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Dose-dependent effects of dietary nitrate on the oxygen cost of moderate-intensity exercise: acute vs. chronic supplementation

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Running head: Acute and chronic effects of dietary nitrate
ABSTRACT

Purpose: To investigate whether chronic supplementation with a low or moderate dose of dietary nitrate (NO₃⁻) reduces submaximal exercise oxygen uptake (\( \dot{V}_{O_2} \)) and to assess whether or not this is dependent on acute NO₃⁻ administration prior to exercise. Methods: Following baseline tests, 34 healthy subjects were allocated to receive 3 mmol NO₃⁻, 6 mmol NO₃⁻ or placebo. Two hours following the first ingestion, and after 7, 28 and 30 days of supplementation, subjects completed two moderate-intensity step exercise tests. On days 28 and 30, subjects in the NO₃⁻ groups completed the test 2 h post consumption of a NO₃⁻ dose (CHR+ACU) and a placebo dose (CHR). Results: Plasma nitrite concentration ([NO₂⁻]) was elevated in a dose-dependent manner at 2 h, 7 days and 28-30 days on the CHR+ACU visit. Compared to pre-treatment baseline, 6 mmol NO₃⁻ reduced the steady-state \( \dot{V}_{O_2} \) during moderate-intensity exercise by 3% at 2 h \((P = 0.06)\), 7 days and at 28-30 days (both \( P < 0.05 \)) on the CHR+ACU visit, but was unaffected by 3 mmol NO₃⁻ at all measurement points. On the CHR visit in the 6 mmol group, plasma [NO₂⁻] had returned to pre-treatment baseline, but the steady-state \( \dot{V}_{O_2} \) remained reduced. Conclusion: Up to ~4 weeks supplementation with 6 but not 3 mmol NO₃⁻ can reduce submaximal exercise \( \dot{V}_{O_2} \). A comparable reduction in submaximal exercise \( \dot{V}_{O_2} \) following chronic supplementation with 6 mmol NO₃⁻ can be achieved both with and without the acute ingestion of NO₃⁻ and associated elevation of plasma [NO₂⁻].

Key Words: nitrate supplementation; nitrite; nitric oxide; exercise efficiency; O₂ uptake
Abbreviations: ANOVA, analysis of variance; ATP, adenosine triphosphate; CHR, effect of chronic nitrate supplementation alone assessed; CHR + ACU, combined effect of chronic and acute nitrate supplementation assessed; GET, gas exchange threshold; NO, nitric oxide; NO₂⁻, nitrite; NO₃⁻, nitrate; O₂, oxygen; PLA, placebo; Rpm, revolutions per minute; \( \dot{V}_O_2 \), oxygen uptake; \( \dot{V}O_2_{\text{peak}} \), peak oxygen uptake; W, watts; Δ, change.
1. Introduction

Dietary nitrate (NO$_3^-$) has been reported to influence several physiological processes via its reduction to nitrite (NO$_2^-$), and subsequently to the physiological signaling molecule nitric oxide (NO) [1–3]. Indeed, acute ingestion (1-3 h) and chronic supplementation (3-15 days) with dietary NO$_3^-$ has been found to increase plasma NO$_2^-$ concentration [NO$_2^-$], and, in some studies, to reduce the oxygen (O$_2$) cost of submaximal exercise [4–6] and improve exercise tolerance [5–7] in healthy, young adults (see [8] for review). However, the influence of different supplementation strategies on the physiological effects of NO$_3^-$ ingestion is still not fully understood.

Whilst a reduction in the O$_2$ cost of submaximal exercise has been reported 1-3 h post consumption of 5.2-16.8 mmol NO$_3^-$ in some studies [6,9,10], reports on the effect of ingesting a lower dose (i.e. 4.2 mmol NO$_3^-$ or below) are ambiguous [6,11]. It has been suggested that chronic supplementation with NO$_3^-$ may represent a more effective supplementation strategy [8,10]. Indeed, 3-7 days of NO$_3^-$ supplementation has been reported to improve mitochondrial efficiency [12] and contractile function [13] by altering the expression of mitochondrial [12] and contractile [13] proteins in skeletal muscle. These structural adaptations are likely to be responsible, in part, for the reduction in submaximal O$_2$ uptake ($\dot{V}O_2$) after chronic NO$_3^-$ supplementation, but would unlikely be manifest within 1-3 h after the ingestion of a single NO$_3^-$ bolus. Given this potential benefit of a chronic NO$_3^-$ supplementation period, it may be reasoned that a significant and consistent reduction in submaximal exercise $\dot{V}O_2$ could be achieved with a low dose of NO$_3^-$ if consumed daily for an extended period of time. However, the effect of chronic supplementation with a low dose of NO$_3^-$ (i.e. 4.2 mmol or lower) on the O$_2$ cost of submaximal exercise has not yet been investigated. Every nutritional supplement has an associated risk-benefit relationship and
financial cost. Determining if a low dose of NO$_3^-$ consumed chronically can elicit beneficial physiological effects is therefore important to guide supplementation procedures and ensure individuals do not consume more NO$_3^-$ than is required.

