Title: Beetroot supplementation improves the physiological responses to incline walking

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Abstract

Purpose: We investigated the effects of an acute 24-h nitrate-rich beetroot juice supplement (BR) on the energy cost, exercise efficiency and blood pressure responses to intermittent walking at different gradients. **Methods:** In a double-blind, cross-over design, eight participants were provided with a total of 350 ml of nitrate-rich (~20.5 mmol nitrate) BR or placebo (PLA) across 24-h before completing intermittent walking at 3 km/h on treadmill at gradients of 1%, 5%, 10%, 15% and 20%. **Results:** Resting mean arterial pressure (MAP) was ~4.1% lower after BR (93 *vs.* 89 mmHg; P = 0.001), as well as during exercise (102 *vs.* 99 mmHg; P = 0.011) and recovery (97 *vs.* 94 mmHg; P = 0.001). Exercising (1227 *vs.* 1129 ml/min P < 0.001) and end-stage (1404 *vs.* 1249 ml/min; P = 0.002) oxygen uptake ($\dot{V}O_2$) was lower in BR compared to PLA, which was accompanied by an average reduction in phase II $\dot{V}O_2$ amplitude (1067 *vs.* 940 ml/min; P = 0.025). Similarly, recovery $\dot{V}O_2$ (509 *vs.* 458 ml/min; P = 0.001) was lower in BR. Whole-blood potassium concentration increased from pre-post exercise in PLA (4.1 ± 0.3 *vs.* 4.5 ± 0.3 mmol/L; P = 0.013) but not BR (4.1 ± 0.31 *vs.* 4.3 ± 0.2 mmol/L; P = 0.188). **Conclusions:** Energy cost of exercise, recovery of $\dot{V}O_2$, MAP and blood markers were ameliorated after BR. Previously reported mechanisms explain these findings, which are more noticeable during less efficient walking at steep gradients (15-20%). These findings have practical implications for hill-walkers.

Key words: Nitrate; efficiency; energy cost; blood pressure.

Abbreviations

Adenosine triphosphate (ATP)

Beetroot juice (BR)

Blood lactate concentration (B[La])

Blood potassium concentration ($[K^+]$)

Blood pressure (BP)

Coefficient of variation (CV%)

Diastolic (DP)

Energy cost of walking (Cw)

Glucose concentration ([Glu])

Gross efficiency (GE%)

Heart rate (HR)

Mean arterial pressure (MAP)

Nitrate (NO_3^-)

Nitrite (NO_2^-)

Nitric oxide (NO)

Oxygen uptake ($\dot{V}O_2$)

Placebo (PLA)

Rating of perceived exertion (RPE)

Sarcoplasmic reticulum calcium ($SR\text{-}Ca^{2+}$)

Systolic pressure (SP)

Ventilatory threshold (V_T)

Introduction

Supplementation of inorganic nitrate (NO_3^-), in the form of a highly-concentrated beetroot juice (BR) supplement (5.2 - 8.2 mmol of NO_3^- per day), improves endurance performance (Bailey et al. 2009, 2010, 2015; Bryan et al. 2008; Lansley et al. 2011a; Wylie et al. 2013). These effects are thought to be facilitated by the oxygen independent reductions of NO_3^- to nitrite (NO_2^-) and nitric oxide (NO) (Bailey et al. 2012). The NO_3^--NO_2^--NO cascade increases circulating NO_2^-, which can be further reduced to NO under certain physiological conditions (Bryan et al. 2008). Thus, the availability of NO can be preserved when enzymatic synthesis of NO via nitric oxide synthase (NOS) has been compromised (Bryan et al. 2008). The role of the NO_3^--NO_2^--NO pathway is potentiated during intense exercise, in particular, owing to the mild hypoxic and/or acidic conditions induced in the working skeletal muscle (Jones et al. 2016).

Nitric oxide is a key regulator of blood pressure (BP) and a signalling molecule for smooth muscle surrounding the endothelium (Gilchrist et al. 2011). These mechanisms permit vasodilation of blood vessels and enhance blood flow to the working musculature (Ferguson et al. 2013). Accordingly, reduced BP is common after NO_3^- supplementation during rest (Bailey et al. 2009; Bond et al. 2013, 2014; Kapil et al. 2010; Vanhatalo et al. 2010; Webb et al. 2008) and exercise (Bond et al. 2013, 2014; Choi et al. 2016; Kenjale et al. 2011; Oggioni et al. 2017). The combination of increased muscle blood flow and reduced oxygen extraction at the site of the working musculature leads to enhanced exercise tolerance (Bailey et al. 2010). The latter finding has been attributed to an enhanced efficiency of oxidative adenosine triphosphate (ATP) turnover in the mitochondria (Larsen et al. 2011). Mammalian studies have demonstrated that other factors, such as improved sarcoplasmic reticulum calcium (SR-Ca^{2+}) handling, might also contribute to improved exercise tolerance following NO_3^-

supplementation (Hernández et al. 2011). However, recent studies have demonstrated that longer (> 7 days) supplementation periods would be necessary to induce such changes in calcium handling proteins and point to alternative mechanisms, such as increased emission of mitochondrial reactive oxygen species and their possible effects on muscle contractile apparatus (Whifield et al. 2016). Nevertheless, these mechanisms contribute to a decreased ATP turnover for a given power output (Bailey et al. 2010), thus improving exercise economy across a variety of work-rates, with a notable 12 % reduction in the O_2 cost ($\dot{V}O_2$ amplitude) of walking at fixed gradients of 1% (Lansley et al. 2011b) and preferable alterations in the pulmonary oxygen uptake ($\dot{V}O_2$) kinetics of older adults (Kelly et al. 2013).

BR supplementation has not improved endurance performance or lowered exercising $\dot{V}O_2$ in all cases, particularly when administered to elite endurance athletes (Christensen et al. 2012; Peacock et al. 2012; Boorsma et al. 2014). The inconsistent findings have been ascribed to the aerobic fitness level of the athletes (Porcelli et al. 2015) and might relate to the lower reliance on the NO_3^--NO_2^--NO pathway or the presumed higher proportions of type I muscle fibre among well-trained endurance athletes. This is consistent with the evidence that the effects of NO_3^- are potentiated in type II muscle fibre (Hernández et al. 2011; Ferguson et al. 2013). As such, certain forms of locomotion that mandate the recruitment of type II muscle fibre could be affected differently by BR supplementation compared to those without, particularly when performed by non-elite athletes.

