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Short Communication

Platelet Reactivity in Male Smokers Following the Acute Consumption of a Flavanol-Rich Grapeseed Extract

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ABSTRACT Epidemiological studies suggest that a high dietary intake of flavanols, a subclass of flavonoids, is associated with reduced risk of vascular disease. Clinical studies have also shown that the consumption of certain flavanol-rich foods (*e.g.*, cocoa, tea, red wine), as well as intake of the individual flavanol (-)-epicatechin, can result in improvement in a number of parameters associated with vascular disease, including improved endothelial function, reduced platelet reactivity, and reduced oxidative stress. The present study assessed the effects of a flavanol-rich supplement on platelet reactivity and plasma oxidant defense in a group of smokers, a population at an elevated risk for vascular disease. Male smokers were randomly assigned to a placebo ($n = 10$) or a flavanol-rich grapeseed extract (FRGSE; $n = 13$) group, and after an overnight fast, blood samples were collected before and at 1, 2, and 6 hours following consumption of the placebo or supplement. The FRGSE supplement, but not the placebo, significantly decreased ADP-stimulated platelet reactivity at 1, 2, and 6 hours following intake ($P < .05$) compared to baseline levels. Similarly, the supplement, but not the placebo, decreased epinephrine-stimulated platelet reactivity 2 hours following consumption. Plasma antioxidant capacity (total radical trapping antioxidant potential), lipid oxidation (plasma thiobarbituric acid-reactive substances), and serum uric acid concentrations were not affected in either group. Thus smokers may obtain some health benefits from the consumption of certain flavanol-rich foods, beverages, and supplements.

KEY WORDS: • ***cardiovascular disease*** • ***dimers*** • ***epicatechin*** • ***flavanols*** • ***platelet function*** • ***procyanidin*** • ***vascular function***

INTRODUCTION

Recent and past epidemiological evidence strongly suggests that the intake of flavanol-rich foods is associated with a reduced risk for cardiovascular disease.[1–4] Flavanol-induced reductions in oxidative stress, platelet reactivity, endothelial function, blood pressure, and measures of glucose sensitivity and insulin resistance have all been reported following the incorporation of flavanol-rich food products into the diet.[5–9] It is therefore reasonable to speculate that readily absorbed flavanol-rich foods or beverages can augment vascular function, particularly in at-risk populations such as those with hypertension, high cholesterol, diabetes, or metabolic syndrome and smokers.

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The average daily flavonoid intakes have been estimated to be around 240 mg/day, though this can vary widely depending on fruit, vegetable, cocoa, tea, and red wine intakes.[3] Flavanols are a subset of flavonoids, and recent data suggest that typical daily intake of flavanols range from 10 to 40 mg/day.[3,4] Flavanols consist of monomers (*e.g.*, catechins and epicatechins) which are relatively well absorbed,[4] and larger oligomers, which are thought to be poorly absorbed.[4,10,11] Supplementation with the isolated flavanol, (-)-epicatechin, can enhance endothelial function and increase circulating levels of nitric oxide (NO) species in healthy adult subjects to extents similar to those observed with a flavanol-rich cocoa extract.[12] It is currently thought that long-chain flavonoids can only be absorbed if they are degraded into monomer or dimer units. Specifically, for flavanols and their longer-chained procyanidin (dimer and longer) oligomers, radiolabeled monomers, dimers, and trimers were transported across a layer of Caco-2 cells at a rate approximately 10-fold greater than six-unit oligomers.[13]

Perfusion for 90 minutes of a flavanol-rich cocoa extract high in dimers through the rat small intestine resulted in conversion of more than 95% of total passing flavanols to be the unconjugated monomer, epicatechin.[10]