To fully understand the physiological effects of a chronic and acute supplementation strategy, it is important to examine the effects of each strategy independently. In all previous studies assessing the influence of chronic NO$_3^-$ supplementation on the physiological responses to exercise, subjects have been instructed to consume their final dose of NO$_3^-$ 1-3 hours prior to final exercise testing, to ensure a significant elevation in plasma [NO$_2^-$] [e.g. 4,5,7,9]. However, this experimental design only allows for the combined chronic and acute effect of NO$_3^-$ ingestion to be investigated. Indeed, it is possible that: a) any chronic adaptions to daily NO$_3^-$ intake would be detectable in the absence of an acute NO$_3^-$ dose and thus, no significant elevation in plasma [NO$_2^-$]; and b) any chronic effects of NO$_3^-$ supplementation may be augmented if an acute NO$_3^-$ dose is consumed. The adoption of an experimental approach in which the chronic effects of NO$_3^-$ exposure can be isolated from those of the acute effects is therefore warranted. This will provide important practical information to guide athletes on optimal supplementation strategies before competition after a period of chronic NO$_3^-$ supplementation.

The purpose of this study was therefore twofold: firstly, to compare the effects of acute (2 h), 7 d and ~30 d supplementation, with a low dose (3 mmol) and a moderate dose (6 mmol; positive control) of dietary NO$_3^-$ on the O$_2$ cost of moderate-intensity exercise; and, secondly, to compare the effect of ~30 day supplementation on the O$_2$ cost of moderate-intensity exercise with and without the acute consumption of NO$_3^-$ 2 h prior to assessment. To achieve the latter, subjects completed an experimental visit on day 28 and 30 of supplementation: on
one visit subjects consumed an acute dose of NO$_3^-$ 2 h prior to testing (CHR + ACU); and on the other visit, exercise was initiated 24 h post consumption of the most recent NO$_3^-$ dose (CHR), allowing plasma [NO$_2^-$] to return to pre-NO$_3^-$ treatment baseline [6]. It was hypothesized that: a) supplementation with 3 mmol NO$_3^-$ and 6 mmol NO$_3^-$ would result in a lower O$_2$ cost of submaximal exercise at 7 d and 28-30 d on the CHR + ACU visit; and, b) any effects observed with CHR + ACU would still be present, albeit to a lesser extent, with CHR.

2. METHODS

2.1. Subjects

Thirty-four healthy, recreationally-active subjects (19 male, mean ± SD age = 21 ± 3 yr, stature = 1.74 ± 0.09 m, body mass = 73.5 ± 14.1 kg) volunteered to participate, gave written informed consent, and completed this study that was approved by the Institutional Research Ethics Committee. All subjects were nonsmokers and none were taking any nutritional supplements in the four months preceding the start of the study. Throughout the experimentation, subjects were asked to adhere to their normal exercise routine and diet. However, subjects were instructed to avoid foods rich in NO$_3^-$ (such as green leafy vegetables and beetroot) and asked to record their diet and exercise in the 48 h preceding the first laboratory visit and to repeat this prior to all subsequent visits. Subjects were instructed to arrive at the laboratory in a rested and fully hydrated state, at least 2 h postprandial, and to avoid strenuous exercise in the 24 h preceding each testing session. Each subject was also asked to avoid caffeine and alcohol 6 and 24 h before each test, respectively. In addition, subjects were asked to abstain from using antibacterial mouthwash and chewing gum for the duration of the study since this inhibits the reduction of NO$_3^-$ to NO$_2^-$ in the oral cavity [14]. All laboratory visits were scheduled at the same time of day (± 1 h) for each subject.
2.2. Pre-treatment tests

Subjects were required to report to the laboratory on two separate occasions prior to beginning 30 days of supplementation with 3 mmol NO₃⁻, 6 mmol NO₃⁻, or placebo (PLA). All exercise tests were performed on an electronically-braked cycle ergometer (Lode Excalibur Sport, Groningen, The Netherlands). During visit 1, subjects completed a ramp incremental exercise test to the limit of tolerance for the determination of peak $\dot{V}O_2$ ($\dot{V}O_{2\text{peak}}$) and gas exchange threshold (GET). The subjects cycled at a constant self-selected pedal rate (80-90 rpm), and this pedal rate, along with saddle and handle bar height configuration, was recorded and reproduced in subsequent tests. Initially, subjects performed 3 min of baseline cycling at 20 W, after which the work rate increased at a rate of 30 W·min⁻¹ in a linear fashion until the limit of tolerance. The test was terminated when the pedal rate fell by > 10 rpm below the chosen pedal rate, despite strong verbal encouragement. The power output achieved at the point of exhaustion was recorded as the peak power output (PPO). Pulmonary gas exchange was continuously collected breath-by-breathe during the incremental tests and averaged over consecutive 10-s periods. The $\dot{V}O_{2\text{peak}}$ was calculated as the highest 30-s mean value attained before the subject’s volitional exhaustion. The GET was determined as described previously [15,16]. The work rate that would require 80 % of the GET (moderate exercise) was then calculated, with account taken of the mean response time for $\dot{V}O_2$ during ramp exercise (i.e. two-thirds of the ramp rate was deducted from the work rate at GET; [17]).

During visit 2, subjects completed a series of PLA-controlled, baseline measurements. All subjects were asked to consume a 50 mL dose of PLA containing negligible NO₃⁻ (see description under Supplementation procedures for further details), 2 h prior to arrival at the laboratory. Upon arrival a venous blood sample was obtained for the measurement of plasma
[NO₃⁻] and [NO₂⁻]. Subjects then completed two step transitions to moderate-intensity cycling at 80% GET, with each bout separated by 5-min of passive recovery. Each step transition was preceded by 3-min of baseline cycling at 20 W and each bout lasted 5 min. Pulmonary \( \dot{V}O_2 \) was measured breath-by-breath throughout the test and averaged over 10-s periods. The data from the two moderate-intensity bouts were time aligned and averaged to improve signal-to-noise ratio [18]. The O₂ cost of moderate-intensity exercise was then calculated. All measurements made during this visit were used as pre-treatment baseline data.

