Beetroot juice and exercise: pharmacodynamic and dose-response relationships

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Abstract

Dietary supplementation with beetroot juice (BR) containing ~5-8 mmol of inorganic nitrate (NO_3^-) increases plasma nitrite concentration ([NO_2^-]), reduces blood pressure, and may positively influence the physiological responses to exercise. However, the dose-response relationship between the volume of BR ingested and the physiological effects invoked has not been investigated. In a balanced crossover design, 10 healthy males ingested 70, 140 or 280 ml of concentrated BR (containing 4.2, 8.4 and 16.8 mmol NO_3^-, respectively) or no supplement to establish the effects of BR on resting plasma [NO_3^-] and [NO_2^-] over 24 h. Subsequently, on six separate occasions, 10 subjects completed moderate-intensity and severe-intensity cycle exercise tests 2.5 h post-ingestion of 70, 140 and 280 ml BR, or NO_3^--depleted BR as placebo (PL). Following acute BR ingestion, plasma [NO_2^-] increased in a dose-dependent manner, with the peak changes occurring at ~2-3 h. Compared to PL, 70 ml BR did not alter the physiological responses to exercise. However, 140 and 280 ml BR reduced the steady-state $\dot{V}_{O2}$ during moderate-intensity exercise by 1.7% (P=0.06) and 3.0% (P<0.05), whilst time to task failure was extended by 14% and 12% (both P<0.05), respectively, compared to PL. The results indicate that, while plasma [NO_2^-] and the O_2 cost of moderate-intensity exercise are altered dose-dependently with NO_3^--rich BR, there is no additional improvement in exercise tolerance after ingesting BR containing 16.8 compared to 8.4 mmol NO_3^-. These findings have important implications for the use of BR to enhance cardiovascular health and exercise performance in young adults.

Key words: nitrate; nitrite; nitric oxide; blood pressure; exercise economy; O_2 uptake, exercise tolerance

Introduction

Nitric oxide (NO) is a gaseous signalling molecule which modulates human physiological function via its role in, for example, the regulation of blood flow, neurotransmission, immune function, glucose and calcium homeostasis, muscle contractility and mitochondrial respiration (9, 36). NO is generated through the oxidation of the amino acid, L-arginine, in a reaction catalysed by nitric oxide synthase (NOS) with nitrite (NO_2^-) and nitrate (NO_3^-) being products of this reaction (30). It is now appreciated that, under appropriate physiological conditions, NO can also be produced via the reduction of NO_2^-, a process which may be particularly important in situations where O_2 availability is low and/or NOS function is impaired (12). Interestingly, administration of dietary inorganic nitrate (NO_3^-) has been shown to increase plasma NO_2^- concentration ($[NO_2^-]$) and to produce NO-like bioactivity (19, 23, 39). Up to 25% of ingested NO_3^- enters the entero-salivary circulation and is concentrated in the saliva whereupon facultative anaerobic bacteria in the oral cavity reduce the NO_3^- to NO_2^- (30). When swallowed into the acidic environment of the stomach, some of the NO_2^- is further converted into NO, whilst the remainder is absorbed to increase circulating plasma $[NO_2^-]$. This NO_2^- may be reduced further to NO and other reactive nitrogen intermediates, particularly in tissues which may be relatively hypoxic, such as contracting skeletal muscle (30).

We and others have demonstrated that NO_3^- ingestion, either in the form of nitrate salts or via the consumption of high-nitrate vegetable products such as beetroot juice, profoundly and consistently reduces resting blood pressure (BP), (3, 19, 23, 37, 39). Consequently, dietary nitrate supplementation has emerged as a potential nutritional agent for the prevention and treatment of hypertension and cardiovascular disease (30). Webb et al. (39) assessed the effects of acute beetroot juice (BR) consumption (~23 mmol NO_3^-) on plasma $[NO_2^-]$ and BP over 24 h. Plasma $[NO_2^-]$ peaked 3 h post-ingestion, remained close to peak values until 5 h post-ingestion, and returned to baseline after 24 h (39). The systolic and diastolic BP and the mean arterial pressure (MAP) were significantly reduced by approximately 10, 8 and 8 mmHg, respectively, at 2.5 to 3 h after BR intake. The same research group later reported a dose-dependent increase in plasma $[NO_3^-]$ and $[NO_2^-]$, and reduction in BP following ingestion of

potassium nitrate (KNO_3) (19). In this study, plasma [NO_2^-] rose by approximately 1.3-, 2.0- and 4.0-fold following consumption of 4, 12 and 24 mmol of KNO_3, respectively. The peak rise in plasma [NO_2^-] was accompanied by significant reductions in both systolic BP (of approximately 2, 6 and 9 mmHg, respectively) and diastolic BP (of approximately 4, 4 and 6 mmHg, respectively). However, since BR contains polyphenols and antioxidants, which can facilitate the synthesis of NO from NO_2^- in the stomach (30), it is unclear whether BP is similarly impacted when different doses of BR are ingested compared to equivalent doses of nitrate salts. Given the growing interest in dietary nitrate supplementation in the form of BR amongst athletes and the general population, it is important to determine the pharmacokinetic-pharmacodynamic relationship between different volumes of BR consumption and changes in plasma [NO_2^-] and BP, in order to establish an optimal dose for beneficial effects.

Recent investigations suggest that dietary NO_3^- supplementation has the potential to influence human physiology beyond the above hemodynamic effects (3, 26). Specifically, we and others have demonstrated that 3-6 days of dietary NO_3^- supplementation reduces the O_2 cost of moderate-intensity exercise and may enhance exercise tolerance in healthy young adults (2, 3, 22, 25, 26). It appears that these effects are related to NO_2^- or NO-mediated enhancements of muscle contractile function (2, 17) and/or mitochondrial efficiency (24), and/or enhanced muscle blood flow especially to type II fibres (14). Importantly, a reduction of the O_2 cost of exercise (25, 37) and improved exercise performance (21) has also been reported as early as 2.5 h following a single dose of dietary NO_3^-, which is consistent with the time required for the peak plasma [NO_2^-] to be attained (39). However, since all exercise performance studies completed to date with BR have administered approximately 5-8 mmol of NO_3^-, it is unclear whether a dose-response relationship exists between acute NO_3^- intake and the physiological responses to exercise. Establishing the dose-response relationship between NO_3^- intake and the physiological responses to exercise, and ascertaining the optimal NO_3^- dose for enhancing exercise performance, is important given the increasing popularity of BR supplementation in both basic research and applied exercise settings.

Therefore, the purpose of the present study was twofold: firstly, to characterize the plasma [NO_3^-] and [NO_2^-] pharmacokinetics and the changes in BP after ingestion of three different quantities of NO_3^- -rich BR; and secondly, to investigate the dose-response relationship between BR/NO_3^- intake and the physiological responses to exercise. In two separate experiments, we administered a BR concentrate which enabled a substantial NO_3^- load to be ingested quickly and easily. We investigated: 1) the influence of acute NO_3^- doses of 4.2, 8.4 and 16.8 mmol consumed in 70, 140 and 280 ml concentrated BR on plasma [NO_3^-] and [NO_2^-] and BP over a 24 h period; and 2) the physiological responses to step transitions to moderate- and severe-intensity exercise 2.5 h post-ingestion of the same NO_3^- doses. We hypothesized that the effects of dietary inorganic NO_3^- on plasma [NO_3^-] and [NO_2^-], BP, the O_2 cost of moderate-intensity exercise and exercise tolerance (assessed as the time to task failure) during severe-intensity exercise would be dose-dependent.

