Consumption of orange fermented beverage improves antioxidant status and reduces peroxidation lipid and inflammatory markers in healthy humans

Blanca Escudero-López,[a] Ángeles Ortega,[a,b] Isabel Cerrillo,[a,c] María-Rosario Rodríguez-Griñolo,[d] Rocío Muñoz-Hernández,[e] Hada C. Macher,[f] Franz Martín,[a,b] Dámaso Hornero-Méndez,[g] Pedro Mena,[h] Daniele Del Rio[h] María-Soledad Fernández-Pachón[a,c*]

a *Área de Nutrición y Bromatología, Departamento de Biología Molecular e Ingeniería Bioquímica, Universidad Pablo de Olavide, Sevilla, Spain*

b *CIBER de Diabetes y Enfermedades Metabólicas Asociadas (CIBERDEM), Universidad Pablo de Olavide, Sevilla, Spain*

c *Investigador Asociado, Facultad de Ciencias de la Salud, Universidad Autónoma de Chile, Santiago de Chile, Chile*

d *Área de Estadística e I.O., Departamento de Economía, Métodos Cuantitativos e Historia Económica, Universidad Pablo de Olavide, Sevilla, Spain*

e *Instituto de Biomedicina de Sevilla (IBiS), Hospital Universitario Virgen del Rocio/CSIC/Universidad de Sevilla, Laboratorio de Hipertensión Arterial e Hipercolesterolemia, Sevilla, Spain.*

f *Servicio de Bioquímica Clínica, Hospital Virgen del Rocío, Sevilla, Spain.*

g *Departamento de Fitoquímica de Alimentos, Instituto de la Grasa-CSIC, Sevilla, Spain*

h *Human Nutrition Unit, Department of Food & Drug, University of Parma, Parma, Italy*

This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1002/jsfa.8774

** Correspondence to: M.S. Fernández-Pachón, Área de Nutrición y Bromatología, Departamento de Biología Molecular e Ingeniería Bioquímica, Universidad Pablo de Olavide, Sevilla, Spain. E-mail: msferpac@upo.es*

Abstract

BACKGROUND: Alcoholic fermentation of fruits has generated novel products with high concentrations of bioactive compounds and moderate alcohol content. The aim of this study was to evaluate the potential effect on cardiovascular risk factors of the regular consumption by healthy humans of a beverage obtained by alcoholic fermentation and pasteurization of orange juice.

RESULTS: Thirty healthy volunteers were enrolled in a randomized controlled study. The experimental group (n=15) drank 500 mL orange beverage (OB) day^{-1} for 2 weeks (intervention phase), followed by a 3-week washout phase. Blood samples were collected at baseline (E-T0) and at the end of the intervention (E-T1) and washout (E-T2) phases. Controls (n=15) did not consume OB during a 2-week period. OB intake significantly increased ORAC (43.9%) and reduced uric acid (-8.9%), CAT (-23.2%), TBARS (-30.2%), and C-reactive protein (-2.1%) (E-T1 *vs*. E-T0). These effects may represent longer-term benefits, given the decreased uric acid (-8.9%), CAT (-34.6%), TBARS (-48.4%), and oxidized LDL (-23.9%) values recorded after the washout phase (E-T2 *vs*. E-T0).

CONCLUSION: The regular consumption of OB improved antioxidant status and decreased inflammation state, lipid peroxidation, and uric acid levels. Thus, OB may protect the cardiovascular system in healthy humans and be considered a novel functional beverage.

Keywords: Orange fermented beverage; bioactive compounds; antioxidant status; lipid peroxidation; inflammation status; healthy humans

INTRODUCTION

Over recent years, there has been increasing interest in the consumption of foods with potential beneficial health effects beyond their nutritional impact.[1] Orange juice consumption has become a worldwide dietary habit, accounting for 60% of all fruit juice and juice-based drinks consumed in Western Europe.[2] Orange juice is valued for its nutritional content[3] and as a rich source of bioactive compounds such as flavanones (hesperetin and naringenin, predominantly as glycosides),[4] carotenoids (xanthophylls, cryptoxanthins, carotenes),[5] and melatonin.[6] These compounds have been found to exert antioxidant, hypotensive, vasodilator, anti-inflammatory, and hypolipidemic effects, among others.[7-9] Furthermore, numerous epidemiological and intervention studies have demonstrated the protective and therapeutic role of regular orange juice consumption against chronic diseases, including cardiovascular disorders, diabetes, obesity, and cancer.[3,10-12] Observations of these beneficial effects of orange juice have increased research interest in technological treatments to preserve or enhance the bioactive compounds it contains.[13,14] Alcoholic fermentation of various types of fruit,[15,16] has generated new products with moderate alcohol content and a higher concentration of bioactive compounds than in the original substrate. Thus, our group found that a novel fermented orange beverage (OB) possessed a larger amount of flavanones, carotenoids, provitamin A, and melatonin and a greater antioxidant capacity in comparison to the original juice.[17-19] Intervention studies in mice with induced metabolic syndrome and healthy mice suggested that regular consumption of OB might provide greater protection against cardiovascular risk factors *versus* the original juice through possible synergistic effects between the bioactive compounds and moderate alcohol contents.[20,21] The above results support the status of OB as a new functional beverage. The aim of the present study was to evaluate the potential beneficial effects of regular OB consumption

on cardiovascular risk factors by determining the antioxidant status, lipid metabolism, lipid peroxidation, endothelial function, and inflammatory response in healthy humans before and after their consumption of 500 mL OB per day for 14 days.

MATERIALS AND METHODS

Reagents and chemicals

Reagents and chemicals were purchased from Sigma-Aldrich (St. Louis, MO), while hesperetin-7-*O*-rutinoside, naringenin-7-*O*-rutinoside, isosakuranetin-7-*O*-rutonoside, and apigenin-8-*C*-glucoside were purchased from Extrasynthese (Genay Cedex, France).

Orange beverage: Production and composition

Grupo Hespérides Biotech S.L. and Mitra Sol Technologies S.L. produced the OB using a commercial orange juice from *Citrus sinensis* L. var. *Navel late* (Huelva, Spain). Its controlled alcoholic fermentation was carried out for 10 days at 22 ºC in 100 L stainless steel tanks (semi-industrial scale) under aseptic conditions using *Saccharomycetaceae* var. *Pichia kluyveri*, previously isolated from the natural microbiota in orange fruit. This yeast strain ferments reducing sugars alone, giving the final product a low alcohol content (1% v/v) and sweet taste. The fermented juice was then pasteurized (25 L h^{-1}) at 80 °C for 30 s in a semi-industrial tubular pasteurizer (Mipaser Prototype, Murcia, Spain) and then cooled to 10 °C in an ice-water-bath. Next, the beverage underwent carbonation up to a pressure of 0.44 x 10^5 Pa and was aseptically poured into aluminum containers (250 mL), which were stored at 4 °C until their consumption. The quality parameters, nutritional composition, bioactive compound profile, and antioxidant activity of OB were obtained using the methodology described by *Escudero-López et al.*,[17] Cerrillo *et al.*,[18] OIV,[22] Collado-González *et al.*,[23] and Escudero-López *et al.*[24]

Subjects

Thirty healthy volunteers (9 men and 21 women) with mean±SEM age of 33.9 ± 6.9 years and body mass index (BMI) of 23.6 ± 3.6 kg m^{-2} were enrolled in the study after the application of study eligibility criteria. Evaluation of the health status of volunteers was based on their medical history, anthropometric measurements, and routine laboratory tests. A lifestyle questionnaire and a 3-day food record were used to examine the healthy habits of the participants. Exclusion criteria were: 1) the presence of chronic diseases (cardiovascular diseases, diabetes, cancer, chronic obstructive pulmonary disease, metabolic syndrome), overweight, and/or kidney or liver failure; 2) abnormal baseline blood test results; 3) intake of any medication or nutritional supplement during the previous 4 weeks; 4) any smoking habit; and 5) alcohol consumption of >2 drinks day^{-1}. All participants gave their oral and written informed consent to participate in the study, which was conducted in accordance with the principles of the Declaration of Helsinki and approved by the Clinical Research Ethical Committee of Virgen del Rocío Hospital (IEC 2013PI/022).

Experimental design

A randomized, controlled trial was conducted, studying experimental and control groups in parallel (Fig. 1). The experimental group (n=15) was instructed to drink 500 mL day^{-1} (two 250-mL cans) of OB during a two-week period (intervention phase) followed by a three-week period with no OB intake (washout phase). Blood samples were drawn after overnight fasting at the beginning (E-T0) and end (E-T1) of the intervention phase and at the end of the washout phase (E-T2). The control group (n=15) did not consume OB for two weeks, and blood samples were drawn after overnight fasting at the beginning (C-T0) and end (C-T1) of this period. The volunteers were told not to change their lifestyles, diet, or level of physical activity during the

intervention but to limit their beer or wine intake to a maximum of 6 portions per week and to avoid any intake of spirits over the study period.