2.3. Supplementation procedures

After these pre-treatment visits, subjects were assigned in a group-matched fashion for moderate-intensity end-exercise \( \dot{V}O_2 \), PPO, GET and \( \dot{V}O_{2\text{peak}} \), to receive either ~3 mmol NO₃⁻ (administered as 9.75 g of dry beetroot extract [PepsiCo, USA] diluted in 50 mL of water), ~6 mmol NO₃⁻ (administered as 19.5 g of dry beetroot extract [PepsiCo, USA] diluted in 50 mL of water), or a PLA (containing ~0.01 mmol of NO₃⁻, administered as 9.5 g of sucrose and red shade coloring [PepsiCo, USA] diluted in 50 mL of water) per day, for 30 days. Subject characteristics are provided in Table 1.

Subjects reported to the laboratory on days 1, 7, 28 and 30 of supplementation to complete the full experimental protocol from visit 2 (Fig. 1). On supplementation days 1 and 7, subjects were asked to consume their 1st and 7th dose of supplement, respectively, 2 h prior to arrival at the laboratory. This pre-test consumption time was selected in order to coincide with the peak plasma [NO₂⁻]. While we have previously established that peak plasma [NO₂⁻] is attained 2 h post beetroot juice consumption [6], it could not be assumed that this time-to-peak is consistent after consumption of other NO₃⁻ containing supplements because the kinetics of plasma [NO₂⁻] may be altered by the form in which the NO₃⁻ is administered [19].
We therefore undertook a pilot study which indicated that peak plasma [NO$_2^-$] occurred at 2 h post consumption of the dry beetroot extract (*unpublished observation*). On non-experimental days, each subject was instructed to consume their allocated supplement at their test time from visit 2 (± 10 min), such that the subject would consume one dose of supplement every 24 h.

To assess the effect of chronic NO$_3^-$ supplementation on the O$_2$ cost of submaximal exercise with and without the acute consumption of NO$_3^-$ 2 h prior to exercise, a randomised counter-balanced design was adopted from day 28 of supplementation for subjects in the 3 mmol and 6 mmol groups (Fig. 1). Two hours prior to arrival on day 28 of supplementation, subjects in the 3 mmol and 6 mmol groups were asked to consume either a PLA beverage [i.e. effect of chronic NO$_3^-$ supplementation alone (CHR) was assessed] (3 mmol: n = 6; 6 mmol: n = 6) or their allocated NO$_3^-$-rich beverage [i.e. combined effect of chronic and acute NO$_3^-$ supplementation (CHR + ACU) was assessed] (3 mmol: n = 5; 6 mmol: n = 6). Ten-minutes post completion of the test procedures on day 28, subjects in the 3 mmol and 6 mmol group who consumed PLA prior to the test, consumed a dose of their allocated NO$_3^-$-rich beverage, and those that consumed their NO$_3^-$-rich beverage consumed a PLA beverage. Two hours prior to arrival at the laboratory on day 30, subjects consumed the opposite beverage to that consumed prior to their laboratory visit on day 28 [e.g. if PLA was consumed prior to arrival on day 28, then their allocated NO$_3^-$-rich beverage was consumed prior to arrival on day 30]. Therefore, all subjects were assessed for the CHR + ACU and CHR effects of NO$_3^-$ supplementation. In all cases, subjects in the 3 mmol and 6 mmol groups consumed 3 mmol NO$_3^-$ or 6 mmol NO$_3^-$, respectively, per day. Subjects in the PLA group were instructed to continue their normal supplementation routine and consume one dose of PLA 2 h prior to arrival at the laboratory on both day 28 and 30 of supplementation.
Subjects in the 3 mmol and 6 mmol groups were deliberately misinformed that the effects of two different NO₃⁻ supplements on the physiological responses of exercise would be tested after different supplementation periods. Subjects in the PLA group were informed that the effect of a NO₃⁻ supplement would be tested over different supplementation periods. All subjects were asked to not comment on the taste or appearance of the supplements to the study investigators. Follow up verbal interviews after completion of the study confirmed that subjects were unaware of the actual research hypothesis.

2.4. Measurements and data analysis

During all exercise tests, pulmonary gas exchange and ventilation were measured breath-by-breath, with subjects wearing a nose clip and breathing through a low dead space, low-resistance mouthpiece and impeller turbine assembly (Jaeger Triple V, Hoechberg, Germany). The inspired and expired gas volume and gas concentration signals were continuously sampled at 100 Hz, the latter using paramagnetic (O₂) and infrared (CO₂) analyzers (Jaeger Oxycon Pro, Hoechberg, Germany) via a capillary line connected to the mouthpiece. The gas analyzers were calibrated before each test with gases of known concentration, and the turbine volume transducer was calibrated with a 3-liter syringe (Hans Rudolph, Kansas City, MO). Pulmonary gas exchange and ventilation were calculated and displayed breath-by-breath.

The breath-by-breath gas exchange data collected during the two identical, moderate-intensity step tests performed at pre-treatment baseline on visit 2 and on days 1, 7, 28 and 30 of supplementation, were averaged over 10-s periods, and time aligned to the start of exercise and ensemble-averaged. The end-exercise $\dot{V}_{O_2}$ was defined as the mean value measured over the final 60 s of exercise.
Venous blood samples were drawn into lithium-heparin vacutainers (7.5 ml Monovette lithium heparin; Sarstedt, Leicester, UK). Within 1 min of collection, samples were centrifuged at 4,000 rpm and 4 °C for 8 min. Plasma was extracted and immediately frozen at -80 °C for later analysis of [NO3⁻] and [NO2⁻] as previously described [6].