METHODS

The study was conducted in two phases (Study 1 (S_1) Pharmacokinetics and Study 2 (S_2) Dose-Response) with the results generated in S_1 being used to inform the experimental design in S_2. There was distinct subject recruitment for each experiment. Ten healthy, recreationally active men volunteered for each experiment (mean ± SD: S_1; age 23 ± 5 yr, height 1.79 ± 0.07 m, body mass 79 ± 9 kg; S_2 ; age 22 ± 5 yr, height 1.77 ± 0.05 m, body mass 74 ± 8 kg). None of the subjects in S_1 and S_2 was a tobacco smoker or user of dietary supplements. All subjects recruited for S_2 were fully familiar with laboratory exercise testing procedures, having previously participated in studies employing cycle ergometry in our laboratory. The procedures employed in S_1 and S_2 were granted full ethics approval by the Institutional Research Ethics Committee. All subjects gave their written informed consent to participate after the experimental procedures, associated risks, and potential benefits of participation had been explained in detail.

All subjects in S_1 and S_2 were instructed to keep a food and physical activity diary in the 24 h preceding their first laboratory visit and to replicate food consumption and physical activity in the 24 h preceding subsequent visits. The subjects were required to arrive at the laboratory in a rested and fully hydrated state, following an overnight

fast, and to avoid strenuous activity in the 24 h preceding each testing session. Subjects were instructed to refrain from caffeine and alcohol-containing drinks for 6 and 24 h prior to each laboratory visit, respectively, and to abstain from using antibacterial mouthwash and chewing gum throughout the study because these are known to eradicate the oral bacteria that are necessary for the conversion of nitrate to nitrite (16).

S_1: Pharmacokinetics and Pharmacodynamics

Procedures

All subjects reported to the laboratory on four separate occasions over a period of three weeks. Upon arrival to the laboratory, resting BP was measured, and a venous blood sample was obtained for the measurement of plasma [NO_2^-] and [NO_3^-]. Subjects then consumed an acute dose of either 70, 140 or 280 ml of concentrated NO_3^--rich BR (organic beetroot juice containing ~4.2, ~8.4 or ~16.8 mmol of NO_3^-, respectively, Beet It; James White Drinks, Ipswich, UK) or 140 ml water (control; CON) in addition to a standardized breakfast (72 g porridge oats with 180 ml of semi-skimmed milk). BP was measured and a venous blood sample was obtained 1, 2, 4, 8, 12 and 24 h post ingestion. For each 24 h period of data collection subjects were provided with a standardised low nitrate diet. The quantity and timing of food and drink intake was recorded on visit 1, and replicated in subsequent visits. A washout period of at least 3 days separated the laboratory visits.

Measurements

The BP of the brachial artery was measured using an automated sphygmomanometer (Dinamap Pro: GE Medical Systems, Tampa, FL) with the subjects in a seated position. After arrival at the laboratory and following 10 min of rest in an isolated room, four measurements were recorded, and the mean of the final three measurements was used for data analysis.

Venous blood samples were drawn into lithium-heparin tubes (7.5 ml Monovette lithium heparin; Sarstedt, Leicester, UK). Samples were centrifuged at 4,000 rpm and

4 °C for 7 min, within 1 min of collection. Plasma was subsequently extracted and immediately frozen at -80 °C for later analysis of [NO_2^-] and [NO_3^-].

All glassware, utensils, and surfaces were rinsed with deionized water to remove residual [NO_2^-] and [NO_3^-] prior to blood analyses. The [NO_2^-] of the undiluted (non-deproteinized) plasma was determined by its reduction to NO in the presence of glacial acetic acid and 4% (w/v) aqueous NaI. The spectral emission of electronically excited nitrogen dioxide product, from the NO reaction with ozone, was detected by a thermoelectrically cooled, red-sensitive photomultiplier tube housed in a Sievers gas-phase chemiluminescence NO analyzer (Sievers NOA 280i. Analytix Ltd, Durham, UK). The [NO_2^-] was determined by plotting signal (mV) area against a calibration plot of 100 nM to 1 μM sodium nitrite. Prior to determination of [NO_3^-], samples were deproteinized using zinc sulfate/sodium hydroxide precipitation. 400 μL of 10% (w/v) aqueous $ZnSO_4$ and 400 μL of 0.5 M NaOH were added to 200 uL of sample and vortexed for 30 s before being left to stand at room temperature for 15 min. Thereafter, samples were centrifuged at 4000 rpm for 5 min, and the supernatant was removed for subsequent analysis. The [NO_3^-] of the deproteinized plasma sample was determined by its reduction to NO in the presence of 0.8% (w/v) VCl_3 in 1 M HCl. The production of NO was detected using the chemiluminescence NO analyzer, as described above.

To more precisely determine the time-to-peak plasma [NO_2^-] following NO_3^- ingestion, a one-compartment model with first-order absorption and elimination kinetics was used, as described in the following equation:

$$Y = (\exp(-Ke*X/(Ke/Ka)-\exp(-Ke*X))/(Ke/Ka-1)$$

where Y represents fraction absorbed; X represents time; and, Ka and Ke represent the first order absorption and elimination rate constants, respectively.

Statistical Analysis

Two-way repeated-measures ANOVA was used to assess the difference across conditions (4.2, 8.4, 16.8 mmol NO_3^- and control) and across time (0, 1, 2, 4, 8, 12

and 24 h) for plasma [NO_2^-] and [NO_3^-], and BP. Significant main or interaction effects were further analysed using simple contrasts. One-way repeated measures ANOVA was used to assess the differences in time-to-peak plasma [NO_2^-]. Relationships between plasma [NO_2^-] and BP were analysed using Pearson product moment correlation coefficients. Statistical significance was accepted at $P<0.05$. Results are presented as mean ± SD unless stated otherwise.

S_2: Dose-Response

Protocol

Subjects were required to report to the laboratory on seven separate occasions, over a 4- to 5-wk period. During the first visit to the laboratory, subjects completed a ramp incremental exercise test for determination of the peak $\dot{V}o_2$ ($\dot{V}o_{2peak}$) and gas-exchange-threshold (GET). All tests were performed on an electronically braked cycle ergometer (Lode Excalibur Sport, Groningen, The Netherlands). Initially, each subject completed 3 min of "unloaded" baseline cycling; then the work rate was increased by 30 W/min until the subject was unable to continue. The subjects cycled at a self-selected pedal rate (70-90 rpm), and this pedal rate, along with the saddle and handlebar height and configuration, was recorded and reproduced in subsequent tests. The breath-by-breath pulmonary gas exchange data were collected continuously during the incremental tests and averaged over consecutive 10-s periods. $\dot{V}o_{2peak}$ was taken as the highest 30-s mean value attained prior to the subject's volitional exhaustion. The GET was determined as described previously (3, 37). The work rates that would require 80% of the GET (moderate-intensity exercise) and 75% Δ (75% of the difference between the power output at GET and $\dot{V}o_{2peak}$ plus the power output at GET; i.e., severe-intensity exercise) were subsequently calculated.

