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Discrete physiological effects of beetroot juice and potassium nitrate supplementation following 4 weeks sprint interval training

Christopher Thompson[1], Anni Vanhatalo[1], Stefan Kadach[1], Lee J. Wylie[1], Jonathan Fulford[2], Scott K. Ferguson[3], Jamie R. Blackwell[1], Stephen J. Bailey[1,4], Andrew M. Jones[1].

Affiliations: *[1]Sport and Health Sciences, University of Exeter, Exeter, United Kingdom; [2]University of Exeter Medical School and NIHR Exeter Clinical Research Facility, Exeter, United Kingdom; [3]Cardiovascular and Pulmonary Research Laboratory, Department of Medicine, University of Colorado Denver, Anschutz Medical Campus, Aurora, Colorado; and [4]Sport, Exercise and Health Sciences, Loughborough University, Loughborough, United Kingdom.*

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Address for correspondence:

Andrew M. Jones, Ph.D.

University of Exeter, St. Luke's Campus

Exeter, Devon, EX1 2LU, UK.

E-mail: a.m.jones@exeter.ac.uk

Tel: 01392 722886; Fax: 01392 264726

Abstract

The physiological and exercise performance adaptations to sprint interval training (SIT) may be modified by dietary nitrate (NO_3^-) supplementation. However, it is possible that different types of NO_3^- supplementation evoke divergent physiological and performance adaptations to SIT. The purpose of this study was to compare the effects of 4 weeks SIT with and without concurrent dietary NO_3^- supplementation administered as either NO_3^--rich beetroot juice (BR) or potassium NO_3^- (KNO_3). Thirty recreationally-active subjects completed a battery of exercise tests before and after a 4 week intervention in which they were allocated to one of three groups: 1) SIT undertaken without dietary NO_3^- supplementation (SIT); 2) SIT accompanied by concurrent BR supplementation (SIT+BR); or 3) SIT accompanied by concurrent KNO_3 supplementation (SIT+KNO_3). During severe-intensity exercise, $\dot{V}O_{2peak}$ and time to task failure were improved to a greater extent with SIT+BR than SIT and SIT+KNO_3 (P<0.05). There was also a greater reduction in the accumulation of muscle lactate at 3-min of severe-intensity exercise in SIT+BR compared to SIT+KNO_3 (P<0.05). Plasma [NO_2^-] fell to a greater extent during severe-intensity exercise in SIT+BR compared to SIT and SIT+KNO_3 (P<0.05). There were no differences between groups in the reduction in the muscle phosphocreatine recovery time constant from pre- to post-intervention (P>0.05). These findings indicate that 4 weeks SIT with concurrent BR supplementation results in greater exercise capacity adaptations compared to SIT alone and SIT with concurrent KNO_3 supplementation. This may be the result of greater NO-mediated signalling in SIT+BR compared to SIT+KNO_3.

Keywords: nitric oxide; fitness; diet; endurance; ergogenic aid; nutrition.

Introduction

Over the last decade, dietary supplementation with inorganic nitrate (NO_3^-) has emerged as a popular pre-competition nutrition strategy to enhance athletic performance. This practice originates from evidence that dietary NO_3^- supplementation can enhance a range of physiological processes, including skeletal muscle metabolic (2, 35, 62), contractile (11, 28, 29, 66) and vascular function (16, 17) with attendant implications for improved performance in a range of exercise settings (5, 33, 58, 60, 69).

Favourable effects on resting blood pressure (BP) have been observed following NO_3^- ingested in the form of both NO_3^--rich beetroot juice (BR; 31, 64) and NO_3^- salts (KNO_3 or $NaNO_3$; 1, 32, 34). However, some recent studies suggest that BR/NO_3^--rich vegetables and NO_3^- salts can evoke disparate physiological effects (18, 30). The improvement in some physiological responses in the contracting skeletal muscle following NO_3^- supplementation has been attributed to enhanced nitroso signalling facilitated by elevated nitrite (NO_2^-), s-nitrosothiol and/or NO bioavailability (40, 57, 64). Importantly, the co-ingestion of the betacyanins and polyphenols found in BR may increase the capacity for NO synthesis from NO_2^- (21, 50). Moreover, chlorogenic acid, a constituent of BR, has been shown to promote NO release by human saliva at the acidic pH of the stomach (50). Therefore, ingesting NO_3^- as BR, which is accompanied by the co-ingestion of compounds that might facilitate the NO_3^- - NO_2^- -NO pathway, has the potential to enhance NO-mediated physiological signalling compared to a similar dose of NO_3^- administered as NO_3^- salts.

In addition to enhanced physiological and performance responses following NO_3^- supplementation in an acute exercise setting, recent evidence suggest that NO_3^- supplementation in the form of BR (59), a high NO_3^- gel (44) or $NaNO_3$ (14) can modulate some of the physiological and performance adaptations to sprint interval training (SIT).

