mL each of 5% formic acid in water and 5% methanol in water. The anthocyanins were eluted from the cartridge using 1 mL of methanol. The eluate was collected and dried under a stream of nitrogen. The

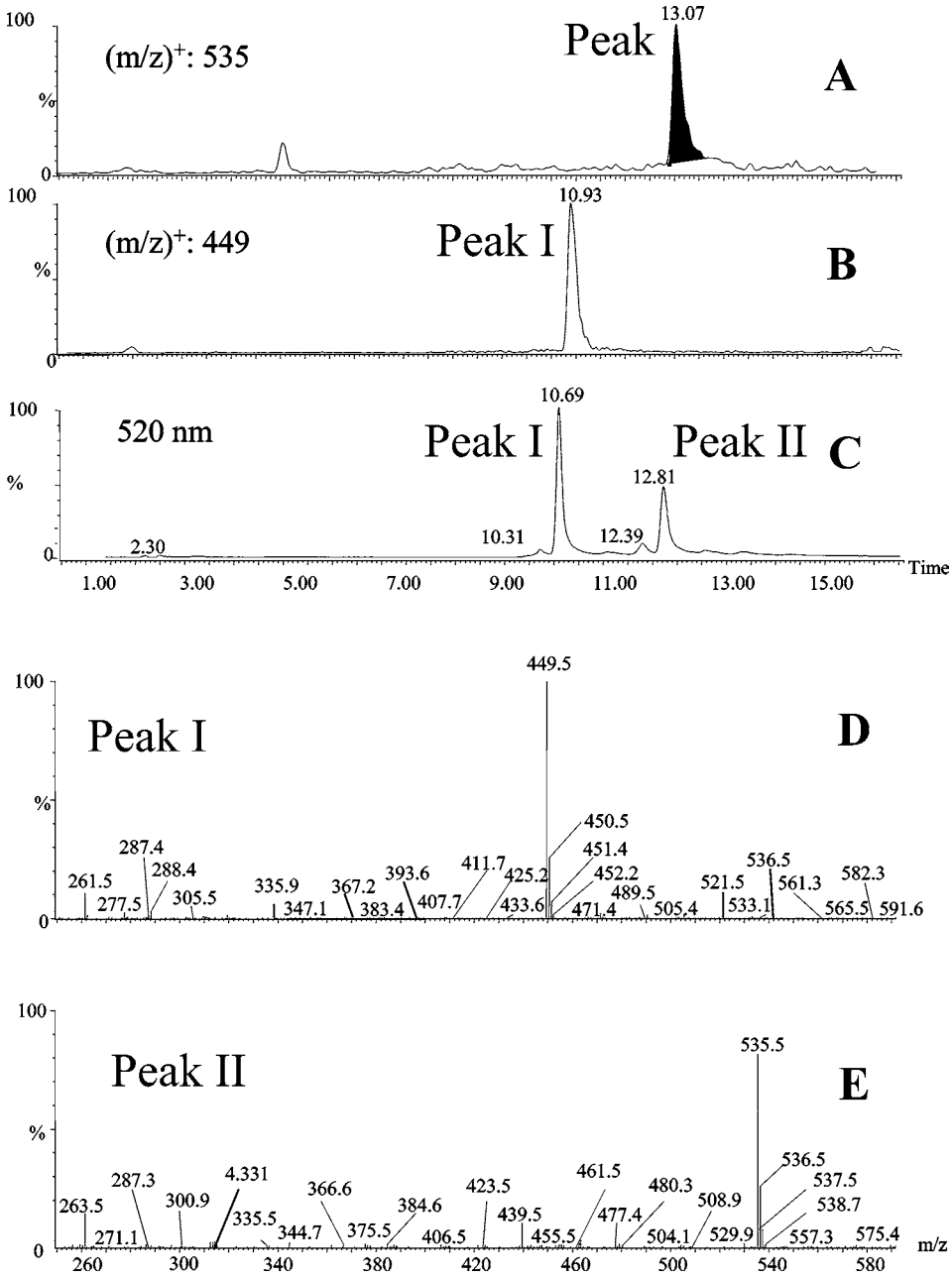

Figure 1. LC-DAD-MS chromatograms of red orange juice anthocyanins: ion cromatograms extracted at (**A**) *m*/*z* 535 (peak II, cyanidin-glucoside-6″-malonyl), (**B**) *m*/*z* 449 (peak I, cyanidin-glucoside), and (**C**) 520 nm. Mass spectra corresponding to peaks I and II are shown in panels **D** and **E**, respectively. ESI ionization was performed in positive mode, and MS conditions were as follows: capillary, 3.3 kV; cone, 20 V; source, 130 °C; desolvation, 350 °C; desolvation gas, 500 L/h; cone gas, 50 L/h; and *m*/*z* acquisition 100–800 amu.

resulting residue was reconstituted with 100 μL of methanol:formic acid (95:5) and stored at −20 °C.

The HPLC system was an Alliance 2695 (Waters) coupled to a Quattro Micro triple quadrupole mass spectrometer (Micromass) equipped with an orthogonal electrospray source. The analytical column was a 5 μm Symmetry C_{18} (150 mm × 2.1 mm, i.d., Waters), and the flow rate was fixed to 200 μL/min. The eluents were 0.1% formic acid (A) and acetonitrile (B), and the separation was carried out with a linear solvent gradient starting at 10% B and ramping to 40% B in 20 min. The column was thermostated at 30 °C. The mass spectrometer was operated in the positive ion mode with the capillary voltage set to 3.3 kV, the cone voltage was 25 