Beetroot juice and exercise: pharmacodynamic and dose-response relationships

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Beetroot juice and exercise: pharmacodynamic and dose-response relationships. J Appl Physiol 115: 325–336, 2013. First published May 2, 2013; doi:10.1152/japplphysiol.00372.2013.— Dietary supplementation with beetroot juice (BR) containing approximately 5–8 mmol inorganic nitrate ([NO3]−) increases plasma nitrate concentration ([NO3]−), 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 men ingested 70, 140, or 280 ml concentrated BR (containing 4.2, 8.4, and 16.8 mmol NO3−, respectively) or no supplement to establish the effects of BR on resting plasma [NO3]− and [NO2]− over 24 h. Subsequently, on six separate occasions, 10 subjects completed moderate-intensity and severe-intensity cycle exercise tests, 2.5 h postingestion of 70, 140, and 280 ml BR or NO3−-depleted BR as placebo (PL). Following acute BR ingestion, plasma [NO2]− increased in a dose-dependent manner, with the peak changes occurring at approximately 2–3 h. Compared with PL, 70 ml BR did not alter the physiological responses to exercise. However, 140 and 280 ml BR reduced the steady-state oxygen (O2) uptake during moderate-intensity exercise by 1.7% (P = 0.06) and 3.0% (P < 0.05), whereas time-to-task failure was extended by 14% and 12% (both P < 0.05), respectively, compared with PL. The results indicate that whereas plasma [NO2]− and the O2 cost of moderate-intensity exercise are altered dose dependently with NO3−-rich BR, there is no additional improvement in exercise tolerance after ingesting BR containing 16.8 compared with 8.4 mmol NO3−. These findings have important implications for the use of BR to enhance cardiovascular health and exercise performance in young adults.

nitrate; nitrite; nitric oxide; blood pressure; exercise economy; O2 uptake; exercise tolerance

Nitric oxide (NO) is a gaseous signaling molecule that 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 catalyzed by NO synthase (NOS), with nitrite (NO2−) and nitrate (NO3−) being oxidation products of NO (30). It is now appreciated that under appropriate physiological conditions, NO can also be produced via the reduction of NO3−, a process that may be particularly important in situations where oxygen (O2) availability is low, and/or NOS function is impaired (12). Interestingly, administration of dietary inorganic NO3− has been shown to increase plasma NO2− concentration ([NO2−]) and to produce NO-like bioactivity (19, 23, 39). Up to 25% of ingested NO3− enters the enterosalivary circulation and is concentrated in the saliva, whereupon facultative, anaerobic bacteria in the oral cavity reduce the NO3− to NO2− (30). When swallowed into the acidic environment of the stomach, some of the NO2− is converted further into NO, whereas the remainder is absorbed to increase circulating plasma [NO2−]. This NO2− may be reduced further to NO and other reactive nitrogen intermediates, particularly in tissues that may be relatively hypoxic, such as contracting skeletal muscle (30).

We (3, 37) and others (19, 23, 39) have demonstrated that NO3− ingestion, either in the form of NO3−-rich BR, reduces resting blood pressure (BP) profoundly and consistently. Consequently, dietary NO3− 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 BR consumption (~23 mmol NO3−) on plasma [NO2−] and BP over 24 h. Plasma [NO2−] peaked 3 h postingestion, remained close to peak values until 5 h postingestion, and returned to baseline after 24 h (39). The systolic and diastolic BP and the mean arterial pressure (MAP) were reduced significantly, by ~10, ~8, and ~8 mmHg, respectively, at 2.5–3 h after BR intake. The same research group later reported a dose-dependent increase in plasma [NO3−] and [NO2−] and reduction in BP following ingestion of potassium NO3− (KNO3) (19). In this study, plasma [NO3−] rose by ~1.3-, approximately two-, and approximately fourfold following consumption of 4, 12, and 24 mmol KNO3, respectively. The peak rise in plasma [NO3−] was accompanied by significant reductions in both systolic BP (of ~2, ~6, and ~9 mmHg, respectively) and diastolic BP (of ~4, ~4, and ~6 mmHg, respectively). However, since BR contains polyphenols and antioxidants, which can facilitate the synthesis of NO from NO3− in the stomach (30), it is unclear whether BP is similarly impacted when different doses of BR are ingested compared with equivalent doses of NO3− salts. Given the growing interest in dietary NO3− 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 [NO2−] and BP to establish an optimal dose for beneficial effects.

Recent investigations suggest that dietary NO3− supplementation has the potential to influence human physiology beyond...
the above hemodynamic effects (3, 26). Specifically, we (2, 3, 22) and others (6, 24–26) 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. It appears that these effects are related to NO$_3^-$ 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 fibers (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$_3^-$] to be attained (39). However, since all exercise-performance studies completed to date with BR have administered approximately 5–8 mmol NO$_3^-$, it is unclear whether a dose-response relationship exists between acute NO$_3^-$ intake and the physiological responses to exercise. The establishment of the dose-response relationship between NO$_3^-$ intake and the physiological responses to exercise and the ascertainment of the optimal NO$_3^-$ dose for enhancing exercise performance are 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 BR 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 that 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 postingestion 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 S$_2$, dose response], with the results generated in S$_1$ 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 (BM) 79 ± 9 kg; S$_2$, age 22 ± 5 yr, height 1.77 ± 0.05 m, BM 74 ± 8 kg]. None of the subjects in S$_1$ and S$_2$ were a tobacco smoker or user of dietary supplements. All subjects recruited for S$_2$ were fully familiar with laboratory exercise-testing procedures, having participated previously in studies using cycle ergometry in our laboratory. The procedures used 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 before 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 NO$_3^-$ to NO$_2^-$ (16).

S$_1$: Pharmacokinetics and Pharmacodynamics

**Procedures.** All subjects reported to the laboratory on four separate occasions over a period of 3 wk. Upon arrival at the laboratory, resting BP was measured, and a venous blood sample was obtained for the measurement of plasma [NO$_3^-$] and [NO$_2^-$]. Subjects then consumed an acute dose of 70, 140, or 280 ml NO$_3^-$-rich BR (organic BR containing ∼4.2, ∼8.4, or ∼16.8 mmol 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 semiskimmed milk). BP was measured, and a venous blood sample was obtained, 1, 2, 4, 8, 12, and 24 h postingestion. For each 24-h period of data collection, subjects were provided with a standardized, low NO$_3^-$ diet. The quantity and timing of food and drink intake were 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 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, with 1 min of collection. Plasma was subsequently extracted and immediately frozen at −80°C for later analysis of [NO$_3^-$] and [NO$_2^-$].

