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Consumption of both low and high (−)-epicatechin apple puree attenuates platelet reactivity and increases plasma concentrations of nitric oxide metabolites: A randomized controlled trial

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ABSTRACT

We hypothesised that consumption of flavanol-containing apple puree would modulate platelet activity and increase nitric oxide metabolite status, and that high flavanol apple puree would exert a greater effect than low flavanol apple puree. 25 subjects consumed 230 g of apple puree containing 25 and 100 mg epicatechin (low and high flavanol apple puree, respectively) and aspirin (75 mg) in random order. Measurements were made at baseline, acutely after treatment (2, 6 and 24 h), and after 14 d of treatment. Low flavanol apple puree significantly attenuated ADP and epinephrine-induced integrin-β3 expression 2 h and 6 h after consumption and ADP and epinephrine-induced P-selectin expression within 2 h of consumption. High flavanol apple puree attenuated epinephrine and ADP-induced integrin-β3 expression after 2 and 6 h. ADP and epinephrine-induced integrin-β3 expression was significantly attenuated 2, 6 and 24 h after consumption of aspirin, whilst 14 d aspirin consumption attenuated collagen-induced P-selectin expression only. The plasma total nitric oxide metabolite conc. was significantly increased 6 h after consumption of both low and high flavanol apple purees. In conclusion, consumption of apple purees containing ⩾25 or 100 mg flavanols transiently attenuated ex vivo integrin-β3 and P-selectin expression and increased plasma nitric oxide metabolite conc. in healthy subjects, but the effect was not enhanced for the high flavanol apple puree.

Introduction

Cardiovascular disease (CVD) is one of the major causes of mortality worldwide; for example in both the US and the UK CVD accounts for around 35% of all deaths. The aetiology of CVD is multi-factorial but there is accumulating evidence from epidemiological investigations to suggest an association between improved cardiovascular health and a diet rich in flavanols [1,2]. Flavanols are one of the 6 sub-classes of flavonoids and comprise monomeric flavanols or 'catechins' (catechin and epicatechin, and gallo and galloylated derivatives such as epigallocatechin gallate) and oligo-/polymeric flavanols collectively known as proanthocyanidins. Numerous short and long term intervention studies using healthy volunteers and at risk groups have reported on the effects of a variety of flavanol-rich plant foods and extracts on risk markers for CVD, and flavanols have been ascribed with various cardio-protective properties including anti-inflammatory [3] and anti-platelet activities [4,5], and the ability to reduce LDL oxidation [6] and improve endothelial function [7]. In a meta-analysis of randomized controlled trials designed to test the effects of flavonoids on established markers of CVD risk [8], cocoa and chocolate were shown to significantly lower blood pressure and improve flow mediated dilation (FMD)[1] [9] of the brachial artery. FMD, a measure of endothelial function in humans, is almost exclusively mediated by

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[1] *Abbreviations used:* ASA, aspirin; CRP, C-reactive protein; ET-1, endothelin-1; HF-apple puree, high flavanol apple puree; LF-apple puree, low flavanol apple puree; FMD, flow mediated dilation; TXA_2, thromboxane A_2; MPA, meta-phosphoric acid.

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nitric oxide (NO) [10], a potent vasodilator. Cocoa consumption has been shown to cause acute increases in endothelial NO production [11] and there is evidence to show that this is likely mediated by epicatechin [7].

Platelet activation and subsequent aggregation is a critical event occurring in the pathogenesis of CVD [12]. Platelet aggregation is mediated by a surface fibrinogen receptor (integrin-β3). Once activated integrin-β3 binds fibrinogen and von Willebrand factor which are secreted in response to endothelial cell damage. The activated platelets in turn synthesize and secrete thromboxane A_2 (TXA_2) and intracellular ADP which, combined with conformational changes in the glycoprotein complex, result in platelet aggregation. Furthermore, P-selectin (an adhesion molecule situated in the membrane of the platelet α-granule) is mobilised to the platelet surface where it mediates platelet-leukocyte adhesion. Anti-platelet therapies such as aspirin, which suppresses the production of TXA_2, and integrin-β3 inhibitors, are well recognized therapeutic regimes used in the prevention and treatment of cardiovascular disorders. Flavanol-rich foods and beverages such as green tea, cocoa (and cocoa products) and grape juice have also been shown to modulate platelet function [4,13–17]. Evidence for the anti-aggregatory effects of flavanol rich foods are arguably strongest for cocoa which has been shown to lower ADP and epinephrine activated platelet aggregation within 2–6 h of consumption [15,18], the effects of which were shown to be associated with a reduction in the expression of the surface protein integrin-β3. Flavanols mediate platelet function through a variety of mechanisms. Purple grape juice [17] and cocoa [19] for example have been shown to inhibit platelet protein kinase C and human platelet 12-lipoxygenase activity. Additionally, chocolate consumption has been shown to favourably affect eicosanoid synthesis providing evidence that flavanols may possess anti-inflammatory properties [20].

