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MUSCLE PHYSIOLOGY

Influence of dietary nitrate supplementation on human skeletal muscle metabolism and force production during maximum voluntary contractions

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Abstract Dietary nitrate supplementation, which enhances nitric oxide (NO) bioavailability, has previously been shown to contribute to improved exercise performance by reducing both oxygen cost and energy expenditure. In contrast, previous studies have indicated that NO can lower force production in vitro. To examine the role of dietary nitrates in regulating force generation under normal physiological conditions, we undertook an extended nitrate supplementation regime and determined force output and energy cost with a repeated isometric maximum voluntary contraction (MVC) protocol. In a double-blind, randomized, crossover design, eight participants received 0.5 l/day of nitrate-rich (BR) or nitrate-depleted (PL) beetroot juice for 15 days and completed an exercise protocol consisting of 50 MVCs at 2.5 h, 5 days and 15 days after the beginning of the supplementation period. No significant reduction in force output was determined for BR relative to PL for the peak contraction, the mean or the end force, and no significant time effect was found over the course of the supplementation period. There was a reduction in the mean PCr cost of exercise averaged over the BR supplementation trials, but this did not reach statistical significance for end exercise (BR 15.10 ± 4.14 mM, PL 17.10 ± 5.34 mM, $P = 0.06$) or the mean throughout the protocol (BR 15.96 ± 4.14 mM, PL 17.79 ± 4.51 mM, $P = 0.06$). However, a significant reduction in PCr cost per unit force output was found for BR at end exercise ($P = 0.04$). These results indicate that, under normal physiological conditions, increased NO bioavailability is not associated with a reduction of force-generating capability in human skeletal muscle and confirm that nitrate supplementation reduces the PCr cost of force production.

Keywords Dietary nitrate · ^{31}P-MRS · Exercise · Force

Physiological relevance: these results indicate that, under normal physiological conditions, dietary nitrate supplementation is not associated with a reduction of force-generating capability in human skeletal muscle but reduces the PCr cost of force production.

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Introduction

Much interest has recently arisen regarding the presence of nitrates within the diet and their potential as a source of nitric oxide (NO). NO itself plays an important regulatory role in a range of physiological processes, such as vasodilatation, blood pressure regulation, mitochondrial respiration, cell signalling and mitochondrial biogenesis [13, 37, 38, 44]. The classic mechanism for NO generation via the oxidation of L-arginine, in a reaction catalysed by nitric oxide synthase (NOS), is well documented [22]. However, an additional NO production pathway has been identified whereby dietary inorganic nitrate (NO_3^-) can be reduced to nitrite (NO_2^-) and subsequently NO [18]. This pathway may be of particular importance in situations where generation of NO via NOS is limited, for example, under conditions of hypoxia or ischaemia. That inorganic NO_3^- can act as a potential NO source arises from the action of commensal gram-negative bacteria on the tongue [45] which reduce NO_3^- to NO_2^- within the saliva. Subsequent nitrite reduction to NO may then be carried out by various proteins, enzymes

and compounds with redox potential such as deoxymyoglobin [44], haemoglobin [33], deoxyhaemoglobin [13], xanthine oxidase [53], vitamin C, polyphenols [23] and potentially enzymes within the mitochondrial respiratory chain [44].

Recent studies have shown that increasing dietary NO_3^- intake can have a positive impact on exercise performance. Specifically, a reduction in the oxygen cost of exercise has been reported [7, 11, 29, 30, 51], indicative of an improvement in the efficiency of either oxidative metabolic or contractile processes. Consistent with this are reports of an associated reduction in the ATP turnover rate [6] and of an improvement in mitochondrial efficiency [28]. However, the effect of NO on muscle force production is equivocal. Studies have shown that NO depresses force output during isometric contractions in single-muscle fibres [9, 17, 27, 41, 42] and in muscle fibre bundle preparations [39]. Likewise, in isolated muscle fibres, NOS inhibitors increase the force generated during submaximal tetanic contractions [5, 10], and correspondingly, NO donors reduce submaximal force [5, 10, 27]. However, these latter findings of an NO-induced decline in force production during submaximal contractions were not accompanied by changes in maximal tetanic force generation. In a human exercise model, treatment with NO donors has been shown to result in an increase in the force generated during maximum voluntary contractions (MVC) but a decrease in force at submaximal frequencies [16]. Thus, it would appear that the impact of NO is dependent upon the mode, intensity and duration of muscle contraction [36]. In addition, the types of NO donors and the magnitude of the associated NO flux may play an important role in dictating the physiological impact [1, 14, 31].

When extended force-generating protocols are considered, muscle fatigue and the consequent capacity to repeatedly generate force may have an increasingly complex dependence upon NO concentration. This results from the role of NO in the regulation of blood vessel relaxation [13, 34]. Hence, an accelerated muscle fatigue following the administration of a NOS inhibitor may result from the dysregulation of blood flow [2] or increased vascular resistance [3] rather than from the potential direct effects of NO on force production. Consistent with this, NOS inhibition has been shown to promote fatigue in vivo [2]; however, for in vitro muscle preparations, neither NOS blockade nor endothelial NOS (eNOS) deficiency has been shown to influence fatigue development [21, 27].

