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Breese, B., McNarry, M., Marwood, S., Blackwell, J., Bailey, S. & Jones, A. (2013). Beetroot juice supplementation speeds O2 uptake kinetics and improves exercise tolerance during severe-intensity exercise initiated from an elevated metabolic rate. *AJP: Regulatory, Integrative and Comparative Physiology, 305*(12), R1441-R1450.
http://dx.doi.org/10.1152/ajpregu.00295.2013

Beetroot juice supplementation speeds O_2 uptake kinetics and improves exercise tolerance during severe-intensity exercise initiated from an elevated metabolic rate

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Running Head: Nitrate and $\dot{V}_{O2}$ kinetics

Abstract

Recent research has suggested that dietary nitrate (NO_3^-) supplementation might alter the physiological responses to exercise via specific effects on type II muscle. Severe-intensity exercise initiated from an elevated metabolic rate would be expected to enhance the proportional activation of higher-order (type II) muscle fibers. The purpose of this study was therefore to test the hypothesis that, compared to placebo (PL), NO_3^--rich beetroot juice (BR) supplementation would speed the phase II $\dot{V}\text{o}_2$ kinetics (τ_p) and enhance exercise tolerance during severe-intensity exercise initiated from a baseline of moderate-intensity exercise. Nine healthy, physically-active subjects were assigned in a randomized, double-blind, crossover design to receive BR (140 mL/day, containing ~8 mmol of NO_3^-) and PL (140 mL/day, containing ~0.003 mmol of NO_3^-) for 6 days. On days 4, 5 and 6 of the supplementation periods, subjects completed a double-step exercise protocol that included transitions from unloaded-to-moderate intensity exercise (U→M) followed immediately by moderate-to-severe-intensity exercise (M→S). Compared to PL, BR elevated resting plasma nitrite concentration (PL: 65 ± 32 vs. BR: 348 ± 170 nM, $P<0.01$) and reduced the $\dot{V}\text{o}_2$ τ_p in M→S (PL: 46 ± 13 vs. BR: 36 ± 10 s, $P<0.05$) but not U→M (PL: 25 ± 4 vs. BR: 27 ± 6 s, $P>0.05$). During M→S exercise, the faster $\dot{V}\text{o}_2$ kinetics coincided with faster NIRS-derived muscle [deoxyhemoglobin] kinetics (τ; PL: 20 ± 9 vs. BR: 10 ± 3 s, $P<0.05$) and a 22% greater time-to-task failure (PL: 521 ± 158 vs. BR: 635 ± 258 s, $P<0.05$). Dietary supplementation with NO_3^--rich BR juice speeds $\dot{V}\text{o}_2$ kinetics and enhances exercise tolerance during severe-intensity exercise when initiated from an elevated metabolic rate.

Key Words: nitric oxide, muscle oxygenation, fatigue, phase II time constant, motor unit recruitment.

Introduction

A step increment in skeletal muscle force production mandates an immediate increase in ATP turnover within the contracting myocytes. However, following an initial cardiodynamic phase (phase I), pulmonary O_2 uptake ($\dot{V}o_2$) rises in an exponential fashion following the onset of exercise with similar response kinetics (denoted by the phase II time constant, τ_p) to that of muscle $\dot{V}o_2$ (28, 38). In order to compensate for this relative lag in oxidative energy transfer, the energy yield from phosphocreatine (PCr) breakdown and 'anaerobic' glycolysis is increased until a steady-state in $\dot{V}o_2$ is attained, at which time the oxidative reconstitution of ATP is coupled to the rate of muscle ATP utilization (56). While a $\dot{V}o_2$ steady-state is attained within ~2-3 min following the onset of moderate-intensity exercise (i.e. below the gas exchange threshold, GET), a supplementary $\dot{V}o_2$ slow component emerges during exercise above the GET that delays the attainment of steady-state within the heavy-intensity exercise domain (i.e., above the GET but below the critical power, CP) or results in the attainment of the maximal O_2 uptake ($\dot{V}o_{2max}$) during severe-intensity exercise (> CP) when this is continued to the limit of tolerance (57, 66, 69). The $\dot{V}o_2$ slow component develops concomitantly with a progressive reduction in muscle [PCr] (59, 60), reflecting a reduction in contractile efficiency as constant-work-rate exercise is continued (34). Interventions that reduce τ_p or the rate of development of the $\dot{V}o_2$ slow component would be expected to positively impact on exercise tolerance (16).

Dietary supplementation with inorganic nitrate (NO_3^-), which undergoes a stepwise reduction to nitrite (NO_2^-) and then nitric oxide (NO) and other reactive nitrogen species (48), has been reported to reduce the O_2 cost of submaximal exercise (2, 5, 18, 44-47, 63) in association with a lower ATP cost of muscle force production (2) and an increase in the mitochondrial ratio of phosphate radicals esterified to atoms of oxygen consumed (P/O ratio; (45)). Muscle oxygenation is greater in contracting skeletal muscle following NO_3^- ingestion (5), while intravenous nitrite infusion has been shown to increase skeletal muscle blood flow at rest and during exercise (25). These physiological effects likely account, at least in part, for the improved exercise tolerance (2, 5, 35, 44, 46) and exercise performance (18, 43) that has been reported following NO_3^- supplementation. Recent studies have indicated that NO_3^- treatment might particularly alter metabolic and vascular control in type II muscles or muscle fibers (23, 24, 32). Specifically, contractile force, rate of force development and sarcoplasmic reticulum calcium release were improved in type II but not type I muscle in mice supplemented with NO_3^- (32), while augmented blood flow, predominantly within locomotor muscles comprising a greater proportion of type II fibers, was reported in rats fed NO_3^- rich beetroot juice (23). However, the potential muscle fiber-type dependency of NO_3^- supplementation on the physiological responses to exercise has not been investigated in humans.

