Dietary nitrates supplementation to enhance exercise capacity in hypoxic COPD: EDEN-OX, a double-blind, placebo-controlled, randomised cross-over study

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ABSTRACT
Rationale Dietary nitrates supplementation improves skeletal muscle oxygen utilisation and vascular endothelial function. We hypothesised that these effects might be sufficient to improve exercise performance in patients with COPD and hypoxia severe enough to require supplemental oxygen.

Methods We conducted a single-centre, double-blind, placebo-controlled, cross-over study, enrolling adults with COPD who were established users of long-term oxygen therapy. Participants performed an endurance shuttle walk test, using their prescribed oxygen, 3 hours after consuming either 140 mL of nitrate-rich beetroot juice (BRJ) (12.9 mmol nitrate) or placebo (nitrate-depleted BRJ). Treatment order was allocated (1:1) by computer-generated block randomisation.

Measurements The primary outcome was endurance shuttle walk test time. The secondary outcomes included area under the curve to isotime for fingertip oxygen saturation and heart rate parameters during the test, blood pressure, and endothelial function assessed using flow-mediated dilatation. Plasma nitrate and nitrite levels as well as \( F_{E_{186}} \) were also measured.

Main results 20 participants were recruited and all completed the study. Nitrate-rich BRJ supplementation prolonged exercise endurance time in all participants as compared with placebo: median (IQR) 194.6 (147.5–411.7) s vs 159.1 (121.9–298.5) s, estimated treatment effect 62 (33–106) s (p<0.0001). Supplementation also improved endothelial function: NR-BRJ group +4.1% (−1.1% to 14.8%) vs placebo BRJ group −5.0% (−10.6% to −0.6%) (p=0.003).

Conclusion Acute dietary nitrates supplementation increases exercise endurance in patients with COPD who require supplemental oxygen.

Trial registration number ISRCTN14888729.

INTRODUCTION
People with COPD may develop hypoxaemia as the condition becomes more severe, impacting on their ability to perform day-to-day activities. Mechanisms include ventilation perfusion mismatch, reduced cardiac output due to hyperinflation and pulmonary vascular limitation, as well as reduced muscle efficiency. In individuals who are sufficiently hypoxaemic, long-term oxygen therapy (LTOT) improves survival, and in many individuals ambulatory oxygen therapy (AOT) improves exercise performance.

Nitric oxide (NO) has potential as a modulator of exercise performance. A ubiquitous signalling molecule, NO is involved in a number of processes at a tissue and cellular level, including mitochondrial and cellular respiration, 7, 8 glucose uptake into the skeletal muscle, 9 skeletal muscle contraction, 10, 11 neurotransmission 12 and fatigue development. 13 NO is produced both by oxygen-dependent NO synthases catalysing its production from L-arginine and an alternative nitrate (NO \(_3^-\))–nitrite (NO \(_2^-\))–NO pathway. 14 The latter can be influenced by supplementation with exogenous dietary NO \(_3^-\) and is enhanced in conditions of hypoxia and low pH as found in exercising skeletal muscle. 15

Dietary NO \(_3^-\) supplementation has been shown to reduce the oxygen cost of exercise in healthy individuals in normoxic conditions 16–17 and in conditions of hypoxia. 18–22 Recently our research group has shown that it augments improvement in exercise capacity seen in people with COPD following pulmonary rehabilitation. 24, 25 We have also previously shown that dietary NO \(_3^-\) supplementation reduces the oxygen cost of exercise during...
endurance cycle ergometry in COPD. However, that study, which excluded patients who required supplemental oxygen, did not demonstrate an improvement in exercise capacity.

The aim of the present study was therefore to assess the acute effect of dietary supplementation in the form of nitrate-rich beetroot juice (NR-BRJ) on exercise performance in individuals with COPD who require supplemental oxygen on exertion, hypothesising that this would increase exercise capacity, measured as endurance shuttle walk time (ESWT), as well as improve endothelial function in people with this specific phenotype.

MATERIALS AND METHODS

Study design

The EDEN-OX (Effect of Dietary Nitrate Supplementation on Exercise Performance in Hypoxia) study was a single-centre, double-blind, placebo-controlled, randomised cross-over trial comparing the effects of dietary NO\textsuperscript{−} supplementation with a matched placebo in individuals with COPD who require LTOT and use AOT during exercise. All participants provided informed consent. The study was registered prospectively in a publicly accessible database. The data presented here relate only to the planned COPD cohort in that study.

People with Global Initiative for Chronic Obstructive Lung Disease grade II–IV COPD\textsuperscript{27} who were established users of LTOT, in accordance with the NICE guidelines,\textsuperscript{28} were recruited from the outpatient clinical services of Royal Brompton and Harefield NHS Foundation Trust (North West London) between 4 November 2016 and 8 August 2017, with the last participant’s final visit completed on 15 January 2018.

Exclusion criteria for the study included clinical instability (ie, less than 1 month after an exacerbation), significant comorbidity limiting exercise tolerance, significant renal impairment (estimated glomerular filtration rate <50 mL/min), hypotension (systolic blood pressure <100 mm Hg), pregnancy, use of NO\textsuperscript{−}-based medicine or phosphodiesterase V inhibitors, or presence of other conditions that might be influenced by NO\textsuperscript{−} supplementation (ie, ischaemic heart disease or peripheral vascular disease). These conditions were assessed at the screening visit through review of clinical history and assessment of relevant clinical data.

Methods

Interventions

The intervention was a commercially available concentrated NO\textsuperscript{−}-rich BRJ (NR-BRJ) (98%) drink cut with organic lemon juice (2%) containing 0.8 g, 12.9 mmol NO\textsuperscript{−} (140 mL Beet-It Sport Shot, James White Drinks, Ipswich, UK). The placebo beetroot juice (PL-BRJ), produced by James White, was 140 mL of the same beverage in which NO\textsuperscript{−} was removed by a standardised method of passing the juice, prior to pasteurisation, through an ion exchange column, containing Purolite A520E, which exchanges NO\textsuperscript{−} against chloride.\textsuperscript{29} The PL-BRJ is identical in appearance, packaging, taste and smell, and also causes beeturia (orange to red discolouration of urine).

Study conduct

At an initial baseline visit, COPD Assessment Test, Hospital Anxiety and Depression Scale, and Medical Research Council Dyspnoea Scale scores were recorded. Body composition was measured by bioelectrical impedance analysis using a Bodystat 4000 device (Bodystat, Isle of Man, UK). Participants then performed two incremental shuttle walk tests to determine the walking speed to be used for the ESWT\textsuperscript{30} and then a practice ESWT. All walking tests throughout the study were performed on the participant’s usual AOT flow rate, and the method for carrying the AOT was recorded to ensure the same method was always used (online supplemental appendix figure E1).

Prior to the two subsequent intervention visits and throughout the study period, participants were asked to avoid the use of antimicrobial mouthwash and chewing gum, as these have been shown to reduce the oral facultative bacteria whose NO\textsuperscript{−} reductase activity is essential for the metabolism of an oral NO\textsuperscript{−} load.\textsuperscript{31} They were asked to consume the same meal on the morning of each study assessment. This was to create as standardised conditions as possible, reducing differing levels of dietary NO\textsuperscript{−} consumption as a source of variation within individuals, while not altering their usual diet greatly. They were also asked to match caffeine consumption to standardise any ergogenic effect arising from it\textsuperscript{32} and to avoid strenuous exercise in the 24-hour period prior to the intervention visits.

