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Ingestion of a nitric oxide enhancing supplement improves resistance exercise performance --Manuscript Draft--

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Abstract:	<p>Studies have established that supplementation of nitrate increases nitrous oxide which in turn improves exercise performance. The current study aimed to investigate the effects of nitrate ingestion on performance of bench press resistance exercise till failure. Twelve recreationally active (age, 21 ± 2 yrs, height, 177.2 ± 4.0 cm, weight, 82.49 ± 9.78 kg) resistance trained males participated in the study. The study utilised a double blind randomized cross-over design, where subjects ingested either 70 ml of "BEET It Sport ®" nitrate shot containing 6.4 millimoles (mmol/L) or 400 mg of nitrate; or a blackcurrant placebo drink. Participants completed a resistance exercise session, consisting of bench press exercise at an intensity of 60% of their established 1 repetition maximum (1-RM), for three sets until failure with 2 minute rest interval between sets. The repetitions completed, total weight lifted, local and general rate of perceived exertion (RPE), and blood lactate were all measured. The results showed a significant difference in repetitions till failure ($p < 0.001$) and total weight lifted ($p < 0.001$). However there were no significant difference between blood lactate over the two trials ($p = 0.238$), and no difference in Local ($p = 0.807$) or general ($p = 0.420$) indicators of fatigue as measured by RPE. This study demonstrates that nitrate supplementation has the potential to improve resistance training performance and work output compared to a placebo.</p>
Response to Reviewers:	<p>The Journal of Strength and Conditioning Research Response to Reviewer's comments We would like to thank the two reviewers for their positive comments and hope, given our responses below, they are now happy with the manuscript. We fully appreciate the time and effort that reviewers put into the reviewing process and we can confirm that</p>

we have taken their comments in a serious and positive manner. Again, many thanks. We provide direct responses to the reviewer's comments in this document marked by bullet points and changes to the revised manuscript have been highlighted in red font. Reviewer #1: General Comments:

Thank you a well written manuscript. In general, the greatest concern is the detailing of your placebo control and subject/researcher blinding. Because this has the potential to be a "fatal flaw" in the study design the reader needs more detailed information. It is not enough to refer to another manuscript in trials such as this. The reader needs to know how well controlled the placebo and blinding protocol was.

Introduction:

Page-1, Line 18 Is there a reference for absorption in the upper GI tract?

- We have now added the following reference to support our statement regarding absorption via the upper GI tract.
- Wagner DA, Schultz DS, Deen WM, Young VR, Tannenbaum SR. Metabolic fate of an oral dose of N-15-labeled nitrate in humans – effect of diet supplementation with ascorbic-acid. Cancer Res 1983; 43: 1921–25.

Methods: How did you control for differences in diet between subjects specifically in regards to nitrate rich foods. Also, you controlled for diet 24 hrs. prior to testing but not during the 6 day ingestion periods (again, the concern being nitrate rich foods).

- We have added a sentence to confirm that participants were required to continue their normal diet which was confirmed verbally. (page 5). Furthermore, they were also instructed to adhere to a diet which was typical of the foods that they normally consume. Participants acted as their own controls due to the experimental design and there were no order effects observed.

The details of blinding both the participants and individuals administering the tests need to be provided. What were subjects told about the hypothesis? How did you control for the testers being made aware of which treatment the subject was involved in (discussing taste etc...)?

- We have now included additional information regarding the administration of the nitrate and placebo and how this was not known by the investigators at the time of the data collection (page 5).

For 1 RM testing the authors state "using a previously validated protocol" please include a reference.

- We have now added the reference to the method for the 1 RM protocol.
- Baechle, TR, Earle, RW, and Wathen, D. Resistance training. In Essentials of Strength Training and Conditioning, edited by Baechle, TR, and Earle, RW, 381-412. Champaign, IL, Human Kinetics. 2008.

What was the rationale for 6 days of ingestion but only 72 hours of a wash out period? You need to justify the timing and evidence that it was adequate.

- Several previous studies have used a similar ingestion period (Cermak et al., 2012a) but not in this type of exercise modality. We have used a slightly lower dose than these authors. Personal communications with AM Jones also confirmed that this strategy would be potentially suitable (obviously this isn't admissible as part of our manuscript). We have included 72 hr washout as a minimum in our original manuscript, but in reality, the vast majority of participants had a 7 day wash out period but for logistical reasons a minority of the participants were only able to complete a 72 hr washout. This is much more in line with previous studies methods. We acknowledge that this may have weakened our method, but the lack of trial order differences (now added to the revised manuscript) suggest that the nitrate supplementation strategy we have used is

still effective.

