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

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ABSTRACT

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

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

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

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

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

The ubiquitous signalling molecule nitric oxide (NO) plays a modulatory role in a host of key physiological processes, including mitochondrial and platelet function, host defence mechanisms [1, 2], neurotransmission, peripheral and cerebral vaso-dilation [3, 4], and the neurovascular coupling of neural activity to local cerebral blood flow (CBF) [5-7]. In most tissues NO is synthesised from L-arginine and is rapidly oxidised to nitrite (NO$_2^-$) and nitrate (NO$_3^-$) [8]. However, evidence suggests that circulating nitrite can also be reduced back to NO by a wide range of proteins and enzymes in blood and tissue, including deoxygenated haemoglobin, myoglobin, xanthine oxidase, aldehyde oxidase, neuroglobin, cytochrome P450 and NO synthase [9]. Furthermore, nitrite has also been identified as a cellular signalling molecule, independent of its relationship with NO [10].

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

The ingestion of nitrate, including from dietary sources, is associated with a number of effects consistent with increased levels of endogenous NO synthesis, including reductions in blood
pressure [16-20]. This effect has been demonstrated as early as three hours after a single dose of nitrate rich beetroot juice, with a concomitant protection of forearm endothelial function and *in vitro* inhibition of platelet aggregation [13]. Dietary nitrate has also been shown to reduce the overall oxygen cost of sub-maximal exercise 2.5 hours after ingestion [21] and after three or more days administration [17, 21-23]. Similarly, an increase in peak power and work-rate [21], a speeding of VO$_2$ mean response time in healthy 60-70yr olds [19] and delayed time to task failure during severe exercise [22, 23] have also been reported following the consumption of nitrate rich beetroot juice daily for 4 to 15 days. Nitrate related reductions have also been demonstrated with regards the rate of adenosine-5'-triphosphate (ATP) turnover using magnetic resonance spectroscopy [22], whilst improved oxygenation [23] has been confirmed directly in the muscle during exercise using Near-Infrared Spectroscopy (NIRS).

NO plays a pivotal role in cerebral vasodilation and the neurovascular coupling of local neural activity and blood-flow [24] and enhanced cerebral blood perfusion has been observed in the prefrontal cortex in response to increased circulating levels of dietary nitrate [11]. Several studies have probed the effects of dietary nitrate derived from beetroot or spinach on brain function, including three studies that have included some form of cognitive testing either as an additional measure [19, 20], or as the primary focus of the project [25]. Whilst these studies demonstrated modulation of a number of physiological parameters they did not provide evidence of cognitive improvements, possibly due to comparatively small sample sizes and other methodological factors. Two studies have also investigated the effects of dietary nitrate on cerebral blood-flow parameters. In the first of these, Presley et al. [11] demonstrated, using arterial spin labelling magnetic resonance imaging (MRI), that a diet high in nitrate consumed for 24 hours increased regional white matter perfusion in elderly humans, but with this effect restricted to areas of the frontal cortex. More recently Aamand et al.
(2013), investigated the effects of 3 days administration of dietary nitrate (sodium nitrate) on the haemodynamic response in the visual cortex elicited by visual stimuli, as assessed by functional MRI (fMRI). They demonstrated a faster, smaller and less variable blood-oxygen-level dependent (BOLD) response following nitrate, which they interpreted as indicating an enhanced neurovascular coupling of local CBF to neuronal activity. As the BOLD response simply reflects the contrasting magnetic signals of oxygenated and deoxygenated haemoglobin (with increased activity imputed from an assumed relative decrease in deoxyhaemoglobin as local activation engenders a greater influx of blood borne oxygenated – Hb), it cannot disentangle the contributions of changes in blood-flow and changes in oxygen consumption to the overall signal. The current study therefore utilised Near-Infrared Spectroscopy (NIRS), a brain imaging technique that has the advantage over fMRI BOLD in that it measures both concentration changes in deoxy-Hb and overall local CBF (changes in oxy-Hb and deoxy-Hb combined).