One major limitation with the majority of studies examining the role of nitrite and NO on muscle force production and fatigue is that they have been carried out under unphysiological conditions. Thus, the aim of the present study was to examine the effect of supplementing the diet of human volunteers with nitrate-rich beetroot juice (compared to supplementing with a nitrate-depleted beetroot juice placebo) on skeletal muscle force-generating ability over a repeated isometric MVC protocol. It was hypothesized that, if there was an NO-induced effect on maximum force output, this would become apparent over the initial contractions. In contrast, any NO-mediated impact on fatigue would be manifested as a greater rate of decrease in attainable force over time. To examine the influence of NO_3^- supplementation on muscle efficiency, force measurements were undertaken simultaneously with ^{31}P-magnetic resonance spectroscopy (MRS). This allowed us to assess whether NO_3^- supplementation resulted in the sparing of phosphocreatine (PCr), which may be indicative of reduced energy expenditure per unit of force output. To address the question of whether an extended period of NO_3^- supplementation might affect muscle function via mitochondrial biogenesis or function [37, 38], measurements of muscle force and intramuscular metabolites were made at 2.5 h, 5 days and 15 days of supplementation.

Methods

Ethical approval

Participants were informed of the benefit and possible risks associated with the study and provided written consent. The study was approved by the University of Exeter research ethics committee and was conducted in accordance with the Declaration of Helsinki.

Participants

Eight healthy, physically active, but not highly trained, males (mean ± SD, age 24 ± 4 years old, body mass 76 ± 8 kg) volunteered to participate in this study. Participants were instructed to maintain their normal diet and exercise routine throughout the course of the experiment but requested to avoid strenuous exercise in the 24 h preceding each testing session. In addition, each participant was also asked to refrain from caffeine and alcohol 6 and 24 h prior to each test, respectively.

Prior to the commencement of data collection (typically 1 week), participants were fully familiarized with all facets of the exercise protocol and given the opportunity to practice until they were comfortable with the requirements. This minimized any possible learning effects during the study and allowed an estimation of suitable work rates required for the part of the protocol assessing PCr recovery kinetics.

Procedures

The participants were required to attend the MRI facility on seven separate occasions. On one occasion, acting as the

control, no supplementation was administered. During the supplementation period, with both nitrate-depleted beetroot juice placebo (PL) and nitrate-rich beetroot juice (BR), participants attended the facility on three occasions: 2.5 h, 5 days and 15 days after the beginning of supplementation. The study used a double-blind, randomized, crossover design. Participants consumed 0.25 l of beetroot juice twice a day, once in the morning and once in the evening, for a period of 15 days, with the exception that, on testing days, the entire daily 0.5 l supplement was taken 2.5 h prior to the commencement of the exercise protocol.

A minimum 14-day washout period was enforced between supplementation periods and between supplement and control measurements. The order of supplementation was randomized such that four participants undertook the BR trial first and four the PL. The juice for the PL and BR trials was organic beetroot (Beet it, James White Drinks Ltd., Ipswich, UK) with a NO_3^- concentration of 0.17 and 10.2 mM for PL and BR, respectively. Nitrate-depleted juice was manufactured by exactly the same process as the nitrate-rich juice, but with an additional nitrate filtering step prior to pasteurization at the end of the manufacturing process, consisting of passing the juice through a Purolite A520E ion-exchange resin which selectively removes nitrate.

During each visit to the laboratory, prior to beginning the exercise protocol, participants provided a venous blood sample for determination of plasma [NO_2^-]. Venous blood samples (~6 ml) were drawn into lithium–heparin tubes (6 ml Vacutainer Lithium Heparin, Becton Dickinson & Co, NJ, USA), and samples were centrifuged at 4,000 rpm and 4 °C for 10 min, within 3 min of collection. Plasma was collected and immediately frozen at -80 °C, for later analysis of [NO_2^-]. Samples were analysed for plasma [NO_2^-] using a modification of the chemiluminescence technique [8], as described previously [7].

Exercise protocol

The entire exercise protocol was undertaken within the bore of a 1.5-T superconducting magnet (Intera, Philips, The Netherlands) at the Peninsula Magnetic Resonance Research Centre (Exeter, UK). Prior to exercise, baseline measurements were undertaken to determine muscle metabolite concentrations. Two different exercise regimes were then carried out to examine different facets of muscle function. Firstly, PCr recovery kinetics was examined by undertaking short bouts of continuous exercise. Secondly, after a recovery period, a repetitive MVC protocol was undertaken to assess force-generating capabilities while simultaneously undertaking ^{31}P MRS for the determination of in vivo skeletal muscle energetics.

Absolute baseline concentrations of metabolites were established via a technique similar to that described previously [25] using a 6 cm ^{31}P transmit/receive surface coil. Participants were positioned within the scanner head first in a prone position, with the coil placed within the scanner bed and positioned such that the participant's right quadriceps muscle was centred directly over the coil and a phosphoric acid source was directly beneath the coil. After initially acquiring images to confirm that the muscle was positioned correctly relative to the coil, spatially localized spectroscopy was undertaken to determine the relative signal intensities obtained from the phosphoric acid source and inorganic phosphate from the participant's quadriceps. After the participant had been removed from the scanner, scans were obtained, comparing the signals from the same phosphoric acid standard and an external inorganic phosphate solution of known concentration, where the localized voxel sampled within the external solution was of the same dimensions and distance from the coil as for the muscle, allowing the calculation of muscle Pi concentration following corrections for relative coil loading. Absolute concentrations of PCr and ATP were subsequently calculated via the ratio of Pi/PCr and Pi/ATP.

