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The effects of flavanone-rich citrus juice on cognitive function and cerebral blood flow: an acute, randomised, placebo controlled crossover trial in healthy young adults

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Abstract

One plausible mechanism underlying flavonoid-associated cognitive effects is increased cerebral blood flow (CBF). However, behavioural and CBF effects following flavanone-rich juice consumption have not been explored. The aim was to investigate whether consumption of flavanone-rich juice is associated with acute cognitive benefits and increased regional CBF in healthy young adults. An acute, single-blind, randomised crossover design was applied with two 500ml drink conditions; high flavanone (HF; 70.5mg) and an energy, vitamin C matched zero flavanone control. Twenty four healthy young adults aged 18-30 underwent cognitive testing at baseline and two hours post drink consumption. A further sixteen healthy young adults were recruited for fMRI assessment whereby CBF was measured with arterial spin labelling during conscious resting state at baseline, and two and five hours post drink consumption. The HF drink was associated with significantly increased regional perfusion in the inferior and middle right frontal gyrus at two hours relative to baseline and the control drink. In addition, the HF drink was associated with significantly improved performance on the Digit Symbol Substitution Test at two hours relative to baseline and the control drink, but no effects were observed on any other behavioural cognitive tests. These results demonstrate that consumption of flavanone-rich citrus juice in quantities commonly consumed can acutely enhance blood flow to the brain in healthy young adults. However, further work is required to establish a direct causal link between increased cerebral blood flow and enhanced behavioural outcomes following citrus juice ingestion.

1. Introduction

Studies investigating the neuro-protective effects of foods and beverages containing flavonoids suggest that they may lead to benefits for memory and learning by improving neuronal functioning and promoting neuronal protection and regeneration[1]. In rodents, dietary flavanone supplementation (e.g. hesperidin) over several weeks is associated with significant improvements in spatial working memory. Moreover, these cognitive improvements correlate with increased expression of signalling proteins involved in learning and memory, and increased brain derived neurotrophic factor (BDNF) in the hippocampus[2,3]. These are important findings since increased expression of BDNF is associated with benefits for cognitive function in humans such as slower onset of Alzheimer's disease[4]. This supports the presence of mechanistic pathways by which citrus fruit based flavanones may have positive effects on the brain.

Epidemiological data showing an association between flavanone consumption and crystallized intelligence[5] is supported by positive effects from several human intervention studies indicating cognitive benefits in adults following chronic consumption of flavanone-rich fruits and vegetables, for reviews see[6,7]. For example, improved memory function in older adults with mild cognitive impairment (MCI) has been observed following daily consumption of concord grape juice (CGJ) for twelve weeks[8] and sixteen weeks[9]. Of particular relevance here is a recent finding that eight weeks daily consumption of flavanone-rich orange juice was associated with improvements in executive function and episodic memory in healthy older adults aged 60-81 years[10]. This indicates that consumption of fruit juices which contain flavanones as the predominant flavonoid may lead to benefits for the human brain, even in healthy adults.

Neuro-imaging studies in young human adults have demonstrated that consumption of flavanol-rich cocoa can acutely enhance peripheral and cerebral blood flow (CBF)[11,12]. Furthermore, promising associations have been observed between increased neuronal activity and behavioural benefits following chronic flavanol-rich cocoa supplementation. Enhanced activation in the dentate gyrus (measured with a fMRI blood oxygenation level-dependent (BOLD) signal) and simultaneous improvements in spatial working memory were reported in healthy older adults following consumption of flavanol-rich cocoa for three months relative to a low flavanol control[13].

However, other chronic flavanol interventions have failed to report concomitant cognitive benefits in the presence of enhanced neuronal activation. For example, increased steady state evoked potentials (assessed using Steady State Probe Topography) in posterior parietal and central-frontal regions were observed in middle-aged adults following thirty days daily consumption of 250mg or 500mg cocoa flavanol drinks relative to placebo, however, there were no effects for behavioural measures of spatial working memory[(14)]. Similarly, enhanced activation was observed in various brain regions during performance of an attention switching task following five days consumption of 172mg cocoa flavanols. However, changes in the BOLD signal were not associated with performance on the attention switching task[(12)].

To summarise, the evidence suggests that flavonoid consumption can enhance vasodilation in the periphery and lead to increased blood flow in specific regions of the brain in the acute postprandial period. Daily flavonoid consumption over several weeks is associated with cognitive benefits, but as yet, there is only weak evidence supporting a coupling between increased CBF with improved performance on neuropsychological tests. The current research builds upon these findings by investigating whether the aforementioned positive cognitive effects of daily flavanone consumption over several weeks[(10)] are supported by acute cognitive benefits in the immediate postprandial phase. It is reasonable to hypothesise that acute cognitive benefits are underpinned by changes in CBF. Therefore, in addition to assessing behavioural outcomes, the present research examined the effects of flavanone-rich juice on CBF using fMRI arterial spin labelling (ASL). We chose a commercially available citrus-based juice given that flavanones are naturally found in high concentrations in citrus fruits such as orange and grapefruit. This also reflects the quality and quantity of juice consumed by the general population. In sum, the aim of the present research was to investigate the effects of flavanone-rich juice on acute cognitive function and CBF in healthy young adults by adopting a placebo matched, crossover, randomized, single-blind, design.