The size principle of Henneman and Mendell (29) posits that skeletal muscle fibers are recruited in a hierarchical manner during exercise according to the requirements for muscle force production. A protocol that has been employed to interrogate the metabolic response of different muscle fiber populations to exercise is the “work-to-work” step exercise test (14, 22, 33). In this protocol, transitions to a higher metabolic rate are divided into two increments in work rate (i.e. lower step and upper step) to manipulate motor unit recruitment and hence reveal the metabolic response profiles of different segments of the motor unit pool. For example, a transition from unloaded cycling to a moderate-intensity work rate (U→M) would be expected to mandate the recruitment of muscle fibers that are positioned low in the recruitment hierarchy (i.e. type I fibers) whereas a subsequent transition from a moderate- to a severe-intensity work rate (M→S) would be expected to require the recruitment of muscle fibers positioned higher in the recruitment hierarchy (i.e. type II fibers) (42). Compared to U→M, the $\dot{V}O_2$ τ_p during M→S is greater (i.e., $\dot{V}O_2$ kinetics are slower) (22, 68). Moreover, compared to a transition from unloaded cycling to a severe-intensity work rate (U→S), the $\dot{V}O_2$ τ_p during M→S is greater and the amplitude of the $\dot{V}O_2$ slow component is truncated, such that the overall response reverts towards being ‘first-order’ (20-22, 67, 68). It is possible that the slower $\dot{V}O_2$ kinetics in M→S compared to U→M reflects a relative imbalance in muscle O_2 supply relative to demand. Consistent with this, it has been reported that microvascular $P\text{O}_2$ (which reflects the dynamic balance between muscle O_2 delivery and muscle O_2 utilization) declines more rapidly during contractions in predominantly type II compared to type I muscle (10, 51). Given that NO_3^- supplementation has been reported to increase both the absolute and relative distribution of blood flow toward contracting type II muscle (23), this might be expected to improve the local matching of O_2 delivery relative to muscle $\dot{V}O_2$ and therefore to speed phase II $\dot{V}O_2$ kinetics during M→S. While NO_3^- supplementation does not reduce the $\dot{V}O_2$ τ_p during either U→M or U→S (2, 5, 44), the effect of NO_3^- supplementation on the $\dot{V}O_2$ τ_p during M→S has yet to be investigated.

Therefore, the purpose of this study was to investigate the effects of short-term dietary NO_3^- supplementation on $\dot{V}O_2$ kinetics during work-to-work exercise transitions, i.e. U→M followed immediately by M→S. We used the muscle deoxyhemoglobin concentration ([HHb]) signal from near infrared spectroscopy (NIRS) measurements to explore the mechanistic bases for any NO_3^--induced changes in phase II $\dot{V}O_2$ dynamics. The kinetics (τ) of muscle [HHb] following the onset of exercise resembles that of mixed venous [O_2] (28, 38) and approximates the reduction in microvascular P_{O_2} during transitions from rest-to-electrically stimulated contractions (36). The [HHb] signal is therefore considered to provide an index of local O_2 extraction (19, 27) and hence to reflect the balance between muscle O_2 delivery and muscle O_2 utilization. We hypothesized that NO_3^- supplementation would reduce the $\dot{V}O_2$ τ_p and increase the muscle [HHb] τ in M→S but not U→M. We also hypothesized that these kinetic changes following NO_3^- supplementation would enhance severe-intensity exercise tolerance.

Methods

Participants

Nine healthy subjects (4 male: mean ± SD age 30 ± 6 years; body mass 77 ± 11 kg; stature 1.78 ± 0.06 m, and 5 female: mean ± SD age 30 ± 6 years; body mass 58 ± 4 kg; stature 1.66 ± 0.02 m) volunteered to participate in the study. The participants were all recreationally active, but not highly trained. Prior to testing, participants were informed of the protocol and risks and gave written consent to participate in the study. All procedures were approved by Swansea University ethics committee and were conducted in accordance with the Declaration of Helsinki. Participants were asked to arrive at the exercise physiology laboratory at Swansea University in a rested state, at least two hours postprandial and to avoid strenuous exercise in the 24 h preceding each testing session. Participants were also asked to refrain from caffeine and alcohol for 6 and 24 h before each test, respectively. The participants also refrained from the use of antibacterial mouthwash throughout the duration of the study (26). All tests were performed at the same time of day (± 0.5 h).

Procedures

Participants were required to visit the laboratory on seven occasions over a 4-week period. On the first visit, participants completed a ramp incremental exercise test for determination of the $\dot{V}o_{2peak}$ and GET. The test included 3-min of baseline cycling at 15W, after which the work rate was increased at a rate of 20 W·min^{-1} for females and 30 W·min^{-1} for males until the limit of tolerance. The participants were asked to maintain a cadence of 70–80 rpm. Breath-by-breath pulmonary gas-exchange data were collected continuously during the incremental tests and averaged over consecutive 5-s periods (Oxycon Pro, Jaeger, Germany). The $\dot{V}o_{2peak}$ was taken as the highest 10-s mean value attained before the subject's volitional exhaustion in the test. The GET was determined using the V-slope method (9) as the first disproportionate increase in CO_2 production ($\dot{V}co_2$) relative to the increase in $\dot{V}o_2$, and subsequently verified by an increase in the ventilatory equivalent for $\dot{V}o_2$ ($\dot{V}_E$ /$\dot{V}o_2$) with no increase in $\dot{V}_E$ /$\dot{V}co_2$. The work rates that would require 90% of the GET (moderate-intensity exercise) and 70% of the difference (Δ) between the GET and $\dot{V}o_{2peak}$ (severe-intensity exercise, Δ70%) were subsequently determined, with account taken of the mean response time for $\dot{V}o_2$ during ramp exercise [i.e. two thirds of the ramp rate was deducted from the work rate at the GET and peak $\dot{V}o_2$ (65)].

Following the ramp incremental test, participants were randomly assigned in a crossover, double-blind design to receive 6 days of dietary supplementation with NO_3^--rich beetroot juice (BR) (140 mL/day; ~ 8 mmol NO_3^-; Beet It, James White Drinks, Ipswich, UK) or NO_3^--depleted BR as a placebo (PL; 140 mL/day; 0.0034 mmol NO_3^-; Beet It, James White Drinks, Ipswich, UK). The placebo NO_3^--depleted BR beverage was identical in color, taste, smell and texture to the experimental NO_3^- -rich BR beverage. The PL beverage was created by passage of the juice, before pasteurization, through a column containing Purolite A520E ion exchange resin, which selectively removes NO_3^- ions. Five participants began with the BR condition, and the other four participants began with the PL condition. The subjects were instructed to consume the beverages (70 mL in the morning and afternoon) on days 1-3 of the supplementation period. On days 4-6, the subjects were instructed to consume the beverages over a 10-min period, 2 h prior to the start of the exercise test (see below), based on recent evidence that plasma [NO_2^-] peaks at approximately 2-2.5 h post-administration of BR containing 8.4 mmol NO_3^- (70). A 7-day washout period separated each supplementation period. Throughout the study, subjects were instructed to maintain their normal daily activities and food intake.