Increased exposure to NO_3^- or NO_2^- has been reported to activate PGC1-α (51) and AMPK (42) which initiate signalling cascades that promote adaptive skeletal muscle remodelling to exercise (23). However, while consumption of BR or NO_3^--rich vegetables can lower the O_2 cost of submaximal exercise (18) and BP (30) compared to an equivalent dose of $NaNO_3$, it has yet to be determined whether equimolar doses of inorganic NO_3^- administered through different supplementation vehicles elicit comparable or divergent physiological and performance adaptations to an exercise training program.

As well as serving as an NO precursor, BR contains a number of compounds with antioxidant properties (55, 67). The most abundant betacyanin present in BR, betanin, has been shown to possess high antioxidant activity (9). There is some evidence to suggest that supplementation with antioxidant compounds during an exercise training program can blunt some of the skeletal muscle adaptive responses to exercise training (26, 43, 48). It has been reported that BR contains a high total antioxidant capacity relative to other vegetable juices (67), although this would be substantially lower than administered in training studies reporting compromised training adaptations in which high doses of vitamin C (1 g) and vitamin E (up to 294 mg) have been administered (43, 48). Therefore, while BR has the potential to augment NO-mediated signalling, and by extension the activation of transcription pathways integral to muscle remodelling to exercise, it remains possible that this benefit might be offset by the antioxidant effects of BR supplementation and the potential suppression of exercise-evoked adaptive signalling cascades. Therefore, further research is required to address the influence of the NO_3^- administration vehicle on the adaptations to an exercise training program.

The purpose of this study was to compare the physiological and exercise performance adaptations to 4 weeks SIT accompanied by concurrent supplementation with BR (SIT+BR) or potassium NO_3^- (KNO_3) (SIT+KNO_3) or SIT undertaken without dietary NO_3^-

supplementation (SIT). It was hypothesised that exercise performance, muscle oxidative capacity measured *in vivo* using ^{31}phosphorus magnetic resonance spectroscopy (^{31}P-MRS), and muscle metabolic adaptions measured from muscle biopsy samples would be improved following all SIT interventions, but that these variables would be improved more in the SIT+BR group compared to the SIT+KNO_3 and SIT groups due to enhanced NO-mediated physiological signalling in SIT+BR.

Methods

Subjects

Eighteen male (mean ± SD: age 25 ± 6 years, height 1.82 ± 0.07 m, body mass 85 ± 12 kg, $\dot{V}O_{2peak}$ 46.6 ± 7.5 mL·kg^{-1}·min^{-1}) and 12 female (mean ± SD: age 22 ± 3 years, height 1.71 ± 0.08 m, body mass 66 ± 10 kg, $\dot{V}O_{2\ peak}$ 39.9 ± 3.9 mL·kg^{-1}·min^{-1}) volunteers were recruited. The subjects were involved in team and/or endurance sports but were not highly trained. Following an explanation of the experimental procedures, associated risks, potential benefits and likely value of the possible findings, subjects gave their written informed consent to participate. The study was approved by the Institutional Research Ethics Committee and conformed to the code of ethics of the Declaration of Helsinki.

Experimental design

Subjects initially visited the laboratory on 3 separate occasions over a 5 day period. On visit 1, subjects completed an incremental exercise test on a cycle ergometer for the determination of $\dot{V}O_{2peak}$ and gas exchange threshold (GET). The work rates requiring 80% of the GET (moderate exercise) and 85%Δ (GET plus 85% of the difference between the work rate at GET and $\dot{V}O_{2peak;}$ severe exercise) were calculated and adjusted for mean response time for $\dot{V}O_2$ during incremental exercise (65). Following this, subjects were familiarized to the exercise testing procedures, including completion of a severe-intensity bout of cycle

ergometry until exhaustion. On visit 2, subjects completed the *^{31}P-MRS protocol* (see below) before a 5-min bout of moderate-intensity cycling and an incremental exercise test were performed. On visit 3, subjects completed 2 bouts of severe-intensity cycling, the first for 3-min and the second until task failure.

A third party (not associated with data collection and analysis) received the baseline characteristics of each participant before assigning participants, in a group characteristic matched manner, to one of three SIT groups. Thereafter, in a double-blind, independent-groups design, subjects were enrolled onto a 4-week supervised SIT program and assigned to receive either NO_3^- rich BR (SIT+BR; age 25 ± 7 years, height 1.76 ± 0.11 m, body mass 80 ± 19 kg), KNO_3^- (SIT+KNO_3: age 25 ± 3 years, height 1.76 ± 0.09 m, body mass 75 ± 13 kg) or water (SIT: age 22 ± 3 years, height 1.79 ± 0.08 m, body mass 78 ± 12 kg) for 28 days. The SIT+BR and SIT+KNO_3 groups were deliberately misinformed that they might be consuming supplements that were either active (NO_3^--rich) or placebo alternatives. All three groups consisted of 6 male and 4 female subjects. Each group consumed 1 x 70 mL of their allocated supplements (SIT+BR; ~6.4 mmol of NO_3^- per 70 mL; Beet it, James White Drinks Ltd., Ipswich, UK; SIT+KNO_3: ~6.4 mmol of NO_3^- per 70 mL; Minerals-Water.ltd, Purfleet, UK) in the morning and 1 x 70 mL in the evening of each day for the duration of the training period. This approach is expected to result in elevated plasma [NO_3^-] and [NO_2^-] for each 24 h period (68). On experimental visits following the intervention period, subjects consumed 2 x 70 mL of their allocated supplement 2.5 h prior to the exercise tests. Compliance to the supplementation procedures was confirmed by the return of empty bottles each week and via the completion of questionnaires during and following the intervention period.