2. Experimental Methods

Different participants were recruited for the behavioural cognitive arm (n=28) and the ASL imaging arm (n=16) of the study (see Table 1), however, inclusion and exclusion criteria were identical for both arms. Participants were not permitted to take part in both arms. At the time of designing the study, there was an absence of published data concerning the effects of flavanone consumption in humans on cognitive function, cardiovascular outcomes, or cerebral blood flow. Therefore, we considered it important to create an experimental design in which cognitive and cerebral blood flow effects could be examined in isolation. For, example, it is important to establish if effects on CBF are observed independently of behavioural effects. Furthermore, in light of the absence of experimental support for a specific behavioural task sensitive to flavanone consumption in humans, it was considered that a range of cognitive functions should be assessed. Incorporating a comprehensive cognitive battery into the fMRI sequencing schedule posed significant practical difficulties. Therefore, a decision was taken to recruit separate cohorts for the behavioural and imaging arms. Healthy young adults aged 18-30 years were recruited from the University of Reading and surrounding area via community advertising with posters, leaflets and emails. Twenty four participants (four males) completed the behavioural cognitive arm (four participants dropped out due to work commitments or illness) and all sixteen participants completed the ASL arm (eight males). Inclusion criteria were BMI 19-25kg/m^2 and fluent English speaker whilst exclusion criteria were signs of mild cognitive impairment (Mini Mental State Examination Score <26), smoking, alcohol consumption >15 units/week, orange juice consumption >250ml/day, fruit/vegetable consumption >4 portions/day, caffeine intake >3 drinks/day, actively pursuing weight loss through a dietary intervention, clinical diagnosis of mental illness, neurological disease, chronic fatigue, kidney disease, liver disease, thyroid dysfunction, diabetes mellitus, myocardial infarction or hypertension, and consumption of medication for lipids, hypertension, hypotension or anticoagulation. Recruitment commenced March 2011 and terminated August 2011. Our sample size was based on previous research reporting significant cognitive effects of berry flavonoids in older adults with sample sizes ranging from nine to twenty one[(8,9,15)] and improvements in CBF following cocoa flavanols in sixteen young adults[(12)].

[Table 1 here]

2.1 Design

An acute single-blind, randomised cross-over design was applied with two drink conditions; high flavonoid (HF) and control (CT). Cognitive behavioural testing and ASL measurements were performed prior to and post consumption of the drink at each visit (see procedure). The 500ml HF drink was a commercially available 100% juice (Tropicana Ruby Breakfast Juice, PepsiCo Inc.) which naturally contained 70.5mg flavonoids (42.15mg hesperidin, 17.25mg naringin, 6.75mg narirutin, 4.3mg caffeic acid; analysed by the University of Reading), 225kcal, 48.5g sugars, 4g protein, 0g fat, 3.5g fibre, and 150mg vitamin C. The Tropicana Ruby Breakfast Juice contained juices from oranges and grapefruits. The 500ml CT drink was a commercially-available concentrated cordial product (Lemon Barley Squash, Sainsbury's, UK) which was prepared with 240mls of concentrate and 260mls of mineral water (Buxton Spring still mineral water) containing zero flavonoids, 230kcal, 48g sugars, 0.7g protein, 0g fat, 0.3g fiber, and 130mg vitamin C. Our dose of 70.5mg flavonoids could be considered low relative to previous research[6], however, it is important to examine whether cognitive benefits are associated with consuming concentrations of flavanones which are present in the habitual diet. Therefore, the 500ml juice serving provided an acceptable balance between a suitable flavonoid concentration and an achievable volume of consumption within the context of the habitual diet. The drinks were stored at 4^{o}C and prepared and served by the experimenter. Each 500ml portion was served in two 250ml opaque flasks and consumed through an opaque straw, thus participants could not see the drink and remained blinded. The randomisation order was determined by an independent statistician. For the behavioural cognitive arm, twelve participants consumed the HF drink at visit 1 and twelve consumed the CT drink at visit 1, whilst for the ASL arm eight participants consumed the HF drink at visit 1 and eight consumed the CT drink at visit 1.

2.2 Procedure

In summary, participants attended three separate visits; one screening visit and two test day visit. The behavioural arm test days included two cognitive test time points (baseline and two hour post) and the ASL arm visit days included three time points (baseline, two hour post and five hour post). The screening visit and each test day visit were separated by a one week washout. Initially telephone screening interviews were performed and volunteers who met the inclusion criteria were invited to attend the University of Reading Hugh Sinclair Nutrition Unit for a screening visit. At screening, data on height, weight, health status, medication and blood pressure was collected and participants completed the Mini Mental State Examination (MMSE), a diet and lifestyle questionnaire and a fruit and vegetable questionnaire, data from which was used to corroborate the inclusion/exclusion criteria. For each test day visit, participants arrived at 08:00 having fasted from alcohol for 48 hours and all other food and drink (except water) for twelve hours. At screening, participants were provided with low-nitrate bottled water for consumption during the fast. Prior to each test day visit, participants were also instructed to avoid polyphenol-rich foods for 24 hours (including berries, fruits, fruit juices, jams and preserves, red wine, black, green and fruit teas, coffee, cocoa, soy products, caffeinated energy drinks and vegetables except potatoes) and were provided with standardised typed instructions identifying which foods to avoid. The evening prior to each test day, participants consumed (at home) a low fat standardized chicken and rice meal provided by the research team (350kcal, 6.9g fat of which 3g saturates, 52.1g carbohydrate of which 9.7g sugars, 19g protein, 1.4g fiber, 0.9g salt) to avoid second-meal cognitive effects[16]. On each test day participants were required to orally confirm that they had adhered to the aforementioned dietary restrictions. Following a fifteen minute rest, blood pressure measurements were taken (on behavioural visit days only) on the left upper arm by a validated blood pressure monitor (Omron MX2 automatic digital upper arms BP monitor, Milton Keynes, UK) and recorded as the average of three consecutive measurements. At 08:30 hrs, participants consumed a standardised breakfast within fifteen minutes (88g croissant, 25g cream cheese and 120ml bottled mineral water containing 51g fat, 14g protein, 64g carbohydrates, 777kcal). For the behavioural test days, baseline cognitive testing commenced at 08:45 hrs, followed by consumption of the drink (either HF or CT) at 09:45 hrs. Participants were informed that the drink was a fruit-based beverage available in most UK supermarkets and which must be consumed within fifteen minutes. Blood pressure was measured at 11:40 hrs (behavioural arm only) and lunch, identical to breakfast in both content and amount, was provided fifteen minutes prior to the two-hour post-drink cognitive battery which commenced at 12:00 hrs. An assessment at this time point was based on previous data demonstrating cognitive effects 2 hours following an acute flavonoid dose[12]. For the ASL visit days, the timings were identical to the behavioural cognitive visit days, such that ASL measurements were performed at 08:45 hrs (baseline), 12:00 hrs (two hours) and 15:00 hrs (five hours). The behavioural cognitive visits took place in individual cubicles at the University of Reading Hugh Sinclair Nutrition Unit and the ASL visits took place at the Centre for Integrative Neuroscience and Neurodynamics (CINN). Participants remained within the Nutrition Unit or the CINN for the entire test visit during which only water consumption was permitted (notwithstanding the test day foods and drinks). Participants received a £120 honorarium upon completion. This study was conducted according to the guidelines laid down in the Declaration of Helsinki and all procedures involving human subjects were approved by the School of Psychology and Clinical Languages Ethics Committee. Written and verbal informed consent was obtained and formally recorded.

