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Flavanone-rich citrus beverages counteract the transient decline in postprandial endothelial function in humans: a randomized, controlled, double-masked, crossover intervention study

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Running head: Flavanones and endothelial function

ABSTRACT

Specific flavonoid-rich foods/beverages are reported to exert positive effects on vascular function; however, data relating to effects in the postprandial state are limited. The present study investigates the postprandial, time-dependent (0 -7 h) impact of citrus flavanone intake on vascular function. An acute, randomized, controlled, double-masked, crossover intervention study was conducted in middle aged healthy men (30-65 yrs, n=28) to assess the impact of flavanone intake (Orange Juice: 128.9 mg; flavanone-rich Orange Juice: 272.1 mg; homogenized whole orange: 452.8 mg; isocaloric control: 0 mg flavanones) on postprandial (double meal delivering a total of 81 g of fat) endothelial function. Endothelial function was assessed by flow-mediated dilatation (FMD) of the brachial artery at 0, 2, 5 and 7 h. Plasma levels of naringenin / hesperetin metabolites (sulphates and glucuronides) and nitric oxide species (NO_x) were also measured. All flavanone interventions were effective at attenuating transient impairments in FMD induced by the double meal (7 h post intake; $P<0.05$), but no dose response effects were observed. The effects on FMD coincided with the peak of naringenin/hesperetin metabolites in circulation (7 h) and sustained levels of plasma nitrite. In summary, citrus flavanones are effective at counteracting the negative impact of a sequential double meal on human vascular function, potentially through the actions of flavanone metabolites on NO.

Key words: citrus flavanones, endothelial function, high-fat meal, nitric oxide, postprandial

Introduction

A transient impairment of vascular function is known to occur in the postprandial or fed state [1-4] and is widely believed to impact on endothelial dysfunction and lifetime cardiovascular disease risk [5-8]. In particular, endothelial function has been shown to be transiently impaired (2-8 h) following ingestion of moderate to high fat meals (36-80 g of fat) [9-13], potentially driven by hyperglycemia and hypertriglyceridemia which occurs during the postprandial state [1, 14, 15]. Observational data have highlighted that the consumption of diets rich in flavonoids might lead to an improved cardiovascular prognosis [16-20]. Indeed, flavonoid-rich foods and beverages are well reported to improve endothelial function in humans both acutely [21-24], short-term [25-27] and long-term [28-31]. However, most of the acute interventions were undertaken with volunteers in the fasted state, which is considered less representative of the free living state, whilst data relating to flavonoid potential to ameliorate acutely postprandial endothelial impairments are more scarce [32-34].

Although, the precise mechanisms by which absorbed flavonoids and their circulating metabolites mediate beneficial vascular effects remain unclear, there is evidence to suggest that the modulation of circulating NO levels might be involved [22-24, 35-38]. Notably, flavanol-rich cocoa has been consistently shown to improve endothelium-dependent vasodilation in healthy individuals [24, 27, 31, 39, 40], smokers [23, 26], patients with coronary artery disease (CAD) [41], hypertension [42], or diabetes [43]. Particularly, acute vascular improvements have been shown to coincide with the appearance of flavanol metabolites in the circulation and with peak plasma NO levels [23, 24, 26, 42]. Furthermore, flavanol-induced improvement in vascular function are inhibited following co-administration of an eNOS inhibitor, suggesting a cause-and-effect relationship between flavonoid intake, plasma NO levels and vascular function [23, 24]. Although less studied, flavanones from citrus, have also been shown to exert beneficial effects on human vascular function [28, 44]. In particular, chronic interventions with orange juice, or the pure flavanone,

hesperedin, resulted in a decrease in blood pressure in overweight volunteers and acute (6 h) improvements in micro-vascular reactivity [45]. Short-term intake of pure hesperedin also resulted in significant improvements in endothelial function (as measured by brachial artery FMD) in volunteers with metabolic syndrome [46].

In the present study, we assessed the impact of an acute intervention with increasing doses of orange flavanones (sourced from differently processed orange beverages) on human vascular function in the postprandial state. A sequential double meal (breakfast and lunch, delivering a total of 81 g of fat) was used to simulate the fed state and investigate the postprandial time-dependent effects of flavanone intake on endothelial function as measured by brachial artery FMD.

SUBJECTS AND METHODS

Ethics

The clinical trial was registered at clinicaltrials.gov (NCT01963416) and conducted according to the Declaration of Helsinki following Good Clinical Practice (GCP). It was approved for conduct by the University of Reading's Research Ethics Committee (ethics reference number 12/06). All volunteers signed an informed consent form before commencing the study.

Intervention study volunteers

Volunteers were recruited from the University of Reading and surrounding area by use of the Hugh Sinclair Unit volunteers' database, poster advertisement within the university and local community via local websites (April-Dec 2012). Fifty-nine male healthy volunteers, aged 30-65 years old were assessed for screening and selected according to the following inclusion criteria: 1) fasting lipids in the upper half

of the normal range (triacylglycerol 0.8-3.2 mmol/l and total cholesterol 6.0-8.0 mmol/l) or BMI 25-32 kg/m^2; 2) Non-smoker; 3) Not diabetic (diagnosed or fasting glucose > 7 mmol/l) or suffer from endocrine disorders; 4) hemoglobin and liver enzymes levels within the normal range; 5) Not having suffered a myocardial infarction/stroke in the past 12 months; 6) Not suffering from renal or bowel disease or have a history of choleostatic liver or pancreatitis; 7) Not on drug treatment for hyperlipidemia, hypertension, inflammation or hyper-coagulation; 8) Not taking any fish oil, fatty acid or vitamin and mineral supplements; 9) No history of alcohol misuse; 10) Not planning or on a weight reducing regime; 11) Not having taken antibiotics in the 6 months prior to the study; 12) Not being able to consume the study meals. Those selected for the study were instructed not to alter their usual dietary or fluid intake. Volunteers were asked for 24 h prior to, and during, the study to refrain from the following: 1) consumption of polyphenol-rich foods including fruits (including citrus fruits) vegetables, cocoa, chocolate, coffee, tea, fruit juices and wine; 2) consumption of foods-rich in nitrates, including beetroot, spinach, lettuce, rocket, celery, parsley, cabbage (defined as containing more than 50 mg nitrates/ 100 g fresh weight [47]; 3) participating in vigorous exercise and 4) consuming any of alcohol beverage. Volunteers were further asked to fast for 12 hours before each study visit and during that period only consume low-nitrate water provided. The same standard meal, low in polyphenols and nitrates, was also provided for dinner for the day before each visit. Written informed consent was obtained from all eligible volunteers prior to their participation in the study.