All groups completed the same exercise tests (at the same absolute work rates) and physiological assessments both before and after the 28-day intervention period. Laboratory

visits were scheduled at the same time of day (± 2 h). Subjects were asked to maintain their normal dietary and exercise behavior throughout the study. However, subjects were instructed to record their diet during the 24 h preceding the first laboratory visit and to repeat this for all subsequent laboratory visits. On training days, subjects were asked to arrive at the training venue ≥1 h post-prandial and to complete a 5 min self-paced warm up before training commenced. On experimental days, subjects were instructed to arrive at the laboratory ≥3 h post-prandial having avoided strenuous exercise and the consumption of alcohol and caffeine in the 24 h preceding each exercise test. For the duration of the study, subjects were asked to refrain from taking other dietary supplements and to avoid the use of antibacterial mouthwash as this inhibits the reduction of NO_3^- to NO_2^- in the oral cavity by eliminating commensal bacteria (27).

Training intervention

During the training sessions, all subjects completed a series of 30-s "all-out" sprints (i.e. Wingate test) against a resistance equivalent to 7.5% body mass on a mechanically-braked cycle ergometer (model 814E, Monark, Stockholm, Sweden). Each sprint was separated by a 4-min period of rest in which subjects cycled at a low cadence against a light resistance to reduce venous pooling and sensations of nausea. During weeks 1 and 2 of training, subjects performed 4 x 30-s sprints three times per week, while during weeks 3 and 4, subjects performed 5 x 30-s sprints four times per week. Following a 5-min warm up of cycling against a light resistance, subjects were given a 10-s count down and instructed to pedal maximally for 2 s before the appropriate load was applied. Subjects were verbally encouraged to maintain maximal cadence throughout each 30-s sprint. All groups completed a total of 14 supervised training sessions over a 4-week period, with at least 24 h separating each training session. The post-intervention laboratory tests were performed at least 48 h following, but

within 4 days of the completion of the final training session. All subjects completed 100% of the training sessions and 100% of the sprints during the intervention.

Incremental exercise tests

Subjects completed all ramp incremental exercise tests on an electronically braked cycle ergometer (Lode Excalibur Sport, Groningen, Netherlands). The self-selected cadence (75-90 rpm), saddle and handle bar height and configuration for each subject were recorded on the first visit and reproduced in subsequent visits. Initially, subjects performed 3-min of baseline cycling at 20 W, after which the work rate was increased by 30 W/min until task failure. Breath-by-breath pulmonary gas exchange data (Oxycon Pro, Jaeger, Hoechberg, Germany) were collected continuously throughout all incremental tests and were averaged over 10-s periods. $\dot{V}O_{2peak}$ and GET were determined as previously described (61).

Step exercise tests

A 5-min moderate-intensity "step" test was performed on the first laboratory visit before and following the intervention. This was completed 10 min before the ramp incremental test protocol was initiated. On the second laboratory visit before and following the intervention, two severe-intensity step tests were performed, separated by a 20 min period of rest; the first until 3-min, and the second until task failure. The time to task failure was recorded once the pedal rate fell by >10 rpm below the target cadence. All step tests began with 3-min of pedaling at 20 W before a sudden transition to the target work rate. Muscle biopsies were obtained before and following the 3-min severe-intensity exercise bout and again at task failure in the second bout. Breath-by-breath pulmonary gas exchange data were collected continuously throughout all step tests.

^{31}P-MRS protocol

Before the initial experimental visit, participants were familiarised to the ^{31}P-MRS protocol using a custom-built, non-ferrous ergometer in the bore of a purpose built 'mock' magnetic resonance scanner. Before and following the intervention, subjects completed four bouts of single-leg knee extension exercise (over a distance of ~0.22 m) in a prone position within the bore of a 1.5 T superconducting magnet (Gyroscan Clinical Intera, Philips, The Netherlands) using a custom-built, non-ferrous ergometer at the University of Exeter Magnetic Resonance Research Centre (Exeter, UK) as previously described (20, 62). Each 24-s bout of high-intensity exercise (20 ± 4 W) was separated by 4 min. ^{31}P-MRS was used for the assessment of muscle PCr recovery kinetics with data acquired every 1.5 s leading to a spectrum every 6 s via a four phase cycle protocol. Due to technical issues with the scanner at the start of data collection the subject number included in the analyses was restricted within each group to n = 9.