On test days, subjects arrived at the laboratory at ~8 a.m. A venous blood sample was drawn for measurement of plasma [NO_2^-] and [NO_3^-]. Subjects then ingested either 70, 140 or 280 ml of concentrated NO_3^--rich BR (containing 4.2, 8.4 or 16.8 mmol of NO_3^-, respectively) (Beet It, James White Drinks, Ipswich, UK) or 70, 140 or 280 ml of concentrated NO_3^--depleted beetroot juice as a placebo (PL70, PL140, PL280; containing ~0.04. ~0.08 or ~0.12 mmol of NO_3^-) (Beet it, James White Drinks Ltd.,

Ipswich, UK). All BR and PL doses were administered using a randomized, double blind, crossover design. Subjects were asked to consume the beverage within a 5 min period and after doing so were served a standardized breakfast (72 g porridge with 180 ml of semi-skimmed milk). A washout period of at least 72 h separated each visit.

After ingestion of the beverage, subjects were given a period of 2.5 h during which they were allowed to leave the laboratory but were asked to refrain from strenuous physical activity. Subjects were also asked to fast during this time, although water was permitted *ad libitum.* Following this 2.5 h period, a second venous blood sample was drawn for measurement of plasma [NO_2^-] and [NO_3^-]. Subjects then completed "step" exercise tests from a 20-W baseline to moderate-intensity (93 ± 11 W) and severe-intensity (258 ± 23 W) work rates for the determination of pulmonary $\dot{V}o_2$ dynamics. On each visit, subjects completed two 5-min bouts of moderate-intensity exercise and one bout of severe-intensity exercise that was continued until task failure as a measure of exercise tolerance. All bouts of exercise on each day were separated by 5-min of passive rest. The time to task failure was recorded when the pedal rate fell by > 10 rpm below the self-selected pedal rate. In the severe-intensity bouts, the subjects were verbally encouraged to continue for as long as possible.

Measurements

During all exercise tests, pulmonary gas exchange and ventilation were measured breath-by-breath with subjects wearing a nose clip and breathing through a low dead space (90 ml), low-resistance (0.75 mmHg.1^{-1}.s^{-1} at 15 l.s^{-1}) mouthpiece and impeller turbine assembly (Jaeger Triple V, Jaeger GmbH, Hoechberg, Germany). The inspired and expired gas volume and gas concentration signals were continuously sampled at 100 Hz, the latter using paramagnetic (O_2) and infrared (CO_2) analyzers (Oxycon Pro; Jaeger GmbH, Hoechberg, Germany) via a capillary line connected to the mouthpiece. These analyzers were calibrated before each test with gases of known concentration, and the turbine volume transducer was calibrated using a 3-litre syringe (Hans Rudolph, Kansas City, MO). The volume and concentration signals were time aligned by accounting for the delay in capillary gas transit and analyzer rise time relative to volume signal. Oxygen uptake, carbon dioxide output, and minute ventilation were calculated using standard formulae and displayed breath-by-breath. Heart rate (HR)

was measured using short-range radiotelemetry (model RS-400 Polar Electro Oy, Kempele, Finland).

Capillary blood samples were collected from the fingertip into a capillary tube during the baseline preceding each step transition in work rate, during the final 30 s of each moderate-intensity exercise bout, and following exhaustion in the severe-intensity exercise bout. These samples were analyzed immediately to determine blood [lactate] (model YSI 1500; Yellow Springs Instruments, Yellow Springs, OH). Venous blood samples were treated and analyzed as described in S_1.

The breath-by-breath data from each exercise test were linearly interpolated to provide second-by-second values, and the two identical moderate-intensity repetitions performed on each visit were time aligned to the start of exercise and ensemble averaged. $\dot{V}o_{2baseline}$, $\dot{V}co_{2baseline}$ and respiratory exchange ratio (RER) at baseline were defined as the mean values measured over the final 90 s of baseline pedaling. The end-exercise $\dot{V}o_2$, $\dot{V}co_2$ and RER were defined as the mean values measured over the final 30 s of exercise. The amplitude of the $\dot{V}o_2$ response was calculated by subtracting $\dot{V}o_{2baseline}$ from $\dot{V}o_2$ at the end of exercise. Subsequently, the functional gain of the entire response was calculated by dividing the $\dot{V}o_2$ amplitude by the change (Δ) in work rate. The amplitude of the $\dot{V}o_2$ slow component during the severe-intensity exercise bout was estimated by subtracting the mean $\dot{V}o_2$ at 3-min from the mean $\dot{V}o_2$ at 6-min.

Statistical Analysis

Two-way repeated-measures ANOVA was used to assess the difference in pulmonary gas-exchange variables, blood [lactate] and HR across dose (70, 140 and 280 ml) and treatment (PL and BR). Differences in pre and post plasma [NO_2^-] and [NO_3^-] were assessed separately in PL and BR, across dose and time (pre and post) using two-way repeated-measures ANOVAs. Significant main and interaction effects were further analysed using simple contrasts. Statistical significance was accepted at $P<0.05$. Results are presented as mean ± SD unless stated otherwise.

RESULTS

Ingestion of BR was tolerated well by all subjects in S_1 and S_2. Subjects did, however, report beeturia (red urine) and red stools, consistent with previous studies (3,39). The absolute NO_3^- doses employed in S_1 and S_2 (4.2, 8.4 and 16.8 mmol) were equivalent to approximately 0.05 ± 0.01 (range: 0.05-0.07), 0.11 ± 0.01 (range: 0.09-0.13) and 0.22 ± 0.03 mmol (range: 0.19-0.26) NO_3^- per kg BM, respectively.

S_1: Pharmacokinetics and Pharmacodynamics

The effects of different volumes of BR (and therefore different amounts of ingested NO_3^-) on plasma [NO_3^-] and [NO_2^-] are presented in Figure 1. There were significant main effects by dose and time, and an interaction effect, for both plasma [NO_3^-] (Figure 1A; all $P < 0.01$) and plasma [NO_2^-] (Figure 1B; all $P < 0.01$).

At resting baseline, prior to the ingestion of any beverage, plasma [NO_3^-] was not significantly different between doses (Figure 1A; all $P > 0.05$). ANOVA analyses revealed significant dose-dependent increases in plasma [NO_3^-] following BR supplementation ($P < 0.05$). The peak elevation above baseline in plasma [NO_3^-] occurred 1 h post administration of 4.2 (160 ± 43 μM) and 8.4 mmol NO_3^- (269 ± 92 μM), and 2 h post administration of 16.8 mmol NO_3^- (581 ± 209 μM), (Figure 1A; all $P < 0.05$). Plasma [NO_3^-] remained elevated above baseline and CON at all time points after administration of 4.2, 8.4 and 16.8 mmol NO_3^- ($P < 0.05$).

At baseline, prior to ingestion of any beverage, plasma [NO_2^-] was not significantly different between doses (Figure 1B; $P > 0.05$). ANOVA analyses revealed significant dose-dependent increases in plasma [NO_2^-] following BR supplementation ($P < 0.05$). The peak elevation above baseline in plasma [NO_2^-] occurred 2 h post administration of 4.2 (220 ± 104 nM) and 8.4 mmol NO_3^- (374 ± 173 nM), and 4 h post administration of 16.8 mmol NO_3^- (653 ± 356 nM; Figure 1B; all $P < 0.05$). Kinetic analyses revealed that plasma [NO_2^-] peaked significantly later (198 ± 64 min; range: 130-367 min) following ingestion of 16.8 mmol relative to both 8.4 mmol (146 ± 38 min; range: 77 ± 213 min; $P < 0.05$) and 4.2 mmol BR (106 ± 39 min; range: 63-192

min; $P < 0.05$). Peak plasma [NO_2^-] following ingestion of 8.4 mmol tended to occur later compared to 4.2 mmol ($P = 0.06$). Plasma [NO_2^-] remained elevated above baseline and CON at 1, 2, 4 and 8 h after administration of 4.2, 8.4 and 16.8 mmol NO_3^- (all $P < 0.05$). At 12 h, plasma [NO_2^-] remained elevated above baseline and 4.2 mmol BR following ingestion of 8.4 and 16.8 mmol NO_3^- (all $P < 0.05$). In addition, plasma [NO_2^-] remained elevated at 24 h following administration of 16.8 mmol NO_3^-, compared to all other doses ($P < 0.05$).