The two intervention visits began at the same time of day (±2 hours), with a minimum of 7-day washout period and a maximum 1-month gap between them. Participants were randomly assigned to the order in which they received NR-BRJ or PL-BRJ using a computer-generated block randomisation list, with a block size of 10, produced by an independent statistician. The researchers responsible for enrolment and outcome measurements remained blinded throughout the study and during data analysis. Following their arrival, after a 10 min rest period, participants were observed consuming either the NR-BRJ or the PL-BRJ, and empty bottles were collected and recorded. All outcome measures were undertaken 3 hours after ingestion of either NR-BRJ or PL-BRJ.

Outcomes

Exercise capacity

The primary outcome was ESWT compared between treatment conditions. Given the cross-over design and taking 65 s (95% CI 45 to 85) to be the minimal clinically important difference (MCID) in ESWT\textsuperscript{30} and a pooled mean difference within individuals of 26 s for repeat testing, to have an 80% statistical power, with a significance level of 0.05, 16 participants would be required to reject the null hypothesis that the active intervention was not superior to placebo. To allow for a 25% withdrawal rate, a sample size of 20 was chosen.

Plasma nitrate/nitrite levels and markers of oxidative stress

Plasma NO\textsuperscript{−} and NO\textsubscript{2} levels were used as a combined biomarker of NO\textsuperscript{−} ingestion, metabolism and NO availability.\textsuperscript{33} Plasma samples were obtained on arrival and 3 hours after consumption of NR-BRJ or PL-BRJ (see online supplemental appendix for full details).

Oxidative stress biomarkers were assessed in plasma samples by a combination of three distinct readouts, including antioxidant potential, that is, measurement of the ferric-reducing ability of plasma (FRAP),\textsuperscript{35} lipid oxidation products by thiobarbituric acid reactive substances (TBARS)\textsuperscript{36} and total free thiols with normalisation for protein\textsuperscript{37} (see online supplemental appendix for full details).

Fractional exhaled nitric oxide

FE\textsubscript{NO} was measured as a steady exhalation rate of 50 mL/s with a NIOX Mino (Aerocrine Systems, Solna, Sweden) at the screening visit and then at the intervention visits at baseline prior to NR-BRJ/PL-BRJ consumption and then at six further intervals (30, 60, 90, 120, 150 and 180 min). Both the study participant

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Endothelial function
Endothelial function was assessed by flow-mediated dilatation (FMD) of the brachial artery 3 hours after NR-BRJ/PL-BRJ consumption \(^{38}\) using a high-resolution Doppler ultrasound to measure at baseline and sequentially over a period of 120 s after release of circulatory arrest of the upper arm. \(^{39}\) All measurements were performed by a single trained operator (see online supplemental appendix for full details).

Continuous oxygen saturation and heart rate analysis
For each ESWT performed, pulse oximetry values were recorded (Pulsox 300i Pulse Oximeter, Konica Minolta, Tokyo, Japan) throughout until the participant had recovered (recovery was defined by return of Borg Dyspnoea Scale to that recorded prior to the ESWT). To maintain blinding, the pulse oximeter display was covered throughout the testing and the data were downloaded by an independent researcher, not directly involved with the trial, who uploaded the data to a password-protected database. These data were only available to the researchers following unblinding of the trial.

Statistical analysis
Data are presented as mean (SD), or if not normally distributed as median and IQR. Differences in response between treatment conditions were assessed using a paired t-test or a Wilcoxon signed-rank test as appropriate. Treatment effect was estimated using the Hodges-Lehmann estimate of shift parameters. The process of determining the Hodges-Lehmann estimator entails estimating the average difference in outcomes \((x-y)\) for every possible \(n(n+1)/2\) pair and then deriving the overall median of all averages (the Hodges-Lehmann estimator). A distribution-free CI is estimated using large-sample approximation. Analysis was performed using SPSS V.24 for Windows and Stata V.16.1 for Windows.

To compare continuous oxygen saturations \((\text{SpO}_2)\) and heart rate \((\text{HR})\) between the two treatment conditions, individual ESWT data periods were subjected to a 30 s rolling average using MATLAB (MATLAB and Statistics Toolbox Release V.2017a, The MathWorks, Natick, Massachusetts, USA) and then expressed as percentages of isotime (defined as the duration of the shortest of the two ESWT). These individual responses were then grouped to allow analysis of HR and SpO\(_2\) against the percentage of isotime (plotted at the midpoint of each 10th percentile of isotime). The area under the curve \((\text{AUC})\) was assessed for each individual participant and the two treatment conditions compared using Wilcoxon signed-rank test. Figures were prepared using GraphPad Prism V.6.0 for Windows (GraphPad Software, San Diego, California, USA). A p value of \(<0.05\) was considered statistically significant.

RESULTS
We screened 67 people for eligibility (figure 1); 31 declined to participate, 7 had a comorbidity precluding participation and 9 were not using supplementary oxygen. Of the 20 participants enrolled in the study, 10 were randomised to receive PL-BRJ first and 10 NR-BRJ first. All participants completed the study. Table 1 shows their baseline characteristics, which were well matched between the two order allocation groups. There were no serious adverse effects reported, although all participants reported beeturia. The average time between each intervention visit was 7 days.

Exercise outcomes
Exercise endurance time was longer for all study participants after NR-BRJ compared with PL-BRJ (figure 2): median (IQR) ESWT: NR-BRJ 194.6 (147.5–411.7) s vs PL-BRJ 159.1

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**Figure 1** CONSORT diagram for recruitment and trial completion. CONSORT, Consolidated Standards of Reporting Trials; NR-BRJ, nitrate-rich beetroot juice; PL-BRJ, placebo beetroot juice.
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(121.9–298.5) s, estimated treatment effect 62.5 (95% CI 33 to 106) s, p = 0.000089. There was no evidence of an intervention order effect (online supplemental appendix figure E2).

There was one individual who was a clear outlier for exercise endurance response. However, a sensitivity analysis removing their data led to a slight change in primary study outcome but not the overall statistical significance: median (IQR) ESWT: NR-BRJ 193.8 (145–389.6) s vs PL-BRJ 158.2 (121.6–236.6) s, estimated treatment effect 56.5 (95% CI 30 to 88) s, indicating a significant increase in ESWT with NR-BRJ (p = 0.0001) (online supplemental appendix results E1).

Pulse oximetry data were available for only 18 participants because recording failed for 2 of them. The average AUC for SpO₂ was higher in the NR-BRJ group compared with the PL-BRJ group. These differences were more apparent at isotime and peak exercise, with no difference at rest, during warm-up or recovery (figure 3A and online supplemental appendix table E1). The estimated treatment effect was also statistically significant: 43.69 (29.09–58.28) s (p < 0.0001). The AUC for HR response to NR-BRJ or PL-BRJ did not show any difference. The estimated treatment effect was also not statistically significant (−41.17 (−116.74 to 34.40) s, p = 0.27) (figure 3B and online supplemental appendix table E1).

