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Influence of dietary nitrate supplementation on physiological and cognitive responses to incremental cycle exercise.

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1. Introduction

The determinants of human exercise tolerance remain obscure but, depending on the subject population and the exercise modality, intensity and duration, may be related to both central and peripheral factors and to the balance between oxidative and nonoxidative contributions to energy turnover (Knicker et al., 2011). For endurance type exercise, it has been proposed that the interaction between exercise efficiency or economy and the peak oxygen uptake ($\dot{V}O_{2\text{peak}}$) may be a critical determinant of performance (Coyle, 1995). Therefore, interventions which enhance exercise efficiency or $\dot{V}O_{2\text{peak}}$ are of considerable interest to athletes and coaches.

The increase in $\dot{V}O_2$ per unit increase in work rate (i.e., the response 'gain') is often used to characterise muscle efficiency during exercise (Whipp et al., 1981). For constant-work-rate cycle exercise performed below the lactate threshold (LT), the gain approximates 10 ml/min/W and has been considered to be essentially unaffected by differences in the age, health or fitness status of the subjects tested or by interventions such as training, prior exercise, hypoxia or hyperoxia (Poole and Richardson, 1997). It is of significant interest, therefore, that several recent studies have reported that dietary nitrate supplementation either with nitrate salts or nitrate-rich beetroot juice consumption, enhances muscle efficiency (Bailey et al., 2009, 2010; Larsen et al., 2007; Vanhatalo et al., 2010a,b). Dietary nitrate intake results in a significant elevation of plasma [nitrite] which may be reduced to nitric oxide (NO) and other reactive nitrogen intermediates under appropriate physiological conditions (Lundberg et al., 2008). This NO production pathway is believed to supplement NO production from the oxidation of L-arginine catalysed by nitric oxide synthase (NOS), and may be particularly important in conditions of lowered tissue PO_2 or pH (Cosby et al., 2003; Shiva et al., 2007) such as would be extant in skeletal muscle during exercise. It has been reported that during <LT exercise, the steady-state $\dot{V}O_2$ is reduced by 3–5% following nitrate supplementation (Bailey et al., 2009; Larsen et al., 2007; Vanhatalo et al., 2010a), and that during >LT exercise, the $\dot{V}O_2$ slow component is attenuated and T_{lim} is extended (Bailey et al., 2009; Lansley et al., 2011). The mechanistic bases for these effects on muscle efficiency remain unclear but may be linked to an NO-mediated reduction in the ATP cost of muscle force production (Bailey et al., 2009), altered calcium handling and force development in type II fibres (Hernández et al., 2012), and/or improved mitochondrial efficiency (i.e., enhanced P/O ratio; Larsen et al., 2011).

In addition to the direct effects of NO or nitrite on muscle efficiency, it is possible that the increased NO bioavailability following nitrate supplementation may enhance bulk muscle blood flow, improve blood-muscle O_2 driving pressure or result in a better matching of local O_2 supply to metabolic rate (Ferguson et al., 2012, 2013) factors which might also predispose to improved exercise tolerance. Alterations in vascular tone following nitrate supplementation are reflected in significant reductions in resting blood pressure (Webb et al., 2008) and

in greater muscle oxygenation at rest and during moderate exercise, as demonstrated by changes in total haemoglobin, oxyhemoglobin or deoxyhemoglobin concentrations in the area of interrogation, as measured with near infra-red spectroscopy (NIRS), (Bailey et al., 2009; Kenjale et al., 2011; Masschelein et al., 2012).

Given recent suggestions that changes in cerebral blood flow during high-intensity exercise might be related to the fatigue process (Nybo, 2008; Rooks et al., 2010; Rupp and Perrey, 2008) it is pertinent to note that dietary nitrate supplementation has been reported to enhance cerebral perfusion in brain areas associated with executive functioning in older adults at rest (Presley et al., 2011). Specifically, a nitrate-rich diet increased regional cerebral perfusion in frontal lobe white matter, particularly between the anterior cingulate cortex (ACC) and dorso-lateral prefrontal cortex (Presley et al., 2011). The ACC is purported to be affected by mental fatigue (Cook et al., 2007) and its activity has been shown to be related to the perception of effort during exercise (Williamson et al., 2001, 2002, 2006). The ACC and pre-frontal cortex have also been associated with processing fatigue-related feedback, emotion, arousal states and decision making (Senn, 2002; Walton et al., 2003, 2006) and in adjusting descending command, including motor control, during an ongoing task (Liu et al., 2003). Therefore an enhancement of cerebral blood flow during exercise, through dietary nitrate supplementation, might better maintain these functions, reducing mental fatigue and effort perception, and aiding cognition. This might, in turn, contribute to improved exercise tolerance given that a raised RPE due to mental fatigue has been suggested to lead to an earlier withdrawal from exercise (Marcora et al., 2009).

The purpose of the present study, therefore, was to investigate the influence of dietary nitrate supplementation on cerebral and skeletal muscle oxygenation, exercise efficiency, effort perception, mental fatigue and cognitive function at rest and over a range of exercise intensities (50%, 70% and 90% $\dot{V}O_{2peak}$). We employed this comprehensive approach to enable simultaneous assessment of the central and peripheral effects of nitrate supplementation on brain and muscle function. We hypothesised that, relative to a placebo condition, dietary nitrate supplementation would: (1) elevate plasma [nitrite] and reduce blood pressure at rest; (2) improve cerebral and muscle oxygenation at rest and during exercise; (3) reduce cerebral and muscle fractional O_2 extraction and lower pulmonary $\dot{V}O_2$ during exercise; (4) improve performance on a battery of cognitive tests at rest and during exercise; (5) attenuate mental fatigue and effort perception; and (6) enhance exercise tolerance.

