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1 **Dietary nitrate modulates cerebral blood flow parameters and cognitive performance in**
2 **humans: a double-blind, placebo-controlled, crossover investigation.**

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17

18 **Running title:** Dietary nitrate, cognition and cerebral blood-flow

19 **Clinicaltrials.gov ID:** NCT01169662

20 **Sources of Support:** The research described herein was internally funded, and all materials
21 were purchased on the open market.

22 **Keywords:** Beetroot, Near-Infrared Spectroscopy, cerebral blood flow, nitric oxide, nitrite
23 (NO_2^-), nitrate (NO_3^-), cognition.

24

25 **ABSTRACT**

26 **Background:** Nitrate derived from vegetables is consumed as part of a normal diet and is
27 reduced endogenously via nitrite to nitric oxide. It has been shown to improve endothelial
28 function, reduce blood pressure and the oxygen cost of sub-maximal exercise, and increase
29 regional perfusion in the brain.

30 **Objectives:** The current study assessed the effects of dietary nitrate on cognitive performance
31 and prefrontal cortex cerebral blood flow (CBF) parameters in healthy adults.

32 **Design:** In this randomised, double-blind, placebo-controlled, parallel-groups study 40
33 healthy adults received either placebo or 450 ml beetroot juice (~5.5 mmol nitrate). Following
34 a 90 minute drink/absorption period, participants performed a selection of cognitive tasks that
35 activate the frontal cortex for 54 minutes. Near-Infrared Spectroscopy (NIRS) was used to
36 monitor CBF and hemodynamics, as indexed by concentration changes in oxygenated and
37 deoxygenated-haemoglobin, in the frontal cortex throughout. The bioconversion of nitrate to
38 nitrite was confirmed in plasma by ozone-based chemi-luminescence.

39 **Results:** Dietary nitrate increased levels of nitrite, and modulated the hemodynamic response
40 to task performance, with an initial increase in CBF at the start of the task period, followed by
41 consistent reductions during the least demanding of the three tasks utilised. Cognitive
42 performance was improved on the Serial 3s subtraction task.

43 **Conclusions:** These results show that single doses of dietary nitrate can modulate the CBF
44 response to task performance and improve cognitive performance, and suggest one possible
45 mechanism by which vegetable consumption may have beneficial effects on brain function.

46

47

48 INTRODUCTION

49 The ubiquitous signalling molecule nitric oxide (NO) plays a modulatory role in a host of key
50 physiological processes, including mitochondrial and platelet function, host defence
51 mechanisms [1, 2], neurotransmission, peripheral and cerebral vaso-dilation [3, 4], and the
52 neurovascular coupling of neural activity to local cerebral blood flow (CBF) [5-7]. In most
53 tissues NO is synthesised from L-arginine and is rapidly oxidised to nitrite (NO_2^-) and nitrate
54 (NO_3^-) [8]. However, evidence suggests that circulating nitrite can also be reduced back to
55 NO by a wide range of proteins and enzymes in blood and tissue, including deoxygenated
56 haemoglobin, myoglobin, xanthine oxidase, aldehyde oxidase, neuroglobin, cytochrome P
57 450 and NO synthase [9]. Furthermore, nitrite has also been identified as a cellular signalling
58 molecule, independent of its relationship with NO [10].

59 Endogenous levels of nitrate, produced as a by-product of the L-arginine/NO pathway, can be
60 augmented by direct sequestration from dietary sources, most notably by eating vegetables
61 high in nitrate; e.g. spinach, lettuce, broccoli and beetroot [11]. Circulating nitrate from both
62 endogenous and dietary sources is actively sequestered and concentrated into saliva before
63 being converted to nitrite by commensal salivary bacteria in the mouth [12]. Entero-salivary
64 recirculation of additional dietary nitrate therefore leads to a sustained increase in circulating
65 nitrite. Following ingestion, nitrate levels peak in plasma following ~90 minutes and nitrite
66 reaches a peak after ~2.5 hours [13]. The reduction of nitrite to NO is particularly prevalent in
67 hypoxic conditions [14], but also takes place in normoxic conditions wherein conversion rates
68 can be modulated by the presence of reducing agents, the local oxygen tension and pH levels
69 [8, 15].

70 The ingestion of nitrate, including from dietary sources, is associated with a number of effects
71 consistent with increased levels of endogenous NO synthesis, including reductions in blood

72 pressure [16-20]. This effect has been demonstrated as early as three hours after a single dose
73 of nitrate rich beetroot juice, with a concomitant protection of forearm endothelial function
74 and *in vitro* inhibition of platelet aggregation [13]. Dietary nitrate has also been shown to
75 reduce the overall oxygen cost of sub-maximal exercise 2.5 hours after ingestion [21] and
76 after three or more days administration [17, 21-23]. Similarly, an increase in peak power and
77 work-rate [21], a speeding of VO_2 mean response time in healthy 60-70yr olds [19] and
78 delayed time to task failure during severe exercise [22, 23] have also been reported following
79 the consumption of nitrate rich beetroot juice daily for 4 to 15 days. Nitrate related reductions
80 have also been demonstrated with regards the rate of adenosine-5'-triphosphate (ATP)
81 turnover using magnetic resonance spectroscopy [22], whilst improved oxygenation [23] has
82 been confirmed directly in the muscle during exercise using Near-Infrared Spectroscopy
83 (NIRS).

84 NO plays a pivotal role in cerebral vasodilation and the neurovascular coupling of local neural
85 activity and blood-flow [24] and enhanced cerebral blood perfusion has been observed in the
86 prefrontal cortex in response to increased circulating levels of dietary nitrate [11]. Several
87 studies have probed the effects of dietary nitrate derived from beetroot or spinach on brain
88 function, including three studies that have included some form of cognitive testing either as
89 an additional measure [19, 20], or as the primary focus of the project [25]. Whilst these
90 studies demonstrated modulation of a number of physiological parameters they did not
91 provide evidence of cognitive improvements, possibly due to comparatively small sample
92 sizes and other methodological factors. Two studies have also investigated the effects of
93 dietary nitrate on cerebral blood-flow parameters. In the first of these, Presley et al. [11]
94 demonstrated, using arterial spin labelling magnetic resonance imaging (MRI), that a diet high
95 in nitrate consumed for 24 hours increased regional white matter perfusion in elderly humans,
96 but with this effect restricted to areas of the frontal cortex. More recently Aamand et al.