All glassware, utensils, and surfaces were rinsed with deionized water to remove residual [NO$_3^-$] and [NO$_2^-$] before blood analyses. The [NO$_3^-$] of the undiluted (nondeproteinized) plasma was determined by its reduction to NO in the presence of glacial acetic acid and 4% (w/v) aqueous sodium iodide. 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 (NOA; Sievers NOA 280i; Analytix, Durham, UK). The [NO$_2^-$] was determined by plotting signal (mV) area against a calibration plot of 100 nM–1 μM sodium NO$_2^-$.

Before determination of [NO$_3^-$], samples were deproteinized using zinc sulfate (ZnSO$_4$)/sodium hydroxide (NaOH) precipitation. Aqueous ZnSO$_4$ [400 μl 10% (w/v)] and 400 μl 0.5 M NaOH were added to 200 μl of sample and vortexed for 30 s before being left to stand at room temperature for 15 min. Thereafter, samples were centrifuged at 4,000 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) vanadium trichloride in 1 M HCl. The production of NO was detected using the chemiluminescence NOA, as described above.

To determine more precisely the time-to-peak plasma [NO$_3^-$] following NO$_3^-$ ingestion, a one-compartment model with first-order absorption and elimination kinetics was used, as described in the following equation

\[
Y = \exp(-Ke \times X)/(Ke/Ka) - \exp(-Ke \times X)/(Ke/Ka - 1)
\]

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, and 16.8
mmol NO₃⁻ and CON) and across time (0, 1, 2, 4, 8, 12, and 24 h) for plasma [NO₂⁻] and [NO₃⁻] and BP. Significant main or interaction effects were analyzed further using simple contrasts. One-way repeated-measures ANOVA was used to assess the differences in time-to-peak plasma [NO₂⁻]. Relationships between plasma [NO₂⁻] and BP were analyzed using Pearson product moment correlation coefficients. Statistical significance was accepted at P < 0.05. Results are presented as mean ± SD unless stated otherwise.

S₂: 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 peak O₂ uptake (V˙O₂peak) 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 were 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. V˙O₂peak was taken as the highest 30-s subsequent tests. The breath-by-breath pulmonary gas-exchange data were collected continuously during the incremental tests and averaged over consecutive 10-s periods. V˙O₂peak was taken as the highest 30-s mean value attained before 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% of the difference between the power output at GET and V˙O₂peak plus the power output at GET, i.e., severe-intensity exercise (Δ) were subsequently calculated.

On test days, subjects arrived at the laboratory at ∼8 AM. A venous blood sample was drawn for measurement of plasma [NO₂⁻] and NO₃⁻. Subjects then ingested 70, 140, or 280 ml NO₃⁻-rich BR (containing 4.2, 8.4, or 16.8 mmol NO₃⁻, respectively; Beet It) or 70, 140, or 280 ml NO₃⁻-depleted BR as a placebo (PL70, PL140, or PL280; containing ∼0.04, ∼0.08, or ∼0.12 mmol NO₃⁻; Beet It). 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. After doing so, were served a standardized breakfast (72 g porridge with 180 ml semiskimmed 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 [NO₂⁻] and NO₃⁻. Subjects then ingested 70, 140, or 280 ml NO₃⁻-rich BR (containing 4.2, 8.4, or 16.8 mmol NO₃⁻, respectively; Beet It) or 70, 140, or 280 ml NO₃⁻-depleted BR as a placebo (PL70, PL140, or PL280; containing ∼0.04, ∼0.08, or ∼0.12 mmol NO₃⁻; Beet It). 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. After doing so, were served a standardized breakfast (72 g porridge with 180 ml semiskimmed milk). A washout period of at least 72 h separated each visit.

RESULTS

Ingestion of BR was tolerated well by all subjects in S₁ and S₂. Subjects did, however, report beeturia (red urine) and red stools, consistent with previous studies (3, 39). The absolute NO₃⁻ doses used in S₁ and S₂ (4.2, 8.4, and 16.8 mmol) were equivalent to ∼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) NO₃⁻/kg BM, respectively.

S₁: Pharmacokinetics and Pharmacodynamics

The effects of different volumes of BR and therefore, different amounts of ingested NO₃⁻ on plasma [NO₂⁻] and [NO₃⁻] are presented in Fig. 1. There were significant main effects by dose and time and an interaction effect for both plasma [NO₂⁻] (Fig. 1A; all P < 0.01) and plasma [NO₃⁻] (Fig. 1B; all P < 0.01).

At resting baseline, before the ingestion of any beverage, plasma [NO₂⁻] was not significantly different between doses (Fig. 1A; all P > 0.05). ANOVA analyses revealed significant dose-dependent increases in plasma [NO₂⁻] following BR supplementation (P < 0.05). The peak elevation above baseline in plasma [NO₂⁻] occurred 1 h postadministration of 4.2 (160 ±
administration of 16.8 mmol NO₃⁻.

Kinetic analyses revealed that plasma [NO₂⁻] following consumption of water (control; ●) and 4.2 (●), 8.4 (■), and 16.8 (●) mmol NO₃⁻ (group mean ± SE). Plasma [NO₃⁻] and [NO₂⁻] rose significantly in a dose-dependent manner. See text for further details. *Significant difference from presupplementation baseline (P < 0.05); †significant difference from control (P < 0.05); ‡significant difference from 4.2 mmol NO₃⁻ (P < 0.05); §significant difference from 8.4 mmol NO₃⁻ (P < 0.05).

43 μM) and 8.4 mmol NO₃⁻ (269 ± 92 μM) and 2 h postadministration of 16.8 mmol NO₃⁻ (581 ± 209 μM; Fig. 1A; all P < 0.05). Plasma [NO₃⁻] remained elevated above baseline and CON at all time points after administration of 4.2, 8.4, and 16.8 mmol NO₃⁻ (P < 0.05).