Endothelial function and blood pressure

Two participants declined the FMD assessment; therefore, data were available for 18 participants. At 180 min following dosing, Table 1 Characteristics of cross-over allocation groups: NR-BRJ or PL-BRJ received first

<table>
<thead>
<tr>
<th>Measurement</th>
<th>NR-BRJ first (n=10)</th>
<th>PL-BRJ first (n=10)</th>
<th>P value</th>
<th>Whole group (N=20)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex (% female:male)</td>
<td>30:70</td>
<td>50:50</td>
<td>1.0</td>
<td>40:60</td>
</tr>
<tr>
<td>Age (years)</td>
<td>68 (62–73)</td>
<td>67 (64–76)</td>
<td>0.7</td>
<td>67.6 (8.5)</td>
</tr>
<tr>
<td>Caucasian (%)</td>
<td>100</td>
<td>100</td>
<td>1</td>
<td>100</td>
</tr>
<tr>
<td>Smoking (pack years)</td>
<td>28 (14–50)</td>
<td>64 (57–96)</td>
<td>0.006</td>
<td>52 (21.6)</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>24.3 (20.9–29.0)</td>
<td>26.2 (21.4–30.1)</td>
<td>0.5</td>
<td>25.2 (4.7)</td>
</tr>
<tr>
<td>FFMI (kg/m²)</td>
<td>18.4 (15.8–20.1)</td>
<td>18.0 (14.0–20.2)</td>
<td>0.8</td>
<td>18.1 (15.8–19.9)</td>
</tr>
<tr>
<td>Inhaled medications</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SABA (%)</td>
<td>91</td>
<td>100</td>
<td>0.4</td>
<td>95</td>
</tr>
<tr>
<td>LABA-ICS (%)</td>
<td>91</td>
<td>78</td>
<td>0.4</td>
<td>95</td>
</tr>
<tr>
<td>LAMA (%)</td>
<td>91</td>
<td>100</td>
<td>0.4</td>
<td>85</td>
</tr>
<tr>
<td>Baseline resting oxygen saturation FiO₂ % (median)</td>
<td>92 (89–94)</td>
<td>92 (89–93)</td>
<td>0.8</td>
<td>92</td>
</tr>
<tr>
<td>Pre-LTOT prescription baseline pO₂ (kPa)</td>
<td>6.9 (6.0–7.3)</td>
<td>6.6 (6.4–7.2)</td>
<td>0.6</td>
<td>6.8</td>
</tr>
<tr>
<td>Oxygen prescription (L/min)</td>
<td>4 (2–6)</td>
<td>2 (2–4)</td>
<td>0.2</td>
<td>3.0 (2.0–6.0)</td>
</tr>
<tr>
<td>CAT score</td>
<td>20 (18–29)</td>
<td>19 (15–28)</td>
<td>0.6</td>
<td>21 (8.0)</td>
</tr>
<tr>
<td>MRC Dyspnoea Score</td>
<td>4 (4–4)</td>
<td>4 (4–4)</td>
<td>1.0</td>
<td>4 (4–4)</td>
</tr>
<tr>
<td>HADS-A score</td>
<td>4 (3–7)</td>
<td>7 (2–10)</td>
<td>0.3</td>
<td>4.0 (2.3–8.8)</td>
</tr>
<tr>
<td>HADS-D score</td>
<td>4 (4–5)</td>
<td>5 (4–7)</td>
<td>0.7</td>
<td>4.5 (4.0–5.6)</td>
</tr>
<tr>
<td>Systolic BP (mm Hg)</td>
<td>139 (123–149)</td>
<td>135 (115–139)</td>
<td>0.1</td>
<td>137 (121–143)</td>
</tr>
<tr>
<td>Diastolic BP (mm Hg)</td>
<td>76 (66–83)</td>
<td>70 (65–80)</td>
<td>0.4</td>
<td>73 (65–82)</td>
</tr>
<tr>
<td>MAP (mm Hg)</td>
<td>94 (91–104)</td>
<td>91 (85–94)</td>
<td>0.2</td>
<td>92 (80–100)</td>
</tr>
<tr>
<td>Lung function</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FEV₁ (L)</td>
<td>0.7 (0.6–1.0)</td>
<td>0.7 (0.3–1.0)</td>
<td>0.3</td>
<td>0.7 (0.6–1.0)</td>
</tr>
<tr>
<td>FVC (L)</td>
<td>2.7 (1.9–3.1)</td>
<td>1.6 (1.4–3.2)</td>
<td>0.3</td>
<td>2.7 (1.6–3.1)</td>
</tr>
<tr>
<td>FEV₁/FVC ratio</td>
<td>0.3 (0.3–0.3)</td>
<td>0.3 (0.2–0.4)</td>
<td>1.0</td>
<td>0.3 (0.3–0.3)</td>
</tr>
<tr>
<td>RV %predicted</td>
<td>211 (181–235)</td>
<td>212 (188–233)</td>
<td>0.8</td>
<td>212 (186–233)</td>
</tr>
<tr>
<td>TLCO %predicted</td>
<td>33 (19–45)</td>
<td>36 (28–44)</td>
<td>0.9</td>
<td>32 (19–44)</td>
</tr>
<tr>
<td>GOLD stage</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>III (%)</td>
<td>22</td>
<td>45</td>
<td>1.0</td>
<td>35</td>
</tr>
<tr>
<td>IV (%)</td>
<td>78</td>
<td>55</td>
<td>1.0</td>
<td>65</td>
</tr>
<tr>
<td>ISWT distance (m)</td>
<td>300 (280–360)</td>
<td>370 (220–280)</td>
<td>0.04</td>
<td>279 (70)</td>
</tr>
<tr>
<td>ESWT (s)</td>
<td>172 (137–267)</td>
<td>181 (158–193)</td>
<td>0.6</td>
<td>179 (152–193)</td>
</tr>
</tbody>
</table>

Data in order of intervention, either NR-BRJ or PL-BRJ first. Data shown are median (IQR), mean (SD) or percentage (%). P value is for independent t-test or Mann-Whitney test comparing groups. BMI, body mass index; BP, blood pressure; CAT, COPD Assessment Test; ESWT, endurance shuttle walk test; FFMI, fat-free mass index; FiO₂, fraction of inspired oxygen; GOLD, Global Initiative for Chronic Obstructive Lung Disease; HADS-A, Hospital Anxiety Depression Scale-Anxiety; HADS-D, Hospital Anxiety Depression Scale-Depression; ICS, inhaled corticosteroid; ISWT, incremental shuttle walk test; LABA, long-acting beta agonist; LAMA, long-acting muscarinic agonist; LTOT, long-term oxygen therapy; MAP, mean arterial pressure; MRC, Medical Research Council; NR-BRJ, nitrate-rich beetroot juice; PL-BRJ, placebo beetroot juice; RV, residual volume; SABA, short-acting beta agonist; TLCO, transfer Factor for carbon monoxide corrected for haemoglobin.
FMD increased: NR-BRJ 4.1% (−1.1% to 14.8%) compared with placebo −5.0% (−10.6% to −0.6%), estimated treatment effect −11.9% (95% CI −18.9 to −7.15) (p=0.0003) (figure 4).

