Dietary nitrate supplementation attenuates the reduction in exercise tolerance following blood donation

Original Article

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Running head: Nitrate, blood donation and exercise performance

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ABSTRACT

We tested the hypothesis that dietary nitrate-rich beetroot juice (BR) supplementation could partially offset deteriorations in O_2 transport and utilization, and exercise tolerance, after blood donation. Twenty-two healthy volunteers performed moderate-intensity and ramp incremental cycle exercise tests prior to and following the withdrawal of ~450 mL of whole blood. Before donation, all subjects consumed 7 x 70 mL of nitrate-depleted beetroot juice shots (PL) in the 48 h preceding the exercise tests. During the 48 h after blood donation, subjects consumed 7 shots of either BR (each containing 6.2 mmol nitrate; n=11) or PL (n=11) before repeating the exercise tests. [Hemoglobin] and hematocrit were reduced by ~8-9% following blood donation (P<0.05), with no difference between the BR and PL groups. When compared with pre-donation, steady-state $\dot{V}O_2$ during moderate-intensity exercise was ~4% lower post-donation in BR (P<0.05) but was unchanged in PL. The ramp test peak power decreased from pre-donation (PL: 341 ± 70 *vs.* BR: 331 ± 68 W) to post-donation (PL: 324 ± 69 *vs.* BR: 322 ± 66 W) in both groups (P<0.05). However, the decrement in performance was significantly less in BR (2.7%) compared with PL (5.0%; P<0.05). Nitrate supplementation reduced the O_2 cost of moderate-intensity exercise and attenuated the decline in ramp incremental exercise performance following blood donation. These results have implications for improving functional capacity following blood loss.

New and Noteworthy: Dietary nitrate supplementation with beetroot juice lowered the O_2 cost of moderate-intensity exercise, better preserved muscle oxygenation and attenuated the decline in incremental exercise test performance following donation of 450 mL whole blood. These results have implications for improving functional capacity following blood loss.

Key words: blood withdrawal; beetroot juice; O_2 transport; O_2 uptake; exercise performance; nitric oxide

INTRODUCTION

The peak rate of pulmonary oxygen uptake ($\dot{V}O_{2peak}$) is an important determinant of exercise capacity and is influenced by the interaction of several central and peripheral factors (6, 53, 64). $\dot{V}O_{2peak}$ and exercise performance can be altered by manipulating the capability of the cardiovascular system to transport O_2 to contracting skeletal muscles during exercise (5, 11, 18, 51, 57, 67). For example, interventions involving the infusion of erythrocytes (18, 19) or the stimulation of erythropoiesis (57, 67) to enhance hemoglobin concentration ([Hb]), increase $\dot{V}O_{2peak}$ during maximal exercise. Conversely, limiting O_2 transport to working muscle by reducing [Hb] via whole blood withdrawal consistently results in a lowered $\dot{V}O_{2peak}$ (11, 18, 47, 54). During sub-maximal exercise, however, Panebianco et al. (47) reported no change in $\dot{V}O_2$ at two and seven days post 450 mL blood donation, despite significant reductions in [Hb]. Compensatory adjustments in cardiovascular control, such as increases in heart rate (HR) and cardiac output ($\dot{Q}$), offset the lower [Hb] and enable muscle O_2 delivery to be maintained during low-intensity exercise after blood donation (19, 27, 51).

The gaseous physiological signaling molecule, nitric oxide (NO), plays a key role in the regulation of vascular tone. NO can be synthesised via the oxidation of L-arginine in a reaction catalysed by the NO synthases (NOS; 32) or it can be produced via the reduction of nitrate (NO_3^-) to nitrite (NO_2^-) and subsequently NO (8). Recently, dietary NO_3^- supplementation has been employed to augment plasma [NO_2^-] and the potential for O_2-independent NO synthesis (4, 38, 65). This NO_3^--NO_2^--NO pathway may be particularly important when NOS activity is compromised (20, 42), O_2 availability is limited (14, 25, 34,

35) and pH is low (44). Limitations in systemic O_2 transport can result in tissue hypoxia and greater metabolic perturbation (41, 60), which can contribute to reduced exercise tolerance (1), as is commonly observed at altitude (2) and in a number of disease states (35, 68). There is evidence to suggest that NO and NO_2^- can combat an insufficient muscle O_2 supply by increasing muscle blood flow via hypoxia-induced vasodilatation (13, 61). Therefore, it is possible that dietary NO_3^- supplementation could ameliorate deteriorations in exercise performance when 'normal' O_2 availability is reduced, during for example, high-intensity exercise, in hypobaric hypoxia or after blood donation.

We and others have reported that, in healthy subjects, dietary NO_3^- supplementation can significantly impact the physiological responses to exercise (4, 15, 38, 59). Specifically, a reduction in the O_2 cost of moderate-intensity exercise has been reported after supplementation with both sodium NO_3^- (38, 39, 40) and NO_3^--rich beetroot juice (BR; 3, 4, 15, 59, 69). In addition, a significantly increased time to task failure (TTF), indicating improved exercise tolerance, has been reported following BR ingestion when recreationally-active, but not highly trained, subjects completed severe-intensity (3, 4, 37) and ramp incremental exercise (59). These alterations may be due to a NO_2^- or NO-related reduction in the ATP cost of muscle contraction (3), greater mitochondrial efficiency (40), changes in muscle redox status (66), and/or enhanced muscle blood flow, particularly to type II fibres (21, 22). Such changes could be particularly advantageous after whole blood withdrawal when [Hb] is reduced and O_2 transport is challenged (11, 18, 54). Indeed, BR supplementation has been shown to reduce muscle metabolic perturbation during exercise in normobaric hypoxia and to restore exercise tolerance and oxidative function to the values observed in normoxia (60, 61). In addition, it has been reported that, when the fraction of inspired O_2 is lowered to 11-13%, BR supplementation can improve muscle oxygenation

status (43), reduce $\dot{V}O_2$ during sub-maximal exercise (34, 46), and enhance TTF during incremental exercise (43). BR supplementation has also been reported to increase arterial O_2 saturation following dynamic apnea (i.e., breath-hold diving), which supports an O_2 sparing effect of NO_3^- ingestion (48). Collectively, these studies suggest that NO_3^- ingestion may enhance the physiological response to exercise when O_2 availability is limited, by sparing muscle O_2 demand and/or better preserving muscle O_2 supply. However, it is not known whether the reductions in O_2 carrying capacity and exercise performance subsequent to the withdrawal of whole blood can be offset by BR supplementation. If so, this may have important implications for clinical conditions in which [Hb] is lowered, for example in anemia, following surgery or involuntary blood loss, or in athletes wishing to donate blood without compromising training.

The purpose of the present study was to determine whether 48 h of BR supplementation following 450 mL of whole blood withdrawal alters the physiological responses to sub-maximal and maximal intensity cycle exercise. It was hypothesized that BR supplementation would lower the O_2 cost of moderate-intensity exercise, improve muscle oxygenation status, and attenuate the expected reduction in TTF during ramp incremental exercise following blood donation.

METHODS

Subjects

Twenty-two recreationally active and pre-registered National Health Service (NHS) blood donors (males, n = 14; females, n = 8) volunteered to participate in this study, which was approved by the Institutional Research Ethics Committee and conformed to the ethical principles of the Declaration of Helsinki. None of the subjects were tobacco smokers or

habitual users of dietary supplements. All subjects provided written informed consent prior to the commencement of the study, after the experimental procedures, associated risks and potential benefits of participation had been explained.

Subjects were instructed to arrive at the laboratory in a rested and fully hydrated state, at least 3 h postprandial, and to avoid strenuous exercise in the 24 h preceding each visit. In addition, subjects were asked to avoid alcohol consumption, chewing gum and antibacterial mouthwash throughout each supplementation period and to avoid caffeine intake in the 3 h preceding each laboratory visit. Each subject recorded habitual diet and exercise undertaken during the first supplementation period and were asked to replicate these habits during the second supplementation period. Prior to data collection, subjects were fully familiarized with the exercise testing procedures. This minimized any possible learning effects during the study. Exclusion criteria were the presence of known cardiovascular disease, hypertension and anemia, the use of antihypertensive medication and antibiotics, and having major surgery or giving blood within 6 months of the study commencing.

