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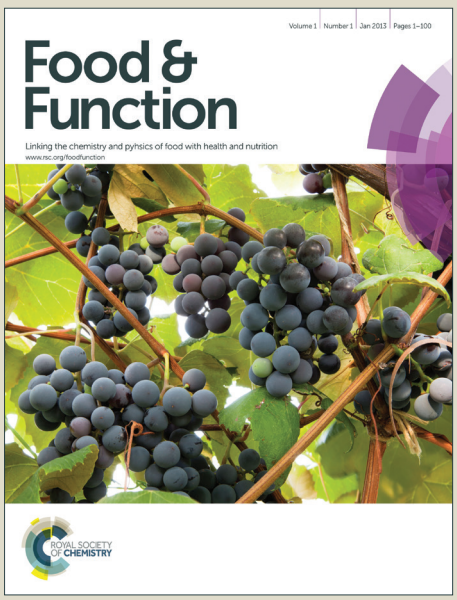

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The acute effect of flavonoid-rich apples and nitrate-rich spinach on cognitive performance and mood in healthy men and women

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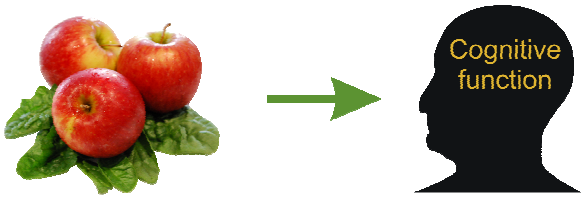
Flavonoid-rich apples and nitrate-rich spinach augment NO status acutely with no concomitant improvements or deterioration in cognitive function and mood.



ABSTRACT

Flavonoids and nitrate in a fruit and vegetable diet may be protective against cardiovascular disease and cognitive decline through effects on nitric oxide (NO) status. The circulating NO pool is increased via distinct pathways by dietary flavonoids and nitrate. Our aim was to investigate the acute effects of apples, rich in flavonoids, and spinach, rich in nitrate, independently and in combination on NO status, cognitive function and mood in a randomised, controlled, cross-over trial with healthy men and women (n=30). The acute effects of four energy-matched treatments (control, apple, spinach and apple+spinach) were compared. Endpoints included plasma nitric oxide status (determined by measuring *S*-nitrosothiols+other nitroso species (RXNO)), plasma nitrate and nitrite, salivary nitrate and nitrite, urinary nitrate and nitrite as well as cognitive function (determined using the Cognitive Drug Research (CDR) computerized cognitive assessment battery) and mood. Relative to control, all treatments resulted in higher plasma RXNO. A significant increase in plasma nitrate and nitrite, salivary nitrate and nitrite as well as urinary nitrate and nitrite was observed with spinach and apple+spinach compared to control. No significant effect was observed on cognitive function or mood. In conclusion, flavonoid-rich apples and nitrate-rich spinach augmented NO status acutely with no concomitant improvements or deterioration in cognitive function and mood.

Keywords: nutrition, flavonoids, nitrate, nitric oxide, cognitive function, mood

Introduction

Diet has a significant impact on cardiovascular disease and neurodegenerative disorders. With the increasing prevalence of these diseases, the identification of components of a healthy diet that can prevent or reduce their severity is of mounting scientific and public importance. A higher intake of fruit and vegetables has been linked to reduced risks of both cardiovascular disease [1-3] and cognitive decline [4-6]. Not fully understood are the components of fruit and vegetables responsible for these benefits. Flavonoids [7] and nitrate [8] are two candidates that could mediate their beneficial effects through augmentation of nitric oxide (NO) status both chronically and acutely.

NO plays a critical role in vascular health via effects on vasodilation and blood flow [9]. It is also an important neurotransmitter [10]. An imbalance of NO is associated with a number of cardiovascular disorders [11] as well as pathological conditions in the brain [10]. In addition, cardiovascular disease or the presence of its risk factors appears to contribute to cognitive decline [12]. Whether this is related to alterations in NO homeostasis in both conditions is unknown.

NO is derived from both endogenous [13] and exogenous sources [8]. Flavonoids may augment endogenous endothelial-derived NO [7, 14, 15] and nitrate is the primary source of exogenous NO [16-18]. Flavonoids and dietary nitrate augment NO status with concomitant functional effects including a reduction in blood pressure and improvement of endothelial function [19]. These are major risk markers for cardiovascular disease. NO also plays a key role in cerebral blood flow and cognitive function, mediating the neurovascular coupling of neuronal activity to increased blood supply [20, 21]. The increase in NO status following consumption of flavonoids and dietary nitrate could improve measures of cognitive function and mood.

Apples are an important contributor to total flavonoid intake [22, 23] and green leafy vegetables, including spinach, are high in dietary nitrate [24, 25]. Evidence suggests that flavonoids and nitrate alone and in combination could increase NO production [7, 26-28]. The aim of this study, therefore, was to investigate the acute effects of apples, rich in flavonoids, and spinach, rich in nitrate, independently and in combination on NO status, blood pressure, endothelial function, cognitive function and mood in healthy men and women. The effect of apple and spinach on plasma RXNO, blood pressure and endothelial function has previously been reported [19]. Here we report the acute effect of apples, rich in flavonoids, and spinach, rich in nitrate, independently and in combination on NO status, cognitive function and mood in healthy men and women. We hypothesized that the flavonoids in apple and the nitrate in spinach would both augment NO status and that this would contribute to acute improvements in cognitive function and mood.