To examine PCr recovery kinetics, participants were positioned in the scanner in a prone position, secured to the scanner bed via Velcro straps at the thigh, buttocks, lower back and middle back to minimize extraneous movement, and asked to perform one-legged knee extension exercise. A custom-designed ergometer was used, which consisted of a nylon frame that fitted onto the bed in alignment with the participant's feet and a base unit that was positioned behind the bed. Cuffs with Velcro straps were secured to the participant's foot and attached to a rope which passed around pulleys housed within the frame to the base unit where they were attached to non-magnetic weights. Prior to the beginning of exercise, images were acquired to confirm that the quadriceps muscle was positioned directly above the 6 cm ^{31}P coil. The coil was sufficiently small that the majority of the signal acquired during exercise would originate from the rectus femoris, with some additional contribution from vastus lateralis, vastus intermedius and vastus lateralis. A number of pre-acquisition steps were carried out to optimize the signal from the muscle under investigation. Matching and tuning of the coil was performed, and an automatic shimming protocol was undertaken within a volume that defined the quadriceps muscle. A baseline spectrum before exercise was then acquired with long repetition time (TR = 20 s), allowing the relative unsaturated peak amplitudes to be determined.

The exercise protocol to assess PCr recovery kinetics required the lifting of weights in accordance with a visual cue at a frequency of 0.67 Hz over a distance of ~0.22 m for two bouts of 24 s separated by a recovery period of 4 min. In

order to obtain a significant PCr depletion (~ 40 %), thereby maximizing the accuracy of the recovery data fitting, exercise was undertaken with weights of mass 1 kg less than the maximum each participant was able to attain during incremental exercise in the previous practice session. As the rate of PCr recovery is pH dependent [24, 49], exercise bouts were limited to 24 s as we have determined that exercise for that period does not lead to a measurable reduction in pH. During exercise and recovery, data were acquired every 1.5 s, and phase cycling with four phase cycles was employed, leading to a spectrum being acquired every 6.0 s.

For the MVC protocol, the ergometer used for the PCr recovery kinetics assessment was replaced by one suitable for performing isometric contractions while the participant remained in the scanner. The ergometer consisted of a fixed foot plate angled at approximately 45° to the scanner bed upon which the participant rested their foot/lower leg. Between the plate and the scanner bed was a magnetically compatible calibrated strain gauge (Interface Force Measurements, Crowthorne, England, model SSM-HN-250) which worked under compression and recorded the forces applied as the participant pushed their foot down towards the bed with a sampling frequency of 10 Hz. Once the participant's foot was comfortably placed upon the plate, the strain gauge force output was zeroed. During exercise and recovery, MRS data were acquired every 1.1 s with averaging over four phase cycles, leading to a spectrum being acquired every 4.4 s. The exercise protocol consisted of 112.2 s of baseline, followed by 50 MVC of 6.6 s interleaved with 2.2 s of rest and then 352 s of resting recovery. Contractions were cued via an audible signal initiated via a trigger from the scanner to ensure time alignment between contractions and spectrum acquisition.

As spectra were acquired every 4.4 s, the exercise protocol translated into 25 baseline spectra, 100 spectra during exercise and 80 during recovery. Because of the intermittent nature of the exercise, the spectra acquired during the exercise period could originate from a mixture of exercise and recovery phases. As a result, the first 'exercise' spectrum was timed to consist of the final 2.2 s of baseline and the initial 2.2 s of the first contraction, whereas the second spectrum was the final 4.4 s of that contraction. Subsequent 4.4 s spectra were alternately the sum of the 2.2 s rest and first 2.2 s of exercise and the last 4.4 s of exercise. The former was subsequently discarded, and the latter was retained for analysis.

Data analysis

^{31}P data

The acquired spectra were quantified via peak fitting, using the jMRUI (version 3) software package employing the AMARES fitting algorithm [50]. Spectra were fitted by assuming the presence of the following peaks: P_i, phosphodiester, PCr, α-ATP (two peaks, amplitude ratio 1:1), γ-ATP (two peaks, amplitude ratio 1:1) and β-ATP (three peaks, amplitude ratio 1:2:1). Intracellular pH was calculated using the chemical shift of the P_i spectral peak relative to the PCr peak [47, 48]. End-exercise values for PCr, Pi and pH were calculated by averaging the individual values over the last five contractions. The mean PCr cost for each contraction of the exercise protocol was determined by subtracting the PCr value at that time point from the initial baseline concentration. End-exercise PCr cost was calculated by averaging the PCr costs of the last five contractions.

For the PCr values following the 24 s exercise period, PCr recovery was fitted with Prism 5 software (GraphPad Software Inc., La Jolla, CA, USA) by a single exponential of the form

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

where PCr_{end} is the value at the end of exercise, $PCr_{(0)}$ is the difference between the PCr at end exercise and fully recovered, t is the time from exercise cessation and τ is the time constant for the exponential recovery of PCr. Each 24 s recovery period was fitted individually, and the time constants were determined for each, before being averaged to give the value quoted for the trial.

Force data

Force data per contraction were analysed to determine the mean force output over the entire 6.6 s and the maximum force output over any 0.9 s period within the contraction. The latter was determined by undertaking a nine-point moving average over the entire contraction and selecting the maximum output. Subsequently, for each participant, under each supplementation condition, the force output from the contraction with the greatest force generation, the mean contraction force over the entire protocol, the end-exercise mean force over the last five contractions and the relative percentage of end-exercise force relative to the maximum were calculated. In all cases, this was determined by considering both the entire 6.6 s contraction and the 0.9 s interval within each contraction with the maximum force output. For the 6.6 s contraction data, a fatigue index was calculated based on the ratio of the mean force over the first 10 contractions to the mean force over the last 10 contractions, multiplied by 100 %.

Combined ^{31}P and force data

To assess whether an increased force output was obtained per unit of energy expenditure (as indicated by the extent of

PCr depletion), the ratio of force generation to PCr cost was determined over all 50 contractions. Group data were then compared for the mean over the entire protocol and for the last five contractions of the exercise bout.

