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

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 disease1–3 and cognitive decline.4–6 Not fully understood are the components of fruit and vegetables responsible for these benefits. Flavonoids5 and nitrate6 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.8 It is also an important neurotransmitter.9 An imbalance of NO is associated with a number of cardiovascular disorders10 as well as pathological conditions in the brain.11 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 endogenous13 and exogenous sources.8 Flavonoids may augment endogenous endothelial-derived NO7,14,15 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 and green leafy vegetables, including spinach, are high in dietary nitrate. Evidence suggests that flavonoids and nitrate alone and in combination could increase NO production. 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. 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⁻¹, weight gain or loss >6% body weight within previous 6 months of the study, >30 g per 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 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 one 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. 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 different forms of quercetin present in apples, as well as flavonoid/low nitrate intervention four hours 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.
toasted white bread sandwich with chicken (skinless, 60 g), mild cheese (30 g) and mayonnaise (15 mL).\textsuperscript{22}

**Measurement of flavonoids in apple and nitrate in spinach**

The polyphenolic compounds of the apple were extracted using a modified method described previously.\textsuperscript{24} Flavonoid composition of the apple samples was determined using high performance liquid chromatography as previously described.\textsuperscript{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.\textsuperscript{19}

Nitrate concentration in the spinach was determined using a previously published gas chromatography-mass spectrometry (GC-MS) method.\textsuperscript{28} Briefly, internal standards \([^{15}\text{N}]\) sodium nitrite (6 ng) and \([^{15}\text{N}]\) sodium nitrate (40 ng) 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\textsubscript{2} for 35 min and the remaining aqueous phase extracted with isoicoulo-toluene. 1 μl of the organic extract was analysed using an Agilent 6890 gas chromatograph coupled to a 5973 mass spectrometer with a cross-linked silicone column (25 m × 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 \([^{15}\text{N}]\) sodium nitrite and \([^{15}\text{N}]\) 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 \([^{15}\text{N}]\) nitrate respectively and \(m/z = 46\) and 47 for nitrite and \([^{15}\text{N}]\) nitrite respectively. The spinach active contained 182 mg of nitrate and the control, rice milk, contained less than 5 mg nitrate.\textsuperscript{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\textsuperscript{28} described above.

**Measurement of plasma S-nitrosothiols and other nitrosocompounds (RXNO) and nitrite**

The concentrations of S-nitrosothiols and other nitrosocompounds (RXNO) and nitrite in plasma were determined using a previously described gas-phase chemiluminescence assay.\textsuperscript{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).\textsuperscript{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\textsuperscript{35-37} as well as acute changes in cognitive function due to natural substances.\textsuperscript{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’.\textsuperscript{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.\textsuperscript{39} A short description of these tests appears in ESI.\textsuperscript{†}

**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.\textsuperscript{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\textsuperscript{29} and literature values\textsuperscript{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) 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