Experimental Overview

Subjects were asked to report to the laboratory on three separate occasions over a ten day period. The first visit included a 5 min bout of moderate-intensity cycle exercise at 80 W, followed by a ramp incremental test to task failure with no dietary supplementation. This served as the pre-intervention familiarization test. Hematocrit (Hct), [Hb], plasma [NO_3^-] and [NO_2^-], pulmonary $\dot{V}O_2$ dynamics, muscle oxygenation status, HR, blood lactate concentration ([lactate]), blood glucose concentration ([glucose]) and TTF during ramp incremental exercise were measured during the first visit and repeated during each visit to the laboratory. Prior to visit 2, subjects consumed 7 shots of NO_3^--depleted beetroot juice (PL)

over ~48 h. On the final day of supplementation, subjects completed the same moderate-intensity exercise bout and ramp incremental test on a cycle ergometer as was performed at pre-intervention. Two days before the final visit to the lab, subjects attended a National Health Service (NHS) blood donation clinic. Each subject lay supine on a bed before ~450 mL of whole blood was drawn from an antecubital vein over a 15 min period. The blood withdrawal was performed by the NHS as part of the national blood donation service. Following blood donation, each subject was randomly assigned, in a double-blind, placebo controlled fashion to consume 7 shots of either NO_3^--rich beetroot juice (BR; *n* = 11; mean ± SD; females, *n* = 4: age 23 ± 3 years, body mass 67 ± 4 kg, height 1.76 ± 0.05 m; males, *n* = 7: age 26 ± 5 years, body mass 81 ± 12 kg, height 1.80 ± 0.10 m) or NO_3^--depleted beetroot juice as a placebo (PL; *n* = 11; mean ± SD; females, *n* = 4: age 22 ± 3 years, body mass 77 ± 11 kg, height 1.75 ± 0.10 m; males, *n* = 7: age 28 ± 7 years, body mass 77 ± 8 kg, height 1.79 ± 0.10 m) over the next ~48 h. Visit 3 occurred on the final day of supplementation with the exercise tests conducted 2 h following final supplement ingestion. All tests were performed at the same time of day (± 2 h) to minimise diurnal variation on the physiological variables under investigation.

Exercise tests

During the first visit to the laboratory subjects performed a short bout of low-intensity exercise at 80 W, followed by a ramp incremental exercise test to task failure on an electrically-braked cycle ergometer (Lode Excalibur Sport, Gronigen, The Netherlands) for determination of $\dot{V}O_{2peak}$ and gas exchange threshold (GET). The protocol began with 3 min of 'unloaded' baseline cycling at 20 W, followed by 5 min at 80 W and 10 min of passive rest. Subsequently, 3 min of baseline cycling at 20 W was performed and then the power output was increased linearly by 30 $W \cdot min^{-1}$ until the subject was unable to continue. The

subjects cycled at a self selected cadence (~80 rpm), and this cadence, along with saddle and handle bar configuration, was recorded and replicated for subsequent tests. Pulmonary gas exchange was measured breath-by-breath and averaged into 10-s bins. $\dot{V}O_{2peak}$ was taken as the highest 30-s mean value attained during the test. The GET was determined as described previously (59). The work rate that would require 80% of the GET (moderate-intensity exercise) was calculated, taking into account the mean response time for $\dot{V}O_2$ during ramp exercise (59).

Subjects returned to the laboratory on two further occasions. The second visit was preceded by PL supplementation (n = 22) and the third visit, ~48 h post blood donation, was preceded by 2 days of either BR (n = 11) or PL (n = 11) supplementation. The final visit was conducted 48 h post donation to allow restoration of total blood volume (23) and to minimize the risk of a syncopal episode occurring during maximal exercise. On each of these two laboratory visits, subjects completed a single 5-min bout of moderate-intensity exercise (at 80 % of the GET) and a ramp incremental test to task failure, separated by 10 min of passive rest. The incremental test was terminated when cadence fell more than 10 rpm below the chosen cadence, despite strong verbal encouragement. TTF was recorded to the nearest second and the power output achieved at the point of test termination was recorded as the peak power output (PPO). Feedback on performance was only provided once all experimentation for the entire study had been completed.

Measurements

During each visit to the laboratory, a venous blood sample (~4 mL) was drawn from an antecubital vein into lithium-heparin tubes (Vacutainer, Becton-Dickinson, NJ, USA) and centrifuged for 10 min at 3000 *g* and 4˚C, within 2 min of collection. Subsequently, the

plasma was extracted and frozen at -80˚C for later determination of [NO_3^-] and [NO_2^-] using a modified chemiluminescence technique (7) as previously described (69). Blood samples from a pre-warmed fingertip were collected into four 30 μl heparinized microhematocrit tubes (Hawksley and Sons Ltd, Lancing, Sussex, England) which underwent microcentrifugation for 1 min for the determination of Hct (1560 Micro-haematocrit reader, Hawksley and Sons Ltd, Lancing, Sussex, England). In addition, blood from the same fingertip was collected into four microcuvettes for determination of [Hb] (HemoCue AB, Ängelholm, Sweden).

Pulmonary gas exchange and ventilation were measured breath-by-breath throughout all exercise tests. Subjects wore a nose clip and breathed through a mouthpiece and impeller turbine assembly (Jaeger Triple V). The inspired and expired gas volume and gas concentration signals were sampled continuously at 100 Hz, with the latter using paramagnetic (O_2) and infrared (carbon dioxide; CO_2) analyzers (Oxycon Pro, Jaeger, Hoechberg, Germany) via a capillary line connected to the mouthpiece. These analyzers were calibrated before each test with gases of known concentration, and the turbine volume transducer was calibrated using a 3-litre syringe (Hans Rudolph, Kansas City, MO, USA). 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. Pulmonary O_2 uptake ($\dot{V}O_2$), CO_2 output ($\dot{V}CO_2$), minute ventilation ($\dot{V}E$) and respiratory exchange ratio (RER) were calculated and displayed breath-by-breath. HR was measured at rest and during all cycle tests using short-range radiotelemetry (Polar S610, Polar Electro Oy, Kempele, Finland). A fingertip blood sample was collected into a capillary tube over the 20 s preceding the step transition in work rate to moderate-intensity exercise and the incremental test. Capillary samples were also collected during the final 20 s of the moderate-intensity exercise bout and following exhaustion in the ramp test. These samples were analyzed within 60 s of

collection to determine blood [lactate] (YSI *2300*, Yellow Springs Instruments, Yellow Springs, OH, USA).

The oxygenation status of the *m. vastus lateralis* of the right leg was monitored using near-infrared spectroscopy (NIRS; model NIRO 300, Hamamatsu Photonics KK, Hiugashi-ku, Japan). Four different wavelength laser diodes provided the light source (776, 826, 845 and 905 nm) and a photomultiplier tube in the spectrometer was used to detect the light returning from the tissue. The intensity of incident and transmitted light was recorded continuously throughout exercise at 2 Hz and used to estimate the change in concentration from baseline for oxygenated, deoxygenated, and total tissue Hb and myoglobin. The NIRS data therefore represent a relative change based on the optical density measured in the first data point collected. The deoxyhemoglobin concentration ([HHb]) was assumed to represent the balance between local O_2 supply and utilization and therefore to provide an estimate of changes in O_2 extraction within the field of interrogation (28, 36). Prior to the cycling exercise, the right leg was cleaned and shaved around the belly of the muscle, the probes were placed in the holder and attached to the skin with an adhesive 20 cm above the fibular head. An elastic bandage was wrapped around the subject's leg to secure the holder and wires in place and to minimize the possibility of extraneous light influencing the signal. Pen marks were made around the probe holder to allow for precise reproduction of the position of the probe in subsequent tests. The probe gain was set at rest with the subject in a seated position and the leg extended at down stroke on the cycle ergometer. NIRS data were collected continuously throughout the moderate-intensity and incremental exercise tests.