Methods

Participants

Healthy volunteers (n=30) were recruited by newspaper advertisement from the Perth general population. Screening was conducted prior to enrolment within the University of Western Australia, School of Medicine and Pharmacology located at Royal Perth Hospital and consisted of a standard medical history questionnaire, routine laboratory analysis of a fasting blood sample, electrocardiography, height, weight, body mass index (BMI) and blood pressure measurement. Volunteers were excluded according to the following criteria: current smoking, BMI <18 or >35 kg/m^2, systolic blood pressure (SBP) <100 or > 160 mmHg, diastolic blood pressure (DBP) <50 or > 100 mmHg, history of cardiovascular or peripheral vascular disease, use of antihypertensive medication, any major illness such as cancer, psychiatric illness, diagnosed diabetes, non-diabetic individuals with fasting plasma glucose concentrations ≥ 5.5 mmol/L, weight gain or loss >6% body weight within previous 6 months of the study, > 30g/day alcohol consumption or woman who were pregnant, lactating or wishing to become pregnant during the study. The screening visit also involved completion of the Cognitive Drug Research (CDR) computerised assessment system[29] test battery twice in order to familiarise participants with the test procedure as well as control for practice effects. Participants were asked to avoid the use of mouth wash for the duration of the study period starting on week prior to their first visit. The study was carried out in accordance with the Declaration of Helsinki and was approved by the University of Western Australia Human Research Ethics Committee. Participants provided written informed consent before inclusion in the study. The trial was registered with the Australian New Zealand Clinical Trials Registry (ACTRN: 12609000425291).

Study design

The study followed a randomised controlled cross-over (latin-square) design. Study participants were assigned to an intervention plan via block randomisation using computer-generated random numbers devised by a statistician. Each participant completed four visits with a minimum washout period of 1-week. The evening meal before each study visit was consistent across all study days. Due to the different absorption kinetics of the different forms of quercetin present in apples, the two apple interventions (the low flavonoid apple control and the high flavonoid apple active) were consumed with breakfast and with lunch. Breakfast and lunch were timed so that flavonoid concentrations would peak in the blood stream during the testing period[30, 31]. On the morning of the study visits, breakfast comprised a low flavonoid / low nitrate meal together with an apple intervention. Study participants were provided with a standard low flavonoid / low nitrate lunch together with the randomly allocated nitrate / flavonoid intervention four hours post breakfast. Adherence to study protocol was verified with a food diary. A saliva sample was taken 120 min post lunch / intervention for analysis of salivary nitrate and nitrite. A plasma sample was taken 140 min post lunch / intervention for analysis of plasma *S*-nitrosothiols and other nitroso species (RXNO), nitrate and nitrite. Cognitive function and mood measures were performed 150 min post lunch / intervention. Urine was collected from breakfast to the end of the study period (8 hour sample) for analysis of urinary nitrate and nitrite.

Interventions

Participants were provided with four interventions in random order: (1) Control: low flavonoid apple control and low nitrate control; (2) Apple: high flavonoid apple active and low nitrate control; (3) Spinach: low flavonoid apple control and nitrate-rich spinach active; (4)

Apple+spinach: high flavonoid apple active and nitrate-rich spinach active. Apple flavonoids, particularly quercetin and (-)-epicatechin, are located in high concentrations in the apple skin. The apple active intervention, rich in flavonoids, was prepared by homogenising apple skin (80 g) and apple flesh (120 g). The low flavonoid apple control consisted of apple flesh only. The total flavonoid content as well as flavonoid structures could be altered by cooking. To account for this, half of each dose was provided raw and the other half was provided cooked. After preparation, the apples were frozen at -20^{o}C and thawed prior to use. Both control and active apple interventions had a total energy of approximately 500 kJ. All apples used in this study were Cripps Pink marketed as Pink Lady® and were derived from two batches. 200 g spinach (204 kJ) was the spinach active intervention and was consumed with lunch. To account for variation in nitrate concentrations with season, method of cultivation and storage conditions, spinach was taken from a single batch of frozen spinach from a commercial supplier, and thawed prior to use. The energy matched low nitrate control for spinach was rice milk, also consumed with lunch. The same evening meal and breakfast was eaten by all participants consumed before each visit. Breakfast was a selection from three low flavonoid / low nitrate meals: oats and milk / non-fruit yoghurt; rice-bubbles and milk / non-fruit yoghurt or white bread toast with butter and a mild cheese. A low flavonoid / low nitrate lunch was provided with the intervention and consisted of a toasted white bread sandwich with chicken (skinless, 60g), mild cheese (30g) and mayonnaise (15 mL) [32].

Measurement of flavonoids in apple and nitrate in spinach

The polyphenolic compounds of the apple were extracted using a modified method described previously [33]. Flavonoid composition of the apple samples was determined using high performance liquid chromatography as previously described [19]. The apple active (apple flesh plus

skin) provided 184 mg of total quercetin glycosides and 180 mg of (-)-epicatechin. The apple control (apple flesh) provided less than 5 mg of total quercetin glycosides and (-)-epicatechin [19].