Was the order of the treatments the same or different (was placebo always first for example). If different how was the order determined? Did you find any differences with the order of testing?

- Participants were randomly assigned to complete the experimental conditions by an experienced laboratory technician that ensured that half of the participants completed the nitrate trial first and half completed the placebo trials first. We have now added this to the method in the revised manuscript (page 5) and have also now confirmed that there was no order effect (in the statistical analysis section – page 6) and in the results section (page 7).

Were there any adverse events during the 6 day ingestion period (for example, gastrointestinal distress)? This is significant to note especially if chronic ingestion is being advocated.

- We did not observe any adverse gastrointestinal responses in any of the participants due to ingestion of either the nitrate shots or the placebo. We have now added this information to the method (page 5).

Discussion:

Page-7, Line 44 Change sunstantial to substantial

Page-8, Line 26 Change couplin to coupling

- Both of these suggestions have now been amended.

Practical Applications:

Your study did not test prolonged use so the suggestion that it could be used on a regular basis may be overstated especially since prolonged supplementation may have adverse side effects that have not yet been detected.

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We thank the reviewer for highlighting this issue, and we acknowledge that we did overstate this in the original manuscript. We have now removed the sentence and added “use and possible adaptations” in the final sentence.

Reviewer #2:

Overall, well done. A few minor adjustments need attention.

P 5 line 14 = "a" should be "at"

P 5 line 38 = utilised = utilized (z vs. s)

P 5 line 54 = press, delete "es"

P 8 line 26 = excitation contraction coupling

P8 line 35 = action-myosin (no capitalization needed)

- All of the above comment have now been amended on the relevant pages of the revised manuscript.

Ingestion of a nitric oxide enhancing supplement improves resistance exercise performance

Original Investigation

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Abstract

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2 Studies have established that supplementation of nitrate increases nitrous oxide which in turn
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4 improves exercise performance. The current study aimed to investigate the effects of nitrate ingestion
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6 on performance of bench press resistance exercise till failure. Twelve recreationally active (age, $21 \pm$
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8 2yrs, height, 177.2 ± 4.0 cm, weight, 82.49 ± 9.78 kg) resistance trained males participated in the study.
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10 The study utilised a double blind randomized cross-over design, where participants ingested either 70
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12 ml of "BEET It Sport [®]" nitrate shot containing 6.4 millimoles (mmol/L) or 400 mg of nitrate; or a
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14 blackcurrant placebo drink. Participants completed a resistance exercise session, consisting of bench
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16 press exercise at an intensity of 60% of their established 1 repetition maximum (1-RM), for three sets
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18 until failure with 2 minute rest interval between sets. The repetitions completed, total weight lifted,
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20 local and general rate of perceived exertion (RPE), and blood lactate were all measured. The results
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22 showed a significant difference in repetitions to failure ($p < 0.001$) and total weight lifted ($p < 0.001$).
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24 However there were no significant difference between blood lactate over the two trials ($p = 0.238$),
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26 and no difference in Local ($p = 0.807$) or general ($p = 0.420$) indicators of fatigue as measured by RPE.
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28 This study demonstrates that nitrate supplementation has the potential to improve resistance training
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30 performance and work output compared to a placebo.
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Key words: Nitrate, Nitric oxide, Exercise performance, Resistance training, Supplementation.