Statistical analysis

Two-way repeated measures ANOVA was used to assess the differences between the PL and BR conditions and across time (2.5 h, 5 days and 15 days) for plasma [NO_2^-], force and ^{31}P data. In addition, to examine any supplement effect independent of the length of the supplementation period, the 2.5 h, 5 day and 15 day data were averaged, and a comparison was made between PL and BR conditions. Paired *t*-tests were used to compare group data averaged over supplement and between data pairs at specific time points. For the PCr depletion analysis, as we hypothesized a reduction in PCr cost following nitrate supplementation, a one-tailed analysis was employed. Significance was accepted as $P < 0.05$, and data are reported as mean ± standard deviation. In order to estimate the sample size required to obtain statistically valid results for selected parameters, an α-level of 0.05 and a power (1-β) of 0.8 were selected. Reciprocally, the power value for the results obtained within the present study with a sample size of eight was calculated by assuming an α-level of 0.05.

Results

None of the participants reported adverse effects from the supplements, and all reported compliance to the pre-test restrictions on diet and exercise.

Plasma nitrite concentration

Group mean plasma [NO_2^-] results are presented in Table 1. A supplement main effect indicated that plasma [NO_2^-] was elevated in BR across the supplementation period relative to PL ($P < 0.01$), although no significant time effect was found. In addition, all three time points were significantly different from the control condition (2.5 h BR, $P = 0.01$; 5 day BR, $P = 0.02$; 15 day BR, $P = 0.01$). No significant changes were observed for the PL [NO_2^-] values over time or in comparison to the control baseline value.

Muscle force

The time courses for contraction forces are illustrated in Fig. 1, expressed as the mean forces over the entire 6.6 s contraction, with supplement comparisons given in Table 2. No significant BR supplementation effect was found relative to PL for the peak contraction, the mean force, the end force, the end force as a percentage of the maximum or the fatigue index ($P > 0.05$ for all comparisons). No significant time effect was found for either BR or PL condition over the course of the supplementation period for any of the variables.

^{31}P data

^{31}P spectroscopy values are given in Table 3. The PCr cost of muscle contraction during the exercise protocol is illustrated in Fig. 2 for all conditions. No significant differences were found for baseline concentrations of PCr, Pi and ATP or baseline pH either between supplementation conditions, across time or relative to the control condition. Likewise, no significant BR effects relative to PL were found for end-exercise PCr (averaged over the last five contractions), end-exercise Pi, end-exercise pH, PCr cost at end exercise or the mean PCr cost throughout the protocol ($P > 0.05$ for all comparisons). No significant time effects were found for either BR or PL over the course of the supplementation time period. PCr recovery time constants following the 24-s exercise protocol are also given in Table 3. No significant differences were found either between supplementation conditions or across time within each condition.

Combined force and ^{31}P data

The data describing the PCr cost of force generation are presented in Table 4. No significant time effect was found for either BR or PL over the course of the supplementation period, and no significant BR supplementation effect was

Table 1 Mean ± SD plasma nitrite concentrations across supplementation time course

	Control condition	Placebo condition			Nitrate condition		
		2.5 h	5 days	15 days	2.5 h	5 days	15 days
Plasma [NO_2^-] (nM)	212 ± 36	225 ± 37	193 ± 31	232 ± 49	418 ± 101	380 ± 114	408 ± 131
Change from control in plasma (%)	–	6	-9	9	97	79	92

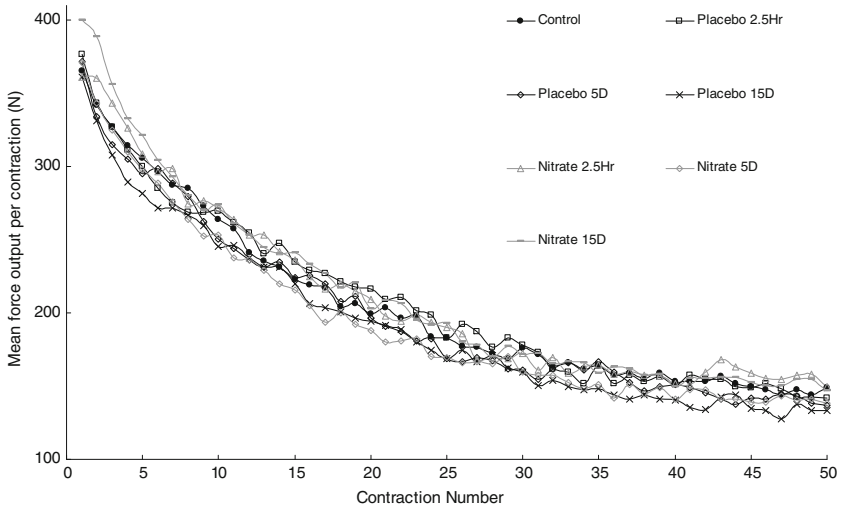

Fig. 1 Time course of mean force output per 6.6 s contraction over the protocol of 50 maximum voluntary contractions for control and following supplementation with nitrate or placebo for 2.5 h, 5 days and 15 days averaged over all subjects

found relative to PL for either the mean force/PCr depletion at end exercise or over the entire exercise protocol ($P > 0.05$ for both comparisons).

Force and ^{31}P data for BR and PL averaged over time

Given that no statistically significant time effects were obtained over the supplementation period for any of the force or ^{31}P data, the BR and PL data were averaged over time (2.5 h, 5 days and 15 days). An illustration of the mean force profile for BR and PL over the 50 contractions is presented in Fig. 3 for the full 6.6 s contraction. The mean force generation values of the entire 6.6 s contraction are presented in Table 5. No significant BR effect was found relative to PL for the peak contraction, mean of the final five contractions, mean over all contractions or fatigue index ($P > 0.05$ for all comparisons). No significant difference for any of the force parameters was found between the supplementation conditions (BR and PL) and the control condition ($P > 0.05$ for all comparisons).