Capillary blood samples were collected from a fingertip into a capillary tube during the 20 s preceding each step transition in work rate and within the final 20 s of each moderate-intensity bout. These samples were subsequently stored on ice and analyzed to determine blood [lactate] (YSI 1500, Yellow Springs Instruments, Yellow Springs, OH), within 5 min of collection.

2.5. Statistical analysis

A mixed-model ANOVA was used to assess differences in change from pre-treatment baseline, in all variables across treatment (PLA, 3 mmol NO3⁻ and 6 mmol NO3⁻) and time (2 h and 7 d, and the CHR + ACU visit on day 28 or 30 of supplementation). Differences over time within each group were analyzed by a single factor repeated-measures ANOVA. Before these analyses, all data collected on days 28 and 30 of supplementation in the PLA group were averaged together to form one data point at days 28 and 30 of supplementation. The effect of CHR on all variables in 3 mmol and 6 mmol was analyzed separately using a single factor repeated measures (pre-treatment baseline, CHR and CHR + ACU) ANOVA. In all cases, significant effects were further explored using simple contrasts. All data analyses were performed using the SPSS (version 22; SPSS, Chicago, IL) statistical package, with statistical significance accepted at \( P<0.05 \). Results are presented as mean ± SD unless stated otherwise.
3. RESULTS

Self-reported compliance to the supplementation regime was 100% in all treatment groups. Subjects reported that their diet and exercise habits prior to each experimental test were consistent.

3.1. Plasma [NO3\(^-\)] and [NO2\(^-\)]

Plasma [NO3\(^-\)] and [NO2\(^-\)] responses in PLA, 3 mmol and 6 mmol groups are presented in Figures 2 and 3.

3.1.1. 2 h, 7 d and 28-30 day (CHR + ACU)

At pre-treatment baseline, plasma [NO3\(^-\)] was not significantly different between treatment groups (PLA: 33 ± 11 μM; 3 mmol: 46 ± 24 μM; 6 mmol: 36 ± 11 μM; \( P > 0.05 \)). The change in plasma [NO3\(^-\)] from pre-treatment baseline at 2 h, 7 d and on the CHR + ACU visit after 28-30 d is shown in Fig. 2A. Plasma [NO3\(^-\)] was significantly elevated above pre-treatment baseline and PLA at all time points in the 3 mmol and 6 mmol groups (all \( P < 0.05 \); Fig. 2A). The elevation in plasma [NO3\(^-\)] above pre-treatment baseline in the 6 mmol group was greater than in the 3 mmol group at all time points (all \( P < 0.05 \); Fig. 2A). The mean increase in plasma [NO3\(^-\)] from pre-treatment baseline, in the 3 mmol and 6 mmol groups across the three sample points was 313% and 867%, respectively (3 mmol: 144 ± 23 μM; 6 mmol: 312 ± 68 μM; both \( P < 0.05 \); Fig. 2A). No changes in plasma [NO3\(^-\)] were observed in the PLA group at any time point (all \( P > 0.05 \)). The rise in plasma [NO3\(^-\)] from pre-treatment baseline was not significantly different between the three sampling time points within any treatment group (all \( P > 0.05 \)).
At pre-treatment baseline, plasma [NO$_2$] was not significantly different between treatment groups (PLA: $43 \pm 19$ nM; 3 mmol: $80 \pm 69$ nM; 6 mmol: $48 \pm 22$ nM; $P > 0.05$). The change in plasma [NO$_2$] from pre-treatment baseline at 2 h, 7 d and on the CHR + ACU after 28-30 d is shown in Fig. 2B. In the 3 mmol and 6 mmol groups, plasma [NO$_2$] was significantly elevated at all time points (all $P < 0.05$; Fig. 2B), relative to pre-treatment baseline and PLA. In addition, the rise in plasma [NO$_2$] was greater in the 6 mmol group than in the 3 mmol group at all time points (all $P < 0.05$; Fig. 2B). Across the three sample points, plasma [NO$_2$] rose by 165% and 579%, above pre-treatment baseline, in the 3 mmol ($132 \pm 49$ nM) and 6 mmol ($278 \pm 155$ nM; all $P < 0.05$; Fig. 2B) groups, respectively. No changes in plasma [NO$_2$] were observed in the PLA group at any time point (all $P > 0.05$). The rise in plasma [NO$_2$] from pre-treatment baseline was not significantly different between the three sampling time points within any treatment group (all $P > 0.05$).

**3.1.2. Comparison between chronic and chronic + acute supplementation**

The change in plasma [NO$_3$] from pre-treatment baseline was significantly lower on the CHR visit compared to the CHR + ACU visit in both the 3 mmol and 6 mmol groups ($P < 0.05$; Fig. 3A). In the 3 mmol group, plasma [NO$_3$] had returned to pre-treatment baseline on the CHR visit ($P > 0.05$; Fig. 3A). In the 6 mmol group, the plasma [NO$_3$] remained elevated above pre-treatment baseline by $34 \pm 36$ μM ($P < 0.05$; Fig. 3A) on the CHR visit.