Incline walking is a popular and effective mode of exercise for physical fitness (Ainslie et al. 2002, 2005; Gottschall and Kram 2006) but will present a challenge to certain populations or vocations. The energy cost of walking (Cw) increases as a function of surface incline (Minetti et al. 2006). The external work done to walk on gradients between 1% and 20% comprises a

combination of negative and positive contributions (Minetti et al. 2006). On extreme uphill slopes (20%), this work becomes entirely positive and reaches a mechanical efficiency (i.e. ratio of mechanical work for vertical displacement to the energy expended) equivalent to concentric muscle contraction (Margaria et al. 1963). Negative work is more efficient (~ 80%), yet diminishes as a function of walking gradient, meaning there is a greater reliance on less efficient (~ 25%) positive work (Minetti et al. 2006). When walking uphill at progressively steeper inclines, muscle recruitment strategies are proportionally altered to coincide with the leading leg performing longer and more forceful concentric muscle contractions (i.e. more positive work) (Gottschall and Kram 2006). Consequently, greater lower-limb muscle activation (Vernillo et al. 2017), anaerobic metabolism (Sloniger et al. 1997; Staab et al. 1992) and perception of effort (Kolkhorst et al. 1996) is evident during uphill locomotion, compared to level exercise at the same speed. These observations reflect a change in the muscle recruitment patterns of the lower limbs and a logical reliance on type II muscle fibres to support the more forceful and concentrically-biased muscle contraction.

Whilst it is known that dietary NO_3^- can enhance muscle efficiency in humans, rodent studies have shown that NO_3^- supplementation can improve muscle O_2 delivery in musculature with a high type II fibre distribution (Ferguson et al. 2013), where it also improves SR-Ca^{2+} storage and release (Hernández et al. 2011). It has recently been proposed that the ergogenic properties of NO_3^- are fibre type dependent (Jones et al. 2016). In support of this, Breese et al. (2013) reported faster $\dot{V}O_2$ kinetics and improved time to exhaustion during step-exercise, designed to elicit the recruitment of higher order motor units. It is therefore feasible that consumption of NO_3^- can improve the efficiency of uphill walking, owing to the constrained changes in gait pattern toward a less efficient form of locomotion and an increased reliance on type II muscle fibres as the gradient increases. Indeed, a recent study demonstrated

lowered $\dot{V}O_2$ after BR supplementation during loaded uphill walking at 10% gradients in hypoxic (fraction of inspired O_2 = 11.7%) conditions (Shannon et al. 2017). However, the effects of BR supplementation on the physiological responses to incline walking at different gradients have not been systematically investigated among unloaded subjects in normoxic conditions. Accordingly, this study aimed to assess the effects of an acute 24-h NO_3^--rich BR supplement on the energy cost, exercise efficiency and BP responses to intermittent walking at different gradients. We hypothesized that the BR supplement would reduce the cost of exercise as a function of the exercise intensity and ameliorate recovery between exercise bouts, including a reduction in BP during rest and exercise.

Methods

Participants and design

Eight participants (5 male (age 27 ± 2 years; stature 1.74 ± 0.09 m; body mass 81.4 ± 7.3 kg; $\dot{V}O_{2max}$ 56.8 ± 7.8 ml/kg/min) and 3 female (age 29 ± 5 years; stature 166 ± 4 cm; body mass 60.0 ± 6.1 kg; $\dot{V}O_{2max}$ 38.0 ± 10.4 ml/kg/min) provided written informed consent to take part in this study. The participants were all recreationally active and regularly hill-walked as part of their occupation or leisure activities. The participants were all naive to the effects of beetroot juice on exercise. After baseline measures and familiarisation, in a double-blind, randomized cross-over design, the participants were either supplemented with a total of 350 ml of BR, in the form of beetroot shots, which were consumed at three evenly-spaced periods in the 24-h before the exercise trial, or a placebo (PLA), one week apart. After 24-h of supplementation, the participants completed a randomized, intermittent, inclined walking protocol on a treadmill, while their physiological ($\dot{V}O_2$, blood lactate concentration (B[La]), blood potassium concentration ($[K^+]$), heart rate (HR) and continuous BP), perceptual (rating

of perceived exertion; RPE) and gross mechanical work was measured. The participants were instructed to arrive at least 3-h post prandial, abstain from heavy exercise, caffeine intake, anti-bacterial mouthwash and NO_3^--rich foods in the 48-h before and during the study. Food diaries were completed to ensure compliance with these instructions. This study was granted institutional ethical approval and was conducted in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki.

Procedure

Baseline measurements

The participants visited the laboratory on three occasions. On their first visit, the participants were screened to ensure low-risk stratification, including assessment of resting BP and the completion of extended health questionnaires (Exercise Sport Science Australia). Resting BP was measured from the left brachial artery, using an automated sphygmomanometer (Omron M7 BP Monitor, OMRON Healthcare Europe, Hoofddrop, Netherlands) after sitting for 15-min in a quiet room. The average systolic (SP), diastolic (DP) and mean arterial pressures (MAP) of three measurements was recorded, calculated as: (MAP) (MAP = DBP + 0.33 (SBP–DBP)). This method of BP measurement was adopted at baseline, for screening purposes, whereas continuous measurements were performed for all subsequent visits. A HR monitor was strapped across the participants' chest and synchronized with a wrist watch (V800, Polar, Kempele, Finland) to record resting HR. Whilst seated in the same position, baseline venous blood was drawn from the participants' right arm at the antecubital fossa, using a hypodermic needle and a lithium-heparinized vacutainer (4 ml). The whole blood was immediately tested for blood glucose ([Glu]) and $[K^+]$ concentrations using a mobile analyser (iSTAT analyser, Abbott Point of Care Inc, Princeton, NJ) with a manufacturer-reported coefficient of variation (CV%) of 1.1%. The remaining and whole blood was then centrifuged

at 4,000 r/min and 4 °C for 10-min, after which the plasma was immediately stored at -80 °C. Blood plasma was later thawed and analysed for NO_3^- using a colorimetric assay, with an intra-assay CV% of 2.7, and an ELISA with an intra-assay CV% of 6.3, respectively (see http://www.abnova.com/products/products_detail.asp?catalog_id=KA1342). Plasma insulin was measured using a solid phase two-site enzyme immunoassay, with an intra-assay CV% of 6.4 and an inter-assay CV of 8.5 (see http://www.abnova.com/products/products_detail.asp?catalog_id=KA0921) .

The participants also completed an incremental, maximal treadmill test on their first visit, to establish $\dot{V}O_{2max}$ and ventilatory threshold (V_T). An adapted Bruce protocol was performed on a calibrated motorized treadmill (Mercury® Med, hpcosmos sports & medical gmbh; Nussdorf-Traunstein, Germany), beginning at 2.7 km/h and 0% gradient and increasing by speed and gradient every 3-min until volitional exhaustion. This test was selected to familiarize the participants to gradient walking, as well as incorporating a warm-up stage into the test and providing a measurement of $\dot{V}O_{2max}$. Pulmonary gas was measured continuously using a breath-by-breath gas analyser (Jaegar, Oxycon Pro, Viasys Healthcare, Hoechberg, Germany). The gas analyser and flow turbine were calibrated before each test using a known gas mixture (15% O_2 and 5% CO_2) and a 3-L syringe, respectively (Hans Rudolph, Kansas City, KS). $\dot{V}O_{2max}$ was determined as the mean value recorded over the final 30-s of the test. The same gas analyser was used throughout the study and calibrated identically, with a CV% of 4.3. The V_T was determined using the V-slope method, which has previously been used during 3-min incremental stages (Amman et al. 2004). The vertical mechanical power output (W) was calculated at the V_T according to Minetti et al. (2006) as:

$$\dot{W}_{vert} = gv \sin(\arctan[i]) \quad \text{[Eq. 1]}$$

where g = gravity (m/s/s), v = treadmill velocity (m/s) and i = the treadmill gradient.