An illustration of the PCr cost of exercise during the isometric protocol averaged over all supplement conditions is given in Fig. 4. The values for mean PCr depletion over all contractions and over the final five contractions are given in Table 5. No significant BR effect was found relative to PL for the mean of all contractions or the mean of the final five contractions ($P > 0.05$ for all comparisons).

For the combined force and PCr data, the mean force/PCr depletion over all 50 contractions is illustrated in Fig. 5. No significant BR effect was found relative to PL for the ratio of mean force/mean PCr depletion over the entire protocol. However, the ratio of mean force/mean PCr depletion at the end of exercise was significantly greater for BR than PL ($P = 0.04$).

Discussion

The main novel finding of this study was that dietary NO_3^- supplementation, which presumably increased NO bioavailability, did not adversely influence skeletal muscle

Table 2 Contraction force values across supplementation time course. In all cases, this is given for both the full 6.6 s contraction and the 0.9 s period within the contraction which generates the maximum force value. Values are mean ± SD. $P > 0.05$ for all comparisons between supplement groups

	Control condition	Placebo condition			Nitrate condition		
		2.5 h	5 days	15 days	2.5 h	5 days	15 days
Mean force of peak contraction (N)	369 ± 105	382 ± 143	387 ± 119	365 ± 115	368 ± 90	380 ± 65	408 ± 110
Mean force during maximal 0.9 s period of peak contraction (N)	416 ± 102	431 ± 149	434 ± 131	416 ± 132	418 ± 100	430 ± 71	457 ± 114
Mean force for the last 5 contractions (N)	147 ± 45	146 ± 38	141 ± 32	133 ± 32	155 ± 49	140 ± 29	151 ± 40
Mean force during maximal 0.9 s period for the last 5 contractions (N)	173 ± 55	177 ± 50	166 ± 38	162 ± 41	184 ± 62	168 ± 35	185 ± 54
Mean force over all contractions (N)	205 ± 55	207 ± 55	199 ± 42	192 ± 47	209 ± 58	194 ± 41	212 ± 54
Mean force, for peak 0.9 s, over all contractions (N)	236 ± 66	243 ± 65	233 ± 53	226 ± 57	239 ± 68	226 ± 43	248 ± 66
Fatigue index (%)	50.5 ± 12.9	51.0 ± 9.6	48.4 ± 9.4	48.3 ± 9.5	52.7 ± 16.1	50.1 ± 15.1	49.6 ± 13.9

Table 3 Phosphorous metabolite values across supplementation time course. Values are mean ± SD. $P > 0.05$ for all comparisons between supplement conditions

	Control condition	Placebo condition			Nitrate condition		
		2.5 h	5 days	15 days	2.5 h	5 days	15 days
Baseline PCr (mM)	32.9 ± 3.4	33.0 ± 2.4	32.5 ± 2.0	33.2 ± 2.9	32.0 ± 3.7	32.7 ± 3.4	31.4 ± 3.8
Baseline Pi (mM)	4.3 ± 0.7	4.1 ± 1.0	4.3 ± 0.7	4.3 ± 0.7	4.0 ± 0.5	3.8 ± 0.7	4.0 ± 0.9
Baseline ATP (mM)	9.3 ± 0.9	7.9 ± 1.4	8.7 ± 0.5	8.8 ± 1.0	8.2 ± 1.1	8.5 ± 0.9	8.5 ± 0.7
Baseline pH	7.03 ± 0.02	7.03 ± 0.03	7.02 ± 0.02	7.02 ± 0.02	7.04 ± 0.03	7.01 ± 0.01	7.03 ± 0.02
End-exercise Pi (mM)	15.0 ± 8.6	14.4 ± 4.9	17.6 ± 6.7	14.5 ± 6.7	14.8 ± 4.2	16.5 ± 8.8	14.7 ± 8.2
End-exercise pH	6.82 ± 0.12	6.88 ± 0.22	6.90 ± 0.16	6.92 ± 0.16	6.90 ± 0.14	6.91 ± 0.15	6.81 ± 0.15
Mean PCr cost over the last 5 contractions (mM)	18.0 ± 5.2	17.6 ± 7.2	17.3 ± 6.0	16.4 ± 5.2	16.1 ± 4.8	15.5 ± 5.4	13.7 ± 3.5
Mean PCr depletion over all contractions (mM)	19.4 ± 5.4	18.5 ± 6.7	17.2 ± 5.1	17.6 ± 4.5	16.8 ± 4.8	16.0 ± 4.7	15.0 ± 3.6
24 s PCr recovery constant (s)	25.4 ± 5.0	25.4 ± 4.7	25.3 ± 5.0	25.7 ± 5.1	25.4 ± 4.3	24.5 ± 4.5	25.7 ± 5.0

force production in a human exercise model. The assessment of muscle energy metabolism during exercise revealed, however, that the PCr cost of maximal force production was reduced following NO_3^- supplementation at the end of 50 consecutive maximal voluntary contractions.

Plasma nitrite concentration

The NO_3^- supplementation protocol was successful in elevating plasma [NO_2^-], consistent with previous reports [6, 7, 51]. There was no further increase in plasma [NO_2^-] with chronic, compared to acute, supplementation, a finding in line with a previous study employing the same supplementation protocol over 15 days [51].

