**Beetroot juice supplementation speeds O2 uptake kinetics and improves exercise tolerance during severe-intensity exercise initiated from an elevated metabolic rate**

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**Running Head: Beetroot juice and O2 kinetics**

**Abstract**

Recent research has suggested that dietary nitrate (NO3-) 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), NO3--rich beetroot juice (BR) supplementation would speed the phase II o2 kinetics (τp) 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 NO3-) and PL (140 mL/day, containing ~0.003 mmol of NO3-) 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 o2 τp 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 o2 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 NO3--rich BR juice speeds o2 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 O2 uptake (o2) rises in an exponential fashion following the onset of exercise with similar response kinetics (denoted by the phase II time constant, τp) to that of muscle o2 ([28](#_ENREF_28), [38](#_ENREF_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 o2 is attained, at which time the oxidative reconstitution of ATP is coupled to the rate of muscle ATP utilization ([56](#_ENREF_56)). While a o2 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 o2 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 O2 uptake (o2max) during severe-intensity exercise (> CP) when this is continued to the limit of tolerance ([57](#_ENREF_57), [66](#_ENREF_66), [69](#_ENREF_69)). The o2 slow component develops concomitantly with a progressive reduction in muscle [PCr] ([59](#_ENREF_59), [60](#_ENREF_60)), reflecting a reduction in contractile efficiency as constant-work-rate exercise is continued ([34](#_ENREF_34)). Interventions that reduce τp or the rate of development of the o2 slow component would be expected to positively impact on exercise tolerance ([16](#_ENREF_16)).

Dietary supplementation with inorganic nitrate (NO3-), which undergoes a stepwise reduction to nitrite (NO2-) and then nitric oxide (NO) and other reactive nitrogen species ([48](#_ENREF_48)), has been reported to reduce the O2 cost of submaximal exercise ([2](#_ENREF_2), [5](#_ENREF_5), [18](#_ENREF_18), [44-47](#_ENREF_44), [63](#_ENREF_63)) in association with a lower ATP cost of muscle force production ([2](#_ENREF_2)) and an increase in the mitochondrial ratio of phosphate radicals esterified to atoms of oxygen consumed (P/O ratio; ([45](#_ENREF_45))). Muscle oxygenation is greater in contracting skeletal muscle following NO3- ingestion ([5](#_ENREF_5)), while intravenous nitrite infusion has been shown to increase skeletal muscle blood flow at rest and during exercise ([25](#_ENREF_25)). These physiological effects likely account, at least in part, for the improved exercise tolerance ([2](#_ENREF_2), [5](#_ENREF_5), [35](#_ENREF_35), [44](#_ENREF_44), [46](#_ENREF_46)) and exercise performance ([18](#_ENREF_18), [43](#_ENREF_43)) that has been reported following NO3- supplementation. Recent studies have indicated that NO3- treatment might particularly alter metabolic and vascular control in type II muscles or muscle fibers ([23](#_ENREF_23), 24, [32](#_ENREF_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 NO3- ([32](#_ENREF_32)), while augmented blood flow, predominantly within locomotor muscles comprising a greater proportion of type II fibers, was reported in rats fed NO3- rich beetroot juice ([23](#_ENREF_23)). However, the potential muscle fiber-type dependency of NO3- 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](#_ENREF_14), [22](#_ENREF_22), [33](#_ENREF_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](#_ENREF_42)). Compared to U→M, the o2 τp during M→S is greater (i.e., o2 kinetics are slower) ([22](#_ENREF_22), [68](#_ENREF_68)). Moreover, compared to a transition from unloaded cycling to a severe-intensity work rate (U→S), the o2 τp during M→S is greater and the amplitude of the o2 slow component is truncated, such that the overall response reverts towards being ‘first-order’ ([20-22](#_ENREF_20), [67](#_ENREF_67), [68](#_ENREF_68)). It is possible that the slower o2 kinetics in M→S compared to U→M reflects a relative imbalance in muscle O2 supply relative to demand. Consistent with this, it has been reported that microvascular *P*O2 (which reflects the dynamic balance between muscle O2 delivery and muscle O2 utilization) declines more rapidly during contractions in predominantly type II compared to type I muscle ([10](#_ENREF_10), [51](#_ENREF_51)). Given that NO3- supplementation has been reported to increase both the absolute and relative distribution of blood flow toward contracting type II muscle ([23](#_ENREF_23)), this might be expected to improve the local matching of O2 delivery relative to muscle o2 and therefore to speed phase II o2 kinetics during M→S. While NO3- supplementation does not reduce the o2 τp during either U→M or U→S ([2](#_ENREF_2), [5](#_ENREF_5), [44](#_ENREF_44)), the effect of NO3- supplementation on the o2 τp during M→S has yet to be investigated.