Nitrate concentration in the spinach was determined using a previously published gas chromatography-mass spectrometry (GC-MS) method [34]. Briefly, internal standards [^{15}N] sodium nitrite (6ng) and [^{15}N] sodium nitrate (40ng) were used to spike a blended spinach sample. Acetone and PFB-Br were used to derivatize the sample at 50°C for 40 min. The acetone was removed by evaporation under N_2 for 35 min and the remaining aqueous phase extracted with isooctane/toluene. 1 µl of the organic extract was analysed using an Agilent 6890 gas chromatograph coupled to a 5973 mass spectrometer fitted with a cross-linked silicone column (25 m x 0.20 mm, 0.33-mm film thickness, HP5-MS) using negative-ion chemical ionization. Peaks were identified using retention time and mass spectra with [^{15}N] sodium nitrite and [^{15}N] sodium nitrate as internal standards. Calibration curves from authentic and labelled standards were used to quantify the samples. Ion monitored were m/z = 62 and 63 for nitrate and [^{15}N] nitrate respectively and m/z = 46 and 47 for nitrite and [^{15}N] nitrite respectively. The spinach active contained 182 mg of nitrate and the control, rice milk, contained less than 5 mg nitrate [19].

Measurement of plasma nitrate, salivary nitrate and nitrite, urinary nitrate and nitrite

Plasma nitrate as well as nitrite and nitrate concentrations in saliva and urine were determined in frozen samples using a previously published gas chromatography-mass spectrometry (GC-MS) method [34] described above.

Measurement of plasma S-nitrosothiols and other nitroso species (RXNO) and nitrite

The concentrations of *S*-nitrosothiols and other nitroso species (RXNO) and nitrite in plasma were determined using a previously described gas-phase chemiluminescence assay [19].

Cognitive function and mood assessment

Cognitive performance was assessed using a tailored version of the Cognitive Drug Research battery (Bracket, Goring-on-Thames, UK)[29]. The CDR assessment battery has previously been found to be a particularly sensitive measure for the detection of changes to cognitive function associated with chronic nutraceutical and dietary interventions [35-37] as well as acute changes in cognitive function due to natural substances[38]. Presentation was via laptop computers and all responses were recorded via two-button (YES/NO) response boxes with the exception of the written word recall task. This test battery took approximately 20 minutes to complete, with the primary outcome measures being three cognitive factors 'Quality Working Memory', 'Power of Attention', and 'Continuity of Attention'[37]. The administered tests were word presentation, simple reaction time, digit vigilance, choice reaction time, spatial working memory, numeric working memory and delayed word recognition. In addition, participants completed the Bond–Lader mood scale[39]. A short description of these tests appears in supplementary information.

Other biochemical analyses

Routine biochemical analyses were performed at screening in the PathWest laboratory at Royal Perth Hospital, Western Australia. Serum total cholesterol, HDL cholesterol and triglycerides were measured using a routine enzymatic colorimetric test with a fully automated analyser (Roche Hitachi 917, Roche Diagnostics Australia Pty. Ltd., Castle Hill, New South Wales,

Australia). LDL cholesterol concentrations were calculated using the Friedewald formula [40]. Serum glucose was measured using an ultraviolet test with a fully automated analyser (Roche Hitachi 917).

Statistics

Plasma RXNO as the primary endpoint was used to calculate sample size. Based on our previous studies [7] and literature values [41] we expected that the SD for RXNO measurement would be approximately 15. Thirty subjects provided >80% power (at $\alpha = 0.05$) to detect a 12 nM equivalents difference in RXNO with a SD of 15. Thirty subjects also provided >80% power at $\alpha = 0.05$ to detect a 0.55 SD difference between interventions in salivary and urinary nitrate and nitrite as well as measures of cognitive performance and mood. For example, there was >80% power to detect a 27 mms difference in simple reaction time, a 0.12 unit difference in spatial memory and a 7 unit difference in Alertness. Statistical analyses were performed using SPSS 15.0 (SPSS Inc, Chicago, IL) and SAS 9.2 (SAS institute Inc., Cary, NC, USA). Non-normally distributed data were log-transformed prior to analysis. Participant characteristics are presented as mean±SD. Results in the text and tables are presented as mean (95% CIs) or geometric mean (95% CIs) for non-normally distributed variables. Results in figures are presented as mean±SEM or geometric mean (95% CIs) for non-normally distributed variables. Outcome variables were analysed with mixed models in SAS using the PROC MIXED command. Subject was included as a random factor in all models. All included fixed effects for intervention group (Control, Apple, Spinach, Apple+Spinach), intervention order and intervention period (1, 2, 3, 4). The models also included post-hoc adjustment for multiple comparisons using Tukey's adjustment. The effect of gender on outcomes was investigated by including gender as a class variable. Gender had no significant effect on the responses observed and was therefore not included in final models.

Results

Baseline and descriptive data

Recruitment began June 2009 and the study ended April 2010. Thirty participants (6 males, 24 females) completed the study (Fig.1). The characteristics of the study participants are shown in Table 1.

Nitrate, nitrite and RXNO

S-nitrosothiols and other nitro species (RXNO) were measured in plasma. Relative to control, all interventions resulted in higher RXNO 140 min post lunch/intervention (control: 33 nmol/L 95%CI: 26, 42; apple: 51 nmol/: 95%CI: 40,65; (p=0.004); spinach: 86 nmol/L 95%CI: 68, 110; (p<0.001); apple+spinach: 69 nmol/L 95%CI: 54, 88; (p<0.001)) (complete results presented in [19]).

