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

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22 Abstract

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

42 1. Introduction

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

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

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

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

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

99 **2. Experimental Methods**

100 Different participants were recruited for the behavioural cognitive arm (n=28) and the ASL
101 imaging arm (n=16) of the study (see Table 1), however, inclusion and exclusion criteria
102 were identical for both arms. Participants were not permitted to take part in both arms. **At the**
103 **time of designing the study, there was an absence of published data concerning the effects of**
104 **flavanone consumption in humans on cognitive function, cardiovascular outcomes, or**

105 cerebral blood flow. Therefore, we considered it important to create an experimental design
106 in which cognitive and cerebral blood flow effects could be examined in isolation. For,
107 example, it is important to establish if effects on CBF are observed independently of
108 behavioural effects. Furthermore, in light of the absence of experimental support for a
109 specific behavioural task sensitive to flavanone consumption in humans, it was considered
110 that a range of cognitive functions should be assessed. Incorporating a comprehensive
111 cognitive battery into the fMRI sequencing schedule posed significant practical difficulties.
112 Therefore, a decision was taken to recruit separate cohorts for the behavioural and imaging
113 arms. Healthy young adults aged 18-30 years were recruited from the University of Reading
114 and surrounding area via community advertising with posters, leaflets and emails. Twenty
115 four participants (four males) completed the behavioural cognitive arm (four participants
116 dropped out due to work commitments or illness) and all sixteen participants completed the
117 ASL arm (eight males). Inclusion criteria were BMI 19-25kg/m² and fluent English speaker
118 whilst exclusion criteria were signs of mild cognitive impairment (Mini Mental State
119 Examination Score <26), smoking, alcohol consumption >15 units/week, orange juice
120 consumption >250ml/day, fruit/vegetable consumption >4 portions/day, caffeine intake >3
121 drinks/day, actively pursuing weight loss through a dietary intervention, clinical diagnosis of
122 mental illness, neurological disease, chronic fatigue, kidney disease, liver disease, thyroid
123 dysfunction, diabetes mellitus, myocardial infarction or hypertension, and consumption of
124 medication for lipids, hypertension, hypotension or anticoagulation. Recruitment commenced
125 March 2011 and terminated August 2011. Our sample size was based on previous research
126 reporting significant cognitive effects of berry flavonoids in older adults with sample sizes
127 ranging from nine to twenty one^(8,9,15) and improvements in CBF following cocoa flavanols in
128 sixteen young adults⁽¹²⁾.

129 [Table 1 here]

130 2.1 Design

131 An acute single-blind, randomised cross-over design was applied with two drink conditions;
132 high flavonoid (HF) and control (CT). Cognitive behavioural testing and ASL measurements
133 were performed prior to and post consumption of the drink at each visit (see procedure). The
134 500ml HF drink was a commercially available 100% juice (Tropicana Ruby Breakfast Juice,
135 PepsiCo Inc.) which naturally contained 70.5mg flavonoids (42.15mg hesperidin, 17.25mg
136 naringin, 6.75mg narirutin, 4.3mg caffeic acid; analysed by the University of Reading),

137 225kcal, 48.5g sugars, 4g protein, 0g fat, 3.5g fibre, and 150mg vitamin C. The Tropicana
138 Ruby Breakfast Juice contained juices from oranges and grapefruits. The 500ml CT drink
139 was a commercially-available concentrated cordial product (Lemon Barley Squash,
140 Sainsbury's, UK) which was prepared with 240mls of concentrate and 260mls of mineral
141 water (Buxton Spring still mineral water) containing zero flavonoids, 230kcal, 48g sugars,
142 0.7g protein, 0g fat, 0.3g fiber, and 130mg vitamin C. Our dose of 70.5mg flavonoids could
143 be considered low relative to previous research⁽⁶⁾, however, it is important to examine
144 whether cognitive benefits are associated with consuming concentrations of flavanones which
145 are present in the habitual diet. Therefore, the 500ml juice serving provided an acceptable
146 balance between a suitable flavonoid concentration and an achievable volume of
147 consumption within the context of the habitual diet. The drinks were stored at 4°C and
148 prepared and served by the experimenter. Each 500ml portion was served in two 250ml
149 opaque flasks and consumed through an opaque straw, thus participants could not see the
150 drink and remained blinded. The randomisation order was determined by an independent
151 statistician. For the behavioural cognitive arm, twelve participants consumed the HF drink at
152 visit 1 and twelve consumed the CT drink at visit 1, whilst for the ASL arm eight participants
153 consumed the HF drink at visit 1 and eight consumed the CT drink at visit 1.