The power output was subsequently used to delineate moderate and heavy exercise domains. The aim of the final experimental protocol was to exercise participants below the heavy exercise domain, at fixed gradients and speeds. As such, the power output at V_T was measured to ensure that it was higher than the highest power output used in the final test protocol. The mean of the highest power output to be performed during the experimental trial was calculated *a-priori* as 108 ± 18 W, while the mean power output at V_T was 165 ± 29 W, with no participants achieving a V_T power output lower than the highest power output in the experimental protocol. Therefore, all participants were exercising below the heavy intensity domain during the study. The mean $\%\dot{V}O_{2max}$ during the highest intensity (20 % gradient) trials was 52 ± 14 % and 48 ± 13 % in the PLA and BR conditions, respectively.

Experimental protocol

At baseline testing, the participants were randomly assigned to one of the two conditions and returned to the laboratory 24-h later (am) to receive their first of three supplements, in the form of two NO_3^--rich BR shots (2 x 70 ml ~ 4.1 mmol NO_3^- per shot; Beet it, James White Drinks Ltd., Ipswich, UK) or PLA (isocaloric fruit juice and maltodextrin mixture). The drinks were opened 10-min before their arrival at the laboratory and provided in identical opaque containers. The drinks were consumed within 30-s by all participants. The participants visited the laboratory to take their supplements on two further occasions across the subsequent 24-h period, with the second beverage (2 x 70 ml ~ 4.1 mmol NO_3^- per shot or PLA) consumed mid-way between the first and final serving. The final drink (1 x 70 ml ~ 4.1 mmol NO_3^- per shot or PLA) was consumed 1.5-h prior to testing (~2-2.5-h prior to exercise).

On the morning of each visit to the laboratory, resting BP, HR was measured and venous blood was drawn after 15-min of seated rest. The participants then mounted the treadmill and were fitted with a continuous BP monitor (Finometer MIDI, Finapres Medical Systems, Amsterdam, The Netherlands), designed to measure brachial BP using photoelectric plethysmography of the finger pressure waveform (Wesseling 1995). The Finapres® device was fitted to the left arm of each participant. Specifically, the device was fitted by securing an inflatable plastic bladder (50 µm and 55 mm length) between the distal and proximal inter-phalangeal joint of the middle finger, which housed an infrared light-emitting diode (950 nm emission) and detector. This was connected to a front-end unit via an air hose. The front-end unit was attached to the left wrist using a Velcro strap. The front end was connected to the main unit and pump inside the Finometer MIDI. A height correction unit was also fitted to the front-end unit to automatically correct for hydrostatic pressure changes due to movement in hand position relative to the heart. During the experimental protocol, the participants walked with a natural arm carriage, with their fingers relaxed and without holding on to the treadmill. A laptop was connected to the Finometer MIDI and the raw 200 Hz data were analysed using the BeatScope® software (Version 1.1, Finapres Medical Systems BV, Arnhem, Netherlands). BeatScope® software was used to reconstruct mean brachial pressure from the finger-pulse pressure waveform. The Finapres® was activated 1-min prior to recording to allow for calibration. The calibration is performed automatically through the Physiocal software in the device, which was activated during the trials. Any interruption of the recording owing to calibration was removed from the data set. No further filtering was applied to the data. MAP was reported during exercising and resting conditions. The inter-day walking MAP CV of this device ranged from 1.6-3.2 %, increasing as a function of exercise intensity (i.e. gradient).

Prior to exercise, a finger-prick capillary sample of blood was then drawn from the index finger of the participant whilst standing on the treadmill, which was measured for B[La] using a hand-held analyser (Lactate Pro; Arkray KDK Corp., Kyoto, Japan), which has a CV% of 3.1. The participants were then fitted with the face mask of the breath-by-breath gas analyser, where 3-min measurements of $\dot{V}O_2$, respiratory exchange ratio (RER), HR, and continuous BP were recorded during quiet standing. These measurements were continued for the remainder of the protocol. After quiet standing measurements, the participants began the intermittent walking trial, which comprised 5 × 5-min bouts of walking at randomized 1%, 5%, 10%, 15% or 20% inclines, at a fixed speed of 3 km/h, with each exercise stage interspersed by 5-min of quiet standing. No talking was permitted by participants during the trial. B[La] was measured 1-min post exercise bout, while RPE was provided by the participants in the final 15-s of each bout. Final venous blood draws were performed no more than 5-min after the final exercise stage.

The rest-recovery configuration of the protocol permitted the measurement of on-pulmonary $\dot{V}O_2$ kinetics during intermittent exercise and prevented interference of $\dot{V}O_2$ responses between repeated exercising bouts. Raw $\dot{V}O_2$ data were filtered to remove errant breaths more than 4 SD from the local mean. After interpolating breath-by-breath data to 1-s values, the $\dot{V}O_2$ on-kinetics were modelled using a non-linear least squares fitting procedure in a custom programme (Microsoft Excel). After excluding the first 20-s of data on-transients, a mono-exponential model was used to characterize all trials, as they were performed below the heavy intensity domain. On-transients were modelled based eq. 2:

$$\Delta\dot{V}O_2(t) = \text{base}\dot{V}O_2 + A\,[1 - e^{-(t - TD)/\tau)}] \quad \text{[Eq. 2]}$$

Where $\Delta\dot{V}O_2(t)$ = absolute $\dot{V}O_2$ at a given time *t*; base$\dot{V}O_2$ = baseline resting $\dot{V}O_2$; τ = fundamental time constant; TD = time delay and A = $\dot{V}O_2$ amplitude (i.e. ΔVO2 > base$\dot{V}O_2$).

The overall mean response time (MRT) was resolved by fitting a mono-exponential model to the $\dot{V}O_2$ data from 0-s to end exercise, without adding the time delay. The external mechanical work (W) was calculated based on published equations (Minetti et al. 2006; see Eq.1) and was used to determine gross efficiency (GE%) as the ratio of mechanical work done and metabolic energy, as measured via gas analysis. The energy cost of walking (Cw) was expressed as in joules (J) / kg body mass / min (J/kg/min), using the thermal equivalent of oxygen (20.9 J/L O_2) (Minetti et al. 2006).

Statistical analyses

All data are presented as means ± SD, unless otherwise stated. A two-way repeated measures analysis of variance was used to evaluate the effects of condition (BR or PLA), gradient (1%, 5%, 10%, 15% and 20%) and their interaction. If tests of Sphericity were violated, the Greenhouse-Giesser correction was used. In the event a statistical difference was identified, a *post-hoc* Bonferroni test was used to identify differences. An alpha level of $P < 0.05$ was set for all analyses. Effect sizes (*d*), calculated as the mean difference divided by the pooled standard deviation of scores, were included for pairwise comparisons to provide information on the magnitude of any effect. Effect sizes were defined as: trivial = 0.2; small = 0.21–0.6; moderate = 0.61–1.2; large = 1.21–1.99; very large > 2.0 (Hopkins et al. 2009). Statistical analysis was conducted through IBM SPSS (Software V22.0, IBM, New York, USA).