Study Design

The study design consisted of an acute, randomized, placebo controlled, double-masked postprandial crossover study (Figure 1). After the initial screening visit to assess the eligibility of volunteers to participate in the study, volunteers were enrolled in the study (by researchers CR and HD) and visited

the Hugh Sinclair Unit at the University of Reading on four separate occasions separated by a two week period (June-Dec 2012). Volunteers were asked to consume either a A) Control drink (C); B) Orange juice beverage (OJ); C) Flavanone-rich orange juice (FROJ) or D) Whole Orange beverage (WO), together with a high fat breakfast (at baseline, t = 0 h), followed by a medium-fat lunch (t = 5.5 h). HD assigned participants to the 3 digit coded drink interventions for their 4 visits according to a random allocation sequence generated by a third party. Details on the flavonoid composition of the interventions, as well as micro and macronutrient composition, can be found on Table 1. Compliance to a 24 hour low-polyphenol intake period and 12 hour fasting was monitored by a 24 hour dietary recall conducted in each study visit. On each visit day, volunteers rested for 30 minutes in a quiet, temperature controlled room before they were cannulated by a qualified research nurse and blood samples were collected in the fasted state (0 h) and at 2, 5, 7 and 24 h after consumption of each intervention drink. Flow mediated-dilation of the brachial artery (FMD) was the primary outcome and it was measured at 0, 2, 5 and 7 h post consumption. Secondary outcomes of the study included systolic and diastolic blood pressure (0, 2, 5, 7 h), plasma flavanone levels (0, 2, 5, 7, 24 h) and Nitric Oxide (NO) plasma levels (0, 2, 5, 7 h). After baseline measurements were taken, the high fat breakfast (Table 2) was consumed with one of the clinical products (C, OJ, FROJ, WO). Volunteers were asked to consume the intervention drink and the high fat meal in 10-15 min. At 5.5 hours from baseline, a medium fat lunch was provided (Table 2). Last measurement of the day was performed at 7 hours and the volunteers were asked to return to the clinical unit the following morning to provide a 24 h blood sample (fasted). From 7 to 24 h, volunteers were asked to consume the free-polyphenol dinner provided by the research team and to continue on the low-polyphenol diet, as well as refrain from exercise and consuming alcohol. Blood samples for flavonoid analysis were collected in EDTA-containing tubes (Greiner Bio-One Ltd, Stonehouse, UK), immediately centrifuged for 15 min at 4 °C (4000 x g) and the plasma spiked formic acid (1.5% of a 50% water solution) and ascorbic acid (5% of a 10 mM solution) and stored at -80 °C. Blood samples for Nitric

Oxide analysis were collected in heparin-containing tubes, immediately (within 3 min of collection) centrifuged for 15 min at 4 °C (4000 x g) and plasma rapidly collected, aliquoted and stored at -80 °C to reduce inactivation of nitroso species. All procedures involving human volunteers were approved by the University of Reading Research Ethics Committee. The clinical trial was registered at clinicaltrials.gov as NCT01963416.

Sequential double-meal

The sequential double meal protocol was based on the department's extensive experience on postprandial studies, which have been collated into the DISRUPT database [48]. It consisted of two meals 1) high fat breakfast (51 g fat; 14 g protein; 64 g carbohydrates; 777 kcal) administered with the intervention drink and 2) medium-fat lunch (30 g fat; 15 g protein; 80 g carbohydrates; 628 kcal) (Table 2) administered 5.5 hours after the intervention drink. The high fat meal consisted of two butter croissants (47 g of fat) and 5 g of butter (4 g of fat). The medium fat meal consisted of 2 slices of white bread (2 g of fat); 42 g of Philadelphia soft cheese (13 g of fat); a small bag of salted crisps (9 g of fat) and two shortbread biscuits (6 g of fat) (Table 2). The volunteers were asked to consume meals within 10-15 min.

Flavanone-containing interventions

The preparations of the intervention drinks were carried out in accordance to good manufacturing practice as described in HACCP. The control (C) drink was matched for sugars found in the orange beverages and 0.67% citric acid and orange flavoring was added for flavor purposes. The levels of total β-carotenes present in the flavanone-treatments are considered negligible (~ 0.25 mg; 2-RSD: 15%) with regards to endothelial function effects; with a 15 mg dose (6 weeks intervention, in combination with 150 mg of vitamin C) resulting in no significant changes on endothelial biomarkers [49]. (Table 1). The levels of folate present (~ 60 μg; 2-RSD: 16%) can also be considered insignificant in regards to its

potential to impact on endothelial function; folate has been shown to drive small improvements in endothelial function only in long-term interventions (1- 4 months) of at least 5000 – 10000 μg /day of folate, but not with lower doses in the ranges of 400 – 800 μg/day [(50)].

Orange juice intervention (OJ) was a 100% commercial pure orange juice (Tropicana Pure Premium). The flavanone-rich orange juice intervention (FROJ) was Tropicana Pure Premium with added orange pomace. Pomace comprised the edible part of a whole orange which is leftover during the production of Tropicana pure premium orange juice and subjected to particle size reduction. Orange pomace is rich in fiber (40:60 ratio of soluble to insoluble) and contains small amounts of micronutrients and a high proportion of the polyphenols found in whole orange. The whole orange intervention (WO) consisted of lightly blended whole table orange, without the peel. Drinks displayed slightly different viscosities, but specific measurements were no undertaken to assess this. All drinks were stored in individual portions (255 g/ 240 ml) in aluminum canisters, frozen at -20 °C and labeled with a 3-digit code to ensure double-masking. Drinks were defrosted overnight in the fridge (4 ^{0}C) just before being used for each study day. Participants, care-providers and all researchers assessing outcomes were blind until all the data was analyzed. Quantification of flavanones from orange beverages (OJ, FROJ and WO) was performed by UHPLC-MS. Sample preparation was performed by diluting the juice sample with DMSO, the internal standard (IS) solution (10 μg/mL d4-Dimethylphthalate in 50% Acetonitrile/water) and 50% Acetonitrile/water, followed by vortexing and centrifugation (10 min, 2500 rpm). The supernatant was filtered prior to analysis in an Agilent 1290 UHPLC, with a Zorbax Eclipse Plus C18 column (1.8μm, 2.1 mm x 100 mm; linear gradient starting at 100% (A) containing 2% Acetonitrile in water with 0.1% Formic acid, to 90% (B) containing Acetonitrile with 0.1% Formic acid, followed by 100% B). MS detection was performed in ESI positive ion mode, on an Agilent 6530A Q-ToF MS with MassHunter Software for instrument control and data processing. Calibration standards were prepared from analytical grade materials purchased from Indofine Chemical Chromadex or LKT Laboratories. The levels of