At baseline, before ingestion of any beverage, plasma [NO₂⁻] was not significantly different between doses (Fig. 1B; P > 0.05). ANOVA analyses revealed significant dose-dependent increases in plasma [NO₂⁻] following BR supplementation (P < 0.05). The peak elevation above baseline in plasma [NO₂⁻] occurred 2 h postadministration of 4.2 (220 ± 104 nM) and 8.4 mmol NO₃⁻ (374 ± 173 nM) and 4 h postadministration of 16.8 mmol NO₃⁻ (653 ± 356 nM; Fig. 1B; all P < 0.05). Kinetic analyses revealed that plasma [NO₂⁻] 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 [NO₂⁻], following ingestion of 8.4 mmol, tended to occur later compared with 4.2 mmol (P = 0.06). Plasma [NO₂⁻] remained elevated above baseline and CON at 1, 2, 4, and 8 h after administration of 4.2, 8.4, and 16.8 mmol NO₃⁻ (all P < 0.05).

At 12 h, plasma [NO₂⁻] remained elevated above baseline and 4.2 mmol BR following ingestion of 8.4 and 16.8 mmol NO₃⁻ (all P < 0.05). In addition, plasma [NO₂⁻] remained elevated at 24 h following administration of 16.8 mmol NO₃⁻ compared with all other doses (P < 0.05).

The effects of different volumes of BR (and therefore, different amounts of ingested NO₃⁻) on systolic and diastolic BP and MAP are presented in Fig. 2. The changes in systolic BP across all conditions are presented in Fig. 2A. There were significant main effects by dose and time and an interaction effect on systolic BP (all P < 0.05). Systolic BP at baseline, before administration of any beverage, was lower (P < 0.05) in the 16.8-mmol NO₃⁻ condition (118 ± 5 mmHg) relative to CON (121 ± 5 mmHg) but not relative to 4.2 (119 ± 6 mmHg) and 8.4 mmol NO₃⁻ (120 ± 6 mmHg). Compared with baseline, systolic BP was lowered significantly following ingestion of 4.2, 8.4, and 16.8 mmol NO₃⁻ (all P < 0.05). The peak reduction in systolic BP occurred 4 h postadministration of 4.2 (5 ± 5 mmHg), 8.4 (10 ± 5 mmHg), and 16.8 mmol NO₃⁻ (9 ± 4 mmHg), respectively, relative to baseline (all P < 0.05). Systolic BP was reduced relative to baseline, CON, and 4.2 mmol NO₃⁻, at 2, 4, and 8 h postadministration of 8.4 mmol and 16.8 mmol NO₃⁻ (all P < 0.05). There were no differences in systolic BP between 8.4 and 16.8 mmol NO₃⁻ 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 NO₃⁻ (P < 0.05). In contrast, systolic BP was not significantly different than CON or baseline at 24 h postad-
administration of 4.2 and 8.4 mmol NO₃⁻ (P > 0.05). Overall, the mean systolic BP across 24 h, relative to CON, was lowered dose dependently by ~3, ~4, and ~6 mmHg after administration of 4.2, 8.4, and 16.8 mmol NO₃⁻, respectively (all P < 0.05). The change in systolic BP was correlated with the change in plasma [NO₃⁻] (r = −0.27; P < 0.05) and the change in plasma [NO₂⁻] (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 NO₃⁻-rich BR are presented in Fig. 2B. There was a significant interaction effect (dose × time) on diastolic BP (P < 0.05). Diastolic BP at baseline was not significantly different among 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 NO₃⁻ reduced diastolic BP significantly, relative to baseline and CON (all P < 0.05). The peak reduction in diastolic BP from baseline occurred at 4 h postadministration of 8.4 mmol NO₃⁻ (3 ± 3 mmHg) and 2 h postadministration of 16.8 mmol NO₃⁻ (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 NO₃⁻ at any time point (P > 0.05).

The change in diastolic BP was correlated with the change in plasma [NO₃⁻] (r = −0.35; P < 0.05) and the change in plasma [NO₂⁻] (r = −0.39; P < 0.05). Moreover, the peak 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 NO₃⁻-rich BR are presented in Fig. 2C. There were significant main effects by dose and time and an interaction effect on MAP (all P < 0.05). At baseline, before the ingestion of any beverage, MAP was not significantly different among 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 NO₃⁻ relative to baseline and CON (all P < 0.05). Following ingestion of 4.2 mmol NO₃⁻, 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 postadministration of 8.4 and 16.8 mmol NO₃⁻ relative to baseline (P < 0.05). MAP was not different between 8.4 and 16.8 mmol NO₃⁻ at any time point (P > 0.05). Overall, the mean MAP across 24 h, relative to CON, was reduced dose dependently by ~1, ~2, and ~4 mmHg after administration of 4.2, 8.4, and 16.8 mmol NO₃⁻, respectively (all P < 0.05). The change in MAP was correlated significantly with the change in plasma [NO₃⁻] (r = −0.35; P < 0.05) and the change in plasma [NO₂⁻] (r = −0.41; P < 0.05).

S₂: Dose Response

Plasma [NO₃⁻] and [NO₂⁻]. The group mean plasma [NO₃⁻] and [NO₂⁻] responses in the BR and PL conditions are illustrated in Fig. 3, A and B, respectively. Presupplementation plasma [NO₃⁻] was not significantly different between conditions (P > 0.05), and no significant change in plasma [NO₂⁻] was observed following PL supplementation (P > 0.05). ANOVA analyses revealed a significant dose-dependent increase in plasma [NO₃⁻] at 2.5 h following BR supplementation (P < 0.05). An elevation in plasma [NO₃⁻] 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 NO₃⁻ (580 ± 89 μM; P < 0.05). Presupplementation plasma [NO₂⁻] was not significantly different among conditions (P > 0.05), and no significant change in plasma [NO₂⁻] was observed following PL supplementation (P > 0.05). ANOVA analyses revealed a significant dose-dependent increase in plasma [NO₂⁻] at 2.5 h following BR supplementation (P < 0.05). Following administration of 4.2, 8.4, and 16.8 mmol NO₃⁻, plasma [NO₂⁻] was elevated above baseline by 150 ± 73 nM, 291 ± 145 nM, and 425 ± 225 nM, respectively (all P < 0.05). Plasma [NO₂⁻] was significantly greater after ingestion of 16.8 mmol compared with 4.2 mmol NO₃⁻ (P < 0.05) and tended to be greater compared with 8.4 mmol NO₃⁻ (P = 0.06). Plasma [NO₂⁻] was
In addition, there was a trend toward a significant reduction in plasma [NO$_3^-$] from baseline to postingestion of 4.2, 8.4, and 16.8 mmol NO$_3^-$ was correlated with the change in V$\dot{O}_2$ amplitude ($r = -0.38; P < 0.05$). There was no significant difference in V$\dot{O}_2$ amplitude between PL and BR at any dose ($P > 0.05$).