2. Methods

2.1. Subjects

Sixteen healthy, recreationally active males (mean $\pm$ SD; age 24 ± 4 yr, height 1.77 ± 0.07 m, weight 75.6 ± 9.2 kg, $\dot{V}O_{2max}$ 47.3 ± 6.3 ml $kg^{-1} min^{-1}$) volunteered to participate in this study. All subjects reported to be non-smokers, who were not using any prescription medications, illicit social drugs or dietary supplements. The study received ethical approval from the Northumbria University School of Psychology and Sport sciences Ethics Committee and adhered to the principles of the Declaration of Helsinki. All subjects gave their written, informed consent prior to commencement of the study. The subjects were instructed to arrive at the laboratory at 8 am on experimental days, fully hydrated, following an overnight fast, and to avoid strenuous exercise 24-h prior to each visit. Subjects were also asked to refrain from alcohol and caffeine consumption for at least 24-h and 6-h prior to each exercise session, respectively. Subjects were provided with a list of foods rich in nitrate and were asked to abstain from these foods 36-h prior to the experiment beginning and thereafter 36-h prior to each testing session.

2.2. Procedures

The subjects were required to visit the laboratory on three separate occasions to complete exercise tests on an electronically braked cycle ergometer (Velotron, Dynafit Pro, RacerMate Inc., Seattle, USA). During visit one, subjects completed an incremental exercise test to determine $\dot{V}O_{2peak}$. Following a 5-min rest period, subjects completed 3-min of 'unloaded' (20 W) cycling, after which the work rate was increased by 30 W/min until volitional exhaustion. $\dot{V}O_{2peak}$ was determined as the highest 30-s mean $\dot{V}O_2$ value achieved prior to exercise termination. The configuration of the saddle and handlebar position was measured and recorded, and repeated for all subsequent exercise tests.

During visits 2 and 3 (Fig. 1), the subjects received two single-dose dietary supplements in a double-blind, cross-over design which was randomly allocated in a counterbalanced order (Latin square). Subjects consumed 0.5 litres of either a nitrate supplement (450 ml of organic beetroot juice containing 5 mmol nitrate; Beet It, James White Drinks, Ipswich, UK and 50 ml of low calorie blackcurrant cordial) or a placebo (50 ml low calorie blackcurrant cordial, 45 ml pressed apple juice, 405 ml H₂O, containing negligible nitrate). No adverse effects were reported. At the time of the experiment the Beet It beetroot juice and shot placebo products were not available (Lansley et al., 2011). Experimental exercise sessions were separated by at least 7 days.

Prior to the exercise test, baseline measures of plasma nitrite concentration, blood pressure and the cognitive tasks [Rapid visual information processing (RVIP) then Stroop tasks] were taken pre and post-supplementation. Resting cerebral oxygenation (COX) was established prior to beverage ingestion and then monitored throughout the experimental session. Subjects consumed the beverage (over ~10-min) and sat quietly, watching non-arousing DVDs during a 90-min absorption period. Resting measurements of muscle oxygenation (MOX) for the right m. vastus lateralis, pulmonary gas exchange, blood lactate concentration [Lac-], mental fatigue rating, energy level, mood (BRUMS) scores and rating of perceived exertion (RPE, Borg, 1998) were taken after beverage ingestion and before the exercise test began.

The exercise test consisted of 3-min of 'unloaded' (20 W) cycling during which baseline EMG of the m. vastus lateralis was established, followed by two, 20-min incremental stages at work rates required to elicit ~50% and ~70% $\dot{V}O_{2peak}$, and a final work rate corresponding to 90% $\dot{V}O_{2peak}$, which was continued until task failure (defined as a fall in pedal rate of >10 rpm below self-selected cadence). These intensities were selected to be positioned within the moderate, heavy, and severe exercise domains, respectively (Poole and Jones, 2012). During the 50% and 70% $\dot{V}O_{2peak}$ stages, the subjects completed the cognitive tasks while cycling. Heart rate, pulmonary gas exchange, EMG, COX and MOX were measured continuously throughout each exercise stage, while blood [Lac-], mental fatigue rating, mood scores and RPE were recorded at the end of each stage. Following task failure during the 90% $\dot{V}O_{2peak}$ stage, time completed on the stage was recorded, with blood [Lac-] measurement and then cognitive tasks repeated at 5-min and 6-min post-exercise, respectively, followed by a blood pressure measurement at 25-min post-exercise.

Fig. 1. Schematic of the study protocol. CT, cognitive tasks; C_{NIRS} , baseline cerebral NIRS; BP, blood pressure; $[NO_2^-]$, venous blood sample for plasma nitrate analysis; M_{NIRS} , baseline muscle NIRS; $[La]$, capillary blood sample for blood lactate determination; subjective assessments, mental fatigue, energy levels and BRUMS questionnaire.

each exercise stage, while blood $[Lac^-]$, mental fatigue rating, mood scores and RPE were recorded at the end of each stage. Following task failure during the 90% VO_{2peak} stage, time completed on the stage was recorded, with blood $[Lac^-]$ measurement and then cognitive tasks repeated at 5-min and 6-min post-exercise, respectively, followed by a blood pressure measurement at 25-min post-exercise.

2.3. Blood and cardio-respiratory measurements

Blood Pressure (BP) of the brachial artery was measured in a rested, seated position using an automated sphygmomanometer (Omron M5-1, Hoofddorp, The Netherlands). Three measurements were taken at each time point and the mean value of the second and third measurements was used for analysis. Venous blood samples were collected into lithium heparin tubes for determination of plasma [nitrite]. Blood samples were centrifuged at 4000 rpm for 10-min at 4 °C within 3-min of collection. Plasma was collected and subsequently stored at -80 °C until further analysis for [nitrite] by chemiluminescence using the procedures described by Bailey et al. (2009).

During all exercise tests, pulmonary ventilation ($\dot{V}_E$), $\dot{V}O_2$, $\dot{V}CO_2$ and RER were determined from breath-by-breath expired gases measured continuously using indirect open circuit calorimetry (Oxycon Pro, Jaeger, Hoechberg, Germany). Heart rate (HR) was continuously measured during all exercise tests using short-range telemetry (Polar S610i, Polar Electro Oy, Kemple, Finland). Capillary blood samples (20 μ l) taken from the fingertip were collected in a heparinised capillary tube for immediate analysis of blood lactate concentration ($[Lac^-]$) (Biosen C-line, EKF Diagnostic, Barleben, Germany).