Supplementation

After completion of the familiarization test, subjects consumed 7 shots of NO_3^--depleted beetroot juice (PL; beetroot juice containing ~0.04 mmol NO_3^- per 70 mL; Beet It Sport Stamina Shot, James White Drinks, Ltd., Ipswich, UK) over ~48 h before completing the pre-donation control trial (PL-Pre and BR-Pre for the PL and BR groups, respectively). This was done in order to control for the antioxidants and polyphenols that exist in both the NO_3^--rich and NO_3^--depleted beverages. The PL was created by passing NO_3^--rich BR through a Purolite A520E ion-exchange resin which selectively removes NO_3^- (37). After blood donation, subjects were randomly assigned, in a double-blind, placebo-controlled fashion, to consume 7 shots of either NO_3^--rich (BR; beetroot juice containing ~6.2 mmol NO_3^- per 70 mL; Beet It Sport Stamina Shot, James White Drinks, Ltd., Ipswich, UK; *n* =11) or NO_3^--depleted beetroot juice (PL; beetroot juice containing ~0.04 mmol NO_3^- per 70 mL; Beet It, James White Drinks, Ltd., Ipswich, UK;*n* =11) over ~48 h (PL-Post and BR-Post for the PL and BR groups, respectively). During both supplementation periods subjects were instructed to consume 2 x 70 mL of the beverage in the evening (~7 p.m.) two days prior to testing, and 1 x 70 mL in the morning (~10 a.m.) and 1 x 70 mL in the evening (~7 p.m.) one day prior to testing. On each experimental day, subjects consumed a further 2 x 70 mL, 2 h prior to testing and 1 x 70 mL on arrival at the laboratory. The supplementation periods were separated by a mean of 8 days (BR: 7 ± 5 days, PL: 9 ± 5 days).

Data Analyses

The breath-by-breath $\dot{V}O_2$ data collected during the exercise tests were initially examined to exclude errant breaths caused by, for example, coughing, swallowing and sighing, and those values lying more than four standard deviations (SDs) from the local mean were removed. $\dot{V}O_{2baseline}$ was defined as the mean $\dot{V}O_2$ measured over the last 60 s of baseline cycling and end-exercise $\dot{V}O_2$ was defined as the mean $\dot{V}O_2$ measured over the last 30 s of exercise. The

baseline and end-exercise $\dot{V}CO_2$, RER, $\dot{V}E$ and HR values were calculated in the same manner.

To provide information on muscle oxygenation, the changes in [HHb] and the tissue oxygenation index (TOI; calculated as the fraction of oxygenated [Hb] compared to total [Hb]) during moderate-intensity exercise were assessed at baseline (60 s preceding the transition to moderate-intensity exercise), in 10 s time bins surrounding 60 s, 120 s, 240 s, and at end-exercise (mean response over the final 30 s of exercise). During ramp incremental exercise, the changes in [HHb] and TOI were assessed at baseline, in 10 s time bins surrounding 120 s, 240 s, 360 s and at task failure.

Blood lactate accumulation (Δ blood [lactate]) was calculated as the difference between blood [lactate] at end-exercise and blood [lactate] at baseline. Similarly, the change in blood glucose concentration (Δ blood [glucose]) was calculated as the difference between blood [glucose] at end-exercise and blood [glucose] at baseline.

Statistical Analyses

Differences in Hct, [Hb], plasma [NO_3^-] and [NO_2^-], pulmonary $\dot{V}O_2$ dynamics, HR, blood [lactate], NIRS-derived variables and TTF were assessed using a mixed model ANOVA. Significant main and interaction effects were further explored using Fisher's LSD. Independent t-tests were used to assess the relative change between the BR and PL treatment groups. Pearson's product moment correlation coefficient was used to explore relationships between changes in [Hb] and Hct and changes in TTF. Statistical analyses were performed using SPSS version 19.0 (Chicago, IL, USA). Data are presented as mean ± SD, unless otherwise stated. Statistical significance was accepted at $P<0.05$.

RESULTS

Subjects' self-reported adherence to the supplementation regimen prior to and post blood donation was 100%. All subjects reported that their physical activity and dietary patterns were similar throughout each of the supplementation periods. The ingestion of BR and PL supplements were well tolerated and no negative side effects were reported. Subjects did, however, report beeturia (red-stained urine).

[Hb] and Hct

The group mean [Hb] and Hct data prior to and following blood donation and BR or PL ingestion are displayed in Table 1. There was a significant main effect by time for both [Hb] and Hct ($P<0.01$) but no main effect by group and no interaction effect ($P>0.05$). Prior to donation, [Hb] and Hct were not different between the BR and PL treatment groups. [Hb] and Hct were both significantly reduced from pre to post donation ($P<0.05$), with no differences between PL and BR groups ($P>0.05$).

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

The group mean plasma [NO_3^-] and [NO_2^-] pre and post blood donation in the BR and PL groups are shown in Table 1. There was a significant main effect by time and group and an interaction effect on plasma [NO_3^-] and [NO_2^-] ($P<0.01$). Prior to blood donation, neither plasma [NO_3^-] nor [NO_2^-] were different between groups ($P>0.05$). Following blood donation, there was a substantial increase in plasma [NO_3^-] and [NO_2^-] in the BR group ($P<0.05$). A small (~11%) rise in plasma [NO_3^-] ($P<0.05$) was also observed in the PL group but there was no change in plasma [NO_2^-] ($P>0.05$).

$\dot{V}O_2$ response to moderate-intensity and incremental exercise

Moderate-intensity exercise

The pulmonary gas exchange and ventilatory responses to moderate-intensity exercise pre and post blood donation in PL and BR groups are reported in Table 2 and the group mean $\dot{V}O_2$ response profiles in BR and PL groups pre and post blood donation are shown in Figure 1. There was a significant main effect by time (P<0.01) but no main effect by condition and no interaction effect (P>0.05) for the $\dot{V}O_2$ measured during the baseline cycling period and at end-exercise. Prior to donation, there were no differences in baseline or end-exercise $\dot{V}O_2$ between BR and PL groups (P>0.05). Follow-up tests revealed that both baseline $\dot{V}O_2$ (P<0.01) and end-exercise $\dot{V}O_2$ (P<0.05) were reduced in the BR group post-donation compared with pre-donation.

The $\dot{V}CO_2$, $\dot{V}E$, RER, blood [lactate] and blood [glucose] data during moderate-intensity exercise are reported in Table 2. Prior to donation, there were no differences in these variables at baseline or at end-exercise between the BR and PL groups (P>0.05) and there were no significant main effects by condition or time and no interaction effects (P>0.05).

Ramp incremental exercise

The effects of blood donation and BR and PL supplementation on the ramp incremental test parameters are reported in Table 3 and illustrated in Figures 2 and 3.

There was a significant main effect by time on $\dot{V}O_{2peak}$ (P<0.05), but no main effect by condition or an interaction effect (P>0.05). There were no differences between the groups at baseline (P>0.05). Follow-up tests indicated that, from pre to post donation, there was a significant reduction (0.19 $L \cdot min^{-1}$; ~5%) in $\dot{V}O_{2peak}$ in the PL group (P<0.05) but not in the

BR group (0.12 $L \cdot min^{-1}$; ~3%; $P>0.05$). There was a significant main effect by time and an interaction effect ($P<0.05$) but no main effect by condition ($P>0.05$) for PPO and TTF. Post hoc tests revealed a significant reduction in PPO and TTF in both PL and BR groups from pre to post donation ($P<0.01$). There were no differences in PPO or TTF between the groups prior to blood donation ($P>0.05$). However, the reduction in PPO and TTF following blood donation was more pronounced in PL compared with BR (5% vs. 3%; $P<0.05$). The change in [Hb] and Hct from pre to post donation was correlated with the change in TTF during ramp incremental exercise in PL (r = 0.58; $P=0.06$, and r = 0.70; $P<0.05$, respectively) but not BR (r = -0.10; $P>0.05$ and r = -0.41; $P>0.05$, respectively).

There was a significant interaction effect, but no main effects by time or group, for peak $\dot{V}CO_2$. Specifically, peak $\dot{V}CO_2$ was reduced in the PL group ($P<0.05$), but was unaffected in the BR group ($P>0.05$). There was no main effect by time or condition nor an interaction effect for peak $\dot{V}E$ ($P>0.05$). There was a significant main effect by time and an interaction effect for peak RER ($P<0.05$). Despite no difference at baseline, post hoc tests revealed an increase in peak RER in the BR group from pre to post donation ($P<0.01$).