There was no statistically significant difference in measures of oxidative stress following acute consumption of either supplement: FRAP: NR-BRJ 1018 (853.0–1125) µM vs PL-BRJ 930.2 (836.8–1073) µM (p=1.0); TBARS: NR-BRJ 1.499 (0.855–3.209) mM vs PL-BRJ 0.971 (0.766–1.614) mM (p=0.4); total free thiol per protein: NR-BRJ 7.079 (5.961–8.115) µmol/g protein vs PL-BRJ 6.942 (5.768–8.026) µmol/g protein (p=0.5). The results suggest that there was no statistically significant difference in oxidative stress following acute consumption of either supplement: FRAP: NR-BRJ 1018 (853.0–1125) µM vs PL-BRJ 930.2 (836.8–1073) µM (p=1.0); TBARS: NR-BRJ 1.499 (0.855–3.209) mM vs PL-BRJ 0.971 (0.766–1.614) mM (p=0.4); total free thiol per protein: NR-BRJ 7.079 (5.961–8.115) µmol/g protein vs PL-BRJ 6.942 (5.768–8.026) µmol/g protein (p=0.5).

Plasma nitrate and nitrite levels and oxidative stress markers
Paired data on plasma NO3− and NO2− concentrations were available for 19 participants, as 1 individual declined sampling. Following supplementation with NR-BRJ, there was an 84% increase in plasma NO2− and an 887% increase in plasma NO3− at 180 min post supplementation, but no change with placebo (figure 6 and online supplemental appendix table E2). Both the NR-BRJ and PL-BRJ supplements were analysed for NO3− and NO2− content as well (online supplemental appendix table E3). The change in plasma NO3− and NO2− from baseline to 180 min was calculated and used to estimate the treatment effect of NR-BRJ. The treatment effect of NO3− was 550 (461–639) µM. The results suggest that this was higher for NR-BRJ than for PL-BRJ and this change was statistically significant (p=0.0003). The treatment effect of NO2− was 0.248 (0.138–0.408) µM. The results suggest that this was higher for NR-BRJ than for PL-BRJ and this change was statistically significant (p=0.0011).

There was no statistically significant difference in measures of oxidative stress following acute consumption of either supplement: FRAP: NR-BRJ 1018 (853.0–1125) µM vs PL-BRJ 930.2 (836.8–1073) µM (p=1.0); TBARS: NR-BRJ 1.499 (0.855–3.209) mM vs PL-BRJ 0.971 (0.766–1.614) mM (p=0.4); total free thiol per protein: NR-BRJ 7.079 (5.961–8.115) µmol/g protein vs PL-BRJ 6.942 (5.768–8.026) µmol/g protein (p=0.5).
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Table E4). For four participants, there was device failure resulting in no data being recorded. The median (IQR) AUC for when the subjects were on PL-BRJ was 3622.5 (3181.9–4796.9) and the corresponding result for when the subjects were on NR-BRJ was 9440.6 (6273.8–11 831.3), and the treatment effect with its 95% CI was 5407 (3096 to 7576) (p=0.0011). The results suggest that the FENO levels while the subjects were on NR-BRJ were significantly higher than when they were on PL-BRJ.

Figure 4 Effect of dietary nitrate supplementation on blood pressure parameters. Change in blood pressure parameters (sBP, dBP and MAP) relative to baseline blood pressure 3 hours prior to dosing with either NR-BRJ or PL-BRJ. Data presented as 25th–75th percentile with the solid line representing the median value and the whiskers the minimum to maximum values. Wilcoxon signed-rank test was used to compare blood pressure parameters. Median (IQR) change in sBP: NR-BRJ −1.5 (15.0–10.8) mm Hg vs PL-BRJ −0.5 (−10.5 to 6.8) mm Hg (p=1.0). Median (IQR) in dBP: NR-BRJ 4.0 (−14.0 to 7.0) mm Hg vs PL-BRJ −1.0 (−9.3 to 5.0) mm Hg (p=0.481). Median (IQR) change in MAP: NR-BRJ −5.0 (−15.3 to 6) mm Hg vs PL-BRJ −2.5 (−13.5 to 7) mm Hg (p=0.359). sBP, diastolic blood pressure; MAP, mean arterial pressure; NR-BRJ, nitrate-rich beetroot juice; PL-BRJ, placebo beetroot juice; sBP, systolic blood pressure.

Figure 5 Effect of dietary nitrate supplementation on endothelial function. Percentage change in FMD from baseline and 180 min after supplementation with NR-BRJ or PL-BRJ. Data presented at the 25th and 75th percentile boxes with the solid line representing the median value and the whiskers the minimum and maximum values. Wilcoxon signed-rank test was used to compare the percentage change in the NR-BRJ (red) and PL-BRJ (black) dosing conditions. There was a statistically significant difference in the FMD percentage change with an increase in the NR-BRJ group (4.1, −1.1 to 14.8) versus a reduction in the PL-BRJ group (−5.0, −10.6 to −0.6). **p=0.0003. FMD, flow-mediated dilatation; NR-BRJ, nitrate-rich beetroot juice; PL-BRJ, placebo beetroot juice.

Figure 6 Plasma nitrite and nitrate levels. Data presented are median (IQR) with whiskers representing minimum to maximum values. Plasma NO$_2^-$ and NO$_3^-$ concentrations were measured at baseline (0 min) and 180 min after dosing with the interventions. Wilcoxon signed-rank test was used to compare change in plasma NO$_2^-$ and NO$_3^-$ concentrations between the intervention groups. Mann-Whitney U test was used to compare change in plasma NO$_2^-$ and NO$_3^-$ concentrations between the treatment conditions. (A) Changes in plasma NO$_2^-$ concentrations. There was a statistically significant difference between baseline plasma NO$_2^-$ concentration and postdosing with NR-BRJ for plasma NO$_2^-$; predosing plasma NO$_2^-$ concentration 0.306 (0.227–0.402) μM vs postdosing 0.620 (0.488–0.673) μM; ****p=0.000076. There was also a statistically significant difference between postdosing plasma NO$_2^-$ concentrations between NR-BRJ and PL-BRJ dosing conditions; postdose NR-BRJ NO$_2^-$ concentration 0.620 (0.488–0.673) μM vs postdose of PL-BRJ NO$_2^-$ concentration 0.306 (0.227–0.402) μM; †††† p=0.000009. (B) Changes in plasma NO$_3^-$ levels. There was a statistically significant difference between baseline plasma NO$_3^-$ concentration and postdosing with NR-BRJ for plasma NO$_3^-$; predosing plasma NO$_3^-$ concentration 62.59 (41.68–77.29) μM vs postdosing 617 (556.25–725.88) μM; ****p=0.00004. There was also a statistically significant difference between postdosing plasma NO$_3^-$ concentration between NR-BRJ and PL-BRJ dosing conditions; postdose of NR-BRJ NO$_3^-$ concentration 0.620 (0.488–0.673) μM vs postdose of PL-BRJ NO$_3^-$ concentration 0.306 (0.227–0.402) μM; †††† p=5.66×10⁻¹¹. NO$_2^-$, nitrite; NO$_3^-$, nitrate; NR-BRJ, nitrate-rich beetroot juice; PL-BRJ, placebo beetroot juice.