The effects of different volumes of BR (and therefore different amounts of ingested NO_3^-) on systolic and diastolic BP and MAP are presented in Figure 2. The changes in systolic BP across all conditions are presented in Figure 2A. There were significant main effects by dose and time, and an interaction effect, on the systolic BP (all $P < 0.05$). Systolic BP at baseline prior to administration of any beverage was lower ($P < 0.05$) in the 16.8 mmol NO_3^- condition (118 ± 5 mmHg) relative to CON (121 ± 5 mmHg) but not relative to 4.2 (119 ± 6 mmHg) and 8.4 mmol NO_3^- (120 ± 6 mmHg). When compared to baseline, systolic BP was significantly lowered following ingestion of 4.2, 8.4 and 16.8 mmol NO_3^- (all $P < 0.05$). The peak reduction in systolic BP occurred 4 h post administration of 4.2 (5 ± 5 mmHg), 8.4 (10 ± 5 mmHg) and 16.8 mmol NO_3^- (9 ± 4 mmHg), respectively, relative to baseline (all $P < 0.05$). Systolic BP was reduced relative to baseline, CON, and 4.2 mmol NO_3^-, at 2, 4 and 8 h post administration of 8.4 mmol and 16.8 mmol NO_3^- (all $P < 0.05$). There were no differences in systolic BP between 8.4 and 16.8 mmol NO_3^- at any time point ($P > 0.05$). At 24 h, systolic BP remained significantly lower (by 5 ± 5 mmHg) than baseline following consumption of 16.8 mmol NO_3^- ($P < 0.05$). In contrast, systolic BP was not significantly different to CON or baseline at 24 h post administration of 4.2 and 8.4 mmol NO_3^- ($P > 0.05$). Overall, the mean systolic BP across 24 h, relative to CON, was lowered dose-dependently by approximately 3, 4 and 6 mmHg after administration of 4.2, 8.4 and 16.8 mmol NO_3^-, respectively (all $P < 0.05$). The change in systolic BP was correlated with the change in plasma [NO_3^-] ($r = -0.27$; $P < 0.05$) and the change in plasma [NO_2^-] ($r = -0.37$; $P < 0.05$). The peak reduction in systolic BP was not correlated with the baseline systolic BP.

The changes in diastolic BP following the ingestion of different doses of NO_3^- -rich BR are presented in Figure 2B. There was a significant interaction effect (dose x time)

on diastolic BP ($P < 0.05$). Diastolic BP at baseline was not significantly different between conditions (CON: 67 ± 5; 4.2 mmol: 68 ± 4; 8.4 mmol: 68 ± 6; 16.8 mmol: 67 ± 6 mmHg; $P > 0.05$). Follow-up tests revealed that ingestion of 8.4 and 16.8, but not 4.2 mmol NO_3^-, significantly reduced diastolic BP relative to baseline and CON (all $P < 0.05$). The peak reduction in diastolic BP from baseline occurred at 4 h post administration of 8.4 mmol NO_3^- (3 ± 3 mmHg) and 2 h post administration of 16.8 mmol NO_3^- (4 ± 4 mmHg; both $P < 0.05$), relative to baseline (both $P > 0.05$), and returned to near baseline values by 24 h ($P > 0.05$). There were no differences in diastolic BP between 8.4 and 16.8 mmol NO_3^- at any time point ($P > 0.05$). The change in diastolic BP was correlated with the change in plasma [NO_3^-] (r = -0.35; $P < 0.05$) and the change in plasma [NO_2^-] (r = -0.39; $P < 0.05$). Moreover, the change in diastolic BP was correlated with the baseline diastolic BP (r = -0.49; $P < 0.05$).

The changes in MAP following the ingestion of different doses of NO_3^--rich BR are presented in Figure 2C. There were significant main effects by dose and time, and an interaction effect on MAP (all $P < 0.05$). At baseline, prior to the ingestion of any beverage, MAP was not significantly different between conditions (CON: 85 ± 4; 4.2 mmol: 85 ± 4; 8.4 mmol: 85 ± 5; 16.8 mmol: 84 ± 5 mmHg; $P > 0.05$). MAP was significantly lower following ingestion of 4.2, 8.4 and 16.8 mmol NO_3^- relative to baseline and CON (all $P < 0.05$). Following ingestion of 4.2 mmol NO_3^- the peak reduction (2 ± 2 mmHg) in MAP occurred at 1 h, and MAP remained reduced by ~2 mmHg at 2 h, relative to baseline ($P < 0.05$). In contrast, the peak reduction in MAP (5 ± 3 mmHg) occurred 4 h post administration of 8.4 and 16.8 mmol NO_3^- relative to baseline ($P < 0.05$). MAP was not different between 8.4 and 16.8 mmol NO_3^- at any time point ($P > 0.05$). Overall, the mean MAP across 24 h, relative to CON, was reduced dose-dependently by approximately 1, 2 and 4 mmHg after administration of 4.2, 8.4 and 16.8 mmol NO_3^-, respectively (all $P < 0.05$). The change in MAP was significantly correlated with the change in plasma [NO_3^-] (r = -0.35; $P < 0.05$) and the change in plasma [NO_2^-] (r = -0.41; $P < 0.05$).

S_2: Dose-Response

Plasma [NO_3^-] and [NO_2^-]

The group mean plasma [NO_3^-] and [NO_2^-] responses in the BR and PL conditions are illustrated in Figure 3A and 3B, respectively. Pre-supplementation plasma [NO_3^-] was not significantly different between conditions ($P > 0.05$) and no significant change in plasma [NO_3^-] was observed following PL supplementation ($P > 0.05$). ANOVA analyses revealed significant dose-dependent increase in plasma [NO_3^-] at 2.5 h following BR supplementation ($P < 0.05$). An elevation in plasma [NO_3^-] above baseline was apparent following 4.2 (130 ± 17 μM; $P < 0.05$), 8.4 (282 ± 54 μM; $P < 0.05$) and 16.8 mmol NO_3^- (580 ± 89 μM; $P < 0.05$). Pre-supplementation plasma [NO_2^-] was not significantly different between conditions ($P > 0.05$) and no significant change in plasma [NO_2^-] was observed following PL supplementation ($P > 0.05$). ANOVA analyses revealed a significant dose-dependent increase in plasma [NO_2^-] at 2.5 h following BR supplementation ($P < 0.05$). Following administration of 4.2, 8.4 and 16.8 mmol NO_3^-, plasma [NO_2^-] was elevated above baseline by 150 ± 73 nM, 291 ± 145 nM and 425 ± 225 nM, respectively (all $P < 0.05$). Plasma [NO_2^-] was significantly greater after ingestion of 16.8 mmol compared to 4.2 mmol NO_3^- ($P < 0.05$), and tended to be greater compared to 8.4 mmol NO_3^- ($P = 0.06$). Plasma [NO_2^-] was significantly greater following ingestion of 8.4 mmol NO_3^- compared to 4.2 mmol NO_3^- ($P < 0.05$)