V, the source temperature was 130 °C, and the desolvating temperature was 350 °C. The collision energy was 25 eV, and argon 6.0 was used to improve fragmentation in the collision cell. Cyanidin-3-glucoside plasma levels were determined in multiple reaction monitoring mode, by using the transition *m*/*z* 449→287. The mass data were acquired by Masslink 4.0 software (Micromass). The calibration curve was obtained from the cyanidin-3-glucoside mother solution prepared by dissolving 20 mg of standard powder (Extrasynthese, Genay, France) in 100 mL of methanol:formic acid solution (95:5). Plasma cyanidin-3-glucoside concentrations were evaluated using a calibration curve in the range of 0.5−10 ng/mL. The detection limit was 0.25 ng/mL, and the CV was 3.4%.

Plasma Antioxidant Capacity. The total antioxidant capacity of plasma was evaluated by an established method based on the reduction, by antioxidants, of Cu^{2+} to Cu^{+}, as previously described (*15*) (Oxis Research, Portland, OR).

Analysis of Malondialdehyde (MDA) in Plasma. As a marker of lipid oxidation, MDA was quantified by HPLC, following the method of Nielsen et al. (*16*). The extraction was performed on 0.5 mL of plasma. MDA was measured by its formation of an adduct with thiobarbituric acid (TBA), which was then separated by HPLC.

A calibration curve of tetramethoxypropane (in the range of 0.5−2 μmol/L) was used as the standard. The HPLC system consisted of a

Table 1. Plasma Antioxidant Concentrations before and after Each Experimental Period[a]