2.4. Cerebral NIRS

A cerebral near-infrared oximeter (12 channel Oxymon system, Artinis Medical Systems BV, The Netherlands) was used to continuously monitor changes in cerebral oxyhemoglobin concentration ($[HbO_2]$), deoxyhemoglobin concentration ($[HHb]$) and total haemoglobin concentration ($[Hb_{tot}]$) during cognitive tasks

and exercise. NIRS is a method increasingly used to study functional activation of the brain through monitoring changes in haemodynamic properties (Huppert et al., 2006). The ability of NIRS to measure blood volume changes following cerebral activation has been validated by its use in a number of studies (Fallgatter and Strik, 1998; Schroeter et al., 2002; Shibuya-Tayoshi et al., 2007) including following nutritional interventions (Kennedy and Haskell, 2011; Kennedy et al., 2010a). However it must be acknowledged that cerebral NIRS measurements can be affected by near surface blood flow, blood pressure, skull thickness and that there is an assumption that prefrontal/cortical oxygenation originates from the cortical haemodynamic response (Gagnon et al., 2012; Kirilina et al., 2012; Takahashi et al., 2011). To mitigate against variability in NIRS measurements between trials we marked probe sites with marker pen. The experimental protocol was also identical between trials with the exception of the dietary treatment. The system emitted NIR light at two wavelengths (765 and 855 nm), with the absorption properties recorded at 10 Hz. The emitter/detector probes were separated by 4 cm, and the differential pathlength factor was adjusted relative to the participant's age (Duncan et al., 1996; Lloyd-Fox et al., 2010; Shibuya et al., 2004a,b; Strangman et al., 2002). In this study, a two emitter/receiver optode pair configuration was utilised (i.e. 2 channels) positioned over the left and right cortex using a standard optode headband. The probes were placed in a configuration corresponding with the International 10–20 system Fp1 and Fp2 EEG positions. Each emitter optode was at a 16.5 mm distance from the midline and 4 cm laterally from the corresponding receiver optode. This separated the emitter/receiver pairs from each other by 33 mm. Cotton/lycra material was placed over the probes to reduce the influence of extraneous light. Relative concentration changes in $[HbO_2]$, $[HHb]$ and $[Hb_{tot}]$ were calculated by means of a modified Beer–Lambert law using the proprietary software and were continuously monitored during all cognitive tasks.

2.5. Muscle NIRS

Oxygenation of the m. vastus lateralis of the right leg was continuously monitored non-invasively using NIRS (NIRO 200NX, Hamamatsu Photonics KK, Japan). The system uses an emission probe which transmits NIR light at three different wavelengths (735, 810, and 850 nm) to the tissue with the light reflected from the tissue detected by a photomultiplier tube (detection probe). The intensity of the transmitted light was continuously recorded at 2 Hz, and concentration changes from resting baseline were calculated for $[HbO_2]$, $[HHb]$, and $[Hb_{tot}]$ (inclusive of myoglobin), based on a modification of the Beer–Lambert law. The emitter-to-detector distance was 4 cm, and the differential path length factor used was 4.95 (Duncan et al., 1996). The values are reported as the final 3-min of each section of the exercise test.

The NIRS probes were placed in a holder and secured to the skin (which had been cleaned and shaved) over the belly of the right m. vastus lateralis (15 cm above the proximal border of the patella and 5 cm lateral to the midline of the thigh) using double sided adhesive tape. Probe placement was marked using indelible ink to ensure the same location on the subsequent visit. An elasticised, tensor bandage was used to minimise the influence of extraneous light, and to further secure the probes and to avoid movement of the probe relative to the skin, whilst allowing unrestricted movement. Resting measurements were recorded while the subject sat quietly on the ergometer, with the legs relaxed and still (knee joint angle, $\sim 90^\circ$).

2.6. iEMG

Neuromuscular activity of the left m. vastus lateralis was measured using surface EMG. The muscle belly was shaved, cleaned and abraded prior to the application of the electrodes (Medi-trace 100, Kendall, Chicopee,

MA) in a biopolar configuration (inter-electrode distance; 30 mm). A reference electrode was placed over the tibial tuberosity. Electrode placements were marked using indelible ink to ensure the same location on the subsequent visit. To minimise movement artefact during cycling, the electrode and wires were secured using a cotton/lycra sheath placed around the subject's thigh. The EMG signal was recorded during exercise using a data acquisition system (Powerlab) at a sampling frequency of 1000 Hz. The raw EMG data were rectified and integrated (iEMG) for analysis. The iEMG values reported are the average of the final 3-min of each section of the exercise trial and expressed relative to the average measured during unloaded cycling.

2.7. Cognitive assessment

Cognitive tasks were used to exacerbate mental fatigue and to assess cognitive performance. All cognitive tasks were computerised and incorporated a computer screen which was positioned at eye level, and a control button box for participant responses which was securely mounted onto the handlebars of the cycle ergometer to allow for the completion of the cognitive tasks during exercise. All cognitive tests were delivered using the Computerised Mental Performance Assessment System (COMPASS), a purpose designed software application for the flexible delivery of randomly generated parallel versions of standard and novel cognitive assessment tasks that has previously been shown to be sensitive to nutritional interventions (Haskell et al., 2010; Kennedy and Haskell, 2011; Kennedy et al., 2010a,b). Tasks were chosen based on their ability to activate the pre-frontal cortex (Lawrence et al., 2002; Schroeter et al., 2002). The tasks comprised of the rapid visual information processing (RVIP) and Stroop tests. The tasks were completed five times in total: at baseline before ingestion of the supplement; during the pre-exercise period; whilst cycling at 50% $\dot{V}O_{2peak}$; whilst cycling at 70% $\dot{V}O_{2peak}$; and 6-min following the termination of exercise at

90% $\dot{V}O_{2peak}$. The tasks were identical in each of these periods and were completed in the same order, and differed only in their duration (baseline tasks were 2-min duration and all other tasks were 9-min duration).