154 2.2 Procedure

155 In summary, participants attended three separate visits; one screening visit and two test day
156 visit. The behavioural arm test days included two cognitive test time points (baseline and two
157 hour post) and the ASL arm visit days included three time points (baseline, two hour post and
158 five hour post). The screening visit and each test day visit were separated by a one week
159 washout. Initially telephone screening interviews were performed and volunteers who met the
160 inclusion criteria were invited to attend the University of Reading Hugh Sinclair Nutrition
161 Unit for a screening visit. At screening, data on height, weight, health status, medication and
162 blood pressure was collected and participants completed the Mini Mental State Examination
163 (MMSE), a diet and lifestyle questionnaire and a fruit and vegetable questionnaire, data from
164 which was used to corroborate the inclusion/exclusion criteria. For each test day visit,
165 participants arrived at 08:00 having fasted from alcohol for 48 hours and all other food and
166 drink (except water) for twelve hours. At screening, participants were provided with low-
167 nitrate bottled water for consumption during the fast. Prior to each test day visit, participants
168 were also instructed to avoid polyphenol-rich foods for 24 hours (including berries, fruits,
169 fruit juices, jams and preserves, red wine, black, green and fruit teas, coffee, cocoa, soy

170 products, caffeinated energy drinks and vegetables except potatoes) and were provided with
171 standardised typed instructions identifying which foods to avoid. The evening prior to each
172 test day, participants consumed (at home) a low fat standardized chicken and rice meal
173 provided by the research team (350kcal, 6.9g fat of which 3g saturates, 52.1g carbohydrate of
174 which 9.7g sugars, 19g protein, 1.4g fiber, 0.9g salt) to avoid second-meal cognitive
175 effects⁽¹⁶⁾. On each test day participants were required to orally confirm that they had adhered
176 to the aforementioned dietary restrictions. Following a fifteen minute rest, blood pressure
177 measurements were taken (on behavioural visit days only) on the left upper arm by a
178 validated blood pressure monitor (Omron MX2 automatic digital upper arms BP monitor,
179 Milton Keynes, UK) and recorded as the average of three consecutive measurements. At
180 08:30 hrs, participants consumed a standardised breakfast within fifteen minutes (88g
181 croissant, 25g cream cheese and 120ml bottled mineral water containing 51g fat, 14g protein,
182 64g carbohydrates, 777kcal). For the behavioural test days, baseline cognitive testing
183 commenced at 08:45 hrs, followed by consumption of the drink (either HF or CT) at 09:45
184 hrs. Participants were informed that the drink was a fruit-based beverage available in most
185 UK supermarkets and which must be consumed within fifteen minutes. Blood pressure was
186 measured at 11:40 hrs (behavioural arm only) and lunch, identical to breakfast in both content
187 and amount, was provided fifteen minutes prior to the two-hour post-drink cognitive battery
188 which commenced at 12:00 hrs. An assessment at this time point was based on previous data
189 demonstrating cognitive effects 2 hours following an acute flavonoid dose¹². For the ASL
190 visit days, the timings were identical to the behavioural cognitive visit days, such that ASL
191 measurements were performed at 08:45 hrs (baseline), 12:00 hrs (two hours) and 15:00 hrs
192 (five hours). The behavioural cognitive visits took place in individual cubicles at the
193 University of Reading Hugh Sinclair Nutrition Unit and the ASL visits took place at the
194 Centre for Integrative Neuroscience and Neurodynamics (CINN). Participants remained
195 within the Nutrition Unit or the CINN for the entire test visit during which only water
196 consumption was permitted (notwithstanding the test day foods and drinks). Participants
197 received a £120 honorarium upon completion. This study was conducted according to the
198 guidelines laid down in the Declaration of Helsinki and all procedures involving human
199 subjects were approved by the School of Psychology and Clinical Languages Ethics
200 Committee. Written and verbal informed consent was obtained and formally recorded.