Measurements

Blood pressure

Before and following the intervention the BP at the brachial artery was measured using an automated sphygmomanometer (Dinamap Pro: GE Medical Systems, Tampa, FL). Following 10 min seated rest in an isolated room, three measurements were recorded. The means of the systolic and diastolic measurements were used for data analysis.

Blood analysis

Venous blood was sampled at rest (baseline) before each experimental test. Blood samples were also obtained at 1-min, at 3-min and at exhaustion during the severe-intensity exercise bout. The blood samples collected during the severe-intensity exercise bout were drawn from a cannula (Insyte-WTM, Becton Dickinson, Madrid, Spain) inserted into the subject's antecubital vein and were collected into lithium-heparin vacutainers (Becton Dickinson, New

Jersey, USA). Blood [lactate] and [glucose], as well as plasma [NO_2^-] were analyzed in all samples (square brackets denote concentration). 200 μL of blood was immediately extracted from the lithium-heparin vacutainers and hemolysed in 200 μL of Triton X-100 solution (Triton X-100, Amresco, Salon, OH) before blood [lactate] and [glucose] were measured (YSI 2300, Yellow Springs Instruments, Yellow Springs, OH). The remaining whole blood from each sample was centrifuged at 4000 rpm for 8 min at 4 ºC within 2 min of collection. Plasma was immediately extracted, frozen at -80 ºC and subsequently analyzed for [NO_2^-] using chemiluminescence, as previously described (68).

Muscle biopsy

Muscle samples were obtained from two incisions from the medial region of the *m. vastus lateralis* under local anesthesia (1% lidocaine) using the percutaneous Bergström needle biopsy technique with suction (4). Muscle samples were taken at three different time points before and following the intervention: at rest; following 3-min of severe-intensity exercise; and at task failure during severe-intensity exercise. The post-exercise biopsies were taken while subjects remained on the cycle ergometer and were typically collected within 10 s of the completion of the exercise bout. Biopsy samples were immediately frozen in liquid nitrogen and stored at -80 °C for subsequent analysis.

Muscle metabolites

Following a freeze-drying process, samples were dissected to remove visible blood, fat, and connective tissue. Approximately 2 mg aliquots of isolated muscle fibers were weighed on fine balance scales (Mettler Toledo XS105, Leicester, UK) and stored in 500 μL microcentrifuge tubes at -80 °C. Prior to metabolite analysis, 200 μL of 3 M perchloric acid was added to ~2 mg dry weight (d.w.) muscle tissue. Following 3 min centrifugation and 30 min incubation on ice, 170 μL of supernatant was transferred to a fresh microcentrifuge tube

and 255 µL of cooled 2 M potassium bicarbonate ($KHCO_3$) was added. This was centrifuged, and the supernatant analyzed for [PCr], [ATP] and [lactate] by fluorometric assays as previously described (39).

Muscle glycogen and pH

Glycogen was extracted from ~2 mg d.w. muscle in 500 µL of 1 M hydrochloric acid (HCl) and hydrolyzed at 100 °C for 3 h to glycosyl units, which were measured using an automated glucose analyser (YSI 2300, Yellow Springs Instruments, Yellow Springs, OH) to determine muscle [glycogen]. Muscle pH was measured using a micro-pH meter (Sentron SI600, Roden, The Netherlands) following homogenization of ~1 mg d.w. muscle in 500 µL of a non-buffering solution (145 mM KCl, 10 mM NaCl and 5 mM NaF).

Muscle fiber type

Approximately 20 mg of tissue obtained from each resting muscle biopsy sample was embedded in Tissue-Tek® O.T.C.™ compound (Sakura Finetek Europe BV Zoeterwoude, The Netherlands), rapidly frozen in liquid nitrogen-cooled isopentane, and stored at -80 °C for subsequent histochemical analysis of myocellular characteristics. Serial cross sections (~10 µM thick) were cut in a cryostat (Cryostar NX50, Thermo Scientific, USA**)** maintained at -16 °C. Sections were mounted on 3 separate slides and pre-incubated at pH values of 4.3, 4.6 and 10.3. According to the lability to the acid and alkaline pre-incubation, the fibers were stained for myofibrillar ATPase, identified as type I, IIa, or IIx and counted under an Olympus CKX41 microscope with cellSens Dimension software (Olympus Corporation, Tokyo, Japan).

Data analysis procedures

Oxygen uptake. The breath-by-breath $\dot{V}O_2$ data from each step exercise test were initially examined to exclude values lying more than four SDs from the local mean. The filtered data

were subsequently linearly interpolated to provide second-by-second values and time-aligned to the start of exercise for each individual. The baseline $\dot{V}O_2$ was defined as the mean $\dot{V}O_2$ measured over the final 60 s of the 3-min baseline period. The end-exercise $\dot{V}O_2$ was defined as the mean $\dot{V}O_2$ measured over the final 30 s of exercise.