Thorax: first published as 10.1136/thoraxjnl-2021-217147 on 1 December 2021. Downloaded from http://thorax.bmj.com/ on November 9, 2022 by guest. Protected by copyright.
that we observed was accompanied by less desaturation during exercise capacity compared with placebo. The improvement in ESWT supplementation in COPD with inconsistent results, 24–26 this in response was not statistically significant.

Although studies have previously considered dietary NO3− supplementation in COPD with inconsistent results, 24–26 this is the first stratified medicine approach focusing on the specific phenotype of individuals with COPD with hypoxaemia requiring LTOT. Our previous study, in non-hypoxaemic patients with COPD, found that there was a reduction in the oxygen cost of exercise during cycle ergometry yet no improvement in exercise capacity.26 In conditions of hypoxia, the L-arginine–nitric oxide synthase pathway is compromised, while the NO3−–NO2−–NO pathway is facilitated due to a lesser inhibition of NO2− bioactivation by oxygen.14 20 22 As such, dietary NO3− supplementation could be expected to have more impact in hypoxic rather than normoxic individuals, both through effects on the skeletal muscle and impacts on the pulmonary vasculature.

The mechanism by which ESWT lengthened is likely to involve multiple synergistic pathways. The finding of relatively preserved SpO2 during exercise in the NO3−–supplemented condition could reflect more efficient oxygen utilisation peripherally, a beneficial impact on central haemodynamics associated with/reduced hypoxia-induced pulmonary vasoconstriction, or a combination of the two. Despite each participant using their prescribed oxygen for each walk test, there was an observed desaturation in the placebo arm. This could mean that their oxygen prescription may have been insufficient and that a higher flow rate might also have increased exercise capacity. This finding of the attenuation of desaturation by NR-BRJ may well be explained by the enhancement of the NO3−–NO2−–NO pathway in conditions of hypoxia.

The observation that NO3− supplementation was associated with improvements in endothelial function assessed using FMD is likely to be relevant to the acute mechanism of benefit from NO3− supplementation, but also raises the possibility that longer-term dosing might reduce the risk of vascular events which are common in COPD. The effects seen are almost certainly not COPD-specific and work is needed to investigate possible benefits in other long-term lung conditions associated with hypoxia, including interstitial lung diseases and the various categories of pulmonary hypertension.

The estimated treatment effect of dietary NO3− supplementation on ESWT found in this study was 62.5 s, which falls fractionally short of the MCID defined in pharmacotherapy trials as 65 s.30 However, in pharmacological trials where the ESWT is the outcome, interventions are typically administered over weeks or months. The demonstration of an effect of similar magnitude in a single-dose study is therefore encouraging, although further studies of longer-term use will be needed before any clinical recommendations can be made.

**Study limitations**

The use of a robust placebo strengthens the reliability of the findings, as does the fact that the improvement in walking time was accompanied by an appropriate physiological response (lower HR and higher SpO2). An additional strength was the use of a walking test rather than a cycling test, which is of clinical relevance to patients as it reflects most individuals’ main form of exercise and daily physical activity. This was a single-dose study and therefore questions remain as to the impact that regular dosing might have and whether this would translate into meaningful clinical effects. The dose used was selected based on previous studies, but future work should investigate whether there is a dose response or ceiling effect. We have also shown that the NR-BRJ does indeed contain a higher quantity of NO3− and provide independent confirmation that NO3− is only present at very low levels in the placebo juice used in our study.

**CONCLUSION**

BRJ is cheap and readily accessible and has the potential to be used widely as a dietary supplement if effective in specific patient groups. Its beneficial effects appear to be mediated by inorganic NO3−, without affecting plasma redox status, on acute administration. Further mechanistic work is needed to work out the relative impact of the possible mechanisms, in particular the impact of muscle versus pulmonary or cardiac/systemic circulation effects, and longer-term studies will be needed to establish if the effects on exercise performance and endothelial function observed here translate into clinically meaningful benefits.

**Acknowledgements**

The authors would like to thank all the participants who took part in this study.

**Contributors**

NSH and MJP developed the original idea for the research study, NSH and MJP designed and wrote the study protocol. WASB designed the statistical analysis.
analysis plan. MJP, AL and SCB undertook patient visits and collected trial data. MF, BOF and MM-L undertook plasma analysis. MJP analysed the data and wrote the first draft of the manuscript. All authors edited and contributed to the final manuscript. NSH is the guarantor.

Funding
The study was funded by a grant from Moulton Charitable Foundation.

Competing interests
None declared.

Patient consent for publication
Not required.

Ethics approval
The study was approved by the London - Chelsea Research and Ethics Committee (ref. 15/L01/0975) and conducted in line with the principles of the Declaration of Helsinki.

Provenance and peer review
Not commissioned; externally peer reviewed.

Data availability statement
Data are available upon reasonable request. Individual participant data that underlie the results in the article after de-identification (text, tables, figures and appendices) will be made available from the corresponding author upon request. The study protocol and statistical analysis plan will also be available. Data will be available indefinitely.

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REFERENCES
SUPPLEMENTARY APPENDIX

Dietary nitrate supplementation to improve exercise capacity in hypoxic COPD

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Figure E1. Study flow diagram

Abbreviations: NO₃⁻ – Nitrate; BP – Blood Pressure; FMD – Flow Mediated Dilatation; NO₂⁻ – Nitrite; F₂NO – Fractional Exhaled Nitric Oxide; E SWT – Endurance Shuttle Walk Test; BRJ – Beetroot Juice; PL – Placebo; GOLD – Global Initiative for Chronic Obstructive Lung Disease; COPD – Chronic Obstructive Pulmonary Disease; eGFR – Estimated Glomerular Filtration Rate; sBP – Systolic Blood Pressure; IS WT – Incremental Shuttle Walk Test; HR – Heart Rate; SaO₂ – Oxygen Saturations

Pre Intervention Visit Guidance
- Avoid strenuous exercise in the 24 h before testing
- Avoid a cooked breakfast & eat the same meal on the day of testing
- Match caffeine consumption
- Avoid the use of mouthwash or chewing gum
- Intervention visits occur at the same time of day (+/- 2 h)

Intervention Visit 1 (within 7 days of baseline visit)
- Pre-dosing (0 minutes):
  - Resting BP
  - FMD
  - Plasma NO₃⁻/NO₂⁻ sampling
  - F₂NO
- Post-dosing (180 minutes)
  - Resting BP
  - FMD
  - Plasma NO₃⁻/NO₂⁻ sampling
  - F₂NO (every 30 minutes post dosing)
  - E SWT

Intervention Visit 2
- Pre-dosing (0 minutes):
  - Resting BP
  - FMD
  - Plasma NO₃⁻/NO₂⁻ sampling
  - F₂NO
- Post-dosing (180 minutes)
  - Resting BP
  - FMD
  - Plasma NO₃⁻/NO₂⁻ sampling
  - F₂NO (every 30 minutes post dosing)
  - E SWT

