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Regular Article

Both red and blond orange juice intake decreases the procoagulant activity of whole blood in healthy volunteers

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ABSTRACT

Aim: Numerous epidemiological studies suggest that exposure to flavonoid-rich fruits has beneficial influence on risk factors for cardiovascular disease. We investigated whether intake of orange juice (OJ) could affect whole blood (WB) procoagulant activity.
Methods: 17 healthy subjects (aged 31 ± 1.5 SEM 10 males) were randomized to receive, according to a cross-over design, either red or blond OJ, enriched or free of anthocyanins, respectively. After one week run-in period on a controlled diet, the subjects were randomly allocated to receive either type of OJ for 4 weeks, with a 4-week wash-out period. Venous blood was collected on citrate before and at the end of each treatment period. WB was incubated with or without an inflammatory stimulus (tumor necrosis factor-α or bacterial endotoxin LPS). Procoagulant activity was evaluated by a one-stage clotting assay. Tissue factor (TF) and TF pathway inhibitor (TFPI) were measured in plasma by ELISA.
Results: Intake of either type of OJ caused a prolongation of unstimulated and stimulated WB clotting times, without any difference between the two treatments. Intake of OJ did not modify TF levels. On the contrary, an increase in circulating TFPI antigen was detected following either treatment.
Conclusions: Orange juice intake by healthy volunteers decreases procoagulant activity, possibly through mechanisms independent of its anthocyanin content.

Introduction

There is growing evidence that dietary bioactive compounds play an important role in promoting health. In particular, flavonoids and related phenolics, a class of plant-specific bioactive compounds present in fruit, tea, red wine, cocoa and chocolate, have a protective effect on risk factors for cardiovascular disease (CVD) [1–6]. The consumption of citrus fruits has been associated with a lower risk of acute coronary events and ischemic stroke in a general population [7,8] and in post-menopausal women [4].

While citrus juice consumption reportedly decreases plasma concentrations of markers of inflammation and oxidative stress and corrects dyslipidemia [7–21], virtually no data are available on its possible effect on pathways leading to fibrin formation.

In normal hemostasis, a natural balance between the pro- and the anti-coagulant systems takes place [22,23]. By contrast, in pathological conditions the scene moves to concomitant upregulation of procoagulant molecules and impaired fibrinolytic function which, together, would favour an environment prone to thrombotic episodes. Tissue factor (TF) is considered to be the trigger of blood coagulation *in vivo* [24]. Widely distributed in cells of several organs and, particularly, in the adventitia of the blood vessel walls, upon endothelial perturbation, TF is released in blood, where it can form a proteolytically active complex with circulating factor VII/VIIa, leading to factor X activation and ultimately to fibrin formation and deposition, the leading cause of atherothrombosis. At a physiological level, the TF:VIIa proteolytic activity is inhibited by the tissue factor pathway inhibitor (TFPI) [25].

As the whole blood (WB) procoagulant activity, resulting from the balance between TF and TFPI, may be crucial in the development of bleeding or thrombosis, we evaluated the possibility to modulate these parameters with a prolonged intake of two types of orange juice (OJ), in a group of healthy subjects. Both OJ, red and blond, contained similar amounts of total flavonoids, hydrocynnamic acids and vitamin C, while only red OJ contained anthocyanins [26].

We provide here evidence for a reduction in WB procoagulant activity in association with either OJ intake.

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Materials and Methods

Study subjects

The study was carried out in 17 healthy subjects (aged 31 ± 1.5 SEM), 10 males and 7 females. All subjects were of normal height and weight (body mass index between 19 and 26 kg/m^2), apparently healthy without any evidence of chronic disease. Exclusion criteria were as follows: CVD, hypertension, type 2 diabetes, dyslipidemia, metabolic and endocrine diseases, use of medications, antioxidants or vitamin supplements, alcohol consumption (≥ 20 g alcohol/d); intense physical activity (≥ 5 h/wk), gastrointestinal disorders, vegetarian or other restrictive dietary requirements. Subjects presenting with a high consumption of flavonoid-rich beverages, such as tea, herb tea, coffee, wine, cocoa, and fruit juice, were also excluded when the daily consumption of one or more of these products exceeded 500 mL (estimated from a food-frequency questionnaire). The study and all procedures of the protocol were approved by the Rome Catholic University Ethic Committee. Written informed consent was obtained from each of the participating subjects before commencement of the study.

Orange juices

Red OJ was obtained from Moro, Tarocco and Sanguinello varieties, while blond OJ derived from Valencia, Navel and Belladonna varieties, as selected by the Istituto Sperimentale per l'Agrumicoltura (Acireale, Italy) and supplied by Ortogel (Caltagirone, Italy).