Therefore, the purpose of this study was to investigate the effects of short-term dietary NO3- supplementation on o2 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 NO3--induced changes in phase II o2 dynamics. The kinetics (τ) of muscle [HHb] following the onset of exercise resembles that of mixed venous [O2] ([28](#_ENREF_28), [38](#_ENREF_38)) and approximates the reduction in microvascular *P*O2 during transitions from rest-to-electrically stimulated contractions ([36](#_ENREF_36)). The [HHb] signal is therefore considered to provide an index of local O2 extraction ([19](#_ENREF_19), [27](#_ENREF_27)) and hence to reflect the balance between muscle O2 delivery and muscle O2 utilization. We hypothesized that NO3- supplementation would reduce the o2 τp and increase the muscle [HHb] τ in M→S but not U→M. We also hypothesized that these kinetic changes following NO3- 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](#_ENREF_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 o2peak 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 o2peak 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](#_ENREF_9)) as the first disproportionate increase in CO2 production (co2) relative to the increase in o2, and subsequently verified by an increase in the ventilatory equivalent for o2 (E /o2) with no increase in E /co2.The work rates that would require 90% of the GET (moderate-intensity exercise) and 70% of the difference (Δ) between the GET and o2peak (severe-intensity exercise, Δ70%) were subsequently determined, with account taken of the mean response time for o2 during ramp exercise [i.e. two thirds of the ramp rate was deducted from the work rate at the GET and peak o2 ([65](#_ENREF_65))].

Following the ramp incremental test, participants were randomly assigned in a crossover, double-blind design to receive 6 days of dietary supplementation with NO3--rich beetroot juice (BR) (140 mL/day; ~ 8 mmol NO3-; Beet It, James White Drinks, Ipswich, UK) or NO3--depleted BR as a placebo (PL; 140 mL/day; 0.0034 mmol NO3-; Beet It, James White Drinks, Ipswich, UK). The placebo NO3--depleted BR beverage was identical in color, taste, smell and texture to the experimental NO3- -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 NO3- 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 [NO2-] peaks at approximately 2-2.5 h post-administration of BR containing 8.4 mmol NO3- ([71](#_ENREF_71)). 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 o2 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 o2 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 o2 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 NO3-  and NO2-, 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 [NO2-] using a modification of the chemiluminescence technique ([7](#_ENREF_7)). All glassware, utensils, and surfaces were rinsed with deionized water to remove residual NO2- prior to analysis. Following defrosting at room temperature, the [NO2-] 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 [NO2-] 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∙l-1∙s-1 at 15 l∙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 (O2) and infrared (CO2) 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](#_ENREF_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 6of 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 (HbO2) 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](#_ENREF_50), [62](#_ENREF_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 o2 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 o2 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 o2 data.

Δo2(t) = $A\_{1}∙(1-e^{-(t-δ\_{1})/τ\_{1}})$ (Eq. 1)

where ∆o2 is the increase in o2 at time t above the baseline value (calculated as the mean o2 from the first 45-s of the last min of baseline pedaling), and *A*1, δ1 and τ1 are the primary component amplitude, time delay (which was allowed to vary freely), and time constant, respectively. Kinetic variables (*A*1, δ1 and τ1) 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.