The V$\dot{CO}_2$/H11021/baseline measured over the last 90 s of 20 W pedaling, and the end-exercise V$\dot{CO}_2$ measured over the last 30 s of exercise, were affected by dose ($P < 0.05$ for both) but not condition ($P > 0.05$ for both; Table 1). Follow-up tests revealed that V$\dot{CO}_2$/H11021/baseline was increased significantly, as the volume of supplement ingested increased ($P < 0.05$), irrespective of the condition (i.e., PL or BR). Specifically, V$\dot{CO}_2$/H11021/baseline was increased by $\sim7\%$ and $\sim5\%$ 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 V$\dot{CO}_2$ between the ingestion of 70 and 140 ml of supplement ($P > 0.05$). Furthermore, post hoc analysis revealed that the end-exercise V$\dot{CO}_2$ 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 V$\dot{CO}_2$ between ingestion of 140 and 280 ml of supplement ($P > 0.05$).

Baseline and end-exercise RER were 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 increased by $\sim5\%$ and $\sim4\%$, 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 dose, baseline RER tended to be higher (by $\sim3\%$) following administration of 16.8 mmol NO$_3^-$ compared with the respective PL ($P = 0.08$). End-exercise RER was increased significantly by $\sim4\%$ and $\sim3\%$, following consumption of 280 ml compared with 70 and 140 ml of supplement, respectively ($P < 0.05$ for both). In addition, the ingestion of 140 ml increased end-exercise RER compared with ingestion of 70 ml of supplement ($P < 0.05$). The baseline, end-exercise, and change in blood lactate and HR were not altered significantly by dose or condition (Table 2; $P > 0.05$).

Moderate-intensity exercise. The pulmonary gas exchange and ventilatory responses to moderate-intensity exercise across all doses and conditions are summarized in Table 1. The V$\dot{O}_2$ 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 V$\dot{O}_2$, measured over the final 30 s of moderate-intensity exercise, was altered significantly by BR ingestion ($P < 0.05$; Fig. 4A). Follow-up tests indicated that end-exercise V$\dot{O}_2$ was lowered significantly by $\sim3\%$ following administration of 16.8 mmol NO$_3^-$ relative to the respective PL (PL280: 1.65 ± 0.19 vs. BR280: 1.60 ± 0.23 l/min; $P < 0.05$). In addition, there was a trend toward a significant reduction ($\sim2\%$) in end-exercise V$\dot{O}_2$ following administration of 8.4 mmol NO$_3^-$ relative to the respective PL (PL140: 1.67 ± 0.21 vs. BR140: 1.64 ± 0.23 l/min; $P = 0.06$). The change in plasma [NO$_3^-$] from baseline to postingestion of 4.2, 8.4, and 16.8 mmol NO$_3^-$ was correlated with the change in end-exercise V$\dot{O}_2$ ($r = -0.47; P < 0.05$). There was no significant difference in end-exercise V$\dot{O}_2$ following ingestion of 4.6 mmol NO$_3^-$ (BR70) compared with PL70 ($P > 0.05$).

The amplitude of the V$\dot{O}_2$ response (end-exercise V$\dot{O}_2$/H11021/baseline: Table 1) was 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 toward a significant reduction in the V$\dot{O}_2$ amplitude (by $\sim6\%$) after administration of 16.8 mmol NO$_3^-$ compared with 8.4 mmol NO$_3^-$ (BR140: 0.70 ± 0.16 vs. BR280: 0.66 ± 0.16 l/min; $P = 0.06$). If the change in plasma [NO$_3^-$] from baseline to postingestion of 4.2, 8.4, and 16.8 mmol NO$_3^-$ was correlated with the V$\dot{O}_2$ amplitude ($r = -0.38; P < 0.05$). There was no significant difference in V$\dot{O}_2$ amplitude between PL and BR at any dose ($P > 0.05$).

The V$\dot{CO}_2$/H11021/base measured over the last 90 s of 20 W pedaling, and the end-exercise V$\dot{CO}_2$ measured over the last 30 s of exercise, were affected by dose ($P < 0.05$ for both) but not condition ($P > 0.05$ for both; Table 1). Follow-up tests revealed that V$\dot{CO}_2$/H11021/base was increased significantly, as the volume of supplement ingested increased ($P < 0.05$), irrespective of the condition (i.e., PL or BR). Specifically, V$\dot{CO}_2$/H11021/base was increased by $\sim7\%$ and $\sim5\%$ 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 V$\dot{CO}_2$ between the ingestion of 70 and 140 ml of supplement ($P > 0.05$). Furthermore, post hoc analysis revealed that the end-exercise V$\dot{CO}_2$ 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 V$\dot{CO}_2$ between ingestion of 140 and 280 ml of supplement ($P > 0.05$).