Randomisation (1:1)

Baseline Visit
- Baseline ISWT x2, practice E SWT
- Baseline FMD and F₂NO
- Full pulmonary function tests (if not performed within 3 months)
- Resting BP, HR, SaO₂

Screening Visit
Inclusion Criteria:
- GOLD II+ COPD
- Prescribed supplemental oxygen therapy

Exclusion Criteria:
- < 1 month following an exacerbation
- Significant comorbidity limiting exercise
- eGFR < 45 mL/min-1
- sBP < 100 mm Hg
- Pregnancy
- Use of nitrate base medications
- Other benefit from NO₃⁻ supplementation

Crossover
Minimum 7 days and maximum 1 month

140 mL PL-BRJ (NO₃⁻; 0.002 mmol)
140 mL PL-BRJ (NO₃⁻; 12.9 mmol)
140 mL NR-BRJ (NO₃⁻; 12.9 mmol)
140 mL NR-BRJ (NO₃⁻; 0.002 mmol)
SUPPLEMENTARY METHODS

Plasma nitrate/nitrite levels - additional methods

Plasma NO$_3^-$ and NO$_2^-$ levels were used as a combined biomarker of NO$_3^-$ ingestion, metabolism and nitric oxide availability [1, 2]. Plasma samples were obtained on arrival and three hours after consumption of either NR-BRJ or PL-BRJ. Samples were obtained by venesection of 6 mL of venous blood into lithium heparin tubes. Within five minutes of collection the vials were split into 3 mL aliquots, with one mixed with 300 µL of 100 mM stock of N-ethylmaleimide (NEM) solution (final concentration 10 mM). The samples were then centrifuged at 1,000 g for eight minutes at room temperature. Subsequently, 1 mL of the supernatant was aliquoted into 2 mL polypropylene cryotubes, snap frozen with liquid nitrogen and stored at -80°C. Plasma nitrate and nitrite concentrations were measured following protein precipitation with methanol (1:1 v/v) by a dedicated high-performance liquid chromatography (HPLC) system equipped with an anion-exchange column, an in-line Cd/Cu reduction column and a post-column diazo coupling reactor coil (Griess reaction) (Eicom NOx analyser, ENO-20, San Diego, USA) [3].

Oxidative stress – additional methods

Ferric-reducing ability of plasma (FRAP)

The FRAP assay is a measure of the antioxidant potential in the extracellular compartment [4]. The same plasma samples used for nitrate/nitrite measurement were also used to process this assay. Briefly, 150 µl of FRAP reagent (containing 300 mM acetate buffer at pH 3.6, 10 mM TPTZ [2,4,6-Tris(2-pyridyl)s-triazine], 20 mM FeCl$_3$ at a ratio of 10:1:1 (v:v:v)) was added to 5 µl of diluted plasma (1:3, v:v) into a 96-well plate containing 15 µl of MQ water in each well. The plate was incubated at 37°C for 30 minutes. The absorbance at 593 nm was taken immediately after incubation using a microplate reader (Spectramax M5, Molecular Devices, California USA). FRAP values for the samples were obtained by comparing the absorbance at 593 nm with the known concentrations in the standards (FeSO$_4$·7H$_2$O).

Thiobarbituric acid-reactive substance (TBARS)

TBARS is a measure of lipid oxidation and is measured using a TBARS assays [5]. The same plasma samples used for nitrate/nitrite measurement were also used to process this assay. In
brief the TBARS assay incorporated the use of an malondialdehyde (MDA) source such as 1,1,3,3 Tetramethoxypropane after hydrolysis as standard, 0.6N trichloroacetic acid as the acid reagent and thiobarbituric acid (0.26g in 50 mL glacial acetic acid) as colour reagent. Prior to analysis, samples were deproteinised by acid precipitation by taking 300 uL samples and adding an equal volume of acid reagent, mixed and incubated for 15 min at room temperature. The supernatant was isolated by 4 min centrifugation at > 12,000 x g. The resulting supernatant was further treated with colour reagent (2:1, v:v), incubated for 1h at 100°C and immediately cooled on ice for 10 min. Treated samples were plated into 96-well microplates and absorbance readings were read at 532 nm using a microplate reader (Spectramax M5, Molecular Devices, California USA). TBARS values for the samples were obtained by comparing the absorbance with known concentrations of MDA standards.

**Total free thiols per protein**

Systemic oxidative stress can be measured as the depletion of the free thiol pool in plasma [6]. The same plasma samples used for nitrate/nitrate measurement were also used to process this assay. Thiol groups were measured as previously described [7, 8]. In brief, 75 µl plasma samples were diluted 1:4 (v:v) with a 0.1 M Tris buffer (pH 8.2) and transferred 90 uL of diluted sample to a 96-well microplate. Using a microplate reader (Molecular Devices Spectramax M5, California, USA), background absorption was measured at 412 nm with a reading at 630 nm for baseline correction. Subsequently, 20 µl 1.9 mM 5,5-Dithio-bis(2-nitrobenzoic acid) [DTNB] in 0.1 M phosphate buffer (pH 7) was added to the samples and standards. Following 20 minutes of incubation at room temperature while mixing, absorption was remeasured at 412 and 630 nm. The concentration of total free thiols in the samples was determined by comparing their absorbance reading to that of an L-cysteine standard before and after addition of DTNB to samples/standards.

**Endothelial function - additional methods**

Endothelial function was assessed using flow medicated dilatation (FMD) of the brachial artery [9] using a high-resolution doppler ultrasound (GE Logiq 3, GE Medical Systems, Milwaukee, Wisconsin, USA) and a 10 MHz multi-frequency linear array probe were used in B-mode. Brachial artery diameter was measured at baseline and sequentially after release of circulatory arrest of the upper arm over a period of 120 seconds [10], three hours after NR-BRJ/PL-BRJ
consumption. All measurements were performed by a single trained operator. Circulatory arrest was generated via a rapid cuff inflation system (Hokanson, Bellevue, WA, USA), which was positioned proximal to the brachial artery and rapidly inflated to 250 mmHg for five minutes. Data were saved for off-line analysis using ImageJ2 software [11].
SUPPLEMENTARY RESULTS

Table E1. Exercise oxygen saturations and heart rate analysis

<table>
<thead>
<tr>
<th>Measure</th>
<th>PL-BRJ (n=18)</th>
<th>NR-BRJ (n=18)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saturations (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rest</td>
<td>96 (90, 97)</td>
<td>96 (92, 97)</td>
</tr>
<tr>
<td>Warm-up</td>
<td>91 (89, 95)</td>
<td>94 (90, 95)</td>
</tr>
<tr>
<td>Isotime</td>
<td>92 (89, 94)</td>
<td>96 (93, 97)</td>
</tr>
<tr>
<td>Peak</td>
<td>88 (86, 92)</td>
<td>94 (91, 96)</td>
</tr>
<tr>
<td>Recovery</td>
<td>97 (92, 98)</td>
<td>98 (96, 98)</td>
</tr>
<tr>
<td>Heart Rate (bpm)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rest</td>
<td>86 (74, 88)</td>
<td>88 (78, 91)</td>
</tr>
<tr>
<td>Warm-up</td>
<td>103 (88, 108)</td>
<td>96 (88, 102)</td>
</tr>
<tr>
<td>Isotime</td>
<td>111 (103, 123)</td>
<td>109 (96, 116)</td>
</tr>
<tr>
<td>Peak</td>
<td>104 (96, 111)</td>
<td>101 (112)</td>
</tr>
<tr>
<td>Recovery</td>
<td>91 (79, 101)</td>
<td>89 (81, 98)</td>
</tr>
</tbody>
</table>

The area under the curve for each treatment group was estimated and reported as mean (SD).