flavanones in the test products are presented in Table 1. Briefly, the total levels of flavanones in a) OJ is 128.88 mg, b) FROJ is 272.14 mg, c) WO is 452.80 mg (Table 1). The flavanone hesperedin is the main flavonoid present in the intervention beverages, ranging from 107.30 mg (OJ) to 352.80 mg (WO).

Flow-mediated dilation (FMD)

FMD of the brachial artery was the primary end point measure of the study and measurements were taken following standard guidelines [51] using an ALT Ultrasound HDI5000 system (ATL Ultrasound, UK) in combination with a semi-automated computerized analysis system (Brachial Analyzer, Medical Imaging Applications-llc, IL, US). Briefly, after 15 minutes supine rest in a quiet air-conditioned room the brachial artery was imaged longitudinally at 2-10 cm proximal to the antecubital fossa. After baseline images were recorded for 60 seconds, a blood pressure cuff placed around the forearm was inflated to 220 mmHg. After 5 min of occlusion, the pressure was rapidly released to allow reactive hyperemia, with image collection continuing for 5 min post release. A single researcher, who was blinded to the measurement details, analyzed all image files and peak diameter was defined as the largest diameter obtained after the occlusion was released. FMD response was calculated as relative diastolic diameter change from baseline as compared to peak diastolic diameter. A total of 28 volunteers were analyzed for their FMD response. Data from 8 volunteers was not analyzed or was excluded due to *i)* measurement of FMD from non-dominant arm (rather than dominant) due to limitations with blood collection (n=2); *ii)* absence of FMD response (n=3); *iii)* technical problems during recording of ultrasound FMD measurements rendered non-analyzable data (n=3).

Blood pressure

Systolic and diastolic blood pressure were measured using an Omron MX2 automatic digital upper arm blood pressure monitor (Omron Healthcare UK Ltd, Milton Keynes, UK). All measurements were taken according to standard practice and by a qualified research nurse, prior to and following each intervention period. Before starting blood pressure measurements the volunteers were seated or laying down quietly for at least 20 min. Measurements were taken in the right arm, before FMD procedure for each time point. The subject's right arm was placed resting on a pillow (on a side table positioned at heart level), slightly flexed with palm upward. Volunteers were asked to refrain from speaking during blood pressure measurements. The measurement were repeated 3 times and blood pressure was considered as the average of these measurements.

Plasma flavanone analysis

Blood samples were collected in EDTA blood tubes and centrifuge at 4 °C for 10-15 min at 4000g. Formic acid (1.5% of a 50% solution) and ascorbic acid (5% of a 10 mM solution prepared fresh everyday) were added to the plasma samples to preserve flavanones before freezing at -80 °C. A subset of 20 volunteers were selected randomly for analysis of their flavanone content. A high throughput analytical method using Ultra-high Performance Liquid Chromatography coupled with tandem mass spectrometry (UHPLC-MS/MS) was developed and validated to measure simultaneously naringenin and hesperetin in human plasma. Enzymatic hydrolysis and methanol extraction was applied as described before [52] with modifications to accommodate *in situ* monitoring of enzyme efficiency and automated sample preparation using a Hamilton Microlab Star liquid handling system (Hamilton, UK). Plasma samples (45 μL) were incubated after addition of β-glucuronidase type VII-A (Sigma, USA) and sulfatase type H-5 (Sigma, USA) for 90 min and 60 min at 37 °C, respectively. To monitor the enzyme activity, every individual sample was spiked with a known concentration of phenolphthalein glucuronide and

potassium 4-nitrophenyl sulfate (Sigma, USA) as enzyme substrates in addition to caffeine-(trimethyl-d9) (Sigma, USA) as internal standard (IS) prior to incubation. The enzyme hydrolyzed samples were subsequently extracted with methanol and centrifuged. The supernatant (6 µL) was analyzed using an Agilent 1290 UHPLC coupled with an Agilent 6490 triple quadrulpole mass spectrometer. Naringenin and hesperetin were separated in a Waters BEH C18 (100 x 2.1 mm, 1.7 micrometre particle size) at a flow rate of 0.6 mL/min in a 6.5 min gradient 99% solvent A (water containing 0.1% formic acid) and 1% solvent B (acetonitrile containing 0.1% formic acid) initially; 70% solvent A at 0.5 minutes; 55% solvent A at 2.5 min; 2% solvent A at 3.0 min; 2% solvent A at 4.0 min; 99% solvent A at 4.5 min followed by post equilibration for 2 minutes). The mass spectrometer was operated in ESI positive ionization mode and Multiple Reaction Monitoring (MRM) mode by monitoring quantifier and qualifier ions for both naringenin and hesperetin. MRM transitions were determined as 204.1/144.0 (m/z) corresponding to caffeine, 495.1/319.1 (m/z) corresponding to phenolphthalein-glucuronide and 217.9/137.9 (m/z) corresponding to potassium 4-nitrophenyl sulfate, as Quantifier ions. MRM transitions were determined as 303.1/153.1 (m/z) corresponding to hesperetin and 273.1/147.1 (m/z) corresponding to naringenin as qualifier ions. Concentrations of hesperetin and naringenin were then calculated based on ratios of their integrated peak area for the quantifier ions to that of IS using two sets of eight point calibration curves. Accuracy of the analysis was monitored by systematic counter-balancing between plasma samples and quality control samples spiked with a known concentration of hesperetin and naringenin. The method was validated for a linear calibration range of 0.0313 µM to 8.02 µM for naringenin and 0.0282 µM to 7.22 µM for hesperetin, respectively. Additionally, limits of detection for naringenin and hesperetin were determined as 2 nM and 7 nM, respectively.