INTRODUCTION

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2 Nitric Oxide (NO) is an influential signalling molecule which rapidly acts on vascular smooth muscle via
3
4 guanylate cyclase (18). The result of this causes increases in cyclic guanosine mono-phosphate, which
5
6 facilitates relaxation effects in smooth muscle, further producing vascular dilatation (13). Dietary
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8 nitrate (NO_3^-) typically obtained from green leafy vegetables and in particular from beetroot, is
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10 reduced to NO_2^- by nitrate reductase (31), causing a sustained increase in circulating NO_2^- levels (23).
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12 It is principally a vasodilator, producing a relaxation effect upon the vascular endothelium (19).
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14 Dietary NO_3^- is 100% bio-available (14, 21) and is readily absorbed in the upper gastrointestinal tract
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16 (28). Dietary nitrate and nitrite can therefore be considered storage pools for NO bioactivity (14, 23).
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19 Dietary supplementation with either sodium nitrate or nitrate-rich beetroot juice (BR) has consistently
20
21 been shown to improve exercise time to exhaustion during both endurance running (20) and cycling
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23 (6). It has also been shown to improve cycling time trial (11) and repeated intermittent rowing
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25 performance (9, 12, 20) as well as improving exercise tolerance at altitude (27). Such improvements
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27 in performance have typically ranged from between 1.2 and 2.9%, but some recent studies with highly
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29 trained participants have observed no improvement in performance following the acute single dose
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31 ingestion of BR (12, 29). However, a relatively common finding in studies examining the ergogenic
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33 effects of nitrate on endurance exercise performance is that heart rate, lactate concentration, CO_2
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35 production, minute ventilation, and respiratory-exchange ratio all show no significant change
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37 between nitrate and placebo groups.
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45 There have been only four studies (4, 17, 22, 26) that have investigated the effects of BR
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47 supplementation or betaine (an amino acid constituent in beetroot juice) on resistance training.
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49 Results regarding the use of betaine are equivocal, but suggest a modest increase in resistance
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51 exercise performance. Bailey *et al.*, (4) recruited seven recreationally active males that consumed
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53 either $0.5 \text{ L}\cdot\text{day}^{-1}$ of BR ($5.1 \text{ mmol}\cdot\text{day}^{-1} \text{ NO}_3^-$) or a placebo for six days. During the last three days of
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55 supplementation, participants completed low and high (15% and 30% maximal voluntary isometric
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57 contractions, respectively) intensity “step” knee extension tests. Their data showed that BR increased
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1 plasma nitrite concentrations by 240% and decreased pulmonary VO_2 by 25% from rest to low-
2 intensity exercise. Beetroot juice consumption also reduced by the amount of phosphocreatine (PCr)
3 degraded during low-intensity exercise and high-intensity exercise by 36% and 59% respectively
4 compared to placebo. Reductions in PCr usage were also accompanied by a reduced total ATP
5 utilization during both high and low-density exercise, suggesting potentially ergogenic effects for this
6 type of exercise. This is the first study that has investigated the effects of a prolonged dose of BR prior
7 to resistance exercise. Therefore the aim of the present study was to investigate the effects of nitrate
8 supplementation on the time to fatigue and subjective ratings of exertion during resistance exercise
9 performance and recovery.
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23 **METHODS**

24 Experimental Approach to the Problem

25 This experimental paradigm was based on the use of a beetroot juice supplement as an ergogenic aid
26 to improve performance of recreational, weight training athletes in terms of fatigue. We decided to
27 measure this by using the number of repetitions until fatigue without increasing physiological as
28 measured by blood lactate or psychological fatigue as measured by RPE indicators.
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37 Participants

38 Twelve recreationally active males (age, $21 \pm \text{SD } 2$ yr, height, 177.2 ± 4.0 cm, weight, 82.5 ± 9.8 kg)
39 were recruited for this study. All participants were familiar with performing resistance exercises, and
40 had a minimum of three years training experience (4.2 ± 0.9 yr), and a minimum weekly training
41 frequency of three days a week (4 ± 1 days), and three hours per week (6 ± 2 hr). Written informed
42 consent was obtained from all participants prior to completing health screening for contraindications
43 to exercise. Participants were required to abstain from ingesting ergogenic supplements during the
44 data collection period. Furthermore, they were also required to replicate dietary intakes for the 24 hr
45 prior to each laboratory visit, avoid exhaustive exercise (9) and limit caffeine consumption (15). Prior
46 to each laboratory visit the participants were instructed to eat no less than three hours prior to the
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1 trials and consume only water in the final hour before testing commenced (9). **Participants were**
2 **requested to continue their normal dietary intake during the study and this was verbally checked**
3 **with each individual prior to testing.** The study was approved by the Departmental Research Ethics
4 Committee.
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7 Procedures

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10 The initial laboratory visit required each participant's bench press one repetition maximum (1-RM) to
11 be determined using a previously validated protocol (3). Participants completed a warm up set of 12
12 repetitions at 10% of estimated 1-RM which was performed using a Smith machine (Hammer Strength,
13 Life Fitness, Ely, UK) followed by a one minute rest period. The load was then increased by 20% and
14 a set of six repetitions was then completed followed by a two minute rest period. A near maximal load
15 was then estimated, this was loaded onto the equipment for the participant to complete three
16 repetitions, followed by a 2-3 min rest period. After these initial warm up sets, the participants
17 estimated 1-RM was established and one attempt was made followed by a 2-4 minute rest. If
18 completed, 1.25 kg, 2.5 kg or 5 kg was progressively loaded to attempt to find the actual 1-RM. The 1-
19 RM was obtained within three to six sets for all participants.
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38 Following the establishment of 1-RM, participants attended the laboratory on two further occasions.