201 2.3 Cognitive Battery

202 The 45-minute cognitive battery consisted of the following tests administered in the
203 respective order: Freiburg Vision Test (v3.6.3), Word Recall (immediate), Logical Memory
204 (immediate recall), Sequence Learning Task, Digit Symbol Substitution (DSST), Stroop Test,
205 Letter Memory Test, Go-NoGo Task, Spatial Delayed Recall, Word Recall (delayed), and
206 Logical Memory (delayed). Where multiple versions of a test were required (see below),
207 parallel versions were presented in a counterbalanced order across conditions and visits. The
208 Freiburg Vision Test assesses visual acuity⁽¹⁷⁾ for which there are two dependent variables:
209 Landholt C and Vernier Threshold. To acquire the Landholt C measurement participants
210 were required to identify the orientation of a horseshoe symbol using the numbers 1-9 on the
211 keyboard keypad (excluding 5). The presentation size of the horseshoe and thus the ease of
212 identifying the orientation randomly varied across trials. Landholt C was subsequently
213 calculated according to the number of correct responses relative to the presentation size. To
214 acquire the Vernier Threshold, participants viewed a stimulus which consisted of two 1cm
215 lines with one directly above the other. Participants pressed the left scroll key if the line
216 above was to the left of the line below, and the right scroll key if the line above was to the
217 right of the line below. The degree to which the lines were aligned varied randomly across
218 trials. The Vernier Threshold was subsequently calculated according to the number of correct
219 responses relative to the horizontal distance between the two lines⁽¹⁷⁾. Verbal Recall involved
220 computerised, individual presentation of thirty words. A response was required (using the
221 keys 'M' for yes, 'Z' for no) according to one of five questions which required visual,
222 phonetic or semantic processing of the target word (e.g. "is the word in capitals", "does the
223 word rhyme with..." or "is the word a type of..."). Upon cessation of the presentation, oral
224 recall of the target words was required (the dependent variable). Within each version of the
225 test, each word was accompanied by the same question for all participants whilst the order of
226 presentation varied randomly. Equal versions were created and matched for frequency,
227 familiarity, imageability, meaningfulness, word length and syllables. Delayed Word Recall
228 involved one attempt to orally recall the words presented thirty minutes prior during
229 Immediate Word Recall. The Logical Memory Test (Wechsler Memory Scale – Revised)
230 requires oral recall of a short paragraph. The paragraphs were presented via cassette tape. The
231 dependent variables for immediate and delayed recall were the number of correctly recalled
232 units. The Sequence Learning Task⁽¹⁸⁾ required participants to immediately press the keys 'V,
233 B, N or M' according to the appearance of a stimulus (a 2mm white dot for 200ms) in one of
234 four 3.5cm x 2cm boxes on the screen. Unbeknownst to participants, the order of stimulus
235 presentation followed a set sequence (one block), thus this test assesses the ability to learn a

236 sequence. The duration of each repetitive sequence varied from 2-4 trials. Each test
237 presentation contained six blocks, with each block consisting of 100 trials. The dependent
238 variable was number of correct responses. The DSST⁽¹⁹⁾ is a pen and paper test which
239 contains a key of nine digit-symbol pairs and an accompanying list of digits. Under each
240 listed digit a space is provided to enter the corresponding symbol. Participants entered as
241 many symbols as possible over 90 seconds. The dependent variable was the number of
242 correct responses. The computerised Stroop Test⁽²⁰⁾ required participants to identify the
243 colour in which a word was presented. There were 120 randomly presented stimuli, each for
244 1650ms, consisting of 60 congruent and 60 incongruent trials (a congruent trial being when
245 the meaning of the word matched the colour in which it was presented). Participants
246 responded with the keys 1-4 which represented the colours green, blue, red and yellow
247 respectively. The dependent variable was reaction time (for correct responses only). The
248 Letter Memory Task⁽²¹⁾ involved serial 2000ms presentation of individual letters. The number
249 of letters per trial varied randomly between 5, 7, 9 and 11 for a total of twelve trials and 48
250 letters. For each trial, at the termination of the presentation phase participants were required
251 to orally recall the final four letters from the presentation. The dependent variable was the
252 total number of correct responses defined as recalling the correct sequence in its entirety. The
253 Go-NoGo is a computerised task assessing inhibition and sustained attention. The present
254 version was adapted from the Go-NoGo paradigm⁽²²⁾. Participants were required to respond
255 to sixty stimuli using one of three specified keyboard keys; 'p' 'q' or 'space bar'. The stimuli
256 consisted of X, Y or a number 'lure'. Initially, there was a 25 stimuli 'Pre-Potent Go' phase.
257 During the Pre-Potent Go phase, X and Y were presented alternately, with the participant
258 required to press 'q' when X appeared and 'p' when Y appeared. The X and Y were known
259 as the 'Go' trials. The Go-NoGo phase followed the Pre-Potent Go phase. During the Go-
260 NoGo phase, the 'Go' trials were interspersed with 'NoGo' trials; these appeared as numbers
261 lures. Pressing the space bar was the required response upon viewing a number lure. During
262 the Go-NoGo phase X and Y were presented randomly, interspersed with number lures, such
263 that the predictable alternating sequence was disrupted. Responses were required only if a Y
264 appeared after an X or vice-versa, and therefore the participant must inhibit the established
265 pre-potent response in all other trials. Reaction Time for correct responses was the dependent
266 variable. The Spatial Delayed Recall Test required participants to recall the location of a
267 white dot on the screen. Each trial commenced with a fixation cross followed by presentation
268 of a white dot for 50ms in a random location. The white dot was replaced by a randomly
269 generated number between 90-99 at which point participants were asked to orally subtract