	before diet	after diet	before juice	after juice
cyanidin 3-glucoside	0.58 (0.49–0.67)[a]	0.63 (0.57–0.69)[a]	0.56 (0.49–0.63)[a]	8.33 (6.01–10.66)[b]
vitamin C	63.8 (58.0–69.6)[a]	67.6 (59.2–76.1)[a]	56.5 (48.8–64.3)[a]	84.5 (75.9–93.0)[b]
β-cryptoxanthin	0.42 (0.31–0.53)[a]	0.25 (0.19–0.30)[a]	0.29 (0.20–0.37)[a]	0.79 (0.66–0.91)[b]
zeaxanthin	0.10 (0.09–0.12)[a]	0.10 (0.09–0.12)[a]	0.10 (0.08–0.11)[a]	0.14 (0.11–0.16)[b]
lutein	0.67 (0.55–0.78)	0.65 (0.55–0.75)	0.60 (0.50–0.70)	0.70 (0.53–0.86)
α-carotene	0.11 (0.07–0.16)	0.10 (0.07–0.14)	0.11 (0.07–0.14)	0.14 (0.08–0.19)
β-carotene	0.33 (0.27–0.39)	0.32 (0.27–0.37)	0.30 (0.24–0.36)	0.35 (0.27–0.42)
lycopene	0.37 (0.28–0.46)[a]	0.28 (0.22–0.34)[b]	0.39 (0.25–0.53)[a]	0.35 (0.25–0.46)[a,b]

[a] Data (μmol/L except for cyanidin 3-glucoside, which is nmol/L) are expressed as means and 95% CI. Values in the same row that do not share a common letter are significantly different ($p < 0.05$). Data of group A and B are matched for each period.

Table 2. Markers of Antioxidant Potential Evaluated before and after Each Experimental Period[a]

	before diet	after diet	before juice	after juice
PAC (mequiv of uric acid)	93.6 (57.9–129.2)	107.8 (72.1–143.6)	77.0 (55.9–98.2)	92.5 (–4.6–189.5)
MDA (μmol/L)	0.72 (0.57–0,87)	0.74 (0.58–0.91)	0.69 (0.59–0.78)	0.77 (0.58–0.96)
11-dehydro tromboxane B_2 (total mg)	134.6 (101.3–167.9)	154.5 (118.0–191.1)	151.4 (102.0–200.8)	153.0 (113.6–192.4)
DNA damage (% DNA in tail)				
group A	56.8 (50.2–63.4)	55.0 (47.4–62.6)	58.0 (50.7–65.3)	21.3 (12.8–29.8)*
group B	49.2 (43.9–54.4)	41.2 (31.4–51.0)	39.1 (30.0–48.2)	34.8 (23.8–45.8)

[a] Data are expressed as means and 95% CI. *$p < 0.05$.

Waters 501 pump, UV−vis spectrophotometer (Waters 486), and a C_{18} Lichosper 5 μm column (4 mm × 4 mm, Merck). The mobile phase was 10 mmol/L phosphate buffer (pH 6.8):methanol (60:40), and the flow rate was 0.5 mL/min. Samples (50 μL) were injected for analysis, and the chromatogram was monitored at 486 nm. The detection limit was 0.1 μmol/L, and the CV was <10%.

Analysis of Urinary 11-Dehydrothromboxane B_2 (11-Dehydro-TXB_2). The urinary excretion of 11-dehydro-TXB_2 was evaluated by immunoassay (Cayman Chemical, Ann Arbor, MI) on aliquots from 24 h urines, following the manufacturer's instructions.

Evaluation of DNA Resistance to Oxidative Damage in Lymphocytes. DNA damage was evaluated by means of the Comet assay, as previously reported in detail (*17*). Briefly, lymphocytes were separated from 70 μL of whole blood by density gradient. The cells were fixed with agarose on fully frosted microscope slides (Richardson Supply Co., London, United Kingdom). For each subject, two slides were subjected to an oxidative treatment (H_2O_2 in PBS 500 μmol/L for 5 min) while others acted as control (PBS for 5 min). Then, all of the slides were placed in cold lysis buffer for 1 h at 4 °C in the dark and then in fresh alkaline electrophoresis buffer, in a horizontal electrophoresis tank (Scoatlab, Coatbridge, United Kingdom), for 40 min, followed by 20 min of electrophoresis (25 V, 300 mA). The slides were subsequently neutralized, stained with ethidium bromide (2 μg/mL, in neutralizing buffer), and washed with PBS, drained, and covered with coverslips.