$\text{S}$-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$^{-1}$ 95% CI: 26, 42; apple: 51 nmol L$^{-1}$ 95% CI: 40, 65; ($p = 0.004$); spinach: 86 nmol L$^{-1}$ 95% CI: 68, 110; ($p < 0.001$); apple + spinach: 69 nmol L$^{-1}$ 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 nmol L$^{-1}$ 95% CI: 297, 483; apple: 214 nmol L$^{-1}$ 95% CI: 168, 272; ($p = 0.003$); spinach: 1972 nmol L$^{-1}$ 95% CI: 1541, 2524; ($p < 0.001$); apple + spinach: 1899 nmol L$^{-1}$ 95% CI: 1490, 2420; ($p < 0.001$)), and salivary nitrite (control: 89 nmol L$^{-1}$ 95% CI: 71, 111; apple: 81 nmol L$^{-1}$ 95% CI: 65, 100; ($p = 0.9$); spinach: 590 nmol L$^{-1}$ 95% CI: 473, 737; ($p < 0.001$); apple + spinach: 605 nmol L$^{-1}$ 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$^{-1}$ 95% CI: 209, 381; apple: 284 µmol L$^{-1}$ 95% CI: 209, 384; ($p = 1.0$); spinach: 651 µmol L$^{-1}$ 95% CI: 479, 885; ($p < 0.001$); apple + spinach: 587 µmol L$^{-1}$ 95% CI: 431, 798; ($p < 0.001$)) and urinary nitrite (control: 2.0 µmol L$^{-1}$ 95% CI: 1.3, 2.9; apple: 1.6 µmol L$^{-1}$ 95% CI: 1.1, 2.4; ($p = 0.8$); spinach: 5.1 µmol L$^{-1}$ 95% CI: 3.4, 7.6; ($p < 0.001$); apple + spinach: 3.8 µmol L$^{-1}$ 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 shown in Table 2. 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 $\text{S}$-nitrosothiols and other nitroso species (RXNO) after consumption of flavonoid-rich apples.$^{43,44}$ 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 distinct pathways and via other enzymes which produce them (xanthine oxidase, lipoxygenase and NADPH oxidase).$^{47,48}$ A recent study observed an increase in FMD and a
NO production through effects on endothelial nitric oxide synthase (eNOS) such as preventing its uncoupling, increasing its activity or enhancing expression. 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. 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 and flavanol-rich cocoa 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. 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. Effects on cerebrovasculature outcomes are thought to underlie the acute benefits of flavonoids on cognitive function. However, improvements in cerebral blood flow are not always associated with concomitant cognitive benefits. Diminished blood flow to the brain, though, is associated with cognitive impairment. 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. Moreover, there is epidemiological evidence to suggest cognitive benefits with long term flavonoid intake. 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. 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. 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. This increase in NO status after consumption of nitrate-rich spinach is associated with concomitant improvements in blood pressure and endothelial function. 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.

Nitrate-rich spinach did not improve cognitive function and mood measures acutely. These results are confirmed by

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**Fig. 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.
Kelly and colleagues who demonstrated no change in brain metabolite concentrations or cognitive function after 3 days of nitrate-rich beetroot juice supplementation. Plasma nitrite concentrations were 1037 nmol L\(^{-1}\) (ref. 66) compared to 99 nmol L\(^{-1}\) observed in this study. 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\(^5\) 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