2.8. Rapid visual information processing (RVIP)

In this test, the participant monitored a continuous series of digits for targets of three consecutive odd or three consecutive even digits. The digits were presented on the computer screen at the rate of 100 per minute in pseudo-random order (eight correct target strings being presented each minute) and the participant responded to the detection of a target string by pressing a button on a button box as quickly as possible. The task was continuous. The task was scored for percentage of target strings correctly detected, average reaction time for correct detections, and percentage of false alarms.

2.9. Stroop test

In this task, a series of colour names ('RED', 'YELLOW', 'GREEN', 'BLUE') appeared on the screen one at a time in different coloured fonts. The participants pressed one of the four coloured response buttons on the control box to select the colour that matched the colour font that the word was written in (e.g., if the word 'RED' was presented in a green font, the correct response would be to press the green button). Therefore the words presented were either 'congruent' (name of colour and colour of ink the same) or 'incongruent' (name of colour and colour of ink different) and were presented randomly. Subjects were asked to respond as quickly and as accurately as possible. The task was scored for percentage accuracy and reaction time.

2.10. Subjective assessments

Standard instructions were given to subjects for overall RPE from the 15-point scale (Borg, 1998). Following completion of each set of cognitive tasks, participants rated their RPE and were then presented with the questions “how mentally fatigued do you feel right now?” and “how energetic do you feel right now?” and asked to rate these by marking a vertical line on a 100 mm line on an A4 piece of paper with the end points labelled ‘not at all’ (left hand end) and ‘extremely’ (right hand end). The value was expressed as a percentage of the scale (100% = extremely). Mood was assessed presupplement, post-supplement but pre-exercise, and post-exercise using the Brunel Mood Scale (BRUMS), a questionnaire which is based on the Profile of Mood States (Terry et al., 2003). The questionnaire contains 24 items which are answered using a Likert scale (0–4; “not at all” to “extremely”). The items are divided into 6 categories (anger, confusion, depression, fatigue, tension, and vigour) and scored from 0 to 16 (raw score total). The scores for fatigue and vigour were subsequently analysed.

2.11. Statistics

All assumptions for using parametric tests were verified. Differences in exercise time to the limit of tolerance between treatments were analysed using two-tailed, paired samples t-tests. A twoway (treatment × epoch or post-exercise level), repeated measures ANOVA was used to determine differences in cardio-respiratory, blood pressure, blood lactate, cerebral and muscle NIRS, EMG and RPE variables. Where significant effects were detected, post hoc analysis was performed using paired samples t-tests. All data are reported as mean ± SD. Statistical significance was accepted at $p < 0.05$. Cohen’s effect size (d) and 90% confidence intervals were calculated. Cerebral and muscle NIRS data were averaged across 3min epochs during the task period and normalised to the baseline concentration during the 10-min pre-treatment period and 5-min pre-exercise/post-absorption period, respectively. Prior to the primary analysis, in order to identify any hemispheric differences, repeated measures ANOVAs were carried out on cerebral NIRS task data (treatment × epoch × hemisphere). No treatment related interactions involving hemisphere as a factor were observed for the task period. Data from both channels were therefore averaged for $[\text{HbO}_2]$, $[\text{HHb}]$ and $[\text{Hb}_{\text{tot}}]$ and then analysed separately across the task period (treatment × task × exercise level × epoch) by repeated measures ANOVAs. Significant treatment related interactions were further investigated by a priori planned comparisons where the active treatment was compared to placebo at each epoch utilising t-tests. Cognitive performance, subjective ratings of mental fatigue, energy levels, mood and RPE were analysed as ‘change from baseline’ by two-way repeated measures ANOVA (treatment × exercise level). Significant treatment effects were further explored with a priori planned comparisons where the active treatment was compared to placebo. Due to a data capture error, only 15 participants were included in the cognitive and subjective analysis. SPSS v19.0 was used for statistical analysis.

3. Results

3.1. Plasma [nitrite] and blood pressure

Plasma [nitrite] was significantly elevated pre- and postexercise with nitrate supplementation compared to placebo ($F_{1,12} = 31.657$, $p < 0.001$). Pre-supplement dietary nitrite values were 143 ± 13 vs. placebo 124 ± 10 nM

($p > 0.05$). Postsupplement/pre-exercise dietary nitrite vs. placebo values were 222 ± 61 vs. 124 ± 43 nM ($p < 0.001$, $d = 1.85$, 90% CI = 63–124 nM) while post-exercise values were 178 ± 44 vs. 133 ± 37 nM ($p = 0.004$, $d = 1.10$, 90% CI = 22–65 nM), respectively. Systolic blood pressure was significantly reduced post-exercise with nitrate compared to placebo (112 ± 8 vs. 116 ± 6 mmHg; $p = 0.032$, $d = 0.65$, 90% CI = -7.7 to -0.1) with a trend also detected post-supplementation/pre-exercise (114 ± 8 vs. 118 ± 5 mmHg; $p = 0.097$, $d = 0.47$, 90% CI = -6.2 to -0.3). No differences in systolic blood pressure were observed between conditions presupplementation. Diastolic blood pressure was not different between conditions at any time point.

3.2. Cerebral and muscle oxygenation at rest and during exercise

Change in $[\text{HHb}]_{\text{cerebral}}$ was significantly lower with nitrate compared to placebo during the pre-exercise RVIP and Stroop cognitive tasks, as well as during work rates eliciting 50% and 70% $\text{VO}'_{2\text{peak}}$ and post-exercise ($F_{6,90} = 2.29$, $p < 0.05$; Fig. 2). Differences in $[\text{HbO}_2]_{\text{cerebral}}$ and $[\text{Hb}_{\text{tot}}]_{\text{cerebral}}$ between treatments did not reach significance. In both treatments, $[\text{HbO}_2]_{\text{cerebral}}$ and $[\text{Hb}_{\text{tot}}]_{\text{cerebral}}$ increased over time during exercise, before falling post-exercise.