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40 The study utilized a double blind, randomized cross-over design requiring participants to complete a
41 six day ingestion period of either a 70 ml BEET It Sport ® nitrate shot, or a blackcurrant placebo drink
42 (6). The nitrate shot contained 6.4 mmol.l⁻¹ or 400 mg of nitrate (2). **The order in which participants**
43 **consumed the nitrate or placebo was administered by a laboratory technician, and this order was**
44 **only divulged to investigators on completion of all of the data collection.** The supplementation
45 strategy required participants to ingest either a 400 mg nitrate shot or placebo on each of the six
46 consecutive days. **No participants reported any adverse gastrointestinal discomfort during either of**
47 **the ingestion periods. Two experimental trials were then performed, one for each of the ingestion**
48 **strategies, which were separated by a minimum wash out period of 72 hours, a strategy which has**
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previously been used in a study with a similar design (30). These trials took place on the last day of ingestion at the same time of day (10). The experimental trials consisted of a warm up of two sets of 12 repetitions of bench **press**. The first set was performed at 10% and the second at 20% of established 1-RM using the Smith machine. This was followed by a one minute rest period that took place after each warm up set. Testing procedures then began performing sets of repetitions to failure using a load of 60% of 1-RM, with each set separated by a two minute rest period (8, 15). Number of repetitions, total weight lifted, local ratings of perceived exertion (RPE) were recorded on completion of each set using 1- 10 scales where 1 represented no exertion at all and 10 being maximal exertion. Pre and post exercise blood lactate was measured from capillary blood samples (Micro-stat P-GM7, Analox, USA).

Statistical Analyses

All data were assessed for normality using standard graphical methods. Between condition analysis for all variables measured after each set were analysed using repeated measures ANOVA. Violations to sphericity were determined using a Mauchly's test. If a significant violation was observed, significance was adjusted using either the Huynh-Feldt or Greenhouse Geisser correction technique, for where for sphericity > 0.75 and < 0.75 respectively. Post hoc analysis was conducted using the Bonferroni pairwise comparison. **Total weight lifted and total number of repetitions were analysed using paired t-tests for both condition effects and order effects.** Statistical significance was set to $p < 0.05$ for all tests. Calculations of effect sizes were done using ηp^2 for ANOVA and Cohen's d for for t-tests. All data were analysed using SPSS v22 (IBM, Portsmouth, UK).

RESULTS

There was a significant improvement in the total number of repetitions in all three sets (Figure 1) in the nitrate trial compared to the placebo ($F = 29.62$, $p < 0.001$, $\eta p^2 = 0.70$). This resulted in significantly greater total repetitions completed (mean difference = 6.92, $t = 5.44$, $p < 0.001$, $d = 0.96$) and total weight lifted (mean difference = 411.3 kg, $t = 5.00$, $p < 0.001$, $d = 0.52$) in the nitrate trials (Figure 1

and Table 1 respectively). **No order effect on resistance exercise performance was observed, since neither total weight lifted nor total repetitions differed between trial 1 and 2 (mean difference = 56.7 kg, $t = -0.41$, $p = 0.69$, $d = 0.16$; and mean difference = 1.6, $t = -0.73$, $p = 0.48$, $d = 0.29$ respectively).** Despite the observed increases in resistance exercise performance in the nitrate condition, there were no significant differences in the lactate responses (Table 1) between the trials ($F = 1.79$, $p = 0.20$, $\eta p^2 = 0.12$), but in both experimental conditions the performance of the bench press protocols did cause significant increases in lactate concentrations ($f = 306.68$, $p < 0.001$, $\eta p^2 = 0.96$), without an observed condition*time interaction ($F = 4.31$, $p = 0.058$, $\eta p^2 = 0.25$). Interestingly, in spite of increases in resistance exercise performance in the nitrate condition, there were no observed differences in either RPE or RPE-L between conditions ($F = 0.69$, $p = 0.42$, $\eta p^2 = 0.05$ and ($F = 0.06$, $p = 0.81$, $\eta p^2 = 0.01$ respectively) although there were significant increases in RPE-L between each of the sets ($F = 24.09$, $p < 0.001$, $\eta p^2 = 0.65$).