Biochemical analysis

The blood samples collected in pre-chilled lithium or heparin tubes were spun (4000 x g; 10-15 min; 4ºC) immediately after collection (within 3 min). Samples were also collected in serum separation tubes (SST) and allowed to stand for 30 min prior to centrifugation (1300 x g; 10 min; 21ºC). All samples were aliquoted and frozen at -80°C until analysis. ***Plasma NO analysis:*** Plasma samples for measurement of total nitroso species (NOx) were aliquoted in 150 µl aliquots to avoid freeze-thawing of the samples for each measurement. Samples were defrosted just before the measurements took place (within 10 min), these were kept on ice throughout. Plasma samples (n=28) were analysed for nitrite, nitrate and other nitroso species (RXNO, including nitrosothiols, nitrosamines, iron-nitrosylhemoglobin and nitrosohemoglobin) by ozone-based chemiluminescence (model 88 AM, Eco Physics) as previously described [53]. In brief, for total NOx measurement (NO derived from nitrate, nitrite and RXNO), one aliquot of plasma was injected in airtight microreaction vessel containing a solution of vanadium (III) chloride (50 mM) dissolved in 1 M HCl, connected to a chemiluminescence analyser. For measurement of nitrite and other RXNO, *i)* one aliquot of plasma was injected in the same apparatus into a glacial acid acetic solution containing 45 mM of potassium iodide and 10 mM of iodide, at 60 ºC actively purged by inert helium, which allowed the detection of NO from both nitrite and RXNO (but no nitrate). *ii)* Subsequently, the plasma sample was treated with acidic sulphanilamide (1 M HCL) to scavenge nitrite, before injection, allowing for quantification of RNXO alone. Nitrite levels in the plasma samples was determined by the difference between these two measurements (*i* and *ii*). Nitrate concentration was determined by subtracting Nitrite + RXNO from total NOx. Samples used for calibration curves were prepared fresh every day and displayed consistent values across days. ***Plasma baseline lipids and glucose:*** Plasma levels of total cholesterol, LDL cholesterol, HDL cholesterol, glucose and triacylglycerol (TAG) were assayed on an ILAB 600 chemistry analyzer (Instrumentation Laboratory, Warrington, UK) using enzyme based colorimetric tests supplied by Instrumentation Laboratory.

Power calculation and statistical analysis

Power calculations were performed for the primary endpoint, change in FMD response. Power was based on the intra-individual variability of the operator that performed the FMD analysis (5% CV, SD=0.3%). Previous measures of variability in a control group estimated the standard deviation within subjects to be 2.3%. At 90% power and 0.05 significance, the number of volunteers required to detect a difference of 1.5% in the response of matched pairs in a crossover study is 25. The statistical analysis was performed using the SPSS Statistics 21 (IBM) package. FMD, blood pressure, plasma levels of Nitric Oxide species (Nitrate, Nitrite and Nitroso species) and plasma levels of flavanones were analyzed using a two-way repeated measures ANOVA within subjects with Time (0, 2, 5, 7 hours) and Treatment (C, OJ, FROJ, WO) as main factors. Post-hoc and Pairwise comparisons were carried out using the Bonferroni correction for multiple comparisons. Significance was defined as $P < 0.05$ (95 % confidence interval) for all outcome measures, with p-values represented in the figures as follows: $*P = 0.01\text{-}0.05$, $**P = 0.001\text{-}0.01$, $***P < 0.001$. Pharmacokinetics parameters were calculated as follows: a) the maximum plasma concentration (C_{max}) and b) the time to reach the maximum plasma concentration (T_{max}) were determined from the individual data obtained from each participant; c) the area under the plasma concentration versus time curve (AUC) was calculated using the trapezoidal method. Multiple regression analysis was used to predict the value of FMD (dependent variable) based on the value of hesperetin and naringenin plasma levels (independent variables). Random allocation sequence was generated by a third party statistician using SAS version 9.1 (procedure plan and seed = 122700). The randomized block design contained 4 blocks and 9 randomized sequences within each block.

RESULTS

Baseline characteristics and tolerance of intervention

The baseline characteristics of volunteers recruited were within the desired ranges, with either triacylglycerol ranging from 0.8-3.2 mmol/l and total cholesterol from 6.0-8.0 mmol/l or/and BMI from 25-32 kg/m^2 (Table 3). All intervention beverages were well tolerated by all volunteers, as well as the high and medium fat meals administered throughout the study. No adverse events were reported.

Flavanone modulation of postprandial FMD

A 2-factor repeated-measures ANOVA for endothelium-dependent brachial artery vasodilation, measured FMD response, revealed a highly significant interaction between the interventions (C, OJ, AOJ, WO) and time of the day (0, 2, 5, 7 h) [$F(9,243)=3.27$, $P<0.0001$], as well as significant main effects of time of the day [$F(3,81)=12.062$, $P<0.0001$] and intervention [$F(3,81)=2.78$, $P<0.05$)]. At baseline (t = 0 h) there were no significant differences in brachial artery FMD between visits with the average baseline levels of FMD for the study population being 4.80 ± 0.03 FMD units. Two hours after intake of the high-fat meal, a significant decrease in % FMD was detected for both control ($P < 0.0001$) and the 3 flavanone interventions ($P < 0.05$) (Figure 2). In particular, in the control group, the % FMD decreased by 0.99 ± 0.17 % FMD after 2 h ($P < 0.0001$) and remained significantly suppressed 5 h ($P < 0.05$) and 7 h ($P < 0.0001$) after intake, relative to baseline levels. In contrast, all orange flavanone interventions resulted in a recovery in % FMD to that of baseline levels between 5-7 h (OJ : 4.51 ± 0.23% FMD; FROJ: 4.74 ± 0.25% FMD and WO: 4.75 ± 0.23% FMD) (Figure 2). At 5 h post intervention, there were no significant differences in % FMD between control and each of the flavanone interventions (OJ, FROJ, WO), whereas at 7 h (following intake of the medium-fat meal at 5.5 h), we observed a significantly higher % FMD for OJ ($P < 0.05$), FROJ ($P < 0.01$) and WO ($P < 0.01$) in comparison to control. There were no significant differences between the flavanone interventions at 7 h, with all three doses of flavanones administered (OJ: 128.8 mg; FROJ: 272.1 mg and WO: 452.7 mg) counteracting the deleterious effect of the double meal challenge on % FMD response to a similar extent. Blood pressure

was not significantly altered following consumption of any of the flavanone interventions, relative to baseline or to the control beverage (Table 4).