Δo2(t) = $A\_{1}∙(1-e^{-(t-δ\_{1})/τ\_{1}}) + A\_{2}∙(1-e^{-(t-δ\_{2})/τ\_{2}})$ (Eq. 2)

Given the failure of the bi-exponential model to adequately describe the o2 response during M→S, the onset of the o2 slow component was determined using purpose designed LabVIEW software which iteratively fits a mono-exponential function to the o2 data until the window encompasses the entire response. The estimated τ for each fitting window was plotted against time and the onset of the o2 slow component was identified as the point at which the estimated τ consistently deviated from the previously “flat” profile ([61](#_ENREF_61)). The amplitude of the o2 slow component was subsequently determined by calculating the difference between the end exercise o2 and the sum of the primary amplitude and baseline o2. This was expressed both in absolute terms and relative to the end-exercise o2. The functional gain of the primary o2 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](#_ENREF_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 o2above. The primary [HHb] amplitude was divided by the phase II o2 asymptote in order to determine the Δ[HHb]/Δo2 ratio as an index of the change in fractional muscle O2 extraction required to elicit a given Δo2 during the primary phase. In addition, we assessed changes in total blood volume by summing the [HbO2] and [HHb] signals to provide an estimate of the total [Hbtot] 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 o2 “slow component”.

***Statistics***

Gaussian distribution was confirmed by the Shapiro-Wilks test. Following this, the pulmonary o2, 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 [NO2-] 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* ≤ 0.05.

**Results**

The subjects’ peak o2 was 3.73 ± 0.46 L∙min-1 for men and 2.69 ± 0.52 L∙min-1 for women with the GET occurring at 2.08 ± 0.41 L·min-1 and 1.71± 0.41 L∙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 [NO2-]**

There was a main effect for ‘supplement’ on plasma [NO2-] 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 [NO2-] 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 [Hbtot] and [HHb] values derived from NIRS interrogation are presented in Table 1. There was no significant main effect for ‘supplement’ on the [Hbtot] 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 BRsupplementation (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 o2 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 o2 τp 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.

**o2 kinetics and exercise tolerance**

The o2 kinetic parameters derived from the mono-exponential fit are presented in Table 3 and the o2 response of a representative subject to U→M and M→S is shown in Fig. 2. The group mean o2 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 o2 kinetics following the onset of exercise (F[1,8] = 18.54, *P* = 0.01). Compared to PL, the τp 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 τp 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 o2 τp 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 o2 amplitude (F[1,8] = 0.01, *P* = 0.91) or primary o2 gain (F[1,8] = 0.05, *P* = 0.83) during U→M or M→S. The emergence of a slow phase in o2 during M→S occurred after a similar time delay and there were no differences in the absolute or relative amplitude of the o2 slow component between PL and BR (both *P* = 0.44). For M→S, there were no differences between PL and BR in the o2 amplitude at end-exercise (F[1,8] = 0.60, *P* = 0.46) or the total o2 gain (F[1,8] = 0.14, *P* = 0.72).

The o2 attained at task failure (PL: 3.12 ± 0.51 vs. BR: 3.09 ± 0.51 L∙min-1) was not different between conditions or when compared to the peak o2 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 o2 τp 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 NO3--rich BR juice speeded pulmonary o2 and muscle [HHb] kinetics and increased the time-to-task failure following the onset of M→S exercise compared to NO3--depleted PL juice. These results suggest that increasing plasma [NO2-], and thus the potential for O2-independent NO generation after BRsupplementation, can speed the o2 τp in M→S such that it is not significantly different from the o2 τp 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 BRsupplementation 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](#_ENREF_23), [32](#_ENREF_32)).