Baseline and end-exercise RER were 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 increased by $\sim5\%$ and $\sim4\%$, 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 dose, baseline RER tended to be higher (by $\sim3\%$) following administration of 16.8 mmol NO$_3^-$ compared with the respective PL ($P = 0.08$). End-exercise RER was increased significantly by $\sim4\%$ and $\sim3\%$, following consumption of 280 ml compared with 70 and 140 ml of supplement, respectively ($P < 0.05$ for both). In addition, the ingestion of 140 ml increased end-exercise RER compared with ingestion of 70 ml of supplement ($P < 0.05$). The baseline, end-exercise, and change in blood lactate and HR were not altered significantly 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 summarized in Table 1. In contrast to the effects observed for moderate-intensity exercise, the V$\dot{O}_2$ and V$\dot{CO}_2$ measured at baseline and at task failure were not altered by dose or treatment (all $P > 0.05$). Moreover, neither the dose nor the treatment altered the V$\dot{O}_2$ slow component amplitude ($P > 0.05$ for both). There was a trend toward 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 $\sim4\%$ and $\sim3\%$ following consumption of 280 ml of supplement compared with 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 altered significantly 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

Fig. 3. Mean ± SE plasma [NO$_3^-$] (A) and [NO$_2^-$] (B) preingestion (black bars) and 2.5-h postingestion (gray bars) of 70, 140, and 280 ml NO$_3^-$-rich beetroot juice (BR) (NO$_3^-$) or NO$_3^-$-depleted BR [placebo (PL)]. See text for further details. *Significant difference from baseline ($P < 0.05$); $\Delta$ significant difference postconsumption of 70 ml NO$_3^-$-rich BR ($P < 0.05$); $\Delta$ significant difference from postconsumption of 140 ml NO$_3^-$-rich BR ($P < 0.05$).

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Fig. 4B). Follow-up tests revealed that consumption of 8.4 mmol NO₃⁻ (BR140) and 16.8 mmol NO₃⁻ (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; Fig. 4B). There was no difference in time-to-task failure between BR70 and PL70 (P > 0.05). The change in plasma [NO₂⁻] from baseline to postingestion of 4.2, 8.4, and 16.8 mmol NO₃⁻ was correlated significantly with the change in time-to-task failure (r = 0.55; P < 0.05). There was no significant difference in time-to-task failure among 4.2, 8.4, and 16.8 mmol BR (all P > 0.05) among PL70, PL140, and PL280 (P > 0.05).

In terms of positive changes in time-to-task failure, there were three “nonresponders” in the 4.2-mmol condition, two in the 8.4-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 [NO₂⁻] from baseline to pre-exercise for the nonresponders was similar to the other subjects who did respond. For example, the three nonresponders at the lowest NO₃⁻ dose had an increase in plasma [NO₂⁻] of 140, 208, and 161 nM compared with a group mean increase of 150 nM. In addition, the nonresponders did not have high baseline values of plasma [NO₂⁻] at 70–121 nM compared with the group mean.

**DISCUSSION**

This study is the first to characterize the pharmacokinetic-pharmacodynamic effects of NO₃⁻-rich BR ingestion and to investigate the dose-response relationship between BR ingestion and the physiological responses to exercise. Specifically, we found how acute ingestion of three different BR volumes (and thus three different NO₃⁻ doses) impacted on plasma [NO₃⁻] and [NO₂⁻], resting BP, the pulmonary gas-exchange responses to moderate- and severe-intensity exercise, and exercise tolerance. Our principal findings were that plasma [NO₃⁻] and [NO₂⁻] increased dose-dependently up to 16.8 mmol NO₃⁻ with there being a dose-dependent peak reduction in BP up to 8.4 mmol NO₃⁻. A NO₃⁻ dose of 16.8 mmol was required to elicit a significant reduction in the O₂ 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 NO₃⁻, with no further benefits observed following the ingestion of 16.8 mmol NO₃⁻.

**S1: BR Pharmacokinetics and Pharmacodynamics—Effects on Plasma [NO₃⁻], [NO₂⁻], and BP**

The results of S1 demonstrated that concentrated BR consumption causes dose-dependent increases in plasma [NO₃⁻] and [NO₂⁻]. Plasma [NO₃⁻] increased by approximately five- and eightfold, 1 h after the ingestion of 4.2 and 8.4 mmol NO₃⁻, and by ~18-fold, 2 h after the ingestion of 16.8 mmol NO₃⁻. In contrast, the increase in plasma [NO₂⁻] occurred later, peaking at approximately 2–2.5 h postadministration of 4.2 and 8.4 mmol NO₃⁻ and ~3 h postadministration of 16.8 mmol NO₃⁻.

**Table 1. Pulmonary gas-exchange variables during moderate- and severe-intensity exercise following supplementation with 3 different volumes of beetroot juice and placebo**