Modulation of plasma flavanones

Total flavanones, naringenin and hesperetin (including glucuronides and sulfates), were not detected in the plasma of volunteers at baseline, indicating compliance to the 24 h low flavonoid diet prior to the study visits. Flavanone metabolites were not detected in the circulation of individuals following intake of the control drink (Figure 3). Significant increases in plasma levels of hesperetin/metabolites were detected at 5 and 7 h ($P < 0.0001$) (Figure 3A), and at 2, 5 and 7 h for naringenin/metabolites ($P < 0.0001$) (Figure 3B). The time to reach T_{max} for hesperetin and naringenin were not significantly different between treatments and coincided with the timeframe of FMD effects (Table 5). At 2, 5 and 7 h, plasma levels of naringenin were significantly higher following WO intake compared to OJ ($P < 0.01$) (Figure 3B). Similarly, at 7 h, both FROJ and WO showed a trend towards higher plasma concentrations of hesperetin, relative to OJ ($P < 0.1$) (Figure 3A). With respect to the C_{max} and AUC (0-24 h) for plasma hesperetin, both FROJ and WO were significantly higher than after OJ ($P < 0.05$), whilst for naringenin, both the C_{max} and AUC were only significantly higher for WO in relation to OJ ($P < 0.005$) (Table 5). No significant differences in plasma flavanone levels were detected between FROJ and WO despite the different levels present in the treatment drinks. At 24 h, the levels of flavanones were not significantly different from baseline, indicating the flavanone metabolites have been cleared from circulation ($P = 0.13$) (Figure 3). A multivariate regression analysis, including both plasma naringenin and hesperetin, showed that hesperetin ($P = 0.001$), but not naringenin ($P = 0.092$), predicted changes in % FMD over the course of 0-7 h. Specifically, at 7 h, at the peak of FMD response, hesperetin significantly predicted the magnitude of FMD increase ($r = 0.32$, $P = 0.005$) following flavanone intake.

Modulation of plasma nitrite, nitrate and RXNO

Levels of nitrate, nitrite and other RXNO (nitrosothiols, nitrosamines, iron-nitrosylhemoglobin and nitrosohemoglobin) were determined in plasma at baseline and 2, 5 and 7 h post treatment (Figure 4). Nitrite plasma levels are known to reflect more accurately endogenous NO production in humans (estimate of 70-80% of plasma nitrite deriving from endothelial nitric oxide synthase activity), whilst the other major source is diet-derived nitrate (by reduction to nitrite). This was the rational for detecting separately levels of nitrite, nitrate and other RXNO species. The average levels of plasma nitrate, nitrite and other RXNO at baseline were 32.1 μmol/L, 68.3 nmol/L and 0.4 nmol/L, respectively, which is in agreement with the values reported in the literature [37, 54]. A significant decrease in plasma nitrate levels was observed at 2, 5 and 7 h for all the interventions, including control ($P < 0.0001$) (Figure 4B). In contrast, plasma nitrite levels remained constant up to 7 h post-treatment (not significantly different from baseline) following OJ, FROJ and WO intake whilst the control group nitrite levels decreased significantly ($P < 0.01$) (Figure 4A). No significant changes were detected in RXNO levels in plasma (NS) (Figure 4C).

DISCUSSION

Considerable evidence suggests that dysregulation of endothelial function in the postprandial state is an important contributing factor for cardiovascular disease risk [2, 5, 7, 8], whilst intake of flavonoid/polyphenol-rich foods, such as cocoa, tea and berries have been shown to exert positive effects on vascular function. In support of this, clinical trial data has indicated that intake of such foods/beverages may lower CVD disease risk, at least partially, through their actions in mitigating fed-state metabolic and vascular disturbances (reviewed in [34]). In the present study, we showed that intervention with orange flavanones, both in juice or whole orange homogenised form, counteracts

impairments in vascular function evoked by a sequential double meal challenge, which reflects a regular eating pattern and a typical dietary intake in the population [48]. Each flavanone intervention tested was effective in reversing vascular impairments, to a physiologically similar degree, despite them containing different levels of flavanones (ranging from 128 mg to 452 mg) and resulting in different concentrations of plasma flavanone metabolites. No changes in blood pressure were observed. The rescue of transient impairments in vascular function, as assessed using brachial artery FMD, coincided with the peak of flavanone metabolites (total sulphates and glucuronides) in the circulation (7 h) and with sustained levels of plasma Nitrite, the latter of which was significantly reduced by the double meal challenge. Thus, our data support the concept that the observed postprandial vascular benefits may be linked to the actions of circulating flavanone metabolites on NO bioavailability.

Our findings are consistent with previous RCT datasets indicating that cocoa flavanols partially counteract the decrease in FMD following high fat meal loading [32]. Furthermore, pure quercetin has also been shown to ameliorate postprandial FMD following maltose overload [33]. To our knowledge this is the first data indicating that citrus flavanones are also capable of attenuating postprandial impairments in endothelial function following a sequential high-medium fat double meal in individuals displaying mild cardio-metabolic risk factors. Furthermore and in support of our findings, previous studies conducted in the fasted state report that chronic interventions with flavanones in at risk groups (e.g. hypertensive, overweight or metabolic syndrome patients) induce positive effects on blood pressure and endothelial function (FMD) [45, 46, 55] and improvements in microvascular reactivity [45]. Most importantly, in the present study, no dose-dependent effects on brachial artery FMD were observed, despite the interventions containing different amounts of flavanones (WO: 3.5 x OJ). This may indicate that at these intake levels a threshold plateau may be reached, similarly to what has been shown previously with other flavonoid-rich interventions [22]. It also further suggests that lower doses of flavanones (approx. 130 mg) can be efficacious at modulating postprandial endothelial function. No changes in blood pressure were

observed in the present study, which is in agreement with previous human intervention trials reporting modulation of blood pressure only after chronic interventions with flavonoid-rich foods, but not in an acute manner [27, 31].