Blood sample collection and handling

Blood samples (20 mL) were drawn from the antecubital vein with subjects in seated position and was immediately collected and centrifuged for 20 min (12000 x *g*; 4 °C). Resulting samples (plasma and erythrocytes) were divided into 0.5 mL aliquots and frozen at -80 °C until analysis. Glucose, insulin, creatinine, glutamic oxaloacetic transaminase (GOT), glutamic-pyruvic transaminase (GPT), hemoglobin, and red blood cell levels were determined by the clinical laboratory of Virgen del Rocío Hospital using routine tests.

Anthropometric measurements

Anthropometric measurements were obtained while participants were barefoot and wearing only nonrestrictive undergarments. A SECA 780 digital scale balance (SECA, Barcelona, Spain) was used to measure body weight and height, from which the BMI (kg m^{-2}) was calculated. A SECA 201 flexible tape (SECA) was used to measure waist circumference (WC) around the narrowest portion of the torso.

Antioxidant Status

Antioxidant status was assessed by determining plasma antioxidant capacity (PAC), levels of endogenous antioxidant compounds (albumin, bilirubin, uric acid, glutathione), and antioxidant enzyme activities (catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (GPx), and glutathione reductase (GR)).

PAC was evaluated by means of the oxygen radical absorbance capacity (ORAC) and ferric reducing antioxidant power (FRAP) assays. The ORAC assay was performed according to Ou *et al.*[25] diluting plasma samples (1:1000) in phosphate buffer (75 mM, pH 7.4), while the FRAP assay followed the method of Delgado-Andrade *et al.*[26]

diluting plasma samples (1:8) in distilled water. PAC measurements were recorded on a Synergy™ HT-multimode microplate reader (Biotek Instruments, Winooski, VT).

Plasma levels of the endogenous antioxidant compounds albumin, bilirubin, and uric acid were measured by enzymatic spectrophotometry using a Roche Cobas C 701 Modular Analyzer (Roche Diagnostics, Madrid, Spain), while glutathione was determined in plasma samples using a commercial kit (Cayman Chemical Company, Midland, MI), recording the measurements on a Synergy™ HT-multimode microplate reader (Biotek Instruments).

CAT, SOD, GPx, and GR activities in red blood cells were determined according to Cohen and Somerson,[27] McCord and Fridovich,[28] Carmagnol *et al.*,[29] and Cribb *et al.*,[30] respectively. Samples were homologized in a 1:4 (v:v) water milliQ solution, vortexed for 5 s, and centrifuged for 30 min (14000 x *g*; 4 °C); resulting supernatants were collected and stored at −80 °C until analysis. Total protein content was assessed by the standard Bradford procedure.[31] Measurements were recorded on a Synergy™ HT-multimode microplate reader (Biotek Instruments).

Lipid metabolism

Plasma levels of total cholesterol (TC), LDL, HDL, and triglycerides (TG) were measured with CHOD-PAP, LDL-C-plus, HDL-C-plus, and GPO-PAP kits (Roche Diagnostics), respectively, using a Roche Cobas 8000 Modular Analyzer (Roche Diagnostics). Plasma concentrations of apolipoproteins (apo) A-I and B were measured using commercial available kits (Cayman Chemical Company and Cloud Clone Corporation, Houston, TX, respectively). Measurements were recorded on a Synergy™ HT-multimode microplate reader (Biotek Instruments).

Lipid peroxidation

Lipid peroxidation was evaluated by determining levels of thiobarbituric acid reactive substance (TBARS) and oxidized LDL (oxLDL). TBARS was measured in hemolyzed erythrocyte samples according to Buege and Aust[32] expressing the results as malondialdehyde (MDA) concentrations. Plasma oxLDL concentrations were measured using a commercial kit (Cell Biolabs Inc., San Diego, CA). TBARS and oxLDL measurements were recorded on a Synergy™ HT-multimode microplate reader (Biotek Instruments).

Endothelial function and inflammation status biomarkers

Plasma concentrations of soluble vascular cell adhesion molecule 1 (sVCAM-1) and soluble intercellular adhesion molecule 1 (sICAM-1) were measured with commercial kits (R&D Systems, Minneapolis, MN). Circulating cell-free DNA (cf-DNA) was automatically extracted from plasma samples (1 mL) using a Compact MagnaPure Instrument (Roche Diagnostics) and was resuspended in a final volume of 90 μL of RNase/DNase-free. The serum cf-DNA template was amplified in a total reaction volume of 20 μL using a qRT-PCR assay for the β-globin sequence on a Light-Cycler-480 instrument (Roche Diagnostics), following the manufacturer's instructions. The β-globin Taqman system uses the following primers: β-globin-354F (5′-GTG CAC CTG ACT CCT GAG GAG A-3′); β-globin-455R (5′-CCT TGA TAC CAA CCT GCC CAG-3′); and a dual-labeled fluorescent Taqman probe β-globin-402T (5′-(FAM) AAG GTG AAC GTG GAT GAA GTT GGT GG (TAMRA)-3′). Amplification was carried out over 48 cycles at 95 ºC for 5 min and at 62 °C for 20 min. A negative reaction and blank control were included to detect any contamination. A conversion factor of 6.6 pg of DNA per cell was used to express the result as copy number or genome equivalents (GE mL^{-1}). Endothelial cell-derives microparticles (EMPs) were measured by incubating plasma samples for 15 min with a monoclonal antibody against FITC labeled

anti-CD31 antibody (BD Bioscience, San Jose, CA) and anti-CD41-Pacific Blue, followed by incubation for 20 min with phycoerythrin-conjugated Annexin V kits according to the manufacturer's instructions (BD Bioscience). CD31-FITC and CD41-Pacific Blue were used to differentiate between CD31+CD41+ platelet-derived microparticles (PMPs) and CD31+CD41– EMPs. The negative control (zero value) was obtained using the isotype antibodies. After the addition of flow count calibrator beads (Beckman Coulter, Villepinte, France), MPs were identified as events with a 0.1-1 μm diameter on forward light scatter (FSC) and side-angle light scatter (SSC) intensity dot plot representation in comparison to flow cytometry calibration beads (Flowcount® calibrator beads, Beckman Coulter, Marseille, France); a BD LSR Fortessa flow cytometer (BD Bioscience) was employed for these analyses. Plasma concentrations of the inflammation status biomarker C-reactive protein (CRP) was determined using a commercial kit (Immundiagnostik AG, Bensheim, Germany), and measurements were recorded on a Synergy^{TM} HT-multimode microplate reader (Biotek Instruments).

Statistical analyses

All measurements were analyzed in triplicate. Data are expressed as mean±SEM. The Shapiro-Wilk test was used to assess the normal distribution. In order to evaluate the effects of OB consumption by the experimental group over time, variables with a normal distribution were compared using a one-way analysis of variance (ANOVA) for repeated measures (at E-T0, E-T1, and E-T2); when a significant main effect was found, the Bonferroni test of multiple comparisons was applied to identify differences between means. Variables with a non-normal distribution were compared using the Friedman test. In the control group, variables were compared between C-T0 and C-T1, using paired t-test when their distribution was normal and the Wilcoxon test when it was not.