The change in plasma [NO$_2$] from pre-treatment baseline was significantly lower on the CHR visit compared to CHR + ACU visit in the 3 mmol and 6 mmol groups ($P < 0.05$; Fig. 3B). Plasma [NO$_2$] had returned to pre-treatment baseline on the CHR visit in both the 3 mmol and 6 mmol groups ($P > 0.05$; Fig. 3B)
3.2. Moderate-Intensity Exercise

At pre-treatment baseline, the $\dot{V}O_2$ measured over the final 60-s of moderate intensity exercise was not significantly different between treatment groups (PLA: $1.46 \pm 0.32$ L·min$^{-1}$; 3 mmol: $1.44 \pm 0.32$ L·min$^{-1}$; 6 mmol: $1.44 \pm 0.30$ L·min$^{-1}$; $P > 0.05$).

3.2.1. 2 h, 7 d and 28-30 day (CHR + ACU)

The change in end-exercise $\dot{V}O_2$ from pre-treatment baseline at 2 h, 7 d and on the CHR + ACU visit after 28-30 d is shown in Fig. 4A. In the 3 mmol and PLA groups, end-exercise $\dot{V}O_2$ was not significantly different from pre-treatment baseline at any time point (all $P > 0.05$; Fig. 4A). Compared to pre-treatment baseline, end-exercise $\dot{V}O_2$ was significantly reduced in the 6 mmol NO$_3^-$ group at 7 d (by ~3%; $0.04 \pm 0.05$ L·min$^{-1}$) and 28-30 d on the CHR + ACU visit (by ~3%; $0.04 \pm 0.04$ L·min$^{-1}$; both $P < 0.05$; Fig. 4A), and tended to be reduced at 2 h (by ~3%; $0.04 \pm 0.02$ L·min$^{-1}$; $P = 0.06$; Fig. 4A). Compared to PLA, end-exercise $\dot{V}O_2$ was significantly reduced in the 6 mmol group at 28-30 d on the CHR + ACU visit ($P < 0.05$; Fig. 4A) and tended to be reduced at 7 d ($P = 0.08$; Fig. 4A). Compared to pre-treatment baseline and PLA, baseline, end-exercise and change in blood [lactate] were not altered by 3 mmol or 6 mmol NO$_3^-$ at 2 h, 7 d or 28-30 d on the CHR + ACU visit ($P > 0.05$).

3.2.2. Comparison between chronic and chronic + acute supplementation

In the 3 mmol group, end-exercise $\dot{V}O_2$ was not different from pre-treatment baseline at 28-30 d on either the CHR or CHR + ACU visits ($P > 0.05$). In the 6 mmol group, end-exercise $\dot{V}O_2$ was significantly lower than pre-treatment baseline at 28-30 days on the CHR visit (by ~3.5%; $0.05 \pm 0.06$ L·min$^{-1}$; $P < 0.05$; Fig. 4B). This reduction in end-exercise $\dot{V}O_2$ was not significantly different from that observed on the CHR + ACU visit ($P > 0.05$; Fig. 4B).
4. DISCUSSION

The principal novel finding of the present study was that neither 7 days nor ~4 weeks of supplementation with 3 mmol NO$_3^-$, per day, reduced the O$_2$ cost of moderate-intensity cycle exercise, despite an increase in plasma [NO$_3^-$] and [NO$_2^-$] throughout the supplementation period. In contrast, the greater rise in plasma [NO$_3^-$] and [NO$_2^-$] with 6 mmol NO$_3^-$ was associated with a significant reduction in the O$_2$ cost of moderate-intensity cycle exercise after 7 days and ~4 weeks of supplementation, and a trend ($P = 0.06$) for a reduction after acute (2 h) ingestion. Interestingly, this reduction in moderate-intensity $\dot{V}O_2$ after ~4 weeks of supplementation was still evident when assessed on a separate occasion without the ingestion of NO$_3^-$ 2 h prior to the exercise test and thus, no elevation in plasma [NO$_2^-$]. This study is the first to demonstrate a lowering of submaximal $\dot{V}O_2$ following chronic NO$_3^-$ supplementation without a significant concomitant increase in systemic [NO$_2^-$].

4.1. The influence of 2 h, 7 d and 28-30 d (Chronic + Acute) NO$_3^-$ supplementation

4.1.1. Plasma [NO$_3^-$] and [NO$_2^-$]

The administration of beetroot extract containing 3 mmol and 6 mmol NO$_3^-$ was successful in dose-dependently elevating plasma [NO$_3^-$] and [NO$_2^-$], a finding consistent with that observed after the ingestion of concentrated NO$_3^-$-rich beetroot juice [6] and pharmaceutical NO$_3^-$ salt [20]. In both the 3 mmol and 6 mmol groups, there was no further increase in plasma [NO$_3^-$] or [NO$_2^-$] after 7 days or 28-30 days supplementation (when an acute bolus of NO$_3^-$ was consumed 2 h prior to assessment), compared to the acute ingestion of a single bolus of NO$_3^-$ on day 1. This finding is also in line with previous observations [9,21].
### 4.1.2. $O_2$ cost of submaximal exercise

