Food and Chemical Toxicology 78 (2015) 78–85

Contents lists available at ScienceDirect

Food and Chemical Toxicology

journal homepage: www.elsevier.com/locate/foodchemtox

Consumption of orange fermented beverage reduces cardiovascular risk factors in healthy mice

Blanca Escudero-López [a], Genoveva Berná [a,b], Ángeles Ortega [a,b], Griselda Herrero-Martín [a], Isabel Cerrillo [a,c], Franz Martín [a,b], María-Soledad Fernández-Pachón [a,c,*]

[a] Area of Nutrition and Food Sciences, Department of Molecular Biology and Biochemistry Engineering, Pablo de Olavide University, Carretera de Utrera Km 1, E-41013 Seville, Spain
[b] Spanish Biomedical Research Centre in Diabetes and Associated Metabolic Disorders (CIBERDEM), Spain
[c] Associated Researcher at Autónoma de Chile University, Av. Pedro de Valdivia 641, Santiago de Chile, Chile

ARTICLE INFO

Article history:
Received 15 November 2014
Accepted 2 February 2015
Available online 7 February 2015

Keywords:
Fermented orange
Antioxidant status
Lipids
Inflammation
Cardiovascular risk

ABSTRACT

The consumption of fruits prevents the risk of cardiovascular diseases. Alcoholic fermentation has been carried out in fruits resulting in products which provide high concentration of bioactive compounds and variable alcohol content. The aim of this study was to assess the potential beneficial effect of an orange beverage obtained by alcoholic fermentation and pasteurization of orange juice on cardiovascular risk biomarkers. For this purpose, four mice groups (n = 8) ingested orange beverage (equivalent volume to 250 mL/day in human), orange juice, alcoholic solution (at the proportional amount of orange beverage) or water during 12 weeks. The equivalent amount to double serving of orange beverage (500 mL/day) was administered to mice in a subsequent intervention, and a control group was also evaluated. Orange beverage consumption increased levels of glutathione and uric acid, improved lipid profile, decreased oxidized LDL and maintained levels of IL-6 and C-reactive protein. Synergistic effects between the bioactive compounds and the alcohol content of orange beverage may occur. The intake of double serving also increased antioxidant enzyme activities, bilirubin content and plasma antioxidant capacity. These results suggest that orange beverage may produce greater protection against cardiovascular risk factors than orange juice in healthy mice.

1. Introduction

Epidemiologic and clinical studies have shown that the intake of fruits and vegetables reduces the risk of cardiovascular diseases. This beneficial effect has been attributed to the presence of bioactive compounds (Liu, 2013). A large number of physiological effects have been attributed to these compounds such as antioxidant and anti-inflammatory activity, lipid profile and blood glucose regulation, improvement of endothelial function, antithrombotic effect or vasodilator action (Tresserra-Rimbau et al., 2014). Orange juice is among the most consumed fruit juices worldwide and it is known to be a rich source of bioactive compounds such as flavonoid, carotenoid, ascorbic acid and melatonin (Sae-Teaw et al., 2013; Tounsi et al., 2011). Numerous studies have shown that orange juice consumption reduces the prevalence of cardiovascular risk factors such as oxidative or inflammatory stress, dyslipidemia, hyperglycemia, endothelial dysfunction, hypertension or obesity (Aptekmann and Cesar, 2013; Buscemi et al., 2012; O'Neil et al., 2012). In recent years, fermentation processes have been carried out in fruit juices (Pérez-Gregorio et al., 2011), resulting in products which provide a higher concentration of bioactive compounds than the respective substrate. Our group has previously described the influence of controlled alcoholic fermentation on the orange juice composition showing that flavanones, carotenoids and melatonin content significantly increased during the process, and the antioxidant capacity was enhanced (Escudero-López et al., 2013; Fernández-Pachón et al., 2014). Thermal treatment in this fermented orange juice has been carried out and the final composition of the beverage will be discussed in the present study. On the other hand, this fermented product of orange presents a moderate alcohol content. Moderate alcohol consumption reduces the risk of cardiovascular diseases (Arranz et al., 2012). Potentially, bioactive compounds and

Abbreviations: PAC, plasma antioxidant capacity; ORAC, oxygen radical absorbance capacity; FRAP, ferric reducing antioxidant power; TEAC, trolox equivalent antioxidant capacity; GSH, reduced glutathione; GSSG, oxidized glutathione; CAT, catalase; SOD, superoxide dismutase; GPx, glutathione peroxidase; GR, glutathione reductase; TC, total cholesterol; LDL-ox, oxidized low-density lipoprotein; TBARS, thiobarbituric acid reactive species; MDA, malondialdehyde; CRP, C-reactive protein.