P<0.05 was considered significant. SPSS version 22 (IBM SPSS Armonk, NY) was used for the statistical analyses

RESULTS AND DISCUSSION

This study is the first long-term randomized trial conducted in humans to evaluate the effects on cardiovascular risk factors of a fermented and pasteurized orange beverage (OB) with low alcohol content (1 % v/v). OB was found to contain a large amount of bioactive compounds, including phenolic compounds (836 nmol L^{-1}) and carotenoids (17.8□□mol L^{-1}) (Table 1). The phenolic profile of OB was characterized by the presence of 8 flavanones, 2 flavones, and one hydroxycinnamate. Hesperidin (445 nmol L^{-1}) and narirutin (133 nmol L^{-1}) were the most abundant phenolic compounds, and the sum of the individual flavanones accounted for the 79.8% of the phenolic content. Total flavones reached a value of 17.1% of the total phenolics. A total of 13 carotenoids were present in OB. The following pigments accounted for principal content: karpoxanthin (1.60 □mol L^{-1}), neochrome (1.41□□mol L^{-1}), auroxantin (4.33□□mol L^{-1}), mutatoxantin (2.77□□mol L^{-1}), (all-E)-zeaxantin (1.81□□mol L^{-1}), □-cryptoxanthin (2.43□□mol L^{-1}), and □-carotene (1.41□□mol L^{-1}) (accounting 88,5% of the total carotenoids). In addition, there is a remarkable presence of carotenoids with provitamin A activity (β-carotene, α-carotene, and β-cryptoxanthin). The provitamin A value obtained for OB was 71 RAEs L^{-1}. On the other hand, ORAC and FRAP values revealed a high antioxidant capacity of OB. Selection of the OB intake for participants (500 mL day^{-1}) was based on a previous study in healthy mice which found that the consumption of OB equivalent to an intake of 500 mL day^{-1} in humans produced a greater enhancement in their antioxidant status in comparison to an intake corresponding to 250 mL day^{-1} in humans.[20]

There were no significant differences in baseline biomarker levels between the experimental (n=15) and control (n=15) groups. All 30 participants complied with all evaluation procedures, and no individual in the experimental group reported any difficulty with the daily intake of OB required or showed any adverse effect from its consumption. Consumption of 500 mL day^{-1} OB represented an additional daily intake of 160 Kcal with 24 g of total sugars (Table 1). Nevertheless, this intake did not produce any significant change over the study period in body weight, WC, plasma concentrations of glucose, insulin, or renal/liver function biomarkers (creatinine, GOT and GPT) (Table 2), in line with previous findings reporting that the daily intake of orange juice does not affect these parameters.[12,33-35] As expected, control individuals, who did not consume OB, showed no significant changes in any of these parameters.

Effect of OB consumption on antioxidant status

Table 3 exhibits values of antioxidant status biomarkers in the experimental group before (E-T0) and after (E-T1) 2 weeks of OB consumption and at the end of the 3-week washout period (E-T2), as well as in the control group (C-T1 *vs*. C-T0). OB intake significantly increased ORAC values (43.9%; $P<0.01$) during the intervention phase (E-T1 *vs*. E-T0). After the washout phase (E-T2), these values were similar to those at baseline (E-T0). No change in ORAC values was observed in the control group. This positive effect of OB may be attributable to the antioxidant activity in plasma of intestinally absorbed flavonoids and carotenoids or their derived metabolites. Hesperidin has been described as responsible for improving PAC.[36] A significant increase in ORAC values was also observed after consumption by healthy humans of 200 mL day^{-1} orange juice for 26 days.[37] ORAC values were also increased in hypercholesterolemic patients after the consumption of 600 mL day^{-1} orange juice for 4 weeks.[38] However, no changes were observed in FRAP values, in agreement with

previous studies on the effects in healthy humans of consuming 500 mL day^{-1} orange juice for 4 weeks[34] or 330 mL day^{-1} of other antioxidant-rich juices for 2 weeks.[39] Findings on antioxidant capacity may differ among the measurement methods, because they are based on distinct types of chemical reaction.[40]

Among endogenous antioxidant compounds, uric acid levels were significantly reduced after OB intake (E-T1 *vs*. E-T0: -8.9%; $P<0.05$) and after washout phase (E-T2 *vs*. E-T0: -8.9%; $P<0.05$) in comparison to baseline values; i.e., the lowering effect of OB intake on uric acid levels persisted over time. No change in uric acid levels was observed in the control group. It was previously reported that uric acid concentrations were significantly reduced after 4 weeks of orange juice consumption (500 mL day^{-1}).[34] This reduction may be related to the intake of vitamin C, given reports of an inverse correlation between plasma vitamin C and uric acid concentrations after orange juice consumption.[2] In addition, it has been observed by other authors that intake of the bioactive compounds naringenin and melatonin may also reduce uric acid levels.[41,42] Uric acid is considered an antioxidant compound, whose actions include the inhibition of DNA damage.[43] It should be noted that the uric acid levels of the subjects drinking OB remained within the normal range throughout the study. On the other hand, elevated serum uric acid concentrations have been associated with cardiovascular diseases,[44] and various mechanisms have been proposed for this effect, including increased platelet adhesiveness and platelet lysis, vascular endothelial cell injury, the formation of free radicals, and oxidative stress. Hence a reduction in plasma uric acid concentrations may contribute to the prevention of cardiovascular disorders. OB consumption did not change albumin, bilirubin, or glutathione concentrations, which were also reported to be unaffected by the consumption of 250 mL day^{-1} apple or pomegranate juice for 4 weeks,[45] 16 fl oz day^{-1} carrot juice for 3 months,[46] or one serving of tomato juice per

day for 2 months.[47] No changes were observed in the control group except for a reduction in glutathione concentrations (C-T1 *vs*. C-T0: -18.2%; $P<0.05$), which may be attributable to inter-individual variability and the sample size.

OB consumption produced a significant decrease in CAT activity (E-T1 *vs*. E-T0: -23.2%; $P<0.05$), and the percentage decrease was higher after the washout phase (E-T2 *vs*. E-T0: -34.6%; $P<0.001$). No change in CAT activity was observed in the control group. A significant decrease in CAT activity was previously reported after the consumption of 500 mL day^{-1} orange juice for 2 weeks by overweight/obese humans[48] or of 600 mL day^{-1} orange juice for 4 weeks by men with mild hypercholesterolemia.[38] Hesperidin in orange juice might decrease superoxide anions and consequently the lipid peroxide and hydrogen peroxide generated during normal cell metabolism, thereby reducing the need for catalase biosynthesis.[49] Constant *et al.*[38] related the reduction in CAT activity to the intake of vitamin C. In another study, orange juice was found to prevent $O_2^{\bullet-}$ production by reducing the over-expression of p47phox and the consequent increase in NADPH oxidase activity, and this effect was attributed to p38MAP kinase inhibition.[50] The greater percentage decrease in CAT after the washout phase suggests that bioactive compounds in OB exert a persistent long-term effect. However, SOD, GPx, and GR activities did not change in either the intervention or control group. It was previously observed that SOD and GPx activities were not modified after the consumption of 600 mL day^{-1} orange juice for 4 weeks by men with mild hypercholesterolemia,[38] or 500 mL day^{-1} orange juice for 2 weeks by obese/overweight humans.[48]

Effect of OB consumption on lipid metabolism

Table 4 compares the values of lipid metabolism biomarkers (TC, LDL, HDL, TG, apo A-1, and apo B) among E-T0, E-T1, and E-T2 in the experimental group and between

C-T0 and C-T1 in the control group. The intake of OB did not lead to significant changes in any parameter. The same results were observed in the control group. It was previously found that TC, LDL, HDL, and TG levels were not modified after the intake by healthy humans of 500-750 mL day^{-1} orange juice for 3-8 weeks.[10,33,34,51] Varied results have been obtained in hypertriglyceridemic/hypercholesterolemic or overweight/obese subjects. Foroudi *et al.*,[12] Kurowska *et al.*[52] and Simpson *et al.*[53] found no significant change in TC, LDL, HDL, TG, apo A-I, or apo B levels after the consumption of 250-592 mL day^{-1} orange juice for 4-13 weeks. However, the consumption of 500-750 mL orange juice for 4 weeks,[38,52] 12 weeks,[48] or 60 days[33] produced, respectively, a significant increase in HDL and improvement in apo A-I, decrease in TG and apoB, or a decrease in TC and LDL. These discrepancies may be related to differences in the volume of orange juice consumed. It is also plausible that orange juice might improve the lipid profile more effectively in subjects with cardiovascular risk factors. On the other hand, some authors obtained dose-dependent increases in TG levels after orange juice consumption due to the triacylglycerol-raising effect of fructose intake.[10,52,54] The reduction in sugar content produced by the alcoholic fermentation process means that OB may be more healthy than the original orange juice in relation to the maintenance of TG levels.