On days 4, 5, and 6 of the supplementation periods, subjects completed a series of step exercise tests for the determination of $\dot{V}o_2$ and muscle [HHb] kinetics. The protocol, which was performed on three consecutive days, consisted of 3-min 'unloaded' pedaling at 15 W, followed by 4-min of moderate-intensity cycling (U→M), and then 6-min of severe-intensity cycling (M→S). The tests were performed on separate days because it is known that prior exercise can alter the $\dot{V}o_2$ response to exercise (3). A schematic illustration of the experimental protocol is shown in Fig 1. On day 6 of each supplementation period, the M→S bout was continued until task failure. The participants were blinded to the elapsed exercise time in both the BR and PL conditions. The time to task failure was used as a measure of exercise tolerance and was recorded when the pedal rate fell by > 10 rpm below the required pedal rate. In total, the participants completed three bouts of U→M and M→S exercise following BR and PL ingestion, with the $\dot{V}o_2$ data being subsequently ensemble-averaged prior to curve-fitting to enhance the signal-to-noise ratio.

Measurements

Venous blood samples (~ 4 ml) were drawn into lithium-heparin tubes (7.5 ml Monovette Lithium Heparin, Sarstedt, Leicester, UK), which have very low levels of NO_3^- and NO_2^-, on each of days 4-6. Within 3 min of collection, the samples were centrifuged at 2700 g and 4°C for 10 min. Plasma was extracted and immediately frozen at -80°C for later analysis of [NO_2^-] using a modification of the chemiluminescence technique (7). All glassware, utensils, and surfaces were rinsed with deionized water to remove residual NO_2^- prior to analysis. Following defrosting at room temperature, 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 nitric oxide 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.

Throughout all exercise tests, participants wore a facemask and breathed through a low dead space (90 ml), low resistance (0.75 $mmHg \cdot l^{-1} \cdot s^{-1}$ at 15 $l \cdot s^{-1}$) impeller turbine assembly (Jaeger Triple V, Hoechberg, Germany). The inspired and expired gas volumes and gas concentration signals were continuously sampled at 100 Hz, the latter using paramagnetic (O_2) and infrared (CO_2) analyzers (Jaeger Oxycon Pro, 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 L 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 the volume signal. Breath-by-breath fluctuations in lung gas stores were corrected for by computer algorithms (8). A Reynolds Lifecard CF digital Holter recorder (Spacelabs Medical Ltd., Hertford, UK) was used to record a three-lead ECG continuously throughout the tests. The ECG leads were positioned in the modified V5, CC5, modified V5R electrode configuration. This system provided ECG data with a sample accuracy of 2.5 µV and 1024 Hz sampling frequency. During one of the U→M and M→S transitions, for both supplementation periods, a blood sample was collected from a fingertip into a capillary tube over the 20 s preceding the step transition in work rate and within the last 20 s of exercise. A capillary blood sample was also collected at the limit of tolerance for the M→S bout performed on day 6 of each supplementation period. The blood samples were subsequently analyzed to determine [lactate] (YSI 1500, Yellow Springs Instruments, Yellow Springs, OH) within 30 s of collection. Blood lactate accumulation was calculated as the difference between blood [lactate] at end-exercise and blood [lactate] at baseline.

NIRS was used to monitor changes in oxygenation status of the *m. vastus lateralis* of the right leg during step exercise (NIRS; OxiplexTS; ISS, Champaign, IL). The NIRS probe was affixed over the midway point between the greater trochanter and lateral epicondyle of the right leg using adhesive tape and secured by elastic Velcro strapping to ensure the device remained stationary and to minimize the interference of extraneous light during exercise. Source (NIR) light was emitted into the muscle at wavelengths of 690 and 830 nm and detection sampled at 2 Hz to measure absolute concentrations (µM) of oxyhemoglobin (HbO_2) and deoxyhemoglobin (HHb) within the microcirculation of the interrogated muscle region. Light source-detector separation distances of 1.50–3.04 cm for each wavelength were used with cell water concentration assumed to be constant at 70%. The NIRS probe was calibrated before each testing session using a calibration block of known absorption and scattering coefficients. Calibration was then cross-checked using a second block of known but distinctly different absorption and scattering coefficients. Each of these procedures was performed according to the manufacturer's recommendations. The contribution of myoglobin (Mb) to the NIRS signal is generally accepted to be relatively small (50, 62) but is currently unresolved. The [HHb] signal reported herein should therefore be considered to reflect the combined concentrations of both deoxygenated Hb and Mb.

Data analysis procedures

The breath-by-breath $\dot{V}o_2$ data from each step exercise bout were initially examined to exclude 'errant' breaths by removing values lying more than four standard deviations from the local mean determined using a 5-breath rolling average. Filtered $\dot{V}o_2$ data were subsequently linearly interpolated to provide second-by-second values and, for each individual, identical repetitions of each exercise condition were time aligned to the start of exercise and averaged together to form a single data set for analysis.

For the U→M transition, the first 20 s of data after the onset of exercise were deleted to remove the phase I (cardio-dynamic) response and a mono-exponential model with time delay (Eq.1) was then fitted to the averaged $\dot{V}o_2$ data.

$$\Delta\dot{V}o_{2(t)} = A_1 \cdot (1 - e^{-(t-\delta_1)/\tau_1}) \qquad \text{(Eq. 1)}$$

where $\Delta\dot{V}o_2$ is the increase in $\dot{V}o_2$ at time t above the baseline value (calculated as the mean $\dot{V}o_2$ from the first 45-s of the last min of baseline pedaling), and A_1, δ_1 and τ_1 are the primary component amplitude, time delay (which was allowed to vary freely), and time constant, respectively. Kinetic variables (A_1, δ_1 and τ_1) and their 95% confidence intervals were determined by least squares non-linear regression analysis (Graphpad Prism, Graphpad Software, San Diego, CA).