Moderate-Intensity Exercise

The pulmonary gas exchange and ventilatory responses to moderate-intensity exercise across all doses and conditions are summarised in Table 1. The $\dot{V}o_2$ measured during the period of baseline cycling at 20 W was not affected by dose or condition ($P > 0.05$). However, the absolute end-exercise $\dot{V}o_2$ measured over the final 30 s of moderate- intensity exercise was significantly altered by BR ingestion ($P < 0.05$; Figure 4A). Follow-up tests indicated that end-exercise $\dot{V}o_2$ was significantly lowered by ~3% following administration of 16.8 mmol NO_3^-, relative to the respective placebo (PL280: 1.65 ± 0.19 vs. BR280: 1.60 ± 0.23 $L \cdot min^{-1}$; $P < 0.05$). In addition, there was a trend towards a significant reduction (~2%) in end-exercise $\dot{V}o_2$ following administration of 8.4 mmol NO_3^-, relative to the respective placebo (PL140: 1.67 ± 0.21 vs. BR140: 1.64 ± 0.23 $L \cdot min^{-1}$; $P = 0.06$). The change in plasma [NO_2^-] from baseline to post-ingestion of 4.2, 8.4 and 16.8 mmol NO_3^- was significantly correlated with the change in end-exercise $\dot{V}o_2$ ($r = -0.47$; $P < 0.05$). There was no significant

difference in end-exercise $\dot{V}o_2$ following ingestion of 4.6 mmol NO_3^- (BR70) compared to PL70 ($P > 0.05$).

The amplitude of the $\dot{V}o_2$ response (end-exercise minus baseline $\dot{V}o_2$; Table 1) was significantly affected by dose ($P < 0.05$) and tended to be affected by condition ($P = 0.07$). Follow-up tests revealed that there was a trend towards a significant reduction in the $\dot{V}o_2$ amplitude (by ~6%) after administration of 16.8 mmol NO_3^- compared to 8.4 mmol NO_3^- (BR140: 0.70 ± 0.16 vs. BR280: 0.66 ± 0.16 $L \cdot min^{-1}$: $P = 0.06$). The change in plasma [NO_2^-] from baseline to post-ingestion of 4.2, 8.4 and 16.8 mmol NO_3^-, was significantly correlated with the change in $\dot{V}o_2$ amplitude ($r = -0.38$; $P < 0.05$). There was no significant difference in $\dot{V}o_2$ amplitude between PL and BR at any dose ($P > 0.05$).

The baseline $\dot{V}co_2$ measured over the last 90 s of 20 W pedalling and the end-exercise $\dot{V}co_2$ measured over the last 30 s of exercise were significantly affected by dose ($P < 0.05$ for both) but not condition ($P > 0.05$ for both) (Table 1). Follow-up tests revealed that $\dot{V}co_2$ at baseline was significantly increased as the volume of supplement ingested increased ($P > 0.05$), irrespective of the condition (i.e. PL or BR). Specifically, baseline $\dot{V}co_2$ was significantly increased by ~7% and ~5% following consumption of 280 ml of supplement relative to 70 and 140 ml, respectively ($P < 0.05$ for both). There were no significant differences in $\dot{V}co_2$ between the ingestion of 70 and 140 ml of supplement ($P > 0.05$). Further post-hoc analysis revealed that the end-exercise $\dot{V}co_2$ was significantly higher following ingestion of both 140 and 280 ml of supplement relative to 70 ml ($P < 0.01$ for both). There was, however, no significant difference in end-exercise $\dot{V}co_2$ between ingestion of 140 and 280 ml of supplement ($P > 0.05$).

Baseline and end-exercise RER were significantly affected by dose ($P < 0.05$ for both) but not condition ($P > 0.05$). The follow-up tests indicated that RER increased as the volume of supplement ingested increased ($P < 0.05$; Table 1). Specifically, RER at baseline was significantly increased by ~5% and ~4% following consumption of 280 ml of supplement relative to 70 and 140 ml, respectively ($P < 0.05$ for both). Although there was no significant interaction effect, or main effect by condition, baseline RER tended to be higher (by ~3%) following administration of 16.8 mmol

NO_3^- compared to the respective placebo ($P = 0.08$). End-exercise RER was significantly increased by ~4% and ~3% following consumption of 280 ml compared to 70 and 140 ml of supplement, respectively ($P < 0.05$ for both). In addition, the ingestion of 140 ml significantly increased end-exercise RER compared to ingestion of 70 ml of supplement ($P < 0.05$). The baseline, end-exercise and change in blood [lactate] and HR were not significantly altered by dose or condition (Table 2; $P > 0.05$).

Severe-Intensity Exercise

The pulmonary gas exchange and ventilatory responses to severe-intensity exercise across all doses and conditions are summarised in Table 1. In contrast to the effects observed for moderate-intensity exercise, the $\dot{V}o_2$ and $\dot{V}co_2$ measured at baseline and at task failure were not significantly altered by dose or treatment (all $P > 0.05$). Moreover, neither the dose nor the treatment significantly altered the $\dot{V}o_2$ slow component amplitude ($P > 0.05$ for both). There was a trend towards significant main effects by dose ($P = 0.09$) and treatment ($P = 0.08$) but no interaction effect on RER at baseline ($P > 0.05$). Follow-up tests revealed that there was a trend toward significant increases in RER at baseline by ~4% and ~3% following consumption of 280 ml of supplement compared to the consumption of 70 ($P = 0.06$) or 140 ml ($P = 0.08$) of supplement, respectively. RER at task failure was not altered by dose or treatment ($P > 0.05$). The baseline, end-exercise and change in blood [lactate] and HR were not significantly altered by dose or condition (Table 2; $P > 0.05$).

There was a significant main effect by condition ($P < 0.05$), but not dose ($P > 0.05$), on time to task failure (Table 1 and Figure 4B). Follow-up tests revealed that consumption of 8.4 mmol NO_3^- (BR140) and 16.8 mmol NO_3^- (BR280) resulted in a significant increase in time to task failure by 71 ± 77 s and 59 ± 61 s, respectively, relative to PL140 and PL280 ($P < 0.05$; Figure 4B). There was no difference in time to task failure between BR70 and PL70 ($P > 0.05$). The change in plasma [NO_2^-] from baseline to post-ingestion of 4.2, 8.4 and 16.8 mmol NO_3^- was significantly correlated with the change in time to task failure ($r = 0.55$; $P < 0.05$). There was no significant difference in time to task failure between 4.2, 8.4 and 16.8 mmol BR (all $P > 0.05$) or between PL70, PL140 and PL280 ($P > 0.05$).

In terms of positive changes in time to task failure, there were three 'non-responders' in the 4 mmol condition, two in the 8 mmol condition and one in the 16.8 mmol condition. Individual subjects who did not respond at lower doses did respond at higher doses. The increase in plasma [NO_2^-] from baseline to pre-exercise for the non-responders was similar to the other subjects who did respond. For example, the three non-responders at the lowest NO_3^- dose had an increase in plasma [NO_2^-] of 140, 208 and 161 nM, compared to a group mean increase of 150 nM. In addition, the non-responders did not have high baseline values of plasma [NO_2^-] (70-121 nM) compared to the group mean.