PCr recovery kinetics. The acquired spectra were quantified using the jMRUI (version 3) software package employing the AMARES peak fitting algorithm as previously described (20, 62). Using Prism 6 software (GraphPad Software Inc., La Jolla, CA, USA), the PCr recovery for each 24 s recovery period was fitted individually by a single exponential of the form

$$PCr_{(t)} = PCr_{end} + PCr_{(0)} (1 - e^{(-t/\tau)})$$

where PCr_{end} is the end exercise PCr value, $PCr_{(0)}$ is the difference between PCr_{end} and full recovery, t is the time from exercise cessation and τ is the time constant for the exponential recovery of PCr. A value of τ was calculated for each trial and then a mean determined from the four individual 24 s recovery periods.

Statistical analyses

Physiological and performance differences consequent to the interventions were assessed using analysis of covariance (ANCOVA) with baseline values used as a covariate. This approach was used to adjust for any small but potentially physiologically important chance imbalances at baseline (54). Significant effects were followed up by Fisher's LSD post hoc tests. Data that were not normally distributed were log transformed before applying the ANCOVA. All values are reported as mean ± SD. Statistical significance was accepted at $P<0.05$.

Results

Plasma [NO_2^-]

Resting plasma [NO_2^-] was increased in SIT+BR (Pre: 39 ± 18 vs. Post: 257 ± 112 nM) and SIT+KNO_3 (Pre: 75 ± 20 vs. Post: 310 ± 123 nM) compared to SIT (Pre: 45 ± 52 vs. Post: 55 ± 83 nM) (P<0.05). There was no difference in the change in resting plasma [NO_2^-] between SIT+BR and SIT+KNO_3 (P>0.05). However, the change in plasma [NO_2^-] during severe-intensity cycling was greater at 3-min in SIT+BR (104 ± 92 nM reduction) compared to SIT+KNO_3 (59 ± 108 nM reduction) (P<0.05; Fig. 1).

Blood Pressure

Systolic BP was reduced in SIT+BR compared to SIT (P<0.05) but not SIT+KNO_3 (P>0.05; Table 1). The change in systolic BP in SIT was not different compared to SIT+KNO_3 (P>0.05). Diastolic BP was reduced in SIT+BR compared to both SIT and SIT+KNO_3 (P<0.05; Table 1). There was no difference in diastolic BP between SIT and SIT+KNO_3 (P>0.05).

Incremental exercise test

None of the interventions resulted in a significant change in body mass (SIT+BR: Pre: 80 ± 19 kg, Post: 80 ± 19 kg; SIT+KNO_3: Pre: 75 ± 13 kg, Post: 74 ± 13 kg; SIT: Pre: 78 ± 12 kg, Post: 77 ± 11 kg; P>0.05). The $\dot{V}O_{2peak}$ was increased to a greater extent in SIT+BR (11% increase) compared to both SIT (6% increase) and SIT+KNO_3 (4% increase) (P<0.05; Table 1). There was no difference in the change in $\dot{V}O_{2peak}$ between SIT and SIT+KNO_3 (P>0.05). The increase in peak WR was not different between groups (P>0.05; Table 1).

Step exercise tests

The steady-state $\dot{V}O_2$ during moderate-intensity exercise was reduced in SIT+BR and SIT+KNO_3 compared to SIT (P<0.05; Table 1). There was no difference in the extent of the

reduction in steady-state $\dot{V}O_2$ between SIT+BR and SIT+KNO_3 (P>0.05). There were no differences in baseline cycling $\dot{V}O_2$ between groups (P>0.05).

The time to task failure during severe-intensity cycling was increased to a greater extent in SIT+BR (71% increase) compared to both SIT (47% increase) and SIT+KNO_3 (42% increase) (P<0.05; Fig. 2). There was no difference in the change in time to task failure between SIT and SIT+KNO_3 (P>0.05). $\dot{V}O_{2peak}$ attained during severe-intensity cycling was not different to that attained during incremental cycling. The change from pre- to post-intervention in blood [lactate] sampled at 1-min of severe-intensity cycling was significantly different in SIT+BR (Pre: 5.1 ± 1.4 vs. Post: 3.5 ± 0.8 mM) compared to SIT (Pre: 3.0 ± 1.4 vs. Post: 1.9 ± 0.9 mM) (P<0.05) but not SIT+KNO_3 (Pre: 3.9 ± 1.7 vs. Post: 2.6 ± 0.7 mM) (P>0.05). There was no difference in blood [lactate] between SIT and SIT+KNO_3 (P>0.05). Blood [glucose] measured during severe-intensity exercise was not different between groups (P>0.05).