NIRS measurements

Moderate-intensity exercise

There were no differences for total Hb (THb) between or within conditions during the moderate-intensity exercise bout. The [HHb] and TOI values measured during moderate-intensity exercise are reported in Table 4. There were no main effects by condition or time and no interaction effect for baseline [HHb] ($P>0.05$). There was a significant main effect by time for [HHb] from pre to post donation at 60 s, 120 s, 240 s and end-exercise ($P<0.05$), but no main effect by condition or an interaction effect at any time point ($P>0.05$). Post hoc tests

revealed a trend toward an increase in [HHb] in the PL group, but not the BR group, from pre to post donation at 120 s and 240 s of moderate exercise ($P<0.10$). There were no main effects by time or interaction effects for TOI at 60 s, 120 s, 240 s and end-exercise ($P>0.05$). However, there was a trend toward a main effect by condition for all time points ($P<0.10$). Follow-up tests revealed that blood donation resulted in reductions in TOI in the PL group at 60 s, 120 s and 240 s during moderate exercise, respectively ($P<0.05$; Table 4).

Ramp incremental exercise

There were no differences for THb between or within conditions during ramp incremental exercise. The [HHb] and TOI values measured during ramp incremental exercise are reported in Table 4 and the [HHb] profile is shown in Figure 4. There was a significant main effect by time ($P<0.05$) but no main effect by condition or an interaction effect ($P>0.05$) for [HHb] at 120 s and 240 s during ramp incremental exercise. Post hoc tests showed that [HHb] increased from pre to post donation at 240 s in PL ($P<0.05$) but not BR ($P>0.05$; Table 4). There was a significant main effect by time ($P<0.05$) and a trend for an interaction effect for [HHb] at 360 s ($P<0.10$) and at end-exercise ($P<0.05$) during the incremental exercise test. Post hoc tests revealed that [HHb] increased significantly from pre to post donation in the PL group at both 360 s and end-exercise ($P<0.05$; Table 4). The change in [HHb] from pre to post donation was higher in PL versus BR at end-exercise ($P<0.05$) and tended to be higher at 360 s ($P<0.10$).

DISCUSSION

The principal original findings in this study, consistent with our hypotheses, were that NO_3^--rich beetroot juice ingestion lowered the O_2 cost of moderate-intensity exercise, better preserved muscle oxygenation during moderate and ramp incremental exercise and attenuated

401 the reduction in ramp incremental exercise test performance and $\dot{V}O_{2peak}$ following blood
402 donation. These results indicate that dietary NO_3^- supplementation can ameliorate decrements
403 in exercise performance in a situation (i.e. reduction in blood O_2-carrying capacity) which
404 would be expected to compromise physiological function during exercise.
405
406 *Effects of blood donation on [Hb] and Hct*
407 The standard NHS blood bank donation (~450mL) reduced [Hb] and Hct by a similar
408 magnitude in the PL and BR groups. These results concur with previous studies that have
409 investigated the influence of whole blood withdrawal on [Hb]. For example, Gordon et al.
410 (27) and Mora-Rodriguez et al. (45) reported ~8% and ~7% reductions in [Hb], 24 and 48 h
411 post blood donation, respectively. The ~8% reduction in Hct in the present study is also
412 similar to the values reported by Burnley et al. (11) and Gordon et al. (27) who reported a ~7-
413 8% decrease in Hct one day after 450 mL blood donation. The reduction in blood O_2 carrying
414 capacity, secondary to the lower [Hb] and Hct, can result in a reduction in muscle O_2 delivery
415 and muscle O_2 diffusing capacity during maximal exercise, with significant implications for
416 exercise performance (5, 11, 18, 47, 54).
417
418 *Effects of nitrate supplementation on plasma [NO_3^-] and [NO_2^-]*
419 The ingestion of NO_3^--rich BR significantly elevated plasma [NO_3^-] and [NO_2^-] when
420 compared with baseline values. These findings are in agreement with earlier studies which
421 also examined the influence of BR supplementation in young, healthy subjects (4, 34, 69).
422 A small but significant rise in plasma [NO_3^-] was also noted in the PL group post
423 donation. This may be explained by a slight hemoconcentration or an upregulation in NOS
424 activity consequent to the reduction in whole body iron concentration after donating blood
425 (62). Plasma [NO_2^-] rose by ~800% in the BR group from pre to post donation, suggesting

appreciably enhanced NO bioavailability. Numerous other studies have also reported increases in plasma [NO_2^-] after BR supplementation, but the percentage increases attained were approximately half of those reported in this study (56, 69). This finding is likely a result of the higher dose of NO_3^- ingested (~43 mmol over 48 h) when compared with previous short-term BR supplementation studies. Interestingly, unlike in some earlier studies (4, 38, 59, 69), BR supplementation did not reduce resting blood pressure (BP) despite the elevated plasma [NO_2^-] (mean arterial pressure, pre- vs. post-donation: 81 ± 7 vs. 80 ± 7 mmHg). Similar BP values pre- vs. post-donation in the PL group indicates that total blood volume was restored 48 h following blood donation. The lack of effect of BR on BP in the present study may be related to the relatively low baseline BP values of the study participants (115/64 mmHg) and the relatively large number of female participants. It has been reported that females are less sensitive than males to the influence of NO_3^- supplementation on BP and that the extent of BP reduction with NO_3^- supplementation is correlated with the baseline BP (33).

Effects of blood donation and nitrate supplementation on the physiological responses to moderate-intensity exercise

The $\dot{V}O_2$ during both the unloaded baseline period and in the steady state of moderate-intensity exercise was significantly reduced (by ~4%) in the BR group, but not the PL group, after blood donation. A similar reduction in the O_2 cost of moderate-intensity exercise has been reported by Bailey et al. (4) after six days of non-concentrated NO_3^--rich BR ingestion and by Larsen et al. (38) after three days of $NaNO_3$ supplementation. The present findings are consistent with those of Kelly et al. (34) who observed that, in hypoxia, BR supplementation resulted in a decrease in both baseline and steady-state $\dot{V}O_2$ when compared with placebo. It has also been reported that acute (46) and 6 days (43) BR ingestion resulted

451 in significant reductions in $\dot{V}O_2$ during submaximal cycling exercise in hypoxia (15% and
452 11% O_2, respectively). Acute BR supplementation has also been reported to better preserve
453 arterial O_2 saturation following dynamic apnea (48).
454
455 The lowering of the O_2 cost of submaximal exercise after NO_3^- supplementation may be due
456 to a number of mechanisms, including a reduction in the ATP cost of muscle force production
457 (4) and/or an improvement in mitochondrial efficiency (40) and/or changes in redox
458 signalling (66). In addition to changes in muscle contractile or metabolic efficiency, muscle
459 O_2 delivery or its intramuscular distribution may be altered following NO_3^- supplementation
460 (21, 22). Exercise, particularly in hypoxia or under conditions that may limit O_2 carrying
461 capacity, such as blood donation, acts as a potent stimulus for vasodilatation and delivery of
462 O_2 to working muscle (12, 13). Both NO and O_2 compete for the binding site at cytochrome-
463 *c* oxidase (COX) in the mitochondrial electron transport chain (9). An elevation in NO
464 availability via NO_3^- supplementation, perhaps especially in conditions limiting O_2 delivery,
465 increases the likelihood of NO binding to COX and therefore inhibiting O_2 consumption at
466 the mitochondrion (10). As a result, NO may modify the intramuscular distribution of O_2 and
467 improve the oxygenation status of muscle fibres that are situated further away from the
468 capillaries (29, 55, 63). Compared to placebo, BR supplementation has been reported to
469 enable a greater maximal rate of mitochondrial ATP resynthesis (Q_{max}) and result in faster
470 muscle phosphocreatine recovery kinetics following exercise in hypoxia (60, 61), indicating
471 improved muscle O_2 availability at least in the immediate post-exercise period (61).
472
473 In the present study, TOI was significantly reduced and [HHb] tended to be higher during
474 moderate-intensity exercise post- compared to pre-donation in the PL group, suggesting that
475 muscle O_2 availability was lower and a greater muscle fractional O_2 extraction was necessary
476 to achieve the required $\dot{V}O_2$ (24, 36). These changes were attenuated in the BR group,

consistent with our hypothesis that BR supplementation would better preserve muscle oxygenation during moderate-intensity exercise when compared with PL. These results are consistent with Masschelein et al. (43) who reported that BR resulted in a greater muscle TOI and lower [HHb] during submaximal exercise in normobaric hypoxia. Collectively, these studies indicate that under conditions which may impair blood O_2 carrying capacity, such as following blood donation (present study) or in normobaric hypoxia (43), BR ingestion promotes a better matching between muscle O_2 delivery and O_2 demand, i.e. less O_2 extraction is required for the same moderate-intensity work rate, perhaps due to the lower exercise $\dot{V}O_2$ (34) or to preferential alterations in muscle perfusion (21, 22, 61). An increased ratio of O_2 delivery to O_2 consumption at a given work rate would be expected to retard the rate of fatigue development and to improve exercise performance.