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

MATERIALS AND METHODS

Participants:

40 healthy adults (mean age 21.28y, range 18-27y) took part in the study. Prior to attending the laboratory all participants refrained from eating for 12 hours, and consumed no vegetables for 36 hours prior to testing. Participants were allowed their usual morning caffeinated
beverages, but consumed no caffeine for a minimum of 2 hours prior to the assessment. The age and physical characteristics of the two groups are shown in Table 1.

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

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

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

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

Physical characteristic data (means plus SEMs) from the placebo and dietary nitrate conditions (n = 20 per group) including pre and post-treatment heart rate, blood pressure and plasma nitrite
measurements. Analysis on the latter measures was by two-way ANOVA with Bonferroni adjusted post-hoc comparisons (* P < 0.05, placebo versus dietary nitrate at that time point).

*Treatments:*

Participants were randomly assigned to receive either:

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

Or

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

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

The drinks were prepared by a neutral third-party according to the computer generated randomisation list and administered double-blind by the researchers. Given the disparity in taste between the treatments the study was run with a between-subjects design and participants were simply informed that the study was investigating the CBF effects of fruit or vegetable drinks. They were given no information on the experimental aims, the identity of the drinks, or the nitrate contents or potential physiological effects of the beetroot juice (other than being informed of the possibility of discoloured urine).
Functional Near-Infrared Spectroscopy (NIRS) is a brain imaging technique that is predicated on the intrinsic optical absorption properties of oxygenated (oxy-Hb) and deoxygenated (deoxy-Hb) haemoglobin following the introduction of near-infrared light through the intact skull. When assessed by NIRS, the increase in CBF in the surface layers of the cortex during localized neural activity is seen as an increase in the total concentration of haemoglobin (total-Hb) and comparative decrease in deoxy-Hb [26] with both parameters corresponding strongly with the functional magnetic resonance imaging (fMRI) blood oxygen level dependent (BOLD) signal [26-28]. NIRS has been used extensively as a technique for multiple-channel imaging of task related brain activity over relevant areas of the head [29], including in groups suffering from potential decrements in CBF [30]. To date, a growing number of pharmacological intervention studies have also used the technique to infer localized brain activity [31] and CBF and oxygenation [32] from changes in haemoglobin concentrations. The paradigm employed here has been shown to be sensitive to both increased [33-35] and decreased [36, 37] CBF in the prefrontal cortex of healthy young volunteers following nutritional interventions.

In the current study relative changes in the absorption of near-infrared light were measured at a time resolution of 10Hz using a 12 channel Oxymon system (Artinis Medical Systems B.V.). The system emitted two nominal wavelengths of light (~765- and 855nm) with an emitter/optode separation distance of 4cm. The differential pathlength factor was adjusted according to the age of the participant. Relative concentration changes in oxy-Hb, deoxy-Hb and total-Hb were calculated by means of a modified Beer-Lambert law [38] using the proprietorial software.
In this study, given the extended recording period and the investigational aims, a simple two emitter/optode pair configuration was utilised (i.e. 2 channels). The emitter/optode pairs were positioned over the left and right frontal cortex using a standard optode holder headband, which separated the pairs from each other by 4 cm. Each pair therefore collected data from an area of prefrontal cortex that included the areas corresponding to the International 10-20 system Fp1 and Fp2 electroencephalogram (EEG) positions.