Effect of OB consumption on lipid peroxidation

Table 4 compares values of lipid peroxidation biomarkers (TBARS and oxLDL) among E-T0, E-T1, and E-T2 in the experimental group and between C-T1 and CT-0 in the control group. TBARS values were significantly decreased by OB intake (E-T1 *vs*. E-T0: -30.2%; $P<0.05$), and were further reduced after the 3-week washout phase (E-T2 *vs*. E-T0: -48.4%; $P<0.001$). oxLDL levels decreased in the experimental group throughout the two phases of the study, and this decrease was significant at the end of

the washout phase (E-T2 *vs*. E-T0: -23.9%; $P<0.05$). There was no change in oxLDL levels in the control group, although a significant decrease in TBARS values was observed between C-T1 and C-T0 (-33.1%; $P<0.05$), which may again be attributable to inter-individual variability and the sample size. Several studies also found that orange juice consumption had a positive effect on lipid peroxidation biomarkers. The intake of 240-750 mL day^{-1} orange juice for 2-8 weeks induced a significant decrease in TBARS levels in healthy humans,[39,55,56] and consumption of 592–750 mL day^{-1} orange juice for 8–13 weeks reduced malondialdehyde levels in overweight humans[56] or those with hypercholesterolemia and hypertriglyceridemia.[12] The bioactive compounds in OB may be responsible for this beneficial effect, which has been previously attributed to flavonoid compounds[57] or carotenoid pigments.[58] These compounds with antioxidant activity may be incorporated into LDL, react with singlet molecular oxygen and peroxyl radicals generated in the process of lipid peroxidation, and become oxidized. In addition, the moderate alcohol content of OB might contribute to its beneficial effect on lipid peroxidation. It has been shown that light alcohol intake by rats and humans upregulates liver paraoxonase 1 mRNA, which inhibits LDL oxidation.[59] The trend towards more pronounced responses in TBARS and oxLDL values during the washout phase than during the intervention period suggests a sustained and possibly long-term effect of the bioactive compounds and/or moderated alcohol content in OB.

Effect of OB consumption on biomarkers of endothelial function and inflammation status

Table 5 compares sVCAM-1, sICAM-1, and CRP levels among E-T0, E-T1, and E-T2 in the experimental group and between C-T1 and C-T0 in the control group. Endothelial function biomarkers sVCAM-1 and sICAM-1 did not change in the experimental or control group. These results are in agreement with findings after the consumption of

500–600 mL day^{-1} orange juice for 4 weeks by healthy[34] and hypercholesterolemic[38] individuals. EMPs and cf-DNA are two recent markers of endothelial dysfunction released into the blood in conditions of systemic endothelial cell damage (apoptosis, necrotic death, or cell lysis), and their levels correlate with the degree of endothelial dysfunction.[60] In clinically healthy individuals, elevated levels of these markers may be an early sign of the presence of other cardiovascular risk factors, including impaired glucose tolerance, elevated blood pressure, dyslipidemia, systemic inflammation, and/or endothelial cell activation.[61] Hence, both markers may indicate the prevalence of subclinical phases of cardiovascular diseases and predict the development of cardiovascular events in asymptomatic patients.[62] In our study, levels of EMPs and cf-DNA were not modified after the intake of OB in the experimental group. In the control group, any change was observed. McFarlin *et al.*[63] also found no changes in EMPs levels of healthy humans after the consumption of a cocoa bar for 4 weeks. However, a significant decrease in EMPs concentrations was reported after the consumption by healthy elderly humans of a Mediterranean diet (high source of bioactive compounds from fruits and vegetables) for 4 weeks,[64] and after the intake of a high-flavanol cocoa drink or cocoa bar (high sources of flavanols) by humans with cardiovascular risk factors for 1 month.[63,65] Results may differ according to the age and prevalence of risk factors in the subjects or the bioactive composition of the food assayed. A significant decrease in the inflammation biomarker CRP was observed after OB intake in comparison to baseline levels (E-T1 *vs*. E-T0: -2.1%; $P<0.05$). This effect was not sustained during the washout period, finding no significant differences between E-T2 and E-T0. No change in CRP levels was observed in the control group. Various studies have demonstrated the beneficial effect of regular orange juice consumption on CRP levels in healthy humans and in those with cardiovascular risk factors. CRP levels were

significantly reduced after the intake by healthy individuals of 500-1000 mL day^{-1} orange juice for 2-8 weeks[35,56,66,67] or the consumption by subjects with increased cardiovascular risk (BMI > 28 and > 2 diagnostic criteria for the metabolic syndrome) of 500 mL day^{-1} orange juice for 1 week[68] or by overweight/obese individuals of 500-750 mL day^{-1} orange juice for 8-12 weeks.[36,56,69] CRP synthesis is stimulated by cytokines such as IL-6, IL-1, and TNF-α, whose expressions are regulated by the oxidative stress-mediated activation of NF-κB. Bioactive compounds of orange juice may act as ROS scavengers, inhibiting NF-κB activation.[70] Further potential mechanisms include the participation of bioactive compounds in the activation of Nrf2, which is involved in the modulation of antioxidant gene transcription.[71] For its part, moderate alcohol consumption has also been reported to reduce systemic inflammation markers, including CRP, in healthy humans and in those with cardiovascular diseases.[72,73] Imhof *et al.*[74] also found an association between light-moderate alcohol consumption and lower CRP levels, suggesting a U-shaped relationship between these parameters in healthy people. Thus, the bioactive compounds and moderate alcohol content in OB may both induce a significant decrease in CRP levels.

Among study limitations, no attempt was made to exclude bioactive compounds from the participants' diet, which was not strictly controlled in order to make the trial more realistic, and this may have increased the inter-individual variability in the evaluated parameters. Investigations with larger sample sizes, targeting those cardiovascular markers altered to a greater extent in the present study, are warranted to obtain more definitive results. The study could also be conducted for longer than 2 weeks (1-2 months) to further explore the effect of the bioactive compounds and moderate alcohol content in OB.

CONCLUSION

In this study, we concluded that the regular consumption of 500 mL OB per day may protect the cardiovascular system in healthy humans by improving antioxidant status through an increase in plasma antioxidant capacity and a decrease in risk factors, e.g., inflammation state, lipid peroxidation, and uric acid levels. Beneficial effects of the bioactive compounds and/or moderate alcohol content of OB may persist over time, given the sustained or more pronounced response in uric acid levels and of lipid peroxidation biomarkers observed three weeks after ending OB consumption. These results indicate that OB can be considered a novel functional beverage for daily human consumption to diminish the likelihood of cardiovascular diseases. Further human studies are required to confirm the potential beneficial effects of this new orange beverage in the treatment of these diseases. In addition, research is under way on the human bioavailability of bioactive compounds in OB in order to establish the profile of circulating metabolites that exert cardio-healthy effects.

ACKNOWLEDGEMENTS

The authors are grateful for the support of the Junta de Andalucía through Projects P09-AGR4814M and Grupo PAI BIO311. They also thank Doctor José Villar (†Deceased) for clinical assistance in the laboratory of Virgen del Rocío Hospital, Grupo Hespérides Biotech S.L. for providing the orange beverage, and Richard Davies for editorial assistance.

REFERENCES

1 Niva M, All foods affect health: understandings of functional foods and healthy eating among health-oriented Finns. *Appetite* **48**:384-393 (2007).

2 Sánchez-Moreno C, Cano MP, de Ancos B, Plaza L, Olmedilla B, Granado F *et al.*, Pulsed electric fields-processed orange juice consumption increases plasma vitamin C and decreases F2-isoprostanes in healthy humans, *J Nutr Biochem* **15**:601-607 (2004).

3 O'Neil CE, Nicklas TA, Rampersaud GC and Fulgoni VL, 100% orange juice consumption is associated with better diet quality, improved nutrient adequacy, decreased risk for obesity, and improved biomarkers of health in adults: National Health and Nutrition Examination Survey, 2003-2006. *Nutr J* **11**:107 (2012).

4 Kelebek H and Selli S, Determination of volatile, phenolic, organic acid and sugar components in a Turkish cv. Dortyol (*Citrus sinensis* L. Osbeck) orange juice. *J Sci Food Agric* **91**:1855-1862 (2011).

5 Meléndez-Martínez AJ, Vicario IM and Heredia FJ, Review: analysis of carotenoids in orange juice. *J Food Compos Anal* **20**:638-649 (2007).

6 Johns NP, Johns J, Porasuphatana S, Plaimee P and Sae-Teaw M, Dietary intake of melatonin from tropical fruit altered urinary excretion of 6-sulfatoxymelatonin in healthy volunteers. *J Agric Food Chem* **61**:913-919 (2013).

7 Landete JM, Updated knowledge about polyphenols: functions, bioavailability, metabolism, and health. *Crit Rev Food Sci Nutr* **52**:936-948 (2012).

8 Jomova K and Valko M, Health protective effects of carotenoids and their interactions with other biological antioxidants. *Eur J Med Chem* **70**:102-110 (2013).

9 Galano A, Tan DX and Reiter RJ, On the free radical scavenging activities of melatonin′s metabolites, AFMK and AMK. *J Pineal Res* **54**:245-257 (2013).

10 Franke AA, Cooney RV, Henning SM and Custer LJ, Bioavailability and antioxidant effects of orange juice components in humans. *J Agric Food Chem* **53**:5170-5178 (2005).

11 Franke SI, Guecheva TN, Henriques JA and Prá D, Orange juice and cancer chemoprevention. *Nutr Cancer* **65**:943-953 (2013).