97 (2013), investigated the effects of 3 days administration of dietary nitrate (sodium nitrate) on
98 the haemodynamic response in the visual cortex elicited by visual stimuli, as assessed by
99 functional MRI (fMRI). They demonstrated a faster, smaller and less variable blood-oxygen-
100 level dependent (BOLD) response following nitrate, which they interpreted as indicating an
101 enhanced neurovascular coupling of local CBF to neuronal activity. As the BOLD response
102 simply reflects the contrasting magnetic signals of oxygenated and deoxygenated
103 haemoglobin (with increased activity imputed from an assumed relative decrease in
104 deoxyhaemoglobin as local activation engenders a greater influx of blood borne oxygenated –
105 Hb), it cannot disentangle the contributions of changes in blood-flow and changes in oxygen
106 consumption to the overall signal. The current study therefore utilised Near-Infrared
107 Spectroscopy (NIRS), a brain imaging technique that has the advantage over fMRI BOLD in
108 that it measures both concentration changes in deoxy-Hb and overall local CBF (changes in
109 oxy-Hb and deoxy-Hb combined).

110 The current double-blind, placebo controlled, parallel groups study investigated the effects of
111 a single dose of dietary nitrate on cognitive performance and the CBF haemodynamic
112 response in the prefrontal cortex during tasks that activate this brain region.

113

114

115 **MATERIALS AND METHODS**

116 *Participants:*

117 40 healthy adults (mean age 21.28y, range 18-27y) took part in the study. Prior to attending
118 the laboratory all participants refrained from eating for 12 hours, and consumed no vegetables
119 for 36 hours prior to testing. Participants were allowed their usual morning caffeinated

120 beverages, but consumed no caffeine for a minimum of 2 hours prior to the assessment. The
 121 age and physical characteristics of the two groups are shown in Table 1.

122 All participants reported themselves to be in good health and free from illicit drugs, alcohol,
 123 prescription medication and herbal extracts/food supplements. Participants who had suffered a
 124 neurological disorder or neuro-developmental disorder were excluded from participation, as
 125 were those who had any relevant food allergies or intolerances, smoked tobacco, drank
 126 excessive amounts of caffeine (more than 6 cups of coffee per day) or took illicit social drugs.

127 The study received ethical approval from the Northumbria University department of
 128 Psychology and Sport Sciences Ethics Committee and was conducted according to the
 129 Declaration of Helsinki (1964). All participants gave their informed consent prior to their
 130 inclusion in the study. Prior to data collection this study was registered on the
 131 clinicaltrials.gov website with the following reference number: NCT01169662.

132

133 **Table 1. Age and physical characteristics of participants**

134

	Placebo n=20		Beetroot n=20		
Age (years)	21.40	0.73	21.15	0.48	
Male/Female	7/13		5/15		
Height (M)	1.71	0.02	1.70	0.02	
Weight (Kg)	74.93	3.43	68.24	3.12	
BMI	25.39	0.80	23.34	0.72	
Heart Rate (bpm)	pre	64.3	2.05	66.85	2.24
	post	59.4	1.54	67.15	2.38*
Systolic BP	pre	115	2.3	114.6	3.16
	post	116.8	2.26	115.7	2.48
Diastolic `BP	pre	74.2	1.86	73.15	1.61
	post	79.05	1.91	76.35	1.59
Nitrite (nM)	pre	228	14.8	226	23.2
	post	246	28.2	598	78.3*

135

136 Physical characteristic data (means plus SEMs) from the placebo and dietary nitrate conditions (n = 20
 137 per group) including pre and post-treatment heart rate, blood pressure and plasma nitrite

138 measurements. Analysis on the latter measures was by two-way ANOVA with Bonferroni adjusted
139 post-hoc comparisons (* $P < 0.05$, placebo versus dietary nitrate at that time point).

140

141 *Treatments:*

142 Participants were randomly assigned to receive either:

143 a) 450 ml organic beetroot juice (including 10% Apple juice - Beet It, James White Drinks,
144 Ipswich, UK) containing 5.5 mmol nitrate [23] plus 50 ml low calorie apple and blackcurrant
145 juice cordial,

146 Or

147 b) A placebo drink with negligible nitrate content composed of 50 ml low calorie apple and
148 blackcurrant juice cordial plus 50 ml apple juice, diluted to 500 ml.

149 The drinks were served chilled in opaque, lidded containers in three equally sized portions
150 (166 ml per portion). Participants were given one third of the drink at the start of the
151 absorption period, with the remaining two thirds of the drink consumed 10 and 20 minutes
152 later. Participants were instructed to drink the drink slowly, through a straw, over each 10
153 minute period.

154 The drinks were prepared by a neutral third-party according to the computer generated
155 randomisation list and administered double-blind by the researchers. Given the disparity in
156 taste between the treatments the study was run with a between-subjects design and
157 participants were simply informed that the study was investigating the CBF effects of fruit or
158 vegetable drinks. They were given no information on the experimental aims, the identity of
159 the drinks, or the nitrate contents or potential physiological effects of the beetroot juice (other
160 than being informed of the possibility of discoloured urine).

161

162 *Near-Infrared spectroscopy:*

163 Functional Near-Infrared Spectroscopy (NIRS) is a brain imaging technique that is predicated
164 on the intrinsic optical absorption properties of oxygenated (oxy-Hb) and deoxygenated
165 (deoxy-Hb) haemoglobin following the introduction of near- infrared light through the intact
166 skull. When assessed by NIRS, the increase in CBF in the surface layers of the cortex during
167 localized neural activity is seen as an increase in the total concentration of haemoglobin
168 (total-Hb) and comparative decrease in deoxy-Hb [26] with both parameters corresponding
169 strongly with the functional magnetic resonance imaging (fMRI) blood oxygen level
170 dependent (BOLD) signal [26-28]. NIRS has been used extensively as a technique for
171 multiple-channel imaging of task related brain activity over relevant areas of the head [29],
172 including in groups suffering from potential decrements in CBF [30]. To date, a growing
173 number of pharmacological intervention studies have also used the technique to infer
174 localized brain activity [31] and CBF and oxygenation [32] from changes in haemoglobin
175 concentrations. The paradigm employed here has been shown to be sensitive to both increased
176 [33-35] and decreased [36, 37] CBF in the prefrontal cortex of healthy young volunteers
177 following nutritional interventions.