An epifluorescence microscope (BX60; Olympus Italia, Milan, Italy) attached to a high sensitivity CCD video camera (Variocam; PCO Computer Optics, Kelheim, Germany) and to a computer provided with an image analysis system was used to visualize DNA damage. One hundred cells for each slide were electronically captured and analyzed for fluorescence intensity. Damaged DNA is recognized as a fluorescent core followed by a tail, which is due to electrophoretic migration of fragments away from the core. DNA damage was calculated as % DNA in the tail. For each subject, mean % DNA in tail of control cells was subtracted from the % DNA in tail of treated cells.

Statistical Analysis. Statistical analyses were performed with the STATISTICA software (Statsoft Inc, Tulsa, OK). A two-way repeated measure analysis of variance (ANOVA) with the sequence of treatments as the independent factor was used to investigate the effect of orange juice intake on the variables under study. When no carryover effect was shown, data of the two groups of subjects were matched. The data of lymphocyte resistance to DNA damage were considered separately, as the analysis was performed after 21 days of juice intake in group B and after 28 days of juice intake in group A, due to technical/experimental problems. Differences between means were further evaluated by the least significant difference test. Differences were considered significant at $p < 0.05$.

RESULTS

Antioxidant Plasma Concentrations. Table 1 reports the concentrations of the main antioxidants considered, before and after each experimental period. The vitamin C plasma concentration increased significantly ($p < 0.0001$) after 3 weeks of blood orange juice supplementation, whereas they remained stable in the period of diet without juice. The mean vitamin C concentration increase was of about 28 μmol/L following the daily intake of the juice. During the washout period, the vitamin C concentration decreased significantly ($p < 0.05$) as compared to the supplementation period.

The cyanidin 3-glucoside plasma concentration was very low and close to the detection limit of the instrument at baseline and after the washout period (about 0.6 nmol/L on average) and increased to ~8 nmol/L after 3 weeks of blood orange juice intake, i.e., an increase of more than 10-fold (**Table 1**). Both the aglycon and the cyanidin-3-glucoside-6″-malonyl were not detected in plasma.

As far as carotenoids were concerned, β-cryptoxanthin, β-carotene, and zeaxanthin concentrations increased significantly ($p < 0.0001$, $p < 0.05$, and $p < 0.001$, respectively) after the supplementation (**Table 1**). Average increases were of 0.5 μmol/L for β-cryptoxanthin and of 0.05 and 0.04 μmol/L for β-carotene and zeaxanthin, respectively. Concentrations of the other carotenoids fluctuated very little, apart from lycopene, which decreased during the period of diet devoid of juice ($p < 0.05$).

Evaluation of Antioxidant Activity and Prothrombotic Potential. Data of the biomarker of oxidative stress are reported in **Table 2**. Plasma antioxidant capacity (PAC) did not increase after the 3 weeks of juice intake and was comparable during the whole study. Concerning the biomarker of lipid oxidation, MDA, its plasma concentration was not affected by the daily intake of the blood orange juice. Also, the urinary excretion of 11-dehydro-TXB_2 did not vary significantly during the different

periods of dietary treatment. Regarding lymphocyte resistance to the ex vivo oxidative stress induced by H_2O_2, data from the two groups of subjects were analyzed separately and are reported in **Table 2**. In fact, because of technical/experimental problems, the Comet assay of group A was performed 1 week later than that of group B (corresponding to 28 days instead of 21 days of juice intake): The ANOVA showed a significant effect of the sequence of treatment ($p < 0.05$). In group B, DNA damage induced by hydrogen peroxide did not significantly decrease after the juice intake (about 16%) whereas that of group A decreased by about 63% (**Table 2**). No modification of DNA damage occurred after the periods of juice-devoid diet and washout. A further analysis revealed an inverse and significant ($R = -0.49$, $p < 0.005$) correlation between plasma vitamin C concentrations and DNA damage in group A.