2.3 Cognitive Battery

The 45-minute cognitive battery consisted of the following tests administered in the respective order: Freiburg Vision Test (v3.6.3), Word Recall (immediate), Logical Memory (immediate recall), Sequence Learning Task, Digit Symbol Substitution (DSST), Stroop Test, Letter Memory Test, Go-NoGo Task, Spatial Delayed Recall, Word Recall (delayed), and Logical Memory (delayed). Where multiple versions of a test were required (see below), parallel versions were presented in a counterbalanced order across conditions and visits. The Freiburg Vision Test assesses visual acuity[17] for which there are two dependent variables: Landholt C and Vernier Threshold. To acquire the Landhold C measurement participants were required to identify the orientation of a horseshoe symbol using the numbers 1-9 on the keyboard keypad (excluding 5). The presentation size of the horseshoe and thus the ease of identifying the orientation randomly varied across trials. Landholt C was subsequently calculated according to the number of correct responses relative to the presentation size. To acquire the Vernier Threshold, participants viewed a stimulus which consisted of two 1cm lines with one directly above the other. Participants pressed the left scroll key if the line above was to the left of the line below, and the right scroll key if the line above was to the right of the line below. The degree to which the lines were aligned varied randomly across trials. The Vernier Threshold was subsequently calculated according to the number of correct responses relative to the horizontal distance between the two lines[17]. Verbal Recall involved computerised, individual presentation of thirty words. A response was required (using the keys ‘M’ for yes, ‘Z’ for no) according to one of five questions which required visual, phonetic or semantic processing of the target word (e.g. “is the word in capitals”, “does the word rhyme with...” or “is the word a type of...”). Upon cessation of the presentation, oral recall of the target words was required (the dependent variable). Within each version of the test, each word was accompanied by the same question for all participants whilst the order of presentation varied randomly. Equal versions were created and matched for frequency, familiarity, imageability, meaningfulness, word length and syllables. Delayed Word Recall involved one attempt to orally recall the words presented thirty minutes prior during Immediate Word Recall. The Logical Memory Test (Wechsler Memory Scale – Revised) requires oral recall of a short paragraph. The paragraphs were presented via cassette tape. The dependent variables for immediate and delayed recall were the number of correctly recalled units. The Sequence Learning Task[18] required participants to immediately press the keys ‘V, B, N or M’ according to the appearance of a stimulus (a 2mm white dot for 200ms) in one of four 3.5cm x 2cm boxes on the screen. Unbeknownst to participants, the order of stimulus presentation followed a set sequence (one block), thus this test assesses the ability to learn a sequence. The duration of each repetitive sequence varied from 2-4 trials. Each test presentation contained six blocks, with each block consisting of 100 trials. The dependent variable was number of correct responses. The DSST[19] is a pen and paper test which contains a key of nine digit-symbol pairs and an accompanying list of digits. Under each listed digit a space is provided to enter the corresponding symbol. Participants entered as many symbols as possible over 90 seconds. The dependent variable was the number of correct responses. The computerised Stroop Test[20] required participants to identify the colour in which a word was presented. There were 120 randomly presented stimuli, each for 1650ms, consisting of 60 congruent and 60 incongruent trials (a congruent trial being when the meaning of the word matched the colour in which it was presented). Participants responded with the keys 1-4 which represented the colours green, blue, red and yellow respectively. The dependent variable was reaction time (for correct responses only). The Letter Memory Task[21] involved serial 2000ms presentation of individual letters. The number of letters per trial varied randomly between 5, 7, 9 and 11 for a total of twelve trials and 48 letters. For each trial, at the termination of the presentation phase participants were required to orally recall the final four letters from the presentation. The dependent variable was the total number of correct responses defined as recalling the correct sequence in its entirety. The Go-NoGo is a computerised task assessing inhibition and sustained attention. The present version was adapted from the Go-NoGo paradigm[22]. Participants were required to respond to sixty stimuli using one of three specified keyboard keys; ‘p’ ‘q’ or ‘space bar’. The stimuli consisted of X, Y or a number ‘lure’. Initially, there was a 25 stimuli ‘Pre-Potent Go’ phase. During the Pre-Potent Go phase, X and Y were presented alternately, with the participant required to press ‘q’ when X appeared and ‘p’ when Y appeared. The X and Y were known as the ‘Go’ trials. The Go-NoGo phase followed the Pre-Potent Go phase. During the Go-NoGo phase, the ‘Go’ trials were interspersed with ‘NoGo’ trials; these appeared as numbers lures. Pressing the space bar was the required response upon viewing a number lure. During the Go-NoGo phase X and Y were presented randomly, interspersed with number lures, such that the predictable alternating sequence was disrupted. Responses were required only if a Y appeared after an X or vice-versa, and therefore the participant must inhibit the established pre-potent response in all other trials. Reaction Time for correct responses was the dependent variable. The Spatial Delayed Recall Test required participants to recall the location of a white dot on the screen. Each trial commenced with a fixation cross followed by presentation of a white dot for 50ms in a random location. The white dot was replaced by a randomly generated number between 90-99 at which point participants were asked to orally subtract three from this number continuously for eight seconds. Once eight seconds had elapsed the number disappeared and the participant was required to indicate (by touching the screen) the location at which the white dot had previously appeared. There were sixteen trials in total and the dependent variable was the distance from the target (mm).