12 Foroudi S, Potter AS, Stamatikos A, Patil BS and Deyhim F, Drinking orange juice increases total antioxidant status and decreases lipid peroxidation in adults. *J Med Food* **17**:612-617 (2014).

13 Bai J, Manthey JA, Ford BL, Luzio G, Cameron RG, Narciso J *et al.*, Effect of extraction, pasteurization and cold storage on flavonoids and other secondary metabolites in fresh orange juice. *J Sci Food Agric* **93**:2771-2781 (2013).

14 Agcam E, Akyildiz A and Akdemir Evrendilek G, Comparison of phenolic compounds or orange juice processed by pulsed electric fields (PEF) and conventional thermal pasteurization. *Food Chem* **143**:354-361 (2014).

15 Mena P, Ascacio-Valdés JA, Gironés-Vilaplana A, Del Rio D, Moreno DA and García-Viguera C, Assessment of pomegranate wine lees as a valuable source for the recovery of (poly)phenolic compounds. *Food Chem* **145**:327-334 (2014).

16 Lim JW, Hwang HJ and Shin CS, Polyphenol compounds and anti-inflammatory activities of Korean black raspberry (Rubus coreanus Miquel) wines produced from juice supplemented with pulp and seed. *J Agric Food Chem* **60**:5121-5127 (2012).

17 Escudero-López B, Cerrillo I, Herrero-Martín G, Hornero-Méndez D, Gil-Izquierdo A, Medina S *et al.*, Fermented orange juice: source of higher carotenoid and flavanone contents. *J Agric Food Chem* **61**:8773-8782 (2013).

18 Cerrillo I, Escudero-López B, Hornero-Méndez D, Martín F and Fernández-Pachón MS, Effect of alcoholic fermentation on the carotenoid composition and provitamin A content of orange juice. *J Agric Food Chem* **62**:842-849 (2014).

19 Fernández-Pachón MS, Medina S, Herrero-Martin G, Cerrillo I, Berna G, Escudero-López B *et al.*, Alcoholic fermentation induces melatonin synthesis in orange juice. *J Pineal Res* **56**:31–38 (2014).

20 Escudero-López B, Berná G, Ortega A, Herrero-Martín G, Cerrillo I, Martín F *et al.*, Consumption of orange fermented beverage reduces cardiovascular risk factors in healthy mice. *Food Chem Toxicol* **78**:78-85 (2015).

21 Escudero-López B, Fernández-Pachón MS, Herrero-Martín G, Ortega A, Cerrillo I, Martín F and Berná G, Orange beverage ameliorates high-fat-diet-induced metabolic disorder in mice. *J Funct Foods* **24**:254-263 (2016).

22 Organisation Internationale de la Vigne et du Vin (OIV), *Recueil des méthodes internationales d´analyse des vins et des moûts*, ed. By OIV, France (2017).

23 Collado-Gonzalez J, Cruz ZN, Medina S, Mellisho CD, Rodriguez P, Galind A *et al.*, Effects of water deficit during maturation on amino acids and jujube fruit eating quality. *Maced J Chem Chem Eng* **33**:105–119 (2014).

24 Escudero-López B, Calani L, Fernández-Pachón MS, Ortega A, Brighenti F, Crozier A *et al.*, Absorption, metabolism, and excretion of fermented orange juice (poly)phenols in rats. *BioFactors* **40**:327-335 (2014).

25 Ou B, Hampsch-Woodill M and Prior RL, Development and validation of an improved oxygen radical absorbance capacity assay using fluorescein as the fluorescent probe. *J Agric Food Chem* **49**:4619–4626 (2001).

26 Delgado-Andrade C, Rufian-Henares JA and Morales FJ, Assessing the antioxidant activity of melanoidins from coffee brews by different antioxidant methods. *J Agric Food Chem* **53**:7832–7836 (2005).

27 Cohen G and Somerson NL, Catalase-aminotriazole method for measuring secretion of hydrogen peroxide by microorganisms. *J Bacteriol* **98**:543–546 (1969).

28 McCord JM and Fridovich I, Superoxide dismutase. An enzymic function for erythrocuprein. *J Biol Chem* **244**:6049–6055 (1969).

29 Carmagnol F, Sinet PM and Jerome H, Selenium-dependent and non-seleniumdependent glutathione peroxidases in human tissue extracts. *Biochim Biophys Acta* **759**:49–57 (1983).

30 Cribb AE, Leeder JS and Spielberg SP, Use of microplate reader in an assay of glutathione reductase using 5-5′-dithiobis (2-nitrobentoic acid). *Anal Biochem* **183**:195–196 (1989).

31 Bradford M, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* **72**:248–254 (1976).

32 Buege JA and Aust SD, Microsomal lipid peroxidation. *Meth Enzymol* **52**:302–310 (1978).

33 Cesar TB, Aptekmann NP, Araujo MP, Vinagre CC and Maranhão RC, Orange juice decreases low-density lipoprotein cholesterol in hypercholesterolemic subjects and improves lipid transfer to high-density lipoprotein in normal and hypercholesterolemic subjects. *Nutr Res* **30**:689-694 (2010).

34 Morand C, Dubray C, Milenkovic D, Lioger D, Martin JF, Scalbert A *et al.*, Hesperidin contributes to the vascular protective effects of orange juice: a randomized crossover study in healthy volunteers. *Am J Clin Nutr* **93**:73-80 (2011).

35 Silveira JQ, Dourado GKZS and Cesar TB, Red-fleshed sweet orange juice improves the risk factors for metabolic syndrome. *Int J Food Sci Nutr* **66**:830-836 (2015).

36 Gorinstein S, Leontowicz H, Leontowicz M, Krzeminski R, Gralak M, Jastrzebski Z *et al.*, Effect of hesperidin and naringin on the plasma lipid profile and plasma antioxidant activity in rats fed a cholesterol-containing diet. *J Sci Food Agric* **87**:1257-1262 (2007).

37 Alvarez-Parrilla E, De La Rosa LA, Legarreta P, Saenz L, Rodrigo-García J and González-Aguilar GA, Daily consumption of apple, pear and orange juice differently affects plasma lipids and antioxidant capacity of smoking and non-smoking adults. *Int J Food Sci Nutr* **61**:369-380 (2010).

38 Constans J, Bennetau-Pelissero C, Martin JF, Rock E, Mazur A, Bedel A *et al.*, Marked antioxidant effect of orange juice intake and its phytomicronutrients in a preliminary randomized cross-over trial on mild hypercholesterolemic men. *Clin Nutr* **34**:1093-100 (2015).

39 Bub A, Watzl B, Blockhaus M, Briviba K, Liegibel U, Müller H *et al.*, Fruit juice consumption modulates antioxidative status, immune status and DNA damage. *J Nutr Biochem* **14**:90-98 (2003).

40 Apak R, Özyürek M, Güçlü K and Çapanoğlu E, Antioxidant activity/capacity measurement. 1. Classification, physicochemical principles, mechanisms, and electron transfer (ET)-based assays. *J Agric Food Chem* **64**:997-1027 (2016).

41 Wang J, Yang Z, Lin L, Zhao Z, Liu Z and Liu X, Protective effect of naringenin against lead-induced oxidative stress in rats. *Biol Trace Elem Res* **146**:354–359 (2012).

42 Cano Barquilla P, Pagano ES, Jiménez-Ortega V, Fernández-Mateos P, Esquifino AI and Cardinali DP, Melatonin normalizes clinical and biochemical parameters of mild inflammation in diet-induced metabolic syndrome in rats. *J Pineal Res* **57**:280–290 (2014).

43 Stinefelt B, Leonard SS, Blemings KP, Shi X and Klandorf H, Free radical scavenging, DNA protection, and inhibition of lipid peroxidation mediated by uric acid. *Ann Clin Lab Sci* **35**:37-45 (2005).

44 Yu TY, Jee JH, Bae JC, Jin SM, Baek JH, Lee MK *et al.*, Serum uric acid: a strong and independent predictor of metabolic syndrome after adjusting for body composition. *Metab Clin Exp* **65**:432–440 (2016).

45 Guo C, Wei J, Yang J, Xu J, Pang W and Jiang Y, Pomenagrate juice is potentially better than apple juice in improving antioxidant function in elderly subjects. *Nutr Res* **28**:72-77 (2008).

46 Potter AS, Foroudi S, Stamatikos A, Patil BS and Deyhim F, Drinking carrot juice increases total antioxidant status and decreases lipid peroxidation in adults. *Nutr J* **10**:96 (2011).