* Corresponding author. Area of Nutrition and Food Sciences, Department of Molecular Biology and Biochemistry Engineering, Pablo de Olavide University, Carretera de Utrera Km 1, E-41013, Seville, Spain. Tel.: +34 954977613; fax: +34 954349813.
E-mail address: msferpac@upo.es (M.-S. Fernández-Pachón).

http://dx.doi.org/10.1016/j.fct.2015.02.006
0278-6915/

moderate alcohol content of this novel fermented orange juice would exert synergistic benefits in the organism, similar to beer or wine (Chiva-Blanch et al., 2012), and increase the health effects already tested with orange juice.

Thus, the aim of the present study was to assess the potential beneficial effect of a novel fermented orange beverage on cardiovascular risk markers in mice evaluating the possible synergistic effects of its bioactive compounds and alcohol content, and the influence of the volume ingested. Therefore, the antioxidant status (plasma antioxidant capacity, endogenous antioxidants content and antioxidant enzyme activities), the lipid profile, the lipid peroxidation (thiobarbituric acid reactive species (TBARS) level and oxidized low-density lipoprotein (LDL-ox) content) and the inflammatory status (IL-6 and C-reactive protein (CRP) levels) have been evaluated after repeated consumption of the fermented orange beverage in healthy mice.

2. Materials and methods

2.1. Chemicals and reagents

Chemicals were purchased from Sigma-Aldrich Quimica (Alcobendas, Spain).

2.2. Orange beverage preparation

The company Grupo Hespérides Biotech S.L. carried out the controlled alcoholic fermentation of commercial orange juice made from *Citrus sinensis* L. var. *Navel late* (Huelva, Spain). The fermentation process was carried out in a 5 L pvc tank at 20 °C for 10 days in repose. The yeast strain *Saccharomycetaceae* var. *Pichia kluyveri* was selected from the natural microbiota present in the orange fruit and used for the inoculation of the fermentation because this yeast strain ferments only reducing sugars, resulting in a final product with low alcohol content and sweet taste. The thermal pasteurization was achieved at 85 °C for 30 s in a semi-tubular pasteurizer 25 L/h (Mipaser Prototype, Murcia, Spain). After treatment, the orange beverage was cooled to 10 °C in an ice-water-bath and stored at –20 °C until its consumption. Quality parameters, bioactive compounds content and antioxidant activity of orange juice and the fermented-pasteurized product (orange beverage) were analyzed as described by Escudero-López et al. (2013), Organisation Internationale de la Vigne et du Vin (OIV) (2014) and Collado-González et al. (2014).

2.3. Animals and experimental design

Eight week-old male OF1 mice were obtained from Charles River Laboratories (Barcelona, Spain) and all methods were performed in accordance with the Legislation for the protection of animals used for scientific purposes (EU Directive 2010/63/EU). All mice were housed and maintained under the same laboratory conditions of temperature (22 °C) and lighting (12 h light–dark cycle) and were given free access to standard nonpurified diet (Scientific Animal Food and Engineering, Spain) and tap water. The mice were acclimated to the laboratory conditions for 1 week before the experiments. The review boards of animal ethics at our University approved this study. We followed the requirements regarding the protection of animals used for experimental and other scientific purposes.

To assess the potential beneficial effects of orange beverage on cardiovascular risk and the possible synergistic effects of its bioactive compounds and alcohol content, a total of thirty-two mice were randomly divided into four groups (n = 8 per group, 1 mice/cage). Each group received different drinks solutions administered *ad libitum* during 12 weeks: (1) Control group (CTRL): water; (2) OJ group: orange juice diluted 1:10 in tap water (this dilution factor was calculated based on a proportional intake of 250 mL/day of orange juice – one serving – in humans which had shown beneficial effects (Johnston et al., 2003); (3) OB group: orange beverage diluted 1:10 in tap water; (4) AS group: aqueous alcohol solution diluted 1:1000 from a 96% ethanol stock (this dilution factor provides the equivalent amount of alcohol of a proportional intake of 250 mL of orange beverage/day in humans).

To investigate whether the effects obtained are dependent on the dose of orange beverage, the equivalent amount to double serving (equal to 500 mL/day in humans) was administered subsequently to 18 mice during 12 weeks (OB-2 group). A control group (n = 18) was also evaluated (CTRL-2).