2.4 fMRI protocol

Scanning was performed at the CINN, University of Reading, UK using a 3.0 Tesla Siemens MAGNETOM Trio MRI scanner with a 12-channel Head Matrix coil. The ASL images were acquired using the PICOREQ2T sequence with the following parameters: number of slices=18, slice thickness=5.0mm, inter-slice gap=1.25mm, TR=2500ms, TE=11ms, TI1=700, Saturation Stop Time=1600, TI2=1800, perfusion mode=PICORE Q2T (pulsed). A high resolution whole-brain three dimensional anatomical image was also acquired using an MPRAGE gradient-sequence with 176 x 1mm thick slices (1*1*1 voxels size, TE: 2.52ms, TR: 2020ms, TI: 1100ms, FOV: 250x250, slice thickness: mm2, Flip Angle: 9deg). FMRI data processing was carried out using FEAT (FMRI Expert Analysis Tool) Version 5.98, part of FSL (FMRIB's Software Library; www.fmrib.ox.ac.uk/fsl). ASL volumes from each scanning session were all registered to the corresponding individual's high resolution structural image using rigid body transformations. In a second step, the images were registered to the Montreal Neurological Institute (MNI) template brain using a 12 degrees of freedom affine transformation algorithm. To allow voxelwise comparisons, each CBF map was individually processed using perfusion signal modelling, which models the differences between control images and tagged (spin labelled) images within a time series. A CBF map was produced for each participant, drink (HF and CT) and time point (baseline, two hours and five hours).

2.5 Statistical Analysis

All analysis and data processing was performed by independent researchers who did not participant in any of the test day procedures and remained blinded to condition. Cognitive test and blood pressure-dependent variables were assessed with a 2x2 repeated measures ANOVA (Drink x Time). Significant main effects and interactions were explored with post hoc t-tests applying Bonferroni corrections for familywise error. Analysis of the cognitive and blood pressure data was performed with SPSS Statistics 21. FMRI data processing was carried out using FEAT (FMRI Expert Analysis Tool) Version 5.98 part of FSL (FMRIB's

Software Library, www.fmrib.ox.ac.uk/fsl). ASL volumes from each scanning session were all registered to the corresponding individual's high resolution structural image using rigid body transformations. In a second step the images were normalised to the Montreal Neurological Institute (MNI) template brain using a 12 degrees of freedom affine transformation algorithm. To allow voxelwise comparisons, we firstly processed each CBF map individually using the perfusion signal modelling, which models the differences between control and tag. We processed a CBF map for each participant, time point (pre and post) and drink (HF & CT). These perfusion flow maps were then given as inputs for the 2nd level analysis (t contrasts) which processed the difference between pre and post for each drink. Specifically these t test contrasts compared the CBF maps at 2 and 5 hours post drink with the pre drink baseline, and had the form of a simple subtraction defined as such: CBF 2 hrs - CBF baseline, and CBF 5 hrs - CBF baseline. The output of this second step was contrast images which corresponded to the actual increase in the perfusion flow post drink consumption. Each of those contrast images was then entered into a 3rd level paired-sample t test which compared the drink interventions. The resulting Z (Gaussianised T/F) statistic image was then cluster thresholded with initial clusters determined using a voxelwise uncorrected height threshold of $Z>2.3$ followed by a cluster significance threshold of $p<0.05$ (corrected for multiple comparisons). Prior to analysis normality checks were performed on all data and outliers were removed.

3. Results

[Figure 1 here]

3.1 ASL CBF

Figure 1 shows significantly greater regional perfusion in the inferior frontal gyrus and middle frontal gyrus of the right hemisphere two hours following consumption of the HF drink compared to the CT drink (988 voxels, co-ordinates: (X=37.9, Y=31.8, Z=17.8), statistics threshold: $Z=3.69$, $p<0.001$. There were no significant differences in regional perfusion between the HF and CT drinks five hours post consumption, and no significant differences in global perfusion were observed between the two conditions at either time point.

3.2 Cognitive Tests

[Figure 2 here]

A significant Drink*Time interaction was observed for the DSST ($F^{1,23}=10.76$, $p<0.01$). As shown in Figure 2, post hoc t-tests revealed that consumption of the HF drink resulted in a significant improvement in DSST performance at two hours relative to baseline ($t=3.84$, $p<0.01$), whereas no significant improvement in performance was observed following the CT drink ($t=0.05$, $p=0.96$). Baseline DSST performance did not differ between the CT and HF drinks ($t=0.02$, $p=0.98$). No significant interactions or main effects were observed for all other cognitive tests (see Table 2).