Salivary and urinary concentrations of nitrate and nitrite post intervention are presented in Fig.2. Relative to control, the spinach, apple+spinach but not apple interventions resulted in higher salivary nitrate (control: 379 µmol/L 95%CI: 297, 483; apple: 214 µmol/: 95%CI: 168,272; (p=0.003); spinach: 1972 µmol/L 95%CI: 1541, 2524; (p<0.001); apple+spinach: 1899 µmol/L 95%CI: 1490, 2420; (p<0.001)), and salivary nitrite (control: 89 µmol/L 95%CI: 71, 111; apple: 81 µmol/: 95%CI: 65,100; (p=0.9); spinach: 590 µmol/L 95%CI: 473, 737; (p<0.001); apple+spinach: 605 µmol/L 95%CI: 487, 753; (p<0.001)) 120 min post meal. Relative to control, the spinach, apple+spinach but not apple interventions resulted in higher urinary nitrate (control: 282 µmol/L 95%CI: 209, 381; apple: 284 µmol/: 95%CI: 209,384; (p=1.0); spinach: 651 µmol/L 95%CI: 479, 885; (p<0.001); apple+spinach: 587 µmol/L 95%CI: 431, 798; (p<0.001)) and

urinary nitrite (control: 2.0 µmol/L 95%CI: 1.3, 2.9; apple: 1.6 µmol/: 95%CI: 1.1,2.4; (p=0.8); spinach: 5.1 µmol/L 95%CI: 3.4, 7.6; (p<0.001); apple+spinach: 3.8 µmol/L 95%CI: 2.5, 5.7; (p=0.02)) in the 8 hour urine sample.

Cognitive function and mood measures

Cognitive Drug Research Battery Scores for each cognitive measure for each intervention are shown in Table 2. Compared to control, no significant differences were observed for Apple, Spinach and Apple+Spinach 150 min post lunch / intervention.

The composite domain scores: Power of attention, Continuity of attention and Quality of working memory for each intervention are represented in Table 3. Again, compared to control, no significant differences were observed for Apple, Spinach and Apple+Spinach 150 min post lunch / intervention.

The mood scores: Alertness, Calmness and Contentedness for each intervention are detailed in Table 4. Compared to control, no significant differences were observed for Apple, Spinach and Apple+Spinach 150 min post lunch / intervention.

Discussion

Our hypothesis was that the flavonoids in apple and the nitrate in spinach would augment NO status via distinct pathways and that this would contribute to acute improvements in cognitive function and mood. The apple, spinach and apple+spinach interventions resulted in augmented NO status, however, no positive or negative effects were observed on measures of cognitive function and mood.

Consumption of flavonoid-rich apples improved plasma NO status. The increase in NO status (the circulating NO pool) is indicated by the increase in plasma *S*-nitrosothiols and other nitroso species (RXNO) after consumption of flavonoid-rich apples [42, 43]. These molecules, which are by-products of endothelial nitric oxide synthase (eNOS) activity, act as a reservoir for NO in that they have the potential to be converted back to NO when required. The mechanism by which NO status is enhanced by flavonoids is unclear, but there is evidence that effects are endothelium-dependent [44]. Recent studies, however, have highlighted potential pathways [45]. Flavonoids may augment NO levels by prevention of NO breakdown. This could occur by a direct reaction with superoxide and other reactive oxygen species [46] and/or inhibition of the enzymes which produce them (xanthine oxidase, lipoxygenase and NADPH oxidase) [47, 48]. A recent study observed an increase in FMD and a concomitant decrease in neutrophil NADPH oxidase activity after blueberry flavonoid intake [49]. Flavonoids may also augment NO production through effects on endothelial nitric oxide synthase (eNOS) such as preventing its uncoupling [50], increasing its activity or enhancing expression [51]. The increase in NO status after flavonoid rich apple consumption has concomitant beneficial effects in the cardiovasculature, with decreases in blood pressure and improvements in endothelial function observed [19]. Whether similar effects, such as improvements in blood flow and perfusion, are observed in the cerebrovasculature after flavonoid

rich apple consumption are unknown. An improvement in cerebral blood flow has been observed after resveratrol[52] and flavanol-rich cocoa [53] consumption. The level of flavanols in the cocoa, however, was more than double given in this study.

In contrast to the spinach and apple+spinach interventions, the apple intervention resulted in an increase in plasma but not urinary nitrite[19]. Possible explanations include the time period for urine collection (8 hours) and possible breakdown of nitrite to nitrate. Additionally the increase in plasma nitrite observed after flavonoid rich apple consumption is likely to only have a minimal impact on urinary nitrite and nitrate levels as they are present at much higher concentrations.

Consumption of flavonoid-rich apples had no acute effect on measures of cognitive function and mood. In only two of four acute studies conducted to date has a significant improvement in cognitive function been observed after flavonoid intake [52, 54-56]. Effects on cerebrovasculature outcomes are thought to underlie the acute benefits of flavonoids on cognitive function [57]. However, improvements in cerebral blood flow are not always associated with concomitant cognitive benefits [52]. Diminished blood flow to the brain, though, is associated with cognitive impairment [58]. The lack of a significant acute effect of flavonoid rich apples on measures of cognitive function observed in this study does not rule out the possibility of cognitive benefits with long term consumption. Indeed, 12 of 15 human randomised controlled trial studies using a flavonoid intervention with a treatment duration ranging 2 weeks to 13 months observed significant improvements in measures of cognitive function [59]. Moreover, there is epidemiological evidence to suggest cognitive benefits with long term flavonoid intake [60, 61]. The mechanisms involved in long-term benefit may or may not relate to increases in NO status.