Both OJ had comparable nutritional characteristics (energy, carbohydrate, sugars, proteins, fats, fiber, vitamin C and sodium) and phenolic composition (total flavonoids and total hydroxycinnamic acids), with the exception of anthocyanins (total, delphinidi-3-glucoside, cyaniding-3-glucoside and cyaniding-3-(6-malonylglucoside)), that were undetectable in blond OJ [26]. Both red and blond OJ were previously characterized as far as anthocyanin urinary levels and cellular markers of cardiovascular risk were concerned [26].

Subjects were instructed to keep the orange juice packages in their home refrigerator at 4 °C until consumption.

Study design

Subjects were enrolled in a controlled, randomized, cross-over intervention trial. After 1 week run-in period on a controlled diet, the subjects were randomized and assigned to two periods of 4 weeks treatment with daily consumption of one liter of either red or blond OJ. The subjects were instructed to distribute the daily OJ dose in three daily doses: at breakfast, at lunchtime and in the evening, in addition to their usual diet, which they were recommended not to change during the intervention.

After a 4 weeks of wash-out, each subject was then crossed over to the other treatment for a further 4-week period.

All the laboratory operators were blinded about the treatment arm, while the study participants were not, for obvious reasons, being the OJ color different from each other.

The degree of compliance was checked weekly by interview, before providing the weekly supply of OJ.

Processing and analysis of biological samples

Blood samples were collected into evacuated tubes containing 0.1 vol of 3.8% sodium citrate/0.15 mol/L NaCl after overnight fasting at baseline (before treatment), after the 4-week treatment, after the 4 weeks of wash-out (before second treatment) and the 4-weeks with the second treatment. Plasma was isolated, processed, and stored at -80 °C until analysis.

Total cholesterol, HDL cholesterol, LDL cholesterol, triglycerides, and glucose were measured according to standard laboratory techniques.

Whole blood procoagulant activity

WB was incubated for 2 h at 37 °C with or without tumor necrosis factor (TNF)-α (100 ng/ml) or bacterial endotoxin (LPS) (100 ng/ml). The optimal agonist concentrations were previously selected on the basis of dose-response curves (not shown). At the end of incubation, WB procoagulant activity (the time taken for recalcified blood to clot) was assessed by a one-stage clotting time. Briefly: 200 μL WB were mixed with 100 μL 25 mM $CaCl_2$ and the time to clot formation was recorded. Results were expressed as clotting time in seconds.

TF and TFPI determination

Platelet poor plasma was prepared by a standard procedure [27] and the presence of TF and TFPI was assessed by enzyme-linked immunosorbent assays (ELISA) as specified by the manufacturers (Imubind TF kit, TFPI total kit, American Diagnostica, Stamford, CT, USA).

Statistical analysis

The results are given as mean ± SEM. The data sets were analyzed using a two-way ANOVA on time and treatment effects. In all statistical tests performed, the null hypothesis (no effect) was rejected at the 0.05 level of probability. A Bonferroni adjustment was used to correct for multiple comparisons. The SAS statistical software package (versions 9.1; SAS Institute Inc, Cary, NC) was used for statistical analysis. The wash-out effect was tested by ANOVA paired test comparing clotting times before and after treatment.

Results

Clinical chemistry parameters

No significant changes were observed after 4 week intake of either OJ preparation in blood glucose, triglycerides and cholesterol levels or in different parameters of blood cell counts (not shown).

Effect of orange juice intake on whole blood procoagulant activity

Fig. 1 reports the mean clotting times of WB recorded in either unstimulated (Fig. 1A), or TNF-α- stimulated (Fig. 1B), or LPS-stimulated (Fig. 1C) experimental conditions. As expected, the clotting time was shortened when WB was stimulated *in vitro* by either inflammatory agonist.

The clotting time test was intra-individually reproducible up to 8 weeks (the time lapse between the start of each of the two treatments).

Following a 4-week ingestion of either OJ, WB clotting times were significantly prolonged both in basal and TNF-α- stimulated conditions (Fig. 1A and B). A non statistically significant prolongation occurred in LPS-stimulated condition; however, if all the results obtained after OJ intake were combined, the OJ intake resulted in a statistical prolongation of clotting time in LPS-stimulated condition too (Fig. 1C).

The effect of OJ intake was reversible since clotting times at the start of the treatment and after wash-out were not different (404.1 ± 37.9 and 405.9 ± 65.0, respectively, $p = 0.90$).