Change in the $[\text{HHb}]_{\text{muscle}}$ was attenuated with nitrate compared to placebo ($F_{1,13} = 5.33$, $p = 0.038$, $d = 0.91$). Planned comparisons showed that changes in $[\text{HHb}]_{\text{muscle}}$ were similar between treatments at rest and pre-exercise. However, changes in $[\text{HHb}]_{\text{muscle}}$ were significantly lower ($p < 0.05$, $d > 0.60$) with nitrate compared to placebo during the RVIP tests at 50% and 70% $\text{VO}'_{2\text{peak}}$, and the Stroop test at 50% $\text{VO}'_{2\text{peak}}$. There were also trends for a smaller change in $[\text{HHb}]_{\text{muscle}}$ at 70% $\text{VO}'_{2\text{peak}}$ during the Stroop test ($p = 0.070$, $d = 0.52$, 90% CI = -4.80 to 0.26) and during the final exhaustive exercise stage ($p = 0.082$, $d = 0.50$, 90% CI = -4.80 to 0.15) (see Fig. 3). For both treatments, the change in

$[\text{HHb}]_{\text{muscle}}$ increased during the exercise protocol ($F_{1,15} = 25.64$, $p = 0.001$, $d = 0.91$).

A trend was detected for an interaction ($F_{1.4,17.8} = 3.458$, $p = 0.069$, $d = 0.64$) for $[\text{HbO}_2]_{\text{muscle}}$. Post hoc comparisons for interaction effects between trials demonstrated a small effect in terms of a trend for a greater change in $[\text{HbO}_2]_{\text{muscle}}$ with nitrate

Fig. 2. Group mean change in concentration of cerebral deoxygenated haemoglobin ([HHb]), oxygenated haemoglobin ([HbO₂]) and total haemoglobin ([Hb_{tot}]) pre-, during- and post-exercise following ingestion of placebo (open circles) or nitrate (closed circles). Each datum on the graph represents a 3 min epoch. Values are mean $\pm$ SE. Treatment $\times$ task $\times$ exercise level $\times$ epoch interaction effects are shown for [HHb], (* p < 0.05, *** p < 0.005, **** p < 0.001). Please note time zero (0 min) in this figure indicates the start of the exercise protocol which equates to 120 min in Fig. 1.

compared to placebo during cognitive tasks at 70% $\dot{V}O_{2peak}$ (RVIP task p = 0.134, d = 0.26, CI -0.21 to 3.10; Stroop task p = 0.146, d = 0.30, 90% CI = -0.20 to 3.80) and at 90% $\dot{V}O_{2peak}$ (p = 0.011, d = 0.34, 90% CI -0.10 to 4.00; Fig. 3). Muscle oxygenation fell at these intensities with both treatments ($F_{1.51,14.96}$ = 7.174, p = 0.015, d = 0.98), but the fall was smaller with nitrate. There was a trend for [Hbdiff]_{muscle} (that is, [HbO₂]_{muscle} – [HHb]_{muscle}) to be greater with nitrate compared to placebo ($F_{1,13}$ = 4.132; p = 0.063).

An interaction was found in the $[Hb_{tot}]_{muscle}$ response ($F_{1.9,24.6} = 3.755$, $p = 0.040$, $d = 0.76$), with a significantly reduced

Fig. 3. Group mean change in concentration of muscle deoxygenated haemoglobin ([HHb]), oxygenated haemoglobin ([HbO₂]), total haemoglobin ([Hb_{tot}]) and integrated EMG (iEMG) during exercise, following ingestion of placebo (open circles) or nitrate (closed circles). Each datum on the graph represents a 3 min epoch. Values are mean $\pm$ SD. * $p < 0.05$, *** $p < 0.005$ vs. placebo. Please note time zero (0 min) in this figure indicates the start of the exercise protocol which equates to 120 min in Fig. 1.

response with nitrate compared to placebo during cognitive tasks in the 50% VO_{2peak} stage (RVIP task $p = 0.005$; Stroop task $p = 0.044$). $[Hb_{tot}]_{muscle}$ increased across the exercise test in both conditions ($F_{1.49,19.31} = 31.103$, $p = 0.001$, $d = 1.64$).

3.3. Cardio-respiratory, EMG, heart rate and blood [Lac-] responses

There was a trend for a reduced $\dot{V}O'_{2peak}$ with nitrate compared to placebo at 50% $\dot{V}O'_{2peak}$ ($p = 0.110$, $d = 0.41$, 90% CI = -0.22 to 0.00 L min⁻¹) but not at other exercise intensities or at rest. Par-

ticipants achieved a similar $\dot{V}O'_{2peak}$ at exhaustion in the final stage of the exercise test. EMG, heart rate and blood [Lac-] increased ($p < 0.001$) as trials progressed but were not significantly different between treatments, except for the post-exercise blood [Lac-] measurement which was significantly elevated with nitrate compared to placebo ($p = 0.016$, $d = 1.04$, 90% CI = 0.31 – 1.50 ; Table 1). A significant main effect for treatment was found for RER ($F_{1,15} = 25.596$, $p < 0.001$; Table 1). RER was slightly but significantly higher with nitrate compared to placebo at rest and at each exercise intensity ($p < 0.02$, $d = 0.40$ – 1.00 ; Table 1).