In the present study, a flavanol-rich grapeseed extract (FRGSE) was produced from grapeseed extract whose flavanol chains were deoligomerized in a process that yielded five times more monomers, 50 times more dimers, and seven times more trimers than the originating grapeseed extract. Acute supplementation of this FRGSE produced significantly higher total plasma phenolics in an experimental animal model, as well as in healthy adult humans, compared to the levels obtained following the intake of the starting grapeseed extract.[14]

Smoking is recognized as a major risk factor for the development of vascular disease. An estimated 30% of coronary heart disease in the United States has been attributed to cigarette smoking.[15] In general, smokers are more predisposed to develop angina, coronary syndromes, sudden death, stroke, and aortic and peripheral atherosclerosis than nonsmokers.[16] A number of smoking-related clinical manifestations, including vascular dysfunction, prothrombotic states, decreased NO availability, systemic inflammation, and modification of lipid profiles, can be modulated by lifestyle and diet, which could in turn reduce vascular-related morbidities and mortality. Acute dietary flavanol supplementation has been reported to improve endothelial dysfunction in smokers,[7] suggesting that a diet rich in high-flavanol foods would likely be beneficial in modulating some effects of smoking on vascular function. Unfortunately, smokers tend to have low intakes of flavonoid- and flavanol-rich fruits, vegetables, and beverages.[17–19] The most appropriate health advice for smokers is to restrict, or better yet eliminate, use of tobacco products. Many health professionals also advise current smokers to consume a dietary supplement containing vitamins and minerals. This may be particularly important for some vitamins such as folate and vitamin C, as the oxidative stress associated with smoking can increase the requirement for these nutrients.[20] Given the reported positive vascular effects associated with the consumption of high flavanol diets, we investigated the effects of an acute dose of an FRGSE on platelet reactivity and plasma antioxidant capacity in male smokers.

MATERIALS AND METHODS

Subjects

Twenty-three male smokers (23.1 ± 1.9 years old, weighing 82.9 ± 2.1 kg, with a body mass index [BMI] of 25.6 ± 0.6 kg/m^2 and smoking 8.1 ± 1.1 cigarettes/day [mean ± SEM]) were recruited from the greater Sacramento metropolitan area using newspaper advertisements and flyers on campus at the University of California, Davis. Participants had no history of cardiovascular disease, thyroid disorder, diabetes mellitus, or other chronic illnesses. They did not report taking antioxidant supplements for at least 1 month prior to the study or consuming flavanol-rich foods or beverages for at least 10 days prior to the experiment. No aspirin was permitted for at least 4 days prior to the study, since aspirin affects platelet-related primary hemostasis, a key outcome measure of the present study. Informed consent and protocols were approved by the Institutional Review Board at the University of California, Davis.

Study design

A randomized, double-blind, parallel-arm study was conducted with two groups. A treatment group consumed 400 mg (2 × 200-mg capsules) of an FRGSE (Oligonol™, Amino Up Chemical Co., Sapporo, Japan), and a placebo group consumed 400 mg (2 × 200-mg capsules) of maltose with identical size and appearance to treatment capsules. Oligonol was prepared by a novel method that yielded higher levels of flavanol monomers and dimers than were found in the starting grapeseed extract material. The underlying principle for processing extracts in this way is that it should, in theory, result in a higher fraction of the extract that is available for absorption. High-performance liquid chromatography analysis showed that two capsules provided approximately 70 mg of flavanol monomers and 60 mg of a combination of dimers plus trimers.[21] Following an overnight fast and completion of a health questionnaire on tobacco usage and dietary intake, baseline (0 hour) blood was collected, after which participants consumed the two treatment or placebo capsules with a small, plain buttered bagel and 8 ounces of purified drinking water. Additional blood samples were collected 1, 2, and 6 hours post-consumption. A 6-ounce turkey and cheese sandwich was provided following the third (2 hours) blood draw, and participants had access to purified drinking water *ad libitum*.