47 Samaras A, Tsarouhas K, Paschalidis E, Giamouzis G, Triposkiadis F, Tsitsimpikou C *et al.*, Effect of a special carbohydrate-protein bar and tomato juice supplementation on oxidative stress markers and vascular endothelial dymanics in ultra-marathon runners. *Food Chem Toxicol* **69**:231-236 (2014).

48 Rangel-Huerta OD, Aguilera CM, Martin MV, Soto MJ, Rico MC, Vallejo F *et al.*, Normal or high polyphenol concentration in orange juice affects antioxidant activity, blood pressure, and body weight in obese or overweight adults. *J Nutr* **145**:1808-1816 (2015).

49 Wilmsen PK, Spada DS and Salvador M, Antioxidant activity of the flavonoid hesperidin in chemical and biological systems. *J Agric Food Chem* **53**:4757–4761 (2005).

50 Ghanim H, Sia CL, Upadhyay M, Korzeniewski K, Viswanathan P, Abuaysheh S *et al.*, Orange juice neutralizes the proinflammatory effect of a high-fat; highcarbohydrate meal and prevents endotoxin increase and toll-like receptor expression. *Am J Clin Nutr* **91**:940-949 (2010).

51 Niv E, Shapira Y, Akiva I, Rokhkind E, Naor E, Arbiv M *et al.*, Effect of levan supplement in orange juice on weight, gastrointestinal symptoms and metabolic profile of healthy subjects: results of an 8-week clinical trial. *Nutrients* **4**:638-647 (2012).

52 Kurowska EM, Spence JD, Jordan J, Wetmore S, Freeman DJ, Piché LA *et al.*, HDL-cholesterol-raising effect of orange juice in subjects with hypercholesterolemia. *Am J Clin Nutr* **72**:1095–1100 (2000).

53 Simpson EJ, Mendis B and Macdonald IA, Orange juice consumption and its effect on blood lipid profile and indices of the metabolic syndrome: a randomized, controlled trial in an at-risk population. *Food Funct* **7**:1884-1891 (2016).

54 Wang DD, Sievenpiper JL, de Souza RJ, Cozma AI, Chiavaroli L, Ha V *et al.*, Effect of fructose on postprandial triglycerides: A systematic review and meta-analysis of controlled feeding trials. *Atherosclerosis* **232**:125-133 (2014).

55 Johnston CS, Dancho CL and Strong GM, Orange juice ingestion and supplemental vitamin C are equally effective at reducing plasma lipid peroxidation in healthy adult women. *J Am Coll Nutr* **22**:519-523 (2003).

56 Dourado GKZS and Cesar TB, Investigation of cytokines, oxidative stress, metabolic, and inflammatory biomarkers after orange juice consumption by normal and overweight subjects. *Food Nutr Res* **59**:28147 (2015).

57 Tirkey N, Pilkhwal S, Kuhad A and Chopra K, Hesperidin, a citrus bioflavonoid, decreases the oxidative stress produced by carbon tetrachloride in rat liver and kidney. *Pharmacol* **5**:2 (2005).

58 Tapiero H, Townsend DM and Tew KD, The role of carotenoids in the prevention of human pathologies. *Biomed Pharmacother* **58**:100–110 (2004).

59 Rao MN, Marmillot P, Gong M, Palmer DA, Seeff LB, Strader DB *et al.*, Light, but not heavy alcohol drinking, stimulates paraoxonase by upregulating liver mRNA in rats and humans. *Metabolism* **52**:1287-1294 (2003).

60 Werner N, Wassmann S, Ahlers P, Kosiol S and Nickenjg G, Circulating CD31+/annexin V+ apoptotic microparticles correlate with coronary endothelial function in patients with coronary artery disease. *Arterioscler Thromb Vasc Biol* **26**:112–116 (2006).

61 Amabile N, Cheng S, Renard JM, Larson MG, Ghorbani A, McCabe E *et al.*, Association of circulating endothelial microparticles with cardiometabolic risk factors in the Framingham Heart Study. *Eur Heart J* **35**:2972–2979 (2014).

62 Song H, Nan Y and Cheng XW, Circulating cf-DNA: a promising, noninvasive tool for assessment of early cardio-metabolic risk. *Atherosclerosis* **233**:307-309 (2014).

63 McFarlin BK, Venable AS, Henning AL, Prado EA, Best Sampson JN, Vingren JL *et al.*, Natural cocoa consumption: potential to reduce atherogenic factors? *J Nutr Biochem* **26**:626–632 (2015).

64 Marin C, Ramirez R, Delgado-Lista J, Yubero-Serrano EM, Perez-Martinez P, Carracedo J *et al.*, Mediterranean diet reduces endothelial damage and improves the regenerative capacity of endothelium. *Am J Clin Nutr* **93**:267–74 (2011).

65 Horn P, Amabile N, Angeli FS, Sansone R, Stegemann B, Kelm M *et al.*, Dietary flavanol intervention lowers the levels of endothelial microparticles in coronary artery disease patients. *Br J Nutr* **111**:1245–1252 (2014).

66 Sánchez-Moreno C, Cano MP, de Ancos B, Plaza L, Olmedilla B, Granado F *et al.*, High-pressurized orange juice consumption affects plasma vitamin C, antioxidative status and inflammatory markers in healthy humans. *J Nutr* **133**:2204-2209 (2003).

67 Asgary S, Keshvari M, Afshani MR, Amiri M, Laher I and Javanmard SH, Effect of fresh orange juice intake on physiological characteristics in healthy volunteers. *ISRN Nutr* http://dx.doi.org/10.1155/2014/405867 [4 March 2014].

68 Buscemi S, Rosafio G, Arcoleo G, Mattina A, Canino B, Montana M *et al.*, Effects of red orange juice intake on endothelial function and inflammatory markers in adult subjects with increased cardiovascular risk. *Am J Clin Nutr* **95**:1089-1095 (2012).

69 Ribeiro C, Dourado G and Cesar T, Orange juice allied to a reduced-calorie diet results in weight loss and ameliorates obesity-related biomarkers: A randomized controlled trial. *Nutrition* **38**:13-19 (2017).

70 de Winther MP, Kanters E, Kraal G and Hofker MH, Nuclear factor κB signaling in atherogenesis. *Arterioscler Thromb Vasc Biol* **25**:904–914 (2005).

71 Mann GE, Rowlands DJ, Li FYL, de Winter P and Siow RCM, Activation of endothelial nitric oxide synthase by dietary isoflavones: role of NO in Nrf2-mediated antioxidant gene expression. *Cardiovasc Res* **75**:261-274 (2007).

72 Sierksma A, van der Gaag MS, Kluft C and Hendriks HFJ, Moderate alcohol consumption reduces plasma C-reactive protein and fibrinogen levels; a randomized, diet-controlled intervention study. *Eu J Clin Nutr* **56**:1130-1136 (2002).

73 Albert MA, Glynn RJ and Ridker PM, Alcohol consumption and plasma concentration of C-reactive protein. *Circulation* **107**:443-447 (2003).

74 Imhof A, Blagieva R, Marx N and Koenig W, Drinking modulates monocyte migration in healthy subjects: a randomized intervention study of water, ethanol, red wine and beer with and without alcohol. *Diabetes Vasc Dis Res* **5**:48-53 (2008).

FIGURE LEGEND

Figure 1. Diagram of randomized, long-term, and controlled intervention study in healthy humans.

Thirty volunteers were enrolled in the study. Experimental (E) (n=15) and control (C) (n=15) groups were studied in parallel. The E group was instructed to drink 500 mL day^{-1} of orange beverage (OB) during a two weeks (intervention phase) followed by a three-week period with no OB intake (washout phase). Blood samples were drawn after overnight fasting at the beginning (E-T0) and end (E-T1) of the intervention phase and at the end of the washout phase (E-T2). The C group (n=15) did not consume OB for two weeks, and blood samples were drawn after overnight fasting at the beginning (C-T0) and end (C-T1) of this period.