Effects of blood donation and nitrate supplementation on the physiological responses to incremental exercise

As expected, blood donation and the associated reduction in O_2 carrying capacity resulted in a significant reduction in PPO and TTF during ramp incremental exercise. Panebianco et al. (47) also reported a significant reduction in PPO during incremental exercise, 2 days post blood donation. An important original finding in the present study was that ingestion of BR in the 48 hours post blood donation partly negated the decrement in performance when compared with PL. Specifically, the reduction in PPO and TTF following blood donation was significantly more pronounced in the PL group compared with BR. Interestingly, the reduction in TTF in the PL group was quite well correlated with the reduction in [Hb] ($r = 0.58$, $P=0.06$) and Hct ($r = 0.70$, $P<0.05$) following blood donation, whereas in the BR group, the correlations were weaker and non-significant ([Hb]: $r = -0.10$; Hct: $r = -0.41$; both $P>0.05$), implying that BR supplementation compensated for the lower [Hb] and Hct. These

findings are consistent with those of Masschelein et al. (43) who reported that, compared to PL, BR ingestion significantly attenuated the reduction in TTF when incremental exercise was performed in hypoxia.

$\dot{V}O_{2peak}$ was reduced by 5% from pre to 48 h post donation in the PL group. Similarly, Burnley et al. (11) reported a 4% decrease in $\dot{V}O_{2peak}$ during severe-intensity exercise 24 h following blood donation. This reduction was proportional to the reduced [Hb] and thus the ability to deliver O_2 to the working skeletal muscle during maximal exercise. In the present study, the reduced $\dot{V}O_{2peak}$ in the PL group following blood donation occurred in conjunction with an increased muscle [HHb], which may be interpreted as an increase in muscle fractional O_2 extraction in an (ultimately unsuccessful) attempt to offset the effects of a reduced [Hb] and lower muscle O_2 delivery (51, 54). In contrast, $\dot{V}O_{2peak}$ and [HHb] during the incremental test were not significantly altered by blood donation in the BR group. These results may indicate that the O_2 sparing effect of BR ingestion (Figure 2B), coupled perhaps with altered perfusion distribution (21, 22, 61), enabled muscle oxygenation to be better preserved during incremental exercise, such that an increased muscle fractional O_2 extraction was not mandated to achieve a given $\dot{V}O_{2peak}$. Ferguson et al. (21, 22) have reported that, in rats, BR supplementation can enhance vascular conductance and blood flow to working muscle and elevate the microvascular partial pressure of O_2 (PO_{2mv}), particularly in type II fibres. If similar effects occur in humans, this may enhance the blood-myocyte O_2 exchange gradient during higher intensity exercise, better preserving muscle oxygenation status, homeostasis and performance. It is also possible that a portion of the preserved ramp incremental test performance following blood donation with BR compared to PL may be attributable to effects of NO_3^- on muscle contractile function (50), perhaps particularly in type II fibers (31).

The mechanistic bases for the positive effects of BR ingestion on vascular and metabolic function in this and other situations warrants further investigation. In particular, while it is widely believed that the effects may be attributed to greater NO bioavailability or bioactivity, it is presently unclear precisely how this NO pool is stored and transported. NO is a highly reactive molecule with a short-half life *in vivo* and its rapid reaction with, for example, O_2 or heme proteins (30) suggests that the free transport of NO may be limited in plasma and within cells. It has been proposed that NO_2^- itself represents a principal means of 'NO' storage and transport, with the one electron reduction of NO_2^- to NO in blood and other tissues being facilitated, amongst many other factors including xanthine oxidoreductase, by deoxyhemoglobin and deoxymyoglobin, which will naturally be present in greater abundance in contracting skeletal muscle (16, 42). However, BR ingestion likely also increases the production and storage of other reactive nitrogen species. In particular, low molecular weight thiol groups may react with nitrogen oxides to yield s-nitrosothiol species (SNOs) which can be transported in the blood as s-nitrosohemoglobin (HbSNO) (17). It has recently been reported that the reduction in blood pressure following NO_3^- or NO_2^- ingestion in a rat model of hypertension was more closely related to plasma [s-nitrosothiol] than to plasma [NO_2^-] (49) and that s-nitrosothiol bioactivity derived through βCys93 may be essential for hypoxic vasodilation by erythrocytes (70). In contrast, in humans, Gladwin et al. (26) reported a significant arterial-venous NO_2^- gradient during forearm exercise and concluded that SNOs and HbSNO do not play a significant role in the regulation of vascular tone. The role of SNOs and HbSNO in the physiological effects of nitrate ingestion in humans remains to be clarified. Equally, the precise mechanisms by which an elevation of tissue [NO_2^-] following NO_3^- ingestion influences metabolic and vascular control at rest and during exercise remains unclear. While it is possible that NO_2^- itself is bioactive (58), unresolved questions include

552 the triggers and time course for the possible reduction of NO_2^- to NO, and the nature of both
553 NO transport to, and storage within, biological targets. Resolution of these issues will likely
554 require synthesis of experimental data deriving from 'competing' hypotheses.

555

556 *Perspectives*

557 This study has shown for the first time that despite a significant reduction in [Hb] post blood
558 withdrawal, BR supplementation lowered the O_2 cost of moderate-intensity exercise, better
559 preserved muscle oxygenation during moderate-intensity and ramp incremental exercise, and
560 attenuated the reduction in $\dot{V}O_{2peak}$ and incremental exercise test performance. These results
561 may have significant implications for athletes who wish to give blood without significant
562 detriment to training, individuals with clinical conditions which reduce blood O_2 carrying
563 capacity, such as anemia, and in conditions resulting in acute blood loss such as surgery or
564 military combat. In this context, it is of interest that transfusion of stored blood may impair
565 vasodilatory capacity, an effect that might be linked to the loss of NO bioavailability that
566 occurs during blood storage (17, 52). Treating banked blood to better maintain NO stores
567 might lead to improved functional outcomes following transfusion. In conclusion, BR
568 supplementation attenuates the decline in functional capacity arising from blood donation.

569

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574

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FIGURE LEGENDS

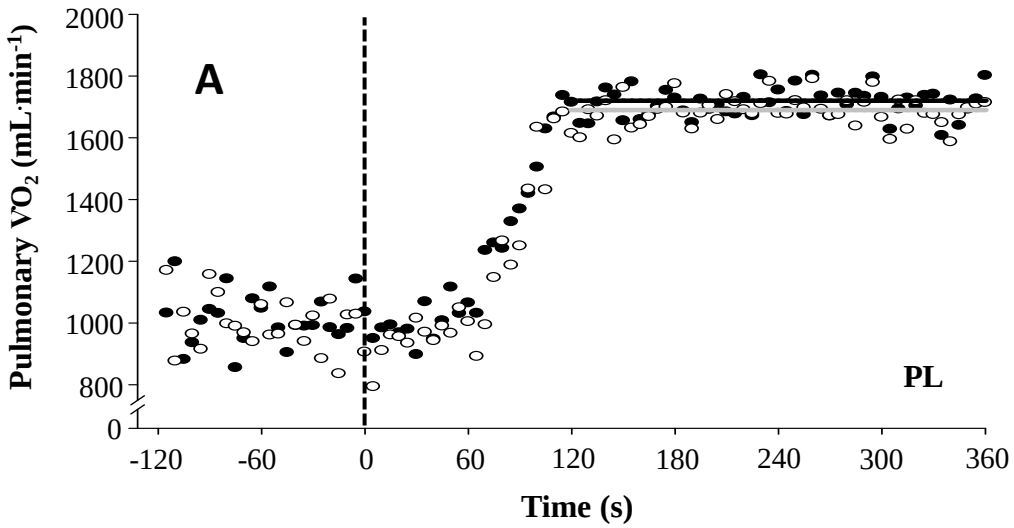
Figure 1: Pulmonary oxygen uptake ($\dot{V}O_2$) response following BR and PL supplementation prior to and following blood donation during a step increment to a moderate-intensity work rate. Responses prior to blood donation are shown as solid, filled circles, while responses post blood donation are shown as open, unfilled circles. The dotted vertical line represents the abrupt imposition of the moderate work rate from a baseline of 'unloaded' cycling. *A:* Group mean $\dot{V}O_2$ response to moderate-intensity exercise following PL ingestion. *B:* Group mean $\dot{V}O_2$ response to moderate-intensity exercise following BR ingestion. *C:* Steady state $\dot{V}O_2$ following PL and BR supplementation relative to pre blood donation baseline. The O_2 cost of moderate-intensity exercise was reduced following BR supplementation and blood donation compared with pre donation values, $*P<0.05$.