270 three from this number continuously for eight seconds. Once eight seconds had elapsed the
271 number disappeared and the participant was required to indicate (by touching the screen) the
272 location at which the white dot had previously appeared. There were sixteen trials in total and
273 the dependent variable was the distance from the target (mm).

274 2.4 fMRI protocol

275 Scanning was performed at the CINN, University of Reading, UK using a 3.0 Tesla Siemens
276 MAGNETOM Trio MRI scanner with a 12-channel Head Matrix coil. The ASL images were
277 acquired using the PICOREQ2T sequence with the following parameters: number of
278 slices=18, slice thickness=5.0mm, inter-slice gap=1.25mm, TR=2500ms, TE=11ms,
279 T11=700, Saturation Stop Time=1600, TI2=1800, perfusion mode=PICORE Q2T (pulsed). A
280 high resolution whole-brain three dimensional anatomical image was also acquired using an
281 MPRAGE gradient-sequence with 176 x 1mm thick slices (1*1*1 voxels size, TE: 2.52ms,
282 TR: 2020ms, TI: 1100ms, FOV: 250x250, slice thickness: mm2, Flip Angle: 9deg). FMRI
283 data processing was carried out using FEAT (FMRI Expert Analysis Tool) Version 5.98, part
284 of FSL (FMRIB's Software Library; www.fmrib.ox.ac.uk/fsl). ASL volumes from each
285 scanning session were all registered to the corresponding individual's high resolution
286 structural image using rigid body transformations. In a second step, the images were
287 registered to the Montreal Neurological Institute (MNI) template brain using a 12 degrees of
288 freedom affine transformation algorithm. To allow voxelwise comparisons, each CBF map
289 was individually processed using perfusion signal modelling, which models the differences
290 between control images and tagged (spin labelled) images within a time series. A CBF map
291 was produced for each participant, drink (HF and CT) and time point (baseline, two hours
292 and five hours).

293 2.5 Statistical Analysis

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

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

320 3. Results

321 [Figure 1 here]

322 3.1 ASL CBF

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

329 3.2 Cognitive Tests

330 [Figure 2 here]

331 A significant Drink*Time interaction was observed for the DSST ($F^{1,23}=10.76$, $p<0.01$). As
332 shown in Figure 2, post hoc t-tests revealed that consumption of the HF drink resulted in a
333 significant improvement in DSST performance at two hours relative to baseline ($t=3.84$,
334 $p<0.01$), whereas no significant improvement in performance was observed following the CT
335 drink ($t=0.05$, $p=0.96$). Baseline DSST performance did not differ between the CT and HF
336 drinks ($t=0.02$, $p=0.98$). No significant interactions or main effects were observed for all
337 other cognitive tests (see Table 2).

338 [Table 2 here]

339 3.3 Blood pressure

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

347 4. Discussion

348 Acute improvement in a measure of executive function (DSST) and increased CBF in the
349 right frontal gyrus during conscious resting state were observed two hours following
350 consumption of 500ml of flavanone-rich citrus juice relative to a zero flavonoid, vitamin C
351 matched, equicaloric control drink. These data indicate that 70.5mg flavonoids (specifically
352 42.15mg hesperidin, 17.25mg naringin, 6.75mg narirutin, 4.3mg caffeic acid) can increase
353 CBF in healthy young adults. However, these data do not provide evidence for a direct
354 association between increased CBF and behavioural benefits. Firstly, cognitive testing and
355 CBF were not assessed simultaneously, and moreover, no effects were observed for the
356 majority of cognitive outcomes.