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

Blood sampling and determination of plasma nitrite levels:

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

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

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

Cognitive tasks:

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

The original verbal Serial 7s test has appeared in many forms, including as part of the Mini-Mental State Examination for dementia screening. In the current study, a modified, 4 minute, computerized version of the Serial Subtraction task was used [47], which consists of 2 minutes of Serial 3s followed by 2 minutes of Serial 7s subtractions. At the start of each 2 minute section, a standard instruction screen informed the participants to count backwards in 3s or 7s, as quickly and accurately as possible, using the keyboard’s linear number keys to enter each response. Participants were also instructed verbally at the outset that if they were to make a mistake they should continue subtracting from the new incorrect number. A random starting number between 800 and 999 was presented on the computer screen, which was
cleared by the entry of the first response. Each 3-digit response was represented on screen by an asterisk. Pressing the enter key signalled the end of each response and cleared the 3 asterisks from the screen. Performance data (total number of subtractions and number of errors) were calculated for the Serial 3s and 7s elements separately. In the case of incorrect responses, subsequent responses were scored as positive if they were correct in relation to the new number.

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

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

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

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

Procedure:

Each participant was required to attend the laboratory on two occasions. The first of these was an initial screening/training visit, and this was followed within 21 days by the active study morning. During the initial visit participants provided written informed consent and were screened with regards the study exclusion/inclusion criteria. Training was given on the cognitive tasks and the compliance requirements for the following visit were explained. On the active study morning participants attended the laboratory between 8.30 and 9.30 am and provided confirmation of their compliance with the inclusion/exclusion requirements. Participants then gave a venous blood sample, completed the Bond-Lader mood scales, made a baseline completion of the three tasks (Serial 3s, 7s, RVIP), and had their blood pressure and heart rate measured. Participants were then fitted with the NIRS headband. After 5 minutes the 10 minute resting baseline period commenced. During this time, and the subsequent absorption period, participants watched a non-arousing DVD. The study drink was presented to the participant in three equal amounts at 10 minute intervals at the start of the 90 minute absorption period. At the end of the absorption period participants were then verbally instructed to start the period of task performance, during which they completed the Bond-
Lader mood scales and then made 6 consecutive repetitions of the Serial Subtractions and RVIP tasks (i.e. 54 minutes of continuous performance). Following task completion they completed the Bond-Lader mood scales for a final time, had their blood pressure and heart rate measured and provided a venous blood sample. The timelines and running order of the testing session are shown in Figure 1.

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

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

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

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

The primary analysis of the averaged NIRS data (total- and deoxy-Hb) was conducted by ANOVAs (treatment group x epoch) performed separately with data from the absorption period and the task period. In order to assess the effects of the differential task demands on haemoglobin concentrations an ANOVA (treatment x task [subtractions/RVIP] x epoch x repetition [1 to 6]) of the task period data was also conducted. Subsequent a priori planned comparisons of data from each 2 minute epoch during both the absorption and cognitive task periods were made between the placebo and dietary nitrate condition using t tests calculated with the Mean Squares Error [50] from the appropriate ANOVA. The planned comparisons were subjected to a Bonferroni adjustment for multiple comparisons. In order to reduce the
potential for Type I errors only those planned comparisons associated with a significant (p < 0.05) main effect of treatment or interaction between treatment and epoch on the primary ANOVA are reported.

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

RESULTS

Plasma nitrite

Plasma levels of nitrite were significantly raised in the beetroot condition (P < 0.01) by the end of the assessment: see right panel of Figure 2 for graphical depiction.
Figure 2. Serial 3 subtraction performance and plasma nitrite levels. Left panel: Adjusted mean (±SEM error bar) number of correct Serial 3s generated in 2 minutes averaged across the 6 post-treatment repetitions of the tasks. Right panel: Mean (±SEM error bar) plasma nitrite levels pre-treatment and at the end of testing (~150 minutes post-treatment). ○ and ○ = placebo; ■ and ● = 450 ml of beetroot juice containing 5.5 mmol nitrate).

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

NIRS parameters

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

Deoxygenated haemoglobin (deoxy-Hb): The initial ANOVAs showed that treatment with dietary nitrate narrowly failed to significantly modulate deoxy-Hb, with a strong trend towards a treatment x epoch interaction (P < 0.1) during the task period. Mean changes in
total-Hb and deoxy-Hb across the absorption and task performance periods are shown in Figure 3. with data from the task period presented in greater detail in Figure 4.