Figure 2: Group mean pulmonary $\dot{V}O_2$ response to incremental exercise prior to blood donation and following BR and PL supplementation after blood donation. Responses prior to blood donation are shown as solid, filled circles, while responses post blood donation are shown as open, unfilled circles. The dotted vertical line represents the onset of the ramp incremental test from a baseline of 'unloaded' cycling. The $\dot{V}O_{2peak}$ was reduced in the PL group ($*= P<0.05$), but not the BR group, after blood donation. TTF was reduced in both groups post donation ($^{\#} = P<0.05$), however, the reduction in TTF was greater in the PL group when compared with the BR group ($^{\$} = P<0.05$).

Figure 3. Group mean time to task failure (TTF) in the ramp incremental test prior to and post blood donation, following BR and PL supplementation. Responses prior to blood donation are shown as solid, filled bars, while responses post donation are shown as open,

unfilled bars. The TTF was reduced in both groups post donation (*= $P<0.05$); however, the reduction in TTF was greater in the PL group when compared with the BR group ($^{\#}=P<0.05$).

Figure 4. Group mean changes in deoxyhaemoglobin ([HHb]) prior to and post blood donation, following BR and PL ingestion. Responses prior to blood donation are shown as solid, filled circles, while responses post blood donation are shown as open, unfilled circles. The dotted vertical line represents the onset of the ramp incremental test from a baseline of 'unloaded' cycling. [HHb] increased significantly from pre to post donation in the PL group at 360 s and end-exercise (*=$P<0.05$). [HHb] was not altered from pre to post donation in the BR group. TTF was reduced in both groups post donation ($^{\#}$ = $P<0.05$), however, the reduction in TTF was greater in the PL group when compared with the BR group ($^{\$}$ = $P<0.05$).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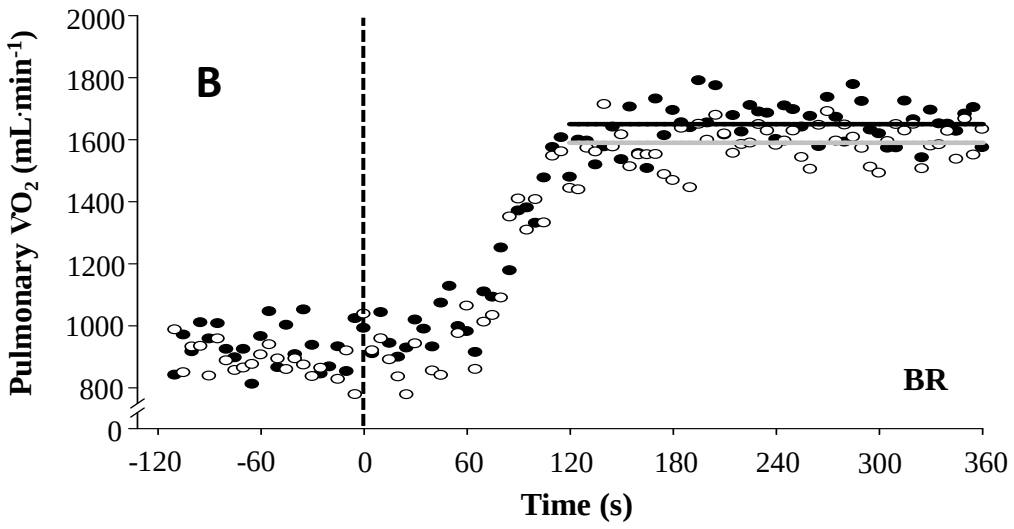
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BR
Pulmonary VO2 (mL·min-1)
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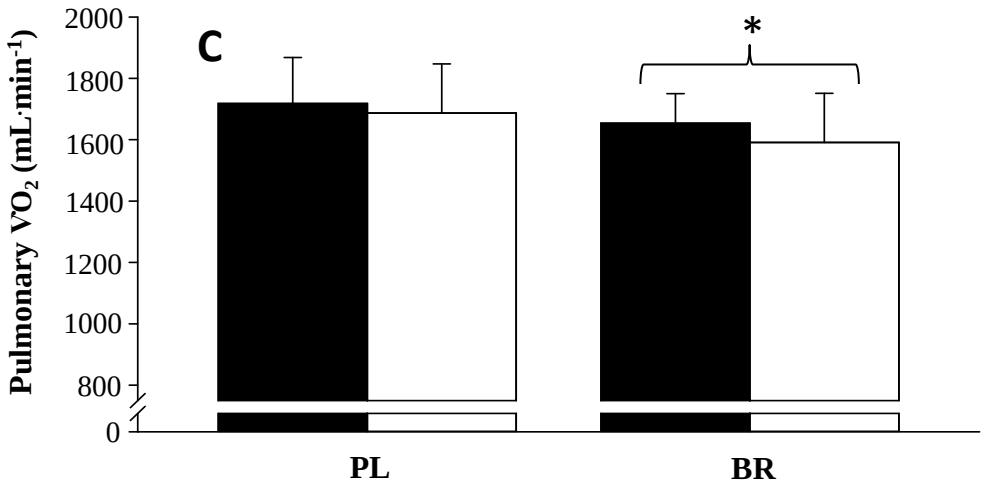
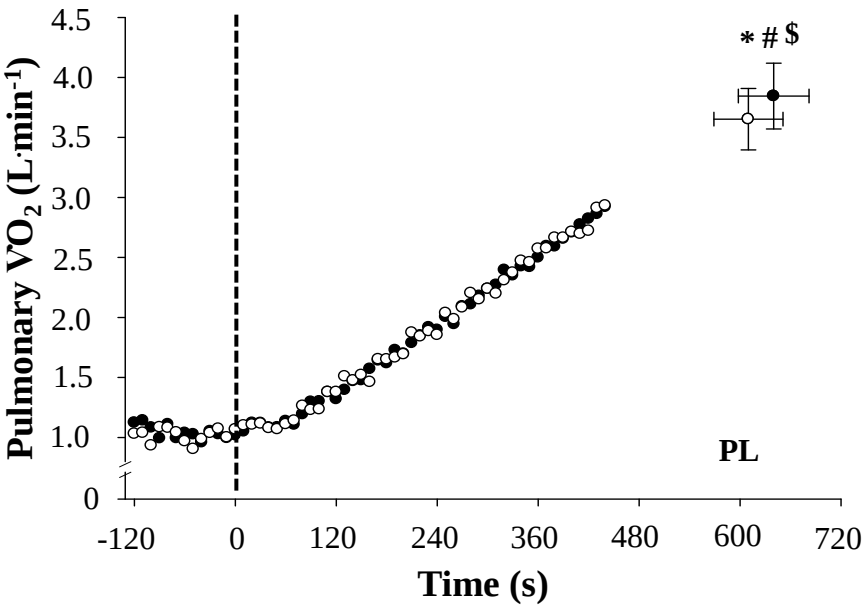
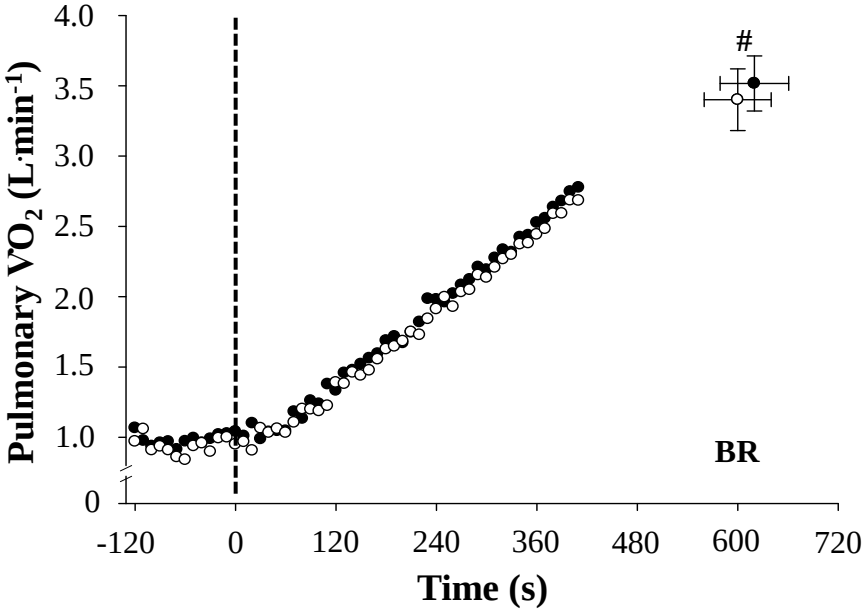
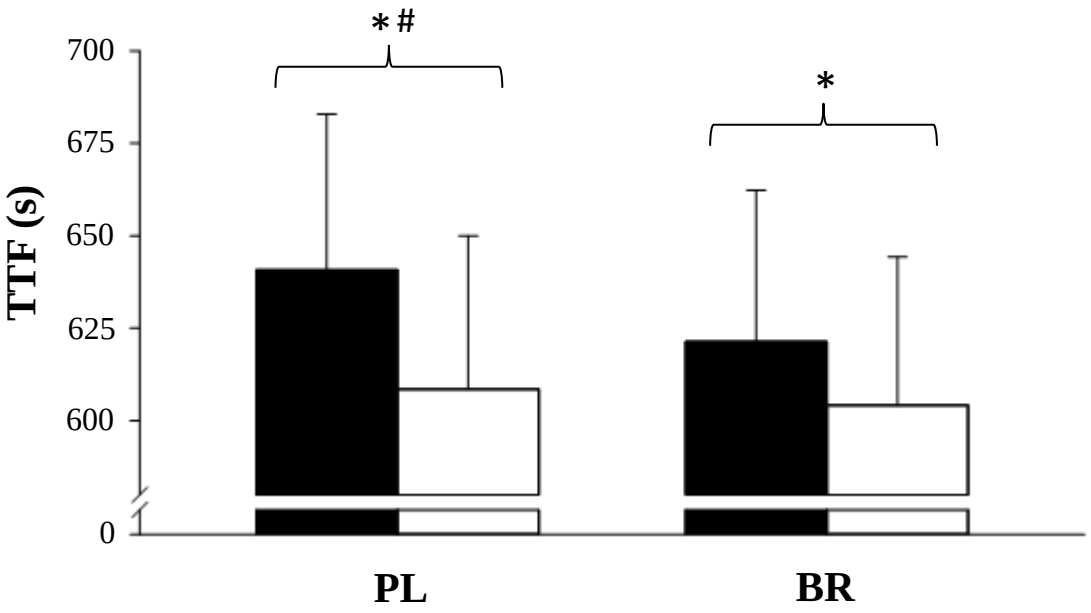
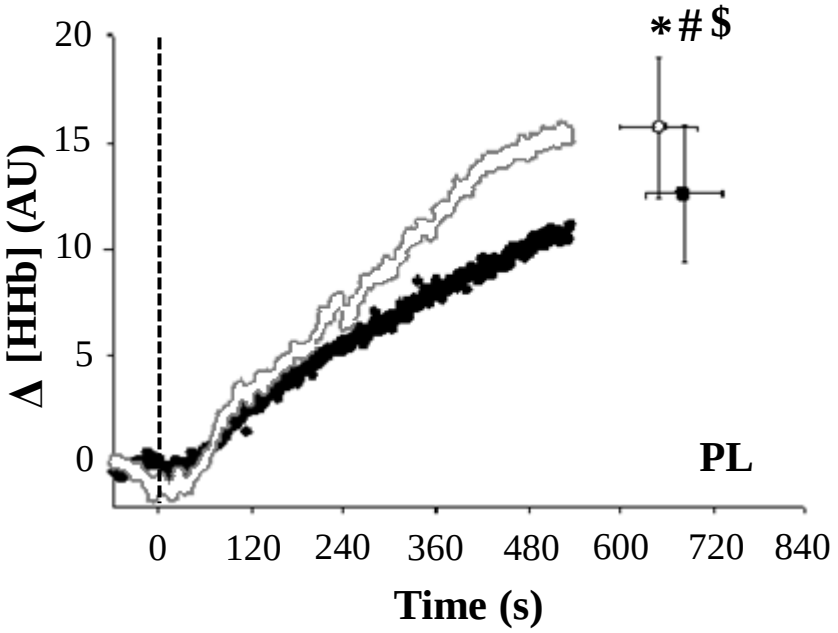
300
360
Time (s)