Figure 1 and Table 1 about Here

DISCUSSION

This study investigated the effects of six days of nitrate supplementation in the form of beetroot juice, on resistance exercise performance. It was hypothesised that nitrate would increase the number of repetitions until fatigue without increasing physiological or psychological fatigue indicators. The principle findings of this study, the first of its kind, established that total work increased in terms of total weight lifted, and repetitions until failure at 60% of 1RM. Both improvements occurred without significantly affecting physiological responses in terms of blood lactate concentration and psychological fatigue indicators of local and general rate of perceived exertion.

Nitrate in the form of beetroot juice has been shown to increase plasma nitrite (6, 21) and these increases have been identified as a significant factor in influencing exercise tolerance (24). This has been investigated by Bailey et al. (4) providing **substantial** evidence that increased plasma levels of nitrate are associated with a 25% increase in intermittent exercise performance during a two legged

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knee extensor exercise protocol. The current study found similar percentage increases in resistance exercise performance to failure with a mean average of 19.4% in total repetitions and mean average of 18.9% in total weight lifted. Although blood plasma nitrite levels were not taken within the current study it is presumed that plasma levels increased due to the reports from previous studies and increased performance within this study. The increased tolerance is a result of increased energy yield through PC stores (7), which is supported by findings from the work of Bailey and colleagues (4). They established that with high intensity exercise, specifically leg extension exercise, there was a reduction in PC degradation and a decrease in the production of Adenosine di-phosphates and inorganic phosphates, which are metabolites that have been linked to fatigue within muscles (8). This work concludes that nitrate supplementation could provide a more sufficient rate of change between these substrates and metabolites, consequently delaying the time **until** they reach critical values. This in turn, enables a greater time until depletion which results in a more efficient usage of PC stores at high intensity exercise, prolonging the tolerance at the given work rate. In relation to the current study's findings, this suggests that PC stores were used more efficiently when performing the repetitions until fatigue within a given set, allowing individuals to sustain muscle contractile performance for longer. This could explain the increase in total repetitions, more specifically increases in repetitions within each set due to the nitrate ingestion.

Dietary nitrate supplementation has also been reported to **excitation-contraction coupling** and efficiency (5, 16). These responses occur through an increase in sarcoplasmic reticulum calcium release and increase in force production per power stroke within type 2 fibres (16). The excitation-contraction coupling occurs when signals at the surface of the cell couple to cause releases in calcium within the sarcoplasmic reticulum, consequently this induces **actin-myosin** interactions resulting in muscle fibre contraction (1). The calcium is then withdrawn by the ATP- dependent pump (25). When multiple burst action potentials occur, calcium is sustained and greater contraction force can occur (25). These components modulate the effectiveness and efficiency of the force that is produced by this process. Greater NO production has been proposed to enhance the efficiency of coupling between

1 the above components to produce this overall effect of greater muscle contractile function (5, 25).
2 Hypothesizing that these given effects are apparent in humans (4) these enhancements in muscle
3 efficiency within type 2 fibres, could result in lower overall energy turnover (5). The above evidence
4 suggests, for our work, that when participants were completing upper body (bench press) exercise
5 until fatigue, more effective muscle contraction was apparent with lower overall energy cost, meaning
6 the nitrate aided in conserving energy over the given sets, thus allowing participants to complete more
7 sets.
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16 **PRACTICAL APPLICATIONS**

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18 Coaches and their athletes alike, could make use of nitrate supplementation since our evidence
19 suggests it, can be used within healthy male resistance trained population to increase exercise
20 tolerance and improve performance. Further studies are necessary to investigate long term **use and**
21 **possible adaptations** to resistance training with longer periods of dosing with nitrate.
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Figure Legend

Figure 1. Mean (\pm SD) number of bench press repetitions for each set and condition totals. (*)

Denotes a significant main effect for condition ($p < 0.05$).