A mono-exponential model was ultimately used for both moderate and severe-intensity exercise because, for the M→S transition, a bi-exponential model (Eq. 2) produced an inferior and ambiguous fit based on analysis of the model residuals.

$$\Delta\dot{V}\text{o}_{2(t)} = A_1 \cdot (1 - e^{-(t-\delta_1)/\tau_1}) + A_2 \cdot (1 - e^{-(t-\delta_2)/\tau_2}) \qquad \text{(Eq. 2)}$$

Given the failure of the bi-exponential model to adequately describe the $\dot{V}\text{o}_2$ response during M→S, the onset of the $\dot{V}\text{o}_2$ slow component was determined using purpose designed LabVIEW software which iteratively fits a mono-exponential function to the $\dot{V}\text{o}_2$ data until the window encompasses the entire response. The estimated τ for each fitting window was plotted against time and the onset of the $\dot{V}\text{o}_2$ slow component was identified as the point at which the estimated τ consistently deviated from the previously "flat" profile (61). The amplitude of the $\dot{V}\text{o}_2$ slow component was subsequently determined by calculating the difference between the end exercise $\dot{V}\text{o}_2$ and the sum of the primary amplitude and baseline $\dot{V}\text{o}_2$. This was expressed both in absolute terms and relative to the end-exercise $\dot{V}\text{o}_2$. The functional gain of the primary $\dot{V}\text{o}_2$ response during U→M and M→S was also calculated by dividing the primary phase amplitude by the change in work rate. Finally, the mean response time (MRT) for both U→M and M→S was calculated by fitting a single exponential curve to the data with no time delay from the onset to the end of exercise.

The NIRS-derived [HHb] response to exercise was also modeled to provide information on muscle oxygenation. The responses to each transition were interpolated to 1 s intervals, time aligned and averaged to produce a single data set. Since the [HHb] signal increased after a short delay in response to step exercise, the time of onset for the exponential-like rise in [HHb] was defined as a 1 SD increase in [HHb] above the mean baseline value (19). The model in Eq. 1 was then used to resolve the [HHb] τ after omitting data points preceding the exponential-like increase. For M→S, the model fitting window was constrained to the onset of the [HHb] slow component determined using the iterative curve fitting procedure as described for $\dot{V}O_2$ above. The primary [HHb] amplitude was divided by the phase II $\dot{V}O_2$ asymptote in order to determine the Δ[HHb]/$\Delta\dot{V}O_2$ ratio as an index of the change in fractional muscle O_2 extraction required to elicit a given $\Delta\dot{V}O_2$ during the primary phase. In addition, we assessed changes in total blood volume by summing the [HbO_2] and [HHb] signals to provide an estimate of the total [Hb_{tot}] in the area under investigation. Specifically, we determined the mean value at baseline (30 s preceding each transition), at 60 s intervals throughout exercise (15 s bins centered on each time point), and at end exercise (final 30 s) to facilitate comparisons between conditions. Finally, heart rate (HR) kinetics was modeled for each condition with the *TD* parameter in Eq. 1 fixed to t = 0 s (i.e. mono-exponential with no delay) and with the fitting window constrained to the onset of the $\dot{V}O_2$ “slow component”.

Statistics

Gaussian distribution was confirmed by the Shapiro-Wilks test. Following this, the pulmonary $\dot{V}O_2$, HR, and NIRS-derived variables were analyzed using two-way repeated measures analysis of variance (ANOVA) with ‘exercise intensity’ (U→M and M→S) and ‘supplement’ (BR vs. PL) included as within-subject factors. Differences in BP and plasma [NO_2^-] were determined using two-way (supplement × time) repeated-measures ANOVA. Subsequent paired samples t-tests were employed as appropriate to identify the location of statistically significant effects. Pearson product moment correlation coefficients were used to analyze the degree of association between key variables. All statistical analyses were conducted using PASW Statistics 18 (SPSS, Chicago, IL). Data are presented as means ± SD. Statistical significance was accepted when $P \leq 0.05$.

Results

The subjects’ peak $\dot{V}O_2$ was 3.73 ± 0.46 $L{\cdot}min^{-1}$ for men and 2.69 ± 0.52 $L{\cdot}min^{-1}$ for women with the GET occurring at 2.08 ± 0.41 $L{\cdot}min^{-1}$ and 1.71± 0.41 $L{\cdot}min^{-1}$, respectively. The peak work rate attained from the incremental test was 327 ± 32 W for men and 263 ± 38 W for women. The work rates calculated to require 90% of the GET and Δ70% were 100 ± 26 and 215 ± 37 W, respectively.

Plasma $[NO_2^-]$

There was a main effect for ‘supplement’ on plasma $[NO_2^-]$ at rest over the last three days of the supplementation period ($F_{[1,8]} = 21.59$, $P = 0.01$). Follow-up paired comparisons revealed that plasma $[NO_2^-]$ was elevated ($P < 0.02$) at each sample point following BR compared to PL ingestion on day 4 (PL: 64 ± 36 vs. BR: 300 ± 141 nM), day 5 (PL: 66 ± 35 vs. BR: 374 ± 149 nM), and day 6 (PL: 65 ± 32vs. BR: 348 ± 170 nM).

Muscle oxygenation

The $[Hb_{tot}]$ and [HHb] values derived from NIRS interrogation are presented in Table 1. There was no significant main effect for ‘supplement’ on the $[Hb_{tot}]$ during U→M and M→S exercise. The [HHb] response during step exercise for a representative subject is illustrated in Fig. 2. Two-way ANOVA revealed a significant interaction effect between ‘exercise intensity’ and ‘supplement’ on [HHb] kinetics following the onset of exercise ($F_{[1,6]} = 15.30$, $P = 0.01$). Specifically, compared to PL, the [HHb] τ was speeded during M→S following BR supplementation (PL: 20 ± 9 vs. BR: 10 ± 3 s, $P = 0.05$) but there were no differences between PL and BR during U→M (PL: 7 ± 3 vs. BR: 10 ± 5 s, $P = 0.17$). The [HHb] τ was significantly slower for M→S compared to U→M in PL ($P = 0.01$) but there was no difference between the upper and lower step in BR ($P = 0.94$) There was no significant main effect for ‘supplement’ on the primary [HHb] amplitude when normalized per unit change in $\dot{V}o_2$ during the fundamental exponential phase ($F_{[1,6]} = 4.81$, $P = 0.07$).