DISCUSSION

This study is the first to characterize the pharmacokinetic-pharmacodynamic effects of NO_3^- -rich BR ingestion and to investigate the dose-response relationship between BR ingestion and the physiological responses to exercise. Specifically, we studied how acute ingestion of three different BR volumes (and thus three different NO_3^- doses) impacted on plasma [NO_3^-] and [NO_2^-], resting BP, the pulmonary gas exchange responses to moderate- and severe-intensity exercise, and exercise tolerance. Our principal findings were that plasma [NO_3^-] and [NO_2^-] rise dose-dependently up to a NO_3^- dose of 16.8 mmol, and there was a dose-dependent peak reduction in BP up to 8.4 mmol NO_3^-. A NO_3^- dose of 16.8 mmol was required to elicit a significant reduction in the O_2 cost of moderate-intensity cycle exercise, although there was a trend ($P = 0.06$) for a reduction with 8.4 mmol. A significant improvement in time to task failure during severe-intensity exercise was evident after ingestion of 8.4 mmol NO_3^-, with no further benefits being observed following the ingestion of 16.8 mmol NO_3^-.

S_1: BR Pharmacokinetics and Pharmacodynamics - Effects on Plasma [NO_3^-], [NO_2^-] and BP

The results of S_1 demonstrated that concentrated BR consumption causes dose-dependent increases in plasma [NO_3^-] and [NO_2^-]. Plasma [NO_3^-] increased by approximately 5-fold and 8-fold 1 h after the ingestion of 4.2 and 8.4 mmol of NO_3^-, and by approximately 18-fold 2 h after the ingestion of 16.8 mmol of NO_3^-. In

contrast, the increase in plasma [NO_2^-] occurred later, peaking at approximately 2-2.5 h post administration of 4.2 and 8.4 mmol NO_3^-, and approximately 3 h post administration of 16.8 mmol NO_3^-. As expected, the rise in plasma [NO_2^-] was smaller compared to plasma [NO_3^-] with peak increases of approximately 2.5-fold, 4-fold and 8-fold, respectively. The delayed peak increases in plasma [NO_2^-] compared to plasma [NO_3^-] reflects the importance of the entero-salivary circulation and subsequent reduction of NO_3^- to NO_2^- by lingual bacteria (16, 39). These pharmacokinetic responses to BR supplementation are consistent with those previously reported following acute ingestion of KNO_3 (19). Together, these data suggest that the pharmacokinetics of plasma [NO_3^-] and [NO_2^-] are dose-dependent when NO_3^- is administered either as nitrate salt or in the form of a natural vegetable supplement.

Ingestion of concentrated BR dose-dependently lowered systolic BP and MAP up to an intake of 8.4 mmol of NO_3^-. More specifically, acute ingestion of 4.2, 8.4 and 16.8 mmol of inorganic NO_3^- administered in the form of BR resulted in peak reductions of systolic BP of approximately 5, 10 and 9 mmHg, and peak reductions of MAP of approximately 2, 5 and 5 mmHg, respectively. Moreover, BR ingestion resulted in a similar 'threshold' effect on diastolic BP with peak reductions of approximately 3 and 4 mmHg following administration of 8.4 and 16.8 mmol NO_3^-; however, ingestion of 4.2 mmol NO_3^- did not significantly reduce diastolic BP. These reductions in BP are similar to those reported by Kapil et al. (19) following acute administration of KNO_3 except that Kapil et al. (19) reported a dose-dependent reduction in BP up to 24 mmol KNO_3. The reason for this discrepancy between studies is unclear. Interestingly, compared to Kapil et al. (19) who reported 6 mmHg and 9 mmHg reductions in systolic BP following the consumption of 12 mmol and 24 mmol KNO_3, respectively, we observed larger reductions in BP following the consumption of BR (e.g., a peak reduction of 10 mmHg in systolic BP with 8.4 mmol NO_3^- contained in 140 ml BR). It is possible that this apparent greater potency of BR compared to nitrate salt in reducing BP is related to the polyphenols and other antioxidants present in BR which may facilitate a more efficient conversion of NO_3^- to NO_2^- (30). Interestingly, although the peak reduction in BP was not significantly different between 8.4 and 16.8 mmol NO_3^-, the mean reduction in BP over 24 h was dose-dependent, with MAP, for example, being reduced by 1, 2 and 4 mmHg following administration of 4.2, 8.4 and 16.8 mmol NO_3^-, respectively.

The results of the present study suggest that BR (and presumably other NO_3^- -rich vegetable) consumption can provide a natural approach to maintaining or improving BP and vascular health in young adults. The reductions in BP evident in the present study are noteworthy. For example, it has been suggested that lowering systolic BP by 10 mmHg may reduce the risk of ischemic heart disease by ~25 % and the risk of stroke by ~35% (27, 28, 29, 31). The beneficial hemodynamic effects of NO_3^- supplementation are thought to be due to the reduction of NO_3^- to NO_2^- and then NO within the blood vessel (13), resulting in arterial dilatation and a reduced peripheral resistance (39). However, it is possible that NO_2^- itself may also exert a direct effect upon the vascular system, independent of NO formation (1). There are several advantages to using inorganic rather than organic NO_3^- for the prevention or treatment of hypertension (33). These include a slow and controlled increase in plasma [NO_2^-] following inorganic NO_3^- intake (due to NO_3^- uptake into the entero-salivary circulation), compared with the more abrupt changes in plasma [NO_2^-] (perhaps to toxic levels) and BP which can occur with organic NO_3^- administration (33). Moreover, unlike the chronic administration of organic NO_3^-, inorganic NO_3^- does not appear to lead to the development of tolerance (37) and endothelial dysfunction (33).

S_2: Dose-Response

The results of S_2 confirm that concentrated BR consumption causes a dose-dependent increase in plasma [NO_3^-] by 334, 778 and 1556 %, and plasma [NO_2^-] by 121, 218 and 338 %, 2.5 h post ingestion of 4.2, 8.4 and 16.8 mmol of NO_3^-, respectively. The magnitude of the increase in plasma [NO_2^-] following consumption of 8.4 and 16.8 mmol of NO_3^- in the present study was much larger than the ~15-150 % rise in plasma [NO_2^-] reported previously following acute (~4-6 mmol; 5, 21, 25, 37) and chronic (~5-6 mmol/day; 2, 3, 22, 26, 37) dietary NO_3^- supplementation. This finding is likely a consequence of the relatively higher NO_3^- doses (8.4 and 16.8 mmol of NO_3^-) administered in the present study. Interestingly, the group mean plasma [NO_3^-] and [NO_2^-] reported in S_2, are somewhat lower than those reported at 2-4 h post-ingestion of BR in S_1. Given that there was distinct subject recruitment for S_1 and S_2, it is likely that this discrepancy is due to individual variations in the pharmacokinetic response to BR consumption. For example, when the individual plasma [NO_2^-]

responses to the ingestion of 16.8 mmol of NO_3^- in S_1 is considered, peak concentrations ranged from 493 to 1523 nM and the time-to-peak concentration ranged from 130 to 367 min. The cause of this wide inter-individual variability in the response of plasma [NO_2^-] to NO_3^- ingestion is unclear although it may depend in part on salivary flow rate; also, it is known that the reduction of NO_3^- to NO_2^- is highly dependent on the activity of sub-lingual bacteria (16, 39). Another consideration is that the absolute NO_3^- doses administered in the present study (4.2, 8.4 and 16.8 mmol in 1, 2 and 4 BR shots, respectively) resulted in somewhat different NO_3^- doses when expressed relative to body mass (0.05-0.07, 0.09-0.13, and 0.19-0.25 mmol NO_3^- per kg BM, respectively).