Flavanols are the major flavonoids consumed by humans, with their contribution to mean total flavonoid intakes estimated at >80% [21]. The main contributors to flavanol intakes in Western Europe are chocolate, apples, red wine and tea. Apples are widely consumed and therefore an important source of flavanols in the diet. In this study we investigated the acute and long term effects of apples (delivered as a puree) containing two different levels of flavanols (25 and 100 mg epicatechin), on platelet function, serum lipid profiles, and plasma concentrations of nitric oxide metabolites, CRP, vitamin C and endothelin-1.

Experimental methods

Unless specifically stated all measurements were conducted at the Institute of Food Research in Norwich.

Chemicals and reagents

The conjugated antibodies CD61 and CD62 were purchased from Invitrogen (Paisley, UK) and Pac-1 from Becton Dickenson (Oxford, UK). ADP, epinephrine and collagen were supplied by Biodata (Alpha laboratories, Hampshire, UK). The Chemiluminescent ET-1 immunoassay kit was supplied by R&D systems (Abingdon, UK). The enzymes β-glucuronidase (*Helix pomatia type H5*), and sulfatase (*H. pomatia type H1*), were purchased from Sigma–Aldrich (Poole, UK) and β-glucuronidase, (*type IX-A from E. coli*) and sulfatase, (*type VIII from Abalone Entrails*) from Sigma (St. Louis, MO, USA). The phenolic standards (taxifolin, (−)-epicatechin, sinapic acid, galangin, (+)-catechin, phloretin) were obtained from Extrasynthese (Genay, France). All other chemicals were of analytical or HPLC grade.

Participants and study design

Forty-seven potential participants were assessed for eligibility on the basis of a health questionnaire and the results of clinical laboratory tests. The exclusion criteria were as follows: smoking; medical conditions such as gastrointestinal disease, history of ulcers and gastro-intestinal bleeding, diabetes, cancer, heart disease, stroke, asthma or hay fever; regular use of aspirin (at least once a week), antacids or laxatives; dietary supplements (unless prepared to cease for 1 month preceding and throughout the study); clinical results at screening judged to affect the study outcome or be indicative of a health problem. Nineteen of the forty-seven potential participants were excluded from this study. Seventeen did not meet the inclusion criteria at eligibility assessment and two, even though eligible, declined to participate with no reason given. Of the twenty-eight participants randomized to treatment, three were withdrawn by the researcher during the study (1 because aspirin consumption was contra-indicated, 1 developed anaemia and 1 developed an allergy) (see Fig. 1 for progress of participants through the phases of the crossover trial). Characteristics of the twenty-five participants (13 men and 12 women) who completed the study were (mean ± SD); weight 77.5 ± 13.8 kg (range 54.8–108.4 kg), BMI 25.9 ± 4.2 kg/m^2 (range 20.9–33.5 kg/m^2) and age 42 ± 11 years (range 23–64 y). This study was conducted in the Human Nutrition Unit at the Institute of Food research, (UK) according to the guidelines laid down in the declaration of Helsinki and all procedures involving human subjects were approved by the Human Research Governance Committee of the Institute of Food Research and the Norfolk Research Ethics Committee (ref no: 06/Q0101/22). Each participant gave written informed consent prior to taking part in the trial. The trial is registered with clinicaltrials.gov (NCT00568152).

The study was a randomized, three-phase crossover design, investigating the acute and long term effects of consuming apple puree containing different amounts of flavanols on risk markers for cardiovascular disease. An aspirin treatment was used as a positive control. Each of the three test phases comprised a 4-week period of intervention followed by a washout period of at least 2 weeks. During each period of intervention participants excluded from the diet food sources that are rich in flavanols (e.g. cocoa, berries, grapes, red wine, apples and legumes) and limited others (e.g. tea and coffee). In addition, consumption of alcohol and oily fish were also limited. However, these foods and beverages were completely excluded from the diet 48 h before blood sampling. A list of authorized and prohibited foods was given and compliance was monitored with the use of food diary records. Participants were assessed at the start (d 1), middle (d 15) and end (d 29) of the intervention period. On d 1 of each intervention period a fasting blood sample was obtained following which participants commenced the low flavanol diet as described above. On d 15 of the intervention period fasted participants had an intravenous cannula inserted and a baseline blood sample (0 h) was obtained. Participants were given a standard breakfast consisting of 2 slices of white toast (72 g) with spread (10 g) followed by either, 230 g apple puree containing 100 mg epicatechin (HF-apple puree), 230 g apple puree containing 25 mg epicatechin (LF-apple puree) or aspirin (75 mg dispersed in 100 mL water). To limit variation in food and drink intakes, participants refrained from drinking and eating for 2 h after the treatment. Further blood samples were collected at 2, 6 and 24 h. For 24 h before and after consumption of the apple puree, participants were instructed to collect all urine passed. Daily consumption of the respective treatment was continued until d 29 of the intervention period following which another fasted blood sample and 24 h urine collection was obtained.

On d 1, 15 and 29 fasting blood samples were collected for assessment of plasma C-reactive protein (CRP), vitamin C, lipid