HR kinetics

The HR responses to step exercise are presented in Table 2. There were no differences in the primary HR τ between PL and BR for U→M or M→S ($F_{[1,8]} = 0.10$, $P = 0.77$). During M→S, the relative change in the $\dot{V}o_2$ τ_p was not correlated with the relative change in HR kinetics between conditions ($r = 0.42$, $P = 0.27$). There were no significant differences in blood [lactate] between conditions.

$\dot{V}_{O_2}$ kinetics and exercise tolerance

The $\dot{V}_{O_2}$ kinetic parameters derived from the mono-exponential fit are presented in Table 3 and the $\dot{V}_{O_2}$ response of a representative subject to U→M and M→S is shown in Fig. 2. The group mean $\dot{V}_{O_2}$ profile during M→S is presented in Fig. 3. Two-way ANOVA revealed a significant interaction effect between ‘exercise intensity’ and ‘supplement’ on phase II $\dot{V}_{O_2}$ kinetics following the onset of exercise ($F_{[1,8]} = 18.54$, $P = 0.01$). Compared to PL, the τ_p was shorter during M→S following BR ingestion (PL: 46 ± 13 vs. BR: 36 ± 10 s, $P = 0.01$) but there were no differences during U→M (PL: 25 ± 4 vs. BR: 27 ± 6 s, $P = 0.25$). For the PL condition, the τ_p was greater in M→S compared to U→M ($P = 0.001$), but there were no significant differences between U→M and M→S in the BR condition ($P = 0.12$). During M→S, the speeding of $\dot{V}_{O_2}$ τ_p was not correlated with the speeding of the primary [HHb] τ after BR compared to PL ($r = -0.16$, $P = 0.76$).

There was no significant main effect for ‘supplement’ on the primary $\dot{V}_{O_2}$ amplitude ($F_{[1,8]} = 0.01$, $P = 0.91$) or primary $\dot{V}_{O_2}$ gain ($F_{[1,8]} = 0.05$, $P = 0.83$) during U→M or M→S. The emergence of a slow phase in $\dot{V}_{O_2}$ during M→S occurred after a similar time delay and there were no differences in the absolute or relative amplitude of the $\dot{V}_{O_2}$ slow component between PL and BR (both $P = 0.44$). For M→S, there were no differences between PL and BR in the $\dot{V}_{O_2}$ amplitude at end-exercise ($F_{[1,8]} = 0.60$, $P = 0.46$) or the total $\dot{V}_{O_2}$ gain ($F_{[1,8]} = 0.14$, $P = 0.72$).

The $\dot{V}_{O_2}$ attained at task failure (PL: 3.12 ± 0.51 vs. BR: 3.09 ± 0.51 $L \cdot min^{-1}$) was not different between conditions or when compared to the peak $\dot{V}_{O_2}$ obtained during the initial ramp incremental test ($P > 0.66$). Compared to PL, the exercise time to task failure was significantly increased during M→S following BR supplementation (PL: 521 ± 158 vs. 635 ± 258 s, $P = 0.02$). The time to task failure was greater in every participant after BR compared to PL (range = 3% to 54%; Fig. 4). During M→S, the increased time to task failure was not correlated with the reduction in the $\dot{V}_{O_2}$ τ_p after BR compared to PL ($r = 0.03$, $P = 0.95$).

Discussion

The principal novel finding of this investigation was that six days of dietary supplementation with NO_3^--rich BR juice speeded pulmonary $\dot{V}o_2$ and muscle [HHb] kinetics and increased the time-to-task failure following the onset of M→S exercise compared to NO_3^--depleted PL juice. These results suggest that increasing plasma [NO_2^-], and thus the potential for O_2-independent NO generation after BR supplementation, can speed the $\dot{V}o_2$ τ_p in M→S such that it is not significantly different from the $\dot{V}o_2$ τ_p in U→M. It is possible that this faster rate of ATP resynthesis through oxidative metabolism can account, at least in part, for the improved exercise tolerance observed during M→S exercise after BR supplementation. Given that M→S would be expected to recruit a population of muscle fibers that are positioned higher in the recruitment hierarchy (i.e., type II) compared to U→M (29, 39), these results suggest that BR supplementation may have specific effects on metabolic and/or vascular control in type II muscle fibers in humans, consistent with previous reports in rodent models (23, 32).

In the present study, short-term dietary supplementation with NO_3^--rich BR juice markedly increased plasma [NO_2^-]. Surprisingly, however, this was not associated with a reduced steady-state $\dot{V}o_2$ during U→M. This finding contrasts with previous studies in young, recreationally-active populations (2, 5, 44-47, 63), but is consistent with other studies in which the participants were well-trained (11, 55). Training status does not provide an explanation for the lack of effect of BR ingestion on steady-state $\dot{V}o_2$ during moderate-intensity exercise in the present study because the participants were not well-trained (48 and 46 $ml \cdot kg^{-1} \cdot min^{-1}$ for males and females, respectively). In a recent study investigating the dose-response relationship between acute NO_3^- intake and the physiological responses to exercise (70), it was reported that steady-state $\dot{V}o_2$ during moderate-intensity exercise was significantly reduced following the consumption of 280 ml of BR (~ 16 mmol NO_3^-) but not 70 ml BR (~ 4 mmol NO_3^-) or 140 ml BR (~ 8 mmol NO_3^-). While this suggests that a higher NO_3^- dose than the 8 mmol employed in the present study might have been required to elicit an altered O_2 cost of exercise, it should be noted that significant reductions in steady-state $\dot{V}o_2$ with 5-8 mmol NO_3^- supplementation (administered as BR) have been reported previously (5, 44, 64). The explanation for the lack of effect of BR on steady-state $\dot{V}O_2$ during moderate-intensity exercise in the present study is therefore obscure.