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

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

365 These data provide evidence that flavonoid sub-classes other than cocoa-flavanols can also
366 have acute effects on CBF within the immediate postprandial period. Increased global CBF
367 across grey matter was observed 2 hours after consumption of a 560mg flavanol drink
368 relative to a control drink⁽¹²⁾, however, regional blood flow was not assessed, most likely due
369 to the small sample size of healthy young adults (n=4). The same authors also reported that a
370 smaller flavanol dose (172mg) was associated with increased regional specific BOLD signal
371 intensity (including medial and lateral prefrontal cortex, parietal cortex, anterior cingulate
372 cortex and the cerebellum) 1.5 hours post consumption in 16 health young adults, although
373 the cocoa drink was consumed for 5 consecutive days prior to the fMRI scan. Direct
374 comparisons between the regions of interest reported by Francis et al.⁽¹²⁾ and the present
375 study are restricted by differences in scanning methods (BOLD or ASL), the flavonoid sub-
376 class and dose (172mg cocoa flavanols or 70.5mg fruit flavanones), duration of consumption
377 (5 days or a single acute dose) and behavioural instructions during imaging; the present study
378 examined conscious resting state whereas Francis et al.⁽¹²⁾ examined neural activity during an
379 executive function task. In addition, a limitation of the present study was the absence of
380 double blinding during data collection which could have introduced experimenter biases.
381 Critically though, data analysis was performed blinded by an independent researcher. Further
382 investigation of the acute effects of flavonoid consumption on regional CBF are required in
383 order to identify whether specific regions appear to particularly reactive to flavonoid
384 ingestion in the postprandial period. For example, increased perfusion in the anterior
385 cingulate cortex and central opercular cortex was recently observed two hours post
386 consumption of 494mg cocoa flavanols⁽²⁵⁾, however, behavioural tasks were not assessed.
387 Studies of neural activation following chronic daily consumption of fruit based flavonoids⁽⁹⁾
388 and flavanol-rich cocoa flavonoids^(13,14) indicate that areas of the brain implicated in memory
389 function such as the hippocampus, specifically the dentate gyrus, are especially sensitive.

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

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

408 Research is required to directly examine the relationship between flavonoid consumption,
409 nitric oxide activity, CBF and cognitive function. Interestingly, increased nitric oxide status
410 in the plasma has been observed two hours post consumption of flavonoid-rich apples,
411 however, no effects were observed for cognitive function⁽³⁰⁾. Kean et al.^[10] reported global
412 cognitive improvements in healthy older adults cognition following daily chronic
413 consumption of flavanone-rich orange juice (305mg/day) over eight weeks, however, nitric
414 oxide status was not examined. This sample of highly educated, healthy young adults, are
415 likely performing close to optimal functioning and therefore, there is greater potential for
416 acutely enhancing cognition in older adults who may be experiencing naturally occurring
417 ageing associated cognitive decline. This may explain why effects were not observed for the
418 majority of cognitive outcomes in the present study, particularly given the relatively small
419 flavanone dose (70.5mg). Previously, positive behavioural effects in healthy young adults
420 have only been observed following high doses of cocoa flavanols e.g. 573mg⁽³¹⁾ and
421 550mg/994g⁽³²⁾. Additionally, it has been argued that flavonoid interventions are more likely
422 to benefit cognition during tasks of high demand³², therefore it is possible that the current
423 cognitive battery was not suitably challenging, however, there was no evidence of ceiling
424 effects.

425 It can be hypothesised that stronger behavioural effects may occur at a later time point given
426 that plasma flavanone metabolites following orange juice consumption have been observed to
427 peak at six hours^(33,34). Indeed, it is a limitation of the present study that cognitive function

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

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

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548 declare. JMF, LTB & JPES designed the research. DJL, DP & AM analysed the data and
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551 **Conflicts of Interest:** None

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552 Table 1 – mean participant characteristics for the behavioural cognitive arm and the arterial
 553 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

554

¹Mini Mental State Examination

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555 Table 2 – Means and standard deviations for each cognitive test and blood pressure data at
 556 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)	

557 **p<0.01

558 1 Digit Symbol Substitution Test correct responses; 2 Freiburg Vision Test Landholt C a higher score indicates better vision; 3 Freiburg
 559 Vision Test Vernier Threshold a higher score indicates better vision; 4 GoNo-Go reaction time (ms); 5 Letter Memory Accuracy; 6 Logical
 560 Memory units recalled; 7 Sequence Learning correct responses; 8 Spatial Delayed Recall Test distance from target (mm), 9 Computerised
 561 Stroop reaction time (ms); Word Recall number of words recalled; Blood Pressure mmHg.