We observed concurrent modulation of FMD, nitrite, and circulating flavanone metabolites (total sulphates and glucuronides) suggesting that the latter may be linked to NO availability and subsequent improvements in vascular function, although we cannot establish a causal relationship at this time. Specifically, both naringenin and hesperetin metabolites peak plasma levels for all three interventions occurs at approximately 7 h, which coincide with significant improvements in endothelial function (at 7 h) and sustained levels of circulating nitrite after flavanone interventions in comparison to control. Plasma levels of flavanone metabolites peaked slightly later than previously reported (4 - 6 h) [45, 56, 57], most likely due to the concomitant intake of fat, which is thought to interfere with flavonoid absorption [58]. On the other hand, no significant differences in time of absorption were detected between the flavanone treatment groups (peak occurs at approx. 7 h for all three treatments).

In support of the link between flavanone intake and human vascular function, we observe that when low or no levels of flavanone metabolites are detected in circulation (e.g 2 h), no differences in postprandial brachial FMD are observed between control and flavanone-rich beverages. Further multiple regression analysis suggests that mainly hesperetin metabolites seem to predict significantly the magnitude of changes in FMD ($r = 0.32$, $P = 0.005$), suggesting an important role of this flavanone in the effects observed. This is corroborated by previous studies showing that pure hesperidin can trigger both acute and chronic improvements in vascular function in humans [45, 46]. It is important to further note that only the sulphated and glucuronidated portion of the flavanone metabolites were quantified in our study and these are likely to account for a fraction (approx. 16%) of the total flavanone metabolites absorbed [59]. As such, we anticipate that gut-derived phenolic compounds might also contribute to the improvements

420 in endothelial function observed. This is supported by our observation that hesperetin metabolites can
421 only significantly predict a small percentage (approx. 30%) of the FMD response observed; therefore it
422 is likely that stronger correlations might be apparent once gut derived small phenolic metabolites are
423 taken into consideration, however such extensive analysis was outside of the scope of our study.
424
425 Our study also indicates that the impairment in postprandial FMD induced by the sequential high fat
426 meal might be linked to decreases in circulating levels of NO species, in particular nitrite and nitrate.
427 Although, the precise mechanisms underlying postprandial endothelium impairments are not established,
428 mechanistic animal studies suggest a role for NO signalling, showing, for example, that endothelial
429 dysfunction induced by fat intake also results in decreases in NO production [60-62]. Importantly, the
430 flavanone interventions only prevented the decrease in nitrite, but not nitrate. Numerous evidence suggest
431 that nitrite reflects more accurately endogenous NO production in humans, with an estimate of 70-80%
432 of plasma nitrite deriving from endothelial nitric oxide synthase (eNOS) activity [63, 64] and also better
433 reflects the degree of endothelial dysfunction in humans [65]. In agreement with our data, previous human
434 clinical data suggests an ability of some flavonoid-rich foods to modulate NO bioavailability [22, 24, 35, 37].
435 In particular, cocoa flavanols induced improvements in FMD have been causally linked to NO production
436 in humans [23]. More recently Bondonno *et al.*, 2012, also showed that apples containing (-) epicatechin
437 and quercetin increased levels of nitrite along with FMD response after 2 h of intake [37]. Additionally
438 and in agreement with the present data, both pure (-) epicatechin and quercetin were shown to specifically
439 increase plasma nitrite, but not nitrate in healthy humans [35]. Supporting *in vitro* mechanistic studies (in
440 endothelial cells) have demonstrated the flavanone hesperetin and some of its *in vivo* metabolites (e.g.
441 7-O-β-D glucuronide) can stimulate NO production *via* activation/expression of eNOS [46, 66] or by
442 decreasing NO degradation through inhibition of nicotinamide adenine dinucleotide phosphate-oxidase
443 (NADPH) [67] and these are possible mechanistic pathways by which flavanone metabolites might

444 modulate postprandial FMD. Although the specific modulation of nitrite by flavanone-containing
445 interventions is an interesting observation in the present study, the interpretation of the temporal
446 dynamics (time course) of flavanone appearance in plasma and levels of plasma nitrite is not
447 straightforward in regards to explaining the effects of nitrite on FMD. This seems to suggest that the
448 impact of flavanone metabolites on FMD response cannot be explained completely by modulation of
449 nitrite (as a measure of NO). Since, the present study was not designed (or powered) to detect changes
450 in NO species, we are limited in our ability to establish a clear-cut link between FMD modulation and
451 NO at this time. However, we believe this preliminary data is very novel and valuable for future, more
452 mechanism-focused, human RCT.
453
454 Interestingly, FROJ intake resulted in similar levels of plasma flavanones to WO, despite lower initial
455 concentrations, which might be related to characteristics of food matrix itself, such as viscosity, which
456 is known to influence the bioavailability of polyphenols (reviewed in [68]). It is possible that the reduced
457 particle size of the pomace in FROJ aided the release of polyphenols from the fiber matrix, making these
458 more accessible for gut microbiota metabolism [69]. It is known that dietary fiber can physically trap
459 polyphenols within the fiber matrix in the fruit tissue reducing the accessibility to enzymes and the gut
460 microbiota [70]. On the other hand, the rate of release of polyphenols from fibrous particles is inversely
461 proportional to the fiber particle size [71], therefore by reducing particle size in the pomace, we are likely
462 to increase the bioavailability of flavanones in FROJ. In order to confirm that this is the case, future
463 studies will focus on measuring accurately total urine excretion (e.g. over a 24 h period). Nonetheless,
464 our study seems to suggest that particle size reduction of fiber-rich orange pomace and the re-introduction
465 of this product into orange juice might be an effective strategy to increase the bioavailability of
466 polyphenols *in vivo*. Importantly, the increased bioavailability of flavanones after FROJ intake did not
467 enhance significantly postprandial FMD in comparison to lower flavanone-containing OJ, again

suggesting that perhaps a certain level of flavanone metabolites is necessary in circulation to trigger postprandial FMD improvements but further increases in flavanone levels may not produce additional benefits [22].