Results

Plasma [NO_3^-]

BR significantly elevated plasma [NO_3^-] (340 ± 58 μM) prior to exercise compared to baseline (90 ± 11 μM, P < 0.001; *d* = 5.3) and PLA (89 ± 12 μM, P = 0.001; *d* = 5.3). After the exercise protocol, plasma [NO_3^-] was significantly reduced in BR (309 ± 45 μM) but remained higher than PLA (86 ± 11 μM, P < 0.001; *d* = 6.0).

******Insert Table 1 here******

MAP during rest and exercise

Resting MAP was ~4.1% lower after BR consumption compared to PLA (P = 0.001, *d* = 1.5). There were also main effects (independent of the exercise stage) for recovery (P = 0.001) and exercise MAP (P = 0.011), which in both cases was lower (average ~3.9% and ~2.4%, respectively) in BR than PLA (Figure 1). The interaction between exercise stage and supplement for exercise MAP approached significance (P = 0.056), with the highest mean difference in MAP present at a gradient of 1% (~4.1%; *d* = 0.98) and lowest at 20% (~1.7%; *d* = 0.3).

******Insert Figure 1 here*******

Cardiorespiratory measurements

There were no differences between BR and PLA for resting (P = 0.699), exercise (P = 0.257) or recovery HR (P = 0.512) (Table 1). Pulmonary $\dot{V}O_2$ kinetics are presented in Table 1 and Figure 2, whilst average $\dot{V}O_2$ during each exercise stage and subsequent recovery is shown in Figure 3. Average (~18%, P < 0.001) and end-stage (~11%, P = 0.002) $\dot{V}O_2$ during each

exercise stage was lower in BR compared to PLA. This was accompanied by an average reduction in phase II $\dot{V}O_2$ amplitude (~12%, P = 0.025), and a trend for a lower phase II time constant (~12%, P = 0.07) with BR in response to exercise. There was an effect of the incline on the MRT (P = 0.04) and a trend for BR (P = 0.08), but no pairwise differences or interactions (P > 0.05) were found. There were also main effects for recovery $\dot{V}O_2$ (P = 0.001), which on average was lower in BR (~10%).

*******Figure 2 here*******

*******Figure 3 here*******

The energy cost of exercise was significantly lower across each exercise stage with BR (~8%, P < 0.001). There was also a significant interaction between exercise gradient and condition for gross efficiency (P < 0.001), which was lower with BR when walking at 10% (P < 0.001; *d* = 1.79), 15% (P = 0.001; *d* = 1.79) and 20% (P = 0.002; *d* = 1.05) gradients, but not different to PLA at any other stage (Figures 4 and 5).

*******Figure 4 here******

*******Figure 5 here*******

Blood measurements

BR had no effect on blood lactate (P = 0.122), which increased across each exercise stage (P = 0.002). Plasma insulin did not change significantly from the start to the end of the exercise protocol (P = 0.104) and was not different between BR and PLA (P = 0.332). Similarly, blood glucose did not change across gradients (P = 0.799) or between conditions (P = 0.379). However, there was a significant interaction (P = 0.005) for K^+, which was higher after exercise (4.5 ± 0.3 mmol/L) than before (4.1 ± 0.3 mmol/L) in PLA (P = 0.013; *d* = 1.33), but did not change in BR from pre-to-post exercise (4.1 ± 0.3 *cf.* 4.3 ± 0.2 mmol/L respectively, P = 0.188).

*******Table 1 here*******

Discussion

The main findings of this study further the current understanding of the physiological responses to exercise mediated by NO_3^- supplementation. In support of our hypothesis, we have demonstrated that the O_2 cost of exercise, the exercise-induced increase in blood [K^+] and both resting and exercising MAP was reduced, alongside favourable alterations in the pulmonary $\dot{V}O_2$ kinetics during rest-to-exercise transitions, after consuming NO_3^--rich BR across a 24-h period. Collectively, these findings support, and extend upon, the reported physiological effects conferred by BR supplementation, which have many potential implications for people walking on inclined surfaces as part of their daily tasks. Furthermore, our findings suggest that the form of locomotion (uphill walking) permits the physiological changes induced by dietary NO_3^- to manifest during exercise, despite the moderate intensity of the task. The MAP/$\dot{V}O_2$ at higher external intensities (i.e. 15-20% inclines) also reveals a phenomenon previously unreported after dietary NO_3^- supplementation.

We have demonstrated that acute supplementation of a NO_3^--rich BR supplement lowered MAP at baseline (rest) and during inclined walking, with seemingly larger effects at gradients of 1% - 10%, as well as during post-exercise recovery (Figure 1). However, there were no differences in HR between conditions (Table 1). These findings imply that the net systemic vasodilatory effect of NO on blood vessels was more active during rest and lower-intensity exercise (Cw < ~7 J/kg/min) than when exercise intensity was increased (Cw = > ~8 J/kg/min) via inclined walking. Indeed, since MAP was also lower during rest, the net increase in MAP during 15% and 20% inclines was actually larger in the BR compared to the PLA condition. We did not anticipate these responses and, consistent with most studies (Bond et al. 2013; Choi et al. 2016; Kenjale et al. 2011), it was hypothesized that MAP would be systematically lower throughout the rest and exercise protocol.

Oggioni et al. (2017) reported very similar findings to that of the current study, albeit among older adults (64.7 ± 3.0 years), where reductions in diastolic BP were observed at lower external cycling power output of 20 W – 80 W after BR consumption. During their subsequent exhaustive trial, both systolic and diastolic BP was equal between PLA and BR conditions. However, other studies have reported a consistent reduction in BP with increasing exercise intensity (Bond et al. 2013; Choi et al. 2016; Kenjale et al. 2011). The reasons for conflicting findings between studies is unclear, but it is noteworthy that each of the studies with contrasting data to ours used manual sphygmomanometry to measure BP responses, which rely on human operation and only provide a single caption of end-exercise values, rather than continuous recordings. Indeed, ambulatory BP recordings have previously been reduced among hypertensive individuals (Kapil et al. 2015), which avoids the technical expertise needed to measure manual exercising BP. One explanation for our MAP

observations could be a greater increase in cardiac output (via stroke volume) in the BR condition at higher gradients; however, changes in cardiac output have not been observed after BR supplementation (Oggioni et al. 2017). Redistribution of blood flow towards the working skeletal musculature has also been demonstrated following BR supplementation (Horiuchi et al. 2017) and the seemingly necessary exercise-induced net increase in central BP might also be explained by a similar mechanism. Further investigations are needed to explore such mechanisms associated with nitrate-induced changes in MAP during different exercise tasks.