The results for Saturations for when the subjects were on placebo beetroot juice were 1161.85 (47.59) and the results for when the subjects on Nitrate-rich beetroot juice were 1205.54 (46.39). The treatment effect was estimated to be 43.69 (29.09 to 58.28) p < 0.0001. The results suggest that on the average the area under the curve for saturations was higher when on Nitrate-rich beetroot juice than when on placebo. These differences tended to show more during the Isotime and peak periods.

The mean (SD) area under the curve for the HR data when the subjects were on placebo beetroot juice was 1299.93 (186.05) for when the subjects were on Nitrate-rich beetroot juice results was 1258.76 (174.01). The estimated treatment effect was -41.17 (-116.74 to 34.40), p=0.27. The results show that while at individual time points the HR was higher for when the subjects were on Placebo, there was no statistically significant difference in the area under the curve.

Abbreviations; bpm – Beats Per Minute; PL-BRJ – Placebo Beetroot Juice; Nitrate-rich Beetroot Juice
Table E2. Between intervention analysis of plasma nitrite and nitrate

<table>
<thead>
<tr>
<th>Measurement</th>
<th>PL-BRJ (n=19)</th>
<th>NR-BRJ (n=19)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baseline Nitrite (µM)</td>
<td>0.32 (0.25, 0.37)</td>
<td>0.34 (0.22, 0.39)</td>
</tr>
<tr>
<td>Baseline Nitrate (µM)</td>
<td>51.89 (38.98, 62.28)</td>
<td>62.59 (41.68, 77.29)</td>
</tr>
<tr>
<td>180 Minute Nitrite (µM)</td>
<td>0.31 (0.23, 0.47)</td>
<td>0.60 (0.48, 0.67)</td>
</tr>
<tr>
<td>180 Minute Nitrate (µM)</td>
<td>45.31 (31.39, 58.84)</td>
<td>617.71 (508.6, 725.88)</td>
</tr>
<tr>
<td>Difference in Nitrite (µM)</td>
<td>0.023 (-0.044, 0.079)</td>
<td>0.276 (0.144, 0.463)</td>
</tr>
<tr>
<td>Difference in Nitrate (µM)</td>
<td>-4.61 (-9.63, 6.23)</td>
<td>543.25 (441.78, 674.23)</td>
</tr>
</tbody>
</table>

Results are reported as median (IQR).

The treatment effect of Nitrate was estimated with the Hodges-Lehman estimate and it was 550 (461 to 639) µM. The results suggest that the was a higher for when the subjects were on Nitrate-rich beetroot juice than when they were on placebo and this change was statistically significant, p=0.0003.

The treatment effect of Nitrite was estimated with the Hodges-Lehman estimate and it was 0.248 (0.138 to 0.408) µM. The results suggest that the was a higher for when the subjects were on Nitrate-rich beetroot juice than when they were on placebo and this change was statistically significant, p=0.0011.

Abbreviations: PL BRJ – Placebo Beetroot Juice; NR-BRJ – Nitrate-rich Beetroot Juice
<table>
<thead>
<tr>
<th>Samples</th>
<th>Nitrite Mean (µM)</th>
<th>Nitrite SD</th>
<th>Nitrite SEM</th>
<th>Nitrite %CV</th>
<th>Nitrate Mean (µM)</th>
<th>Nitrate SD</th>
<th>Nitrate SEM</th>
<th>Nitrate %CV</th>
</tr>
</thead>
<tbody>
<tr>
<td>NEM Stock Solution</td>
<td>0.07</td>
<td>0.03</td>
<td>0.02</td>
<td>45.41</td>
<td>22.37</td>
<td>2.33</td>
<td>1.34</td>
<td>10.40</td>
</tr>
<tr>
<td>Cryotube</td>
<td>0.09</td>
<td>0.02</td>
<td>0.01</td>
<td>17.15</td>
<td>2.44</td>
<td>0.53</td>
<td>0.31</td>
<td>22.33</td>
</tr>
<tr>
<td>PL-BRJ</td>
<td>195.86</td>
<td>2.12</td>
<td>1.22</td>
<td>1.08</td>
<td>55.05</td>
<td>0.68</td>
<td>0.39</td>
<td>1.24</td>
</tr>
<tr>
<td>NR-BRJ</td>
<td>10.75</td>
<td>0.20</td>
<td>0.11</td>
<td>1.83</td>
<td>12041.03</td>
<td>5267.10</td>
<td>3040.96</td>
<td>4.37</td>
</tr>
</tbody>
</table>

Concentration of NO₂⁻ and NO₃⁻ in NEM Stock Solution, Cryotubes, Cryotubes with 0.9% Sodium Chloride, PL-BRJ and NR-BRJ.

*Abbreviations: NEM – N-Ethylmaleimide; SD – Standard Deviation; SEM – Standard Error Mean; %CV – Percentage Coefficient of Variation; PL-BRJ – Placebo Beetroot Juice; NR-BRJ – Nitrate Rich Beetroot Juice; µM – micromole.*
Table E4. Fractional Exhaled Nitric Oxide (FeNO)

<table>
<thead>
<tr>
<th></th>
<th>FeNO post NR-BRJ (ppb)</th>
<th>FeNo post PL-BRJ (ppb)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baseline</td>
<td>18.5 (15.0, 21.5)</td>
<td>18.0 (14.0, 22.5)</td>
</tr>
<tr>
<td>0 Minutes</td>
<td>19.5 (16.0, 22.5)</td>
<td>19.0 (15.0, 22.5)</td>
</tr>
<tr>
<td>30 Minutes</td>
<td>44.5 (27.0, 63.0)</td>
<td>21.0 (17.0, 25.5)</td>
</tr>
<tr>
<td>60 Minutes</td>
<td>49.5 (33.5, 78.5)</td>
<td>21.5 (17.0, 29.0)</td>
</tr>
<tr>
<td>90 Minutes</td>
<td>54.0 (26.5, 90.0)</td>
<td>19.0 (15.0, 27.5)</td>
</tr>
<tr>
<td>120 Minutes</td>
<td>49.0 (32.5, 56.0)</td>
<td>20.5 (16.0, 24.5)</td>
</tr>
<tr>
<td>150 Minutes</td>
<td>59.0 (33.5, 84.0)</td>
<td>20.0 (17.0, 32.5)</td>
</tr>
<tr>
<td>100 Minutes</td>
<td>55.0 (35.0, 76.5)</td>
<td>21.5 (15.0, 27.5)</td>
</tr>
</tbody>
</table>

FeNO levels measured at baseline (visit 1) and subsequently at intervention visits (visits 3 and 4) at time point zero minutes prior to dosing with either PL-BRJ or NR-BRJ and subsequently every 30 minutes until 180 minutes post dosing. Data presented: median (IQR).