Muscle force

The force data were analysed by considering both the mean output over the entire 6.6 s contraction and also over the peak 0.9 s. The rationale behind the two different assessments lay in the concern that such an extended MVC might induce considerable fatigue over the time course of the contraction. Subsequently, the participant's force response averaged over the entire 6.6 s contraction would be a composite reaction to mechanisms impacting upon force-generating capacity and initiating fatigue and hence would not be representative of maximal force-generating capacity. However, no difference in the temporal force response over the exercise protocol between the two analysis methods was apparent, indicative of no additional NO-related fatigue occurring during the contraction itself.

The majority of studies examining force output in isolated muscle fibres have indicated a depressive effect of NO [9, 27, 39, 41]. However, whereas reduced force production may be present for submaximal exercise protocols, this may not be the case for maximal exercise [4, 10, 27]. Indeed, Folland et al. [16] reported an increase in MVC force generation in human skeletal muscle in vivo. Likewise, Evangelista et al. [14]

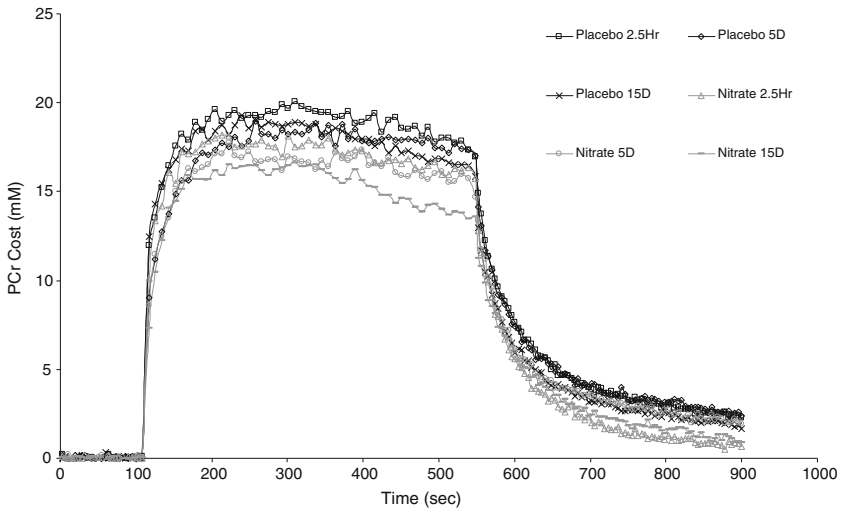

Fig. 2 Time course of mean PCr cost relative to baseline over the protocol of 50 maximum voluntary contractions for control and following supplementation with nitrate or placebo for 2.5 h, 5 days and 15 days averaged over all subjects

Table 4 Combined force and PCr values across supplementation time course. Values are mean ± SD

	Control condition	Placebo condition			Nitrate condition		
		2.5 h	5 days	15 days	2.5 h	5 days	15 days
Mean force at end exercise/PCr depletion at end exercise (N/mM)	9.0 ± 5.1	9.0 ± 2.8	9.1 ± 4.0	8.6 ± 2.1	10.5 ± 4.7	10.6 ± 5.9	11.7 ± 4.0
Mean force over entire exercise/PCr depletion over entire exercise (N/mM)	11.0 ± 3.5	11.7 ± 2.4	12.1 ± 3.2	11.0 ± 1.7	13.3 ± 5.3	13.2 ± 5.3	15.0 ± 5.6

reported that NO donors reduced actin filament velocity but actually increased the isometric force generated by myosin. The present study indicated that NO_3^- supplementation, which increased the circulating NO substrate, NO_2^-, resulted in no significant attenuation of maximum skeletal muscle force output. Heunks et al. [20] have postulated that NO only reduces the rate of cross-bridge cycling during submaximal Ca^{2+} activation, a hypothesis supported by their finding that force development was significantly reduced during submaximal but not maximal Ca^{2+} activation when single fibres were exposed to NO donors. Similarly, in terms of the cross-bridge cycling kinetics, these authors reported only a small effect of NO donors, which was limited to submaximal Ca^{2+} activation with no impact during maximal Ca^{2+} activation. Maréchal and Gailly [31] have postulated that the precise consequences of NO may be dose dependent. This may arise due to a multiplicity of opposing NO effects. Hence, for example, NO may impair Ca^{2+} activation of actin filaments but at the same time increase Ca^{2+} release from the sarcoplasmic reticulum [1, 32, 46].

Over the time course of the exercise protocol, there was no reduction in force output that could be attributed to an NO-mediated acceleration of fatigue development. However, the underlying mechanism for this is not readily apparent. It is possible that NO has no impact upon muscle function, but it is equally possible that it has a range of positive and negative effects that cancel each other out. For example, with a fatiguing exercise protocol, the production of reactive oxygen species (ROS) and their impact on force production may become significant. ROS may alter myofilament structure [19], reduce calcium sensitivity of myofilaments [5], modify cross-bridge kinetics [5] and accelerate the reduction in calcium sensitivity of fatiguing muscle [35]. In contrast, low concentrations of ROS have been shown to increase levels of force production [43]. As a result, the presence of exercise-induced ROS and any subsequent interaction with NO increases the potential complexity of the role of NO during exercise. Therefore, studies reporting reduced force with NO may be observing an NO-related amelioration of the effects of ROS rather than a direct NO-mediated reduction in force per se. In addition, the reaction of NO with superoxide may give rise to peroxynitrite which itself is highly reactive [40]. Hence, whether any modification of the action of ROS by NO during exercise is protective or damaging is debatable and may be dependent on both the specifics of the exercise protocol and the relative concentrations of a variety of different ROS as well as NO.