[Table 2 here]

3.3 Blood pressure

The Drink*Time interactions were not significant for either diastolic ($F^{1,23}=1.19$, $p=0.29$) or systolic blood pressure ($F^{1,23}=0.5$, $p=0.49$). However, main effects of Time revealed that both systolic ($F^{1,23}=4.56$, $p<0.05$) and diastolic ($F^{1,23}=13.38$, $p<0.01$) blood pressure significantly reduced at two hours relative to baseline (see Table 2). To further explore the main effect of Time, post hoc t-tests revealed that consumption of the HF drink significantly reduced diastolic blood pressure at two hours compared to baseline ($t=3.43$, $p<0.01$), whereas this reduction did not reach significance following the CT drink ($t=2.05$, $p>0.05$).

4. Discussion

Acute improvement in a measure of executive function (DSST) and increased CBF in the right frontal gyrus during conscious resting state were observed two hours following consumption of 500ml of flavanone-rich citrus juice relative to a zero flavonoid, vitamin C matched, equicaloric control drink. These data indicate that 70.5mg flavonoids (specifically 42.15mg hesperidin, 17.25mg naringin, 6.75mg narirutin, 4.3mg caffeic acid) can increase CBF in healthy young adults. However, these data do not provide evidence for a direct association between increased CBF and behavioural benefits. Firstly, cognitive testing and CBF were not assessed simultaneously, and moreover, no effects were observed for the majority of cognitive outcomes.

This is the first data to show regional specific increases in human CBF following a flavanone dose. The frontal gyrus has been identified within a network of brain areas which are active during conscious resting state[(23)] which may explain the observed regional specific increased perfusion. The inferior frontal gyrus has typically been implicated in tasks which require inhibition, planning, decision making and other aspects of executive function[(24)], such as the

DSST, for which improvements were observed in this study following the flavanone-rich juice. However, the mechanisms underpinning the right hemispheric lateralisation are unclear.

These data provide evidence that flavonoid sub-classes other than cocoa-flavanols can also have acute effects on CBF within the immediate postprandial period. Increased global CBF across grey matter was observed 2 hours after consumption of a 560mg flavanol drink relative to a control drink[12], however, regional blood flow was not assessed, most likely due to the small sample size of healthy young adults (n=4). The same authors also reported that a smaller flavanol dose (172mg) was associated with increased regional specific BOLD signal intensity (including medial and lateral prefrontal cortex, parietal cortex, anterior cingulate cortex and the cerebellum) 1.5 hours post consumption in 16 health young adults, although the cocoa drink was consumed for 5 consecutive days prior to the fMRI scan. Direct comparisons between the regions of interest reported by Francis et al.[12] and the present study are restricted by differences in scanning methods (BOLD or ASL), the flavonoid sub-class and dose (172mg cocoa flavanols or 70.5mg fruit flavanones), duration of consumption (5 days or a single acute dose) and behavioural instructions during imaging; the present study examined conscious resting state whereas Francis et al.[12] examined neural activity during an executive function task. In addition, a limitation of the present study was the absence of double blinding during data collection which could have introduced experimenter biases. Critically though, data analysis was performed blinded by an independent researcher. Further investigation of the acute effects of flavonoid consumption on regional CBF are required in order to identify whether specific regions appear to particularly reactive to flavonoid ingestion in the postprandial period. For example, increased perfusion in the anterior cingulate cortex and central opercular cortex was recently observed two hours post consumption of 494mg cocoa flavanols[25], however, behavioural tasks were not assessed. Studies of neural activation following chronic daily consumption of fruit based flavonoids[9] and flavanol-rich cocoa flavonoids[13,14] indicate that areas of the brain implicated in memory function such as the hippocampus, specifically the dentate gyrus, are especially sensitive.

The mechanisms by which flavonoids acutely induce vasodilation and enhance CBF are thought to be via increased nitric oxide synthesis in the endothelium (eNOS). Nitric oxide synthesis is a key regulator of angiogenesis and the dilation of cells, and is also synthesised by neurons in response to neuronal activation (nNOS)[26]. As such, nitric oxide is thought to be crucial for the coupling between increased blood supply and neuronal activity[27].

Flavonoid ingestion in humans is known to enhance circulating nitric oxide species[28] in association with beneficial vascular outcomes such as increased flow mediated dilation and augmented microcirculation[11]. Therefore, it is plausible that flavonoid-induced increases in the bioavailability of nitric oxide in the brain may lead to increased blood vessel and neuronal efficiency and, subsequently, improvements in cognitive function. These vascular mechanisms are tentatively supported by the observed reduction in systolic blood pressure following the flavanone-rich juice in the present study, however it should be noted that this was a subtle reduction (3mmHg). Having said that, a large reduction in blood pressure would not be anticipated in this sample of healthy young adults. Research in adults with metabolic syndrome shows that 550mg daily supplementation of the flavanone hesperidin for three weeks can lead to increased flow mediated dilation and endothelial nitric oxide synthesis[29]. This is pertinent to the present findings given that hesperidin was the predominant flavanone within the flavanone-rich citrus juice.