The AUC was calculated for each treatment group and compared to estimate the treatment effect using the Hodges-Lehman estimate. The median (IQR) AUC for when the subjects were on placebo was 3622.5 (3181.9, 4796.9) and the corresponding results for when the subjects were on Nitrate-rich beetroot juice was 9440.6 (6273.8, 11831.3) and the treatment effect with its 95% CI was 5407 (3096 to 7576), p=0.0011. The results suggest that the FeNO levels while the subjects were on Nitrate-rich beetroot juice were significantly higher than when they were on placebo.

Abbreviations: FeNO – Fractional Exhaled nitric Oxide; IQR – Interquartile Range; AUC – area under the curve; ppb – Parts Per Billion
Figure E2. Primary Outcome Order Effect

Change in ESWT time (seconds) when testing for intervention order effect, if PL-BRJ was applied first or NR-BRJ. Data presented median (line) and interquartile range (whiskers) with as individual data points (dots). Mann-Whitney U test, the median (IQR) change in ESWT time if PL-BRJ was applied first was 60.0 (21.8, 88.4) seconds, compared to 43.1 (14.03, 155.3) seconds, if NR-BRJ was applied first; p = 0.82.

Abbreviations: ESWT – Endurance Shuttle Walk Test; PL-BRJ – Placebo Beetroot Juice; NR-BRJ – Nitrate Rich Beetroot Juice
Results E1. Effect of dietary nitrate supplementation on endurance shuttle walk time

There is a clear outlier in this dataset. When this individual’s data was removed from analysis, all individuals still walked further following consumption the NO$_3^-$-rich BRJ. There was a statistically significant difference between the median (IQR) ESWT time with the outlier removed; NO$_3^-$-rich BRJ 193.8 (145.5, 389.6) seconds vs PL 158.2 (121.6, 236.6) seconds; $p = 0.0001$. Regarding this specific individual at baseline assessment their best ISWT distance was 370 meters, using the ESWT conversion table the ESWT speed was calculated as 4.65 km/h which equates to ESWT level 11. All individuals undertook a practice ESWT, this individual’s practice ESWT time was 599 seconds. The ESWT time that this individual achieved following consumption of the placebo beverage was 785 seconds. For both ESWT this individual reported peak Borg Dyspnoea scale of 8. This individual’s data was included in the full analysis as it is felt to be a true representation of this individuals exercise endurance.
Figure E3. Measures of oxidative stress

Panel A

FRAP (uM)

0 minutes (PL-BRJ)
0 minutes (NR-BRJ)
180 minutes (PL-BRJ)
180 minutes (NR-BRJ)

Panel B

TBARS (mM)

0 minutes (PL-BRJ)
0 minutes (NR-BRJ)
180 minutes (PL-BRJ)
180 minutes (NR-BRJ)

Panel C

TFF/Protein (umol/g protein)

0 minutes (PL-BRJ)
0 minutes (NR-BRJ)
180 minutes (PL-BRJ)
180 minutes (NR-BRJ)
Measures of oxidative stress for PL-BRJ and NR-BRJ Dosing conditions. Data presented as median and interquartile range (box) with whiskers representing minimum to maximum values. Plasma samples were measured at baseline (zero minutes) and 180 minutes after dosing. Wilcoxon sign-rank test was used to compare change in measures of oxidative stress between intervention groups. Mann-Whitney U test was used to compare change in measures of oxidative stress between treatment conditions.

Panel A. Ferric reducing ability of plasma (FRAP)

There was no statistically significant difference between interventions for baseline and post intervention FRAP. Baseline FRAP PL-BRJ: 1028 (762.9, 1195) µM vs FRAP NR-BRJ: 927.7 (790.2, 1064) µM; p = 0.7. Post intervention FRAP PL-BRJ: 930.2 (836.8, 1073) µM vs FRAP NR-BRJ: 1018 (853.0, 1125) µM; p = 1.0. (Wilcoxon sign-rank test)

There was no statistically significant difference between baseline FRAP levels and post dosing levels with either PL-BRJ and NR-BRJ. FRAP PL-BRJ: 1028 (762.9, 1195) µM vs post dosing 930.2 (836.8, 1073) µM; p = 0.9. FRAP NR-BRJ: 927.7 (790.2, 1064) µM vs post dosing 1018 (853.0, 1125) µM; p = 0.3. (Mann-Whitney U test).

Panel B. Thiobarbituric acid-reactive substance (TBARS)

There was no statistically significant difference between interventions for baseline and post intervention TBARS. Baseline TBARS PL-BRJ: 1.443 (1.102, 2.940) mM vs TBARS NR-BRJ: 1.450 (1.007, 2.613) mM; p = 0.8. Post intervention TBARS PL-BRJ: 0.971 (0.766, 1.614) mM vs TBARS NR-BRJ: 1.499 (0.855, 3.209) mM; p = 0.4. (Wilcoxon sign-rank test)

There was no statistically significant difference between baseline TBARS levels and post dosing levels with either PL-BRJ and NR-BRJ. TBARS PL-BRJ: 1.443 (1.102, 2.940) mM vs post dosing 0.971 (0.766, 1.614) mM; p = 0.8. TBARS NR-BRJ: 1.450 (1.007, 2.613) mM vs post dosing 1.499 (0.855, 3.209) mM; p = 0.3. (Mann-Whitney U test).
Panel C. Total free thiols (TFT) per protein

There was no statistically significant difference between interventions for baseline and post intervention TFT per protein. Baseline TFT per protein PL-BRJ: 6.754 (6.328, 8.342) µmol.g⁻¹ protein vs TFT per protein NR-BRJ: 7.284 (6.508, 7.960) µmol.g⁻¹ protein; p = 0.9. Post intervention TFT per protein PL-BRJ: 6.942 (5.768, 8.026) µmol.g⁻¹ protein vs TFT per protein NR-BRJ: 7.079 (5.961, 8.115) µmol.g⁻¹ protein; p = 0.5. (Wilcoxon sign-rank test)

There was no statistically significant difference between baseline TFT per protein levels and post dosing levels with either PL-BRJ and NR-BRJ. TFT per protein PL-BRJ: 6.754 (6.328, 8.342) µmol.g⁻¹ protein vs post dosing 6.942 (5.768, 8.026) µmol.g⁻¹ protein; p = 0.1. TFT per protein NR-BRJ: 7.284 (6.508, 7.960) µmol.g⁻¹ vs post dosing 7.079 (5.961, 8.115) µmol.g⁻¹ protein; p = 0.4. (Mann-Whitney U test).

Abbreviations: FRAP - Ferric Reducing Ability of Plasma; TBARS - Thiobarbituric Acid-Reactive Substance; TFT – Total Free Thiols; PL-BRJ – Placebo Beetroot Juice; NR-BRJ – Nitrate-rich Beetroot Juice; mM - millimole
REFERENCES