C
*
Pulmonary VO2 (mL·min-1)
2000
1800
1600
1400
1200
1000
800
0
PL
BR


Pulmonary VO_2 ($L \cdot min^{-1}$)
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0
-120
0
120
240
360
480
600
720
Time (s)
* # $
PL


Pulmonary VO_2 ($L \cdot min^{-1}$)
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0
-120
0
120
240
360
480
600
720
Time (s)
#
BR


*#
*
700
675
650
625
600
0
TTF (s)
PL
BR


*#$
20
15
10
5
0
Δ [HHb] (AU)
PL
0
120
240
360
480
600
720
840
Time (s)

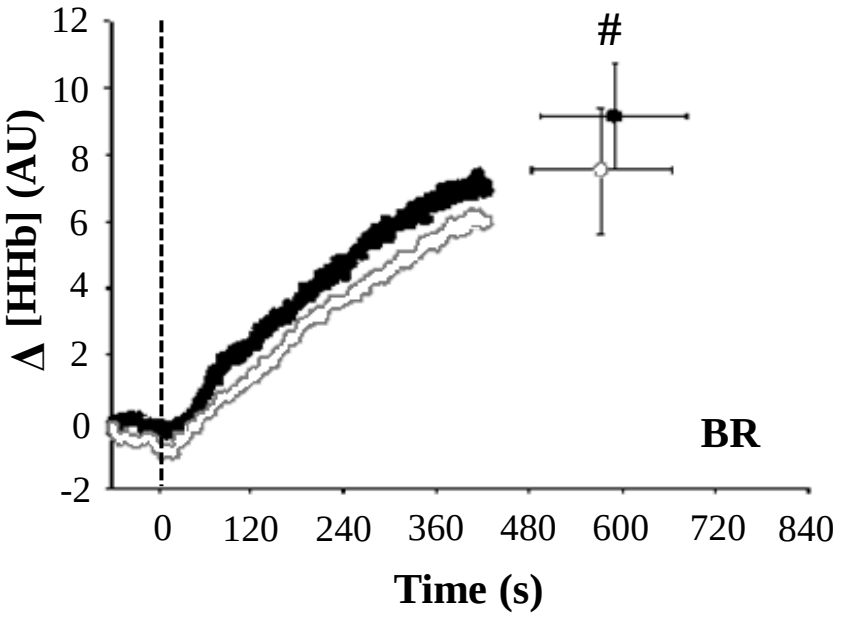

#
12
10
8
6
4
2
0
-2
Δ [HHb] (AU)
BR
0
120
240
360
480
600
720
840
Time (s)

Table 1: Blood pressure, resting heart rate, plasma nitrate and nitrite concentrations, hemoglobin concentration and hematocrit prior to and following blood donation in the PL and BR groups.