In the present study, short-term dietary supplementation with NO3--rich BR juice markedly increased plasma [NO2-]. Surprisingly, however, this was not associated with a reduced steady-state o2 during U→M. This finding contrasts with previous studies in young, recreationally-active populations ([2](#_ENREF_2), [5](#_ENREF_5), [44-47](#_ENREF_44), [63](#_ENREF_63)), but is consistent with other studies in which the participants were well-trained ([11](#_ENREF_11), [55](#_ENREF_55)). Training status does not provide an explanation for the lack of effect of BRingestion on steady-state o2 during moderate-intensity exercise in the present study because the participants were not well-trained (48 and 46 ml∙kg-1∙min-1 for males and females, respectively). In a recent study investigating the dose-response relationship between acute NO3- intake and the physiological responses to exercise ([71](#_ENREF_70)), it was reported that steady-state o2 during moderate-intensity exercise was significantly reduced following the consumption of 280 ml of BR (~ 16 mmol NO3-) but not 70 ml BR (~ 4 mmol NO3-) or 140 ml BR (~ 8 mmol NO3-). While this suggests that a higher NO3-dose than the 8 mmol employed in the present study might have been required to elicit an altered O2 cost of exercise, it should be noted that significant reductions in steady-state o2 with 5-8 mmol NO3- supplementation (administered as BR) have been reported previously ([5](#_ENREF_5), [44](#_ENREF_44), [64](#_ENREF_64)). The explanation for the lack of effect of BR on steady-state o2 during moderate-intensity exercise in the present study is therefore obscure.

While NO2- has traditionally been considered as an inert product of NO oxidation ([53](#_ENREF_53)), recent studies have shown that NO2- can be recycled back into bioactive NO ([48](#_ENREF_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 NO2- to NO is O2-independent ([17](#_ENREF_17)) and is potentiated by acidosis ([52](#_ENREF_52)). Since pH and microvascular *P*O2 decline more rapidly in contracting type II muscle ([10](#_ENREF_10), [51](#_ENREF_51)), NO2- 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 o2 kinetics in M→S exercise whereas, consistent with previous research ([5](#_ENREF_5), [44](#_ENREF_44)), BR did not impact on phase II o2 kinetics during U→M. The intensity-dependent effects of dietary NO3- intake with BR on phase II o2 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](#_ENREF_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](#_ENREF_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% o2max whereas the reverse was true at 50% o2max ([42](#_ENREF_42)). The steady state o2 amplitude in the U→M step in the present study was ~ 54% of o2max, suggesting that type I muscle fibers were principally activated in the lower step transition. Conversely, the longer o2 mean response time and increased total o2 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](#_ENREF_6), [40](#_ENREF_40), [41](#_ENREF_41), [58](#_ENREF_58)). Our findings therefore suggest that the faster o2 kinetics observed following BR supplementation during M→S might be related to specific effects of NO3- treatment on higher-order (i.e. type II) muscle fibers.