<table>
<thead>
<tr>
<th></th>
<th>70 ml Placebo</th>
<th>Nitrate, 4.2 mmol</th>
<th>140 ml Placebo</th>
<th>Nitrate, 8.4 mmol</th>
<th>280 ml Placebo</th>
<th>Nitrate, 16.8 mmol</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Moderate-intensity exercise</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>VO₂</td>
<td>Baseline, l/min</td>
<td>0.94 ± 0.10</td>
<td>0.93 ± 0.09</td>
<td>0.92 ± 0.12</td>
<td>0.94 ± 0.13</td>
<td>0.95 ± 0.12</td>
</tr>
<tr>
<td></td>
<td>End-exercise, l/min</td>
<td>1.64 ± 0.21</td>
<td>1.61 ± 0.21</td>
<td>1.67 ± 0.21</td>
<td>1.64 ± 0.23</td>
<td>1.65 ± 0.19</td>
</tr>
<tr>
<td></td>
<td>Primary amplitude, l/min</td>
<td>0.70 ± 0.16</td>
<td>0.68 ± 0.16</td>
<td>0.74 ± 0.16</td>
<td>0.70 ± 0.16</td>
<td>0.70 ± 0.14</td>
</tr>
<tr>
<td></td>
<td>Primary gain, ml · min⁻¹ · W⁻¹</td>
<td>9.5 ± 1.0</td>
<td>9.2 ± 1.1</td>
<td>10.1 ± 0.9</td>
<td>9.5 ± 0.9</td>
<td>9.6 ± 0.6</td>
</tr>
<tr>
<td>VCVO₂</td>
<td>Baseline, l/min</td>
<td>0.82 ± 0.07</td>
<td>0.81 ± 0.05</td>
<td>0.82 ± 0.09</td>
<td>0.83 ± 0.12</td>
<td>0.86 ± 0.07</td>
</tr>
<tr>
<td></td>
<td>End-exercise, l/min</td>
<td>1.48 ± 0.17</td>
<td>1.45 ± 0.17</td>
<td>1.51 ± 0.17</td>
<td>1.50 ± 0.17</td>
<td>1.52 ± 0.14</td>
</tr>
<tr>
<td>Vr</td>
<td>Baseline, l/min</td>
<td>23 ± 3</td>
<td>22 ± 2</td>
<td>23 ± 3</td>
<td>23 ± 4</td>
<td>24 ± 3</td>
</tr>
<tr>
<td></td>
<td>End-exercise, l/min</td>
<td>37 ± 5</td>
<td>36 ± 5</td>
<td>37 ± 5</td>
<td>37 ± 5</td>
<td>38 ± 5</td>
</tr>
<tr>
<td>RER</td>
<td>Baseline</td>
<td>0.88 ± 0.05</td>
<td>0.88 ± 0.04</td>
<td>0.89 ± 0.04</td>
<td>0.89 ± 0.04</td>
<td>0.91 ± 0.05</td>
</tr>
<tr>
<td></td>
<td>End-exercise</td>
<td>0.91 ± 0.04</td>
<td>0.90 ± 0.04</td>
<td>0.91 ± 0.03</td>
<td>0.92 ± 0.05</td>
<td>0.93 ± 0.04</td>
</tr>
<tr>
<td><strong>Severe-intensity exercise</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>VO₂</td>
<td>Baseline, l/min</td>
<td>1.00 ± 0.10</td>
<td>0.99 ± 0.11</td>
<td>0.99 ± 0.13</td>
<td>0.99 ± 0.11</td>
<td>0.99 ± 0.11</td>
</tr>
<tr>
<td></td>
<td>End-exercise, l/min</td>
<td>3.89 ± 0.40</td>
<td>3.97 ± 0.34</td>
<td>3.96 ± 0.38</td>
<td>3.99 ± 0.40</td>
<td>3.98 ± 0.35</td>
</tr>
<tr>
<td></td>
<td>Overall gain, ml · min⁻¹ · W⁻¹</td>
<td>12.1 ± 0.8</td>
<td>12.5 ± 1.0</td>
<td>12.5 ± 0.9</td>
<td>12.6 ± 1.2</td>
<td>12.6 ± 0.9</td>
</tr>
<tr>
<td></td>
<td>Slow-phase amplitude, 6–2 min; l/min</td>
<td>0.66 ± 0.14</td>
<td>0.65 ± 0.15</td>
<td>0.67 ± 0.17</td>
<td>0.62 ± 0.17</td>
<td>0.75 ± 0.09</td>
</tr>
<tr>
<td>VCVO₂</td>
<td>Baseline, l/min</td>
<td>0.91 ± 0.06</td>
<td>0.90 ± 0.08</td>
<td>0.89 ± 0.08</td>
<td>0.91 ± 0.09</td>
<td>0.92 ± 0.08</td>
</tr>
<tr>
<td></td>
<td>End-exercise, l/min</td>
<td>4.16 ± 0.36</td>
<td>4.17 ± 0.27</td>
<td>4.21 ± 0.38</td>
<td>4.18 ± 0.32</td>
<td>4.20 ± 0.25</td>
</tr>
<tr>
<td>RER</td>
<td>Baseline</td>
<td>0.91 ± 0.05</td>
<td>0.91 ± 0.06</td>
<td>0.91 ± 0.06</td>
<td>0.92 ± 0.06</td>
<td>0.94 ± 0.05</td>
</tr>
<tr>
<td></td>
<td>End-exercise</td>
<td>1.07 ± 0.06</td>
<td>1.05 ± 0.05</td>
<td>1.06 ± 0.05</td>
<td>1.05 ± 0.05</td>
<td>1.06 ± 0.05</td>
</tr>
<tr>
<td>Time-to-task failure(s)</td>
<td>470 ± 81</td>
<td>508 ± 102</td>
<td>498 ± 113</td>
<td>570 ± 153</td>
<td>493 ± 114</td>
<td>552 ± 117</td>
</tr>
</tbody>
</table>

Values are means ± SD. VO₂, oxygen uptake; VCVO₂, expired carbon dioxide; Vr, ventilation; RER, respiratory exchange ratio. *Significantly different from placebo (PL70 (P < 0.05); †Significantly different from PL280 (P < 0.05); ‡Significantly different from beetroot juice (BR140 (P < 0.05); §Significantly different from BR70 (P < 0.05); ‡Significantly different from PL140 (P < 0.05).
lingual bacteria (16, 39). These pharmacokinetic responses to BR supplementation are consistent with those reported previously following acute ingestion of KNO₃ (19). Together, these data suggest that the pharmacokinetics of plasma [NO₃⁻] and [NO₂⁻] are dose dependent when NO₃ is administered, either as NO₃ 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 NO₃. More specifically, acute ingestion of 4.2, 8.4, and 16.8 mmol inorganic NO₃, administered in the form of BR, resulted in peak reductions of systolic BP of ~5, ~10, and ~9 mmHg and peak reductions of MAP of ~2, ~5, and ~5 mmHg, respectively. Moreover, BR ingestion resulted in a similar “threshold” effect on diastolic BP, with peak reductions of ~3 and ~4 mmHg following administration of 8.4 and 16.8 mmol NO₃; however, ingestion of 4.2 mmol NO₃ did not reduce diastolic BP significantly. These reductions in BP are similar to those reported by Kapil et al. (19) following acute administration of KNO₃, except that Kapil et al. (19) reported a dose-dependent reduction in BP up to 24 mmol KNO₃. The reason for this discrepancy between studies is unclear. Interestingly, compared with Kapil et al. (19), who reported 6 mmHg and 9 mmHg reductions in systolic BP following the consumption of 12 mmol and 24 mmol KNO₃, 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 NO₃ contained in 140 ml BR). It is possible that this apparent greater potency of BR compared with NO₃ salt in reducing BP is related to the polyphenols and other antioxidants present in BR, which may facilitate a more efficient conversion of NO₃ to NO₂ (30). Interestingly, although the peak reduction in BP was not significantly different between 8.4 and 16.8 mmol NO₃, the mean reduction in BP over 24 h was dose dependent, with MAP, for example, reduced by 1, 2, and 4 mmHg following administration of 4.2, 8.4, and 16.8 mmol NO₃, respectively.