	PL		BR	
	Pre	**Post**	**Pre**	**Post**
Blood pressure (mmHg)				
Systolic	119 ± 7	118 ± 9	115 ± 11	113 ± 11*
Diastolic	69 ± 7	67 ± 7	64 ± 7	63 ± 7
Mean Arterial	86 ± 6	84 ± 8	81 ± 7	80 ± 7
Resting HR ($b \cdot min^{-1}$)	62 ± 9	66 ± 9	66 ± 11	71 ± 10*
Plasma [NO_3^-] (µM)	45 ± 11	50 ± 14*	47 ± 17	845 ± 350$^{*\$}$
Plasma [NO_2^-] (nM)	73 ± 18	72 ± 21	81 ± 29	619 ± 363$^{*\$}$
[Hb] ($g \cdot L^{-1}$)	149 ± 12	132 ± 18*	148 ± 15	137 ± 19*
Hct (%)	45 ± 2	41 ± 4*	45 ± 3	42 ± 5*

Values are mean ± SD. PL, Placebo group; BR, Nitrate group; Pre, pre-donation; Post, post-donation ; HR, heart rate; [NO_2^-], nitrite concentration; [NO_3^-], nitrate concentration; [Hb], hemoglobin concentration; Hct, hematocrit. *Significantly different from pre in the same condition (P<0.05). $^{\$}$Significantly different from post supplementation value in the PL group (P<0.05).

Table 2: Ventilatory and gas exchange dynamics, and blood lactate and glucose concentrations during moderate-intensity exercise prior to and following blood donation in the PL and BR groups

	PL		BR	
	Pre	**Post**	**Pre**	**Post**
$\dot{V}O_2$ **(L·min^{-1})**				
Baseline	1.01 ± 0.17	0.97 ± 0.20	0.96 ± 0.20	0.87 ± 0.21$^{\#}$
End exercise	1.72 ± 0.50	1.69 ± 0.53	1.65 ± 0.32	1.59 ± 0.34$^{\#}$
$\dot{V}CO_2$ **(L·min^{-1})**				
Baseline	0.88 ± 0.19	0.86 ± 0.19	0.89 ± 0.19	0.81 ± 0.19$^{\#}$
End exercise	1.60 ± 0.52	1.56 ± 0.50	1.53 ± 0.29	1.54 ± 0.29
RER				
Baseline	0.88 ± 0.08	0.90 ± 0.06	0.89 ± 0.05	0.92 ± 0.09
End exercise	0.94 ± 0.06	0.93 ± 0.06	0.93 ± 0.04	0.96 ± 0.06$^{\#}$
$\dot{V}E$ **(L·min^{-1})**				
Baseline	25 ± 5	24 ± 5	24 ± 5	22 ± 5$^{\#}$
End exercise	42 ± 11	40 ± 11	38 ± 6	38 ± 6
Δ **Blood [lactate]** (mM)	0.0 ± 0.3	0.1 ± 0.4	0.1 ± 0.3	0.1 ± 0.4
Δ**Blood [glucose]** (mM)	0.1 ± 0.7	- 0.2 ± 0.7	0.00 ± 0.3	0.1 ± 0.5

Values are mean ± SD. PL, Placebo group; BR, Nitrate group; Pre, pre-donation; Post, post-donation; [Bla], blood lactate concentration; [glu], blood glucose concentration; HR, heart rate. $^{\#}$Significantly different from pre in the same condition (P<0.05).

Table 3: Physiological responses to ramp incremental exercise prior to and following blood donation in the PL and BR groups.

	PL		BR	
	Pre	**Post**	**Pre**	**Post**
$\dot{V}O_2$peak ($L \cdot min^{-1}$)	3.84 ± 0.91	3.65 ± 0.85*	3.52 ± 0.65	3.40 ± 0.73
$\dot{V}O_2$peak ($mL \cdot kg^{-1} \cdot min^{-1}$)	49.9 ± 11.0	47.4 ± 10.0*	46.6 ± 6.0	44.9 ± 6.0
Peak power (W)	341 ± 70	324 ± 69*	331 ± 68	322 ± 66*
GET ($L \cdot min^{-1}$)	1.76 ± 0.40	1.68 ± 0.43	1.64 ± 0.44	1.63 ± 0.44
GET (W)	117 ± 29	109 ± 27	116 ± 35	112 ± 24
$\dot{V}CO_2$peak ($L \cdot min^{-1}$)	4.69 ± 1.12	4.44 ± 0.97*	4.26 ± 0.68	4.36 ± 0.77
RER peak	1.22 ± 0.06	1.22 ± 0.05	1.22 ± 0.06	1.29 ± 0.06*
$\dot{V}E$peak ($L \cdot min^{-1}$)	156 ± 44	150 ± 43*	134 ± 28	137 ± 32
HRpeak ($b \cdot min^{-1}$)	177 ± 16	181 ± 9	178 ± 12	179 ± 10
Δ Blood [lactate] (mM)	6.1 ± 1.4	5.5 ± 1.2	6.1 ± 1.9	6.8 ± 2.5
Δ Blood [glucose] (mM)	-0.2 ± 0.7	0.0 ± 1.1	-0.2 ± 0.4	0.0 ± 1.1

Values are mean ± SD. PL, Placebo group; BR, Nitrate group; Pre, pre-donation; Post, post-donation; GET, Gas exchange threshold; [Bla], blood lactate concentration; [glu], blood glucose concentration; HR, heart rate. *Significantly different from pre in the same condition (P<0.05).

Table 4: Near-infrared spectroscopy-derived [HHb] and TOI dynamics during moderate-intensity and ramp incremental exercise prior to and following blood donation in the PL and BR groups.

	PL		BR	
	Pre	**Post**	**Pre**	**Post**
	Moderate-intensity exercise			
[HHb]				
Baseline (AU)	-4.4 ± 3.0	-2.3 ± 3.1	-3.1 ± 3.7	-1.9 ± 2.5
60 s (AU)	-1.2 ± 2.3	2.3 ± 5.0	-0.1 ± 5.0	0.6 ± 3.9
120 s (AU)	-0.9 ± 3.0	3.5 ± 6.2	-0.1 ± 4.9	1.0 ± 3.7
240 s (AU)	-0.7 ± 3.9	2.3 ± 5.2	0.1 ± 4.9	1.1 ± 3.6
End (AU)	0.0 ± 4.4	2.5 ± 4.9	0.0 ± 4.9	1.0 ± 3.4
TOI				
Baseline (%)	65.3 ± 3.4	63.4 ± 3.3*	68.2 ± 4.3	70.1 ± 5.8
60 s (%)	61.9 ± 4.9	57.7 ± 5.0*	64.6 ± 6.5	65.6 ± 8.5
120 s (%)	61.9 ± 4.8	57.1 ± 5.7*	64.8 ± 6.1	65.6 ± 8.8
240 s (%)	60.7 ± 6.6	58.1 ± 4.8*	64.8 ± 6.5	65.8 ± 8.9
End (%)	61.4 ± 6.4	57.8 ± 5.0	65.3 ± 6.3	65.8 ± 8.9
	Ramp incremental exercise			
[HHb]				
Baseline (AU)	-6.2 ± 4.1	-3.4 ± 3.6	-5.1 ± 4.1	-2.6 ± 2.5
120 s (AU)	-3.3 ± 5.4	-0.1 ± 5.0	-2.7 ± 5.0	-0.7 ± 3.3
240 s (AU)	-0.8 ± 6.2	3.3 ± 5.8*	-0.6 ± 5.8	1.4 ± 4.4
360 s (AU)	2.0 ± 9.4	7.3 ± 9.1*	1.5 ± 6.6	3.4 ± 5.8
End (AU)	6.2 ± 11.3	12.8 ± 10.1*	3.8 ± 7.6	5.3 ± 7.2
TOI				
Baseline (%)	66.5 ± 3.9	67.3 ± 7.1	71.5 ± 3.9	72.5 ± 4.7
120 s (%)	63.3 ± 5.1	64.6 ± 8.6	68.6 ± 5.5	69.5 ± 6.9
240 s (%)	60.8 ± 6.5	60.7 ± 9.2	65.8 ± 7.5	65.9 ± 9.7
360 s (%)	57.3 ± 11.5	55.4 ± 12.3	61.9 ± 8.6	61.7 ± 11.4
End (%)	49.5 ± 12.6	47.6 ± 14.9	57.1 ± 7.0	57.2 ± 10.9

Values are mean ± SD. PL, Placebo group; BR, Nitrate group; Pre, pre-donation; Post, post-donation; [HHb], deoxygenated haemoglobin concentration; TOI, tissue oxygenation index; AU, arbitrary units. *Significantly different from pre in the same condition (P<0.05).