178 In the current study relative changes in the absorption of near- infrared light were measured at
179 a time resolution of 10Hz using a 12 channel Oxymon system (Artinis Medical Systems
180 B.V.). The system emitted two nominal wavelengths of light (~765- and 855nm) with an
181 emitter/optode separation distance of 4cm. The differential pathlength factor was adjusted
182 according to the age of the participant. Relative concentration changes in oxy-Hb, deoxy-Hb
183 and total-Hb were calculated by means of a modified Beer-Lambert law [38] using the
184 proprietary software.

185 In this study, given the extended recording period and the investigational aims, a simple two
186 emitter/optode pair configuration was utilised (i.e. 2 channels). The emitter/optode pairs were
187 positioned over the left and right frontal cortex using a standard optode holder headband,
188 which separated the pairs from each other by 4cm. Each pair therefore collected data from an
189 area of prefrontal cortex that included the areas corresponding to the International 10-20
190 system Fp1 and Fp2 electroencephalogram (EEG) positions.

191 The NIRS data output was time stamped at the start of each task segment to assure that data
192 corresponded to the relevant epoch of task performance.

193

194 *Blood sampling and determination of plasma nitrite levels:*

195 Blood was collected in lithium-heparin vacutainer tubes and was centrifuged at 4,000 rpm at
196 4°C for 10 minutes, commencing within 3 minutes of collection. Plasma was subsequently
197 extracted and immediately frozen at -80°C for later analysis.

198 For the subsequent analysis all glass wear, utensils and surfaces were rinsed with deionised
199 water to remove residual NO_2^- prior to analysis. After thawing at room temperature, plasma
200 samples were initially de-proteinized using cold ethanol precipitation. The ethanol was chilled
201 to 0°C and 1 ml of cold ethanol was added to 0.5 ml of plasma sample, after which the sample
202 was vortexed and left to stand at 0°C for 30 minutes. Thereafter, samples were centrifuged at
203 14000 rpm for 5 minutes and the supernatant was removed. The $[\text{NO}_2^-]$ of the deproteinized
204 plasma samples was determined using a modification [23] of the chemi-luminescence
205 technique [39].

206

207

208 *Blood pressure and heart rate:*

209 Sitting blood pressure and heart rate readings were collected using a Boso Medicus Prestige
210 blood pressure monitor with the subject's arm supported at the level of the heart and with
211 their feet flat on the floor. Readings were taken following completion of the baseline tasks
212 and again following completion of the post-dose tasks.

213

214 *Cognitive tasks:*

215 The 3 tasks used here were previously shown to activate the prefrontal cortex in brain-
216 imaging studies [40-42]. The objective of this collection of tasks was generally to assess the
217 effect of the treatment on speed/accuracy and mental fatigue during continuous performance
218 of cognitively demanding or "effortful" tasks. Multiple completions of the 9 minute battery of
219 tasks (see below) has previously been shown to reliably increase self-ratings of mental fatigue
220 and to be sensitive to many natural interventions [43-46]. The 9 minute battery consists of 4
221 minutes of Serial Subtractions, 5 minutes of Rapid Visual Information Processing (RVIP),
222 and a Mental Fatigue visual analogue scale.

223 The original verbal Serial 7s test has appeared in many forms, including as part of the Mini-
224 Mental State Examination for dementia screening. In the current study, a modified, 4 minute,
225 computerized version of the Serial Subtraction task was used [47], which consists of 2
226 minutes of Serial 3s followed by 2 minutes of Serial 7s subtractions. At the start of each 2
227 minute section, a standard instruction screen informed the participants to count backwards in
228 3s or 7s, as quickly and accurately as possible, using the keyboard's linear number keys to
229 enter each response. Participants were also instructed verbally at the outset that if they were to
230 make a mistake they should continue subtracting from the new incorrect number. A random
231 starting number between 800 and 999 was presented on the computer screen, which was

232 cleared by the entry of the first response. Each 3-digit response was represented on screen by
233 an asterisk. Pressing the enter key signalled the end of each response and cleared the 3
234 asterisks from the screen. Performance data (total number of subtractions and number of
235 errors) were calculated for the Serial 3s and 7s elements separately. In the case of incorrect
236 responses, subsequent responses were scored as positive if they were correct in relation to the
237 new number.

238 The RVIP task has been widely used to study the cognitive effects of psychotropic drugs. The
239 participant monitors a continuous series of single digits for targets of 3 consecutive odd or 3
240 consecutive even digits. The digits are presented on the computer screen at the rate of
241 100/minute in pseudo-random order, and the participant responds to the detection of a target
242 string by pressing the space bar as quickly as possible. The task is continuous and lasts for 5
243 minutes, with 8 correct target strings being presented in each minute. The task is scored for
244 number of target strings correctly detected, average reaction time for correct detections, and
245 number of false alarms.

246 With the mental fatigue visual analogue scale, participants rated their subjective feelings of
247 mental fatigue via an on-screen 100mm visual analogue scale with the endpoints labelled as
248 'not at all' and 'extremely'. The scale was scored as a percentage along the line toward
249 'extremely'.

250 In this instance the tasks described above were repeated six times in succession (i.e. ~54
251 minutes of task performance). The tasks (and mood scales) were presented using the
252 COMPASS cognitive assessment system (Northumbria University, Newcastle, UK).

253

254

255

256 *Mood:*

257 Mood was assessed with Bond-Lader mood scales [48], which have been utilised in numerous
258 pharmacological, psychopharmacological and medical trials. These scales comprise a total of
259 sixteen 100mm lines anchored at either end by antonyms (e.g. ‘alert-drowsy’, ‘calm-excited’).
260 Participants indicate their current subjective position between the antonyms on the line.
261 Outcomes comprise three factor analysis derived scores: ‘Alertness’, ‘Calmness’ and
262 ‘Contentment’.