Consistent with other studies (Bailey et al. 2009; Larsen et al. 2011; Lansely et al. 2011b), we found a reduction in the O_2 cost of exercise, which translated into improvements in GE at 10%, 15% and 20% gradients, among the BR condition compared to PLA (Figure 5). These findings are most comparable with the reported 12%-14% reductions in O_2 cost of exercise (Lansley et al. 2011b), despite the lower treadmill velocity (3 km/h *vs.* 4 km/h). The only other study to use an inclined treadmill protocol (Kenjale et al. 2011) did not increase the incline beyond 6% and reported minimal effects of BR on $\dot{V}O_2$ responses, despite exercising to exhaustion. We attribute our findings to the type of exercise being performed at higher gradients.

When walking uphill at progressively steeper inclines, muscle recruitment strategies are proportionally altered, such that the leading leg performs longer and more forceful concentric muscle contractions (i.e. more positive work) (Gotschall and Kram 2006). Based on Henneman's size principle, the leading legs would require recruitment from a greater number of larger motor units (type II fibre). These muscle fibres provide more force but are less fatigue-resistant and are recruited during a period of increased metabolic demand (Minetti et

al. 2006). Given the lower-limb forces required to walk on inclined terrain, coupled with the above-mentioned changes in gait, it is feasible that intermittent occlusion of blood flow will also result in metabolic perturbation at the working musculature. The increased metabolic cost, intensity and duration of contractions, coupled with an intermittent occlusion of local blood flow that occurs during uphill walking, would lead to a mildly hypoxic and acidic environment, which has been suggested to potentiate the effects of NO at the muscle (Jones et al. 2016).

Plasma insulin and glucose concentrations were unaffected by the BR condition compared to PLA, which has been reported before (Gilchrist et al. 2013), despite the previously described improvements in glucose homeostasis induced by dietary NO_3^- after intense exercise (Wylie et al. 2013). Our findings are most likely related to the lower intensity of the exercise or the health status of the participants, who did not present with symptoms of metabolic disease. There were also no differences in B[La] between conditions after each trial. However, owing to the organization of the protocol, B[La] was recorded in the 1-min after exercise cessation, which is unlikely to permit a larger accumulation of lactate in the blood. There was an interaction between time and condition for $[K^+]$, indicating that BR supplementation attenuated its exercise-induced increase. This confirms the findings of other analyses (Wylie et al. 2013), which were explained by the accelerated NO_3^--NO_2^--NO pathway in acidic environments, potentially blunting the rise in extracellular $[K^+]$ in this state. We also speculate that the increased ATP availability (Larsen et al. 2011) for Na^+/K^+ pumping or increases in muscle blood flow (Ferguson et al. 2013) found with NO_3^- consumption would facilitate clearance of K^+. We are the first to show this during moderate intensity exercise and also attribute this finding to the type of exercise performed. Plasma $[K^+]$ is increased after exercise, reflecting a transient disturbance in muscle ion homeostasis and reduced muscle

excitability. Intermittently walking at 1%-20% inclines for a total of 55-min at an intensity below the heavy domain was, therefore, sufficient to induce K^+ flux into the blood. The recruitment of muscle fibres when fixed into an inefficient gait cycle is likely to be type II preferential, thus relying upon musculature that is more fatigable owing to the lower O_2 muscle tension (Jones et al. 2016). The observed increase in $[K^+]$ in this study can be considered as a marker of peripheral fatigue that is increased during inclined walking and attenuated by BR supplementation. Future studies should explore the mechanisms through which BR exerts this effect.

$\dot{V}O_2$ on-kinetics were improved with BR supplementation. Reductions in the amplitude and non-significant (P = 0.07), yet small effect sizes (d = 0.2 – 0.5), for phase II time constant from rest to exercise support the findings of others during different exercise types (Bailey et al. 2009, 2010; Lansley et al. 2011b). To the best of the authors' knowledge, this is the first study to demonstrate enhanced recovery of pulmonary O_2 after BR supplementation. Recovery was improved by reducing the resting $\dot{V}O_2$ during the intermittent 5-min recovery periods. These results were accompanied by a lower resting MAP, reflecting reduced cardiorespiratory stress in response to exercise and, thus, improved exercise tolerance after BR supplementation.

There are a variety of occupations in which employees are required to regularly perform physically demanding tasks. For example, the daily duties of outdoor occupations, such as military, farming and emergency services, comprise walking activities performed over-ground of varying topography (Ruby et al. 2002). Similarly, physically challenging outdoor activities, such as mountaineering and hiking, are increasingly popular recreational pursuits, requiring prolonged sub-maximal hill-walking (Ainslie et al. 2002, 2005; Gottschall and

Kram 2006). For people engaged in such habitual activities, uphill locomotion is perhaps one of their most challenging tasks and can lead to an earlier onset of fatigue (Vernillo et al. 2017). Indeed, there is a significant metabolic cost associated with hill-walking, which increases proportionally with the gradient of the slope and limits the capacity for mechanical energy transfer (Gottschall and Kram 2006; Minetti et al. 2006). Interventions that increase hill-walking performance and presumably delay the onset of fatigue via reduced metabolic costs delay the onset of fatigue, without the need for long-term training adaptation, are therefore applicable to habitual hill-walkers. Beyond those employed in hazardous occupations, the capacity to improve the physiological responses to daily exercise, such as commuting, might also confer a functional and health benefit. Given its apparent capacity to reduce the appearance of fatigue-related metabolites and lower the O_2 cost of exercise, acute NO_3^- supplementation (BR) could provide a practical intervention for such populations.

Conclusion

The Cw of walking is reduced and muscle efficiency is increased, particularly at steeper gradients (10%-20%), after acute BR supplementation. Intermittent recovery of pulmonary O_2 uptake and MAP is ameliorated and selected blood markers of peripheral fatigue are attenuated by BR supplementation. BP was particularly lowered whilst walking at smaller gradients (1-10%) after BR supplementation but approaches PLA conditions at higher (15-20%) gradients, despite a lower O_2 cost. We collectively attribute these findings to the inefficient form of locomotion that is required to walk at these gradients, necessitating the recruitment of more fatigable muscle fibre types during a period of increased metabolic demand and intermittently hypoxic or mildly acidic environment, know to augment the reduction of NO_2^- to NO. However, further research is required to confirm these suggestions.

These findings have implications for those exercising on terrain of varying topography in their daily tasks.

Compliance with Ethical Standards

Conflict of Interest: The authors declare that they have no conflict of interest.

Ethical approval: All procedures were performed in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Informed consent: Informed consent was obtained from all individual participants included in the study

References

Ainslie PN, Campbell IT, Frayn KN, et al. (2002) Physiological and metabolic responses to a hill walk. J. Appl. Physiol. 92:179–187.

Ainslie PN, Campbell IT, Lambert JP, et al. (2005) Physiological and metabolic aspects of very prolonged exercise with particular reference to hill walking. Sports Med. 35:619–647.

Amman M, Subudhi A, Foster C. (2004) Influence of Testing Protocol on Ventilatory Thresholds and Cycling Performance. Med. Sci. Sports Exerc. 36:613–622.