The results of the present study suggest that BR (and presumably other NO₃-rich vegetable) consumption can provide a natural approach to maintaining or improving BP and

As expected, the rise in plasma [NO₂⁻] was smaller compared with plasma [NO₃⁻], with peak increases of ~2.5-fold, approximately fourfold, and approximately eightfold, respectively. The delayed peak increases in plasma [NO₃⁻] compared with plasma [NO₂⁻] reflect the importance of the enterosalivary circulation and subsequent reduction of NO₃ to NO₂ by

Table 2. Heart rate and blood lactate responses to moderate- and severe-intensity exercise following supplementation with 3 different volumes of beetroot juice and placebo

<table>
<thead>
<tr>
<th></th>
<th>70 ml</th>
<th>140 ml</th>
<th>280 ml</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Placebo</td>
<td>Nitrate, 4.2 mmol</td>
<td>Placebo</td>
</tr>
<tr>
<td><strong>Moderate-intensity exercise</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Heart rate, beats/min</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>89 ± 9</td>
<td>89 ± 8</td>
<td>88 ± 8</td>
</tr>
<tr>
<td>End-exercise</td>
<td>116 ± 11</td>
<td>116 ± 12</td>
<td>115 ± 10</td>
</tr>
<tr>
<td>Blood [lactate], mM</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>1.1 ± 0.3</td>
<td>1.1 ± 0.5</td>
<td>1.1 ± 0.4</td>
</tr>
<tr>
<td>End-exercise</td>
<td>1.2 ± 0.2</td>
<td>1.2 ± 0.5</td>
<td>1.2 ± 0.5</td>
</tr>
<tr>
<td>Δ</td>
<td>0.1 ± 0.2</td>
<td>0.1 ± 0.4</td>
<td>0.1 ± 0.3</td>
</tr>
<tr>
<td><strong>Severe-intensity exercise</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Heart rate, beats/min</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>99 ± 9</td>
<td>100 ± 8</td>
<td>99 ± 9</td>
</tr>
<tr>
<td>End-exercise</td>
<td>186 ± 11</td>
<td>186 ± 12</td>
<td>185 ± 12</td>
</tr>
<tr>
<td>Blood [lactate], mM</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>0.9 ± 0.4</td>
<td>0.9 ± 0.4</td>
<td>0.9 ± 0.4</td>
</tr>
<tr>
<td>Task failure</td>
<td>9.7 ± 1.4</td>
<td>9.4 ± 1.6</td>
<td>9.4 ± 1.6</td>
</tr>
<tr>
<td>Δ</td>
<td>8.8 ± 1.2</td>
<td>8.5 ± 1.7</td>
<td>8.5 ± 1.7</td>
</tr>
</tbody>
</table>

Values are means ± sd. [lactate], lactate concentration; Δ, change.
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% (27–29, 31). The beneficial hemodynamic effects of NO3 supplementation are thought to be due to the reduction of NO3 to NO2 and then to NO within the blood vessel (13), resulting in arterial dilatation and a reduced peripheral resistance (39). However, it is possible that NO2 itself may also exert a direct effect on the vascular system, independent of NO formation (1). There are several advantages to using inorganic rather than organic NO3 for the prevention or treatment of hypertension (33). These include a slow and controlled increase in plasma [NO2-] following inorganic NO3 intake (due to NO3 uptake into the enterosalivary circulation) compared with the more abrupt changes in plasma [NO3-] (perhaps to toxic levels) and BP, which can occur with organic NO3- administration (33). Moreover, unlike the chronic administration of organic NO3-, inorganic NO3 does not appear to lead to the development of tolerance (37) and endothelial dysfunction (33).

S2: Dose Response

The results of S2 confirm that concentrated BR consumption causes a dose-dependent increase in plasma [NO3-] by 334%, 778%, and 1,556% and plasma [NO2-] by 121%, 218%, and 338%, 2.5 h postingestion of 4.2, 8.4, and 16.8 mmol NO3, respectively. The magnitude of the increase in plasma [NO2-] following consumption of 8.4 and 16.8 mmol NO3 in the present study was much larger than the approximate 15–150% rise in plasma [NO2-], reported previously, following acute (approximately 4–6 mmol) (5, 21, 25, 37) and chronic (approximately 5–6 mmol/day) (2, 3, 22, 26, 37) dietary NO3 supplementation. This finding is likely a consequence of the relatively higher NO3 doses (8.4 and 16.8 mmol NO3) administered in the present study. Interestingly, the group mean plasma [NO3-] and [NO2-] reported in S2 are somewhat lower than those reported at 2–4 h postingestion of BR in S1. Given that there was distinct subject recruitment for S1 and S2, it is likely that this discrepancy is due to individual variations in the pharmacokinetic response to BR consumption. For example, when the individual plasma [NO2-] responses to the ingestion of 16.8 mmol NO3 in S1 are considered, peak concentrations ranged from 493 to 1,523 nM, and the time-to-peak concentration ranged from 130 to 367 min. The cause of this wide interindividual variability in the response of plasma [NO2-] to NO3 ingestion is unclear, although it may depend, in part, on salivary flow rate; also, it is known that the reduction of NO3 to NO2 is highly dependent on the activity of oral bacteria (16, 39). Another consideration is that the absolute NO3 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 NO3 doses when expressed relative to BM (0.05–0.07, 0.09–0.13, and 0.19–0.25 mmol NO3/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 NO3 supplementation in humans. We assessed the acute response to three different doses of BR at 2.5 h postingestion, based on the significant dose-dependent elevation in plasma [NO2-] observed at 2–3 h postingestion in S1 (Fig. 1B). The steady-state VO2 measured over the final 30 s of moderate-intensity cycle exercise was unaffected by 4.2 mmol NO3, tended to be lower (~30 ml/min) following administration of 8.4 mmol NO3, and was reduced significantly (by ~50 ml/min) following administration of 16.8 mmol NO3.

The reduction in steady-state VO2 (~3%), observed following acute ingestion of 16.8 mmol NO3 (~0.23 mmol/kg BM), is similar to that reported 2.5 h postingestion of 5.2 mmol NO3 (~0.07 mmol/kg BM) in the form of nonconcentrated BR (37) but is smaller than the 6% reduction reported 1 h postingestion of 0.033 mmol/kg BM sodium nitrate (25). In contrast to acute ingestion, longer-term BR supplementation (3–6 days at approximately 5–7 mmol NO3/day) resulted in an approximate 5–7% reduction in steady-state VO2 during moderate-intensity cycling (3, 26) and running (22).