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

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

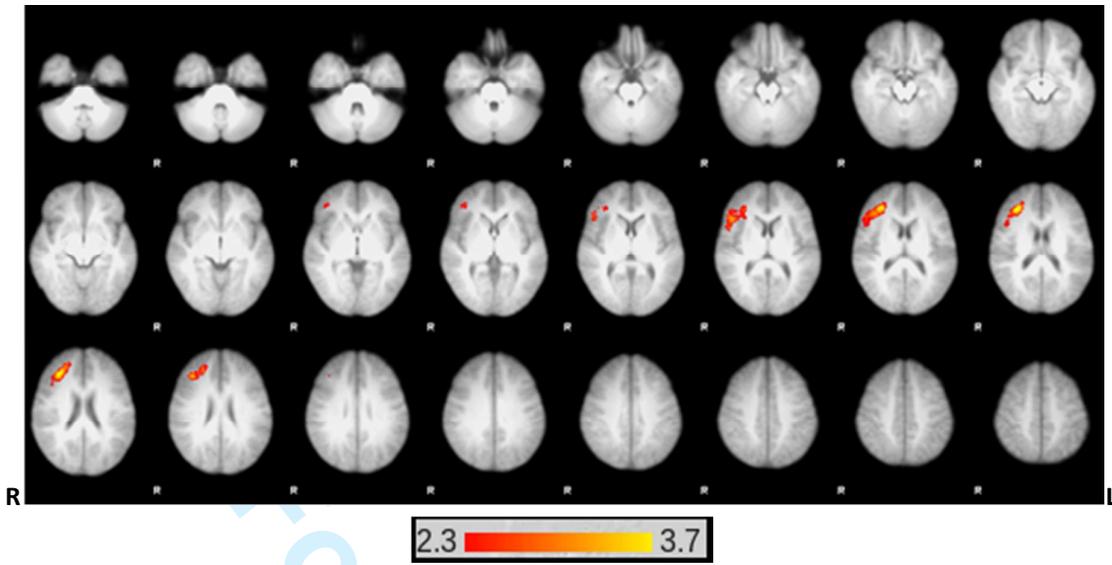
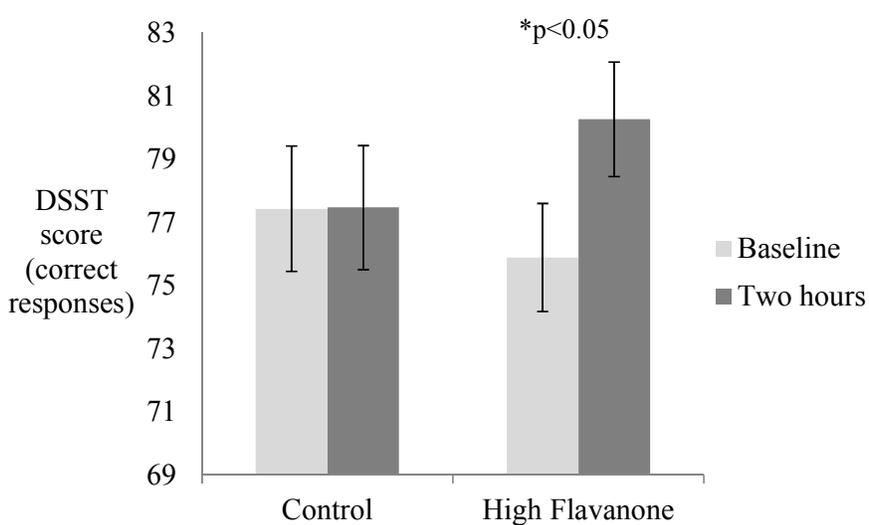


Figure 2 – Digit Symbol Substitution Test mean correct responses and standard errors for the control and high flavanone drink at baseline and two hour post consumption



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