Dose-response: moderate-intensity exercise

This is the first study to assess the acute dose-dependent physiological responses to exercise following dietary NO_3^- supplementation in humans. We assessed the acute response to three different doses of BR at 2.5 h post-ingestion based on the significant dose-dependent elevation in plasma [NO_2^-] observed at 2-3 h post-ingestion in S_1 (Fig. 1B). The steady-state $\dot{V}o_2$ measured over the final 30 s of moderate-intensity cycle exercise was unaffected by 4.2 mmol NO_3^-, tended to be lower (~30 $ml.min^{-1}$) following administration of 8.4 mmol of NO_3^-, and was significantly reduced (by ~50 $ml.min^{-1}$) following administration of 16.8 mmol NO_3^-.

The reduction in steady-state $\dot{V}o_2$ (~3 %) observed following acute ingestion of 16.8 mmol NO_3^- (~0.23 mmol/kg BM) is similar to that reported 2.5 h post ingestion of 5.2 mmol NO_3^- (~0.07 mmol/kg BM) in the form of non-concentrated BR (37), but is smaller than the 6 % reduction reported 1 h post-ingestion of 0.033 mmol/kg BM $NaNO_3$ (25). In contrast to acute ingestion, longer-term BR supplementation (3-6 days at ~5-7 mmol NO_3^- per day) resulted in a ~5-7 % reduction in steady-state $\dot{V}o_2$ during moderate-intensity cycling (3, 26) and running (22).

Previous studies have indicated that the lowering of submaximal exercise $\dot{V}o_2$ following dietary NO_3^- supplementation may result from improved mitochondrial efficiency (25) and/or a reduction in the ATP cost of muscle force production (4). Alterations in protein expression have been proposed as the mechanistic basis for

these effects (17, 24); however, it is unlikely that these alterations occur sufficiently rapidly to explain the effects observed so soon (1-2.5 h) after NO_3^- ingestion (25, 37). Alternatively, NO may acutely and reversibly impact on protein function through post-translational protein modifications. For instance, S-nitrosation of adenine nucleotide translocase or other mitochondrial or calcium-handling proteins (35) may contribute to the acute reduction in O_2 cost of exercise following BR ingestion. The mechanistic basis for the acute changes in the O_2 cost of exercise following BR ingestion warrants further investigation.

An interesting observation was the dose-dependent increase in baseline and end-exercise $\dot{V}co_2$, irrespective of condition (i.e. PL or BR). This small but significant rise in $\dot{V}co_2$ led to a dose-dependent increase in RER that was more pronounced during baseline cycling compared to the exercising steady-state. An elevation in RER is indicative of a shift in substrate utilization towards a relatively greater use of carbohydrate and is likely due to the sugar content of the concentrated BR and PL beverages (~16 g per 70 ml).

Dose-response: severe-intensity exercise

A novel finding of the present study was that 8.4 and 16.8 mmol NO_3^-, but not 4.2 mmol NO_3^-, administered acutely in the form of concentrated BR, significantly improved the time to task failure by 14 % and 12 %, respectively, during severe-intensity exercise. These findings are similar to the 14-16 % improvement in exercise tolerance reported previously following 5-6 days BR supplementation at a lower dose (5-6 mmol NO_3^-) (3, 22). Although the mechanism(s) responsible for the ergogenic potential of NO_3^- supplementation remains uncertain, they are believed to be mediated via a biochemical reduction of ingested NO_3^- to biologically active NO_2^- and NO (4).

NO has been linked to the efficiency of aerobic respiration (9) and the regulation of muscle contraction (35). Indeed, both more efficient mitochondrial oxidative phosphorylation via a reduced proton leak across the inner mitochondrial membrane (24) and a reduced ATP and PCr cost of muscle force production (2, 15) have been reported following dietary NO_3^- supplementation. In addition, recent evidence suggests that BR supplementation results in a marked increase in muscle blood flow

during exercise in rats, with the blood flow being preferentially distributed to muscle groups that principally contain type II fibres which are recruited during severe-intensity exercise (14). Furthermore, NO_3^- supplementation has been shown to increase muscle force production in mice via modulation of intracellular Ca^{2+} handling in fast-twitch fibres (17). It is possible that these mechanisms operate simultaneously and/or synergistically, resulting in enhanced exercise tolerance. It is, however, important to note that the studies which demonstrated effects of NO_3^- supplementation on muscle metabolic and vascular control mechanisms (2, 14, 17, 24) employed chronic, rather than acute, NO_3^- supplementation protocols. On the other hand, Cosby et al. (10) reported acutely increased blood flow to exercising forearm muscle following infusion of nitrite into the brachial artery. It is possible that the improved time to task failure we observed with 8.4 and 16.8 mmol NO_3^- was related to improved blood flow to muscle or to an NO-mediated enhancement of local matching of O_2 delivery to metabolic rate. This would be consistent with reports that BR supplementation results in a preferential distribution of blood flow to type II fibers (14) and improves oxidative function in hypoxic muscle (38). The lack of a further improvement in time to task failure with 16.8 mmol compared to 8.4 mmol NO_3^- mirrors the lack of additional effect of consuming the higher NO_3^- dose on the peak reduction in BP we observed in S_1, suggesting that the acute effects of BR ingestion on exercise tolerance may be related, at least in part, to effects on the vasculature. Further studies are needed to establish which mechanisms may be responsible for the ergogenic potential of NO_3^-, at least at high doses, as early as 2.5 h after ingestion of BR.

The results of the present study indicate a dose-dependent effect of BR supplementation on exercise tolerance up to 8.4 mmol with no further benefit (indeed a small reduction in exercise tolerance) following ingestion of 16.8 mmol of NO_3^-. A possible explanation for this threshold might be a NO-dependent reduction in skeletal muscle force via modulation of excitation-contraction coupling. It has been reported that the opening of the Ca^{2+} release channels of the sarcoplasmic reticulum (SR) are inhibited by NO (32, 35), and highly related to NO availability (35). In addition, Ca^{2+} transport (35), SR Ca^{2+}-ATPase activity (18) and cytochrome-c-oxidase inhibition (9), may be influenced by NO and contribute to a dose-dependent modulation of excitation-contraction coupling. Therefore, whilst an increase in NO bioavailability

may result in a more efficient mitochondrial function (24), and changes to type II fibre contractility (17) and blood flow (14), it is possible that these positive effects may be offset by impairments of mitochondrial or contractile function at higher NO levels that might promote nitrative stress. These suggestions are naturally speculative and await further investigation.