One of the limitations in the design of the present study is related to the composition of the control intervention, which did not take into account the levels of ascorbic acid present in the citrus beverages. Clinical studies suggest that doses up to 500 mg of vitamin C do not impact on biomarkers of endothelial function (e.g.[49, 72]). More specifically, it has been shown in a recent stratified meta-analysis that doses ranging from 90 to 500 mg of ascorbic acid do not produce improvements in endothelial function, both acutely or chronically [73, 74], especially in populations with normal vitamin C status [75, 76]. Furthermore, previous studies reporting acute beneficial effects of ascorbic acid on endothelium dependent vasodilation (within 2- 4 h of intake), deliver doses of at least 2000 mg (e.g. [77-79]) and in many cases positive outcomes are achieved by delivering ascorbic acid intravenously, resulting in supra-physiological plasma levels of vitamin C, which cannot be achieved by oral ingestion [79, 80]. Since, the levels of vitamin C in the present study were approx. 80-120 mg, we are confident that these can be considered negligible with respect to acute effects on endothelial function, as measured by FMD. Therefore, despite these limitations, we can safely argue that our conclusions are reasonable when attributing the FMD response to circulating flavanone metabolites (at 7 h post intake) and that our data are relevant in furthering the understanding of flavonoid-rich foods/beverages impact on postprandial endothelial function.

In summary, our results suggest that acute intake of a beverage containing at least 128 mg of flavanones can be an effective dietary strategy to blunt the acute transient impairment in endothelial function induced by a sequential double meal that reflects a typical intake in the population. Although we cannot draw

firm conclusions regarding the mechanisms by which flavanones elicit vascular responses, our results suggest that these might be linked to an ability of flavanone metabolites to sustain basal circulating NO levels. Collectively these observations have important implications considering that most individuals spend the majority of the day in the postprandial state and such temporary vascular changes repeated on a daily basis can critically impact on long-term vascular health and overall chronic disease risk.

DISCLOSURES

CS works as a Senior Scientist at PepsiCo Inc, LH works as a Senior Director at Global R&D Nutrition at PepsiCo Inc, RLB, MB and YH work as Principle Scientists at PepsiCo Inc. The other authors declare no conflicts of interest.

The views expressed in this manuscript are those of the authors and do not necessarily reflect the position or policy of PepsiCo Inc.

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AUTHORS' CONTRIBUTIONS

CR: coordinated and conducted the study, undertook all FMD measurements, did data analysis and wrote the manuscript; HD: coordinated and conducted the study; CS: design and coordination of the study, writing of the manuscript; LH: design of the study; RLB: conducted study drinks analysis MB, YH: conducted plasma flavanone analysis; GC: conducted NO species measurements and analysis; JL: co-investigator in the study, involved in experimental design; JPES: principal investigator, involved in experimental design and writing of the manuscript. All authors reviewed the manuscript.

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TABLES

Table 1: Compositional analysis of orange flavanone beverages and control beverage used in the acute postprandial study.

		Intervention drink (240 ml)			
Compounds		Control[1]	OJ[2]	FROJ[3]	WO[4]
	2-RSD (%)				
Hesperidin (mg)	5.0	-	107.30	220.46	352.80
Narirutin (mg)	5.0	0.08	15.41	34.54	76.58
Others* (mg)		0.02	6.17	17.14	23.33
Total Flavonoids (mg)		***0.10***	***128.88***	***272.14***	***452.71***
Fructose (g)	4.0	6.38	6.63	6.12	6.89
Glucose (g)	4.0	5.36	5.36	5.10	5.87
Sucrose (g)	2.0	10.20	10.97	11.99	11.48
Total Sugars (g)		***21.93***	***22.95***	***23.21***	***24.23***
Fiber (total) (g)	12.0	-	0.66	5.36	6.30
Ascorbic acid (mg)	8.0	-	105.57	80.17	123.01
Folate (µg)	16.0	-	54.06	65.28	64.77
Total β carotenes (mg)	15.0	-	0.13	0.26	0.35

[1] Control, sugar matched control; [2] OJ, Tropicana pure premium orange juice without pulp; [3] FROJ, Flavanone-rich orange juice: Tropicana pure premium orange juice with added orange pomace; [4] WO, juice made from lightly blended fresh whole orange. * Includes Diosmin, Didymin, Nobiletin, Tangeretin, Sinensetin, Me4-Scutellarein. *2-RSD*: Relative standard deviation of the measurement (expressed in %)

Table 2: Macronutrient composition of double-meal protocol

	Foods	Macronutrient breakdown			
		Fat (g)	Protein (g)	Carbohydrates (g)	**Energy (kcal)**
High-fat breakfast	Butter Croissant (2x)	47	14	64	740
	Butter (5 g)	4	n/a	n/a	37
	Total	***51***	***14***	***64***	***777***
Medium-fat Lunch	2 Slices of sliced white bread (108 g)	***2***	***8.5***	***50***	***237***
	Philadelphia soft cheese (42 g)	13	3.6	n/a	131
	Crisps (25 g)	9	1.5	13	133
	Shortbread biscuit (22 g)	6	1.4	16	127
	Total	***30***	***15***	***80***	***628***

Table 3: Baseline clinical characteristics of the study population.

Baseline characteristics	Mean ± SEM
Age (y)	48 ± 1
BMI (kg/m$^{2)}$	28.4 ± 0.4
Total cholesterol (mmol/L)	5.6 ± 0.2
HDL cholesterol (mmol/L)	1.3 ± 0.3
Triacylglycerol (mmol/L)	1.5 ± 0.1
Fasting Glucose (mmol/L)	4.8 ± 0.1
Haemoglobin (g/dL)	14.9 ± 0.1
Systolic blood pressure (mm Hg)	124.0 ± 1.7
Diastolic blood pressure (mm Hg)	74.9 ± 1.5
Liver Enzymes	
Alanine Aminotransferase (ALT) (IU/L)	42.1 ± 2.1
Gamma-glutamyltransferase (IU/L)	41.7 ± 5.3

Table 4: Acute postprandial effects of orange flavanone beverages on static blood pressure.