To explore the mechanisms responsible for any alterations in o2 τp in M→S, the NIRS-derived muscle [HHb] signal was used to provide information on the dynamic (im)balance between microvasculature O2 delivery and metabolic demand ([19](#_ENREF_19), [27](#_ENREF_27)). For the same o2 kinetics, enhanced muscle O2 supply relative to muscle O2 demand would be expected to result in a longer muscle [HHb] τ, whereas faster o2 kinetics alongside unchanged [HHb] kinetics would be interpreted as a proportionally similar increase in the rate of muscle O2 delivery to o2. However, in the present study, faster o2 kinetics in M→S with BR was accompanied by a *shorter* [HHb] τ during which [Hbtot] (and by inference blood volume) in the interrogated muscle area was not different compared to PL. This suggests that BR may have speeded o2 kinetics, in part, by enhancing muscle O2 extraction. It has been reported that muscle O2 demand exceeds microvasculature O2 delivery in muscle comprised of predominantly type II fibers ([10](#_ENREF_10), [51](#_ENREF_51)) and that BR increases muscle bulk blood flow and promotes a greater distribution of blood flow to type II muscle fibers ([23](#_ENREF_23)). If absolute or relative perfusion of type II fibers was greater after BR ingestion, this might have facilitated enhanced muscle O2 extraction, as suggested by the faster muscle [HHb] kinetics, and therefore permitted faster o2 kinetics in M→S. However, the faster o2 τp with BR compared to PL was not significantly correlated with the reduction in the [HHb] τ. It is therefore also possible that BR speeded o2 kinetics, by altering metabolic control in type II fibers during the transition from M→S. Given that short-term NO3- supplementation does not increase markers of mitochondrial biogenesis in skeletal muscle ([45](#_ENREF_45)), or speed the recovery of [PCr] following intense exercise ([44](#_ENREF_44)) which would reflect increased muscle oxidative capacity ([12](#_ENREF_12)), the faster o2 kinetics in M→S is unlikely to have resulted from an increase in mitochondrial volume. Increased intracellular calcium content [Ca2+]i has been observed during tetanic contractions of type II, but not type I, muscle fibers excised from mice supplemented with NO3- ([32](#_ENREF_32)). As well as activating the muscle contractile apparatus, Ca2+ has also been suggested to signal the activation of oxidative phosphorylation ([30](#_ENREF_30)). Therefore, it is possible that increased [Ca2+]i and parallel activation of the contractile and oxidative metabolic machinery might have contributed to the faster muscle [HHb] and o2 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](#_ENREF_2), [5](#_ENREF_5), [35](#_ENREF_35), [44](#_ENREF_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](#_ENREF_13), [72](#_ENREF_72)), which would also be expected to engender significant recruitment of type II muscle fibers ([39](#_ENREF_39)). It has been reported (using multi-channel NIRS) that there is marked inter-site heterogeneity in matching of O2 delivery to o2 within the quadriceps muscle during high-intensity cycling ([37](#_ENREF_37)). One possibility is that NO might inhibit O2 utilization in some well-oxygenated muscle fibers (15) whereas the hypoxic and acidic environment within and surrounding muscle fibers receiving less O2 might stimulate NO2- reduction to NO and thus increase microvascular O2 supply ([29](#_ENREF_29)). Faster phase II o2 kinetics during M→S after BR might therefore have resulted from a more homogenous distribution of O2 relative to metabolic demand within contracting muscle. Interventions that speed o2 kinetics have been previously shown to improve the tolerable duration of severe-intensity exercise ([3](#_ENREF_3), [4](#_ENREF_4)). A faster adjustment of o2 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](#_ENREF_1), [16](#_ENREF_16), [54](#_ENREF_54)). However, in the present study, whilst an increased time to task failure with BR was accompanied by a shorter o2 τp compared to PL, the two were not significantly correlated.

Dietary supplementation with NO3--rich BR juice has been reported to improve exercise tolerance in concert with attenuated skeletal muscle ATP turnover, PCr hydrolysis, and Pi and ADP accumulation during high-intensity exercise ([2](#_ENREF_2)). Perturbations of skeletal muscle Ca2+ handling and membrane excitability are also hallmarks of skeletal muscle fatigue ([1](#_ENREF_1)). In this respect, it is interesting that mice receiving NO3- treatment had an improved capacity for sarcoplasmic Ca2+ release and increased tetanic force production in type II muscle ([32](#_ENREF_32)). In humans, BR supplementation appears to blunt the accumulation of extracellular K+, possibly preserving muscle excitability, during intense intermittent exercise (72). As discussed earlier, improvements in muscle blood flow and a greater distribution of blood flow to type II muscle fibers with BR ([23](#_ENREF_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 O2 for the O2-binding site at cytochrome-*c* oxidase (COX) in the electron transport chain (15). If NO3- 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 NO3- 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 O2 to diffuse to fibers further from the capillary which might be O2 deficient (thereby increasing ‘global’ oxidative ATP production across a muscle), (29). There is also evidence that greater NO production via NO3- supplementation might improve matching of O2 supply to O2 utilization and increase the O2 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 NO3- 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 NO3--rich BR juice speeded pulmonary o2 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 o2max and peak power output during incremental exercise were increased, and that acute reductions of resting blood pressure and the O2 cost of moderate-intensity exercise were maintained, after 15 days of BR supplementation ([63](#_ENREF_63)). This indicates that subjects do not develop tolerance to inorganic nitrate intake, at least up to 15 days of supplementation.