3.4. Perceptual responses and cognitive tests

There were no significant on-day differences or treatment related differences in RPE, mental fatigue or energy level or mood (fatigue and vigour) prior to treatment or during exercise ($p > 0.05$). However, pooled data indicated a main effect for time for mental fatigue score ($F_{4,56} = 16.29$, $p < 0.001$, $d = 1.16$), energy ($F_{4,56} = 10.61$, $p < 0.001$, $d = 0.76$) and RPE ($F_{2,30} = 195.82$, $p < 0.001$, $d = 13.05$). The mental fatigue score became elevated at 70% $\dot{V}O'_{2peak}$ ($p < 0.05$, see Fig. 4) and stayed elevated until exercise cessation, while perceived energy levels initially increased from pre-exercise to 50% $\dot{V}O'_{2peak}$ but then decreased with each exercise stage ($p < 0.05$). RPE increased linearly with exercise intensity (Fig. 4). There was also a significant main effect for time for fatigue ($F_{2,30} = 15.24$, $p < 0.01$; BRUMS questionnaire), with fatigue increasing from pre-exercise to post-exercise ($p < 0.01$).

A main effect for time (i.e., task repetition) was found for RVIP % Correct ($F_{3,42} = 7.15$, $p < 0.05$) and RVIP false alarms ($F_{3,42} = 3.242$, $p < 0.05$). A significant decrease in RVIP % Correct was detected from 50% to 70% $\dot{V}O'_{2peak}$ ($p < 0.05$) while RVIP false alarms increased from

70% $\dot{V}O'_{2peak}$ to post-exercise ($p = 0.05$). There were no differences in cognitive test performance between treatments.

3.5. Time to the limit of tolerance at 90% $\dot{V}O'_{2peak}$

The time to exhaustion at 90% $\dot{V}O'_{2peak}$ was 16% longer with nitrate compared to placebo (185 ± 122 s vs. 160 ± 109 s, $p = 0.011$, $d = 0.23$, 90% CI = 10 – 41 s) with 13 of 16 subjects increasing their tolerable exercise duration (Fig. 5). The $\dot{V}O'_{2peak}$ measured at exhaustion in the nitrate and placebo conditions (3.46 ± 0.55 and $3.400.60$ L min⁻¹, respectively) was not significantly dif-

ferent from the $\dot{V}O'_{2peak}$ measured in the initial incremental test (3.56 ± 0.55 L min⁻¹).

4. Discussion

This is the first study to investigate the influence of dietary nitrate supplementation on a task involving concomitant physical exercise and fatiguing cognitive tests. The principal findings of this study were that, compared to placebo, the acute consumption of 500 ml of nitrate-rich beetroot juice: elevated plasma [nitrite] and lowered systolic blood pressure at rest and post-exercise; improved muscle oxygenation; reduced cerebral and muscle deoxygenation; and enhanced exercise tolerance. Compared to placebo, nitrate supplementation did not improve cognitive performance at rest or during exercise, nor did it attenuate increases in perceived exertion and mental fatigue as exercise intensity increased. However, there are two principal original findings of the study. Firstly, acute dietary

Table 1
Cardio-respiratory and blood responses, pre-, during-, and post-exercise.

	V_E (L min ⁻¹)	$\dot{V}O_2$ (L min ⁻¹)	RER	Lactate (mmol L ⁻¹)
<i>Dietary nitrate condition</i>				
Pre-supplementation	–	–	–	–
Post-suppl./Pre-exercise (rest)	10.44 ± 1.93	0.35 ± 0.09	0.84 ± 0.09	1.64 ± 0.38
RVIP task 50% $\dot{V}O_{2peak}$	38.07 ± 6.63	1.68 ± 0.29	0.88 ± 0.04*	–
Stroop task 50% $\dot{V}O_{2peak}$	42.80 ± 8.20	1.71 ± 0.28	0.89 ± 0.03*	1.89 ± 0.56
RVIP task 70% $\dot{V}O_{2peak}$	71.53 ± 13.31	2.65 ± 0.40	0.96 ± 0.03*	–
Stroop task 70% $\dot{V}O_{2peak}$	84.55 ± 16.92	2.80 ± 0.40	0.92 ± 0.03	6.25 ± 1.91
No task 90% $\dot{V}O_{2peak}$	135.19 ± 30.98*	3.46 ± 0.54	1.04 ± 0.06*	9.53 ± 2.22
Post-exercise	–	–	–	8.80 ± 2.10*
<i>Placebo condition</i>				
Pre-supplement	–	–	–	–
Post-suppl./Pre-exercise (rest)	9.44 ± 2.44	0.35 ± 0.08	0.80 ± 0.06	1.74 ± 0.94
RVIP task 50% $\dot{V}O_{2peak}$	36.68 ± 6.24	1.60 ± 0.26	0.85 ± 0.04	–
Stroop task 50% $\dot{V}O_{2peak}$	42.26 ± 7.82	1.75 ± 0.26	0.86 ± 0.06	2.11 ± 1.21
RVIP task 70% $\dot{V}O_{2peak}$	70.78 ± 11.92	2.70 ± 0.37	0.93 ± 0.04	–
Stroop task 70% $\dot{V}O_{2peak}$	84.36 ± 13.63	2.84 ± 0.38	0.91 ± 0.03	6.30 ± 2.59
No task 90% $\dot{V}O_{2peak}$	125.19 ± 31.53	3.40 ± 0.60	1.01 ± 0.06	9.13 ± 1.89
Post-exercise	–	–	–	7.90 ± 2.33

Values are reported as mean ± SD. V_E , minute ventilation; $\dot{V}O_2$, peak oxygen consumption; RER, respiratory exchange ratio; RVIP, rapid visual information processing.

* Significantly different from placebo condition ($p < 0.05$).

nitrate supplementation improved high-intensity endurance exercise performance under conditions of significant mental fatigue, which has implications for activities and occupations where significant mental fatigue will occur alongside developing physical fatigue. Secondly, dietary nitrate may modulate the local cerebral haemodynamic response during exercise with concomitant cognitive tasks.