263

264 *Procedure:*

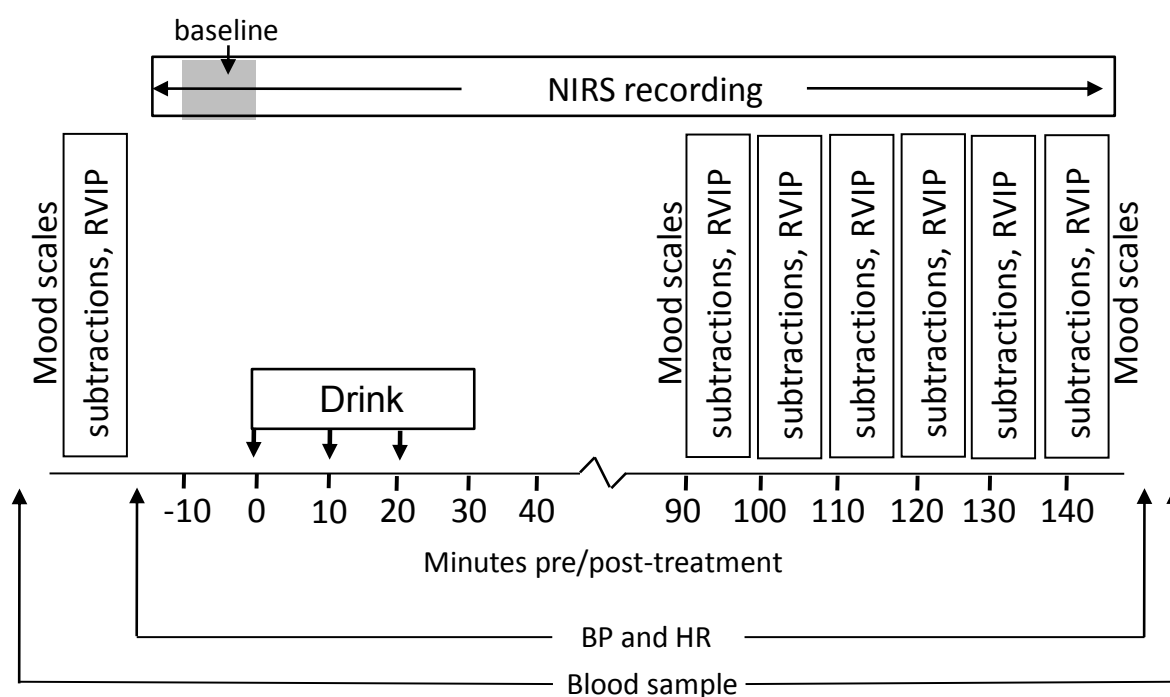
265 Each participant was required to attend the laboratory on two occasions. The first of these was
266 an initial screening/training visit, and this was followed within 21 days by the active study
267 morning. During the initial visit participants provided written informed consent and were
268 screened with regards the study exclusion/inclusion criteria. Training was given on the
269 cognitive tasks and the compliance requirements for the following visit were explained.

270 On the active study morning participants attended the laboratory between 8.30 and 9.30 am
271 and provided confirmation of their compliance with the inclusion/exclusion requirements.

272 Participants then gave a venous blood sample, completed the Bond-Lader mood scales, made
273 a baseline completion of the three tasks (Serial 3s, 7s, RVIP), and had their blood pressure
274 and heart rate measured. Participants were then fitted with the NIRS headband. After 5
275 minutes the 10 minute resting baseline period commenced. During this time, and the
276 subsequent absorption period, participants watched a non-arousing DVD. The study drink was
277 presented to the participant in three equal amounts at 10 minute intervals at the start of the 90
278 minute absorption period. At the end of the absorption period participants were then verbally
279 instructed to start the period of task performance, during which they completed the Bond-

280 Lader mood scales and then made 6 consecutive repetitions of the Serial Subtractions and
 281 RVIP tasks (i.e. 54 minutes of continuous performance). Following task completion they
 282 completed the Bond-Lader mood scales for a final time, had their blood pressure and heart
 283 rate measured and provided a venous blood sample. The timelines and running order of the
 284 testing session are shown in Figure 1.

285



286

287 **Figure 1. Timelines of each assessment.** On arrival participants provided a blood sample, completed
 288 mood scales and one repetition of the cognitive tasks, after which blood pressure and heart rate were
 289 measured. Following a 10 minute resting/baseline period they consumed their day's drink in 3 portions
 290 that were sipped over 30 minutes in total. After a further 60 minutes they completed the mood scales
 291 and the cognitive tasks 6 times in succession (i.e. 54 minutes in total), after which they completed the
 292 mood scales for a final time, had their heart rate and blood pressure measured and provided a further
 293 blood sample. NIRS data was collected throughout the resting/baseline, absorption and cognitive task
 294 periods, with the last three minutes of the pre-treatment resting phase used to baseline adjust all post-
 295 treatment data.

296

297

298

299 *Statistical analyses:*

300 The analyses of NIRS data were conducted with Minitab 15 for Windows (Minitab Inc, State
301 College, PA) and behavioural data with SPSS 16.0 for Windows (SPSS Inc, Chicago, IL).

302 NIRS data was converted to 'change from baseline' (calculated from a 3 minute pre-treatment
303 resting period) and averaged across 2 minute epochs during the 90 minute 'resting/absorption'
304 period, and 2 minute (Serial Subtractions) or 2.5 minute (RVIP, 5 minutes per repetition in
305 total) epochs during the cognitive task performance period. As the duration of each complete
306 epoch of averaged NIRS data entered into the analysis was substantially longer than the
307 potential physiological oscillations that can cause drift in shorter periods of NIRS recording
308 [49] no adjustment was required to control for this phenomenon.

309 Prior to the primary analyses a within subjects Analysis of Variance (ANOVA) was carried
310 out with left/right optode included as a factor (hemisphere x treatment group x epoch) to
311 examine any hemispheric differences in response. As there were no treatment related
312 interactions involving this factor the data from the two channels were averaged across
313 hemispheres for the analysis and figures reported below.

314 The primary analysis of the averaged NIRS data (total- and deoxy-Hb) was conducted by
315 ANOVAs (treatment group x epoch) performed separately with data from the absorption
316 period and the task period. In order to assess the effects of the differential task demands on
317 haemoglobin concentrations an ANOVA (treatment x task [subtractions/RVIP] x epoch x
318 repetition [1 to 6]) of the task period data was also conducted. Subsequent *a priori* planned
319 comparisons of data from each 2 minute epoch during both the absorption and cognitive task
320 periods were made between the placebo and dietary nitrate condition using t tests calculated
321 with the Mean Squares Error [50] from the appropriate ANOVA. The planned comparisons
322 were subjected to a Bonferroni adjustment for multiple comparisons. In order to reduce the

323 potential for Type I errors only those planned comparisons associated with a significant ($p <$
324 0.05) main effect of treatment or interaction between treatment and epoch on the primary
325 ANOVA are reported.

326 Individual task performance data from the Serial 3s and Serial 7s subtraction tasks, the RVIP,
327 and the fatigue scales, were analysed by 2-way mixed Analysis of Covariance (ANCOVA)
328 (treatment x repetition [1 to 6]) using the pre-treatment score as a covariate, with planned
329 comparisons for adjusted data from each repetition as described above. Bond-Lader mood
330 factor scores, heart rate, blood pressure and plasma nitrite level data were analysed by two-
331 way ANOVA (treatment x pre-post treatment) with Bonferroni adjusted *post-hoc*
332 comparisons.