Bailey SJ, Fulford J, Vanhatalo A, et al. (2010) Dietary nitrate supplementation enhances muscle contractile efficiency during knee-extensor exercise in humans. J. Appl. Physiol. 109:135-48.

Bailey SJ, Vanhatalo A Winyard PG, et al. (2012) The nitrate-nitrite-nitric oxide pathway: Its role in human exercise physiology. Eur. J. Sport Sci. 12:4, 309-320.

Bailey SJ, Varnham RL, DiMenna FJ, et al. (2015) Inorganic nitrate supplementation improves muscle oxygenation, O_2 uptake kinetics, and exercise tolerance at high but not low pedal rates. J. Appl. Physiol. 118:1396-405.

Bailey SJ, Winyard P, Vanhatalo A, et al. (2009) Dietary nitrate supplementation reduces the O_2 cost of low-intensity exercise and enhances tolerance to high-intensity exercise in humans. J. Appl. Physiol. 107:1144-55.

Bond V, Curry BH, Adams RG, et al. (2013) Effects of dietary nitrates on systemic and cerebrovascular hemodynamics. Cardiol. Res. Pract. 435629. doi: 10.1155/2013/435629.

Bond V, Curry BH, Adams RG, et al. (2014) Cardiorespiratory function associated with dietary nitrate supplementation. Appl. Physiol. Nutr. Metab. 39: 168–172.

Boorsma RK, Whitfield J, Spriet LL. (2014) Beetroot juice supplenetation does not improve performance of elite 1500-m runners. Med. Sci. Sports. Exerc. 46: 2326-2334.

Breese BC, McNarry MA, Marwood S, et al. (2013) Beetroot juice supplementation speeds O_2 uptake kinetics and improves exercise tolerance during severe-intensity exercise initiated from an elevated metabolic rate. Am. J. Physiol. Regul. Integr. Comp. Physiol. 305:1441–1450.

Bryan NS, Calvert JW, Gundewar S, et al. (2008) Dietary nitrite restores NO homeostasis and is cardioprotective in endothelial nitric oxide synthase-deficient mice. Free Radic Biol Med. 45: 468-474.

Choi H-M, Kim B-H, Nho, H, et al. (2016) Dietary nitrate supplementation attenuates blood pressure in young prehypertensive men during exercise. J Mens. Health. 12: 25-33.

Christensen PM, Nyberg M, Bangsbo J. (2013) Influence of nitrate supplementation on VO_2 kinetics and endurance of elite cyclists. Scand. J. Med. Sci. Sport. 23:21-31.

Ferguson SK, Hirai DM, Copp SW, et al. (2013) Impact of dietary nitrate supplementation via beetroot juice on exercising muscle vascular control in rats. J. Physiol. 591:547-557.

Gilchrist M, Shore AC, Benjamin N. (2011) Inorganic nitrate and nitrite and control of blood pressure. Cardiovasc. Res. 89: 492–498.

Gilchrist M, Winyard PG, Jones AM. (2013) Effects of short-term dietary nitrate supplementation on blood pressure, O2 uptake kinetics, and muscle and cognitive function in older adults. Am. J. Physiol. Regul. Integr. Comp. Physiol. 304:73–83.

Gottschall JS, Kram R. (2006) Mechanical energy fluctuations during hill walking: the effects of slope on inverted pendulum exchange. J. Exp. Biol. 2006; 209: 4895–4900.

Hernández A, Schiffer TA, Ivarsson N, et al. (2011) Dietary nitrate increases tetanic [Ca2]i and contractile force in mouse fast-twitch muscle. J. Physiol. 590: 3575–3583.

Hopkins, WG, Marshall, SW, Batterham, AM et al. (2009) Progressive statistics for studies in sports medicine and exercise science. Med. Sci. Sports. Exerc. 41:3-13.

Horiuchi M, Endo J, Dobashi S, et al. (2017). Muscle oxygenation profiles between active and inactive muscles with nitrate supplementation under hypoxic exercise. Physiol. Rep. 5: e13475; doi:10.14814/phy2.13475

Jones AM, Ferguson SK, Bailey SJ, et al. (2016) Fiber type-specific effects of dietary nitrate. Exerc. Sport. Sci. Rev. 44: 53-60.

Kapil V, Milsom AB, Okorie M, et al. (2010) Inorganic nitrate supplementation lowers blood pressure in humans. Hypertension. 56: 274–281.

Kapil V, Khambata RS, Robertson A, et al. (2015) Dietary nitrate provides sustained blood pressure lowering in hypertensive patients: a randomized, phase 2, double-blind, placebo-controlled study. Hypertension. 65:320–327

Kelly J, Fulford J, Vanhatalo A, et al. (2013) Effects of short-term dietary nitrate supplementation on blood pressure, O_2 uptake kinetics, and muscle and cognitive function in older adults. Am. J. Physiol. Regul. Integr. Comp. Physiol. 304:73–83.

Kenjale AA, Ham KL, Stabler T, et al. (2011) Dietary nitrate supplementation enhances exercise performance in peripheral arterial disease. J. Appl. Physiol. 110: 1582-1591.

Kolkhorst FW, Mittelstadt SW, Dolgener FA. (1996) Perceived exertion and blood lactate concentration during graded treadmill running. Eur. J. Appl. Physiol. Occup. Physiol. 72:272–277.

Lansley KE, Winyard PG, Bailey SJ, et al. (2011a) Acute dietary nitrate supplementation improves cycling time trial performance. Med. Sci. Sport Exerc. 243:1125-1131.

Lansley KE, Winyard PG, Fulford J, et al. (2011b) Dietary nitrate supplementation reduces the O_2 cost of walking and running: A placebo-controlled study. J. Appl. Physiol.110: 591-600.

Larsen FJ, Schiffer TA, Borniquel S, et al. (2011) Dietary inorganic nitrate improves mitochondrial efficiency in humans. Cell. Metab. 13: 149–159.

Margaria R, Cerretelli P, Aghemo P, et al. (1963) Energy cost of running. J. Appl. Physiol. 18: 367–370.

Minetti AE, Moia C, Roi GS, et al. (2002) Energy cost of walking and running at extreme uphill and downhill slopes J. Appl. Physiol. 93:1039-1046.

Oggioni C, Jakovljevic DG, Klonizakis M, et al. (2017) Dietary nitrate does not modify blood pressure and cardiac output at rest and during exercise in older adults: a randomised cross-over study. Int. J. Food. Sci. 31: 1-10.

Peacock O, Tjonna AE, James P, et al. (2012) Dietary nitrate does not enhance running performance in elite cross-country skiers. Med. Sci. Sports. Exerc. 44:2213-2219.

Porcelli P, Ramaglia M, Bellistri G, et al. (2015). Aerobic fitness affects the exercise performance responses to nitrate supplementation. Med. Sci. Sports. Exerc. 47;1643-1651.

Rowell LB. (1993) Central circulatory adjustments to dynamic exercise. In Rowell LB (auth.) Human Cardiovascular Control. Oxford University Press, New York, pp 162-203.