A single 500 ml nitrate-rich beetroot juice beverage was sufficient to significantly increase plasma [nitrite] following the 90-min absorption period, which is in agreement with previous studies (Bailey et al., 2009; Vanhatalo et al., 2010a; Webb et al., 2008). The percent change observed in plasma [nitrite] following the acute nitrate supplementation regimen employed in the present study was similar to that reported after 3–6 days of supplementation (Bailey et al., 2009). A well-established effect of dietary nitrate supplementation is a reduction in systolic blood pressure either with (Larsen et al., 2007; Webb et al., 2008) or without (Bailey et al., 2009) a concomitant reduction in diastolic blood pressure. Consistent with this, we found a significant reduction in systolic blood pressure post-exercise (by ~4 mmHg) with a trend for lower systolic blood pressure also following the absorption period. Webb et al. (2008) reported that peak reductions in blood pressure occurred 2.5 to 3-h post ingestion, with changes in systolic blood pressure persisting for longer than changes in diastolic blood pressure. The reduction in blood pressure has been suggested to result from the reduction of nitrite to NO, which

is known to promote endothelial relaxation via its role in cyclic guanosine monophosphate synthesis (Gruetter et al., 1979).

4.1. Low-intensity exercise

During the cognitive tasks at 50% $\dot{V}O'_{2peak}$ and the RVIP task of the 70% $\dot{V}O'_{2peak}$ stage, $[HHb]_{muscle}$ was significantly reduced with nitrate compared to placebo. The change in $[Hb_{tot}]_{muscle}$ was also lower with nitrate compared to placebo and there was a trend towards a reduced $\dot{V}O'_2$. A lower steady-state $\dot{V}O'_2$ coincident with reduced $[HHb]_{muscle}$ during moderate-intensity exercise following dietary nitrate supplementation has been interpreted as less O_2 extraction being required to support a reduced rate of muscle oxidative energy turnover (Bailey et al., 2009). The similar HR, $\dot{V}E'$ and iEMG responses in the nitrate and placebo conditions in the present study would suggest that the reduction in $\dot{V}O'_2$ was not due to changes in the O_2 cost of cardio-respiratory processes or activation of the m. vastus lateralis. The mechanistic bases for the lower muscle fractional O_2 extraction (as inferred from NIRS measurements; Ferguson et al., 2013; Grassi et al., 2003) and reduced whole-body O_2 cost of exercise (Bailey et al., 2009; Larsen et al., 2007; Vanhatalo et al., 2010a) following nitrate supplementation might be related to improvements in mitochondrial (Larsen et al., 2011) or muscle contractile (Hernández et al., 2012) efficiency. Interestingly, RER was slightly but significantly elevated with nitrate compared to placebo at rest and during exercise at 50% $\dot{V}O'_{2peak}$ which might suggest a subtle shift in substrate utilisation towards increased carbohydrate metabolism. The cause of this increase in RER is not clear but might be related to an increased NO-mediated muscle glucose uptake (Merry and McConell, 2012). However, the magnitude of the change in RER is too small to make a meaningful difference to $\dot{V}O'_2$.

4.2. High-intensity exercise

Significant differences in NIRS-derived indices of muscle oxygenation were observed with nitrate compared to placebo at 70% and 90% $\dot{V}O'_{2peak}$ although there was no significant change in $\dot{V}O'_2$. Specifically, the typical rise in $[HHb]_{muscle}$, which is associated with increasing exercise intensity (Rupp and Perrey, 2008), was attenuated by nitrate supplementation. The smaller change in $[HHb]_{muscle}$ in the nitrate condition is suggestive of reduced muscle fractional O_2 extraction. Given that $\dot{V}O'_2$ was not different between conditions, this in turn implies that muscle O_2 delivery was greater with nitrate compared to placebo during work rates intended to elicit 70% $\dot{V}O'_{2peak}$ (Stroop task) and 90% $\dot{V}O'_{2peak}$. Consistent with this interpretation, an interaction was observed in the $[HbO_2]_{muscle}$ response whereby the typical reduction in $[HbO_2]_{muscle}$ which is apparent as exercise intensity increases (Rupp and Perrey, 2008) was attenuated by nitrate compared to placebo at 70% $\dot{V}O'_{2peak}$ and 90% $\dot{V}O'_{2peak}$. In both treatment conditions, $[Hb_{tot}]_{muscle}$ increased with exercise intensity until 70% $\dot{V}O'_{2peak}$, after which it remained relatively unchanged until the end of exercise. When $[Hb_{tot}]_{muscle}$ is stable, $[Hb_{diff}]$ is a good indicator of muscle oxygenation (Shibuya et al., 2004a,b). Notably, although a fall in $[Hb_{diff}]_{muscle}$ was apparent in both conditions as exercise intensity increased, the fall was attenuated with nitrate compared to placebo, presumably reflecting improved muscle oxygenation following nitrate supplementation. Better muscle oxygenation may reduce phosphocreatine breakdown and anaerobic glycolysis and

Fig. 4. Group mean values $\pm$ SD for subjective measures of mental fatigue, energy levels and rating of perceived exertion (RPE) following ingestion of placebo (open circles) or nitrate (closed circles). * denotes significant change in grand mean value compared to proceeding stage of the trial ($p < 0.05$).

blunt the accumulation of metabolites that have been associated with the fatigue process (e.g., H^+ , P, ADP) (Vanhatalo et al., 2010b).

In turn, these changes might be expected to enhance exercise tolerance, as was indeed observed at the 90% VO_{2peak} work rate. A recent study reported similar effects during incremental exercise in hypoxia (Masschelein et al., 2012). In that study, compared to placebo, six days of beetroot juice consumption resulted in improved muscle oxygenation and greater time-to-exhaustion in hypoxia (11% O₂) compared to normoxia.