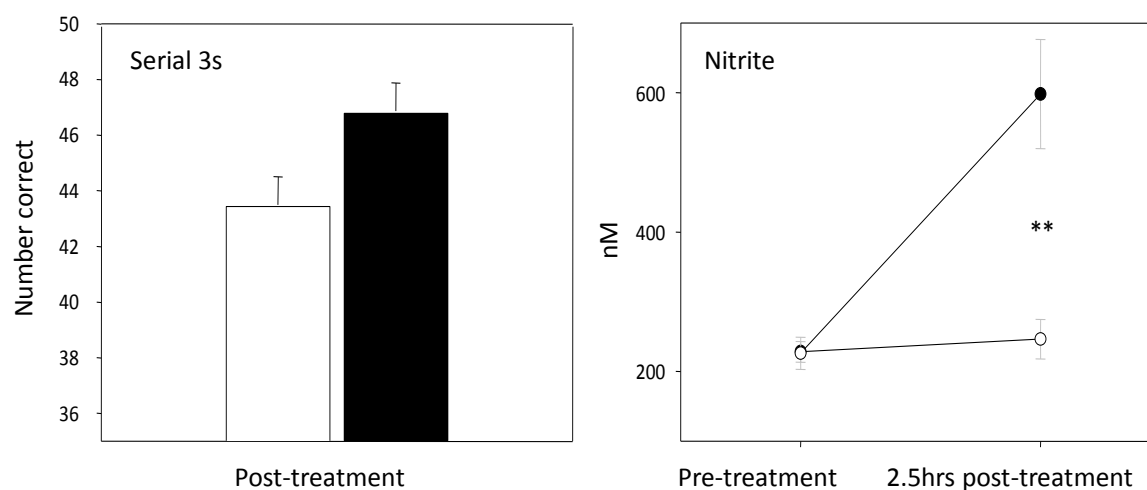
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334

335 RESULTS

336 *Plasma nitrite*

337 Plasma levels of nitrite were significantly raised in the beetroot condition ($P < 0.01$) by the
338 end of the assessment: see right panel of Figure 2 for graphical depiction.



339

340 **Figure 2. Serial 3 subtraction performance and plasma nitrite levels.** Left panel: Adjusted mean
 341 (\pm SEM error bar) number of correct Serial 3s generated in 2 minutes averaged across the 6 post-
 342 treatment repetitions of the tasks. Right panel: Mean (\pm SEM error bar) plasma nitrite levels pre-
 343 treatment and at the end of testing (~150 minutes post-treatment). (\square and \circ = placebo; \blacksquare and \bullet = 450
 344 ml of beetroot juice containing 5.5 mmol nitrate).

345
 346 (Footnote) The study followed a parallel groups design ($n = 20$ per condition). The Serial 3s task was
 347 repeated 6 times in total commencing 90 minutes post-dose. Analysis was by 2-way ANCOVA
 348 (treatment x repetition [1 to 6]) using the pre-treatment score as a covariate. The main effect of
 349 treatment was significant ($P < 0.05$). Blood samples were taken pre-treatment and at the end of the
 350 testing session (~150 minutes post-treatment). Plasma nitrite levels were assessed by ozone-based
 351 chemi-luminescence. Statistical analysis was by ANOVA (pre/post x treatment) with post-hoc
 352 Bonferroni t tests comparisons between means (* = $P < 0.05$).