Research is required to directly examine the relationship between flavonoid consumption, nitric oxide activity, CBF and cognitive function. Interestingly, increased nitric oxide status in the plasma has been observed two hours post consumption of flavonoid-rich apples, however, no effects were observed for cognitive function[30]. Kean et al.[10] reported global cognitive improvements in healthy older adults cognition following daily chronic consumption of flavanone-rich orange juice (305mg/day) over eight weeks, however, nitric oxide status was not examined. This sample of highly educated, healthy young adults, are likely performing close to optimal functioning and therefore, there is greater potential for acutely enhancing cognition in older adults who may be experiencing naturally occurring ageing associated cognitive decline. This may explain why effects were not observed for the majority of cognitive outcomes in the present study, particularly given the relatively small flavanone dose (70.5mg). Previously, positive behavioural effects in healthy young adults have only been observed following high doses of cocoa flavanols e.g. 573mg[31] and 550mg/994g[32]. Additionally, it has been argued that flavonoid interventions are more likely to benefit cognition during tasks of high demand[32], therefore it is possible that the current cognitive battery was not suitably challenging, however, there was no evidence of ceiling effects.

It can be hypothesised that stronger behavioural effects may occur at a later time point given that plasma flavanone metabolites following orange juice consumption have been observed to peak at six hours[33,34]. Indeed, it is a limitation of the present study that cognitive function was exclusively assessed two hours post consumption (in addition to baseline). Recently, benefits for global cognitive function and subjective alertness were observed 2 and 6 hours post consumption of a flavanone rich (272mg) 100% orange juice in healthy young adults, with the effects being more pronounced (relative to the control drink) at 6 hours[35]. Having said that, presently, increased CBF was observed at two hours but not five hours, possibly indicating that the time course by which the flavonoids in orange and grapefruit juice exert their physiological effects may differ relative to 100% orange juice, although the mechanism for this is unclear. Future acute interventions of flavonoid consumption should examine plasma flavonoid metabolites concomitantly with cognitive outcomes to investigate whether peak metabolite concentrations coincide with the hypothesised behavioural effects. Flavanone metabolites are certainly of interest given that they are known to cross the blood brain barrier[36]. Future studies should carefully consider the time span over which circulating flavonoid metabolites may impact cognitive outcomes. Anthocyanin metabolites have been observed in urine up to 5 days following acute ingestion of blueberries[36]. This has implications for the current findings; the 24 hour dietary restriction may not have been sufficient to account for potential confounding effects of habitual flavonoid intake, although it is unclear whether the associated levels of circulating metabolites can acutely affect cognition.

In conclusion, 500ml citrus juice containing 70.5mg flavonoids was associated with increased regional perfusion in the right frontal gyrus in young healthy adults two hours following the flavanone-rich juice in conscious resting state relative to the zero-flavonoid, equicaloric, vitamin C matched control. This data demonstrates that fruit based flavonoids can acutely enhance CBF in healthy adults. Behavioural improvements on a battery of cognitive tests following the flavonoid-rich juice were only observed for one measure of executive function (DSST) in a separate cohort of young adults. Therefore, the present data does not show a clear association between increased CBF and behavioural benefits. Further research should simultaneously examine cognitive performance and respective functional brain activation, regional cerebral blood flow and concentrations of circulating nitric oxide species following consumption of flavonoid-rich juices to further our understanding of underlying mechanisms.

References

1. Spencer JPE (2010) Beyond antioxidants: the cellular and molecular interactions of flavonoids and how these underpin their actions on the brain. *Proceedings of the Nutrition Society* **60**, 244-60.

2. Menze ET, Tadros MG, Abdel-Tawed AM *et al*. (2012) Potential neuroprotective effects of hesperidin on 3-nitropropuinic acid-induced neurotoxicity in rats. *Neurotoxicology* **33**, 1265–1275.

3. Rendeiro C, Vauzour D, Rattray M *et al.* (2013) Dietary levels of pure flavonoids improve spatial memory performance and increase hippocampal brain derived neurotrophic factor. *PLoS One* **8**, e63535.

4. Laske C, Stellos K, Hoffmann N *et al.* (2011) Higher BDNF serum levels predict slower cognitive decline in Alzheimer's disease patients. *Int J Neuropsychop* **14**, 399-404.

5. Butchart C, Kyle J, McNeill G *et al.* (2011) Flavonoid intake in relation to cognitive function in later life in the Lothian Birth Cohort 1936. *Brit J Nutr* **106**, 141-148.

6. Lamport DJ, Dye L, Wightman JD *et al.* (2012) The effects of flavonoid and other polyphenol consumption on cognitive performance: A systematic research review of human experimental and epidemiological studies. *Nutrition and Aging* **1**, 5-25.

7. Lamport DJ, Hoyle E, Lawton CL *et al.* (2011) Evidence for a second meal cognitive effect: glycaemic responses to high and low glycaemic index meals are associated with cognitive the following morning. *Nutr Neurosci* **14**, 66-72.

8. Krikorian R, Nash TA, Shidler MD *et al.* (2010a) Concord grape juice supplementation improves memory function in older adults with mild cognitive impairment. *Br J Nutr* **103**, 730-734.

9. Krikorian R, Boespflug EL, Fleck DE *et al.* (2012) Concord grape juice supplementation and neurocognitive function in human aging. *J Agric Food Chem* **60**, 5736-5742.

10. Kean RJ, Lamport DJ, Dodd GF *et al.* (2015) Chronic consumption of flavanone-rich orange juice is associated with cognitive benefits: an 8-week randomised double-blind placebo-controlled trial in healthy older adults. *Am J Clin Nutr* **101**, 506-514.

11. Schroeter H, Heiss C, Balzer J *et al.* (2006) (-)-Epicatechin mediates beneficial effects of flavanol-rich cocoa on vascular function in humans. *Proc Natl Acad Sci USA* **103**, 1024-9

12. Francis S, Head K, Morris PG *et al.* (2006) The effect of flavanol-rich cocoa on the fMRI response to a cognitive task in healthy young people. *J Cardiovasc Pharmacol* **47**, S215-220.