Sample collection

At each time point, 30 mL of blood was collected by venipuncture into evacuated tubes containing sodium citrate (for whole blood) and either EDTA or sodium heparin (for plasma). Separators were used to obtain serum. Samples were cooled in ice, centrifuged (1,800 *g* at 4°C for 10 minutes), and aliqouted within 1 hour of collection. For determination of plasma thiobarbituric acid-reactive substances (TBARS) (see below), 1 mL of plasma was mixed with 0.25 mL of 4% (wt/vol) butylated hydroxytoluene/ethanol and stored at –80°C until analysis.

Anthropometric measurements

Height was recorded using a wall-mounted stadiometer (model S-100, Ayrtron Corp., Prior Lake, MN), weight was measured using an electronic scale (Scale-tronix™ 6002, Scale-tronix Corp., Carol Stream, IL), and an automated blood pressure (BP) machine (Critikon™ Vital Signs Monitor 1846 SX, Critikon Corp., Tampa, FL) was used to record BP and pulse (average of three measurements).

Clinical laboratory measurements

Serum lipid and uric acid concentrations and whole blood complete blood count (CBC) without differential were measured at the University of California, Davis Medical Center clinical pathology laboratory using a Synchron LX-20 System (Beckman Coulter, Inc., Brea, CA).

Platelet function analysis

Duplicate 1.0-mL whole blood samples were measured for each time point. Platelet function was analyzed using a PFA-100™ (Dade Behring, Inc., Deerfield, IL) as described previously.[22] Briefly, the test measures primary platelet-related hemostasis as the time required for whole blood to occlude an aperture in a collagen membrane cartridge under shear conditions resembling those found near branching points in the human cardiovascular system when first stimulated with ADP or epinephrine (EPI). Blood samples were undisturbed for at least 30 minutes prior to analysis and assayed within 4 hours of collection.

TBARS

Plasma TBARS were determined as previously described.[23] Briefly, 100 μL of plasma, 200 μL of 3% sodium dodecyl sulfate, 800 μL of 0.1 mol/L HCl, 100 μL of 10% (wt/vol) phosphotungstic acid, and 400 μL of 0.7% (wt/vol) 2-thiobarbituric acid were combined, vortex-mixed, and incubated at 95°C for 30 minutes. Samples were cooled in ice and mixed with 1 mL of 1-butanol. After centrifugation (1,800 *g*, 10 minutes, 4°C), 200 μL of the butanol phase was separated and analyzed spectrofluorometrically (excitation at 515 nm; emission at 555 nm) using a plate reader (Cetus, Perkin-Elmer, Norwalk, CT). TBARS were measured as malondialdehyde equivalents, and the final index was calculated relative to serum triglycerides, since TBARS are active in the lipid-soluble fraction of the plasma.

Total radical trapping antioxidant potential (TRAP)

Plasma TRAP was measured as previously described.[23,24] Heparinized plasma was assessed for its ability to inhibit the chemiluminescence produced by a mixture of 2,2′-azobis(2-amidinopropane) dihydrochloride and luminol. Chemiluminescence was measured in a liquid scintillation counter (Wallac 1410, Wallac Oy, Turku, Finland), and Trolox was used as a positive control. Plasma antioxidant capacity was calculated as the lag time prior to the increase in observed chemiluminescence and was considered proportional to the cumulative amount of antioxidant present in the plasma.

Data analysis

Data are presented as mean ± SEM values and were analyzed by SigmaStat for Windows, version 2.03 (SPSS, Chicago, IL). Baseline characteristics were compared using unpaired *t* tests for individual parameters. One-way repeated measures analysis of variance (ANOVA) was performed, and Student's *t* test was used to assess differences between baseline and post-intervention values. Transformations were conducted when appropriate. Differences were considered significant at $P \leq .05$.