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

(Footnote) The study followed a parallel groups design (n = 20 per condition). Data are averaged across 2 minute (absorption period, serial subtractions) or 2.5 minute (RVIP) epochs. Analysis with repeated measures ANOVA showed a significant treatment x epoch interaction (P < 0.05) for total haemoglobin concentrations (i.e. CBF – top panel) during both the absorption and cognitive task periods, with no significant effect for deoxygenated haemoglobin (bottom panel). *A priori* planned comparisons comparing data from each dietary nitrate group to placebo for each epoch were carried out with t tests incorporating Mean Squares Error from the ANOVA with a Bonferroni adjustment for
multiplicity. Significance on the Bonferroni adjusted comparisons between placebo and dietary nitrate during the individual epoch is indicated by * (P < 0.05) and ** (P < 0.01).

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

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

Cognitive performance, mental fatigue and mood
The ANCOVA (using baseline performance as a covariate) showed that participants’ performance improved significantly in terms of the number of correct Serial 3s subtractions following the consumption of dietary nitrate (P < 0.05). There were no other significant improvements seen in terms of the other tasks (Serial 7s, RVIP), the three Bond-Lader mood factors, or ratings of mental fatigue. It should be noted that the dietary nitrate group under-
performed the placebo group prior to treatment (mean correct Serial 3s subtractions: dietary nitrate 35.6, Placebo 50.15). The adjusted mean number of serial 3s subtractions (plus SEMs) are presented graphically in the left panel of Figure 2.

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

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

The primary investigational question here was whether dietary nitrate would modulate haemodynamic responses in the prefrontal cortex during the performance of tasks that activate this area of the brain. The pattern of hemodynamic effects following dietary nitrate was for an initial significant increase in CBF, as indexed by total-Hb, at the very outset of task
performance (i.e. the first Serial 3s), followed by consistent reductions during the RVIP task, culminating in reduced CBF during both the Serial 3s task and RVIP during their last repetitions. The concentration of deoxy-Hb was not significantly modulated here, but it is worth noting that the pattern was for a reduced concentration throughout the task period (See bottom portion of figure 3).

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

Typically, and as in the placebo condition here, performance of the RVIP task results in a smaller increase in CBF than does performance of the Serial Subtraction tasks (see, for instance, Kennedy et al. [52]). This can largely be attributed to the relative cognitive demands of the two tasks, with Serial Subtractions requiring the continuous retention of information in working memory and the active mathematical manipulation of numbers throughout the task, whereas RVIP simply requires the monitoring of rapidly changing digits along with a more passive contribution from working memory (i.e. remembering whether the last two digits were odd or even). The overall pattern of CBF is therefore as expected, but singularly more
exaggerated than normal; a finding which was also observed in Aamand et al. [51] and which they argue represents an “enhanced hemodynamic coupling” between activity and local blood-flow. In this case the accentuated reduction in CBF may potentially represent a more sensitive match between blood flow and activity during the RVIP task. Of course this begs the question as to why blood flow was comparatively unchanged during the more difficult Serial Subtractions. Whilst no clear explanation can be provided, it may be pertinent that these tasks are self-paced (with participants actively performing the subtractions as opposed to passively monitoring digits in the RVIP) and that performance on one of the two serial subtraction tasks was improved.