Bottles were replaced every 2 days to avoid oxidization and precipitate formation, and the liquid volume consumption was also measured (difference between initial and final volumes) every 2 days in both interventions. Body weight of mice was recorded weekly.

2.4. Sample collection

Mice were fasted overnight prior to being sacrificed by cervical dislocation. Blood samples were collected by intracardiac puncture and plasma was separated by centrifugation at 3000 *g* (10 min, 4 °C). Plasma samples were stored at –80 °C for subsequent analysis. Liver was carefully dissected, weighed and immersed in liquid N_2 before storage at –80 °C.

2.5. Plasma antioxidant capacity (PAC)

2.5.1. Oxygen radical absorbance capacity (ORAC) assay

Plasma samples were diluted (1:2000) in phosphate buffer (75 mM, pH 7.4). ORAC assay was performed according to Ou et al. (2001).

2.5.2. Ferric reducing antioxidant power (FRAP) assay

Plasma samples were diluted (1:10) in distilled water. The ferric reducing ability was estimated according to Delgado-Andrade et al. (2005).

2.5.3. Trolox equivalent antioxidant capacity (TEAC) assay

Plasma samples were diluted (1:10) in water/methanol (1:1). TEAC assay was performed following the procedure described by Delgado-Andrade et al. (2005).

2.6. Endogenous antioxidants content

Albumin, bilirubin and uric acid contents were measured in plasma samples using the manufacture's protocols established by Roche Diagnostics. Contents of total glutathione, oxidized (GSSG) and reduced (GSH) were determined in liver samples using a commercial kit (Enzo Life Sciences, Plymouth Meeting, USA). Liver was homogenized using a Thomas-Teflon homogenizer in a solution of 5% metaphosphoric acid 1:20 (w/v), and kept on ice. Homogenates were centrifuged at 15700 *g* (10 min, 4 °C), and the supernatants were used for the analysis.

2.7. Antioxidant enzyme activities

Activities of catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (GPx) and glutathione reductase (GR) were evaluated in liver samples according to Cohen and Somerson (1969), McCord and Fridovich (1969), Carmagnol et al. (1983) and Cribb et al. (1989), respectively. Samples were homogenized in a 1:4 (w:v) buffered solution (100 mM Tris-HCl with 0.1 mM EDTA, 0.1% triton X-100) using a Miccra D-1 homogenizer (Miccra, Germany). The homogenates were centrifuged at 20800 *g* (30 min, 4 °C). The resulting supernatants were collected and stored at –80 °C until analysis. Total protein content was assessed by standard Bradford's procedure (Bradford, 1976).

2.8. Lipid profile

Commercial available kits were used to measure the lipid profile in plasma samples: total cholesterol (TC) (Invitrogen Life Technologies, Oregon, USA), HDL (Roche Diagnostics Systems Inc., New Jersey, USA) and TAG (Thermo Scientific, Middletown, USA). LDL was obtained using the Friedewald's formula (Friedewald et al., 1972): LDL = TC – HDL – (TAG/5).

2.9. Lipid peroxidation

The lipid peroxidation was evaluated by the thiobarbituric acid reactive species level and oxidized low-density lipoprotein content. TBARS was measured in liver homogenates according to Buege and Aust (1978), and the results were expressed as malondialdehyde (MDA) concentration. LDL-ox was measured in plasma samples using a commercial kit (Cusabio Biotech, Wuhan, China).

2.10. Inflammation status

Plasmatic levels of IL-6 and C-reactive protein were measured with ELISA kits purchased from Ray Biotech Inc. (Georgia, USA) and USCN Life Science Inc. (Wuhan, China), respectively.

All markers were recorded on a SynergyTM HT-multimode microplate reader (Biotek Instruments, Winooski, USA) except albumin, bilirubin and uric acid which were recorded on a COBAS Integra 400 Plus biochemistry analyzer (Roche Diagnostics, Indianapolis).

2.11. Statistical analysis

All analyses were in triplicate. The values are given as mean ± SEM. Differences between the CTRL, OB, OJ and AS groups were tested by one-way analysis of variance (ANOVA) followed by Tukey's test. Student's *t* test was applied to establish differences between CTRL-2 and OB-2 groups. A probability value of $p < 0.05$ was adopted as the criteria for significant differences. These analyses were carried out by SPSS 15.0 Software (SPSS Inc., Chicago, USA).

3. Results and discussion

The current study is the first intervention involving an orange juice derivative with low content of alcohol (0.87% v/v) obtained