DISCUSSION

This study shows that 3 weeks of blood orange juice (rich in vitamin C, carotenoids, and anthocyanins such as cyanidin 3-glucoside) consumption by healthy subjects increases plasma antioxidant concentrations but does not exert significant effects on several markers of oxidative stress, with the exception of DNA damage protection observed in one group.

It is noteworthy that data on the absorption and metabolism of polyphenols from polyphenol-rich juices are limited, in particular those on the bioavailability of cyanidin 3-glucoside. The general consensus is that polyphenols are promptly absorbed (reaching peak concentrations within 1−3 h after the intake) and are subsequently rapidly metabolized (*18*). This appears to hold true also for cyanidin 3-glucoside: It can be absorbed intact as glycoside within a few hours, and its plasma clearance is concluded within 6 h from the intake (*19*, *20*). Recently, Prior (*21*) reviewed the results published on anthocyanin absorption and reporting plasma maximum concentration and urine recovery recorded after the consumption of several food sources. Maximum plasma levels range from 1 to 120 nmol/L, depending on the dose (from 0.7 to 10.9 mg/kg body weight). For example, the intake of a single dose of red wine, dealcoholized red wine, or red grape juice (providing 0.7, 0.9, and 1.5 mg/kg body weight of malvidin 3-glucoside, respectively) produced plasma maximum concentrations of about 1.4, 1.5, and 2.8 nmol/L (*20*). However, baseline concentrations are in general assumed to be zero, as they are not detectable in plasma.

In the present study, we tested the hypothesis that daily intakes of low amounts of cyanidin 3-glucoside (∼0.4 mg/kg body weight) from blood orange juice could increase its plasma concentration (measured after an overnight fast) in the medium term. Indeed, 21 days of ingestion produced an increase of about 7.8 nmol/L, suggesting that regular consumption of anthocyanin-rich juices does enhance its concentration. Conversely, Bub et al. (*22*) did not find an increase in plasma polyphenol concentrations after 2 weeks of ingestion of two different juices, one of which contained 210 mg of cyanidin 3-glucoside, that is, about 10 times the dose employed in our study. Many factors might have contributed to this discrepancy, including differences in subjects, compliance, coingestion of food, etc.

The nutritional and biological activity of cyanidin 3-glucoside needs to be thoroughly investigated in vivo, taking into account several parameters. For example, it has been hypothesized that most of the ingested polyphenols are rapidly metabolized in the intestinal tract, where they might exert protective functions prior to their eventual absorption (*23*, *24*).

Concerning carotenoids, we demonstrate that plasma zeaxanthin and, most of all, β-cryptoxanthin concentrations are increased by consumption of blood orange juice. β-Cryptoxanthin is a xanthophyll present in few food sources such as oranges, tangerine, papaya, red peppers, etc. (*25*). To our knowledge, this is the first report on carotenoid bioavailability from blood orange juice. Data on β-cryptoxanthin bioavailability from other food sources are also limited. Xanthophylls in foods are often present as esters, which are likely hydrolyzed before or during absorption, as suggested by Wingerath et al. (*26*), who did not detect esters in chylomicrons and serum of subjects receiving concentrated tangerine juice. Recently, Breithaupt et al. (*27*) found that single oral doses of esterified or nonesterified β-cryptoxanthin from papaya puree had similar bioavailabilities. The blood orange juice that we used contained about 68% β-cryptoxanthin esters; even though we did not specifically measure their availability, our results further support the hypothesis of the presence of an effective enzymatic cleavage system that allows carotenoid absorption (*27*).