In the present study we found that the steady-state $\dot{V}o_2$ measured over the final 60 s of moderate-intensity cycle exercise was unaffected by the acute ingestion of 3 mmol NO$_3^-$ but tended ($P = 0.06$) to be lower by 3% following acute ingestion of 6 mmol NO$_3^-$. Several previous studies have also reported a dose-response relationship, with higher but not lower doses of dietary NO$_3^-$ eliciting physiological effects [6,22,23]. In particular, we have previously reported that the $O_2$ cost of submaximal cycling was unaffected by the acute administration of 4.2 mmol NO$_3^-$, tended to be reduced (by 1.7%) following administration of 8.4 mmol NO$_3^-$, and was significantly reduced (by 3%) following administration of 16.8 mmol NO$_3^-$, administered in the form of concentrated beetroot juice [6]. The reason for the greater relative reduction in submaximal exercise $\dot{V}o_2$ for a given NO$_3^-$ dose in the present study (i.e. 3% reduction with 6 mmol NO$_3^-$) compared to that reported by us previously (i.e. 1.7% reduction with 8.4 mmol NO$_3^-$) [6] is unclear. However, despite this, the results presented herein and by us previously [6] support a dose-dependent effect of acute NO$_3^-$ ingestion on submaximal exercise $\dot{V}o_2$, and suggest that higher doses of NO$_3^-$ ($\geq$ 6 mmol NO$_3^-$) are required to acutely lower the $O_2$ cost of submaximal exercise.

This is the first study to investigate the effect of chronic supplementation with a low dose of NO$_3^-$ on the $O_2$ cost of moderate-intensity exercise. Contrary to our experimental hypothesis, we found that prolonging the supplementation period with 3 mmol NO$_3^-$ to 7 days, and further to ~4 weeks, did not lower the $O_2$ cost of submaximal cycle exercise. These results suggest that a reduction in submaximal exercise $\dot{V}o_2$ cannot be achieved with a low dose of NO$_3^-$ even with an extension of the supplementation period. In contrast, we found that the $O_2$ cost of submaximal exercise was significantly lower than pre-treatment baseline after both 7 days and ~4 weeks of supplementation with 6 mmol NO$_3^-$. These findings are in agreement
with the 3-6% reduction in the O₂ cost of submaximal exercise reported in untrained subjects following 3-15 days of supplementation with 5-8 mmol NO₃⁻ [4,5,9,12,24]. Therefore, these results indicate that the daily ingestion of 6 mmol NO₃⁻ reduces the O₂ cost of submaximal exercise after short duration (7 day) supplementation, and that this effect is maintained over ~4 weeks of continued supplementation with no evidence of a reduced sensitivity to supplementation.

The lowering of submaximal exercise \( \dot{V}o_2 \) may be a result of improved mitochondrial efficiency [12,25, cf. 26] and/or a reduction in the ATP cost of skeletal muscle force production [27]. Alterations in the expression, and therefore content, of mitochondrial [12] and contractile proteins [13] have been proposed as the mechanistic bases for these effects. However, alterations in protein expression are unlikely to occur within 1-3 h of acute NO₃⁻ ingestion, and can therefore only contribute to a reduction in submaximal exercise \( \dot{V}o_2 \) after a period of chronic NO₃⁻ supplementation. Instead, a reduction in \( \dot{V}o_2 \) following acute NO₃⁻ ingestion may be related to an acute and reversible change in protein function through post-translational protein modifications [28]. Given the potential for the protein expression changes to occur after chronic supplementation but not acute ingestion, we hypothesized that the prolonged exposure to elevated concentrations of NO₂⁻ provided by a chronic supplementation regime may reduce the dose of NO₃⁻ required to lower submaximal exercise \( \dot{V}o_2 \). In contrast to this hypothesis we found that extending the duration of supplementation on a low dose of NO₃⁻ to ~4 weeks did not result in a reduction in submaximal exercise \( \dot{V}o_2 \). This observation implies that these structural modifications are not only dependent on the duration of exposure but also the magnitude of the exposure. The influence of different NO₃⁻ doses and durations of supplementation on structural modifications to skeletal muscle warrants further investigation.
4.2. The effect of chronic NO3⁻ supplementation in the absence of acute NO3⁻ ingestion

In all previous studies examining the effect of chronic NO3⁻ supplementation on the physiological responses to exercise, subjects have been instructed to consume their final dose of NO3⁻ 1–3 h prior to testing [e.g. 4,5,9,12,27,29]. This experimental approach has been adopted to ensure a significant elevation in plasma [NO2⁻] (and therefore the potential for O2⁻-independent NO synthesis) at the time of assessment. However, considering that: 1) for a given dose, the rise in plasma [NO2⁻] afforded by this chronic supplementation strategy is not different to that afforded by a single acute bolus of NO3⁻ [9,21]; 2) acute NO3⁻ supplementation may reduce submaximal exercise \( \dot{V}o_2 \) and improve exercise tolerance [6]; and 3) the acute effects of NO3⁻ are believed to be mediated via an elevation in plasma [NO2⁻] [6,9]; it is not possible to fully ascertain if the effects observed after chronic NO3⁻ supplementation would still be evident when an acute NO3⁻ dose is not administered. Therefore, in the present study, we examined the effects of chronic NO3⁻ supplementation both with and without administration of an acute NO3⁻ dose. Specifically, subjects were asked to consume their final dose of NO3⁻ 24 h prior to exercise testing. Importantly, 24 h provided sufficient time for plasma [NO2⁻] to return to pre-treatment baseline, a finding that is in agreement with the pharmacokinetics of plasma [NO2⁻] following acute ingestion of similar NO3⁻ doses [6,20].