Ruby BC, Shriver TC, Zderic TW, et al. (2002) Total energy expenditure during arduous wildfire suppression. Med. Sci. Sports Exerc. 34: 1048–1054.

Shannon OM, Duckworth L, Barlow MJ, et al. (2017). Effects of dietary nitrate supplementation on physiological responses, cognitive function, and exercise performance at moderate and very-high simulated altitude. Front. Physiol. 8:401. doi:10.3389/fphys.2017.00401

Sloniger MA, Cureton KJ, Prior BM, et al. (1997) Anaerobic capacity and muscle activation during horizontal and uphill running. J Appl Physiol. 83:262–269.

Staab JS, Agnew JW, Siconolfi SF. (1992) Metabolic and performance responses to uphill and downhill running in distance runners. Med. Sci. Sports. Exerc. 24:124–127.

Vanhatalo A, Bailey SJ, Blackwell JR, et al. (2010) Acute and chronic effects of dietary nitrate supplementation on blood pressure and the physiological responses to moderate-intensity and incremental exercise. Am. J. Physiol. Regul. Integr. Comp. Physiol. 299:1121-1231.

Vernillo G, Giandolini M, Edwards WB, et al. (2017) Biomechanics and physiology of uphill and downhill running. Sports. Med. 47:615-629.

Webb AJ, Patel N, Loukogeorgakis S, et al. (2008) Acute blood pressure lowering, vasoprotective, and antiplatelet properties of dietary nitrate via bioconversion to nitrite. Hypertension. 51: 784-790.

Wesseling KH. (1995) A century of noninvasive arterial pressure measurement: from Marey to Penaz and Finapres. Homeostasis. 36: 2–3.

Whitfield J, Ludzki A, Heigenhauser GJF, et al. (2016). Beetroot juice supplementation reduces whole body oxygen consumption but does not improve indices of mitochondrial efficiency in human skeletal muscle. J. Physiol. 594: 421–435.

Wylie LJ, Mohr M, Krustrup P, et al. (2013) Dietary nitrate supplementation improves team sport-specific intense intermittent exercise performance. Eur. J. Appl. Physiol. 113:1673-1684.

Figure legends

Figure 1. Mean arterial pressure responses to walking at 1%, 5%, 10%, 15% and 20% inclines and intermittent recovery in beetroot and placebo conditions (n = 8).*Significant (P < 0.05) main effect of BR supplementation

Figure 2. Pulmonary $\dot{V}O_2$ (L/min) responses of a representative subject walking at 1%, 5%, 10%, 20% and 15% inclines and intermittent recovery in beetroot and placebo conditions (n = 8). Data are shown in 5-s averages.

Figure 3. Mean pulmonary $\dot{V}O_2$ (ml/min) responses to walking at 1%, 5%, 10%, 15% and 20% inclines and intermittent recovery in beetroot and placebo conditions (n = 8). *Significant (P < 0.05) main effect of BR supplementation

Figure 4. Energy cost (J/kg/min) of walking at 1%, 5%, 10%, 15% and 20% inclines in beetroot and placebo conditions (n = 8). *Significant (P < 0.05) main effect of BR supplementation

Figure 5. Gross efficiency (%) of walking at 1%, 5%, 10%, 15% and 20% inclines in beetroot and placebo conditions (n = 8). *Significant (P < 0.01) interaction between BR and PLA.

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Table title

Table 1. Cardiorespiratory responses to walking at 1%, 5%, 10%, 15% and 20% inclines in beetroot (BR) and placebo (PLA) conditions (n = 8). *Significant (P < 0.05) main effect of BR supplementation

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Figure 1

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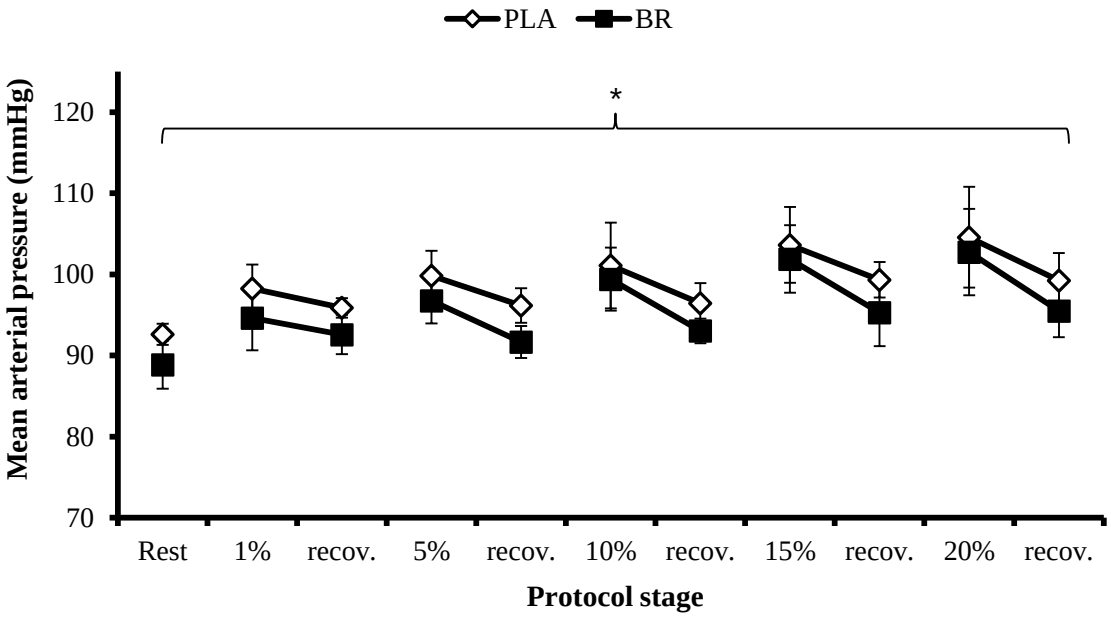