Blood Pressure (mm Hg)		Baseline	2 h	5 h	7 h	*P*
Systolic	Control [1]	125.5 ± 1.9	126.7 ± 1.9	126.7 ± 1.7	128.6 ± 1.5	NS
	OJ [2]	124.8 ± 1.5	125.7 ± 1.2	125.72 ± 1.5	126.4 ± 1.5	
	FROJ [3]	126.1 ± 2.1	125.7 ± 2.0	127.3 ± 2.1	127.1 ± 1.6	
	WO [4]	126.1 ± 1.6	125.1 ± 1.4	124.8 ± 1.5	126.6 ± 1.5	
Diastolic	Control	75.6 ± 1.6	71.5 ± 1.4	74.6 ± 1.4	72.1 ± 1.4	NS
	OJ	74.9 ± 1.5	70.1 ± 1.2	73.7 ± 1.5	70.4 ± 1.5	
	FROJ	75.5 ± 1.5	70.9 ± 1.6	74.6 ± 1.5	72.2 ± 1.4	
	WO	76.0 ± 1.6	69.9 ± 1.4	73.9 ± 1.5	70.9 ± 1.5	

[1] Control, sugar matched control; [2] OJ, Tropicana pure premium orange juice without pulp; [3] FROJ, Flavanone-rich orange juice: Tropicana pure premium orange juice with added orange pomace; [4] WO, juice made from lightly blended fresh whole orange; Results are presented as mean ± SEM (n=36). Baseline levels did not differ between groups. NS: Non significant differences between treatments

Table 5: Pharmacokinetics of the major plasma flavanones, naringenin and hesperetin, after consumption of beverages containing either 128.88 mg (OJ), 272.14 mg (FROJ) or 452.80 mg (WO) of total orange flavanones in healthy middle-aged men.

		Cmax (μM)			***Tmax (h)***			***AUC (0 - 24 h)***		
		OJ	AOJ	WO	OJ	AOJ	WO	OJ	AOJ	WO
Flavanones	Hesperetin	0.1 ± 0.0	0.3 ± 0.1 *	0.2 ± 0.0 *	7.9 ± 1.3	7.6 ± 0.9	9.2 ± 1.4	1.4 ± 0.2	3.5 ± 0.8 *	2.4 ± 0.4 *
	Naringenin	0.05 ± 0.0	0.2 ± 0.0	0.1 ± 0.0 *	6.5 ± 1.0	6.5 ± 0.2	6.3 ± 0.3	0.8 ± 0.1	1.9 ± 0.5 #	1.8 ± 0.2 *

OJ, Tropicana pure premium orange juice without pulp; FROJ, Flavanone-rich orange juice: Tropicana pure premium orange juice with added orange pomace; WO, juice made from lightly blended fresh whole orange. Results are presented as mean ± SEM (n=20). * $P < 0.05$, indicates a significant difference in Cmax / AUC in WO or FROJ in relation to OJ. # $P = 0.058$, indicates a trend in AUC in FROJ in relation to OJ. No significant differences in Tmax were detected between treatments.

FIGURES

Figure 1. CONSORT flow diagram for the postprandial study. CONSORT, Consolidated Standards of Reporting Trials.

Figure 2. Time-course of postprandial FMD following consumption of flavanone beverages containing either 128.88 mg of flavanones (OJ); 272.14 mg of flavanones (FROJ); 452.80 mg of flavanones (WO) or a macronutrient and micronutrient-matched control in middle aged healthy men (n=28). A high-fat breakfast (51 g of fat) was administered at t = 0 h, and a medium-fat lunch (30 g of fat) was administered at t = 5.5 h. Data are presented as mean ± SEM and analyzed using a 2-factor repeated measures ANOVA with time and treatment as the 2 factors [significant main effects of time x treatment ($P < 0.0001$), time ($P < 0.0001$) and treatment ($P < 0.05$)]. Post hoc analysis were conducted using Bonferroni multiple comparisons test. * $P < 0.05$ OJ significantly different from control at the 7 h; ** $P < 0.01$ FROJ and WO significantly different from control at 7 h. $^{\#}$ Significant decrease in FMD response in relation to baseline levels for both control (at 2, 5 and 7 h; $P < 0.0001$, $P < 0.05$, $P < 0.0001$ respectively) and all three flavanone interventions (at 2 h; $P < 0.05$). FMD, flow-mediated dilatation; OJ, orange juice; FROJ, flavanone-rich orange juice; WO, whole blended orange.

Figure 3: Plasma flavanone profile following postprandial consumption of flavanone beverages containing either 128.88 mg of flavanones (OJ); 272.14 mg of flavanones (FROJ); 452.80 mg of flavanones (WO) or a macronutrient and micronutrient-matched control in middle aged healthy men (n=20). A) Hesperetin, B) Naringenin. Data are presented as mean ± SEM and analyzed using a 2-factor repeated measures ANOVA with time and treatment as the 2 factors [significant main effects of time x treatment ($P < 0.0001$), time ($P < 0.0001$) and treatment ($P < 0.001$)]. Post hoc analysis were conducted using Bonferroni multiple comparisons test. Hesperetin levels are significantly higher in all treatments in comparison to control at 5 and 7 h ($^{\$}$ $0.00 < P < 0.02$), whilst Naringenin levels are significantly

higher in all treatments in comparison to control at 2, 5 and 7 h ($^{\$}$ 0.00 < *P* < 0.03). *** *P* < 0.001, ** *P* < 0.01: levels of plasma Naringenin are significantly higher in OJ in comparison to WO. $^{\#}$ *P* < 0.1: levels of plasma Hesperetin in FROJ and WO show a trend towards higher values then OJ. OJ, orange juice; FROJ, flavanone-rich orange juice; WO, whole blended orange.

Figure 4: Plasma Nitric Oxide levels following postprandial consumption of flavanone beverages containing either 128.88 mg of flavanones (OJ); 272.14 mg of flavanones (FROJ); 452.80 mg of flavanones (WO) or a macronutrient and micronutrient-matched control in middle aged healthy men (n=28). A) Nitrite levels (nmol/L), B) Nitrate levels (μmol/L) , C) Nitroso species (RNXO) including nitrosothiols, nitrosamines, iron-nitrosylhemoglobin and nitrosohemoglobin (nmol/L). Data are presented as mean ± SEM and expressed as change from baseline. Data were analyzed using a 2-factor repeated measures ANOVA with time and treatment as the 2 factors [significant main effects of time (*P* < 0.0001)]. Post hoc analysis were conducted using Bonferroni multiple comparisons test. ** *P* < 0.01 Nitrite levels are significantly different from baseline only for the control group at the specified time points. $^{\#}$ Nitrate levels are significantly different from baseline for both control and all three flavanone treatents (*P* < 0.0001) at the specified time points. OJ, orange juice; FROJ, flavanone-rich orange juice; WO, whole blended orange.