An original finding of the present study was that moderate-intensity steady-state \( \dot{V}o_2 \) was significantly lowered following ~4 weeks of supplementation with 6 mmol NO3⁻, even when 24 h had elapsed since the ingestion of the last NO3⁻ dose. To our knowledge, these results are the first to demonstrate a reduction in submaximal exercise \( \dot{V}o_2 \) following NO3⁻ supplementation in the absence of an increase in plasma [NO2⁻]. However, this observation is
consistent with a previous report that the physiological effects of NO\textsuperscript{3−} supplementation can be achieved without an accompanying rise in plasma [NO\textsubscript{2}\textsuperscript{−}] \cite{30}. Specifically, Ferguson et al., \cite{30} showed that despite no increase in plasma [NO\textsubscript{2}\textsuperscript{−}] after 5 days of NO\textsuperscript{3−} supplementation in rats, microvascular O\textsubscript{2} partial pressure was elevated during contraction in fast-twitch skeletal muscle. Interestingly, the magnitude of reduction in $\dot{V}o_2$ was not significantly different to that observed after ~4 weeks of supplementation when an acute bolus of NO\textsuperscript{3−} was consumed prior to testing. These results therefore suggest that following chronic supplementation with 6 mmol NO\textsuperscript{3−}, a reduction in the O\textsubscript{2} cost of submaximal exercise may be preserved up to at least 24 h after the final dose of NO\textsuperscript{3−} is ingested, and that the addition of an acute NO\textsuperscript{3−} dose, and the resulting elevation in plasma [NO\textsubscript{2}\textsuperscript{−}], does not augment the reduction in submaximal exercise $\dot{V}o_2$. The mechanistic basis for this preserved reduction in $\dot{V}o_2$ is currently unclear. However, as briefly discussed earlier, the reduction in submaximal $\dot{V}o_2$ following chronic NO\textsuperscript{3−} supplementation may be mediated, in part, by a change in the content of mitochondrial [12, cf 26] and contractile [13] proteins, and an associated improvement in mitochondrial [12, cf. 26] and contractile [13,27] efficiency. It is likely that such structural adaptations are maintained for some period of time after NO\textsuperscript{3−} supplementation is stopped, resulting in the preserved reduction in steady-state $\dot{V}o_2$. Indeed, the increased expression of mitochondrial proteins following sprint interval training have been observed up to 6 weeks after training was ceased \cite{31}.

Another possible explanation for the preserved reduction in steady-state $\dot{V}o_2$ is that other reactive nitrogen intermediates and/or NO bioavailability remained elevated post NO\textsuperscript{3−} supplementation, despite plasma [NO\textsubscript{2}\textsuperscript{−}] returning to pre-treatment baseline. Previous research in rodents has shown that skeletal muscle tissue acts as an endogenous NO\textsubscript{2}\textsuperscript{−} and NO\textsuperscript{3−} reservoir \cite{32} and that NO\textsubscript{2}\textsuperscript{−} infusion increases the concentration of NO\textsubscript{2}\textsuperscript{−} in heart, liver
and kidney tissue [33]. If this uptake is also true for human skeletal muscle following NO$_3^-$ supplementation, it is possible that the pharmacokinetics of these changes are different to that of plasma [NO$_2^-$] and [NO$_3^-$], and as a result, tissue NO$_2^-$ may accumulate and remain elevated beyond 24 h after the cessation of NO$_3^-$ supplementation. An increase in tissue NO bioavailability may contribute to the reduction in $\dot{V}o_2$ via acutely and reversibly impacting mitochondrial and/or contractile protein function through post-translational protein modifications [28]. The preserved lowering of $\dot{V}o_2$ in the present study may therefore reflect a structural adaptation and/or elevated NO bioavailability in skeletal muscle tissue following ~4 weeks of supplementation with 6 mmol NO$_3^-$. Further research is required to determine how long the reduction in submaximal exercise $\dot{V}o_2$ may be preserved following cessation of NO$_3^-$ supplementation.

It should be noted that although the acute elevation of plasma [NO$_2^-$] following chronic NO$_3^-$ supplementation did not augment the reduction in moderate-intensity $\dot{V}o_2$ in the present study, there are other potential benefits of the systemic rise in plasma [NO$_2^-$] resulting from the ingestion of an acute bolus of NO$_3^-$. Recent evidence suggests that NO$_3^-$ ingestion significantly improves the perfusion and oxygenation of skeletal muscle, particularly type II muscle fibers, during exercise [30,34]. The vascular effects of NO$_3^-$ consumption are believed to be mediated acutely by NO$_2^-$ or its reduction to the potent vasodilator NO [35,36]. Indeed, an increase in blood flow to exercising forearm muscle has been reported following acute NO$_2^-$ infusion [37]. Moreover, the acute increase in plasma [NO$_2^-$] following acute NO$_3^-$ ingestion has been closely coupled to a reduction in blood pressure, with both returning back to pre-treatment baseline values simultaneously at 24 h [6,20]. Collectively, these results suggest that improvements in perfusion and oxygenation following NO$_3^-$ intake would not be preserved once NO$_3^-$ supplementation is ceased and plasma [NO$_2^-$] returns back to baseline.
If this is the case, an acute dose of dietary \( \text{NO}_3^- \) following chronic supplementation may be beneficial before exercise in which improved oxygenation and perfusion in type II muscle fibers may be advantageous, i.e. high-intensity continuous and intermittent exercise. However, it must be acknowledged that a preserved improvement in vascular function could be possible if chronic \( \text{NO}_3^- \) supplementation results in a greater storage of tissue \( \text{NO}_2^- \) (as discussed above) and this tissue \( \text{NO}_2^- \) is released into the circulation during exercise [32]. Therefore, further research is required to determine if any improvement in high-intensity exercise performance following chronic \( \text{NO}_3^- \) supplementation is preserved after supplementation is stopped and plasma [\( \text{NO}_2^- \)] returns to baseline. In the present study, we focused on the effects of \( \text{NO}_3^- \) on submaximal exercise \( \dot{V}\text{O}_2 \) and did not address possible changes in exercise performance.