PCr recovery kinetics

The reduction in muscle PCr recovery time constant following the cessation of 24 s high intensity knee-extension exercise was not different between groups (SIT: Pre: 31.6 ± 10.3 vs. Post: 24.7 ± 6.2 s; SIT+BR: Pre: 28.4 ± 4.9 vs. Post: 25.9 ± 3.5 s; SIT+KNO_3: Pre: 31.7 ± 8.0 vs. Post: 26.7 ± 7.3 s; P>0.05; Fig. 3).

Muscle substrates and metabolites

The concentrations of muscle ATP, PCr and glycogen, and muscle pH measured during severe-intensity cycling were not different between groups (P>0.05; Table 2). However, muscle [lactate] was different between groups at 3-min of severe-intensity cycling (P<0.05; Table 2). Specifically, the accumulation of muscle lactate from rest to 3-min of severe-

intensity cycling was reduced in SIT+BR compared to SIT+KNO_3 (P<0.05; Table 2) but not SIT.

Muscle fiber type

There was no difference in the change in proportion of type I or type IIx muscle fibres between groups (P>0.05). However, the increase in the proportion of type IIa muscle fibers was greater in SIT and SIT+BR compared to SIT+KNO_3 (P<0.05; Table 3).

Discussion

The principal original finding of this study is that SIT with concurrent NO_3^- supplementation in the form of a natural dietary source (BR) results in superior physiological and exercise performance adaptations compared to both SIT alone and SIT with concurrent supplementation of a NO_3^- salt (KNO_3). Specifically, SIT+BR enhanced time to task failure during severe-intensity cycling to a greater extent than SIT+KNO_3 and SIT. Moreover, despite the administration of equimolar NO_3^- doses, SIT+BR resulted in a greater reduction in resting BP and a greater fall in plasma [NO_2^-] during exercise compared to SIT+KNO_3. These findings suggest that BR, but not KNO_3, supplementation may augment the improvements in exercise capacity and some physiological responses to SIT, presumably via increased NO-mediated physiological signalling.

Influence of SIT+BR and SIT+KNO_3 on exercise capacity

In the present study, peak work rate, $\dot{V}O_{2peak}$ and the time to task failure during severe-intensity exercise were increased following the intervention period in all SIT groups. This confirms the efficacy of low volume, high-intensity interval training to increase exercise capacity (7, 24, 38, 52). Interestingly, we found that $\dot{V}O_{2peak}$ and time to task failure were increased to a greater extent when SIT was combined with BR compared to SIT alone. This is

consistent with our previous study showing that 4 weeks SIT combined with BR improved $\dot{V}O_{2peak}$ whereas SIT combined with NO_3^--depleted beetroot juice did not (59). Surprisingly, and in contrast to our previous study (59), the greater increase in VO_{2peak} in SIT+BR was not accompanied by a significantly greater increase in peak WR during incremental exercise in the present study. However, although not significantly different, it is interesting to note that 7 out of 10 participants in SIT+BR improved peak WR to a greater extent than the pooled group mean change (equivalent to an ~8% improvement), whereas fewer participants exhibited this trend in SIT (3/10) and SIT+KNO_3 (2/10).

The greater improvement in $\dot{V}O_{2peak}$ and time to task failure in SIT+BR might be explained by differences in muscle fiber type transformation and/or changes in muscle metabolic responses to exercise between groups. PGC-1α regulates the exercise-stimulated skeletal muscle remodelling from glycolytic type IIx to more oxidative type IIa and type I muscle fibers (36, 47). The transcription of PGC-1α may be stimulated by both low volume SIT (37) and elevated NO bioavailability (42, 45, 46). It is therefore possible that, by activating common signalling pathways, dietary NO_3^- may augment some of the oxidative metabolic adaptations typical of exercise training. Consistent with this, Roberts et al. (51) have recently demonstrated that both exercise and NO_3^- increased the expression of PGC-1α in human and rat muscle. The change from type IIx towards type IIa and type I fibers typically associated with exercise training was observed in both the soleus (comprising ≤20% type IIa + IIx fibers (13)) and gastrocnemius (comprising ~100% type IIa + IIx fibers (13))) muscle of NO_3^- supplemented rats (51). In the present study, we found a significant increase in type IIa muscle fibers following the intervention period in SIT and SIT+BR but not SIT+KNO_3. However, in contrast to recent findings in hypoxia (14) and normoxia (59), we did not find that concurrent dietary NO_3^- supplementation modulated muscle fiber type transformation compared to SIT alone.

SIT+BR was associated with more favorable changes in blood and muscle markers of skeletal muscle metabolism during exercise following the intervention period. Specifically, blood lactate accumulation was reduced to a greater extent at 1-min severe-intensity exercise in SIT+BR compared to SIT and muscle lactate accumulation was reduced to a greater extent at 3-min severe-intensity exercise in SIT+BR compared to SIT+KNO_3. Dietary NO_3^- supplementation may reduce physiological strain during severe-intensity cycling by: 1) lowering the O_2 cost of a given work rate within the severe-intensity domain (see below; 18); 2) reducing the ATP and PCr cost of muscle force production (2, 20); 3) improving Ca^{2+} handling in type II muscle fibers (29); and 4) improving blood flow distribution towards type II muscle and elevating the driving pressure for capillary-myocyte O_2 flux (16, 17).