RESULTS

Subjects

Twenty-three subjects were randomly enrolled in the study; 10 received the placebo, and 13 received the FRGSE. No differences between groups existed at enrollment with respect to age, weight, BMI, systolic and diastolic BP, years smoking, or the number of cigarettes smoked/day (Table 1). Clinical measures of serum total cholesterol (189 ± 12 vs. 185 ± 10 mg/dL), low-density lipoprotein (112 ± 11 vs. 102 ± 12 mg/dL), high-density lipoprotein (44 ± 4.9 vs. 52 ± 5.1 mg/dL), total cholesterol:high-density lipoprotein ratio (4.6 ± 0.5 vs. 3.8 ± 0.3), non–high-density lipoprotein cholesterol (145 ± 12 vs. 136 ± 10 mg/dL), and uric acid (5.9 ± 0.4 vs. 6.2 ± 0.3 mg/dL) were similar in the placebo and FRGSE groups, respectively. All indices of the CBC were normal in all men. The placebo group had significantly higher serum triglycerides at baseline than the FRGSE group (162 ± 80 vs. 102 ± 56 mg/dL; $P = .043$). Reported dietary intakes and vitamin and supplement usage were similar between the two groups (data not shown).

Platelet-related primary hemostasis

No significant differences were measured between the placebo and FRGSE group at baseline for hemostasis stimulated by ADP-collagen (82.8 ± 17 vs. 73.8 ± 20 seconds) and EPI-collagen (146 ± 85 vs. 134 ± 61 seconds), respectively. Compared to baseline values, consumption of 400 mg of the FRGSE was associated with a significant increase in ADP-stimulated clotting time at 1, 2, and 6 hours after baseline: 13.5 ± 4.1% ($P = .01$), 13.1 ± 3.5% ($P = .032$), and 15.6 ± 4.1% ($P = .002$), respectively (Fig. 1). Consumption of the placebo had no effect on platelet-related primary hemostasis. Similarly, consumption of the FRGSE, but not the placebo, resulted in a significant increase in EPI-simulated

Table 1. Baseline Characteristics

	Placebo (n = *10*)	*FRGSE* (n = *13*)
Age (years)	24.3 ± 3.1	21.9 ± 1.3
Weight (kg)	87.4 ± 3.3	79.6 ± 2.8
BMI (kg/m^2)	26.7 ± 1.0	24.8 ± 1.0
BP (mm Hg)		
Systolic	124 ± 3.6	118 ± 4.0
Diastolic	70 ± 2.8	65 ± 2.8
Years of smoking	7.9 ± 3.1	5.3 ± 1.4
Cigarettes/day	7.9 ± 2.0	8.3 ± 1.5

Data are mean ± SEM values.

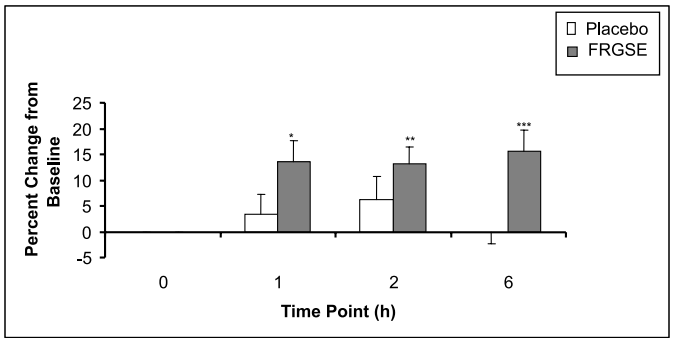

FIG. 1. Percentage change of ADP-collagen-stimulated clotting time. Data are mean ± SEM values for placebo ($n = 10$) and FRGSE ($n = 13$) groups. *$P = .01$, **$P = .032$, ***$P = .002$, significantly different from baseline within treatment.

clotting time of 14.9 ± 7.8% ($P = .032$) at 2 hours post-consumption (Fig. 2).

TBARS

Neither the FRGSE nor the placebo affected the concentration of TBARS when they were calculated relative to serum triglyceride levels.

TRAP

Neither the FRGSE nor the placebo significantly affected plasma TRAP values. Independent of the treatment group, serum uric acid (urate) was significantly related to plasma TRAP values ($R^2 = 0.542$, $P = .001$).