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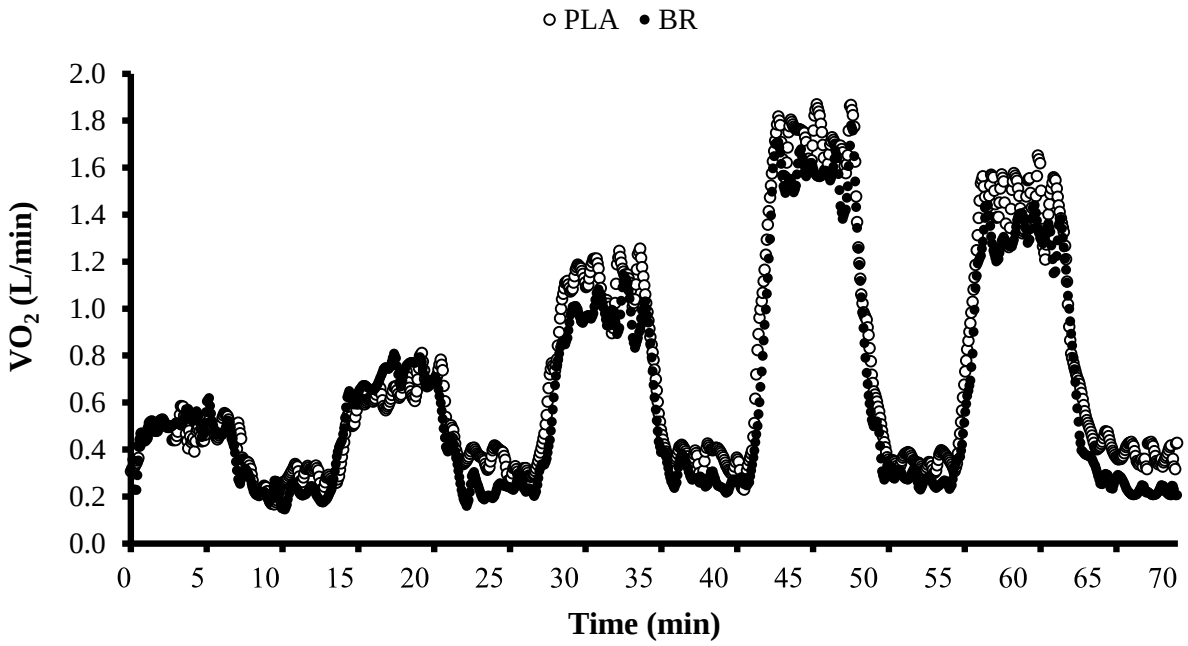

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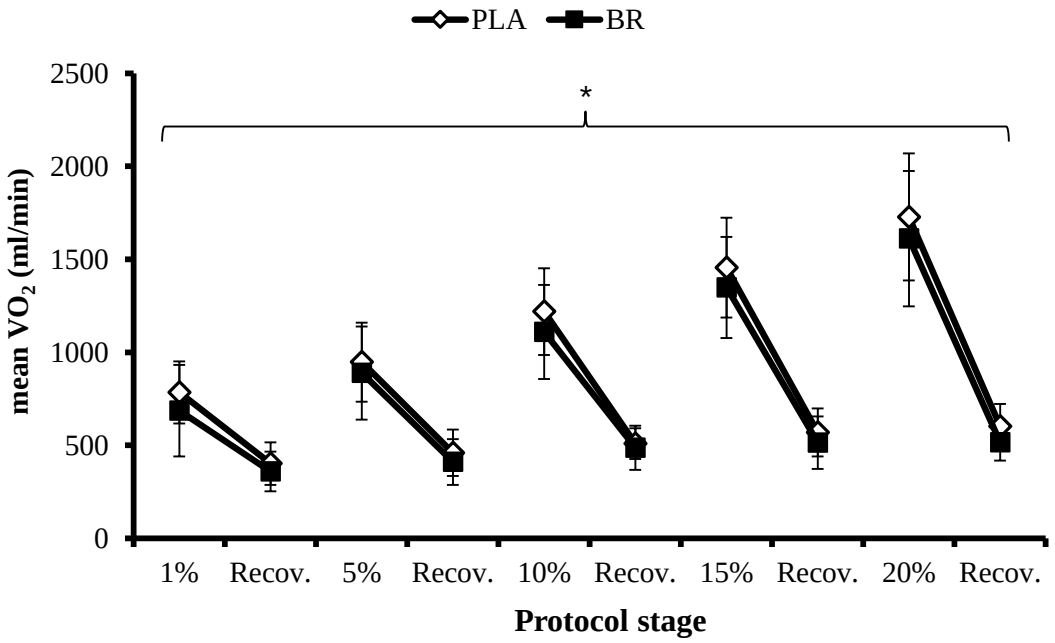

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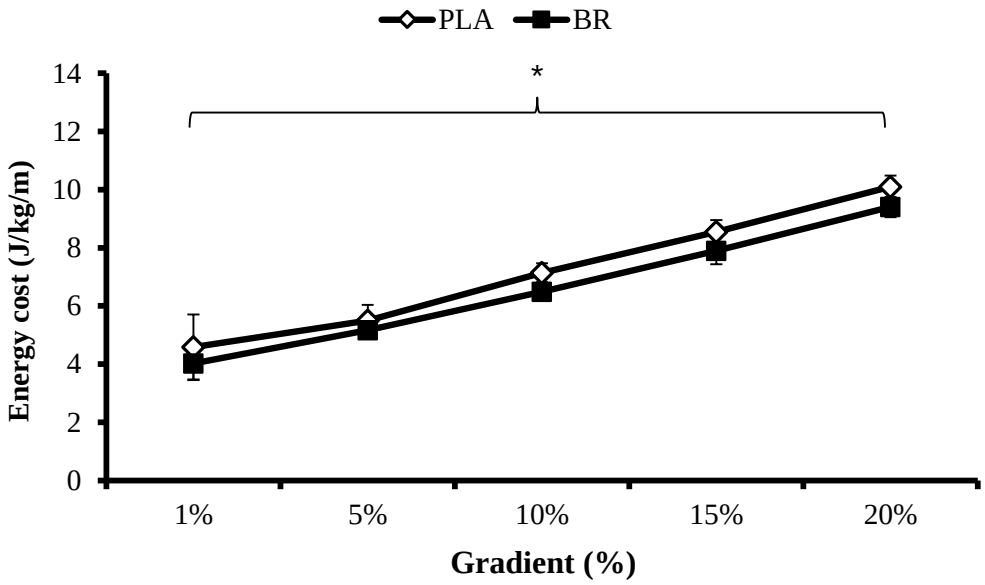

Table 1

	PLA	BR	PLA	BR	PLA	BR	PLA	BR	PLA	BR
	1%		5%		10%		15%		20%	
End exercise VO_2 (ml/min)*	794 ± 204	643 ± 137	1076 ± 277	931 ± 221	1363 ± 285	1249 ± 264	1707 ± 287	1550 ± 310	2083 ± 389	1877 ± 352
On phase II amplitude (ml/min)*	472 ± 125	349 ± 101	742 ± 265	591 ± 237	1078 ± 303	1086 ± 383	1319 ± 291	1158 ± 278	1722 ± 306	1514 ± 368
On phase II time constant (s)	30 ± 16	26 ± 13	35 ± 12	29 ± 7	43 ± 11	38 ± 16	24 ± 10	26 ± 11	37 ± 18	30 ± 11
Mean response time (s)	41 ± 18	39 ± 14	47 ± 12	42 ± 9	56 ± 12	52 ± 16	32 ± 11	37 ± 13	48 ± 18	42 ± 9
Exercising Heart rate (b/min)	80 ± 11	79 ± 12	88 ± 12	86 ± 13	98 ± 13	97 ± 12	113 ± 19	109 ± 20	120 ± 17	116 ± 17
Resting heart rate (b/min)	71 ± 10	68 ± 12	78 ± 12	76 ± 10	81 ± 13	77 ± 11	87 ± 20	79 ± 14	89 ± 18	84 ± 18

MW conceived and designed research. MW, JH and CL conducted experiments. MW, JH and LW analyzed data. MW,JH,LW and AG wrote the manuscript. All authors read and approved the manuscript.