While NO_2^- has traditionally been considered as an inert product of NO oxidation (53), recent studies have shown that NO_2^- can be recycled back into bioactive NO (48). Moreover, in contrast to the generation of NO through the oxidation of L-arginine in a reaction catalyzed by nitric oxide synthase, the reduction of NO_2^- to NO is O_2-independent (17) and is potentiated by acidosis (52). Since pH and microvascular PO_2 decline more rapidly in contracting type II muscle (10, 51), NO_2^- reduction to NO may be a more effective pathway for NO generation in, and within the microvasculature surrounding, type II muscle fibers during contractions.

In this study we have shown for the first time that, compared to PL, BR ingestion speeded phase II $\dot{V}O_2$ kinetics in M→S exercise whereas, consistent with previous research (5, 44), BR did not impact on phase II $\dot{V}O_2$ kinetics during U→M. The intensity-dependent effects of dietary NO_3^- intake with BR on phase II $\dot{V}O_2$ kinetics may be due, at least in part, to differences in muscle fiber activation patterns in U→M and M→S. In accord with an orderly 'size' principle of motor unit recruitment (31), M→S would be predicted to activate a fraction of the total muscle fiber pool positioned higher in the recruitment hierarchy compared to U→M. Empirical evidence to support this postulate is provided by the study of Krustrup et al. (42). These authors reported that PCr and glycogen content were lowered more in type II compared to type I muscle fibers when subjects cycled at an intensity corresponding to 80% $\dot{V}O_{2max}$ whereas the reverse was true at 50% $\dot{V}O_{2max}$ (42). The steady state $\dot{V}O_2$ amplitude in the U→M step in the present study was ~ 54% of $\dot{V}O_{2max}$, suggesting that type I muscle fibers were principally activated in the lower step transition. Conversely, the longer $\dot{V}O_2$ mean response time and increased total $\dot{V}O_2$ gain observed during M→S in the PL condition is consistent with what would be expected if a greater proportional activation of type II muscle fibers occurred in the upper step (6, 40, 41, 58). Our findings therefore suggest that the faster $\dot{V}O_2$ kinetics observed following BR supplementation during M→S might be related to specific effects of NO_3^- treatment on higher-order (i.e. type II) muscle fibers.

To explore the mechanisms responsible for any alterations in $\dot{V}O_2$ τ_p in M→S, the NIRS-derived muscle [HHb] signal was used to provide information on the dynamic (im)balance between microvasculature O_2 delivery and metabolic demand (19, 27). For the same $\dot{V}O_2$ kinetics, enhanced muscle O_2 supply relative to muscle O_2 demand would be expected to result in a longer muscle [HHb] τ, whereas faster $\dot{V}O_2$ kinetics alongside unchanged [HHb] kinetics would be interpreted as a proportionally similar increase in the rate of muscle O_2 delivery to $\dot{V}O_2$. However, in the present study, faster $\dot{V}O_2$ kinetics in M→S with BR was accompanied by a *shorter* [HHb] τ during which [Hb_{tot}] (and by inference blood volume) in the interrogated muscle area was not different compared to PL. This suggests that BR may have speeded $\dot{V}O_2$ kinetics, in part, by enhancing muscle O_2 extraction. It has been reported that muscle O_2 demand exceeds microvasculature O_2 delivery in muscle comprised of predominantly type II fibers (10, 51) and that BR increases muscle bulk blood flow and promotes a greater distribution of blood flow to type II muscle fibers (23). If absolute or relative perfusion of type II fibers was greater after BR ingestion, this might have facilitated enhanced muscle O_2 extraction, as suggested by the faster muscle [HHb] kinetics, and therefore permitted faster $\dot{V}O_2$ kinetics in M→S. However, the faster $\dot{V}O_2$ τ_p with BR compared to PL was not significantly correlated with the reduction in the [HHb] τ. It is therefore also possible that BR speeded $\dot{V}O_2$ kinetics, by altering metabolic control in type II fibers during the transition from M→S. Given that short-term NO_3^- supplementation does not increase markers of mitochondrial biogenesis in skeletal muscle (45), or speed the recovery of [PCr] following intense exercise (44) which would reflect increased muscle oxidative capacity (12), the faster $\dot{V}O_2$ kinetics in M→S is unlikely to have resulted from an increase in mitochondrial volume. Increased intracellular calcium content $[Ca^{2+}]_i$ has been observed during tetanic contractions of type II, but not type I, muscle fibers excised from mice supplemented with NO_3^- (32). As well as activating the muscle contractile apparatus, Ca^{2+} has also been suggested to signal the activation of oxidative phosphorylation (30). Therefore, it is possible that increased $[Ca^{2+}]_i$ and parallel activation of the contractile and oxidative metabolic machinery might have contributed to the faster muscle [HHb] and $\dot{V}O_2$ kinetics reported in this study.

It has been reported previously that the tolerable duration of severe-intensity exercise initiated from an unloaded cycling or resting baseline can be enhanced after a period of BR supplementation (2, 5, 35, 44). The findings of this study extend these earlier reports by showing that the tolerable duration of severe-intensity cycle exercise initiated from a moderate-intensity baseline work rate can also be improved (by ~22% on average). Recent studies show that performance is also enhanced during high-intensity intermittent exercise (13, 71), which would also be expected to engender significant recruitment of type II muscle fibers (39). It has been reported (using multi-channel NIRS) that there is marked inter-site heterogeneity in matching of O_2 delivery to $\dot{V}o_2$ within the quadriceps muscle during high-intensity cycling (37). One possibility is that NO might inhibit O_2 utilization in some well-oxygenated muscle fibers (15) whereas the hypoxic and acidic environment within and surrounding muscle fibers receiving less O_2 might stimulate NO_2^- reduction to NO and thus increase microvascular O_2 supply (29). Faster phase II $\dot{V}o_2$ kinetics during M→S after BR might therefore have resulted from a more homogenous distribution of O_2 relative to metabolic demand within contracting muscle. Interventions that speed $\dot{V}o_2$ kinetics have been previously shown to improve the tolerable duration of severe-intensity exercise (3, 4). A faster adjustment of $\dot{V}o_2$ during M→S would be expected to spare expenditure of the finite anaerobic reserves (i.e. from PCr breakdown and anaerobic glycolysis) and reduce the accumulation of metabolites that have been implicated in the development of skeletal muscle fatigue (1, 16, 54). However, in the present study, whilst an increased time to task failure with BR was accompanied by a shorter $\dot{V}o_2$ τ_p compared to PL, the two were not significantly correlated.