13. Brickman AM, Khan US, Provenzano FA *et al.* (2014) Enhancing dentate gyrus function with dietary flavanols improves cognition in older adults. *Nat NeuroSci* **17**, 1798-1803.

14. Camfield DA, Scholey A, Pipingas A *et al.* (2012) Steady state visually evoked potential (SSVEP) topography changes associated with cocoa flavanol consumption. *Physiol Behav* **105**, 948-957.

15. Krikorian R, Shidler MD, Nash TA *et al.* (2010b) Blueberry supplementation improves memory in older adults. *J Agric Food Chem* **58**, 3996-4000.

16. Lamport DJ, Saunders C, Butler LT *et al.* (2014) Fruits, vegetables, 100% juices and cognitive function. *Nutr Rev* **72**, 774-789.

17. Bach M (1996) The Freiburg Visual Acuity Test – automatic measurement of visual acuity. *Optometry Vision Sci* **73**, 49–53.

18. Stadler MA (1995) Role of attention in implicit learning. *J Exp Psychol Learn* **21**, 674-685.

19. Wechsler D (1981) The psychometric tradition – developing the Wechsler adult intelligence scale. *Contemp Educ Psychol* **6**, 82-5.

20. Stroop JR (1935) Studies of interference in serial verbal reactions. *J Exp Psychol* **18**, 643–662.

21. Morris N, Jones DM (1990) Memory updating in working memory: The role of the central executive. *Br J Psychol* **81**, 111-21.

22. Garavan H, Ross TJ, Stein EA (1999) Right hemispheric dominance of inhibitory control: An event-related functional MRI study. *PNAS* **96**, 8301-8306.

23. Binder JR, Frost JA, Hammeke TA *et al.* (1999) Conceptual processing during the conscious resting state: a functional MRI study. *J Cogn Neurosci* **11**, 80-93.

24. Aron AR, Robbins TW, Poldrack RA (2004) Inhibition and the right inferior frontal cortex. *Trends Cogn Sci* **8**, 170–177.

25. Lamport DJ, Pal D, Moutsiana C *et al*. (2015). The effect of flavanol rich cocoa on cerebral perfusion in healthy older adults during conscious resting state: a placebo controlled crossover acute trial. *Psychopharmacol* **232**, 3227-3224.

26. Berdt DS, Hwang PM, Snyder SH (1990) Localization of nitric oxide synthesis indicating a neural role for nitric oxide. *Nature* **347**, 768-770.

27. Toda N, Ayajiki K, Okamura T (2009) Cerebral blood flow regulation by nitric oxide in neurological disorders. *Can J Physiol Pharmacol* **87**, 581–594.

28. Loke WM, Hodgson JM, Proudfoot JM *et al* (2008) Pure dietary flavonoids quercetin and (-)-epicatechin augment nitric oxide products and reduce endothelin-1 acutely in healthy men. *Am J Clin Nutr* **88**, 1018-25.

29. Rizza S, Muniyappa R, Lantorno M *et al.* (2011) Citrus polyphenol hesperidin stimulates production of nitric oxide in endothelial cells while improving endothelial function and reducing inflammatory markers in patients with metabolic syndrome. *J Clin Endocrinol Metab* **96**, E782-92

30. Bondonno CP, Downey LA, Croft KD *et al.* (2014). The acute effect of flavonoid-rich apples and nitrate rich spinach on cognitive performance and mood in healthy men and women. *Food Funct* DOI: 10.1039/c3fo60590f

31. Field DT, Williams CM, Butler LT (2011) Consumption of cocoa flavanols results in an acute improvement in visual and cognitive functions. *Physiol Behav* **103**, 255–260.

32. Scholey A, French SJ, Morris PJ *et al.* (2010) Consumption of cocoa flavanols results in acute improvements in mood and cognitive performance during sustained mental effort. *J Psychopharmacol* **24**, 1505–1514.

33. Manach C, Scalbert A, Morand C (2004) Polyphenols: food sources and bioavailability. *Am J Clin Nutr* **79**, 27-47.

34. Mullen W, Archeveque MA, Edwards CA *et al* (2008) Bioavailability and metabolism of orange juice flavanones in humans: impact of a full-fat yogurt. *J Agr Food Chem* **56**, 11157-11164.

35. Alharbi MH, Lamport DJ, Dodd G *et al*. (2015) Flavonoid rich orange juice is associated with acute improvements in cognitive function in healthy middle aged males. *Eur J Nutr* DOI:10.1007/s00394-015-1016-9.

36. Youdim KA, Dobbie MS, Kuhnle G *et al.* (2003) J Interaction between flavonoids and the blood-brain barrier: in vitro studies. *Neurochem* **85**, 180-92.

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Conflicts of Interest: None

Table 1 – mean participant characteristics for the behavioural cognitive arm and the arterial spin labelling (ASL) arm (standard deviation)

	Behavioural Cognitive Arm (n=24)	ASL Arm (n=16)	p-value comparison between arms
Age (years)	22 (2.2)	22 (1.9)	0.73
BMI (kg/m^2)	23.2 (3.9)	23.3 (1.7)	0.88
Years in education	16.9 (1.8)	16.6 (1.4)	0.53
MMSE[1] (max 30)	29.3 (1)	29.6 (0.5)	0.19

1Mini Mental State Examination

Table 2 – Means and standard deviations for each cognitive test and blood pressure data at baseline and two hour post consumption for the control and high flavanone drinks