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

<table>
<thead>
<tr>
<th>Measure</th>
<th>Treatment</th>
<th>CDR Score (Means ± S.E.M.)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Simple reaction time (ms)</td>
<td>Control</td>
<td>282 ± 9.16</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>Apple</td>
<td>287 ± 9.22</td>
<td>0.44</td>
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<tr>
<td></td>
<td>Spinach</td>
<td>284 ± 9.25</td>
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<tr>
<td></td>
<td>Apple + spinach</td>
<td>279 ± 9.21</td>
<td>0.66</td>
</tr>
<tr>
<td>Digit vigilance accuracy (%)</td>
<td>Control</td>
<td>98 ± 0.49</td>
<td>—</td>
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<tr>
<td></td>
<td>Apple</td>
<td>99 ± 0.50</td>
<td>0.25</td>
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<td></td>
<td>Spinach</td>
<td>99 ± 0.50</td>
<td>0.43</td>
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<td></td>
<td>Apple + spinach</td>
<td>99 ± 0.50</td>
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<tr>
<td>Digit vigilance reaction time (ms)</td>
<td>Control</td>
<td>426 ± 10.1</td>
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<td>Apple</td>
<td>422 ± 10.1</td>
<td>0.41</td>
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<tr>
<td></td>
<td>Spinach</td>
<td>426 ± 10.1</td>
<td>1.00</td>
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<td>Apple + spinach</td>
<td>419 ± 10.1</td>
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<tr>
<td>Digit vigilance false alarms (number)</td>
<td>Control</td>
<td>0.50 ± 0.18</td>
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<tr>
<td></td>
<td>Apple</td>
<td>0.39 ± 0.18</td>
<td>0.66</td>
</tr>
<tr>
<td></td>
<td>Spinach</td>
<td>0.71 ± 0.18</td>
<td>0.27</td>
</tr>
<tr>
<td></td>
<td>Apple + spinach</td>
<td>0.67 ± 0.18</td>
<td>0.39</td>
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<tr>
<td>Choice reaction time accuracy (%)</td>
<td>Control</td>
<td>97 ± 0.41</td>
<td>—</td>
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<tr>
<td></td>
<td>Apple</td>
<td>97 ± 0.42</td>
<td>0.77</td>
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<tr>
<td></td>
<td>Spinach</td>
<td>97 ± 0.42</td>
<td>0.74</td>
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<tr>
<td></td>
<td>Apple + spinach</td>
<td>96 ± 0.42</td>
<td>0.26</td>
</tr>
<tr>
<td>Choice reaction time (ms)</td>
<td>Control</td>
<td>457 ± 16.2</td>
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<tr>
<td></td>
<td>Apple</td>
<td>461 ± 16.2</td>
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<td>Spinach</td>
<td>460 ± 16.2</td>
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<td></td>
<td>Apple + spinach</td>
<td>458 ± 16.2</td>
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<td>Spatial memory (sensitivity index)</td>
<td>Control</td>
<td>0.87 ± 0.04</td>
<td>—</td>
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<td></td>
<td>Apple</td>
<td>0.89 ± 0.04</td>
<td>0.66</td>
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<td>Spinach</td>
<td>0.79 ± 0.04</td>
<td>0.05</td>
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<td>Apple + spinach</td>
<td>0.85 ± 0.04</td>
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<tr>
<td>Spatial memory reaction time (ms)</td>
<td>Control</td>
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<td></td>
<td>Apple</td>
<td>888 ± 98.3</td>
<td>0.84</td>
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<td>Spinach</td>
<td>905 ± 98.2</td>
<td>0.89</td>
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<td></td>
<td>Apple + spinach</td>
<td>848 ± 97.9</td>
<td>0.30</td>
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<tr>
<td>Numeric working memory (sensitivity index)</td>
<td>Control</td>
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<td>—</td>
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<td></td>
<td>Apple</td>
<td>0.93 ± 0.01</td>
<td>0.69</td>
</tr>
<tr>
<td></td>
<td>Spinach</td>
<td>0.93 ± 0.01</td>
<td>0.90</td>
</tr>
<tr>
<td></td>
<td>Apple + spinach</td>
<td>0.94 ± 0.01</td>
<td>0.34</td>
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<tr>
<td>Numeric working memory reaction time (ms)</td>
<td>Control</td>
<td>695 ± 36.0</td>
<td>—</td>
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<tr>
<td></td>
<td>Apple</td>
<td>684 ± 36.0</td>
<td>0.45</td>
</tr>
<tr>
<td></td>
<td>Spinach</td>
<td>696 ± 36.1</td>
<td>0.93</td>
</tr>
<tr>
<td></td>
<td>Apple + spinach</td>
<td>677 ± 36.0</td>
<td>0.20</td>
</tr>
<tr>
<td>Delayed word recognition (sensitivity index)</td>
<td>Control</td>
<td>0.77 ± 0.05</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>Apple</td>
<td>0.71 ± 0.05</td>
<td>0.28</td>
</tr>
<tr>
<td></td>
<td>Spinach</td>
<td>0.71 ± 0.05</td>
<td>0.29</td>
</tr>
<tr>
<td></td>
<td>Apple + spinach</td>
<td>0.72 ± 0.05</td>
<td>0.34</td>
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<tr>
<td>Delayed word recognition reaction time (ms)</td>
<td>Control</td>
<td>798 ± 39.1</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>Apple</td>
<td>816 ± 39.3</td>
<td>0.46</td>
</tr>
<tr>
<td></td>
<td>Spinach</td>
<td>813 ± 39.4</td>
<td>0.55</td>
</tr>
<tr>
<td></td>
<td>Apple + spinach</td>
<td>806 ± 39.2</td>
<td>0.75</td>
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</table>
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 27,28,29 which can diffuse across the stomach wall and induce local muscle relaxation.28,29 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 but not using the cognitive battery employed here.22 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.

### Funding sources

National Health and Medical Research Council, Australian Research Council, and the Department of Agriculture and Food, Western Australia.

### Acknowledgements

CP Bondonno acknowledges the support of an Australian Postgraduate Award. NC Ward acknowledges the support of a MRF/UWA Fellowship. JM Hodgson was supported by an NHMRC senior research Fellowship. LA Downey is supported by an NHMRC (APP1054279) biomedical fellowship. We also acknowledge the Ada Bartholomew Medical Research Trust. The authors would like to thank Ms Adeline Indrawan for technical assistance and Mr Claude Backory for expert nursing assistance. The authors would like to thank Logan Farms for supplying the spinach.

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