Dietary supplementation with NO_3^--rich BR juice has been reported to improve exercise tolerance in concert with attenuated skeletal muscle ATP turnover, PCr hydrolysis, and P_i and ADP accumulation during high-intensity exercise (2). Perturbations of skeletal muscle Ca^{2+} handling and membrane excitability are also hallmarks of skeletal muscle fatigue (1). In this respect, it is interesting that mice receiving NO_3^- treatment had an improved capacity for sarcoplasmic Ca^{2+} release and increased tetanic force production in type II muscle (32). In humans, BR supplementation appears to blunt the accumulation of extracellular K^+, possibly preserving muscle excitability, during intense intermittent exercise (71). As discussed earlier, improvements in muscle blood flow and a greater distribution of blood flow to type II muscle fibers with BR (23) might also have contributed to the improved exercise performance in this study. The enhanced exercise tolerance observed during M→S in the present study might therefore be consequent to a conflation of alterations in skeletal muscle metabolism, excitation-contraction coupling and perfusion. Additional studies are required to address these issues.

It is of interest that, *in vitro*, NO may inhibit oxidative ATP flux by competing with O_2 for the O_2-binding site at cytochrome-*c* oxidase (COX) in the electron transport chain (15). If NO_3^- supplementation and the associated increased NO production significantly inhibited COX then an increased ATP contribution from anaerobic metabolism would be expected for the same work rate. However, we have reported previously that muscle PCr utilization is reduced and pH is not changed after NO_3^- supplementation (2), which argues against this possibility. NO has many physiological effects and it is possible that any inhibition of COX by NO is offset by other, positive, effects. For example, COX inhibition of fibers nearest a capillary might allow O_2 to diffuse to fibers further from the capillary which might be O_2 deficient (thereby increasing ‘global’ oxidative ATP production across a muscle), (29). There is also evidence that greater NO production via NO_3^- supplementation might improve matching of O_2 supply to O_2 utilization and increase the O_2 driving pressure within contracting muscle (23, 24), increase the mitochondria P/O ratio (45) and improve mitochondrial function in hypoxia (64). Therefore, while the effects of NO on oxidative metabolism are complex, the existing evidence suggests that NO_3^- supplementation has a beneficial rather than a detrimental effect on oxidative function.

Perspectives and significance

In this study we showed that six days of dietary supplementation with NO_3^--rich BR juice speeded pulmonary $\dot{V}o_2$ and muscle [HHb] kinetics and increased the tolerable duration of severe-intensity cycling in M→S compared to PL. It remains to be determined if longer periods of supplementation might elicit greater, or lesser, physiological and performance effects. It has previously been reported that $\dot{V}o_{2max}$ and peak power output during incremental exercise were increased, and that acute reductions of resting blood pressure and the O_2 cost of moderate-intensity exercise were maintained, after 15 days of BR supplementation (63). This indicates that subjects do not develop tolerance to inorganic nitrate intake, at least up to 15 days of supplementation. From the results of the present study, we cannot rule out the possibility that NO_3^-, or its derivatives, might have acted synergistically with other components in the BR juice. For example, ascorbate and polyphenols facilitate the reduction of nitrite to NO (49) which might augment NO production compared to NO_3^- treatment, *per se*. However, since supplementation with NO_3^--depleted BR juice does not impact on blood pressure, $\dot{V}O_2$ responses, blood or exercise tolerance compared to a control condition with no supplementation (44), it appears that NO_3^- is the key active ingredient in BR.

The results of the present study have important implications for competitive sport and also provide insight into the mechanisms by which BR supplementation may improve performance during simulated competition (18, 43), as well as during high-intensity intermittent exercise (13, 71). Continuous athletic events such as cycling and running races are rarely completed at an even pace but are often stochastic with frequent ‘surges’ in speed (i.e., step transitions in metabolic rate) throughout the competition. The results of the present study, which indicate faster $\dot{V}O_2$ kinetics in the transition from a lower to a higher metabolic rate, suggest that BR supplementation has the potential to enhance performance in such events. This provides further support to the notion that short-term BR supplementation may be conducive to exercise performance, at least in recreationally-active participants.

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Figure Legends

Figure 1: Schematic of the step exercise test protocol performed on *days 4-6* of the supplementation period.

Figure 2: NIRS-derived [HHb] of the *m. vastus lateralis* (Panel 1) and pulmonary $\dot{V}o_2$ (Panel 2) in a representative subject during U→M (A) and M→S (B) cycling transitions. Data are normalized relative to the end-exercise amplitude after correcting for [HHb] and $\dot{V}o_2$ during baseline pedaling. The onset of step exercise is indicated by the vertical dotted line. Note the faster [HHb] and $\dot{V}o_2$ dynamics in M→S but not U→M following BR compared to PL supplementation.

Figure 3: Group mean $\dot{V}o_2$ response during M→S exercise following BR and PL supplementation with the group mean ± SEM $\dot{V}o_2$ at task failure also shown. The onset of step exercise is indicated by the vertical dotted line.

Figure 4: Group mean ± SD time to task failure during the upper M→S step bout with individual responses shown (dashed black lines). *Significant difference between supplement conditions ($P<0.05$).

Table 1: Near-infrared spectroscopy-derived $[\mathrm{Hb}_{tot}]$ and [HHb] responses to moderate- and severe-intensity exercise following BR and PL supplementation.