Interestingly, reference to figure 4 demonstrates a nitrate-induced exaggeration of the normal (placebo) CBF response. This sensitivity of NIRS (to oscillating pattern of CBF changes) has also been demonstrated with the stilbene polyphenol (and NO-modulator) resveratrol; where serial subtraction performance consistently increased total- and deoxy-Hb (and to a lesser extent oxy-Hb) across the entire 36 minute post-dose task period, compared to interspersed decreases in response to the RVIP task [52]. In terms of an explanation for these effects, at least two distinct NO-related mechanisms may be involved here. Firstly, these results may represent an exaggeration of the NO-mediated relationship between task-related neural activity and the local neurovascular response. The relationship between increased cognitive workload and augmented CBF has been demonstrated with NIRS previously with Son et al. [53] reporting an amplified CBF response as a result of increasing workload and Shibuya-Tayoshi et al. [54] evidencing a greater CBF response to the difficult, versus the easy, aspect of the Trail-Maker task. Taken together, the RVIP task could be conceived as requiring less cognitive resources (or indeed frontal involvement) than the mental arithmetic serial subtraction tasks.
As well as this exaggerated response, this study also reports reduced CBF during all tasks by the end of the cognitive task period. As such, a second, related, explanation for these results is that both the improved performance during the Serial Subtractions and reduced CBF during the RVIP task reflect improvements in cellular oxygen utilisation driven by NO synthesis, with reduced CBF reflecting a decreased need for additional metabolic substrates. This interpretation is supported by concomitant (non-significant) reductions in concentrations of deoxy-Hb seen during the periods of reduced CBF; suggesting decreased oxygen extraction. In this respect the expected pattern would be for the concentration of deoxy-Hb to increase with decreasing CBF as it became a greater proportion of the overall blood volume, and vice versa (e.g. the opposite pattern is seen during the first 60 minutes of the absorption period, with increased CBF engendering decreased deoxy-Hb).

In terms of mechanisms underlying the effects seen here, as well as acting as a vaso-dilator during local neural activity [5-7] previous research suggests that NO exerts a number of effects that might also impact on overall cellular energy consumption in the brain. These include the inhibition of mitochondrial respiration and therefore oxygen consumption, including via inhibition of cytochrome c oxidase [55, 56] and enhancement of the efficiency of oxidative phosphorylation by decreasing slipping of the proton pumps [57, 58]. In line with this, increased efficiency of oxidative phosphorylation has recently been demonstrated in human mitochondria following nitrate supplementation, with this effect correlating with reduced oxygen cost during exercise [59] and a trend for reduced oxygen uptake during exercise at 50% of VO2 max, without detrimental effects to physical or cognitive performance [20]. Evidence too suggests that nitrite itself may function in respiration as an alternative electron acceptor to oxygen [60] and that it acts as an important cellular signalling molecule independent of its relationship with NO [10].
With regards cognitive performance, improvements were observed in this study but restricted to one of the three tasks (serial 3 subtractions). Differential levels of cognitive demand, speed of performance and the involvement of disparate cognitive domains across these three tasks make global improvements by any intervention unlikely. The serial 3s task itself requires resources in terms of working memory, psychomotor speed, and executive function. It is therefore inextricably linked to frontal cortex function. It should be noted that the dietary nitrate group under-performed placebo at baseline on this task and, as pre-treatment performance was used as a covariate in the ANCOVA, it is possible that this factor contributed to the significant improvement seen at post-dose. Whether the improvements seen here following nitrate were dependent on poor performance, and therefore a greater sensitivity to any benefits derived from the intervention, remains to be investigated further.

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

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

Overall, the findings here suggest that supplementation with dietary nitrate can directly
modulate important physiological aspects of brain function and improve performance on a
cognitive task that is intrinsically related to prefrontal cortex function. Taken alongside a
previous demonstration of increased prefrontal cortex perfusion in elderly humans following
consumption of a high nitrate diet for ~36 hours [67], the results here suggest both a specific
food component and physiological mechanisms that may contribute to epidemiological
observations of relationships between the consumption of a diet rich in vegetables [68, 69]
and polyphenols (which naturally co-occur with nitrate in vegetables) [70, 71] and preserved
cognitive function in later life. Of particular importance, the results here were demonstrated in young humans, who can be assumed to be close to their optimum in terms of brain function [72], and hint at the potential benefits of a healthy, vegetable rich diet across the lifespan.

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