In conclusion, low dose (3 mmol) \( \text{NO}_3^- \) supplementation does not significantly reduce the \( \text{O}_2 \) cost of submaximal cycle exercise acutely, or when the supplementation period is extended to ~4 weeks, despite a significant elevation in plasma [\( \text{NO}_3^- \)] and [\( \text{NO}_2^- \)] throughout the supplementation period. In contrast, the greater elevation in plasma [\( \text{NO}_3^- \)] and [\( \text{NO}_2^- \)] following 6 mmol \( \text{NO}_3^- \) supplementation was accompanied by a reduction in the \( \text{O}_2 \) cost of submaximal cycle exercise after 2 h (\( P = 0.06 \)), 7 days and ~4 weeks (both \( P < 0.05 \)) of supplementation. The present study also demonstrated that the reduction in submaximal exercise \( \dot{V}\text{O}_2 \) after ~4 weeks supplementation with 6 mmol \( \text{NO}_3^- \) is preserved up to 24 h after the latest dose of \( \text{NO}_3^- \) is ingested and thus, in the absence of elevated plasma [\( \text{NO}_2^- \)]. To our knowledge, this study is the first to dissociate the reduction in submaximal exercise \( \dot{V}\text{O}_2 \) from an increase in plasma [\( \text{NO}_2^- \)] following \( \text{NO}_3^- \) supplementation. This novel observation implies that beneficial structural skeletal muscle adaptations had occurred and were preserved, and/or muscle [\( \text{NO}_2^- \)] remained elevated following chronic \( \text{NO}_3^- \) supplementation.
5. GRANTS

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6. DISCLOSURES

Coauthor Lara Nyman is an employee of the Gatorade Sports Science Institute, a division of PepsiCo, Inc. The views expressed in this article are those of the authors and do not necessarily reflect the position or policy of PepsiCo, Inc.

7. REFERENCES


Figure Legends

**Fig. 1.** Schematic illustration of the study design. Following pre-treatment tests, all subjects were allocated to receive 6 mmol NO\textsubscript{3}⁻, 3 mmol NO\textsubscript{3}⁻ or placebo for 28 – 30 days. All subjects completed two moderate-intensity bouts of cycle, 2 h post-ingestion (day 1; acute), and after 7, 28 and 30 days of supplementation. In the 3 mmol and 6 mmol NO\textsubscript{3}⁻ groups, subjects were randomised to receive a dose of placebo or NO\textsubscript{3}⁻ 2 h prior to exercise testing in a counterbalanced order on days 28 and 30 of supplementation. This design enabled the efficacy of chronic NO\textsubscript{3}⁻ supplementation in the absence and presence of an acute NO\textsubscript{3}⁻ dose on the day of exercise testing to be determined. See main text for more details.

**Fig. 2.** Change (Δ) relative to pre-treatment baseline in plasma [NO\textsubscript{3}⁻] (panel A) and [NO\textsubscript{2}⁻] (panel B) following acute (2 h), 7 d and 28-30 d (CHR + ACU) supplementation with 6 mmol NO\textsubscript{3}⁻ (squares), 3 mmol NO\textsubscript{3}⁻ (triangles) and placebo (circles). Values are mean ± SE. * significant difference from pre-treatment baseline (P < 0.05); # significant difference from placebo group (P < 0.05); ¥ significant difference from placebo and 3 mmol NO\textsubscript{3}⁻ group (P < 0.05).

**Fig. 3.** Change (Δ) relative to pre-treatment baseline in plasma [NO\textsubscript{3}⁻] (panel A) and [NO\textsubscript{2}⁻] (panel B) following 28 – 30 d supplementation with 6 mmol NO\textsubscript{3}⁻ (squares) and 3 mmol NO\textsubscript{3}⁻ (triangles) when subjects consumed an acute dose of NO\textsubscript{3}⁻ (CHR + ACU) or placebo (CHR) 2 h prior to assessment. Values are mean ± SE. * significant difference from pre-treatment baseline (P < 0.05).

**Fig. 4.** The effect of NO\textsubscript{3}⁻ supplementation on end-exercise \(\hat{V}o_2\) during moderate-intensity cycle. Panel A: Change (Δ) relative to pre-treatment baseline in end-exercise \(\hat{V}o_2\) following acute (2 h), 7 d and 28-30 d (chronic + acute) supplementation with 6 mmol NO\textsubscript{3}⁻ (squares), 3 mmol NO\textsubscript{3}⁻ (triangles) and placebo (circles). Panel B: Change (Δ) relative to pre-treatment baseline in end-exercise \(\hat{V}o_2\) following 28 – 30-days supplementation with 6 mmol NO\textsubscript{3}⁻ (squares) and 3 mmol NO\textsubscript{3}⁻ (triangles) when subjects consumed a dose of NO\textsubscript{3}⁻ (CHR + ACU) or placebo (CHR) 2 h prior to assessment. Values are mean ± SE. \(\hat{V}o_2\), oxygen uptake. * Significant difference from pre-treatment baseline (P < 0.05); /fixtures trend towards difference from pre-treatment baseline (P < 0.10); # significant difference from placebo group (P < 0.05); ¥ trend towards difference from placebo group (P < 0.10).
Table 1. Subject characteristics. Data expressed as mean ± SD.

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