	PL	BR
Unloaded-to-moderate-intensity exercise		
$[\mathrm{Hb}_{tot}]$		
Baseline (μM)	53.4 ± 27.1	50.4 ± 30.0
120 s (μM)	56.4 ± 29.8	52.9 ± 31.2
End (μM)	57.4 ± 30.3	53.9 ± 31.6
[HHb]		
Baseline (μM)	15.3 ± 10.4	14.9 ± 10.9
Primary time delay (s)	10 ± 3	8 ± 4
Primary time constant (s)	7 ± 3	10 ± 5
Primary amplitude (μM)	3.4 ± 4.0	3.3 ± 4.1
$\Delta[\mathrm{HHb}]/\Delta\dot{V}\mathrm{o}_2$ ($\mu\mathrm{M}\cdot\mathrm{L}\cdot\mathrm{min}^{-1}$)	3.4 ± 3.7	3.3 ± 3.6
End (μM)	18.6 ± 14.4	17.4 ± 13.4
Moderate-to-severe-intensity exercise		
$[\mathrm{Hb}_{tot}]$		
Baseline (μM)	57.4 ± 30.3	53.9 ± 31.6
120 s (μM)	58.7 ± 31.2	54.9 ± 33.2
End (μM)	61.3 ± 31.9	56.6 ± 32.0
[HHb]		
Baseline (μM)	18.6 ± 14.4	17.4 ± 13.4
Primary time delay (s)	1 ± 3*	3 ± 3*
Primary time constant (s)	20 ± 9*	10 ± 3†
Primary amplitude (μM)	4.0 ± 4.7	2.8 ± 3.3
$\Delta[\mathrm{HHb}]/\Delta\dot{V}\mathrm{o}_2$ ($\mu\mathrm{M}\cdot\mathrm{L}\cdot\mathrm{min}^{-1}$)	3.1 ± 3.7	2.4 ± 3.0
End (μM)	24.7 ± 20.9#	23.0 ± 18.8#

Values are mean ± SD. $[\mathrm{Hb}_{tot}]$, total hemoglobin concentration; [HHb], deoxygenated hemoglobin concentration; Δ, change. Significantly different from moderate exercise within condition: *$P < 0.01$, #$P < 0.05$. Significantly different from PL: †$P < 0.05$.

Table 2: Blood [lactate] and heart rate dynamics during moderate- and severe-intensity exercise following BR and PL supplementation.

	PL	**BR**
Unloaded-to-moderate-intensity exercise		
Baseline HR (b·min^{-1})	83 ± 11	82 ± 10
Primary HR time constant (s)	30 ± 9	29 ± 10
End-exercise HR (b·min^{-1})	119 ± 14	118 ± 14
Baseline blood [lactate] (mM)	1.9 ± 0.6	1.7 ± 0.4
End-exercise blood [lactate] (mM)	3.0 ± 0.9	2.6 ± 0.8
Δ blood [lactate] (mM)	1.1 ± 1.4	1.0 ± 0.9
Moderate-to-severe-intensity exercise		
Baseline HR (b·min^{-1})	117 ± 14*	116 ± 13*
Primary HR time constant (s)	48 ± 19*	47 ± 12*
HR at 360-s (b·min^{-1})	170 ± 13*	171 ± 13*
HR mean response time (s)	73 ± 20	67 ± 17
Baseline blood [lactate] (mM)	3.0 ± 0.9*	2.6 ± 0.8*
Blood [lactate] at 360-s (mM)	11.0 ± 3.0	10.7 ± 3.1
Δ blood [lactate] (mM)	8.0 ± 2.2*	8.1 ± 2.4*
Blood [lactate] at exhaustion (mM)	10.8 ± 2.8	10.9 ± 2.3

Values are mean ± SD. HR, heart rate; Δ, change. Significantly different from moderate exercise within condition: *$P < 0.01$, #$P < 0.05$. Significantly different from PL: †$P < 0.05$.

Table 3: Pulmonary O_2 uptake responses to moderate- and severe-intensity exercise following BR and PL supplementation.

	PL	**BR**
Unloaded-to-moderate-intensity exercise		
Baseline $\dot{V}\text{o}_2$ ($\text{L}\cdot\text{min}^{-1}$)	0.76 ± 0.13	0.76 ± 0.15
Phase II time constant (s)	25 ± 4	27 ± 6
Primary amplitude ($\text{L}\cdot\text{min}^{-1}$)	0.91 ± 0.28	0.95 ± 0.33
Primary gain ($\text{mL}\cdot\text{min}^{-1}\cdot\text{W}^{-1}$)	10.8 ± 1.4	11.1 ± 1.3
End-exercise $\dot{V}\text{o}_2$ ($\text{L}\cdot\text{min}^{-1}$)	1.67 ± 0.37	1.70 ± 0.39
Mean response time (s)	40 ± 12	40 ± 6
Moderate-to-severe-intensity exercise		
Baseline $\dot{V}\text{o}_2$ ($\text{L}\cdot\text{min}^{-1}$)	1.66 ± 0.38*	1.69 ± 0.39*
Phase II time constant (s)	46 ± 13*	36 ± 10†
Primary amplitude ($\text{L}\cdot\text{min}^{-1}$)	1.18 ± 0.25	1.14 ± 0.26
Primary gain ($\text{mL}\cdot\text{min}^{-1}\cdot\text{W}^{-1}$)	10.3 ± 1.1	9.9 ± 0.8
Slow phase time delay (s)	163 ± 27	157 ± 21
Slow phase amplitude ($\text{L}\cdot\text{min}^{-1}$)	0.24 ± 0.11	0.26 ± 0.12
Slow phase relative amplitude (%)	17 ± 7	18 ± 8
Total gain ($\text{mL}\cdot\text{min}^{-1}\cdot\text{W}^{-1}$)	12.4 ± 0.9#	12.3 ± 1.2
$\dot{V}\text{o}_2$ at 360-s ($\text{L}\cdot\text{min}^{-1}$)	3.08 ± 0.55*	3.10 ± 0.54*
Mean response time (s)	76 ± 14*	69 ± 11*
$\dot{V}\text{o}_2$ at exhaustion ($\text{L}\cdot\text{min}^{-1}$)	3.12 ± 0.51	3.09 ± 0.51

Values are mean ± SD. Significantly different from moderate exercise within condition: $*P < 0.01$, $\#P < 0.05$. Significantly different from PL: $\dagger P < 0.05$.