4.3. Cerebral oxygenation changes during exercise

A decreased $[\text{HHb}]_{\text{cerebral}}$ was observed with nitrate compared to placebo during the cognitive tests at rest, during each exercise intensity and during the recovery period, suggesting a reduced

Fig. 5. Individual (dashed lines) and group mean $\pm$ SD for time to the limit of tolerance at 90% $\text{VO}'_{2\text{peak}}$ following placebo (open circles) and nitrate (closed circles) ingestion. * $p < 0.05$ vs. placebo.

cerebral O_2 extraction. Notably a recent study by Aamand et al. (2013) reported that the BOLD signal from high resolution functional magnetic imaging was altered by dietary nitrate when a visual stimuli was provided to the subjects (Aamand et al., 2013). A faster and smaller BOLD response, with less variation across local cortex was measured, which the authors suggested was consistent with an enhanced haemodynamic/neurovascular coupling following nitrate intake. In the present study, it is possible that dietary nitrate enhanced haemodynamic coupling in response to the stimuli of the cognitive tasks and the exercise challenge being undertaken. During exercise at 50% $\text{VO}'_{2\text{peak}}$, the trend for a reduced

VO'_2 coincident with reduced $[\text{HHb}]_{\text{cerebral}}$ suggests that nitrate supplementation resulted in less brain O_2 extraction. In addition, at the higher exercise intensities, there was a simultaneous reduction of $[\text{HHb}]_{\text{cerebral}}$ and a greater change and attenuated reduction in $[\text{HbO}_2]_{\text{muscle}}$ suggestive of altered vascular control to support metabolic processes in the brain and skeletal muscles.

$[\text{HbO}_2]_{\text{cerebral}}$ and $[\text{Hb}_{\text{tot}}]_{\text{cerebral}}$ increased in the nitrate and placebo conditions from rest up to and including the 90% $\text{VO}'_{2\text{peak}}$ stage before falling during the recovery period. The similar responses in $[\text{HbO}_2]_{\text{cerebral}}$ and $[\text{Hb}_{\text{tot}}]_{\text{cerebral}}$ between treatments was also found in a recent study which reported that nitrate supplementation did not significantly change cerebral oxygenation in hypoxia (Masschelein et al., 2012). An increase in $[\text{HbO}_2]_{\text{cerebral}}$ and $[\text{Hb}_{\text{tot}}]_{\text{cerebral}}$ during the early stages of incremental exercise is indicative of the local increase in cerebral blood flow required to meet the elevated O_2 requirements of increased neuronal activation (Ide et al., 1999; Rooks et al., 2010; Rupp and Perrey, 2008). These changes in cerebral blood flow maintain cerebral metabolism and function by increasing O_2 and glucose delivery (Rasmussen et al., 2007; Secher et al., 2008).

4.4. Cognitive performance

Cognitive tasks were selected based on their ability to activate the pre-frontal cortex (Lawrence et al., 2002; Schroeter et al., 2002) and to be sensitive to nutritional interventions (Haskell et al., 2010; Kennedy and Haskell, 2011; Kennedy et al., 2010a,b). However, in the present study, no improvements in task performance were observed in the nitrate trial despite evidence of reduced cerebral deoxygenation following dietary nitrate supplementation. Brain metabolism is known to be dependent on the O_2 diffusion gradient and accordingly

cerebral blood flow (Rooks et al., 2010). An effect of strenuous exercise is to disengage the higher order functions of the pre-frontal cortex perhaps to avoid compromise of optimal motor execution (Dietrich and Audiffren, 2011). The lack of differences in performance on the cognitive tasks between treatments might indicate that cerebral oxygenation was either not sufficiently diminished in the placebo trial or enhanced in the nitrate trial to cause a difference between treatments in cognitive performance; or alternatively that any benefit from the reduced $[\text{HHb}]_{\text{cerebral}}$ in the nitrate treatment condition was directed towards maintaining motor function, rather than cognitive function, and may have contributed to the increase in exercise tolerance.

4.5. Mental fatigue during exercise

An interesting finding was that the cognitive tasks, undertaken with concomitant exercise, appear to have led to a significant rise in mental fatigue following the work rate eliciting $70\% \text{VO}'_{2\text{peak}}$ which was maintained until the end of the trials. Participants' mental fatigue scores rose then reached a plateau in both trial conditions at the work rate set to elicit $70\% \text{VO}'_{2\text{peak}}$. These data coincided with a period of prolonged concomitant mental and physical work and compromised executive function (deterioration in performance on the RVIP task) which are all aspects associated with definitions of mental fatigue. Perceived energy levels had also begun to fall at this point. Upon completion of the trials, all subjects reported feeling exhausted both mentally and physically when they were asked "how they felt".

It is plausible that the decision to stop exercising was influenced, in part, by the mental fatigue subjects experienced from the stage eliciting $70\% \text{VO}'_{2\text{peak}}$. A recent study found that mentally fatiguing subjects for 90-min prior to sub-maximal endurance exercise (80% peak power output) reduced their time to exhaustion, despite there being no difference in physiological responses compared to the control condition where mental fatigue was not induced (Marcora et al., 2009). A more recent study observed a reduced time to exercise cessation for a trial containing a concomitant physical (35% MVC static shoulder abductions) and mental workload, compared to a control trial containing only the physical workload component (Mehta and Agnew, 2012). In the current study, exercise tolerance was greater with nitrate compared to placebo, so it would appear that the decision to terminate exercise, despite similar losses in cognitive performance and increases in mental fatigue between conditions, was delayed in the dietary nitrate condition. Therefore an important finding from this study is that nitrate supplementation extends time to exhaustion when participants are experiencing mental fatigue, presumably due to more favourable physiological conditions such as enhanced muscle oxygenation, oxidative efficiency and possibly enhanced cerebral haemodynamic coupling. In practical terms, this has implications for activities and professions requiring people to perform exhausting exercise concomitant with mentally fatiguing tasks.

5. Conclusions

Dietary nitrate supplementation elevated plasma [nitrite], lowered systolic blood pressure, improved muscle oxygenation, reduced cerebral and muscle O_2 extraction and improved exercise tolerance compared to the placebo condition. However, compared to placebo, dietary nitrate did not improve cognitive performance or attenuate the rate of rise in perceived exertion and mental fatigue as exercise intensity increased. Dietary nitrate supplementation can improve subjects' exercise tolerance during endurance exercise under conditions of significant mental fatigue.

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