Previous studies have indicated that the lowering of submaximal exercise VO2, following dietary NO3 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 quickly enough to explain the effects observed so soon (1–2.5 h) after NO3 ingestion (25, 37). Alternatively, NO may acutely and reversibly impact 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 O2 cost of exercise following BR ingestion. The mechanistic basis for the acute changes in the O2 cost of exercise following BR ingestion warrants further investigation.

An interesting observation was the dose-dependent increase in baseline and end-exercise VCO2, irrespective of condition (i.e., PL or BR). This small but significant rise in VCO2 led to a dose-dependent increase in RER that was more pronounced during baseline cycling compared with the exercising steady-state. An elevation in RER is indicative of a shift in substrate use toward a relatively greater reliance on carbohydrate and is likely due to the sugar content of the concentrated BR and PL beverages (~16 g/70 ml).

Dose Response: Severe-Intensity Exercise

A novel finding of the present study was that 8.4 and 16.8 mmol NO3, but not 4.2 mmol NO3, 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 of BR supplementation at a lower dose (5–6 mmol NO3) (3, 22). Although the mechanism(s) responsible for the ergogenic potential of NO3 supplementation remain uncertain, they are believed to be mediated via a biochemical reduction of ingested NO3 to biologically active NO2 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 phosphocreatine cost of muscle force production (2, 15), has 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 preferentially distributed to muscle groups that principally contain type II fibers, 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 calcium ion (Ca$^{2+}$) handling in fast-twitch fibers (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 that demonstrated effects of NO$_3^-$ supplementation on muscle metabolic and vascular control mechanisms (2, 14, 17, 24) used 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 NO$_2^-$ into the brachial artery. It is possible that the improved time-to-task failure that we observed with 8.4 and 16.8 mmol NO$_3^-$ was related to improved blood flow to muscle or to a 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 an additional effect of consuming the higher NO$_3^-$ dose on the peak reduction in BP that we observed in S1, 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 compared with 8.4 mmol) following ingestion of 16.8 mmol 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) is 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, whereas an increase in NO bioavailability may result in a more efficient mitochondrial function (24) and changes to type II fiber 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 8.4 and 16.8 mmol NO$_3^-$ in the present study, were evident without any significant changes in the VO$_2$ response to exercise. Neither the amplitude of the VO$_2$ slow component nor the end-exercise VO$_2$ was influenced by acute ingestion of up to 16.8 mmol 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 VO$_2$ slow-component amplitude by 23% and improved exercise tolerance by $\sim$16%. In contrast, Kelly et al. (20) reported that 3 days of BR supplementation improved exercise tolerance at three different severe intensities by 12–17%, without any accompanying changes in the VO$_2$ response. We found no difference in end-exercise VO$_2$ between BR and PL at any dose. In the severe exercise-intensity domain, the VO$_2$ at the point of volitional exhaustion would be expected to equal the maximum VO$_2$ (VO$_2$ max) (11). Our results are therefore consistent with some (3, 37) but not all (5, 25) previous studies that indicate that NO$_3^-$ supplementation does not reduce VO$_2$ max. Interestingly, there was a disconnect between the effects of BR on steady-state VO$_2$ 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 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 an approximate 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 of BR supplementation (6). A 1% improvement in performance is highly meaningful in elite sport. For example, it could improve 1,500-m running performance by $\sim$2 s or 3,000-m running performance by approximately 4–5 s in international standard athletes. It remains unclear, however, whether elite athletes may confer a performance benefit from NO$_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 fibers in the muscles of highly endurance trained compared with moderately trained subjects (40). It is possible that the dose-response relationship between NO$_3^-$ ingestion and changes in exercise performance are different in elite compared with sub-elite subjects, such that larger NO$_3^-$ doses and/or longer supplementation periods may be 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 interindividual differences in the speed with which ingested NO$_3^-$ is reduced to NO$_2^-$, which may preclude any more specific advice other than to consume NO$_3^-$ some 2–3 h before the start of exercise.
It has been suggested previously that there may be "responders" and nonresponders to dietary NO3− supplementation (40), and there was evidence of this in the present study. Interestingly, the number of nonresponders (in terms of exercise capacity) decreased as the dose ingested increased. For example, there were three nonresponders in the 4.2-mmol condition, two in the 8.4-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 4.2 or 8.4 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 NO3− ingestion. Unlike in our previous study (40), the increase in plasma [NO2−] from baseline to pre-exercise for the nonresponders was not smaller than that measured in other subjects who did respond, and the nonresponders did not have particularly high baseline plasma [NO2−]. In a recent study, we found that the subjects who demonstrated improvement in high-intensity, intermittent exercise performance following dietary NO3− supplementation were those whose plasma [NO2−] fell significantly during exercise (41). We did not measure plasma [NO2−] postexercise in the present study. The explanation for the existence of responders and nonresponders to dietary NO3− supplementation is presently obscure.

In conclusion, dietary supplementation with NO3−-rich BR dose dependently increased plasma [NO3−] and [NO2−] up to 16.8 mmol NO3− and caused peak reductions in systolic BP and MAP dose dependently, up to 8.4 mmol NO3−. These results suggest that the consumption of high NO3− foodstuffs may be an effective strategy for maintaining and perhaps enhancing vascular health in young adults. The present study also demonstrated that the O2 cost of moderate-intensity exercise is reduced dose dependently, up to 16.8 mmol NO3−. Supplementation with 4.2 mmol NO3− did not enhance time-to-task failure relative to PL; however, supplementation with 8.4 mmol NO3− significantly improved time-to-task failure relative to PL, with no further improvement evident following supplementation with 16.8 mmol NO3−. Although the mechanistic bases for the reduction in the O2 cost of submaximal exercise and enhancements in exercise tolerance following acute dietary BR remain unclear, these results provide important, practical information that may underpin the potential use of BR/NO3− supplementation for improving cardiovascular health in the general population and for enhancing exercise performance in athletes.

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AUTHOR CONTRIBUTIONS


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