Consumption of nitrate-rich spinach augmented NO status with increases observed in plasma RXNO, nitrate and nitrite, salivary nitrate and nitrite as well as urinary nitrate and nitrite. Nitrate-rich spinach improves NO status through the recently described enterosalivary nitrate-nitrite-NO pathway [16-18]. While most ingested nitrate is ultimately excreted in urine, approximately 25% is actively extracted from the plasma and secreted in the saliva resulting in levels of nitrate that are 10 to 20 fold higher in saliva than plasma [62]. Our results are consistent with this estimate. The salivary nitrate is converted to nitrite by nitrate reductase enzymes of the oral facultative anaerobic bacteria found mainly on the dorsal surface of the tongue. The nitrite is swallowed and enters the blood stream via the stomach where it is thought to become a circulating storage pool for NO [63, 64]. This increase in NO status after consumption of nitrate-rich spinach is associated with concomitant improvements in blood pressure and endothelial function [19]. Whether improvements in blood flow and perfusion occurs in the cerebrovasculature after nitrate-rich spinach consumption are unknown. An improvement in cerebral blood flow in frontal lobe white matter has been observed in older adults fed a high nitrate diet [65].

Nitrate-rich spinach did not improve cognitive function and mood measures acutely. These results are confirmed by Kelly and colleagues who demonstrated no change in brain metabolite concentrations or cognitive function after 3 days of nitrate-rich beetroot juice supplementation [66]. Plasma nitrite concentrations were 1037 nmol/L[66] compared to 99 nmol/L observed in this study[19]. Although no acute effects on cognitive function were observed, the increase in NO status may have long term benefits as NO plays a significant role in cerebral physiology as well as being a key molecule in learning and memory. The long term benefits of nitrate consumption on cognitive performance have not been measured, though epidemiological evidence suggests

cognitive benefits with cruciferous [67] and green leafy vegetable [5] intake. Whether this is related to their nitrate content is unknown.

The flavonoid-rich apple and nitrate-rich spinach combination augmented NO status and had no effect on cognitive performance. The possibility that simultaneous ingestion of dietary nitrate and flavonoids could have an additive or even synergistic effect on NO status comes from the observation that they both enhance NO production via different mechanisms as well as from studies demonstrating that flavonoids enhance the reduction of nitrite to NO. Dietary nitrate contributes to the circulating pool of nitrite and NO through the nitrate-nitrite-NO pathway. While the exact mechanisms of protective action by flavonoids has yet to be confirmed, evidence suggests that flavonoids modulate NO metabolism through the L-arginine NOS pathway. *In vitro* studies and *in vivo* experiments suggest that flavonoids could also mediate the direct bioconversion of nitrite to NO. These studies have demonstrated that flavonoids, in the acidic conditions of the stomach, can enhance the production of NO from salivary nitrite [27, 68-70] which can diffuse across the stomach wall and induce local muscle relaxation [28, 71]. Since salivary nitrite is increased after nitrate consumption, polyphenols could, theoretically, enhance NO production after a nitrate rich meal. Whether this occurs in the circulation is unknown. Results from this clinical trial did not provide any evidence for additive effects on NO status.

While no positive effects were observed on cognition and mood following flavonoid-rich apple and nitrate-rich spinach consumption, no deleterious effects were observed either. The flavonoid-rich apple and nitrate-rich spinach were well tolerated acutely and thus could be administered repeatedly to determine chronic effects on cognition and mood. Finally we cannot rule out the possibility that cognitive effects may have been evident with different cognitive tasks. It is notable that cocoa flavanol administration was associated with better cognitive function during

relatively effortful cognitive tasks [56] but not using the cognitive battery employed here [72]. Thus the effects of NO-mediated increased endothelial function may only become evident during heavily loaded cognitive processing.

In conclusion, flavonoid-rich apples and nitrate-rich spinach augmented NO status acutely without any concomitant improvements or deterioration in cognitive function and mood. Future studies need to examine the effect of elevated NO status on cognitive performance with long term consumption of flavonoid-rich apples and nitrate-rich spinach as well as the effect on a population with a lower cognitive performance at baseline.

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DISCLOSURES

None

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Figure legends

Figure 1: Participant flow from recruitment through screening and randomisation to trial completion (adapted from [19]).

Figure 2: The effect of interventions on salivary nitrate (A) and nitrite (C) 120 min post meal, and on urinary nitrate (B) and nitrite (D) from an 8 hour sample. Results are expressed as geometric mean (95% CIs). A mixed random-effects linear model (n=30) was used to compare interventions.