353

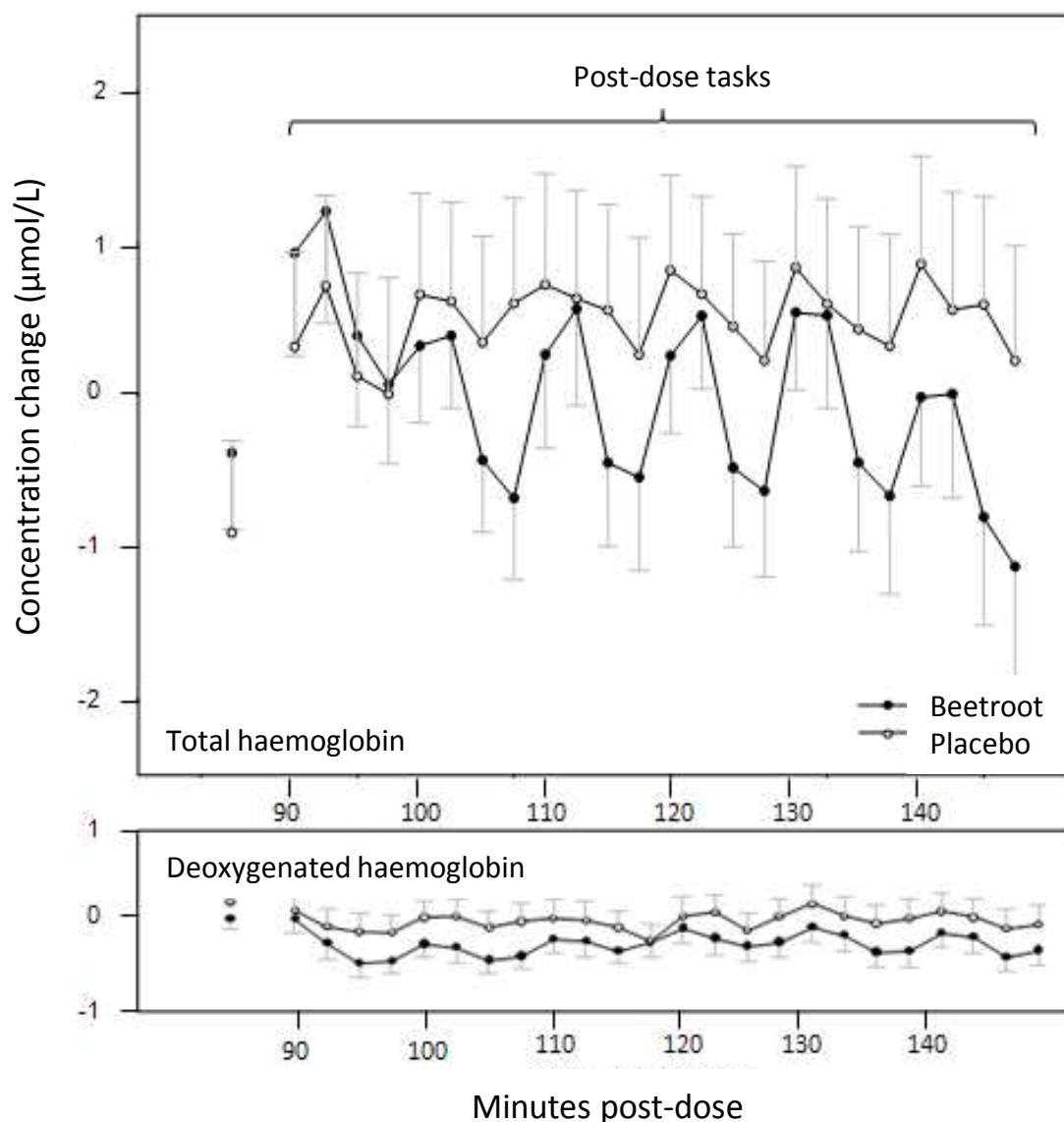
354

355 *NIRS parameters*

356 *Total haemoglobin (total-Hb):* The ANOVA showed that there was a significant interaction
 357 between epoch and treatment ($P < 0.01$) during the 90 minute absorption period. Reference to
 358 the planned comparisons showed that the concentration of total-Hb (and therefore CBF) was
 359 higher following consumption of dietary nitrate throughout the ten epochs spanning 13 to 32
 360 minutes post-dose (all $p < 0.05$). There was also a significant epoch x treatment interaction on
 361 the ANOVA of data from the task period ($P < 0.05$), with the planned comparisons showing
 362 that, following the consumption of dietary nitrate, whereas total-Hb was increased during the
 363 first epoch of task performance (91-92 min (during Serial 3s), $P < 0.05$), it was decreased in
 364 comparison to placebo during both epochs of the last 5 repetitions of the RVIP task (all $P <$
 365 0.01) as well as the final repetition of the serial 3s task ($P < 0.01$). Reference to the secondary
 366 ANOVA (treatment x task x epoch x repetition) assessing task related differences showed that
 367 the treatment x task interaction narrowly failed to reach significance ($P < 0.1$).

368 *Deoxygenated haemoglobin (deoxy-Hb):* The initial ANOVAs showed that treatment with
 369 dietary nitrate narrowly failed to significantly modulate deoxy-Hb, with a strong trend
 370 towards a treatment x epoch interaction ($P < 0.1$) during the task period. Mean changes in

371 total-Hb and deoxy-Hb across the absorption and task performance periods are shown in
 372 Figure 3. with data from the task period presented in greater detail in Figure 4.

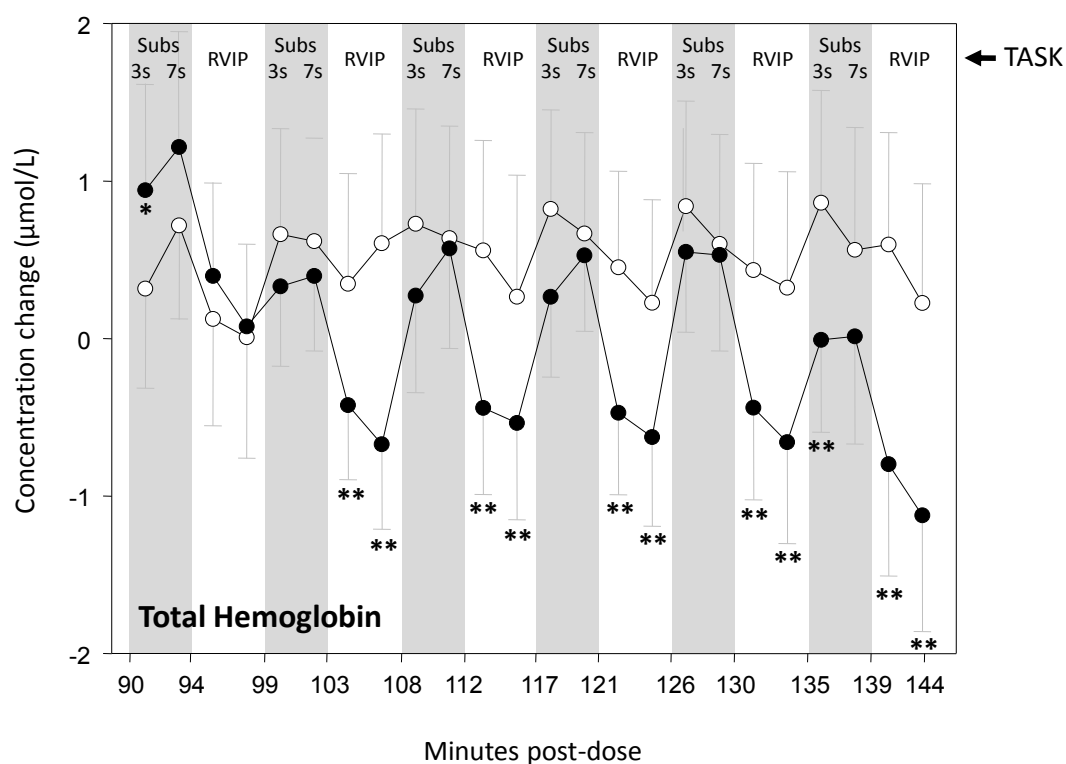


373

374 **Figure 3. Concentration changes in deoxy- and total-Hb.** Graph depicts mean (\pm SEM error bar)
 375 concentration changes in total levels of haemoglobin (total-Hb) and deoxygenated haemoglobin
 376 (deoxy-Hb) during a 90 minute absorption period (averaged to 1 time-point) and subsequent 54
 377 minutes of cognitive task performance, following placebo (\circ), and 450 ml of beetroot juice containing
 378 5.5 mmol nitrate (\bullet). Data in the top and bottom panels are graphed to the same scale.

379 (Footnote) The study followed a parallel groups design ($n = 20$ per condition). Data are averaged
 380 across 2 minute (absorption period, serial subtractions) or 2.5 minute (RVIP) epochs. Analysis with
 381 repeated measures ANOVA showed a significant treatment \times epoch interaction ($P < 0.05$) for total
 382 haemoglobin concentrations (i.e. CBF – top panel) during both the absorption and cognitive task
 383 periods, with no significant effect for deoxygenated haemoglobin (bottom panel). *A priori* planned
 384 comparisons comparing data from each dietary nitrate group to placebo for each epoch were carried
 385 out with t tests incorporating Mean Squares Error from the ANOVA with a Bonferroni adjustment for

386 multiplicity. Significance on the Bonferroni adjusted comparisons between placebo and dietary nitrate
 387 during the individual epoch is indicated by * ($P < 0.05$) and ** ($P < 0.01$).
 388



389

390 **Figure 4. Concentration changes in total-Hb during post-dose cognitive task period.** Graph
 391 depicts mean (\pm SEM) concentration changes in total levels of haemoglobin (tot-Hb) during 54 minutes
 392 of cognitive task performance following placebo (○), and 450 ml of beetroot juice containing 5.5
 393 mmol nitrate (●).