PCr cost of contractions

Recent studies investigating NO_3^- supplementation have shown a reduced ATP turnover during submaximal exercise

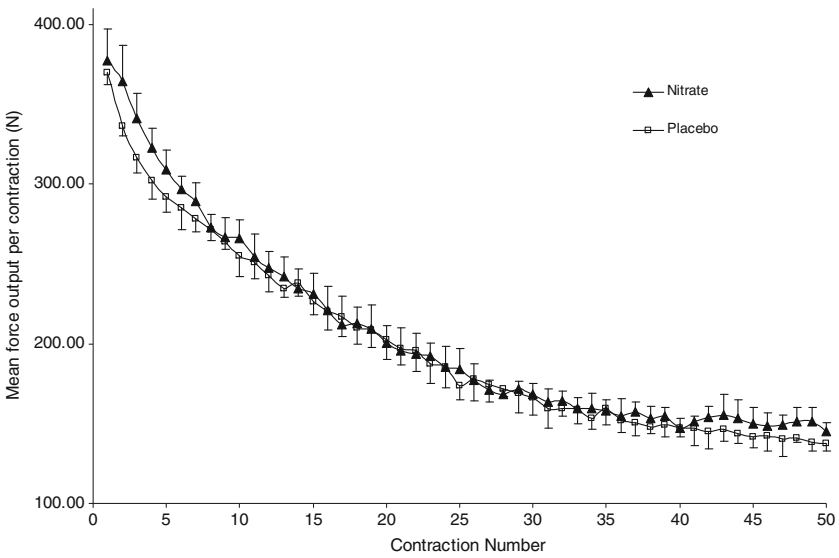

Fig. 3 Time course of mean force applied per 6.6 s for all 50 contraction grouping over all nitrate and placebo supplement times averaged over all subjects. Individual points are mean ± SD. *Upper* and *lower error bars* are shown for nitrate and placebo, respectively

Table 5 Force and PCr values for placebo and nitrate conditions averaged over time trials. Values are mean ± SD

	Placebo condition	Nitrate condition	*P* value
Mean force of the largest contraction (N)	364 ± 120	385 ± 91	0.35
Mean force for the last 5 contractions (N)	140 ± 33	149 ± 38	0.11
Mean force over all contractions (N)	200 ± 46	205 ± 48	0.57
Mean fatigue index (%)	50.8 ± 14.5	49.3 ± 9.0	0.53
PCr depletion at end exercise (mean last 5 contractions) (mM)	17.1 ± 5.3	15.1 ± 4.1	0.06
PCr mean depletion over all contractions (mM)	17.8 ± 4.5	16.0 ± 4.1	0.06
Mean force/PCr depletion for the last 5 contractions (N/mM)	8.7 ± 2.6	10.7 ± 4.3	0.04
Mean force/PCr depletion for all contractions (N/mM)	11.4 ± 1.7	13.7 ± 4.7	0.06

[6] with an associated reduction in O_2 uptake, without any coupled increase in anaerobic energy yield, indicative of improved muscle efficiency [6]. Thus, in the current study, it was anticipated that PCr depletion might be reduced following NO_3^- supplementation while achieving the same MVC force. When values for PCr depletion during exercise were calculated, there was a strong trend (P = 0.06) for reduced PCr depletion with BR supplementation compared to PL as shown in Fig. 4. Power calculations resulted in β = 0.53, corresponding to a sample size requirement of 18 in order to provide sufficient power to detect a statistically significant effect if such an effect exists. In addition, the present study revealed no significant changes in end-exercise pH, indicative of no additional anaerobic contribution to energy production. These results are broadly in line with increased plasma [NO_2^-], leading to an improvement in muscular efficiency as has been reported previously [6, 28].

When the force and PCr responses were combined such that the force production per millimolar of PCr depletion was examined, the analysis confirmed the interpretation that the same force could be produced with a reduced PCr cost in the BR condition. Although the force produced per unit of PCr depletion averaged over the entire protocol was not statistically significant, a strong trend was again present (P = 0.06). Indeed, for each of the 50 MVCs, the group mean force to PCr depletion ratio was higher following BR supplementation (see Fig. 5). Power calculations resulted in β = 0.66, corresponding to a sample size requirement of 22 in order to detect a statistically significant effect. At the end of the MVC protocol, when fatigue would be most pronounced, the mean force to PCr depletion ratio was significantly higher for BR compared to PL (P = 0.04), indicative of a lower PCr cost of force production and therefore improved muscle efficiency. The basis for any potential improvement in muscle efficiency following dietary NO_3^- supplementation has been the subject of much recent speculation. It has been reported that leakage of protons through the inner mitochondrial membrane is reduced following NO_3^- supplementation, thereby improving mitochondrial P/O and reducing the O_2 cost of exercise [28]. Alternatively,