Like other orange juices, blood orange juice contains high amounts of vitamin C, i.e., 40−100 mg/100 mL, depending on industrial supplementation. The bioavailability of this vitamin from orange juices has been investigated only recently (*4*), and no data are available after blood orange juice intake. We now demonstrate that daily intakes of this juice do increase plasma vitamin C concentrations; however, despite the high amount of ascorbate introduced with the juice (about 400 mg/day), the increase was moderate (about 50%) in subjects who exhibited basal concentrations of this vitamin in the normal range. Recently, Sanchez-Moreno et al. (*4*) reported an average 30−60% plasma increase of vitamin C (from about 43 and 56 μmol/L to 68 and 73 μmol/L for men and women, respectively) after 14 days of blond orange juice intake (500 mL/day, providing 250 mg/day vitamin C). These data confirm that vitamin C absorption is not dose-dependent (*28*) and that it is possible that half of the amount of orange juice could have been sufficient to produce a similar increase in plasma ascorbate.

In our study, the consumption of 600 mL/day blood orange juice for 21 days did not affect plasma MDA concentration, selected as a marker of lipid peroxidation. Recently, Bub et al. (*22*) demonstrated that the ingestion of two antioxidant-rich juices did not affect markers of lipid peroxidation, namely, TBARS and FOX_2 detectable lipid hydroperoxydes, and low-density lipoprotein (LDL) resistance to oxidative stress. Furthermore, in agreement with our findings, they did not find an increase in antioxidant status, as evaluated by the FRAP assay.

We also analyzed 11-dehydro-TXB_2 excretion, an in vivo marker of systemic platelet activation and inflammatory status, which has been found to be increased in clinical conditions of cardiovascular risk and oxidative stress, such as hypercholesterolemia and diabetes mellitus (*29*). The intake of the blood orange juice did not produce any effect on this marker. Other studies have reported that the consumption of fruit juices can reduce oxidative stress and reduce atherogenic modifications of LDL cholesterol and platelet aggregation (*30*, *31*).

In conclusion, our results indicate that the consumption of blood orange juice for a short period (21 days) is not sufficient to significantly affect markers of lipid oxidation and, indirectly, of cardiovascular risk. We cannot exclude that longer periods of consumption or larger sample sizes could, instead, produce positive effects. This hypothesis is corroborated by the data obtained on lymphocyte resistance to DNA oxidative damage, as induced ex vivo, which was significantly reduced in the group of subjects (group A) who drank the juice for 28 days, whereas in the group that consumed the juice for 21 days there was a reduction that failed to reach significance. On the basis of this

piece of data, several hypotheses can be formulated. First, we noticed that in group B, the lymphocyte DNA damage induced before the juice intervention was lower than that recorded in group A. Thus, it is conceivable that lymphocytes of group B subjects had higher basal antioxidant protection. We have previously noted that the lower the DNA damage registered at baseline, the lower the eventual protective effect produced by a dietary intervention with antioxidants. Second, an analysis of the correlations between vitamin C concentrations and DNA damage recorded in the two groups demonstrated an inverse relationship between these two variables only in group A, where higher concentrations of vitamin C were reached after 21 days of juice intake. Hence, it can be speculated that several factors contributed to the different results obtained in the two groups of subjects, i.e., duration of blood orange juice supplementation, plasma vitamin C concentrations, and initial lymphocyte resistance to oxidative stress. From our experience, it seems plausible that both the extent of the blood orange juice supplementation and the different initial lymphocyte resistance to oxidative stress may have contributed to the different results obtained in the two groups of subjects. A significant reduction in oxidative DNA damage was also observed by other authors (*22*) after 2 weeks of supplementation with two fruit juices.

In conclusion, we demonstrate that blood orange juice consumption effectively increases plasma antioxidant concentrations, i.e., vitamin C, β-cryptoxanthyn, zeaxanthin, and cyanidin 3-glucoside. However, despite the increased antioxidant concentrations, plasma antioxidant status and lipid peroxidation were unaffected; conversely, an increased protection was afforded against lymphocyte DNA damage. In light of its widespread consumption in Southern Europe as a source of bioavailable antioxidants, the long-term effects of blood orange juice consumption are to be further investigated.

LITERATURE CITED

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