Influence of SIT+BR and SIT+KNO_3 on the O_2 cost of exercise

SIT alone had no effect on the O_2 cost of exercise but submaximal $\dot{V}O_2$ was lowered post-training in both SIT+BR and SIT+KNO_3. This is consistent with several studies that have assessed steady-state $\dot{V}O_2$ after acute and short-term BR supplementation (3, 61, 70) and with two studies following 4 weeks NO_3^- supplementation (59, 70). The one other study that has assessed the effect of KNO_3 supplementation on $\dot{V}O_2$ during exercise found that acute KNO_3 supplementation was ineffective at lowering the O_2 cost of exercise in élite athletes (49). Fleuck et al. (18) previously reported that acute BR ingestion is more effective at reducing the O_2 cost of exercise than an equimolar concentration of NO_3^- administered as $NaNO_3$, perhaps due to differential effects on NO_3^-/NO_2^- metabolism and NO bioavailability evoked by the different vehicles of NO_3^- supplementation (See *Effect of SIT+BR and SIT+KNO_3 on markers of NO bioavailability* below). However, in the present study, both SIT+BR and SIT+KNO_3 reduced submaximal $\dot{V}O_2$ compared to SIT, with no difference in the magnitude of change between the two different types of NO_3^- supplementation.

Influence of SIT+BR and SIT+KNO_3 on markers of NO bioavailability

Systolic and diastolic BP, and plasma [NO_2^-] were unchanged following 4 weeks of SIT. However, despite the administration of equimolar concentrations of NO_3^-, there were disparate effects of SIT+BR and SIT+KNO_3 on BP. Resting plasma [NO_2^-] did not differ between the interventions in which dietary NO_3^- was administered as BR or as NO_3^- salt. However, greater reductions in BP were observed in SIT+BR compared to SIT+KNO_3. Acute supplementation with KNO_3 has been shown to lower systolic and diastolic BP (1, 32) but no previous study has compared the effects of chronic supplementation with KNO_3 to BR. Our results are consistent with Jonvik et al. (30) who reported that systolic and diastolic BP were reduced following BR but were unchanged following the same dose (~800 mg) of NO_3^- administered as a $NaNO_3$-containing beverage. Collectively, these results indicate that dietary NO_3^- consumed as BR is more effective at lowering BP than dietary NO_3^- consumed as a NO_3^- salt. This effect may be linked to the presence of other compounds within the BR beverage which may facilitate the conversion of NO_2^- into bioactive NO and other reactive nitrogen intermediates (21, 50). In this regard, it is pertinent that the relatively high total antioxidant content of BR (67) did not appear to blunt the adaptations to training in the present study. This is in contrast to previous studies in which high doses of vitamins C and E have been administered (43, 48).

A possible greater effect of BR compared to KNO_3 on NO synthesis may be particularly important during exercise to support NO-mediated physiological responses in contracting muscle. In this regard, it is interesting that, plasma [NO_2^-] declined to a greater extent during the severe-intensity exercise test in SIT+BR compared to SIT+KNO_3 (Fig. 1), which is perhaps indicative of enhanced NO synthesis from NO_2^- during exercise in SIT+BR. Given the importance of NO_2^- availability to exercise performance (15), and evidence that the decline in plasma [NO_2^-] during exercise is correlated to improvements in performance (60,

69), differences in the magnitude of NO_2^- reduction may explain the superior improvements in exercise capacity observed in SIT+BR compared to SIT+KNO_3 and SIT in the present study.

We have previously reported that, in the absence of exercise training, exercise capacity improved following 15 days (61) and 28 days (59) of NO_3^- supplementation in the form of BR. However, it remains unclear whether similarly protracted periods of dietary NO_3^- supplementation in the form of a NO_3^- salt, could elicit comparable improvements in exercise performance. It is possible that different levels of exposure to NO bioavailability incurred by the different NO_3^--based supplements evoke discrete skeletal muscle adaptive responses which may have contributed to the differences in exercise capacity reported herein.

Influence of SIT+BR and SIT+KNO_3 on oxidative capacity measured in vivo using ^{31}P-MRS

Elevating NO bioavailability via long term dietary NO_3^- administration may stimulate angiogenesis (22), mitochondrial biogenesis (45, 46) and the transformation towards a more oxidative skeletal muscle fiber type (47, 56), effects which may enhance muscle oxidative capacity. Owing to the equilibrium of the creatine kinase reaction, post-exercise muscle PCr resynthesis is a function of mitochondrial ATP production (53). Therefore, we measured the resynthesis of PCr following brief, high-intensity exercise, where the time constant for the exponential recovery of PCr is proportional to muscle mitochondrial oxidative capacity (10, 41). In contrast to our hypothesis, 4 weeks of SIT combined with dietary NO_3^- supplementation in the form of either BR or KNO_3 did not enhance muscle oxidative capacity to a greater extent than SIT alone.