The improvements in time to task failure during severe-intensity exercise following ingestion of both 8.4 and 16.8 mmol of NO_3^- in the present study were evident without any significant changes in the $\dot{V}O_2$ response to exercise. Neither the amplitude of the $\dot{V}O_2$ slow component nor the end-exercise $\dot{V}O_2$ were influenced by acute ingestion of up to 16.8 mmol of NO_3^-. This finding is consistent with some (20) but not all previous reports (3, 22). For example, Bailey et al. (3) reported that 3 days of BR supplementation reduced the $\dot{V}O_2$ slow component amplitude by 23% and improved exercise tolerance by ~16%. In contrast, Kelly et al. (20) reported that 3 days of BR supplementation improved exercise tolerance at three different severe intensities by 12-17%, without any accompanying changes in the $\dot{V}O_2$ response. We found no difference in end-exercise $\dot{V}O_2$ between BR and PL at any dose. In the severe exercise intensity domain, the $\dot{V}O_2$ at the point of volitional exhaustion would be expected to equal the $\dot{V}O_2$ max (11). Our results are therefore consistent with some (3, 37), but not all (5, 25), previous studies which indicate that NO_3^- supplementation does not reduce $\dot{V}O_2$ max. Interestingly, there was a disconnect between the effects of BR on steady-state $\dot{V}O_2$ during moderate-intensity exercise (where the greatest reduction occurred at the highest dose of NO_3^-) and the effects of BR on exercise tolerance (where the increased time to task failure was similar with 8.4 and 16.8 mmol of NO_3^-). Collectively, these results appear to indicate that the effects of BR on severe-intensity exercise performance may be independent from the effects of BR on the O_2 cost of submaximal exercise.

It should be noted that, while a ~12-14% extension of time to task failure during severe-intensity constant-work-rate exercise following acute BR ingestion may appear impressive, this is likely to translate into no more than a 1-2% reduction in the time to complete a given distance, for example during a short endurance time trial (TT) event (34). This is similar to the magnitude of improvement in performance reported previously for 4 km and 16.1 km TT after acute BR ingestion (21) and for 10 km TT

following 6 days BR supplementation (6). A 1% improvement in performance is highly meaningful in élite sport. For example, it could improve 1500 m running performance by approximately 2 s or 3000 m running performance by approximately 4-5 s in international-standard athletes. It remains unclear, however, whether élite athletes may confer a performance benefit from NO_3^- supplementation. Several studies now indicate that, at least when NO_3^- is ingested acutely, TT performance is not enhanced in highly-trained endurance athletes (7, 8, 40). This may be related to factors such as greater NOS activity, better muscle oxygenation and mitochondrial efficiency, and a lower fraction of type II fibres in the muscles of highly-trained compared to moderately-trained subjects (40). It is possible that the dose-response relationship between NO_3^- ingestion and changes in exercise performance may be different in élite compared to sub-élite subjects such that larger NO_3^- doses and/or longer supplementation periods are required to elicit improved exercise performance. The significant correlation between the change in plasma $[NO_2^-]$ and the change in time to task failure indicates that the dietary NO_3^- intervention must be sufficient to increase plasma $[NO_2^-]$ if performance is to be improved. In this regard, an important consideration may be the timing of supplementation relative to the start of exercise. The present study shows that, on average, plasma $[NO_2^-]$ takes longer to peak when larger doses of NO_3^- are imbibed. However, there are appreciable inter-individual differences in the speed with which ingested NO_3^- is reduced to NO_2^-, which may preclude any more specific advice than to consume NO_3^- some 2-3 hours before the start of exercise.

It has been suggested previously that there may be ‘responders’ and ‘non-responders’ to dietary NO_3^- supplementation (40) and there was evidence of this in the present study. Interestingly, the number of non-responders (in terms of exercise capacity) decreased as the dose ingested increased. For example, there were three non-responders in the 4 mmol condition, two in the 8 mmol condition and one in the 16.8 mmol condition. Two of the subjects who did not respond at the lowest dose, did respond to the larger doses and one subject who did not respond following administration of either 4 or 8 mmol did respond to the 16.8 mmol dose. This suggests that some individuals will require a larger acute dose than others to elicit any positive effects on exercise capacity from dietary NO_3^- ingestion. Unlike in our previous study (40), the increase in plasma $[NO_2^-]$ from baseline to pre-exercise for the non-

responders wasn't smaller than that measured in other subjects who did respond, and the non-responders did not have particularly high baseline plasma [nitrite]. In a recent study, we found that the subjects who demonstrated improvement in high-intensity intermittent exercise performance following dietary NO_3^- supplementation were those whose plasma [NO_2^-] fell significantly during exercise (41). We did not measure plasma [NO_2^-] post-exercise in the present study. The explanation for the existence of responders and non-responders to dietary NO_3^- supplementation is presently obscure.

In conclusion, dietary supplementation with NO_3^--rich BR dose-dependently increased plasma [NO_3^-] and [NO_2^-] up to 16.8 mmol NO_3^-, and caused peak reductions in systolic BP and MAP dose-dependently up to 8.4 mmol of NO_3^-. These results suggest that the consumption of high-NO_3^- foodstuffs may be an effective strategy for maintaining and perhaps enhancing vascular health in young adults. The present study also demonstrated that the O_2 cost of moderate-intensity exercise is reduced dose-dependently up to 16.8 mmol NO_3^-. Supplementation with 4.2 mmol NO_3^- did not enhance time to task failure relative to placebo; however, supplementation with 8.4 mmol NO_3^- significantly improved time to task failure relative to placebo, with no further improvement being evident following supplementation with 16.8 mmol of NO_3^-. Although the mechanistic bases for the reduction in the O_2 cost of sub-maximal exercise and enhancements in exercise tolerance following acute dietary BR remain unclear, these results provide important practical information which may underpin the potential use of BR/NO_3^- supplementation for improving cardiovascular health in the general population, and for enhancing exercise performance in athletes.

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FIGURE LEGENDS

Figure 1: Plasma [nitrate] (Panel A) and [nitrite] (Panel B) following consumption of water (control; ●), and 4.2 (▲), 8.4 (■) and 16.8 mmol (◆) of NO_3^- (group mean ± SE). Plasma [nitrate] and [nitrite] rose significantly in a dose-dependent manner. See text for further details. *Significant difference from pre-supplemention baseline ($P < 0.05$); [a] significant difference from control ($P < 0.05$); [b] significant difference from 4.2 mmol of NO_3^- ($P < 0.05$); [c] significant difference from 8.4 mmol of NO_3^- ($P < 0.05$).

Figure 2: Change (**Δ)** relative to pre-supplementation baseline in systolic blood pressure (BP; Panel A), diastolic BP (Panel B) and mean arterial pressure (MAP; Panel C) following consumption of water (control; ●), and 4.2 (▲), 8.4 (■) and 16.8 mmol (◆) of NO_3^- (group mean ± SE). *Significant difference from pre-supplemention baseline ($P < 0.05$); [a] significant difference from control ($P < 0.05$); [b] significant difference from 4.2 mmol of NO_3^- ($P < 0.05$).

Figure 3: Mean ± SE plasma [nitrate] (Panel A) and [nitrite] (Panel B) pre (black bars) and 2.5 h post ingestion of 70, 140 and 280 ml of nitrate-rich beetroot juice (Nitrate) or nitrate-depleted beetroot juice (Placebo). See text for further details. *Significant difference from baseline ($P < 0.05$); [a] significant difference post consumption of 70 ml of nitrate-rich BR($P < 0.05$); [b] significant difference from post consumption of 140 ml of nitrate-rich BR($P < 0.05$).

Figure 4: Mean ± SE steady-state $\dot{V}o_2$ during moderate-intensity exercise (Panel A) and time to task failure during severe-intensity exercise (Panel B) following consumption of 70, 140 and 280 ml of nitrate-rich beetroot juice (BR; grey bars) or nitrate-depleted beetroot juice (PL: black bars). End-exercise $\dot{V}o_2$ during moderate-intensity exercise was significantly reduced following the ingestion of 280 ml BR. Time to task failure during severe-intensity exercise was extended after consumption of 140 ml BR with no further increase following 280 ml BR. *Significant difference from placebo ($P < 0.05$).