Table1. Baseline characteristics of study subjects (n=30; males n=6; females n=24)

	Mean±SD
Age (years)	47.3±13.6
Weight (kg)	66.4±10.8
Body Mass Index (kg/m^2)	23.6±3.4
Systolic blood pressure (mmHg)	112.2±11.5
Diastolic blood pressure (mmHg)	68.3±7.8
Total cholesterol (mM)	5.1±0.7
Triglyceride (mM)	1.0±0.4
High density lipoprotein cholesterol (mM)	1.6±0.36
Low density lipoprotein cholesterol (mM)	3.1±0.6
Fasting plasma glucose (mM)	5.1±0.4

Table 2: CDR scores for each cognitive measure for each intervention

Measure	Treatment	CDR Score (Means ± S.E.M.)	*p* value
Simple reaction time (ms)	Control	282 ± 9.16	
	Apple	287 ± 9.22	0.44
	Spinach	284 ± 9.25	0.75
	Apple+spinach	279 ± 9.21	0.66
Digit vigilance accuracy (%)	Control	98 ± 0.49	
	Apple	99 ± 0.50	0.25
	Spinach	99 ± 0.50	0.43
	Apple+spinach	99 ± 0.50	0.40
Digit vigilance reaction time (ms)	Control	426 ± 10.1	
	Apple	422 ± 10.1	0.41
	Spinach	426 ± 10.1	1.00
	Apple+spinach	419 ± 10.1	0.14
Digit vigilance false alarms (number)	Control	0.50 ± 0.18	
	Apple	0.59 ± 0.18	0.66
	Spinach	0.71 ± 0.18	0.27
	Apple+spinach	0.67 ± 0.18	0.39
Choice reaction time accuracy (%)	Control	97 ± 0.41	
	Apple	97 ± 0.42	0.77
	Spinach	97 ± 0.42	0.74
	Apple+spinach	96 ± 0.42	0.26
Choice reaction time (ms)	Control	457 ± 16.2	
	Apple	461 ± 16.2	0.55
	Spinach	460 ± 16.2	0.64

	Apple+spinach	458 ± 16.2	0.89
Spatial memory (sensitivity index)	Control	0.87 ± 0.04	
	Apple	0.89 ± 0.04	0.66
	Spinach	0.79 ± 0.04	0.05
	Apple+spinach	0.85 ± 0.04	0.67
Spatial memory reaction time (ms)	Control	898 ± 97.6	
	Apple	888 ± 98.3	0.84
	Spinach	905 ± 98.2	0.89
	Apple+spinach	848 ± 97.9	0.30
Numeric working memory (sensitivity index)	Control	0.92 ± 0.01	
	Apple	0.93 ± 0.01	0.69
	Spinach	0.93 ± 0.01	0.90
	Apple+spinach	0.94 ± 0.01	0.34
Numeric working memory reaction time (ms)	Control	695 ± 36.0	
	Apple	684 ± 36.0	0.45
	Spinach	696 ± 36.1	0.93
	Apple+spinach	677 ± 36.0	0.20
Delayed word recognition (sensitivity index)	Control	0.77 ± 0.05	
	Apple	0.71 ± 0.05	0.28
	Spinach	0.71 ± 0.05	0.29
	Apple+spinach	0.72 ± 0.05	0.34
Delayed word recognition reaction time (ms)	Control	798 ± 39.1	
	Apple	816 ± 39.3	0.46
	Spinach	813 ± 39.4	0.55
	Apple+spinach	806 ± 39.2	0.75

Table 3: Composite domain scores for each intervention

Measure	Treatment	CDR Score (Means ± S.E.M.)	*p* value
Power of attention	Control	1166 ± 32.7	
	Apple	1166 ± 32.8	0.74
	Spinach	1170 ± 32.8	0.70
	Apple+spinach	1156 ± 32.8	0.43
Continuity of attention	Control	92.2 ± 0.37	
	Apple	92.5 ± 0.38	0.49
	Spinach	92.1 ± 0.38	0.84
	Apple+spinach	92.0 ± 0.38	0.60
Quality of working memory	Control	1.87 ± 0.03	
	Apple	1.89 ± 0.03	0.38
	Spinach	1.83 ± 0.03	0.25
	Apple+spinach	1.86 ± 0.03	0.83

Table 4: Mood scores for each intervention

Measure	Treatment	Bond-lader visual analogue scales (Means ± S.E.M.)	*p* value
Alertness	Control	60.0 ± 2.34	
	Apple	58.6 ± 2.37	0.53
	Spinach	56.9 ± 2.38	0.16
	Apple+spinach	58.2 ± 2.36	0.41
Calmness	Control	56.0 ± 2.15	
	Apple	53.9 ± 2.17	0.26
	Spinach	54.5 ± 2.18	0.40
	Apple+spinach	55.5 ± 2.17	0.79
Contentedness	Control	66.8 ± 2.56	
	Apple	65.8 ± 2.58	0.61
	Spinach	62.9 ± 2.59	0.04
	Apple+spinach	64.7 ± 2.58	0.26

Figure 1.