Mitochondrial oxidative capacity can also be estimated by measuring the activity of enzymes such as citrate synthase (12); markers that have been shown to be elevated following SIT (6, 8, 25, 63). However, to our knowledge, this is the first study to demonstrate that 4 weeks SIT

improved oxidative capacity *in vivo* using ^{31}P-MRS. The reduction in the PCr recovery time constant in our study (~16%) was similar to that reported by Forbes et al. (19) in the recovery from moderate-intensity exercise following 2 weeks SIT. Mitochondrial biogenesis might be anticipated following exercise training (37, 38). Furthermore, interventions that elevate NO bioavailability such as dietary NO_3^- supplementation may also increase mitochondrial mass via comparable signaling pathways (42, 45, 46). However, in the present study, the effects of exercise training appear responsible for the speeding of PCr recovery observed in all SIT groups, with no additional benefit afforded by dietary NO_3^- supplementation.

Experimental considerations

A key strength of the present investigation is that, in addition to comparing changes in exercise capacity between conditions, we assessed changes in markers of NO bioavailability, muscle metabolism (by biopsyand ^{31}P-MRS techniques) to explore the mechanistic bases to any functional improvements observed. Furthermore, the inclusion of a SIT only control group allowed us to isolate the effects of SIT from the effects of dietary NO_3^- supplementation alongside SIT in the SIT+BR and SIT+KNO_3 groups. In our study design, we elected to simulate the approach that athletes might adopt during training and in preparation for competition: that is, daily NO_3^- supplementation during training and then an acute NO_3^- dose prior to the criterion exercise trial. However, one disadvantage to this approach is that it complicates differentiation of the effects of training with NO_3^- supplementation, *per se*, from potential effects of acute NO_3^- supplementation on some of the physiological responses to exercise. For this reason, we cannot exclude the possibility that some of the physiological and exercise performance effects observed in the SIT+BR and SIT+KNO_3 groups may have been influenced by the acute NO_3^- ingestion. Our results do, however, indicate that the combination of SIT+BR and acute BR ingestion results in superior physiological and performance outcomes compared to SIT alone or SIT+KNO_3 and acute

KNO_3 ingestion. Further research is necessary to partition out the possible differences in the physiological adaptations to SIT alongside chronic NO_3^- supplementation with and without the addition of acute NO_3^- supplementation prior to post-training performance tests.

The BR and KNO_3^- supplements were consumed by participants in this study as they might be used by athletes in training, i.e., no attempt was made to match the macronutrient, micronutrient or energy content of the supplements. Therefore, it is possible, though we believe unlikely, that the additional carbohydrate and/or energy intake provided by the BR supplement (~31g/day or ~120 kcal/day) might have influenced the adaptations to SIT compared to the other training groups. It should also be acknowledged that, since the total work completed in each of the training programs was not quantified, it is unclear whether the enhanced adaptations in the SIT+BR group relative to the SIT and SIT+KNO_3 groups were a result of a greater overall training load, due to an aggregation of acute ergogenic effects of BR, or to a greater physiological remodeling to the same training load.

Summary

The present study demonstrated that SIT combined with dietary NO_3^- supplementation in the form of BR improved exercise capacity to a greater extent than SIT alone and SIT combined with dietary NO_3^- supplementation in the form of KNO_3. These findings may be linked to greater NO synthesis from NO_2^- or a greater increase in other bioactive nitroso compounds in SIT+BR compared to the other SIT groups. Increased dietary NO_3^- intake, including via supplementation with a natural NO_3^--rich product such as BR, may promote greater exercise capacity adaptations and NO-mediated physiological responses to exercise training. These findings might have important implications for augmenting some of the physiological and performance adaptations to a short-term SIT program.

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Figure Legends

Figure 1. Plasma [NO_2^-] declined to a greater extent from baseline to 3min during severe-intensity exercise in SIT+BR (panel A) compared to SIT+KNO_3 (panel B) (* P<0.05). Open circles represent plasma [NO_2^-] pre-intervention, closed circles represent plasma [NO_2^-] post-intervention. Values presented as mean ± SE.

Figure 2. Percentage increase in time to task failure and $\dot{V}O_{2peak}$ during severe-intensity exercise in SIT (solid grey line), SIT+BR (solid black line) and SIT+KNO_3 (dotted black line). Values presented as mean ± SE. The ANCOVA indicated the changes in time to task failure and $\dot{V}O_{2peak}$ during severe-intensity exercise were greater in SIT+BR compared to SIT (# P<0.05) and compared to SIT+KNO_3 (* P<0.05).

Figure 3. Muscle phosphocreatine (PCr) time constant in the recovery from brief high-intensity exercise. The ANCOVA indicated that change in PCr recovery time constant with training was not different between groups (P>0.05).The dashed lines represent individual responses. The bars represent the mean response for each group (±SE).