		Control Drink	High Flavanone	Drink*Time interaction (p-value)
DSST[1]	Baseline	77.4 (9.7)	75.9 (8.4)	0.003**
	2 hours	77.5 (9.6)	80.3 (8.9)	
FVT Landholt C[2]	Baseline	0.41 (0.03)	0.4 (0.02)	0.19
	2 hours	0.42 (0.04)	0.4 (0.02)	
FVT Vernier[3]	Baseline	21.2 (23.3)	19.9 (20.1)	0.65
	2 hours	19.6 (16.8)	21.3 (14.7)	
GoNo-Go[4]	Baseline	315 (55)	310 (60)	0.86
	2 hours	308 (62)	305 (57)	
Letter Memory[5]	Baseline	77 (16.7)	74.6 (18.4)	0.89
	2 hours	77.1 (12)	74.1 (16.3)	
Logical Memory Imm[6]	Baseline	17.5 (3.6)	18.3 (3.3)	0.97
	2 hours	15.4 (3)	16.1 (3.6)	
Logical Memory Del.[6]	Baseline	16.1 (3.6)	15.8 (3.9)	0.48
	2 hours	14.1 (3.8)	14.6 (3.3)	
Sequence Learning[7]	Baseline	97.8 (1.5)	98 (1.6)	0.52
	2 hours	96.9 (2.1)	97 (2)	
Spatial Memory[8]	Baseline	27.3 (15.8)	28.2 (18)	0.68
	2 hours	28.2 (15.4)	30 (20.6)	
Stroop[9]	Baseline	654 (74)	647 (71)	0.71
	2 hours	626 (84)	623 (67)	
Word Recall Imm[10]	Baseline	7.3 (3.2)	7.3 (3.5)	0.11
	2 hours	7 (2.7)	5.7 (2.5)	
Word Recall Del.[10]	Baseline	5.2 (2.9)	5.2 (3.2)	0.15
	2 hours	4.5 (2.5)	3.2 (2.3)	
Diastolic BP[11]	Baseline	72 (8.4)	71.7 (7.5)	0.49
	2 hours	69.7 (7.8)	68.4 (7.5)	
Systolic BP[11]	Baseline	115.9 (12.4)	116.5 (12.4)	0.29
	2 hours	115.3 (12.3)	113.8 (12.1)	

**p<0.01

1Digit Symbol Substitution Test correct responses; 2 Freiburg Vision Test Landholt C a higher score indicates better vision; 3 Freiberg Vision Test Vernier Threshold a higher score indicates better vision; 4 GoNo-Go reaction time (ms); 5 Letter Memory Accuracy; 6 Logical Memory units recalled; 7 Sequence Learning correct responses; 8 Spatial Delayed Recall Test distance from target (mm), 9 Computerised Stroop reaction time (ms); Word Recall number of words recalled; Blood Pressure mmHg.

Figure 1 Legend: Significantly greater regional perfusion occurred in the inferior frontal gyrus and medial frontal gyrus of the right hemisphere two hours following the high flavanone drink compared to the control drink. Activations are superimposed on axial slices of the MNI template brain and represent perfusion flow in ml/100g tissue/min with yellow indicating greater perfusion. The images were initially thresholded at $Z>2.3$ to identify activation clusters and then a (corrected) cluster significance threshold of $p<0.05$ was applied.

Figure 2 Legend: Following a significant Drink*Time interaction ($F^{1,23}=10.76$, $p<0.01$) post hoc tests revealed that number of correct responses on the Digit Symbol Substitution Test was significantly greater at two hours relative to baseline ($t=3.84$, $p<0.01$) following consumption of the flavanone rich juice.

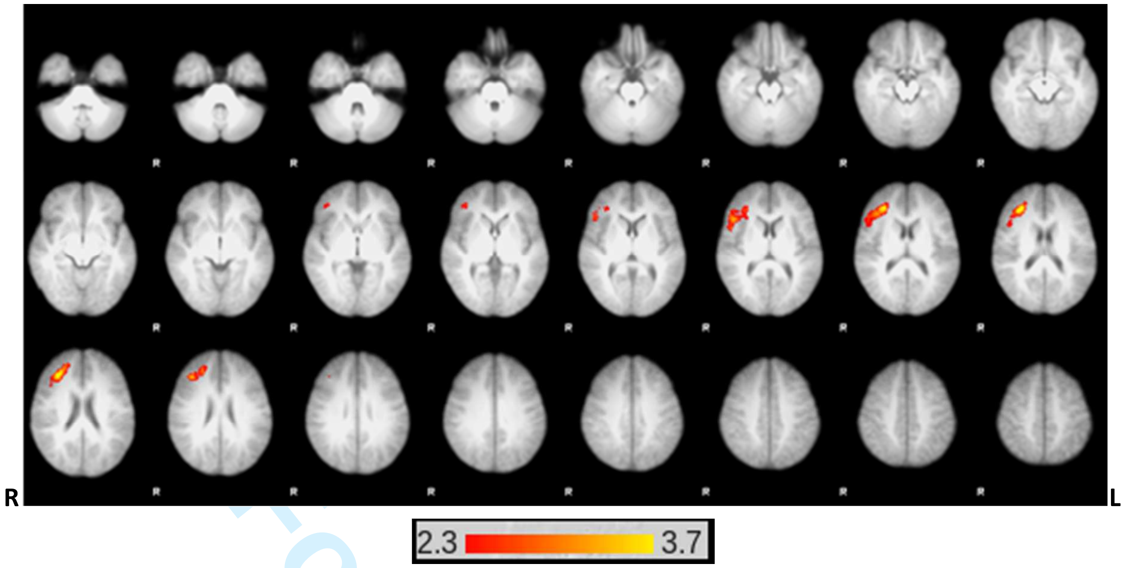
R
L
2.3
3.7

Figure 2 – Digit Symbol Substitution Test mean correct responses and standard errors for the control and high flavanone drink at baseline and two hour post consumption