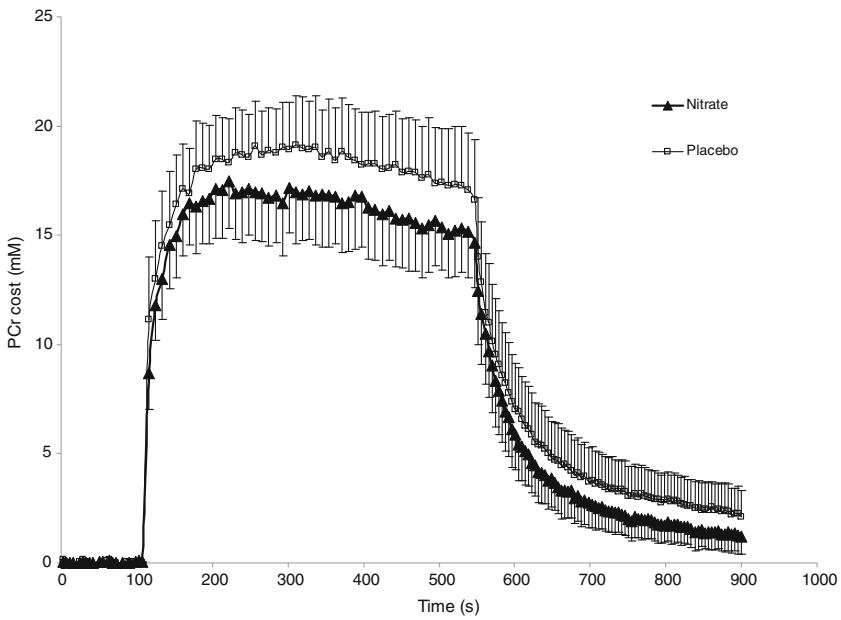

Fig. 4 Time course of PCr cost relative to baseline for all 50 contraction grouping over all nitrate and placebo supplement times averaged over all subjects. Individual points are mean ± SD. *Upper* and *lower error bars* are shown for nitrate and placebo, respectively

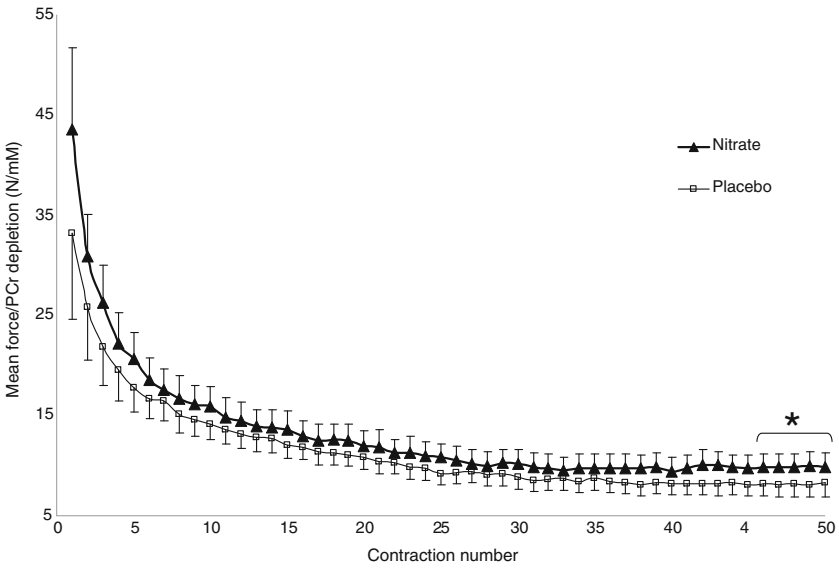

Fig. 5 Time course of ratio of force produced to PCr cost relative to baseline for all 50 contraction grouping over nitrate and placebo supplement times averaged over all subjects. Individual points are mean ± SD. *Upper* and *lower error* bars are shown for nitrate and placebo, respectively. *Difference between nitrate and placebo ($P < 0.05$)

improved efficiency may be related to a reduction in the degree, and hence associated energy cost, of Ca^{2+} cycling via the NO-mediated inhibition processes previously discussed [6]. It is evident from the present study, however, that any improved efficiency did not adversely affect the maximal muscle force-generating capacity in vivo. It should also be recognized that the reduced PCr cost per unit force generated towards the end of the exercise protocol might also be related to a possible enhancement of muscle oxygenation with NO_3^- supplementation. This would be consistent with the recent study of Ferguson et al. [15] which reported an increase in skeletal muscle blood flow in exercising rats following beetroot juice supplementation. An improvement in oxygenation with BR may lead to an increase in oxidative ATP yield relative to PL which, assuming a consistent total ATP cost of force production, will lead to a concomitant attenuation of the fall of PCr.

PCr recovery

The time constant describing the recovery of [PCr] following exercise cessation is considered to be representative of the maximum rate of oxidative metabolism, provided that there is no significant change in pH [26]. The short, intense, 24 s exercise bout was chosen to specifically adhere to these conditions. That there was no change between groups in end-exercise pH or in the time constant for PCr recovery shows that the increased plasma [NO_2^-] had no effect on the maximal rate of oxidative metabolism. If O_2 availability was a limiting factor for oxidative metabolism, then faster recovery kinetics might be anticipated with BR supplementation, potentially as a result of NO's role in vasodilation leading to an increase in blood flow. Such a finding has been reported under hypoxia where the lengthening of the PCr time constant compared to normoxia was attenuated following BR supplementation [52]. However, as the present study examined a young, physically active group, O_2 delivery would not normally be considered a limiting factor during recovery from exercise which challenges only a relatively small muscle mass. There were also no significant changes in PCr recovery time constants over the 15 day BR supplementation period, which should be a sufficiently long time period for any NO-induced mitochondrial biogenesis to become apparent [12, 37, 38]. It can therefore be suggested that BR supplementation over 15 days did not enhance mitochondrial biogenesis relative to PL.

In conclusion, the results of this study suggest that, under normal physiological conditions, up to 15 days of NO_3^- supplementation, which was associated with elevated plasma [NO_2^-], did not result in a reduction of force-generating capability in skeletal muscle. Muscle force-generating capacity was maintained with a reduced PCr cost of contractions following NO_3^- supplementation, indicative of improved muscle efficiency. These results are consistent with the emerging paradigm that dietary NO_3^- supplementation may modify skeletal muscle function.

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