394 (Footnote) Methods and statistics are as per Figure 2. Subs = serial subtractions tasks, RVIP = Rapid
 395 Visual Information Processing task.

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398 *Cognitive performance, mental fatigue and mood*

399 The ANCOVA (using baseline performance as a covariate) showed that participants'
 400 performance improved significantly in terms of the number of correct Serial 3s subtractions
 401 following the consumption of dietary nitrate ($P < 0.05$). There were no other significant
 402 improvements seen in terms of the other tasks (Serial 7s, RVIP), the three Bond-Lader mood
 403 factors, or ratings of mental fatigue. It should be noted that the dietary nitrate group under-

404 performed the placebo group prior to treatment (mean correct Serial 3s subtractions: dietary
405 nitrate 35.6, Placebo 50.15). The adjusted mean number of serial 3s subtractions (plus SEMs)
406 are presented graphically in the left panel of Figure 2.

407 *Blood pressure and heart rate*

408 There was no significant modulation of blood pressure during the single post-dose
409 measurement that was taken following completion of the task period. However, heart rate
410 dropped significantly from pre-treatment levels in the placebo condition but not the beetroot
411 condition ($P < 0.05$).

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413

414 **DISCUSSION**

415 In the current study the consumption of nitrate rich beetroot juice resulted in a modulation of
416 the haemodynamic response in the prefrontal cortex during the performance of tasks that
417 activate this brain area. In this case the pattern following nitrate was most notably of an initial
418 transient rise in CBF at the beginning of the task period, followed by consistent significant
419 reductions in CBF during each repetition of the RVIP task. No significant effects were seen
420 with regards concentrations of deoxy-Hb. Alongside these hemodynamic effects, performance
421 of the serial 3s subtraction task was also improved following dietary nitrate. The absorption of
422 nitrate and subsequent reduction to nitrite seen in previous studies [19, 20, 23] was confirmed.

423 The primary investigational question here was whether dietary nitrate would modulate
424 haemodynamic responses in the prefrontal cortex during the performance of tasks that activate
425 this area of the brain. The pattern of hemodynamic effects following dietary nitrate was for an
426 initial significant increase in CBF, as indexed by total-Hb, at the very outset of task

427 performance (i.e. the first Serial 3s), followed by consistent reductions during the RVIP task,
428 culminating in reduced CBF during both the Serial 3s task and RVIP during their last
429 repetitions. The concentration of deoxy-Hb was not significantly modulated here, but it is
430 worth noting that the pattern was for a reduced concentration throughout the task period (See
431 bottom portion of figure 3).

432 Despite the markedly differing methodologies, the results here could be described as being
433 consistent with those of the Aamand et al. [51] fMRI study, which demonstrated a faster and
434 smaller BOLD response in the visual cortex during the presentation of visual stimuli
435 following nitrate, which the authors interpreted as indicating an enhanced neurovascular
436 coupling of local CBF to neuronal activity. The BOLD signal itself simply represents the
437 contrast between the magnetic signals of oxygenated and deoxygenated haemoglobin, and
438 therefore, as Aamand et al note, it cannot disentangle the contributions of changes of blood-
439 flow/volume and changes in oxygen consumption to the overall signal. In the present study,
440 the predominant finding of reduced blood flow, with the concentration of deoxy-Hb
441 remaining largely unaffected, would most likely have also resulted in a reduced BOLD signal
442 as the overall concentration of deoxy-Hb increased in proportion to the larger decrease in
443 blood volume in the interrogated area.

444 Typically, and as in the placebo condition here, performance of the RVIP task results in a
445 smaller increase in CBF than does performance of the Serial Subtraction tasks (see, for
446 instance, Kennedy et al. [52]). This can largely be attributed to the relative cognitive demands
447 of the two tasks, with Serial Subtractions requiring the continuous retention of information in
448 working memory and the active mathematical manipulation of numbers throughout the task,
449 whereas RVIP simply requires the monitoring of rapidly changing digits along with a more
450 passive contribution from working memory (i.e. remembering whether the last two digits
451 were odd or even). The overall pattern of CBF is therefore as expected, but singularly more

452 exaggerated than normal; a finding which was also observed in Aamand et al. [51] and which
453 they argue represents an “enhanced hemodynamic coupling” between activity and local
454 blood-flow. In this case the accentuated reduction in CBF may potentially represent a more
455 sensitive match between blood flow and activity during the RVIP task. Of course this begs the
456 question as to why blood flow was comparatively unchanged during the more difficult Serial
457 Subtractions. Whilst no clear explanation can be provided, it may be pertinent that these tasks
458 are self-paced (with participants actively performing the subtractions as opposed to passively
459 monitoring digits in the RVIP) and that performance on one of the two serial subtraction tasks
460 was improved.

461 Interestingly, reference to figure 4 demonstrates a nitrate-induced exaggeration of the normal
462 (placebo) CBF response. This sensitivity of NIRS (to oscillating pattern of CBF changes) has
463 also been demonstrated with the stilbene polyphenol (and NO-modulator) resveratrol; where
464 serial subtraction performance consistently increased total- and deoxy-Hb (and to a lesser
465 extent oxy-Hb) across the entire 36 minute post-dose task period, compared to interspersed
466 decreases in response to the RVIP task [52]. In terms of an explanation for these effects, at
467 least two distinct NO-related mechanisms may be involved here. Firstly, these results may
468 represent an exaggeration of the NO-mediated relationship between task-related neural
469 activity and the local neurovascular response. The relationship between increased cognitive
470 workload and augmented CBF has been demonstrated with NIRS previously with Son et al.
471 [53] reporting an amplified CBF response as a result of increasing workload and Shibuya-
472 Tayoshi et al. [54] evidencing a greater CBF response to the difficult, versus the easy, aspect
473 of the Trail-Maker task. Taken together, the RVIP task could be conceived as requiring less
474 cognitive resources (or indeed frontal involvement) than the mental arithmetic serial
475 subtraction tasks.