In addition to containing NO3-, BR is also rich in several other compounds, including betaine, antioxidants, and polyphenols (including quercetin and resveratrol) (70), that might influence the physiological responses to exercise. At present, we cannot rule out the possibility that these compounds might exert independent effects or that NO3− operates synergistically with one or more of them. Moreover, ascorbate and polyphenols facilitate the reduction of nitrite to NO ([49](#_ENREF_49)) which might augment NO production. However, in a previous study, we reported that the physiological responses to exercise and exercise tolerance were only improved when BR contained NO3- (44). When placebo BR (which has negligible amounts of NO3-) was administered, the physiological responses were not different to those measured in a control (non-supplemented) condition (44). It is also important to note that similar effects on plasma [nitrite] and exercising o2 have been reported when subjects have consumed nitrate salts (45, 47). This strongly suggests that the physiological effects of BR consumption can be attributed, in large part, to its high NO3− content. Nevertheless, it would be beneficial for future research to compare the physiological effects of dietary inorganic NO3- supplementation with those of other NO donors, such as nitroglycerin, and for changes in NO availability to be more directly assessed.

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](#_ENREF_18), [43](#_ENREF_43)), as well as during high-intensity intermittent exercise ([13](#_ENREF_13), [72](#_ENREF_72)). 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 o2 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 o2 (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 o2 during baseline pedaling. The onset of step exercise is indicated by the vertical dotted line. Note the faster [HHb] and o2 dynamics in M→S but not U→M following BR compared to PL supplementation.

*Figure 3*: Group mean o2 response during M→S exercise following BR and PL supplementation with the group mean ± SEM o2 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 [Hbtot] and [HHb] responses to moderate- and severe-intensity exercise following BR and PL supplementation.**

|  |  |  |
| --- | --- | --- |
|  | **PL** | **BR** |
| ***Unloaded-to-moderate-intensity exercise*** |
| **[Hbtot]** |  |  |
| **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 |
| **Δ[HHb]/Δhttp://jap.physiology.org/content/vol99/issue6/fulltext/2463/f2.gifo2 (µM∙L∙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*** |
| **[Hbtot]** |  |  |
| **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 |
| **Δ[HHb]/Δhttp://jap.physiology.org/content/vol99/issue6/fulltext/2463/f2.gifo2 (µM∙L∙min-1)** | 3.1 ± 3.7 | 2.4 ± 3.0 |
| **End (µM)** | 24.7 ± 20.9# | 23.0 ± 18.8# |

Values are mean ± SD. [Hbtot], 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 O2 uptake responses to moderate- and severe-intensity exercise following BR and PL supplementation.**

|  |  |  |
| --- | --- | --- |
|  | **PL** | **BR** |
| ***Unloaded-to-moderate-intensity exercise*** |
| **Baseline f2o2 (L·min-1)** | 0.76 ± 0.13 | 0.76 ± 0.15 |
| **Phase II time constant (s)** | 25 ± 4 | 27 ± 6 |
| **Primary amplitude** **(L·min-1)** | 0.91 ± 0.28 | 0.95 ± 0.33 |
| **Primary gain (mL·min-1·W-1)** | 10.8 ± 1.4 | 11.1 ± 1.3 |
| **End-exercise** **f2o2 (L·min-1)** | 1.67 ± 0.37 | 1.70 ± 0.39 |
| **Mean response time (s)** | 40 ± 12 | 40 ± 6 |
| ***Moderate-to-severe-intensity exercise*** |
| **Baseline f2o2 (L·min-1)** | 1.66 ± 0.38\* | 1.69 ± 0.39\* |
| **Phase II time constant (s)** | 46 ± 13\* | 36 ± 10† |
| **Primary amplitude (L·min-1)** | 1.18 ± 0.25 | 1.14 ± 0.26 |
| **Primary gain (mL·min-1·W-1)** | 10.3 ± 1.1 | 9.9 ± 0.8 |
| **Slow phase time delay (s)** | 163 ± 27 | 157 ± 21 |
| **Slow phase amplitude (L·min-1)** | 0.24 ± 0.11 | 0.26 ± 0.12 |
| **Slow phase relative amplitude (%)** | 17 ± 7 | 18 ± 8 |
| **Total gain (mL·min-1·W-1)** | 12.4 ± 0.9# | 12.3 ± 1.2 |
| **f2o2 at 360-s (L·min-1)** | 3.08 ± 0.55\* | 3.10 ± 0.54\* |
| **Mean response time (s)** | 76 ± 14\* | 69 ± 11\* |
| **f2o2 at exhaustion (L·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: †*P* < 0.05.