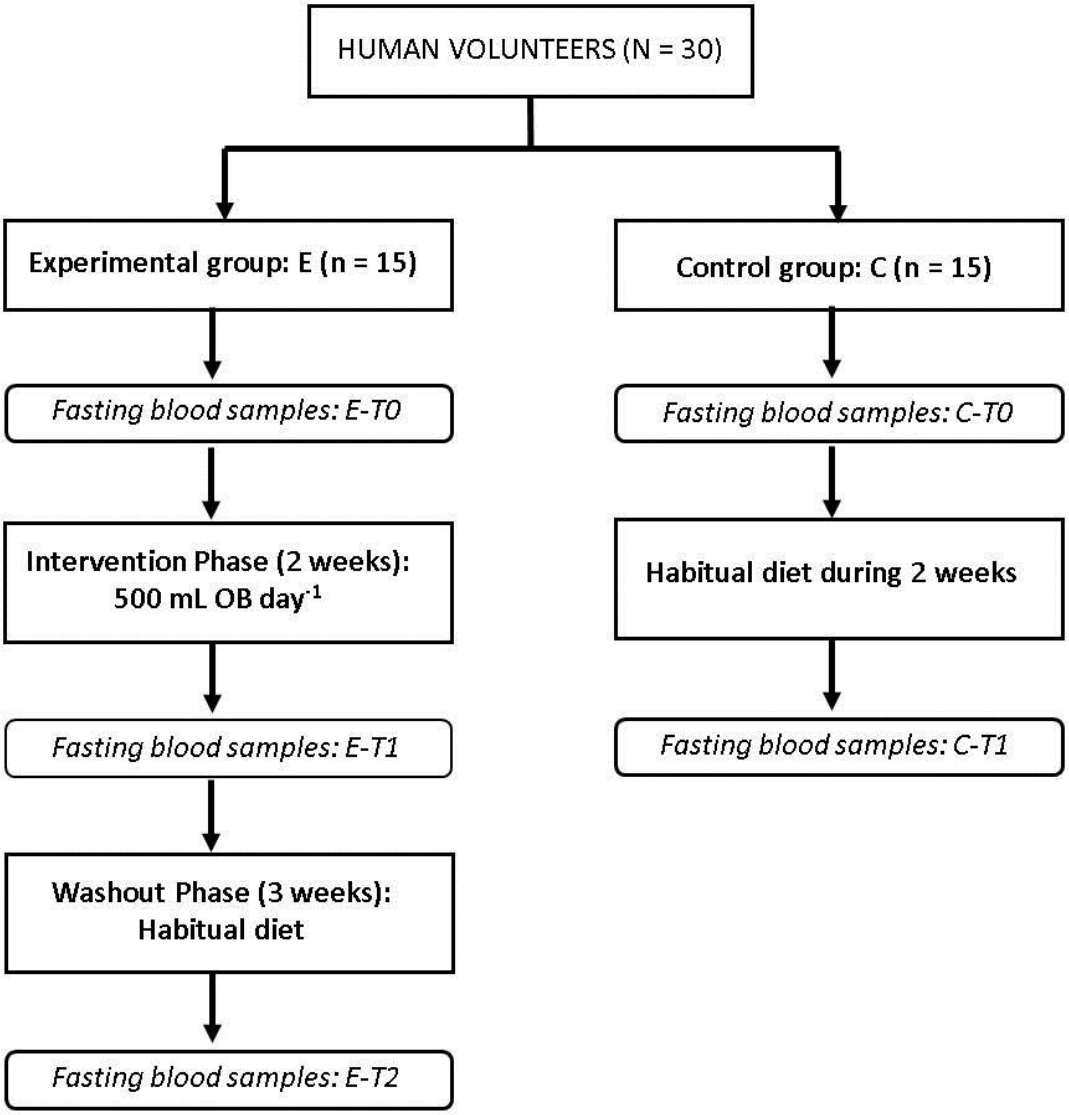

Table 1. Composition of the orange beverage (quality parameters, nutrients, bioactive compounds, and antioxidant activity)

Composition	Orange beverage[a]
pH	3.48 ± 0.03
TA (g citric acid L^{-1})	8.60 ± 0.01
TSS (ºBrix)	9.01 ± 0.14
% Pulp	5.65 ± 0.22
Alcohol (% v/v)	0.90 ± 0.15
Ascorbic acid (mg L^{-1})	197 ± 6.80
Total sugars (g L^{-1})	47.9 ± 4.10
Reducing sugars (g L^{-1})	20.3 ± 2.40
Non-reducing sugars (g L^{-1})	27.1 ± 2.61
Total protein (g L^{-1})	5.10 ± 0.04
Total fat (g L^{-1})	1.12 ± 0.10
Energy (kcal L^{-1})	320 ± 0.25
Total Phenol Index (mg L^{-1})	593 ± 13.9
Phenolic compounds (nmol L^{-1})	
Ferulic acid-4'-O-glucoside	25.7 ± 0.10
Hesperetin-7-O-rutinoside (hesperidin)	445 ± 51.9
Hesperitin-7-O-rutinoside-3'-O-glucoside	11.1 ± 3.00
Naringenin-7-O-rutinoside (narirutin)	133 ± 11.7
Naringenin-7-O-rutinoside-4'-O-glucoside	7.70 ± 0.90
Naringenin-C-diglycoside	26.5 ± 1.90
Eriodictyol-7-O-rutinoside (eriocitrin)	9.70 ± 0.70
Isosakuranetin-7-O-rutinoside	32.4 ± 25.5
Isosakuranetin-O-rutinoside-O-hexoside	1.80 ± 0.10
Apigenin-8-C-glucoside (vitexin)	12.8 ± 0.30
Apigenin-6,8-di-C-glucoside (vicenin-2)	130 ± 8.30
Total individual phenolics	836 ± 53.1
Carotenoid pigments (□mol L^{-1})	
Latochrome	0.38 ± 0.01
Karpoxanthin	1.60 ± 0.04
Neochrome	1.41 ± 0.03
Auroxantin	4.33 ± 0.14
Mutatoxantin	2.77 ± 0.09
(all-E)-Zeaxantin	1.81 ± 0.09
(all-E)-Lutein	0.84 ± 0.04
(9Z)-Lutein	0.08 ± 0.01
(13Z)-Lutein	0.36 ± 0.01
□□□□□□*-Cryptoxanthin*	2.43 ± 0.11
□□□□□□*-Carotene*	1.41 ± 0.07
(all-E)-□-Carotene	0.11 ± 0.00
(all-E)-□-Carotene	0.28 ± 0.01
Total individual carotenoids	17.8 ± 0.61
Provitamin A (RAEs L^{-1})	71.0 ± 3.26
ORAC (μmol L^{-1})	2306 ± 331

FRAP (mmol L^{-1})	5.43 ± 0.19

[a]Values are expressed as Mean (n=3) and SD.

TA, titratable acidity; TSS, total soluble solids; RAEs, Retinol Activity Equivalents; ORAC, oxygen radical absorbance capacity; FRAP, ferric reducing antioxidant power.

Table 2. Anthropometric and biochemical measurements of subjects at baseline, after consumption of orange beverage (OB) for 2 weeks, and after 3 weeks of washout period

Measurements[a]	Experimental Group			Control Group	
	Baseline (E-T0)	Intervention Phase (E-T1)	Washout Phase (E-T2)	Baseline (C-T0)	Final (C-T1)
Anthropometric					
Body weight (kg)	66.0 ± 2.37 a	66.1 ± 2.42 a	65.9 ± 2.39 a	69.3 ± 4.06	69.1 ± 4.01
BMI (kg m^{-2})	22.7 ± 0.46 a	22.7 ± 0.47 a	22.6 ± 0.45 a	24.5 ± 1.24	24.5 ± 1.23
WC (cm)	75.7 ± 1.61 a	75.6 ± 1.63 a	75.2 ± 1.71 a	76.8 ± 3.05	77.7 ± 2.87
Glucose metabolism					
Glucose (mg dL^{-1})	86.3 ± 1.72 a	84.2 ± 1.69 a	85.8 ± 1.78 a	88.7 ± 2.15	84.7 ± 1.80
Insulin (µU mL^{-1})	6.83 ± 0.91 a	7.14 ± 0.70 a	7.99 ± 1.01 a	8.19 ± 0.92	7.99 ± 1.03
Hepatic/kidney function					
GOT (U L^{-1})	17.9 ± 0.96 a	21.3 ± 2.65 a	19.9 ± 2.51 a	17.1 ± 0.82	18.3 ± 1.33
GPT(U L^{-1})	15.8 ± 1.45 a	19.9 ± 3.56 a	18.6 ± 3.60 a	17.1 ± 1.83	19.9 ± 2.78
Creatinine (mg dL^{-1})	0.81 ± 0.04 a	0.78 ± 0.04 a	0.81 ± 0.05 a	0.82 ± 0.04	0.78 ± 0.04
Hematocrit					
Hemoglobin (g L^{-1})	134.8 ± 2.94 a	136.1 ± 3.05 a	133.1 ± 3.46 a	136.1 ± 4.12	134.7 ± 4.41
Erythrocytes (x10^{12} L^{-1})	4.60 ± 0.10 a	4.67 ± 0.11 a	4.55 ± 0.11 a	4.83 ± 0.11	4.79 ±0.11

[a]Values are means ± SEM. Experimental group (n = 15) includes subjects consuming OB during 2-wk intervention period followed by 3-wk washout phase. E-T0: At baseline. E-T1: At end of intervention phase. E-T2: At end of washout phase. Control group (n=15) includes subjects not consuming OB during a 2-wk period. C-T0: At baseline. C-T1: At end of 2-wk period. Values with different letters indicate significant difference (P<0.05) among E-T0, E-T1, and E-T2. *: Significant difference (P<0.05) between C-T0 and C-T1.