DISCUSSION

A reduction in platelet reactivity following the intake of the FRGSE is consistent with previous reports of improved platelet function and platelet-related primary hemostasis following consumption of flavanol-rich foods,[5] flavanol-rich cocoa,[25,26] chocolate,[11] purple grape juice,[27] and grape seed and grape skin extract.[28,29] In addition to decreased platelet reactivity, flavanols have been reported to trigger other improvements in vascular function, including enhancement of endothelial function after acute[9] and chronic intake,[30,31] protection against oxidized low-density lipoprotein damage to vascular endothelial cells,[32] MP-dependent vasodilation,[33,34] and reductions in BP.[35] Our observation that ADP- and EPI-stimulated primary platelet hemostasis was not related to changes in plasma antioxidant activity or lipid oxidation suggests that the effects of the FRGSE on platelet function may be acting through non–redox-related mechanisms.

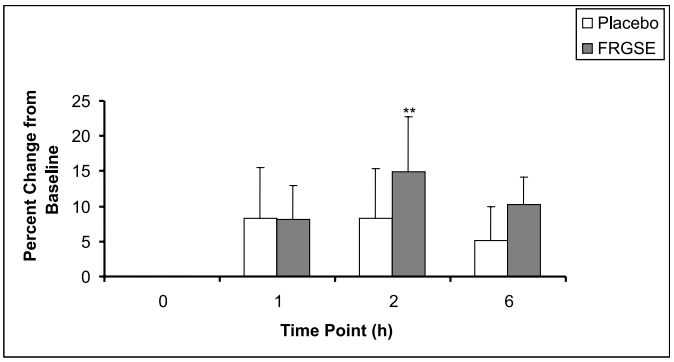

FIG. 2. Percentage change of EPI-collagen-stimulated clotting time. Data are mean ± SEM values for placebo ($n = 10$) and FRGSE ($n = 13$). **$P = .032$, significantly different from baseline within treatment.

Consistent with the results of the present study, intake of flavanol-rich cocoa has been shown to reduce ADP- and EPI-stimulated platelet reactivity within a 2–6-hour period in nonsmokers.[11,26,36] The anti-aggregatory effects of flavanols were associated with a reduction in ADP- and EPI-induced expression of platelet GPIIa/IIIb surface protein to an extent similar to that achieved by low-dose (81 mg) aspirin.[26,37]

Flavanols may function in a number of ways, involving both redox- and non–redox-related processes. As antioxidants, flavanol-rich extracts from tea,[38] cocoa,[38–40] and grapeseed[41] can scavenge free radicals *in vitro* and *in vivo*. Smoking is associated with increased oxidative stress and decreased antioxidant capacity,[42–44] and vitamin C supplementation in smokers for 4 weeks has been reported to decrease plasma TBARS.[45] However, in the present study we did not observe any changes in plasma antioxidant capacity as measured by plasma TBARS and TRAP following the acute consumption of the FRGSE. In contrast, a significant decrease in plasma TBARS was reported in healthy nonsmokers ingesting a flavanol-rich chocolate[36] and in smokers ingesting a grapeseed extract.[46] Potential explanations for the differences between studies include different amounts of total and individual flavanols and different time periods tested.

In conclusion, the addition of flavanol-rich food and supplements to one's diet may be health-promoting, improving vascular parameters such as endothelial function and NO availability.[34,47] In the present study, male smokers consuming a novel FRGSE showed favorable changes in platelet-related primary hemostasis. The amount of flavanols delivered by the FRGSE did not influence plasma antioxidant capacity on an acute basis. A higher dose of flavanols or a different combination of flavanols may be preferable to the extract that was tested in this study. Supplementation of flavanols by at-risk populations (*e.g.*, smokers, diabetics, postmenopausal women), in addition to healthy subjects, warrants further study.

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