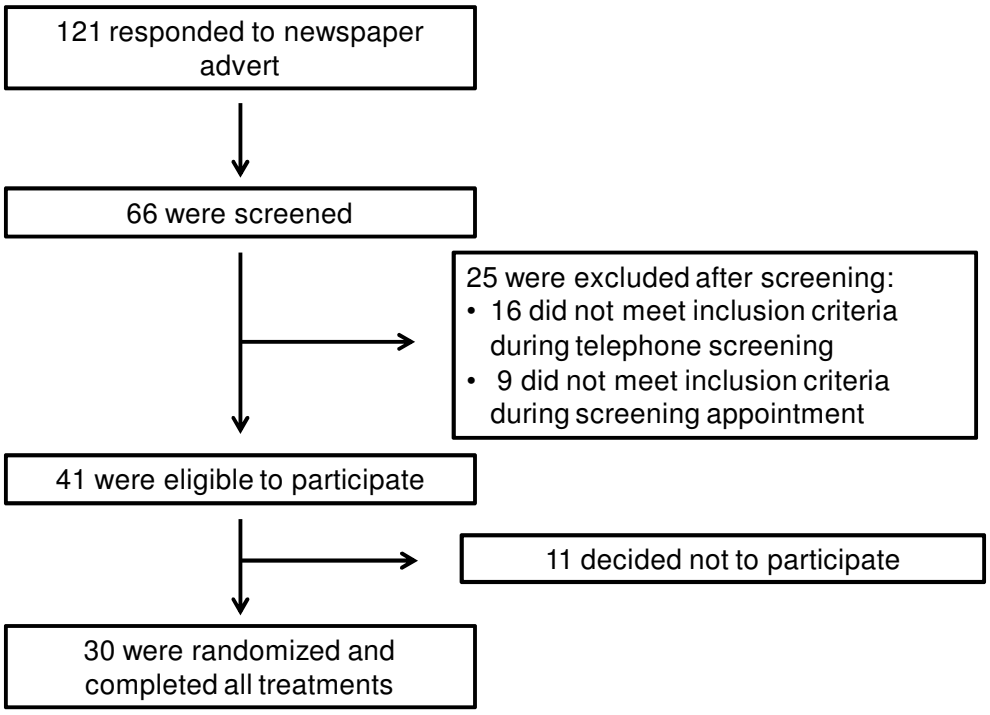
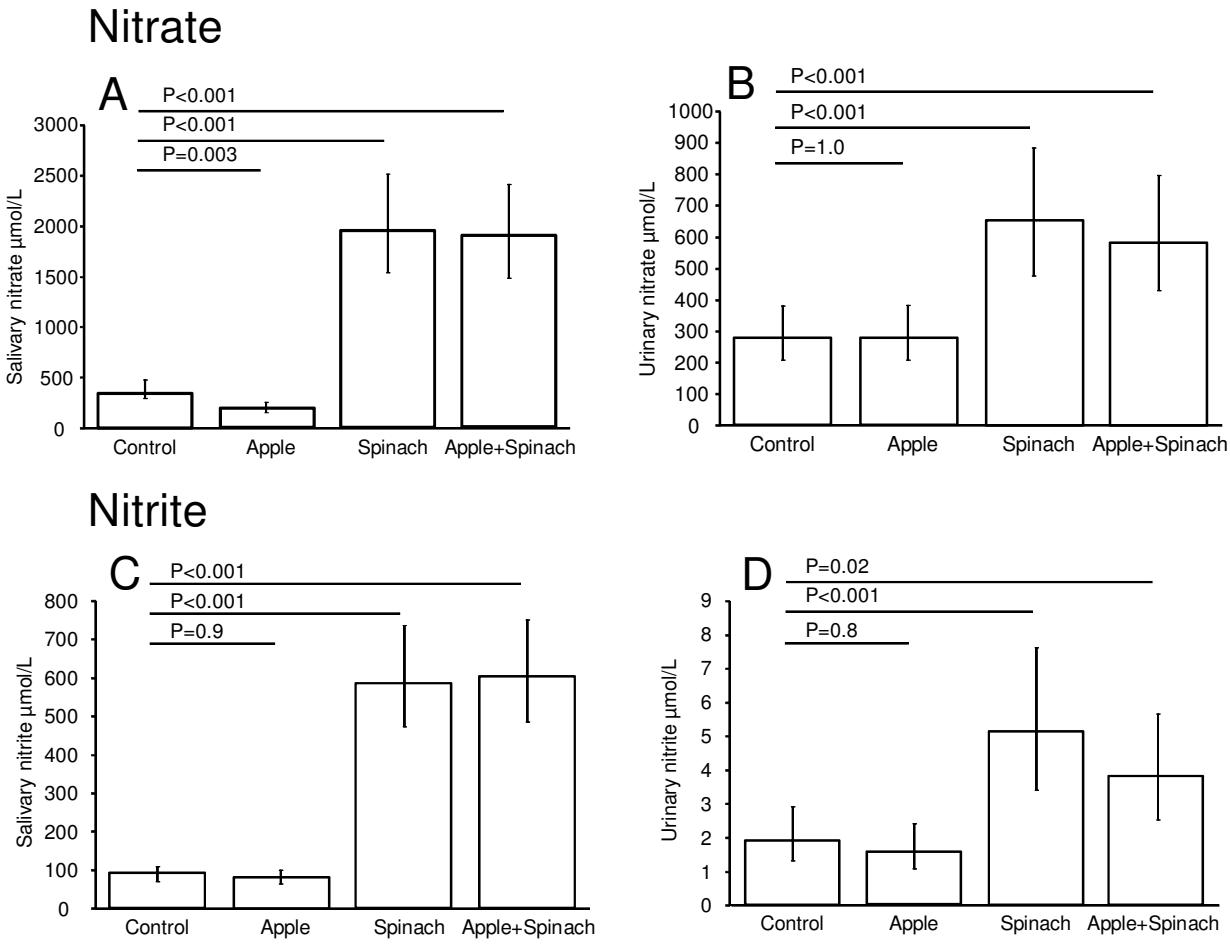


Figure 2.