BMI, body mass index; WC, waist circumference; GOT, glutamate oxaloacetate transaminase; GPT, glutamate pyruvate transaminase.

Table 3. Antioxidant status of subjects at baseline, after consumption of orange beverage (OB) for 2 weeks, and after 3 weeks of washout period

Antioxidant status[a]	Experimental Group			Control Group	
	Baseline (E-T0)	Intervention Phase (E-T1)	Washout Phase (E-T2)	Baseline (C-T0)	Final (C-T1)
Plasma antioxidant capacity					
ORAC (mmol L^{-1})	11.1 ± 0.79 a	19.8 ± 2.08 b	11.4 ± 1.18 a	14.9 ± 1.25	17.3 ± 1.45
FRAP (mmol L^{-1})	1.42 ± 0.07 a	1.39 ± 0.04 a	1.49 ± 0.03 a	1.30 ± 0.04	1.30 ± 0.04
Endogenous antioxidant compounds					
Albumin(mg dL^{-1})	4.35 ± 0.10 a	4.37 ± 0.06 a	4.33 ± 0.80 a	4.27 ± 0.08	4.06 ± 0.07
Bilirubin(mg dL^{-1})	0.74 ± 0.10 a	0.76 ± 0.15 a	0.84 ± 0.15 a	0.45 ± 0.04	0.47 ± 0.05
Uric acid (g dL^{-1})	4.53 ± 0.33 a	4.12 ± 0.31 b	4.14 ± 0.32 b	3.81 ± 0.28	3.79 ± 0.34
Glutathione (µmol L^{-1})	112.8 ± 6.42 a	101.2 ± 7.64 a	78.8 ± 6.31 a	122.7 ± 7.08	100.4 ± 7.04*
Antioxidant enzymes activities					
CAT (U mg $prot^{-1}$)	17.2 ± 0.67 a	13.2 ± 0.77 b	11.3 ± 0.52 b	18.1 ± 0.86	16.7 ± 1.30
SOD (U mg $prot^{-1}$)	27.5 ± 2.62 a	27.0 ± 2.72 a	22.7 ± 1.95 a	28.8 ± 3.17	30.2 ± 3.05
GPx (mU mg $prot^{-1}$)	11.7 ± 1.59 a	10.0 ± 1.44 a	11.2 ± 1.43 a	13.7 ± 1.88	11.7 ± 2.28
GR (mU mg $prot^{-1}$)	1.70 ± 0.06 a	1.57 ± 0.05 a	1.63 ± 0.09 a	1.62 ± 0.09	1.61 ± 0.07

[a]Values are means ± SEM. Experimental group (n = 15) includes subjects consuming OB during 2-wk intervention period followed by 3-wk washout phase. E-T0: At baseline. E-T1: At end of intervention phase. E-T2: At end of washout phase. Control group (n=15) includes subjects

not consuming OB during a 2-wk period. C-T0: At baseline. C-T1: At end of 2-wk period. Values with different letters indicate significant difference ($P<0.05$) among E-T0, E-T1, and E-T2. *: Significant difference ($P<0.05$) between C-T0 and C-T1.

ORAC, Oxygen Radical Absorbance Capacity; FRAP, Ferric Reducing Antioxidant Power; CAT, catalase; SOD, superoxide dismutase; GPx, glutathione peroxidase; GR, glutathione reductase; prot, proteins.

Table 4. Lipid metabolism and lipid peroxidation of subjects at baseline, after consumption of orange beverage (OB) for 2 weeks, and after 3 weeks of washout period

Lipid markers[a]	Experimental Group			Control Group	
	Baseline (E-T0)	Intervention Phase (E-T1)	Washout Phase (E-T2)	Baseline (C-T0)	Final (C-T1)
Lipid metabolism					
TC (mg dL^{-1})	178.8 ± 10.1 a	179.1 ± 9.04 a	180.7 ± 9.25 a	191.3 ± 10.2	188.9 ± 11.1
LDL (mg dL^{-1})	92.8 ± 9.32 a	97.2 ± 7.36 a	100.5 ± 7.16 a	110.1 ± 11.0	105.1 ± 10.6
HDL (mg dL^{-1})	59.9 ± 4.94 a	64.7 ± 4.08 a	67.7 ± 4.32 a	61.7 ± 6.06	60.5 ± 5.95
TG (mg dL^{-1})	65.8 ± 5.56 a	77.6 ± 8.31 a	62.7 ± 5.97 a	85.0 ± 9.10	90.7 ± 10.4
Apo A-I (mg dL^{-1})	116.3 ± 18.2 a	94.9 ± 9.92 a	109.1 ± 13.1 a	91.4 ± 28.8	105.4 ± 19.5
Apo B (mg dL^{-1})	237.4 ± 50.2 a	237.0 ± 44.6 a	235.0 ± 41.7 a	122.8 ± 7.32	133.9 ± 9.06
Lipid peroxidation					
TBARS (nmol mL^{-1})	7.31 ± 0.30 a	5.10 ± 0.52 b	3.77 ± 0.16 c	7.07 ± 0.17	4.73 ± 0.43*
oxLDL (μg mL^{-1})	244.1 ± 34.4 a	218.4 ± 21.4 ab	185.8 ± 14.3 b	232.7 ± 25.2	252.9 ± 25.6

[a]Values are means ± SEM. Experimental group (n = 15) includes subjects consuming OB during 2-wk intervention period followed by 3-wk washout phase. E-T0: At baseline. E-T1: At end of intervention phase. E-T2: At end of washout phase. Control group (n=15) includes subjects not consuming OB during a 2-wk period. C-T0: At baseline. C-T1: At end of 2-wk period. Values with different letters indicate significant difference (P<0.05) among E-T0, E-T1, and E-T2. *: Significant difference (P<0.05) between C-T0 and C-T1.

TC, total cholesterol; TG, triglycerides; Apo, apolipoprotein; TBARS, thiobarbituric acid reactive species; oxLDL, oxidized low-density lipoproteins cholesterol.

Table 5. Endothelial function and inflammation biomarkers of subjects at baseline, after consumption of orange beverage (OB) for 2 weeks, and after 3 weeks of washout period

	Experimental Group			Control Group	
Endothelial function and inflammation markers[a]	Baseline (E-T0)	Intervention Phase (E-T1)	Washout Phase (E-T2)	Baseline (C-T0)	Final (C-T1)
sVCAM-1(ng mL^{-1})	638.8 ± 66.0 a	633.2 ± 66.9 a	574.0 ± 51.3 a	492.6 ± 22.8	505.4 ± 23.6
sICAM-1 (ng mL^{-1})	133.1 ± 7.15 a	122.4 ± 9.55 a	134.7 ± 11.9 a	157.8 ± 13.0	184.7 ± 16.9
cf-DNA (GE mL^{-1})	221.5 ± 31.1 a	229.3 ± 41.3 a	296.2 ± 27.9 a	266.8 ± 25.1	234.1 ± 35.2
EMPs (U □L^{-1})	165.4 ± 30.8 a	193.5 ± 30.4 a	182.8 ± 31.5 a	169.6 ± 27.4	177.7 ± 34.5
CRP (mg L^{-1})	1.93 ± 0.78 a	1.89 ± 0.80 b	2.47 ± 1.14 ab	1.44 ± 0.43	3.84 ± 2.02

[a]Values are means ± SEM. Experimental group (n = 15) includes subjects consuming OB during 2-wk intervention period followed by 3-wk washout phase. E-T0: At baseline. E-T1: At end of intervention phase. E-T2: At end of washout phase. Control group (n=15) includes subjects not consuming OB during a 2-wk period. C-T0: At baseline. C-T1: At end of 2-wk period. Values with different letters indicate significant difference (P<0.05) among E-T0, E-T1, and E-T2. *: Significant difference (P<0.05) between C-T0 and C-T1.

sVCAM-1, soluble vascular cell adhesion molecule 1; sICAM-1, soluble intercellular adhesion molecule 1; cf-DNA, circulating cell-free DNA; GE, genome equivalents; EMPs, endothelial cell-derives microparticles; CRP, C-reactive protein.