476 As well as this exaggerated response, this study also reports reduced CBF during all tasks by
477 the end of the cognitive task period. As such, a second, related, explanation for these results is
478 that both the improved performance during the Serial Subtractions and reduced CBF during
479 the RVIP task reflect improvements in cellular oxygen utilisation driven by NO synthesis,
480 with reduced CBF reflecting a decreased need for additional metabolic substrates. This
481 interpretation is supported by concomitant (non-significant) reductions in concentrations of
482 deoxy-Hb seen during the periods of reduced CBF; suggesting decreased oxygen extraction.
483 In this respect the expected pattern would be for the concentration of deoxy-Hb to increase
484 with decreasing CBF as it became a greater proportion of the overall blood volume, and vice
485 versa (e.g. the opposite pattern is seen during the first 60 minutes of the absorption period,
486 with increased CBF engendering decreased deoxy-Hb).

487 In terms of mechanisms underlying the effects seen here, as well as acting as a vaso-dilator
488 during local neural activity [5-7] previous research suggests that NO exerts a number of
489 effects that might also impact on overall cellular energy consumption in the brain. These
490 include the inhibition of mitochondrial respiration and therefore oxygen consumption,
491 including via inhibition of cytochrome c oxidase [55, 56] and enhancement of the efficiency
492 of oxidative phosphorylation by decreasing slipping of the proton pumps [57, 58]. In line with
493 this, increased efficiency of oxidative phosphorylation has recently been demonstrated in
494 human mitochondria following nitrate supplementation, with this effect correlating with
495 reduced oxygen cost during exercise [59] and a trend for reduced oxygen uptake during
496 exercise at 50% of VO₂ max, without detrimental effects to physical or cognitive
497 performance [20]. Evidence too suggests that nitrite itself may function in respiration as an
498 alternative electron acceptor to oxygen [60] and that it acts as an important cellular signalling
499 molecule independent of its relationship with NO [10].

500 With regards cognitive performance, improvements were observed in this study but restricted
501 to one of the three tasks (serial 3 subtractions). Differential levels of cognitive demand, speed
502 of performance and the involvement of disparate cognitive domains across these three tasks
503 make global improvements by any intervention unlikely. The serial 3s task itself requires
504 resources in terms of working memory, psychomotor speed, and executive function. It is
505 therefore inextricably linked to frontal cortex function. It should be noted that the dietary
506 nitrate group under-performed placebo at baseline on this task and, as pre-treatment
507 performance was used as a covariate in the ANCOVA, it is possible that this factor
508 contributed to the significant improvement seen at post-dose. Whether the improvements seen
509 here following nitrate were dependent on poor performance, and therefore a greater sensitivity
510 to any benefits derived from the intervention, remains to be investigated further.

511 It is important to note that beetroot contains a plethora of other, potentially bioactive,
512 phytochemicals including the nitrogenous betalains, a range of phenolics, including multiple
513 flavonoids and flavonols [61] and folates [62]. Given the ability of similar phytochemicals to
514 modulate peripheral endothelial function [63, 64], CBF parameters [52] and cognitive
515 function [65] the possibility that any effects are related to high levels of these other
516 compounds cannot be ruled out. It is also notable that the $\text{NO}_3^-/\text{NO}_2^-/\text{NO}$ pathway is reported
517 to be most prevalent during hypoxic conditions and in the presence of reducing agents such as
518 vitamin C and polyphenols [8]. Having said this, recent evidence from a study directly
519 comparing nitrate rich beetroot juice to nitrate depleted (but otherwise identical) beetroot
520 juice suggests that the effects seen on blood pressure and the O_2 cost of exercise are directly
521 attributable to the nitrate content of the juice rather than to any other bioactive components
522 (although synergies cannot be ruled out) [66]. Given the potential for both phytochemicals
523 and gustatory factors to impact on CBF, an extension of the current study using these nitrate

524 rich and depleted interventions may be able to resolve the question of the direct contribution
525 of nitrate to the cognitive and CBF effects seen here.

526 Notably, the consistent reductions in blood pressure following dietary nitrate reported
527 elsewhere [16, 17, 22] were not seen here. Further, the significant drop in heart rate in the
528 placebo group from pre-dose to post-assessment was not matched in the dietary nitrate group.
529 The difference in experimental paradigm between the current and aforementioned studies may
530 provide an explanation for this less clear-cut effect. Previous studies either involved
531 participants who naturally consume a diet high in levels of dietary nitrate (i.e. Japanese) or
532 assessed the effects of dietary nitrate during exercise; which, as stated above, enhances the
533 reductive pathway of nitrate to NO [14]. Taken together, the effects of nitrate (and NO) on the
534 peripheral vasculature might therefore not be expected in sedentary humans after an acute
535 dose of dietary nitrate. This lack of an effect on blood pressure could also be attributed to
536 these measures being taken within the period of atypical physiological arousal following a
537 venous blood sample and completion of demanding cognitive tasks, rather than reflecting a
538 treatment related effect, or lack of the same in the case of blood pressure. Future studies might
539 therefore bear this in mind and incorporate longer periods of rest between potentially stressful
540 or arousing events and the taking of physiological readings.

541 Overall, the findings here suggest that supplementation with dietary nitrate can directly
542 modulate important physiological aspects of brain function and improve performance on a
543 cognitive task that is intrinsically related to prefrontal cortex function. Taken alongside a
544 previous demonstration of increased prefrontal cortex perfusion in elderly humans following
545 consumption of a high nitrate diet for ~36 hours [67], the results here suggest both a specific
546 food component and physiological mechanisms that may contribute to epidemiological
547 observations of relationships between the consumption of a diet rich in vegetables [68, 69]
548 and polyphenols (which naturally co-occur with nitrate in vegetables) [70, 71] and preserved

549 cognitive function in later life. Of particular importance, the results here were demonstrated in
550 young humans, who can be assumed to be close to their optimum in terms of brain function
551 [72], and hint at the potential benefits of a healthy, vegetable rich diet across the lifespan.

552 In summary, dietary nitrate, administered as beetroot juice, modulated CBF in the prefrontal
553 cortex during the performance of cognitive tasks that activate this brain region, with this
554 effect most consistently seen as reduced CBF during the easiest of three tasks; RVIP.
555 Cognitive performance was improved on a further task; serial 3 subtractions. These results
556 suggest that a single dose of dietary nitrate can modify brain function, and that this is likely to
557 be as a result of increased NO synthesis leading to an exaggerated neurovascular response to
558 activity or improved efficiency of cellular metabolism.

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572

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