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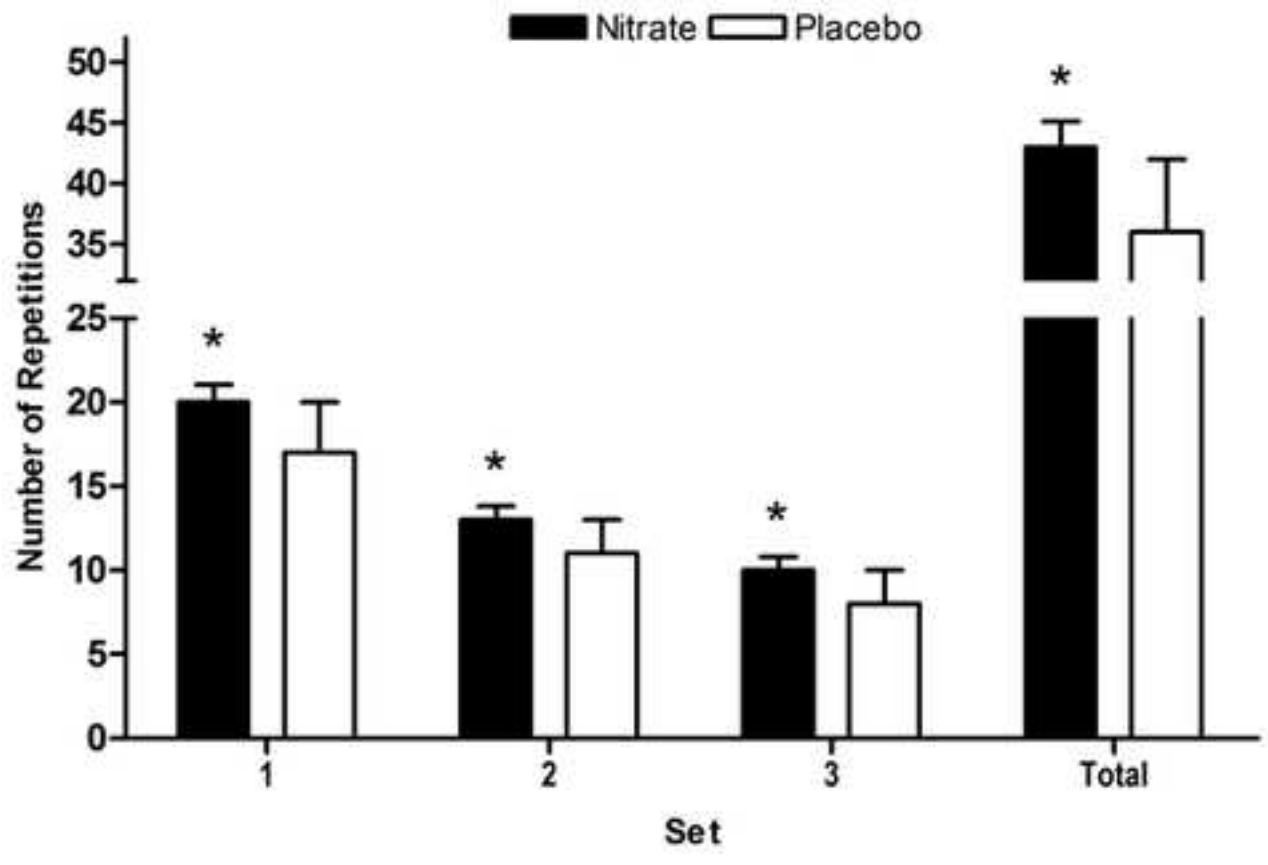


Table 1. Mean (\pm SD) physiological and perceived exertional responses.

	Condition	
	Nitrate	Placebo
Total Weight Lifted (kg)	2582.8 \pm 863.9‡	2171.5 \pm 720.5
Lactate (mmol.l ⁻¹)		
Pre	1.1 \pm 0.5	1.1 \pm 0.3
Post	4.2 \pm 0.8*	3.8 \pm 0.6*
RPE		
Set 1	4 \pm 2	4 \pm 1
Set 2	5 \pm 2	4 \pm 2
Set 3	5 \pm 2	5 \pm 2
RPE-L		
Set 1	5 \pm 2	5 \pm 2
Set 2	6 \pm 2	6 \pm 2
Set 3	7 \pm 2	7 \pm 2

(‡) Denotes a significant main effect for condition ($p < 0.001$).

(*) Denotes a significant main effect for time ($p < 0.05$).