Electronic Supplementary Information

Cognitive function and mood assessment – short description of administered tests

Word presentation. A list of 15 words, matched for frequency and concreteness, was presented in a random sequence on the centre of the screen at a rate of one word every 2 s for the participant to remember.

Simple reaction time. The participant was instructed to press the YES response button as quickly as possible every time the word 'YES' was presented on the screen. Thirty stimuli were presented with a varying inter-stimulus interval of between 1 and 4 s. Reaction times were recorded in milliseconds (ms).

Digit vigilance. A target digit was randomly selected and displayed to the right of the screen. A series of digits were presented in the centre of the screen at the rate of 2.5 digits per second. The participant was required to press the YES button as quickly as possible every time the digit in the series matched the target digit. The task measures were the percentage of targets detected (accuracy), the average reaction time (ms) and the number of false-positives (false alarms) made.

Choice reaction time. Either the word 'YES' or the word 'NO' was presented on the screen and the participant was instructed to press the corresponding YES/NO button as quickly as possible. There were 30 trials with a varying inter-stimulus interval of between 1 and 4 s. The task measures were the percentage of correct responses (accuracy) and the average reaction time to the stimuli (ms).

Spatial working memory. A simple pictorial representation of a house was presented on the screen with four of its nine windows illuminated. The participant was instructed to memorize the

positions of the illuminated windows. For each of the subsequent presentations of the house the participant had to decide whether or not the one window which was illuminated was also illuminated in the original presentation. Participants recorded their response by pressing the YES or NO button as appropriate and as quickly as possible. The measures were the percentage of correctly identified stimuli (accuracy) and the average reaction time (ms).

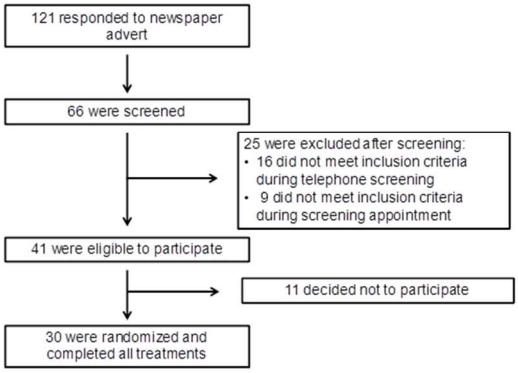
Numeric working memory. A series of five digits was presented one after the other for the participant to hold in their memory. This was followed by a series of 30 probe digits. For each digit the participant was required to decide whether or not the digit was from the original series and indicate their choice by pressing either the YES or NO button as appropriate and as quickly as possible. The measures were the percentage correctly identified stimuli (accuracy) and the average reaction time (ms).

Delayed word recognition. The original 15 words (presented in *Word presentation*) plus 15 distracter words were presented one at a time in a randomized order. For each word the participant indicated whether they recognized the word as being from the original list of words by pressing the YES or NO button as appropriate. The measures were the percentage of correctly identified words (accuracy) and the average reaction time (ms).

From these tests three composite domain scores can be derived: Power of Attention (a measure of attention and psychomotor/information processing speed; by summing reaction times from the Simple reaction time, Choice reaction time and Digit vigilance tasks), Continuity of Attention (a measure of attentional accuracy; by summing accuracy and error measures from the Choice reaction time and Digit vigilance tasks), Quality of Working Memory (a measure of working

memory accuracy; by summing the accuracy scores from the Numeric and Spatial working memory tasks)[37].

In addition, participants completed the Bond–Lader mood scale[39], which is made up of 16 × 100 mm visual analogue mood scales (VAMS) with the end-points anchored by antonyms. Three mood measures are derived as recommended by the authors, with scores on each ranging from 0 to 100: 'alertness' (from individual VAMS of alert-drowsy, attentive-dreamy, lethargic-energetic, muzzy-clearheaded, well-coordinated-clumsy, mentally slow-quick witted, strong-feeble, interested-bored, incompetent-proficient); 'calmness' (calm-excited, tense-relaxed); and 'contentedness' (contented-discontented, trouble-tranquil, happy-sad, antagonistic-friendly, withdrawn-sociable). These measures of mood have been shown to be sensitive to changes induced by acute consumption of flavonoid rich natural substances[73].



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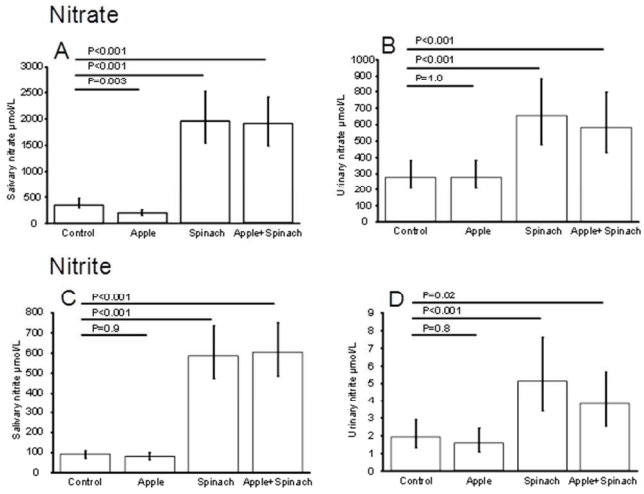

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TABLE OF CONTENTS ENTRY

Flavonoid-rich apples and nitrate-rich spinach augment NO status acutely with no concomitant improvements or deterioration in cognitive function and mood.